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Mutation Research 588 (2005) 129–135

MR

Genetic Toxicology and Environmental Mutagenesis

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In silico assessment of chemical mutagenesis in comparison with results of Salmonella microsome assay on 909 chemicals

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Received 12 June 2005; received in revised form 20 September 2005; accepted 26 September 2005
Available online 28 October 2005

Abstract

Genotoxicity is one of the important endpoints for risk assessment of environmental chemicals. Many short-term assays to evaluate genotoxicity have been developed and some of them are being used routinely. Although these assays can generally be completed within a short period, their throughput is not sufficient to assess the huge number of chemicals, which exist in our living environment without information on their safety. We have evaluated three commercially available in silico systems, i.e., DEREK, MultiCASE, and ADMEWorks, to assess chemical genotoxicity. We applied these systems to the 703 chemicals that had been evaluated by the Salmonella/microsome assay from CGX database published by Kirkland et al. [1]. We also applied these systems to the 206 existing chemicals in Japan that were recently evaluated using the Salmonella/microsome assay under GLP compliance (ECJ database). Sensitivity (the proportion of the positive in Salmonella/microsome assay correctly identified by the in silico system), specificity (the proportion of the negative in Salmonella/microsome assay correctly identified) and concordance (the proportion of correct identifications of the positive and the negative in Salmonella/microsome assay) were increased when we combined the three in silico systems to make a final decision in mutagenicity, and accordingly we concluded that in silico evaluation could be optimized by combining the evaluations from different systems. We also investigated whether there was any correlation between the Salmonella/microsome assay result and the molecular weight of the chemicals: high molecular weight (>3000) chemicals tended to give negative results. We propose a decision tree to assess chemical genotoxicity using a combination of the three in silico systems after pre-selection according to their molecular weight.
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Keywords: In silico; (Quantitative) structure-activity relationship; (Q)SAR; Chemical genotoxicity; Decision tree

1. Introduction

It is said that more than 20,000 chemicals are in use in Japan. Among them, only approximately 10% are thought to have been assessed for human hazard based on data from in vitro and in vivo bioassays. According to the "Law Concerning the Evaluation of Chemical Substances and Regulation of Their Manufacture, etc." [2], the Salmonella/microsome (Ames) assay, in vitro chromosomal aberration assay (or alternatively mouse lymphoma TK assay), and 28-day repeat dose toxicity test in rodents are obligatory to notify new chemicals for production/import at a level of more than 10 t per year.

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doi:10.1016/j.mrgentox.2005.09.009

To screen the remaining 18,000 chemicals for human hazard by application of this three-test battery is not realistic from the time and economical point of view. We need a much higher-throughput system to assess these chemicals, at least for prioritization of those chemicals that should be submitted to biological testing. To assess human hazard for regulatory purposes, in silico systems are now beginning to be used [3]. Here, we evaluated three commercially available in silico (quantitative) structure-activity relationship ((Q)SAR) systems and tried to construct a decision tree for prioritization of which chemicals need in vitro and/or in vivo testing. Also, within the drug discovery process, integrated computational analysis has been proposed to be incorporated as a toxicity prediction tool [4].

Kirkland et al. [1] published a database (CGX database, see http://www.lhasalimited.org/cgx) for nearly 1000 carcinogens and non-carcinogens with results of representative in vitro genotoxicity assays, i.e., Salmonella/microsome assay (Ames), mouse lymphoma TK assay using L5178Y cells (MLA), and in vitro chromosomal aberration assay or in vitro micronucleus assay (CA/MN). We used 703 chemicals that had been assessed in the Ames assay for evaluation of the three in silico systems, i.e., DEREK, MultiCASE (MCase), and ADMEWorks (AWorks). We also used a database (the ECJ database) that we constructed from chemicals existing in Japan that had recently been assessed in the Ames assay, in vitro chromosomal aberration assay, and 28 day repeat dose rodent toxicity test and/or reproductive and developmental toxicity test for their safety evaluation under GLP compliance. The ECJ database consisted of 206 chemicals but only 26 chemicals were positive by the Ames assay. Initially we evaluated both sensitivity and specificity of these three systems using the ECJ database of 206 chemicals [5].

We selected these three in silico systems because of their different modes of analysis. DEREK is a rule-based system [6], MCase [7] is a database/substructure based system, and AWorks is a QSAR. We applied these systems individually to assess gene-mutation induction on the 703 and 206 chemical sets described above and evaluated their sensitivity, specificity, concordance, and applicability (how many chemicals could be assessed), independently.

It is known that high molecular weight polymers tend not to induce gene mutation and chromosomal aberrations mainly because they cannot enter the target cells to react with DNA, or other bio-molecules necessary for genetic stability. We analyzed 194 Ames positive chemicals (confidential source) for the effect of molecular weight.

2. Materials and methods

2.1. Data sources for chemicals assessed

Of about 1000 chemicals, 703 that had been assessed in the Ames test were chosen from the CGX database published by Kirkland et al. [1]. All chemical structures were re-drawn using Chemdraw Ultra (Cambridge Soft Corporation, USA) and converted to MOL files before application to each system. We also used the database of 206 chemicals evaluated in the MHLW project "Safety Examination of Existing Chemicals and Safety Programmes in Japan" (ECJ database). The test summary for each of these chemicals can be seen at http://wwwdb.mhlw.go.jp/ginc/html/db1.html. In addition, we collected 194 Ames positive chemicals from a confidential source and investigated the relationship between gene mutation induction and molecular weight, with identification of any active side chain that might have contributed to the positive result in the Ames assay.

2.2. In silico systems used and definition of positive and negative responses

We used DEREK (Lhasa Ltd., UK) version 8.0.1. When the system gave an evaluation as "certain", "probable" or "plausible" we considered this as "positive", and when the system gave "equivocal", "doubted", "improbable", "impossible", or "no alert" we considered this as "negative". We used MCase (Multicase Co. Ltd.) version mc4pc. When the system gave "active" or "marginal" we considered this as "positive", and when the system gave "Inactive" we considered this as "negative". In the case of AWorks (Fujitsu Kitakyushu, Co. Ltd., version 2.0), we considered as "positive" when system evaluation was "positive", and considered as "negative" when the system evaluation was "negative". We excluded chemicals from further analysis when DEREK or AWorks gave no answer, or the evaluation was "inconclusive" by MCase.

2.3. Definition of sensitivity, specificity, concordance, and applicability

We calculated sensitivity, specificity, concordance, and applicability as follows:

$$\text{sensitivity} = \frac{N_{A+S+}}{N_{A+}} \times 100, \qquad \text{specificity} = \frac{N_{A-S-}}{N_{A-}} \times 100,$$

$$\text{concordance} = \frac{N_{A+S+} + N_{A-S-}}{N_{eval}} \times 100,$$

$$\text{applicability} = \frac{N_{eval}}{N_{all}} \times 100$$

where N_{A+} is number of chemicals revealing positive in Ames assay; N_{A-} is number of chemicals negative in Ames assay; N_{A+S+} is number of chemicals revealing positive by both Ames assay and in silico evaluation; N_{A-S-} is number of chemicals negative in both Ames assay and in silico evaluation; N_{eval} is

Table 1
Performance of in silico systems

	Ames result	+	−	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
CGX database								
DEREK	+	288	64	352				
	−	69	267	336	81.8	79.5	80.7	97.9
	Total	357	331	688				
MCase	+	235	32	267				
	−	6	249	255	88.0	97.6	92.7	74.3
	Total	241	281	522				
AWorks	+	267	89	356				
	−	149	187	336	75.0	55.7	65.6	98.4
	Total	416	276	692				
ECJ database								
DEREK	+	19	7	26				
	−	21	159	180	73.1	88.3	86.4	100.0
	Total	40	166	206				
MCase	+	13	7	20				
	−	13	133	146	65.0	91.1	88.0	80.6
	Total	26	140	166				
AWorks	+	19	7	26				
	−	54	124	178	73.1	69.7	70.1	99.0
	Total	73	131	204				

MCase: MultiCASE; AWorks: ADMEWorks.

number of chemicals evaluated; and N_{all} is total number of chemicals subjected.

3. Results

Among the set of 703 CGX chemicals with published Ames data, 358 were positive and 345 were negative. The results of the in silico evaluation are summarized in Table 1. The highest sensitivity, specificity, and concordance with Ames assay results was provided by MCase, then followed by DEREK. However, the systems that showed the best applicability were AWorks and (almost the same) DEREK, then followed by MCase. For the database of 206 ECJ chemicals, 26 were positive and 180 were negative. The outcomes of the in silico analyses are summarized in Table 1. The pattern of performance was very similar to that with the 703 chemicals in the CGX database.

Fig. 1 shows the cumulative percent of Ames positive chemicals against molecular weight. It can be seen that 87.1% of those positive chemicals had molecular weights less than 1000, and 96.4% had molecular weights less than 3000; in other words, only 3.6% of the chemicals with a molecular weight >3000 gave a positive response in the Ames assay. Seven of 194 Ames positive chemicals had a molecular weight >3000 and four of these seven polymers had epoxy groups.

When we combined the in silico systems, the performance was different from that when assessed individually (Table 2). If we considered the in silico mutagenicity as positive (or negative) when two or more systems gave positive (or negative) evaluations, 87.8

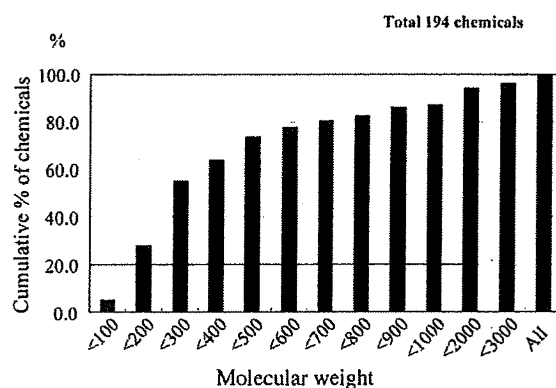

Fig. 1. Cumulative percentage of chemicals based on their molecular weight. 194 Ames positive chemicals were analyzed. 7/194 chemicals were more than 3000 molecular weight and Ames positive and 4/7 contained epoxy groups.

Table 2
Performance of in silico systems after combined

CGX database

Ames \ In silico	++ or +++	-- or ---	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
+	279	40	319				
-	42	249	291	87.8	85.6	86.7	86.8
Total	321	289	610				
	+++	---					
+	166	1	167				
-	3	127	130	99.4	97.7	98.7	42.2
Total	168	129	297				

ECJ database

Ames \ In silico	++ or +++	-- or ---	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
+	19	7	26				
-	23	147	170	73.1	86.5	84.7	95.1
Total	42	154	196				
	+++	---					
+	13	2	15				
-	5	94	99	86.7	94.9	93.9	55.3
Total	18	96	114				

Table 3
Performances of DEREK and MCase in several published papers.

Target compounds	In silico system	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)	Reference
394 Drugs	DEREK	52	75	74	94[a]	[11]
	MCase	48	93	90	91[a]	
217 Non-drugs	DEREK	86	50	81	100[a]	[10]
	MCase	91	62	83	100[a]	
520 Drug candidates	DEREK	28	80	72	100	[13]
	MCase	50	86	81	41	
	DEREK + MCase	29	95	88	29	
	DEREK + Mcase + TOPKAT	75	96	95	15	
123 Drug candidates	DEREK	8[b]	31[c]	61	100[d]	[4]
	MCase (A2H)	13[b]	15[c]	72	97[d]	
	Topcat (Ames Mut)	18[b]	15[c]	67	98[d]	
	DEREK + MCase	6[b]	19[c]	75	97[d]	
	DEREK + Mcase + TOPKAT	5[b]	9[c]	86	46[d]	
94 Non-drugs	DEREK	63	81	76	100	[13]
	MCase	40	90	76	75	
	DEREK + MCase	47	100	85	56	
	DEREK + Mcase + TOPKAT	55	100	86	37	
516 Non-drugs	DEREK	6[b]	24[c]	70	100[d]	[4]
	MCase (A2H)	7[b]	12[c]	81	98[d]	
	Topcat (Ames Mut)	25[b]	19[c]	56	97[d]	
	DEREK + MCase	2[b]	16[c]	82	98[d]	
	DEREK + Mcase + TOPKAT	7[b]	10[c]	83	43[d]	

[a] Calculated by us
[b] % False negative.
[c] % False positive.
[d] (1-Indeterminate).

and 73.1% sensitivity, 85.6 and 86.5% specificity, 86.7 and 84.7% concordance, and 86.8 and 95.1% applicability were obtained for the CGX and ECJ databases, respectively. If we considered the in silico mutagenicity as positive (or negative) only when all three systems gave positive (or negative) evaluations, all performance measures (sensitivity, specificity, etc.) increased up to 98.7 and 93.9%. However, applicability decreased to 42.2 and 55.3%, which meant only about half of the chemicals in the CGX and ECJ databases could be evaluated. One chemical, *o*-phenylphenol [90-43-7], was positive in the Ames test but negative by all three in silico systems and three chemicals, carboxymethylnitrosourea [60391-92-6], methidathion [950-37-8], 1-nitroso-3,5-dimethyl-4-benzoylpiperazine [61034-40-0], were negative in the Ames test although all three in silico system gave positive evaluation for mutagenicity in the CGX database. When we used the ECJ database, 2-amino-1-naphthalenesulfonic acid [81-16-3] and 2-vinylpyridine [100-69-6] were positive in the Ames test but negative by all three in silico systems and there was no chemical that was negative in the Ames assay and all positive in in silico system. These exceptional chemicals are listed in Table 3 together with such chemicals taken from literatures.

4. Discussion

It is important to construct a strategy for efficient evaluation of the toxicity of a large number of existing chemicals. Even so-called short-term assays, e.g., Ames assay and in vitro chromosomal aberration assay, can practically assess only 100 chemicals per year according to our experiences in Japan. In this case, it will take 180 years to assess the outstanding 18,000 existing chemicals for genotoxicity, and it will take even longer when repeat dose toxicity tests are also performed, as these are not short-term assays. We therefore need higher-throughput systems to assess chemical safety, or at least to set priorities for those chemicals that should be tested in in vitro and/or in vivo tests. In silico systems have the capability for high throughput but have not yet been well validated for assessment of human hazard, although some regulatory bodies have started to use these methods.

Correlation between the Ames assay result and molecular weight could be explained by the lack of membrane permeability of high molecular weight chemicals, making it more difficult for them to reach target molecules such as DNA and proteins that contribute to the fidelity of cell division. Therefore, only a few chemicals with molecular weight >3000 gave positive responses in the Ames assay. This phenomenon is also true for induction of chromosomal aberrations in vitro (data not shown). The other important issue is the contribution of epoxy group in the polymer. Although of molecular weight >3000, some polymers with an epoxy group gave positive results in both the Ames and chromosomal aberration assays. Epoxy embedding reagents employed in electron microscopy (e.g., epon and araldite) have been reported as mutagenic in the Ames assay [8]. According to these findings, we should include a step to evaluate molecular weight and existence of any epoxy groups in the molecule.

In the present study, we used the CGX database recently published by Kirkland et al. [1] for microbial mutagenicity data on 358 carcinogens and 345 non-carcinogens for validation of three commercially available in silico (Q)SAR systems. When applied individually, MCase gave high sensitivity, specificity, and concordance compared to other two systems. One of the reasons may be because the CGX database contained many results from the U.S. National Toxicology Program (NTP), and the learning dataset of MCase would have used many of the same results. Therefore, some of them were evaluated by direct matching. Moreover, the applicability of MCase was relatively low compared with the other systems in this study (Table 1). MCase judged 119 chemicals as inconclusive and one chemical as marginal, and could not evaluate 67 chemicals. Such selectivity in MCase may contribute to the high concordance. On the other hand, the other systems were not influenced directly by the NTP data. We applied the in silico systems to another dataset, the ECJ database, that does not contain the NTP data and we obtained similar patterns of sensitivity, specificity, etc.

Each in silico system showed different outcomes on some chemicals complimentary by some extent. These different evaluation patterns were mainly due to the different evaluation rules. The DEREK is a rule-based system, AWorks is a discriminant-based system mainly depending on physicochemical descriptors, and MCase is a hybrid system based on a database. Therefore, we concluded that in silico evaluation could be optimized by combining the evaluations from the three systems. Sensitivity, specificity and concordance were increased when we combined the three in silico systems to make a final conclusion of mutagenicity (Table 1). Concordance was much higher after combining but the applicability became poor (42.2%). When two of the in silico systems gave the same evaluations, the applicability (86.8%) was good but the concordance was lower (86.7%) than when all three were combined (98.7).

Recently, several in silico studies for prediction of mutagenicity have been conducted on drugs or non-

Table 4
Exceptional chemicals that showed Ames test gave positive but all three in silico systems (DEREK, MCase, TOPKAT/AWorks) gave negative and Ames test gave negative but all three systems gave positive

Compound	CAS	Ames test	DEREK	MCase	TOPKAT/Aworks	Source[a]
Bupropion	34911-55-2	+	–	–	–	1
Citalopram	59729-33-8	+	–	–	–	1
Naloxone	465-65-6	+	–	–	–	1
Oxcarbazepime	28721-07-5	+	–	–	–	1
Quetiapine	111976-69-7	+	–	–	–	1
Rabeprazole	117976-89-3	+	–	–	–	1
Zolmitriptan	139264-17-8	+	–	–	–	1
2-(2-Methylpropyl) thiazole	18640-74-9	+	–	–	–	2
2-Chloropyridine	109-09-1	+	–	–	–	2
Pyrogallol	87-66-1	+	–	–	–	2
o-Phenylphenol	90-43-7	+	–	–	–	3
2-Amino-1-naphthalenesulfonic acid	81-16-3	+	–	–	–	3
2-Vinylpyridine	100-69-6	+	–	–	–	3
Fosfomycin	23155-02-4	–	+	+	+	1
Toremifene	89778-26-7	–	+	+	+	1
Poly (2-hydroxypropyl methacrylate)	25703-79-1	–	+	+	+	2
Carboxymethylnitrosourea	60391-92-6	–	+	+	+	3
Methidathion	950-37-8	–	+	+	+	3
1-Nitroso-3,5-dimethyl-4-benzoylpiperazine	–	+	+	+	3	

[a] 1: Synder et al. [11] (with TOPKAT), 2: White et al. [13] (with TOPKAT), 3: this study (with AWorks).

drug chemicals with commercially available programs, e.g., DEREK, MCase or TOPKAT, or newly developed computational approaches [4,9–12]. The performances of DEREK and MCase in several of these studies are summarized in Table 4. Generally, similar performance in sensitivity, specificity, concordance, and applicability were shown between DEREK and MCase but with some exceptions, e.g., sensitivity in 520 drug candidates [13], specificity in 516 non-drugs [4], and applicability in 520 pharmaceutical drug candidates and 94 non-drugs [13]. These differences might be due to the chemical class of target compounds in each database. However, there was no remarkable difference in performance whether the chemical was intended for use as a pharmaceutical, agricultural, or industrial agent. Our results on performance of in silico systems showed similarity with the published analyses. With respect to the combination of in silico prediction systems, White et al. [13] reported that combination improved the overall accuracy and specificity, but sensitivity was barely above the 50% level (Table 4). On the other hand, their analysis showed quite low applicability in the combination of three prediction systems, DEREK, MCase and TOPKAT. Our analysis of the combination of DEREK, MCase and AWorks showed good improvements in sensitivity, specificity and concordance, but applicability was low, especially in the 3-system combination.

Exceptional chemicals that gave positive Ames results but were negative in all three in silico systems (DEREK, MCase, TOPKAT/AWorks), and those that were negative in the Ames test but gave positive evaluations in all three systems, are summarized in Table 4. This table, which includes data from Synder et al. [11] and White et al. [13] shows there are 19 exceptional chemicals from both drug and non-drug families. Although it would be unrealistic to expect zero exceptions using this approach, further improvement of the prediction systems is needed. We do not have good reasons to explain the discordance, therefore we will verify the results from both sides, i.e., in silico system and Ames test.

Considering these outcomes, we propose a decision tree (Fig. 2), in order to evaluate chemical induction of gene mutation. We may use the decision tree to prioritize chemicals to be assayed by in vitro and/or in vivo tests. A final goal being that eventually, chemical mutagenicity will be evaluated by in silico systems alone for regulatory use. The decision tree consists of three steps; namely to assess the molecular weight, the existence of epoxy groups, and the in silico evaluation for genotoxicity. Based on the purpose of the in silico evaluation, the tree might be altered by the different final call of the in silico evaluation, i.e., regarding as positive (negative) all three systems show positive (negative). The choice of definition for final call applying to the decision tree should be based on the balance between accuracy of eval-

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Toxicology and Applied Pharmacology 217 (2006) 375–383

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The expression of Hedgehog genes (*Ihh*, *Dhh*) and Hedgehog target genes (*Ptc1*, *Gli1*, *Coup-TfII*) is affected by estrogenic stimuli in the uterus of immature female rats

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Received 3 August 2006; revised 29 September 2006; accepted 2 October 2006
Available online 6 October 2006

Abstract

The objective of this study was to investigate the effects of estrogen receptor (ER) agonists and an ER antagonist on the expression of Hedgehog genes (Indian hedgehog: *Ihh*; Desert hedgehog: *Dhh*) and Hedgehog target genes (Patched 1: *Ptc1*; glioma-associated oncogene homolog 1: *Gli1*; chicken ovalbumin upstream promoter transcription factor II: *Coup-TfII*) in the rat uterus. Immature female rats were administered once with 17α-ethynyl estradiol (EE, an ER agonist), propyl pyrazole triole (PPT, an ERα-selective agonist), diarylpropionitrile (DPN, an ERβ-selective agonist), or ICI 182,780 (an ER antagonist). Expression of mRNA for *Ihh*, *Dhh*, and *Ptc1* was dose-dependently downregulated by EE in the uterus of immature rats, mediated by ER as confirmed by coadministration of ICI 182,780. The mRNA expression levels of *Ptc1*, *Gli1*, and *Coup-TfII* were simultaneously downregulated during the period in which the mRNA expression levels of *Ihh* and *Dhh* were downregulated in the uterus after administration of EE. PPT downregulated the transcription of *Ihh*, *Dhh*, *Ptc1*, *Gli1*, and *Coup-TfII*, indicating that expression of these genes was regulated by the ERα-dependent pathway. DPN also downregulated the transcription of *Ihh* and *Dhh*, although the effect was weaker than that of PPT, indicating that the regulation of uterine *Ihh* and *Dhh* transcription was also affected by the ERβ-dependent pathway. These results suggest that the expression of Hedgehog genes (*Ihh*, *Dhh*) and Hedgehog target genes (*Ptc1*, *Gli1*, *Coup-TfII*) is affected by estrogenic stimuli in the uterus of immature female rats.
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Keywords: Estrogen receptor; Uterus; Indian hedgehog; Desert hedgehog

Introduction

Estrogen is a steroid hormone that plays an important role in the regulation of mammalian reproduction by regulating the transcription of specific genes. The transcriptional regulation of estrogen is mediated by two types of specific nuclear receptors, estrogen receptor alpha (ERα) (Green et al., 1986) and estrogen receptor beta (ERβ) (Kuiper et al., 1996; Mosselman et al., 1996). The uterus contains both ERα and ERβ, however, ERα is the predominant receptor (Couse et al., 1997; Rosenfeld et al., 2001). The study of *ERβ* knockout mice suggests that ERβ plays a role in modulation of the effects of ERα in the uterus. For example, in both the wild-type and ERβ knockout female mice, 17β-estradiol (E2) induced the expression of progesterone receptor (PR) in the stroma and glandular epithelial cells of the uterus. However, in the luminal epithelium, E2 inhibited the expression of PR in wild-type mice but had no effect on PR expression in these cells in ERβ knockout female mice, indicating that induction of PR is an ERα-mediated event and inhibition of epithelial PR is ERβ mediated (Weihua et al., 2000). ERα and ERβ regulate the expression of specific target genes by functioning as ligand-induced transcriptional factors to exert various physiological functions (Tasset et al., 1990;

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0041-008X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2006.10.003

Mangelsdorf et al., 1995). Therefore, it is considered that the characterizations of the gene expression change by estrogenic stimuli provide important clues for understanding the influence of the estrogenic compounds.

Hedgehog proteins are involved in the regulation of cellular events at various stages of development from embryonic to adult in humans and many animal species (Ingham, 1998; Walterhouse et al., 1999; Ingham and McMahon, 2001; Walterhouse et al., 2003). *Drosophila* has a single Hedgehog gene, but higher vertebrates have three Hedgehog genes: Sonic hedgehog (*Shh*); Indian hedgehog (*Ihh*); and Desert hedgehog (*Dhh*) (Pathi et al., 2001). Regulation of diverse processes by Hedgehog proteins requires activation or suppression of the intracellular Hedgehog signaling pathway and the Hedgehog target genes, which finally leads to cell proliferation, growth arrest, differentiation, programmed cell death, or cell migration (Ingham, 1998; Ingham and McMahon, 2001; Oldak et al., 2001).

The Hedgehog signaling pathway is tightly regulated, and the signal is transmitted by two receptors, the twelve-transmembrane protein Patched (PTC) and the seven-transmembrane protein Smoothened (SMO). In the absence of Hedgehog proteins, PTC acts as a negative regulator of SMO. When present, Hedgehog proteins bind to PTC, and SMO is released from the inhibitory effect of PTC, then transcription factor GLI is translocated into the nucleus, where it regulates the transcription of Hedgehog target genes (Ingham and McMahon, 2001; Cohen, 2003). Dysregulation of the Hedgehog signaling pathway during development has been associated with significant birth defects, including basal cell nevus syndrome (Johnson et al., 1996) and holoprosencephaly (Belloni et al., 1996). In addition, dysregulation of the Hedgehog signaling pathway during postnatal life has been associated with cancers, including basal cell carcinoma (Xie et al., 1998) and sarcomas (Stein et al., 1999).

In the mammalian Hedgehog family, *Ihh* gene was reported as a progesterone-upregulated gene in the mouse uterus (Matsumoto et al., 2002; Takamoto et al., 2002). Estrogen and progesterone are steroid hormones that play a pivotal role in the regulation of female reproduction (DeMayo et al., 2002), and there is considerable biological evidence for cross-talk between the ER and PR signaling pathways (Kraus et al., 1995; Katzenellenbogen, 2000). For example, liganded PR-A and PR-B can each suppress E2-stimulated ER activity, with the magnitude of repression dependent on the PR isoform, ligand type (agonist or antagonist), PR levels, and ligand concentration (Kraus et al., 1995; Katzenellenbogen, 2000). Therefore, we hypothesized that the expression of Hedgehog-related genes may also be affected by estrogenic stimuli. However, only limited information is available whether estrogenic compounds influence the regulation of such gene expression.

Immature female rats are frequently used to investigate the regulatory actions of sex steroid hormones on various functions in the uterus. In the rat, the concentration of endogenous estrogen is consistently low throughout prepubertal development and starts to increase after 28 days of age. However, exposure to estrogenic compounds during the prepubertal period can induce an uterotrophic response (Noda et al., 2002; Naciff et al., 2003). This uterotrophic response in immature rats is being used as one of the standard assays to estimate estrogenic activity of different compounds *in vivo* (Owens and Ashby, 2002; Yamasaki et al., 2002; Kanno et al., 2003). Furthermore, it is considered that the immature female rats are suitable animal model to examine the influence of test compounds on the uterine gene expression via the ER (Naciff et al., 2003; Naciff et al., 2004; Lee et al., 2005; Katayama et al., 2006). Therefore, we used sexually immature female rats corresponding to a stage of high sensitivity to exogenous estrogenic stimulation to investigate the effects of ER agonists and an ER antagonist on the expression of Hedgehog genes and Hedgehog target genes in the uterus. Furthermore, we used four types of ER ligands: 17α-ethynyl estradiol (EE, an ER agonist used medically in oral contraceptives); propyl pyrazole triole (PPT, an ERα-selective agonist); diarylpropionitrile (DPN, an ERβ-selective agonist); and ICI 182,780 (ICI, an ER antagonist). PPT is a potent ERα agonist that has 410-fold higher binding affinity for ERα than ERβ and has been demonstrated to have almost no binding affinity to ERβ (Kraichely et al., 2000; Stauffer et al., 2000). In contrast, DPN is a potency-selective agonist for ERβ with a more than 70-fold higher binding affinity for ERβ than ERα (Meyers et al., 2001). We report here that ER agonists downregulate transcription of Hedgehog genes (*Ihh*, *Dhh*) and Hedgehog target genes (*Ptc1*, *Gli1*, *Coup-TfII*) in the uterus of immature female rats, predominantly via ERα-dependent pathway.

Materials and methods

Chemicals. 17α-Ethynyl estradiol (EE) and corn oil were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Propyl pyrazole triole (PPT), diarylpropionitrile (DPN), and ICI 182,780 (ICI) were obtained from Tocris Cookson Inc. (Ellisville, MO, USA). Ethanol was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Corn oil containing 1% ethanol was prepared as the vehicle solution.

Animals. Thirteen-day-old, female Crj:CD (SD) IGS rats were obtained from Charles River Japan, Inc. (Kanagawa, Japan) with lactating maternal animals. After arrival, immature and maternal animals were acclimated for 5 days. Weaning and group assignment were performed on the day before administration (at 18 days old) to minimize the stress associated with weaning. One hundred animals in total were assigned to 20 groups (Experiment 1: 5 groups; Experiment 2: 12 groups; Experiment 3: 3 groups) to give similar intergroup variations in body weight among the resulting groups in each experiment. Animals were housed in polycarbonate cages (380 × 330 × 175 mm [width × depth × height], CLEA Japan, Inc., Tokyo, Japan). During the quarantine period, animals were accommodated in cages in groups of 10 immature animals and one maternal animal per cage. After group assignment, immature animals were accommodated at 5 animals per cage. The animal room was maintained at a temperature of 19.0 °C to 25.0 °C, with a relative humidity of 35.0% to 75.0%, and at a 12-h light/dark cycle. The animals were allowed free access to a pellet diet for experimental animals (MF, Oriental Yeast, Co., Ltd., Tokyo, Japan) and sterilized water. The animals were cared for in accordance with 'The Guidelines for Animal Experimentation' for our laboratory, Mitsubishi Chemical Safety Institute Ltd.

Study design. The study design was shown in Fig. 1. Rats were used for all experiments at 19 days of age. In Experiment 1 (dose-dependent response), animals were treated once by oral gavage with the vehicle or EE (0.3, 1, 3 μg/kg). In addition, to determine whether the gene expression changes caused by EE

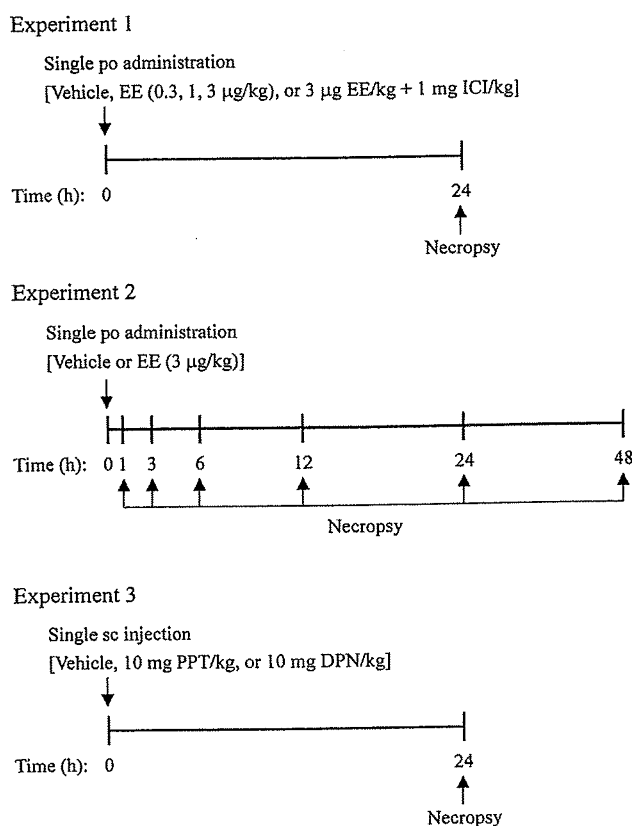

Fig. 1. Study design. Vehicle: corn oil containing 1% ethanol, EE: 17α-ethynyl estradiol (an ER agonist), ICI: ICI 182,780 (an ER antagonist), PPT: propyl pyrazole triole (an ERα-selective agonist), DPN: diarylpropionitrile (an ERβ-selective agonist).

were ER-mediated reactions, a sub-group was treated with ER antagonist (1 mg ICI/kg) and 3 μg EE/kg. In Experiment 2 (time-dependent response), animals were treated once by oral gavage with either the vehicle or 3 μg EE/kg. In Experiment 3, animals were treated once by subcutaneous injection with the vehicle, 10 mg PPT/kg, or 10 mg DPN/kg. The dose and administration route of EE and ICI were selected based on the results of preliminary experiment and published data (Kanno et al., 2001; Katayama et al., 2006). The dose and administration route of PPT and DPN were judged to be suitable to evaluate whether a certain phenomenon was predominantly regulated via either ERα or ERβ, based on the results of preliminary experiment and published data (Harris et al., 2002; Frasor et al., 2003; Tena-Sempere et al., 2004; Lee et al., 2005). Body weights were measured using an electronic balance (PM3000, Mettler Toledo K.K., Tokyo, Japan). The administration volume was 10 mL/kg, and was adjusted individually based on the body weight immediately before administration. Five animals were included in each treatment group. Animals were sacrificed by CO_2 asphyxiation in Experiments 1 and 3, at 24 h after administration; and in Experiment 2, at 1, 3, 6, 12, 24, and 48 h after administration. The uterus was removed from the body, placed on gauze, and cut at several sites to discharge gently the fluid in the uterus. The blotted uterine weight (weight of the uterus excluding the inner fluid) was measured using an electronic balance (Model AE260, Mettler Toledo K.K.). The uterus was submerged in the RNA preservative reagent RNA*later* (Ambion, Inc., Austin, TX, USA), kept at 4 °C overnight, and then was stored at −20 °C until processing for RNA isolation.

Isolation of total RNA. The uterus was homogenized in the dissolving and absorption liquid containing 1% 2-mercaptoethanol (TOYOBO CO., LTD., Osaka, Japan) for 300 s at −20 °C using an automatic sample preparation system (Twist Crusher HMX-2000, TOYOBO CO., LTD.). Total RNA was isolated using MagExtractor-RNA- (TOYOBO CO., LTD.) and an automatic nucleic acid extraction system (MagExtractor System MFX-2000, TOYOBO CO., LTD.) according to the manufacturer's recommended protocol, and was subsequently DNase-treated with RNase-free DNase I (TAKARA BIO INC., Shiga, Japan) for 30 min at 37 °C in the presence of RNase OUT (Invitrogen Corporation, Carlsbad, CA, USA). The amount of total RNA was determined using the RiboGreen RNA Quantitation kit (Molecular Probes, Inc., Eugene, OR, USA) or spectrophotometer (DU-7400, Beckman Coulter, Inc., Fullerton, CA, USA). The absence of genomic DNA contamination in the total RNA samples was confirmed by real-time PCR for each RNA sample without reverse transcriptase using TaqMan Rodent GAPDH Control Reagents VIC™(Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommended protocol.

Real-time reverse transcription-PCR. One-step real-time reverse transcription (RT)-PCR was performed to determine changes in the gene expression using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Total RNA (0.5 to 20 ng) isolated from each uterus was added to a reaction mixture containing forward primer, reverse primer, TaqMan probe, and TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems) in a final volume of 50 μL according to the manufacturer's instruction. Rat-specific primers and TaqMan probes were designed for the Hedgehog genes (*Ihh, Dhh*) and Hedgehog target genes (*Ptc1, Gli1, Coup-TfII*) using Primer Express software (Applied Biosystems) (Table 1). The mRNA expression of *Shh* was determined using primer and probe sets in the TaqMan Gene Expression Assays for rat *Shh* (Applied Biosystems). In this study, we selected 18S rRNA as a stable endogenous reference gene to normalize the target mRNA expression in the uterus (Katayama et al., 2006). TaqMan probes were labeled with a fluorescent reporter dye (FAM or VIC) at the 5′ end and a quencher dye (TAMRA) at the 3′ end. Thermal cycling conditions were as follows: 1 cycle of 30 min at 48 °C for reverse transcription; 1 cycle of 10 min at 95 °C for activation of DNA polymerase; 40 cycles of 15 s at 95 °C for denaturation and 1 min at 60 °C for annealing/extension. The expression levels of target gene and 18S rRNA in each sample were calculated based on the standard curve generated with the rat total RNA for the uterus (UNITECH. Co.,Ltd., Chiba, Japan) and ovary (Ambion, Inc.), or Rat Universal Reference Total RNA (BD Biosciences Clontech, Palo Alto, CA, USA). The expression level of target gene was then normalized by the expression level of 18S rRNA using TaqMan Ribosomal RNA Control Reagents (Applied Biosystems) to control the quantity of the isolated RNA. Real-time RT-PCR analyses were performed in duplicate on all five animals in each treatment group.

Statistical analysis. Data were expressed as the mean±S.D. for each group. Fold changes for uterine weights and gene expression data were expressed as the ratio of the mean value of the test compound group to the mean value of the vehicle group. Statistical analyses were performed using the SAS Proprietary Software Release 8.2 (SAS Institute, Inc., Cary, NC, USA). In experiment 1, statistical analysis among multiple groups was performed using Bartlett's test for homogeneity of variance. When the variance was homogenous, the data were analyzed by one-way analysis of variance test. When the differences were significant by one-way analysis of variance test, the data were analyzed by Williams' test. When the variance was not homogenous, the data were analyzed by Kruskal–Wallis test. When the differences were significant by Kruskal–Wallis test, the data were analyzed by the Shirley–Williams' test. In the all experiment, statistical analyses between 2 groups were performed using the *F*-test for homogeneity of variance (Experiment 1: 3 μg EE/kg vs 3 μg EE/kg + 1 mg ICI/kg; Experiment 2: vehicle vs 3 μg EE/kg; Experiment 3: vehicle vs 10 mg PPT/kg, vehicle vs 10 mg DPN/kg). When the variance was homogeneous, the data were analyzed by Student's *t*-test. When the variance was not homogeneous, the data were analyzed by the Aspin–Welch's *t*-test. The *F*-test and Bartlett's test were conducted at the significance level of 5%. Williams' test and the Shirley–Williams' test were conducted at the one-tailed significance level of 5%. The other tests were conducted at the two-tailed significance level of 5%.

Results

In this study, *Shh* mRNA was not detected by the real-time RT-PCR analysis in the uterus of immature female rats,

Table 1
Sequences of primer and probe sets used for real-time RT-PCR analysis

Gene	Sequence	Accession no.	Amplicon size (bp)
Hedgehog gene			
Ihh			
Forward primer	5′-ACCCCACCTTCAGCGATGT-3′	XM_343590	78
Reverse primer	5′-GAGTCTCGATGACCTGGAAAGC-3′		
Probe	5′-CATTTTCCTGGACCGTGAGCCAAACAG-3′		
Dhh			
Forward primer	5′-CGTTACGTGCGCAAGCAA-3′	XM_343327	69
Reverse primer	5′-GGTCCGCTCGGGCATACT-3′		
Probe	5′-TGTGCCTCTGCTCTACAAGCAGTTTGTGC-3′		
Hedgehog target gene			
Ptc1			
Forward primer	5′-AGCGTACCTCCTAGGTAAGCCTC-3′	NM_053566	122
Reverse primer	5′-CGGCTTTATTCAGCATTTCCTC-3′		
Probe	5′-CGGTGGACAAACTTTGACCCCTTGG-3′		
Gli1[a]			
Forward primer	5′-TCTCCACAGTGGAGCCCAA-3′	XM_345832	76
Reverse primer	5′-CCCTCCGGCACAGTCAGT-3′		
Probe	5′-TGCTCTCTTCCCTGCCGGATCCT-3′		
Coup-TfII			
Forward primer	5′-CAATCAACTAGCCCTGAGCCA-3′	NM_080778	77
Reverse primer	5′-GCGCGCCGCCTTTT-3′		
Probe	5′-CCTCTCGCACCCTCGCACACAC-3′		

[a] Sequence of probe for *Gli1* was designed by the antisense strand.

regardless of the presence of EE, ICI, PPT, or DPN (data not shown). Therefore, *Ihh* and *Dhh* mRNAs were selected to evaluate the effects of estrogenic compounds on the expression of Hedgehog genes in immature rat uterus.

Experiment 1

To determine whether ER agonist (EE) and ER antagonist (ICI) influence the expression of Hedgehog genes (*Ihh*, *Dhh*) and Hedgehog target gene (*Ptc1*) in the uterus, immature female rats were treated once with the vehicle, EE (0.3, 1, 3 μg/kg), or EE (3 μg/kg) plus ICI (1 mg/kg).

The blotted uterine weights significantly increased in a dose-dependent manner by EE, in the dose range of 0.3–3 μg/kg, as compared with the vehicle group (Fig. 2A). The effect of EE (3 μg/kg) on the blotted uterine weight was significantly blocked by coadministration with ICI (1 mg/kg), indicating that the effect was ER-mediated (Fig. 2A).

The mRNA expression of *Ihh*, *Dhh*, and *Ptc1* significantly decreased in a dose-dependent manner by EE, in the dose range of 1–3 μg/kg, as compared with the vehicle group (Figs. 2B, C, D). The effects of EE (3 μg/kg) on the mRNA expression of *Ihh*, *Dhh*, and *Ptc1* were significantly blocked by coadministration with ICI (1 mg/kg), indicating that these effects were ER-mediated (Figs. 2B, C, D). However, coadministration with ICI was not able to fully block the inhibitory effect of EE on the mRNA expression of *Dhh* and *Ptc1*, while the effect on the mRNA expression of *Ihh* was completely abrogated by ICI. These results suggest that EE influences the mRNA expression of *Dhh* and *Ptc1* through the ER-dependent and ER-independent mechanisms.

Experiment 2

To determine the time-course changes in the expression of Hedgehog genes (*Ihh*, *Dhh*) and Hedgehog target genes (*Ptc1*, *Gli1*, *Coup-TfII*) in the uterus exposed to ER agonist, immature female rats were treated once with the vehicle or EE (3 μg/kg).

The blotted uterine weights significantly increased between 6 and 48 h after treatment with EE as compared with those in the time-matched vehicle groups, indicating that the treatment with EE at 3 μg/kg was enough to stimulate the immature rat uterus (Fig. 3A).

The mRNA expression of *Ihh* significantly decreased between 6 and 48 h after treatment with EE as compared with those in the time-matched vehicle groups (Fig. 3B). The decrease in the mRNA expression of *Ihh* in the EE group (3 μg/kg) reached maximum at 24 h (0.05-fold) after administration. In addition, the mRNA expression of *Ihh* fluctuated after treatment with the vehicle, indicating that it is important to set the time-matched vehicle group in time course experiment of gene expression profiling.

The mRNA expression of *Dhh* transiently and slightly increased 3 h (1.70-fold) after treatment with EE, but no statistical significance was noted in this change as compared with that in the time-matched vehicle group (Fig. 3C). Thereafter, the mRNA expression of *Dhh* decreased from 12 to 48 h after treatment with EE, as compared with those in the time-matched vehicle groups (Fig. 3C). A statistically significant difference was noted in the mRNA expression of *Dhh* 12 h (0.54-fold) and 48 h (0.39-fold) after treatment with EE as compared with those in the time-matched vehicle groups.

The mRNA expression of *Ptc1* significantly decreased between 3 and 48 h after treatment with EE as compared

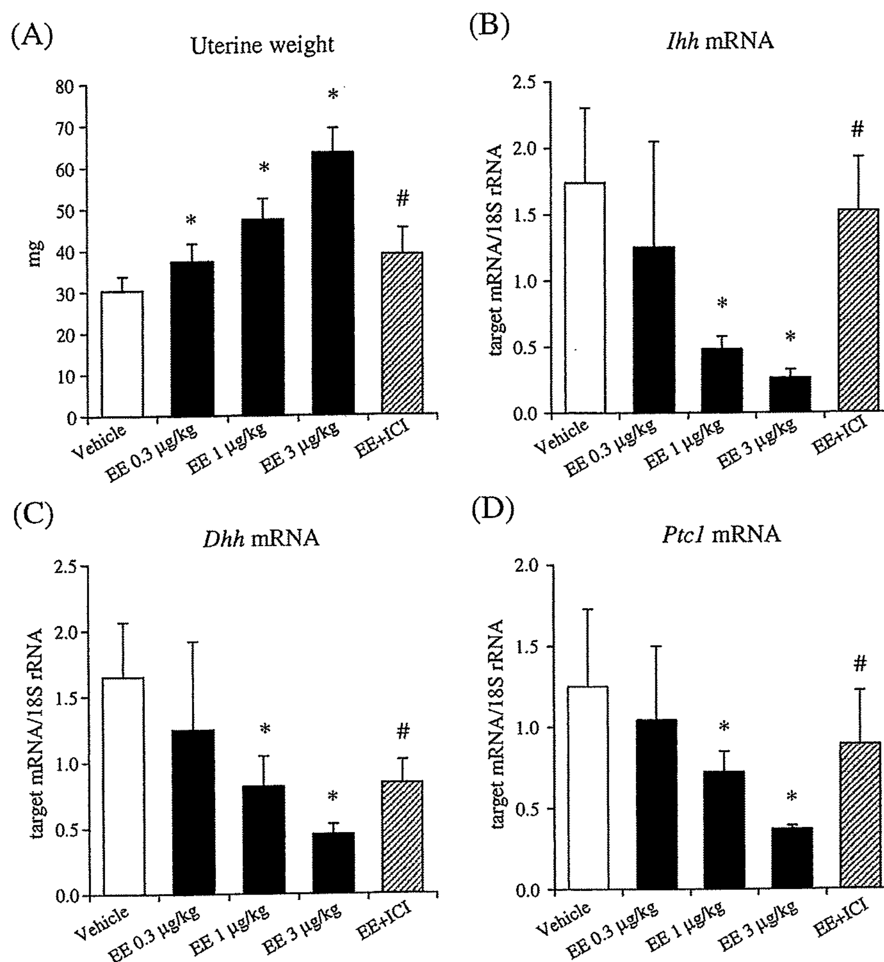

Fig. 2. Effects of EE and ICI on the uterine weights (A), the expression of Hedgehog genes (B, *Ihh*; C, *Dhh*), and Hedgehog target gene (D, *Ptc1*) of immature female rats. Each value represents the mean±S.D. of five animals per group. Vehicle: corn oil containing 1% ethanol, EE: 17α-ethynyl estradiol (an ER agonist), ICI: ICI 182,780 (an ER antagonist), EE+ICI: 3 μg EE/kg+1 mg ICI/kg. *: Significantly different from the vehicle group ($p<0.05$). #: Significantly different from the 3 μg EE/kg group ($p<0.05$).

with those in the time-matched vehicle groups (Fig. 3D). The decrease in the mRNA expression of *Ptc1* in the EE group (3 μg/kg) reached maximum at 24 h (0.31-fold) after administration.

The mRNA expression of *Gli1* transiently and significantly increased 3 h (3.72-fold) after treatment with EE as compared with that in the time-matched vehicle group (Fig. 3E). However, thereafter, the mRNA expression of *Gli1* significantly decreased more than those of the time-matched vehicle groups between 12 h (0.29-fold) and 48 h (0.23-fold) after administration (Fig. 3E).

The mRNA expression of *Coup-TfII* significantly decreased from 6 h after treatment with EE as compared with that in the time-matched vehicle group, and continued to decrease until 48 h after administration (Fig. 3F). Significant differences were noted between 6 h and 24 h (the lowest: 0.37-fold at 12 h) after administration.

Experiment 3

To determine whether a particular ER subtype is involved in the altered expression of Hedgehog genes (*Ihh*, *Dhh*) and Hedgehog target genes (*Ptc1*, *Gli1*, *Coup-TfII*) in the uterus exposed to ER agonists, immature female rats were treated once with the vehicle, PPT (10 mg/kg, ERα-selective agonist) or DPN (10 mg/kg, ERβ-selective agonist).

The blotted uterine weights significantly increased after treatment with either PPT or DPN as compared with the vehicle group (Fig. 4A). These results suggest that the increase of uterine weight by estrogenic compounds can occur through either ERα- or ERβ-dependent mechanisms. However, the magnitude of response caused by PPT (2.43-fold) was larger than that caused by DPN (1.32-fold).

The mRNA expression of *Ihh* and *Dhh* significantly decreased after treatment with either PPT or DPN as compared with the vehicle group (Figs. 4B, C). These results suggest that the downregulation of the mRNA expression of *Ihh* and *Dhh* by estrogenic compounds can occur through either ERα- or ERβ-dependent mechanism. However, the magnitude of response caused by PPT (*Ihh*: 0.08-fold; *Dhh*: 0.44-fold) was larger than those caused by DPN (*Ihh*: 0.60-fold; *Dhh*: 0.77-fold).

The mRNA expression of *Ptc1*, *Gli1*, and *Coup-TfII* significantly decreased after treatment with PPT (*Ptc1*: 0.26-fold; *Gli1*: 0.29-fold;

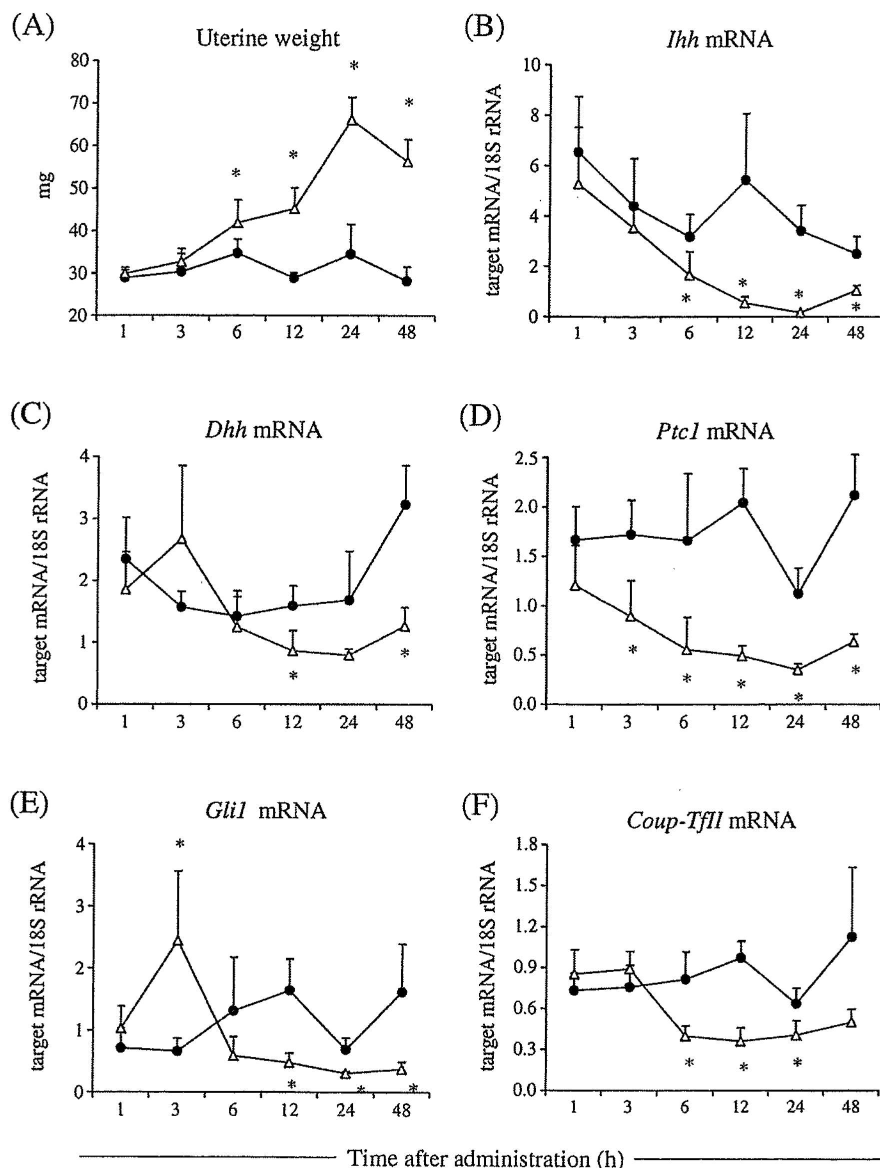

Fig. 3. Time-course changes in the uterine weights (A), the expression of Hedgehog genes (B, *Ihh*; C, *Dhh*), and Hedgehog target genes (D, *Ptc1*; E, *Gli1*; F, *Coup-TfII*) in the uterus of immature female rats exposed to EE. Each value represents the mean±S.D. of five animals per group. Vehicle: corn oil containing 1% ethanol, EE: 17α-ethynyl estradiol (an ER agonist). Closed circles indicate groups treated with the vehicle. Open triangles indicate groups treated with 3 μg EE/kg. *: Significantly different from the time-matched vehicle group ($p<0.05$).

Coup-TfII: 0.64-fold), but not with DPN, as compared with the vehicle group (Figs. 4D, E, F). These results suggest that the downregulation of the mRNA expression of *Ptc1*, *Gli1*, and *Coup-TfII* by estrogenic compounds can occur predominantly through the ERα-dependent mechanism.

Discussion

To understand the molecular events associated with the estrogenic activity of different chemicals, much information on gene expression changes after treatment with estrogenic compounds was reported in the rat uterus using microarray technology (Naciff et al., 2002; Naciff et al., 2003; Naciff et al., 2004). However, only limited information was available on whether estrogenic compounds influence the expression of Hedgehog-related genes. In this study, we demonstrated that estrogenic compounds affected the mRNA expression of Hedgehog genes and Hedgehog target genes in the uterus of immature rats.

In previous research, the *Ihh* gene was reported as a progesterone-upregulated gene (Matsumoto et al., 2002; Takamoto et al., 2002). Takamoto et al. (2002) demonstrated that the mRNA level of *Ihh* was induced within 3 h after a single injection of progesterone to ovariectomized mice, and that the induced *Ihh* mRNA and protein were localized to the luminal and glandular epithelial compartment of the endometrium. Matsumoto et al. (2002) used *in situ* hybridization to determine whether progesterone and estrogen differentially regulated the expression of *Ihh*. In ovariectomized mice, *Ihh* mRNA was expressed in uterine epithelial cells within 6 h after progesterone injection, and persisted until 24 h. On the other hand, estrogen was not effective

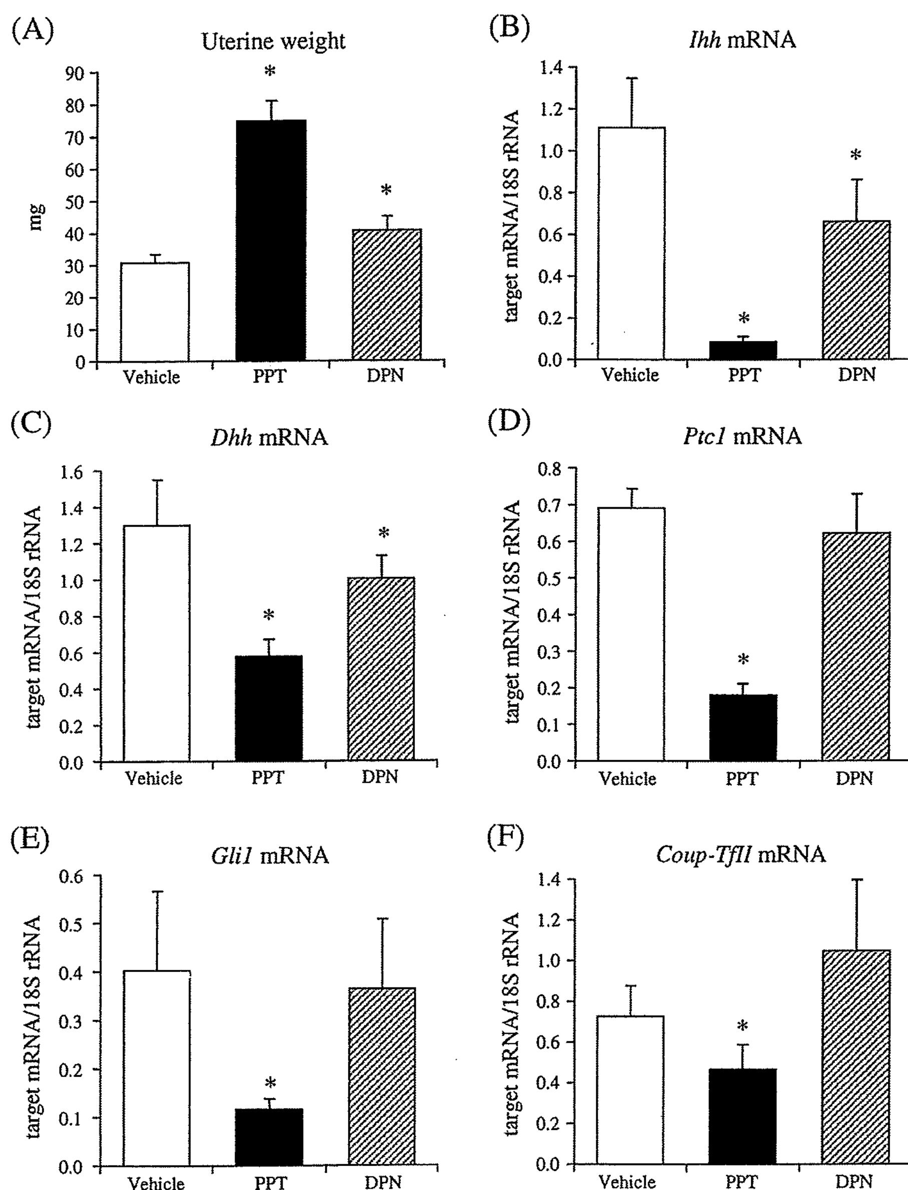

Fig. 4. Effects of PPT (10 mg/kg) and DPN (10 mg/kg) on the uterine weights (A), the expression of Hedgehog genes (B, *Ihh*; C, *Dhh*), and Hedgehog target genes (D, *Ptc1*; E, *Gli1*; F, *Coup-TfII*) in the uterus of immature female rats. Each value represents the mean±S.D. of five animals per group. Vehicle: corn oil containing 1% ethanol, PPT: propyl pyrazole triole (an ERα-selective agonist), DPN: diarylpropionitrile (an ERβ-selective agonist). *: Significantly different from the vehicle group ($p<0.05$).

in inducing this gene, and was not synergistic with it, but rather inhibited the progesterone-upregulated *Ihh* expression when the two hormones were given at the same time (Matsumoto et al., 2002). Recent work suggested that IHH signaling might play a role in preparation of the uterus for implantation during the peri-implantation period (Takamoto et al., 2002).

In this study, we demonstrated that the mRNA expression of *Ihh* and *Dhh* was dose-dependently downregulated by EE in the uterus of immature rats, and that these reactions were mediated by ER as confirmed by coadministration with ICI. PPT downregulated *Ihh* and *Dhh* mRNAs, indicating that uterine *Ihh* and *Dhh* mRNAs were tightly regulated by the ERα-dependent pathway. In addition, DPN also downregulated *Ihh* and *Dhh* mRNAs although these effects were weaker than those of PPT, indicating that the regulation of uterine *Ihh* and *Dhh* mRNAs were also affected by the ERβ-dependent pathway. In previous research, it was unclear whether the mRNA expression of *Ihh* and *Dhh* in the uterus was regulated by estrogenic compounds. However, the downregulation of *Ihh* and *Dhh* mRNAs in response to estrogenic stimuli was considered to be an important clue for understanding the uterine Hedgehog signaling pathway affected by ER agonists. To address this issue, we analyzed the promoter sequence using the 5′-flanking region of the rat *Ihh* and *Dhh* gene obtained from NCBI Rat Genome Resources (http://www.ncbi.nih.gov/genome/guide/rat/). These results indicated that the rat *Ihh* and *Dhh* promoter regions contained half-estrogen response element, half-cAMP response element, and GC box (data not shown). These ER-interacting

transcription factor-binding sequences are well known to involve the activation of gene transcription. However, in this study, the expression of *Ihh* and *Dhh* mRNAs in the uterus was downregulated by the ER agonist, especially the ERα-selective agonist. The GC box is the core sequence of binding site for specificity protein 1 (Sp1) and Kruppel-like factors (KLFs) family. Sp1 is the transcriptional activator that binds to GC-rich promoter elements. On the other hand, although KLFs bind to the promoter elements similar to Sp1, this family of proteins regulates the expression of various target genes by functioning as an activator of transcription, a repressor, or both (Cook et al., 1999; Lomberk and Urrutia, 2005). For example, it has been reported that KLF10 (also known Transforming growth factor-β Inducible Early Gene 1), a transcriptional repressor that binds to the GC box, was rapidly induced after treatment with 17β-estradiol in ER-positive human fetal osteoblastic cells (Tau et al., 1998). Therefore, we assumed that the ER signal activates the expression of some kinds of inhibitory transcription factor as the mechanism to downregulate the mRNA expression of *Ihh* and *Dhh* by the ERα-selective agonist (Zhang and Dufau, 2004; Lomberk and Urrutia, 2005). In this study, although the precise mechanisms of the ERα-selective agonist action on the mRNA expression of *Ihh* and *Dhh* remain to be elucidated, further study is needed to examine whether the transcriptional repressors such as KLFs are involved in the regulation of uterine *Ihh* and *Dhh* gene expression in response to estrogenic stimuli. In our study, the expression level of *ERβ* mRNA was about 100 times lower than that of *ERα* mRNA in the uterus of immature rats (Katayama et al., 2006). Therefore, a predominantly lower expression level of ERβ than ERα was considered as one of the reasons why the ERβ-selective agonist did not downregulate the mRNA expression level of *Ihh* and *Dhh* at the same level as the ERα-selective agonist.

If ER agonists interfere with Hedgehog signaling pathway through the alteration of the expression of Hedgehog ligands, then the mRNA expression of downstream target genes of Hedgehog should also be altered in the uterus of immature rats. In this study, we selected three Hedgehog target genes as follows: *Ptc1* (Ingham and McMahon, 2001; Yoon et al., 2002), *Gli1* (Lee et al., 1997; Ingham and McMahon, 2001; Dunaeva et al., 2003), and *Coup-TfII* (Krishnan et al., 1997a; Krishnan et al., 1997b). GLI1 (Sasaki et al., 1997; Teh et al., 2002; Yoon et al., 2002) and COUP-TFII (Pereira et al., 1999; Pereira et al., 2000; Zhang and Dufau, 2004; Takamoto et al., 2005) are transcription factors known to be involved in multiple signaling pathways by regulating the expression of various downstream target genes. In this study, the mRNA expression of *Ptc1* and *Coup-TfII* was downregulated on similar time courses to those of *Ihh* mRNA after EE administration. The time course of the mRNA expression of *Gli1* after EE administration was different from those of *Ptc1* and *Coup-TfII*, partially. The early phase change of *Gli1* mRNA was similar to that of *Dhh* mRNA, but not *Ihh* mRNA. DPN, a highly potent ERβ agonist, failed to downregulate the transcription of *Ptc1*, *Gli1*, and *Coup-TfII* mRNAs. However, PPT, an ERα agonist that poorly activates ERβ, downregulated the mRNA expression of *Ptc1*, *Gli1*, and *Coup-TfII*, indicating that these mRNA expressions were predominantly regulated by ERα-dependent pathway.

The Hedgehog signaling pathway is one of the key regulators of diverse cellular events. Therefore, it is necessary to precisely control the expression of Hedgehog gene through the various stages of development from embryonic to adult. Our findings in this study suggest that the expression of Hedgehog genes (*Ihh*, *Dhh*) and Hedgehog target genes (*Ptc1*, *Gli1*, *Coup-TfII*) is affected by estrogenic stimuli in the uterus of immature female rats. The experiment in immature female rats gives useful information to understand whether an ER ligand interferes with the expression of morphogenesis-related gene such as the Hedgehog gene. The obtained finding will provide the clues to predict the action of environmental estrogenic compounds *in vivo*.

Acknowledgments

The authors thank Mr. T. Sato, Mr. T. Shinozuka, and Ms. K. Tsutsumi for their excellent technical assistance, and Dr. G.J. Wishart, University of Abertay Dundee, United Kingdom, for kindly reviewing the manuscript.

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TOXICOLOGICAL SCIENCES **91(2)**, 419–430 (2006)
doi:10.1093/toxsci/kfj167
Advance Access publication March 21, 2006

Differential Expression Patterns of *Wnt* and β-Catenin/TCF Target Genes in the Uterus of Immature Female Rats Exposed to 17α-Ethynyl Estradiol

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Received December 15, 2005; accepted March 13, 2006

To characterize the effects of an estrogen receptor (ER) agonist on the gene expressions in the uterus, immature female rats were administered once orally with 17α-ethynyl estradiol (EE, 3 μg/kg), a potent ER agonist. We focused on four categories of sex steroid hormone receptor genes: well-known estrogen target genes, *Wnt* genes, and β-catenin/T-cell factor (TCF) target genes. *ERα*, *ERβ*, progesterone receptor, and androgen receptor mRNAs were all downregulated at 24 and/or 48 h after EE administration. Complement C3 and insulin-like growth factor 1 mRNAs were markedly induced after EE administration. Although the time courses of *Wnt4*, *Wnt5a*, and *Wnt7a* mRNA status varied until 12 h after EE administration, all of them were simultaneously downregulated at 24 and 48 h. The remarkable downregulation of *Wnt7a* mRNA in response to EE was considered to be important to understand the various uterine phenomena affected by ER agonists. In the β-catenin/TCF target genes, the downregulation of anti-Mullerian hormone type 2 receptor and bone morphogenetic protein 4 mRNA after EE administration appeared to be closely related to the downregulation of *Wnt7a*. The upregulation of cyclin D1 and follistatin mRNA at the early phase after EE administration was considered to have been affected by the upregulation of *Wnt4*. These results indicate that an ER agonist influences not only the mRNA expression of sex steroid hormone receptor genes and well-known estrogen target genes but also *Wnt* genes (*Wnt4*, *Wnt5a*, *Wnt7a*) and β-catenin/TCF target genes in the uterus of immature rats, indicating that their molecules are the potential players affected by estrogenic stimuli.

Key Words: estrogen receptor agonist; uterus; *Wnt* genes; β-catenin/TCF target genes.

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The evidence that several synthetic compounds released into the environment may cause developmental and reproductive abnormalities in wildlife by disrupting normal endocrine functions has increased the concern about potential adverse human health effects from such endocrine disruptors (Colborn *et al.*, 1993; Kavlock *et al.*, 1996). Among the compounds considered as endocrine disruptors, many of them have estrogenic activity (Kanno *et al.*, 2001, 2003; Owens and Ashby, 2002). However, there are still many unresolved aspects about the molecular mechanism in the process to induce abnormal differentiation of the female reproductive tract exposed to estrogenic compounds.

Estrogen is a steroid hormone that plays a pivotal role in the regulation of mammalian reproduction and acts by regulating the transcription of specific genes through the specific nuclear receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (DeMayo *et al.*, 2002). Therefore, changes in the expression of estrogen target genes are considered to be a useful index for evaluating the estrogenic activity of synthetic compounds. However, it is difficult to predict the full range of effects of estrogenic compounds from changes of the expression level of only the well-known estrogen target genes. Therefore, we considered whether there was any possible candidate with a further broad range of effects among the genes responding to estrogenic compounds.

Wnt genes encode a large family of secreted cysteine-rich proteins that play key roles as intercellular signaling molecules in embryonic development (Wodarz and Nusse, 1998). In the Wnt family, Wnt4, Wnt5a, and Wnt7a play important roles in the female reproductive system (Heikkila *et al.*, 2001). Wnt4 expression is crucial for the formation of Mullerian ducts, and thus both male and female *Wnt4*-deficient mice completely lack Mullerian ducts (Vainio *et al.*, 1999). *Wnt5a*-deficient mice die at birth due to a failure to complete anteroposterior body axis development (Yamaguchi *et al.*, 1999). Mericskay *et al.* (2004) demonstrated that Wnt5a is required to appropriately establish the development of the posterior region of the

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female reproductive tract. Furthermore, although the oviduct, uterine, and cervical compartments of the female reproductive tract developed in the absence of *Wnt5a*, the mutant uterus failed to form glands that are essential for adult function (Mericskay *et al.*, 2004). *Wnt7a* is expressed in the luminal epithelial cells of the fetal Mullerian tracts and is maintained at high levels in the adult uterine luminal epithelium (Miller *et al.*, 1998b). *Wnt7a*-deficient mice are viable, but *Wnt7a* mutant males are infertile due to the consequences of ectopic Mullerian ducts. Mutant females are sterile because of the abnormal development of the oviduct and uterus, both of which are Mullerian duct derivatives (Miller and Sassoon, 1998; Parr and McMahon, 1998). The abnormal female reproductive tract caused by the deficiency of *Wnt7a* closely resembles the abnormalities in female humans and mice prenatally exposed to diethylstilbestrol (Miller *et al.*, 1998a).

In the Wnt signaling pathway, at least three kinds of the Wnt/β-catenin pathway, the Wnt/c-Jun N-terminal kinase pathway (Wnt/JNK pathway), and the Wnt/Ca^{2+} pathway are identified (Kuhl *et al.*, 2000; Veeman *et al.*, 2003; Willert and Nusse, 1998). The Wnt/β-catenin pathway regulates the transcription of various target genes via stabilized β-catenin and T-cell factor/lymphoid enhancer factor (TCF/LEF) family members. If a certain estrogenic compound influences the expressions of *Wnt* genes and/or β-catenin/TCF target genes, then the compound may cause altered development of the female reproductive tract. However, only limited information is available whether estrogenic compounds influence the regulation of such gene expression.

The objective of this study was to investigate the effects of ER agonist on the expression of *Wnt* genes and β-catenin/TCF target genes. In this study, we used 17α-ethynyl estradiol (EE) as an ER agonist. EE is the estrogenic chemical used medically in oral contraceptive. It is estimated that each year about 3% of women in the United States and Europe who use oral contraceptives become pregnant accidentally, primarily because of missed pills (Thayer *et al.*, 2001; Timms *et al.*, 2005). Oral contraceptive pills may be taken for many months until the unplanned and unexpected pregnancy is discovered (Li *et al.*, 1995; Timms *et al.*, 2005). Therefore, there is concern about the effect of EE on the reproductive tract including uterus because it has the possibility of becoming an unexpected source of exposure of human fetuses to estrogenic compounds. We report here that EE influences the mRNA expression of *Wnt* genes (*Wnt4*, *Wnt5a*, *Wnt7a*) and β-catenin/TCF target genes in the uterus of immature rat.

MATERIALS AND METHODS

Chemicals. EE (purity: 99%) and corn oil were obtained from Sigma-Aldrich Co. (St. Louis, MO). Ethanol was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Corn oil containing 1% ethanol was prepared as the vehicle solution.

Animals. Thirteen-day-old, female Crj:CD(SD) IGS rats were obtained from Charles River Japan, Inc. (Kanagawa, Japan) with lactating maternal animals. After arrival, immature and maternal animals were acclimated for 5 days. Weaning and group assignment were performed on the day before administration (at 18 days old) to minimize the stress associated with weaning. Sixty animals were assigned to 12 experimental groups to give similar intergroup variations in body weight among the resulting groups. Animals were housed in polycarbonate cages (380 × 330 × 175 mm [width × depth × height], CLEA Japan, Inc., Tokyo, Japan). During the quarantine period, animals were accommodated in cages in groups of 10 immature animals and one maternal animal per cage. After group assignment, immature animals were accommodated at five animals per cage. The animal room was maintained at a temperature of 19.0–25.0°C, with a relative humidity of 35.0–75.0%, and at a 12 h light/dark cycle. The animals were allowed free access to a pellet diet for experimental animals (MF, Oriental Yeast, Co., Ltd., Tokyo, Japan) and sterilized water. The animals were cared for in accordance with "The Guidelines for Animal Experimentation" for our laboratory, Mitsubishi Chemical Safety Institute Ltd.

Study design. We used sexually immature female rats in which significant ovarian estrogen synthesis and regulation by the hypothalamic-pituitary-gonadal axis had not been initiated. Rats were used for the experiment at 19 days of age. Animals were administered once by oral gavage with either the vehicle or 3 μg/kg EE. The dose of EE was selected at a dose known to cause hypertrophy of the uterus in a 3-day uterotrophic assay (Kanno *et al.*, 2001). Initial body weight before the administration and final body weight before the necropsy were measured using an electronic balance (PM3000, Mettler Toledo K.K., Tokyo, Japan). The administration volume was 10 ml/kg and was adjusted individually based on the initial body weight before administration. Five animals were included in each treatment group. Animals were euthanized by CO_2 asphyxiation at 1, 3, 6, 12, 24, and 48 h after administration. The uterus was removed from the body, placed on gauze, and cut at several sites to discharge gently the fluid in the uterus. The absolute "blotted uterine weight" (weight of the uterus excluded the inner fluid) was measured using an electronic balance (Model AE260, Mettler Toledo K.K.). In addition, the relative weight of the uterus from each animal was calculated by dividing the absolute blotted uterine weight by final body weight before the necropsy. The uterus was submerged in the RNA preservative reagent RNAlater (Ambion, Inc., Austin, TX), kept at 4°C overnight, and then was stored at −20°C until processing for RNA isolation.

Isolation of total RNA. The uterus was homogenized in the dissolving and absorption liquid containing 1% 2-mercaptoethanol (TOYOBO Co., Ltd., Osaka, Japan) for 300 s at −20°C using an automatic sample preparation system (Twist Crusher HMX-2000, TOYOBO Co., Ltd.). Total RNA was isolated using MagExtractor-RNA- (TOYOBO Co., Ltd.) and an automatic nucleic acid extraction system (MagExtractor System MFX-2000, TOYOBO Co., Ltd.) according to the manufacturer's recommended protocol and was subsequently DNase treated with RNase-free DNase I (TAKARA BIO INC., Shiga, Japan) for 30 min at 37°C in the presence of RNase OUT (Invitrogen Corporation, Carlsbad, CA). The amount of total RNA was determined using the RiboGreen RNA Quantitation kit (Molecular Probes, Inc., Eugene, OR) or spectrophotometer (DU-7400, Beckman Coulter, Inc., Fullerton, CA). The absence of genomic DNA contamination in the total RNA samples was confirmed by real-time polymerase chain reaction (PCR) for each RNA sample without reverse transcriptase using TaqMan Rodent GAPDH Control Reagents VIC (Applied Biosystems, Foster City, CA), according to the manufacturer's recommended protocol.

Real-time reverse transcription–PCR. One-step real-time reverse transcription (RT)–PCR was performed to determine changes in gene expression using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Total RNA (0.5–20 ng) isolated from each uterus was added to a reaction mixture containing forward primer, reverse primer, TaqMan probe, and TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems) in a final volume of 50 μl according to the manufacturer's instruction. Rat-specific

TABLE 1
Sequences of Primer and Probe Sets Used for Real-Time RT-PCR Analysis

Gene		Sequence (5′–3′)	Accession number	Amplicon size (bp)
ERα	Forward primer	ccaccgagtcctggacaaga	X61098	69
	Reverse primer	tgcagagtcaggccagctt		
	Probe	cacagacactttgatccacttgatggcc		
ERβ	Forward primer	gggtgattgcgaagagtggta	NM_012754	91
	Reverse primer	ctgatgtgcctgacgtgagaa		
	Probe	cgactggccaacctcctgatgcttc		
PR	Forward primer	caacacaaagcccgacacttc	NM_022847	108
	Reverse primer	cggaaacctggcagagactta		
	Probe	ttgctgaccagtctcaaccaactaggcg		
AR	Forward primer	atgaccagatggcagtcattcag	NM_012502	141
	Reverse primer	acttgtgcatgcgatactcattg		
	Probe	aggatgctctactttgcacctgacctggt		
Complement C3	Forward primer	agttctttaacgtgggacttatcca	NM_016994	138
	Reverse primer	acatttcattgtggcacagctt		
	Probe	atgcacccggttctatcatccggaga		
Igf1	Forward primer	gtgatctgaggaggctggagat	X06043	71
	Reverse primer	ggcccggatggaacga		
	Probe	actgtgctccgctgaagcctacaaagtc		
Wnt4	Forward primer	gaaccggcgctggaact	NM_053402	70
	Reverse primer	ggtcccttgtgtcaccacctt		
	Probe	ttccacactggactccctgcctgtc		
Wnt5a	Forward primer	gcgtggctatgaccagtttaaga	NM_022631	75
	Reverse primer	ttgacatagcagcaccagtgaaa		
	Probe	cagaccgaacgctgccattgcaa		
Wnt7a	Forward primer	gcccacctttctgaagatcaag	XM_342723	103
	Reverse primer	tgggtcctcttcacagtaattgg		
	Probe	ccgtaagcccatggacactgatctgg		
Amhr2	Forward primer	ggctctactactgtgggagatcct	NM_030998	90
	Reverse primer	tctgcctcataagccaattgaa		
	Probe	tgttccgatttgaggcctgaccaca		
Bmp4	Forward primer	tggacacctcatcacacgactac	NM_012827	111
	Reverse primer	tgcttttcccgggtccat		
	Probe	ttcgatgtgagccctgcagtccttc		
Fgf9[a]	Forward primer	acccaggtggtcacttagcaa	NM_012952	114
	Reverse primer	atggctcccttaggtgaagttg		
	Probe	cggtaccgcgtcctgcacacc		
Cyclin D1	Forward primer	cctctcctgctaccgcacaa	NM_171992	111
	Reverse primer	cgcaggcttgactccagaag		
	Probe	cggactgcctccgtgcctgc		
Follistatin	Forward primer	cggcgtactgcttgaagtga	NM_012561	100
	Reverse primer	agggaagctgtagtcctggtctt		
	Probe	tccggatcttgcaactccatctcgg		
Fibronectin	Forward primer	tgcgctccattccaccttat	NM_019143	110
	Reverse primer	tccctggcttggtcgtacac		
	Probe	cgctccaaggattggcttcaagctg		
Mmp7	Forward primer	ctctaggccatgcctttgca	NM_012864	140
	Reverse primer	acccagagagtggccaagttc		
	Probe	tcaggagtgaacttcctgtttgttgcca		

[a]Sequences of primer and probe sets for *Fgf9* were designed by the antisense strand.

primers and TaqMan probes were designed for the genes of interest (Table 1) using Primer Express software (Applied Biosystems). The mRNA expression of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was determined using primer and probe sets in the TaqMan Rodent GAPDH Control Reagents VIC (Applied Biosystems). TaqMan probes were labeled with a fluorescent reporter dye (FAM or VIC) at the 5′ end and a quencher dye (TAMRA) at the 3′ end. Thermal cycling conditions were as follows: 1 cycle of 30 min at 48°C for reverse transcription, 1 cycle of 10 min at 95°C for activation of DNA polymerase, 40 cycles of 15 s at 95°C for denaturation, and 1 min at 60°C for annealing/extension. The expression levels of target gene and 18S rRNA in each sample were calculated based on the standard curve generated with the rat total RNA for the uterus (UNITECH Co., Ltd., Chiba, Japan) or ovary (Ambion, Inc.). The expression level of target gene was then normalized by the expression level of 18S rRNA using TaqMan Ribosomal RNA Control

Reagents (Applied Biosystems) to control the quantity of the isolated RNA. Real-time RT-PCR analyses were performed in duplicate on all five animals in each treatment group.

Statistical analysis. The data of each group were expressed as the means ± SDs. Fold changes for uterine weights and gene expression data were expressed as the ratio of the mean value of the group treated with EE (3 μg/kg) to the mean value of the time-matched vehicle group. Differences of body weights, uterine weights, and gene expression data in the EE treatment group from those in the time-matched vehicle group were analyzed for statistical significance. F-test was applied to analyze the homogeneity of the variance. When the variance was homogeneous, Student's t-test was performed. When the variance was not homogeneous, Aspin-Welch' t-test was performed. The statistical analysis was performed with SAS Proprietary Software Release 8.2 (SAS Institute, Inc., Cary, NC). Significance was determined by a two-tailed significance level of 5%.

RESULTS

Effects of EE on the Body Weight in Immature Female Rats

No statistically significant differences were noted in body weights between any of the 3-μg/kg EE groups and the time-matched vehicle groups (Table 2). Since there were no abnormal clinical signs observed in any groups, 3 μg/kg of EE was considered not to produce serious toxicity in the animals.

Effects of EE on the Uterine Weight in Immature Female Rats

Uterine weights significantly increased between 6 and 48 h after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups (Fig. 1, Table 3). An increase of the relative uterine weight in the 3-μg/kg EE group reached a peak at 24 h (1.99-fold) after administration.

Effects of EE on the Expression of Reference Genes in the Uterus of Immature Female Rats

Reference genes, which are often referred to as housekeeping genes, are frequently used to normalize mRNA levels between different samples. However, the expression level of these genes may vary in different tissues, different cell types, and different disease stages, so the selection of the reference genes is critical for the interpretation of the expression data. Therefore, to determine a stable endogenous reference gene in the uterus after treatment with ER agonist, time-course changes were measured in the expressions of commonly used reference genes, *Gapdh* mRNA and 18S rRNA. Firstly, the expression of *Gapdh* mRNA and 18S rRNA were normalized with an amount of total RNA. The expression of *Gapdh* mRNA normalized with total RNA significantly increased between 3 and 12 h after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups (Fig. 2B). However, no statistically significant differences were noted in the expression of 18S rRNA normalized with total RNA between any of the 3-μg/kg EE groups and the time-matched vehicle groups (Fig. 2A). Therefore, 18S rRNA was judged to be suitable as a stable endogenous reference gene

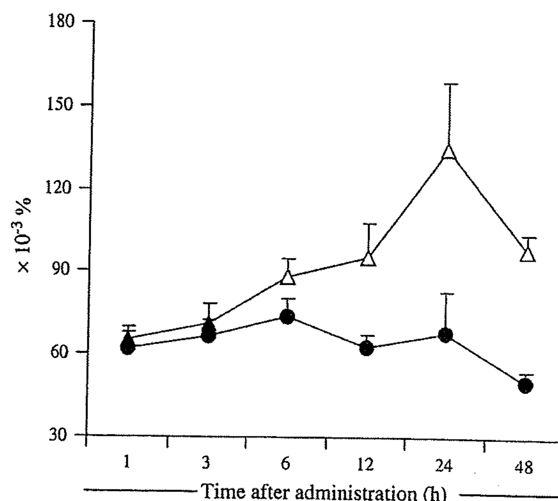

FIG. 1. Effects of EE on the uterine weight in immature female rats. Wet weight of uterus was normalized to final body weight (absolute blotted uterine weight/final body weight × 10^{-3}%). Each value represents the mean ± SD of five animals per group. Closed circles indicate groups treated with the vehicle. Triangles indicate groups treated with EE at 3 μg/kg. Open triangles indicate a statistical significance from the time-matched vehicle group ($p < 0.05$).

TABLE 2
Effects of EE on the Body Weight in Immature Female Rats

	Initial body weight before the administration[a]		Final body weight before the necropsy[b]	
Group	Vehicle	EE (3 μg/kg)	Vehicle	EE (3 μg/kg)
1 h[c]	43.9 ± 3.1	44.4 ± 2.1	46.9 ± 3.5	46.1 ± 3.3
3 h	43.9 ± 2.8	44.4 ± 2.1	45.4 ± 3.0	46.1 ± 3.0
6 h	44.1 ± 2.3	44.4 ± 2.2	47.0 ± 2.8	47.3 ± 3.5
12 h	44.3 ± 2.1	44.4 ± 2.3	46.1 ± 2.3	47.5 ± 2.2
24 h	44.4 ± 2.0	44.3 ± 2.3	51.1 ± 3.5	49.8 ± 4.7
48 h	44.4 ± 2.0	44.4 ± 2.2	56.0 ± 4.9	57.5 ± 3.1

Note. Each value represents the mean ± SD of five animals per group. No values were significantly different from those in the time-matched vehicle group.
[a]19 days old (referring to the date of their birth as day 0).
[b]19 days old (1-, 3-, 6-, and 12-h groups), 20 days old (24-h group), and 21 days old (48-h group).
[c]Time after administration.

TABLE 3

Time-Course Changes in Organ Weight and Gene Expression in the Uterus of Immature Female Rats Treated with EE

	Time after administration (h)											
Endpoint	1		3		6		12		24		48	
Uterine weight	1.05		1.07		1.19	+	1.52	+	1.99	+	1.95	+
Sex steroid hormone receptor gene												
ERα	1.24		1.23		0.99		0.80		0.63	–	0.57	–
ERβ	2.01	+	1.75		0.25	–	0.69		0.61	–	0.46	
PR	1.27		1.27		0.71		0.60	–	0.40	–	0.51	–
AR	2.16		1.93	+	1.01		0.92		0.96		0.50	–
Well-known estrogen target gene												
Complement C3	0.86		1.73		7.17	+	16.80	+	43.90	+	77.82	+
Igf1	1.95		9.73	+	3.68	+	3.90	+	1.77		0.52	–
Wnt gene												
Wnt4	0.83		2.39		1.71	+	1.95	+	0.51	–	0.43	–
Wnt5a	1.06		2.12	+	1.04		1.08		0.45	–	0.39	–
Wnt7a	0.70	–	0.70		0.56	–	0.48	–	0.15	–	0.28	–
β-catenin/TCF target gene												
Amhr2	1.08		0.91		0.14	–	0.23	–	0.18	–	0.25	
Bmp4	1.01		0.90		0.25	–	0.13	–	0.65	–	0.22	–
Fgf9	0.76		0.69		0.90		1.06		0.40	–	0.66	–
Cyclin D1	1.32		1.89	+	1.87	+	1.67	+	0.45	–	0.79	
Follistatin	1.90	+	3.28	+	4.18	+	0.38	–	0.32	–	0.29	–
Fibronectin	2.26	+	3.06	+	0.71		0.97		1.09		0.57	
Mmp7	0.56		8.08		41.93	+	26.43	+	9.26		53.13	+

Note. Each value represents the ratio of the mean value of the group treated with EE (3 μg/kg) to the mean value of the time-matched vehicle group; "+," significantly increase from the time-matched vehicle group ($p < 0.05$); and "–," significantly decrease from the time-matched vehicle group ($p < 0.05$).

to normalize the target mRNA expression in the uterus after treatment with ER agonist.

The expression of *Gapdh* mRNA normalized with 18S rRNA significantly increased between 3 and 24 h after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups (Fig. 2C). An increase of the *Gapdh* mRNA in the 3-μg/kg EE group reached a peak at 12 h (9.64-fold) after administration.

Effects of EE on the Expression of Sex Steroid Hormone Receptor Genes in the Uterus of Immature Female Rats

ERα, *ERβ*, progesterone receptor (*PR*), and androgen receptor (*AR*) mRNAs were selected to evaluate the effect of EE on the expression of sex steroid hormone receptor genes (Diel *et al.*, 2000; Waters *et al.*, 2001).

The expression level of *ERα* mRNA was about 100 times higher than that of *ERβ* mRNA in the uterus of immature rats. The expression of *ERα* mRNA significantly decreased from 24 h after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups and kept decreasing until 48 h (0.57-fold) (Fig. 3A, Table 3).

The expression of *ERβ* mRNA significantly increased at 1 h (2.01-fold) after treatment with 3 μg/kg EE, but thereafter it decreased more than those of the time-matched vehicle groups (the lowest: 0.25-fold at 6 h) (Fig. 3B, Table 3).

The expression of *PR* mRNA significantly decreased from 12 h after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups and kept decreasing until 48 h (the lowest: 0.40-fold at 24 h) (Fig. 3C, Table 3).

The expression of *AR* mRNA significantly increased at 3 h (1.93-fold) after treatment with 3 μg/kg EE as compared with that in the time-matched vehicle group but significantly decreased more than that of the time-matched vehicle group at 48 h (0.50-fold) (Fig. 3D, Table 3).

Since ERα, ERβ, PR, and AR are transcription factors to exert physiological functions specific to each ligand (Mangelsdorf *et al.*, 1995; Tasset *et al.*, 1990), it is suggested that estrogenic compounds may affect the expressions of their downstream target genes.

Effects of EE on the Expression of Well-Known Estrogen Target Genes in the Uterus of Immature Female Rats

Complement C3 (Diel *et al.*, 2000; Sundstrom *et al.*, 1989) and insulin-like growth factor 1 (*Igf1*: Klotz *et al.*, 2000) mRNAs were selected to evaluate the effect of EE on the expression of well-known estrogen target genes.

The expression of complement C3 mRNA significantly increased between 6 and 48 h after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups