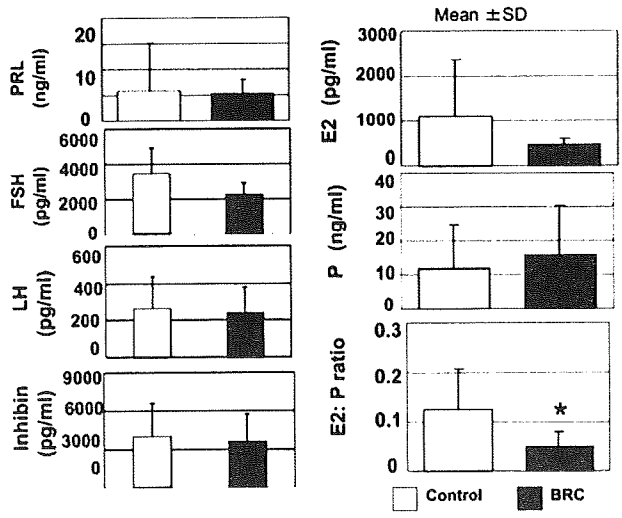

Fig. 3. Serum hormone profiles at 15 months of age, the end of the study. White column (Control): the control group. Black column (BRC): 1 mg/kg BRC-treated group. The E2: P ratio was calculated as E2 (pg/ml) divided by P (ng/ml). Although the FSH, LH, inhibin and progesterone levels were not different between the control and BRC-treated groups, the E2: P ratio was significantly lower in the BRC-treated group. The E2 levels of the rats in the BRC-treated group tended to increase, but there was not significant difference. *P<0.05.

in the present study. The precise mechanism, however, was not determined in the present study. The Donryu strain rat used here is a useful animal model for endometrial adenocarcinoma in the uterine corpus, particularly the endometrioid type [4]. This strain of rat is not only a high yield strain, but also has the following 3 similarities to humans [1, 4]: 1) multi-step development from atypical hyperplasia of the glandular epithelium to adenocarcinoma; 2) change in morphological and gene expression profiles; and 3) consistent elevation of the serum E2: P ratio manifested by early occurrence of atrophic ovaries with cystic atretic follicles and lack of a corpus luteum, resulting in persistent estrus (PE) on vaginal cytology [2, 3]. These features indicated that ovarian hormonal imbalance is crucial for uterine carcinogenesis in rats as well as humans [2–4]. Many previous studies have provided evidence that delayed onset of PE and/or depression of the serum E2 level can prevent adenocarcinoma development in rats [31–33]. The present study supports our hypothesis that an increased E:P ratio is very important for promoting effects on uterine carcinogenesis. There are a number of studies showing that PRL, E2 and/or their receptors control each other through endocrine and autocrine/paracrine mechanisms [34–36]. While the BRC treatment did not affect estrous cyclicity in the present study, the decrease in the serum E2: P ratio and tendency of the serum E2 levels to decrease might be related to the inhibitory effect on uterine cancer development. Subcutaneous treatment with 1 mg/kg body weight BRC did not modulate regular estrous cyclicity in the present study. The sensitivity of the response to BRC treatment in the aged ovary under PE conditions might be different from that in adults with normal estrous cyclicity.

BRC might act directly on the uterus in related to uterine carcinogenesis, because PRLR-L is predominantly located in the rat uterus [19]. The effects of PRL and BRC on the uterine proliferating lesions in the uteri of mice are controversial [23]. Mori *et al.* reported that an increase in the plasma level of PRL induces adenomyosis in mice through increased expression of PRL receptor mRNA in the uterus [20–22]. On the other hand, BRC-treatment for 30 days induces proliferation of endometrial epithelial cells in mice [37]. The present study did not provide any clear evidence showing long-term BRC treatment has any direct action on uterine carcinogenesis in rats.

When rodent model data are extrapolated for human carcinogenicity predictions, it is very important to pay attention to the differences in modes of action between rodents and humans. The functional effect of PRL on the rat ovary is quite different from that in humans. Whereas PRL directly leads to atrophic corpora lutea or luteolysis in rodents, this does not occur in women [38, 39]. Although BRC has been used clinically for therapy in patients with prolactinomas for long-term, there is little information available showing thatt therapeutic BRC affects ovarian and uterine function, and this suggests that the influence observed in the present study may be restricted to the rat. However, there remain unclear points in regard to the effects of BRC on uterine carcinogenesis in the rat model. Therefore, further investigation is necessary to clarify the differences between the data collected from animals and women.

In conclusion, the present results indicate that long-term BRC treatment inhibits uterine cancer development. The major pathway to the inhibitory effect could not be determined; however, there is a very plausible link to ovarian hormonal alterations resulting in a decrease in the serum E: P ratio.

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Letter

Sexing of postimplantation rat embryos in stored two-dimensional electrophoresis (2-DE) samples by polymerase chain reaction (PCR) of an *Sry* sequence

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ABSTRACT — Proteomic analysis of developmental toxicity by two-dimensional electrophoresis (2-DE) may detect gender-related toxic effects in embryos without visible gender characteristics. In the present study, we explored sexing of rat embryo stored in frozen 2-DE samples by polymerase chain reaction (PCR) of a male-specific gene sequence, sex determining region Y (*Sry*). The embryo proper and yolk sac membrane at gestation day 11 from Wistar rats were used for stored embryonic 2-DE samples. The embryonic 2-DE samples were desalted and their total DNA was extracted. The *Sry* sequence in the extracted DNA was amplified by PCR and the product was analyzed by agarose gel electrophoresis. The embryos with the PCR product of *Sry* were determined as male, and those without the product were determined as female. It was concluded that stored embryonic 2-DE samples could be used for retrospective examination of gender-related effects in proteomic analysis of developmental toxicity.

Key words: Developmental toxicity, Embryo, PCR, Sexing, *Sry*, 2-DE

INTRODUCTION

It has been shown that some chemicals have gender differences in their developmental toxicities when examined in rat fetuses or neonates. Prenatal exposure to procarbazine on day 14 of gestation induced cleft palate and microgenia more frequently in males than in females of rat fetuses (Malek *et al.*, 2003). It has been suggested that perinatal exposure to polychlorinated biphenyls affects behavior and cerebellar development of males more severely than those of females in rat neonates (Nguon *et al.*, 2005). However, developmental toxicity studies of chemicals with early postimplantation rat embryos both *in vivo* and *in vitro* have often been performed irrespective to their gender. This is because there are no visible gender characteristics yet, although sex determination and gonadal differentiation begin around this developmental stage in rodent embryos (Hunter, 1995).

Recent advances in analytical techniques revealed gender differences related to birth defects in early postimplantation rodent embryos. For example, neural tube defects, a major congenital malformation, are more frequent in female embryos than in male ones as early as day 10.5 of gestation in p53-null mice (Chen *et al.*, 2008). It is therefore likely that comprehensive biochemical and molecular analyses, such as proteomics by two-dimensional electrophoresis (2-DE), of early postimplantation rat embryos (Usami *et al.*, 2007b), might detect gender-related toxic effects when the data is analyzed according to the embryonic gender.

The gender of rat embryos can be determined by polymerase chain reaction (PCR) of a sequence in *Sry*, a male-specific gene on the Y chromosome (Poletti *et al.*, 1998), in DNA samples from the embryonic tissues, i.e., the embryo proper or yolk sac membrane. There are, however, no reported methods applicable to comprehensive proteomic analyses of early postimplantation rat embryos, where both embryo proper and yolk sac membrane are

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used for 2-DE in order to examine tissue-specific responses (Usami *et al.*, 2007a, 2008).

In the present study, we attempted sexing of postimplantation rat embryos stored in frozen 2-DE samples by PCR of an *Sry* sequence. Stored embryonic 2-DE samples were desalted with spin columns, and their DNA was extracted with an extraction kit. The extracted DNA was used for PCR. A sequence of beta actin gene (*Actb*) was also amplified simultaneously as a control. The embryos with the PCR product of *Sry* were determined as male, and those without the product were determined as female.

MATERIALS AND METHODS

Collection of tissue samples

Wistar rats (Crlj: WI, Charles River Laboratories Japan, Kanagawa, Japan) were used. Day 11 (plug day = day 0) *in vivo* or equivalent *in vitro* embryos cultured for 24 or 48 hr (Usami and Ohno, 1996) were collected in Hanks' balanced salt solution. The embryo proper and yolk sac membrane from the embryos were washed three times with ice-cold buffer (10 mM Tris-HCl, pH 7.0, 150 mM NaCl), and stored at −80°C in 1.5 ml Eppendorf tubes individually with a minimum amount of the buffer. Kidneys were removed from adult male and female rats for the preparation of male and female DNAs working as references. All the animal experiments were performed according to the guideline for animal experiments in National Institute of Health Sciences.

Desalting of embryonic 2-DE samples

Embryonic 2-DE samples prepared as follows and stored after the analyses at −30°C were used. The embryo proper and yolk sac membrane of the day 11 embryos were lysed in 300 μl/embryo of rehydration buffer, i.e., 2-DE lysis buffer, by pulsed sonication immediately after the addition of the buffer (Usami *et al.*, 2007b). The 2-DE samples (20-50 μl) were applied to spin columns (Micro Bio-Spin P-30 Tris, Bio-Rad, Hercules, CA, USA) and centrifuged at 1,000 x g for 4 min at 20°C. The filtrates obtained were directly used for extraction of DNA.

Extraction of DNA

Total DNA was extracted from sample tissues with an extraction kit (DNeasy Blood & Tissue Kits, QIAGEN, Valencia, CA, USA). Lysis buffer (180 μl) of the extraction kit was added into 1.5 ml Eppendorf tubes containing the desalted 2-DE sample (20 μl), entire embryo proper, entire yolk sac membrane or adult kidney (approximately 1 mm^3), and thereafter the samples were processed according to the manufacturer's instruction to obtain 100 or 200 μl of total DNA solutions. The concentration of extracted DNA was determined by absorbance at 260 nm (ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA). For determination of the detection limit of the PCR, extracted DNA was serially diluted with the extraction buffer prior to PCR analysis.

PCR analysis of extracted DNA

An *Sry* sequence was amplified by PCR with a forward primer, 5'- TAC AGC CTG AGG ACA TAT TA -3', and a reverse primer, 5'- GCA CTT TAA CCC TTC GAT GA -3', to yield a 317-bp product (Poletti *et al.*, 1998). As a control, a sequence of an autosomal gene, beta actin gene (*Actb*, Chr. 12p11), was also amplified by PCR with a forward primer, 5'- AGC CAT GTA CGT AGC CAT CC -3', and a reverse primer, 5'- TGT GGT GGT GAA GCT GTA GC -3', the sequences of which were obtained from the NCBI UniSTS database (UniSTS: 270076, PMC102156P1), to yield a 220-bp product. PCR was performed in tubes containing 2 μl of the extracted DNA sample and 23 μl of a master mixture (10 mM Tris-HCl, pH9.0, 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 200 μM dATP, dGTP, dCTP and dTTP, 0.625U Ampli-Taq polymerase (Promega, Madison, WI, USA), and 0.2 μM all of the above primers). PCR conditions were: denaturation at 95°C for 2 min, followed by 35 cycles of amplification at 95°C for 1 min, 52°C for 1 min and 72°C for 1 min, and then extension at 72°C for 5 min with a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA, USA). For analysis of PCR products, the PCR mixture (10 μl) diluted with loading buffer (4 μl) was subjected to electrophoresis in a 3% agarose gel (NuSieve 3:1 Agarose, Biowhittaker Molecular Applications, Rocklane, ME, USA) for 70 min at 50 V. The gel was stained with SYBR Green I (FMC BioProducts, Rockland, ME, USA) for visualization of the products, and was photographed with a CCD camera system (DIANA, Raytest, Straubenhardt, Germany).

RESULTS

Extraction of DNA from the embryonic samples

The size of the embryos and concentrations of their extracted DNA are shown in Table 1. The embryos in the 2-DE samples include those treated with test chemicals in selected *in vitro* toxicity studies and therefore their sizes are smaller than those of usual day 11 embryos. In addition to the effects of test chemicals, varied sample volumes from the 2-DE samples caused large variations of the DNA concentrations. In the intact tissues, variations of the DNA concentrations also appeared to be large due

Table 1. Sizes and extracted DNA concentrations of day 11 rat embryos used for sexing

	2-DE sample	Intact tissue
No. of embryos	23	10
Embryo size		
Crown-rump length (mm)[a]	3.92 ± 0.42	4.32 ± 0.25
Yolk sac diameter (mm)[a]	4.44 ± 0.48	4.89 ± 0.38
Extracted DNA (ng/μl)[a]		
Embryo proper (ng/μl)[a]	36.6 ± 14.3 [5.12-58.9]	80.4 ± 32.5 [32.5-154]
Calculated content[b] (μg)[a]	48.3 ± 23.7 [7.68-88.4]	11.7 ± 5.57 [6.22-20.4]
Yolk sac membrane (ng/μl)[a]	6.29 ± 3.48 [2.28-13.9]	50.4 ± 35.4 [15.0-111]
Calculated content[b] (μg)[a]	8.44 ± 5.79 [2.99-20.8]	6.32 ± 2.97 [3.00-11.1]

[a]: Mean ± S.D. is shown. [b]: DNA content per embryo calculated from the dilution factor.
Values in the brackets indicate ranges.

to the non-quantitative extraction procedure. In spite of their large concentration variations, these extracted DNA samples could be used for the PCR without adjustment of their concentrations. When DNA content per embryo was calculated from the dilution factor, the calculated content in the 2-DE samples and that of the yolk sac membrane in the intact tissues were comparable to the DNA content reported for rat embryos at similar developmental stages (Snell and Mullock, 1987), indicating that DNA was extracted effectively. The lower calculated content for the embryo proper in the intact tissues, on the other hand, is considered due to overload of the samples in the extraction of DNA.

PCR of the *Sry* sequence in the extracted DNA

PCR of the *Sry* sequence yielded a 317-bp product in the adult male kidney, but not in the female one, as a reference and the same product was observed in some embryos, which were determined as male (Fig. 1A). PCR of the *Actb* sequence yield a 220-bp product in all the samples, confirming successful PCR. Therefore, the embryos without the PCR product of *Sry* were determined as female. There were no differences between the products from the 2-DE samples and intact embryonic tissues, indicating validity of the present method in the analysis of 2-DE samples.

Reliability and accuracy of the sexing

Serial dilution of an extracted DNA from a male embryonic 2-DE sample showed that the PCR product of *Sry* could be detected together with that of *Actb* in the range as broad as from 0.5 to 50 ng/μl DNA (Fig. 1B). At 0.05 ng/μl or less DNA, only the PCR product of *Actb* was detected and therefore embryonic gender might be misdetermined as female. However, DNA concentrations in the 2-DE samples from postimplantation rat embryos were sufficient for the detection of the PCR product of *Sry* as shown in Table 1.

As an index of accuracy of the sexing, embryonic genders determined by the embryo proper and yolk sac membrane samples were compared, since genetic genders are the same between them. In all the embryos of which both embryo proper and yolk sac membrane samples were used for sexing, their gender was consistent between the embryo proper and yolk sac membrane (Table 2). It was thus considered that embryonic genders determined by the present method were accurate.

DISCUSSION

The present results indicate that 2-DE samples stored after the analyses can be used for sexing of the rat embryos. This means that it is possible to examine gender-related effects retrospectively in proteomic analyses by 2-DE of postimplantation rat embryos in developmental toxicity studies. This also means that both embryo proper and yolk sac membrane can be used for proteomics by 2-DE when their sexing is necessary. If embryonic 2-DE samples require cleanup treatments, such as protein precipitation and nucleic acid removal (Rabilloud and Chevallet, 1999), they should be aliquoted after lysis but before these treatments, because the sexing by PCR can be performed with a very small amount of embryonic samples.

The present sexing method consists of desalting, DNA extraction and PCR. The desalting with spin columns effectively removes constituents of the 2-DE lysis buff-

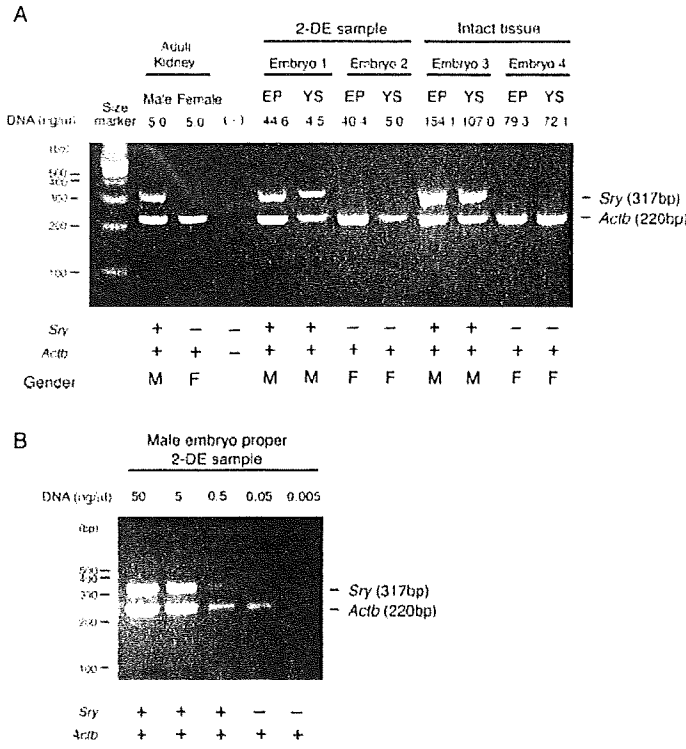

Fig. 1. Analysis of PCR products by agarose gel electrophoresis. (A) PCR products from embryonic 2-DE samples and intact tissues. Male and female adult kidneys were used as a reference. EP, embryo proper; YS, yolk sac membrane; M, male; F, Female. (B) PCR products from a serially diluted male embryo proper 2-DE sample.

Table 2. Consistency between embryonic genders determined with samples from the embryo proper and yolk sac membrane

		Yolk sac membrane	
		Male	Female
Embryo proper	Male	14	0
	Female	0	19

The number of embryos is shown. Consistency is statistically significant by the Fisher's exact test at $P = 1.22 \times 10^{-9}$.

er, which would interfere subsequent DNA extraction, from embryonic 2-DE samples stored after the analyses. Use of a DNA extraction kit makes this method convenient for many samples. The robustness of the present PCR procedure for a wide range of DNA concentrations obviates procedures for concentration adjustment. Thus, the present method is suitable for sexing of embryonic 2-DE samples in developmental toxicity studies, where many samples need to be processed.

For DNA extraction, a rapid method for PCR-sexing of embryos has been reported in mice (McClive and Sinclair, 2001), and the same method may be applicable in rats. We used, instead, a DNA extraction kit, which is considered to be a basis for the robustness of subsequent PCR because of higher purity of DNA. The use of a DNA extraction kit will also make it easier to perform the method in developmental toxicology studies since its protocol is well established.

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