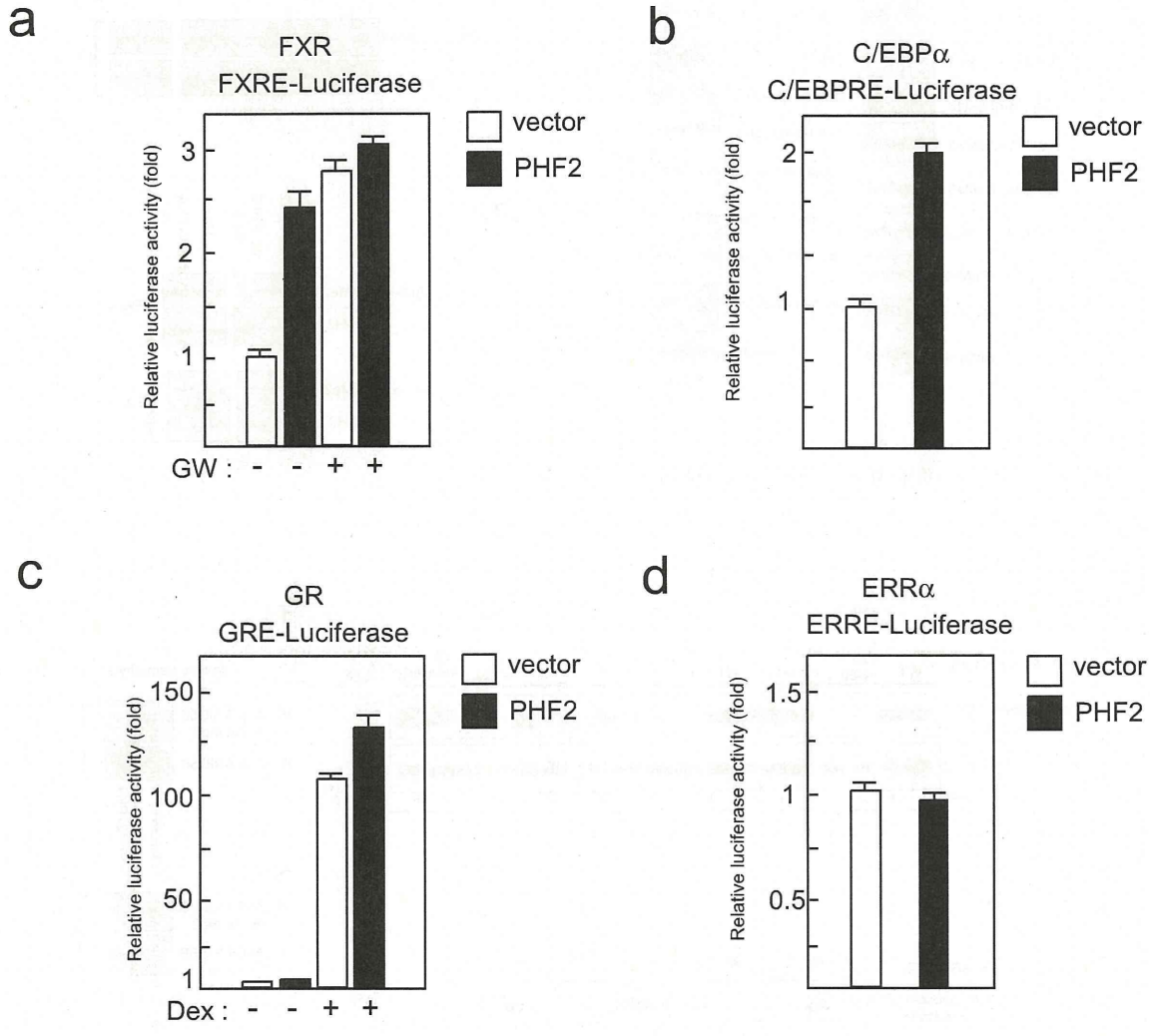


Figure S7 Co-activator activity of PHF2 for known gluconeogenic-related transcription factors. 293F cells were transfected with the indicated expression vectors and reporter plasmids with or without PHF2. Cells were treated with FSK for 24 hrs, then Luciferase assay were performed as indicated.

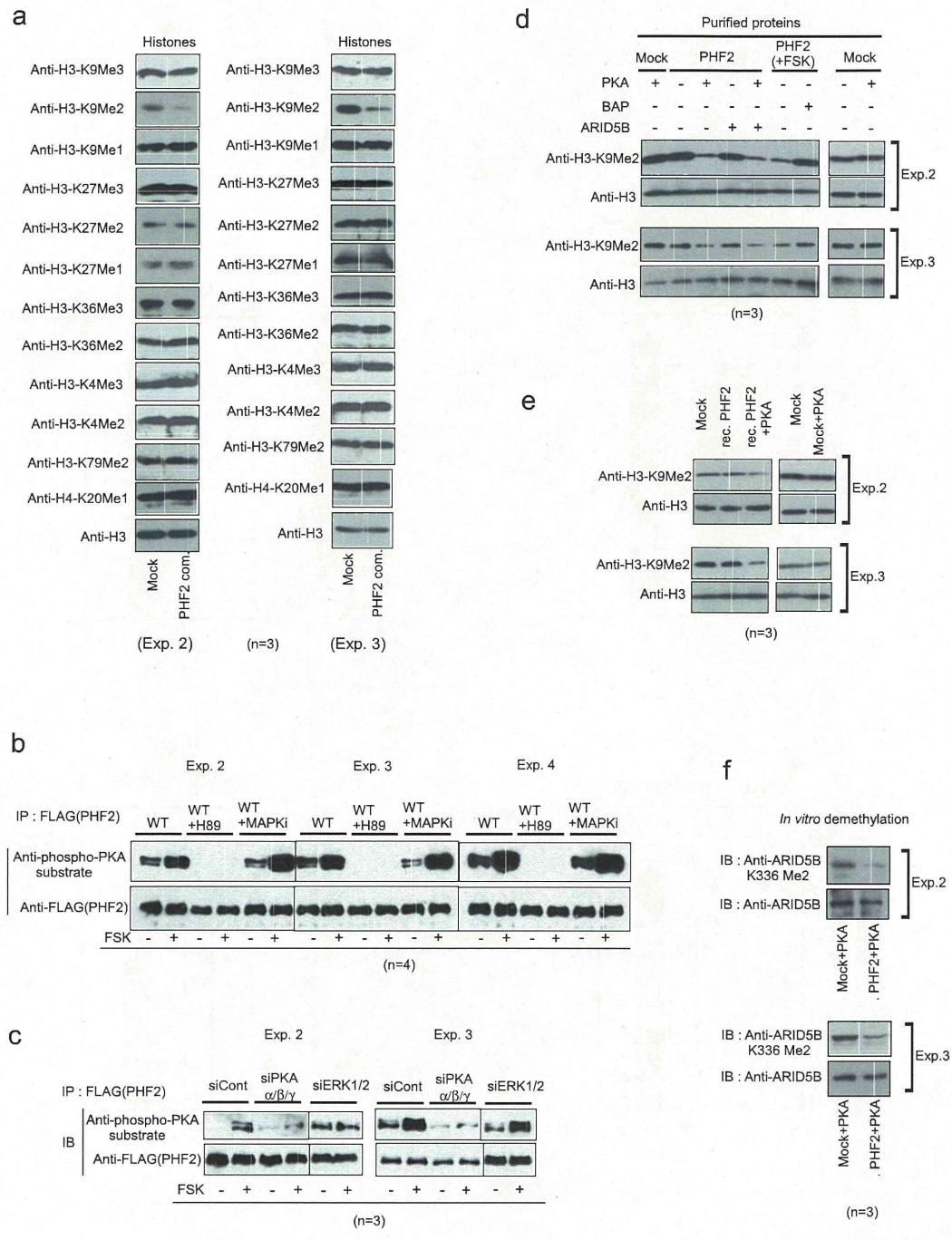
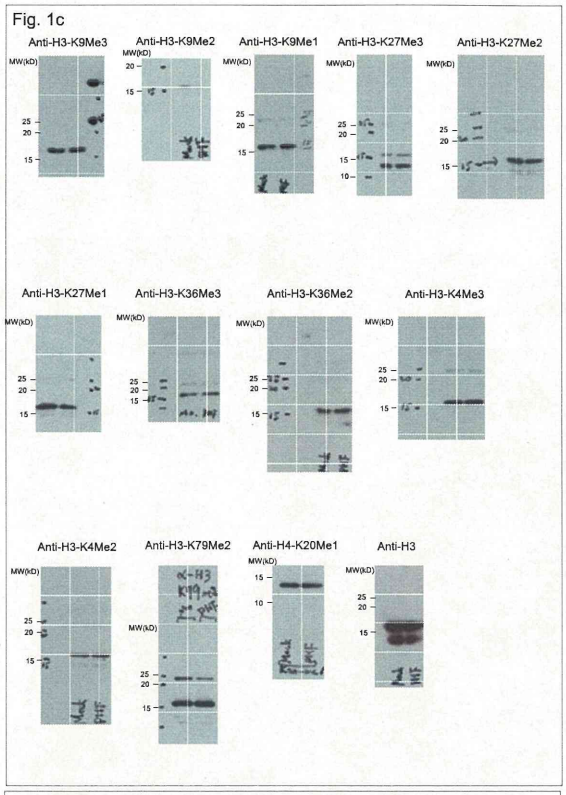
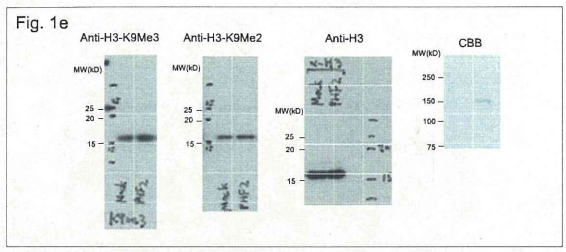
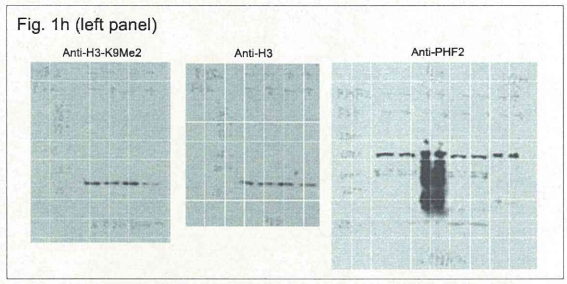
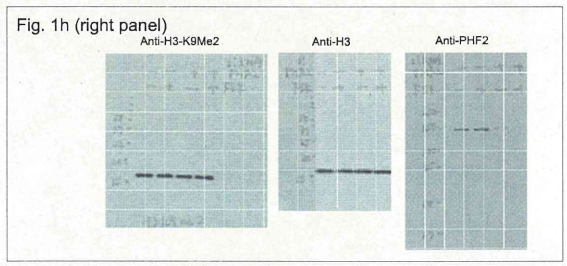


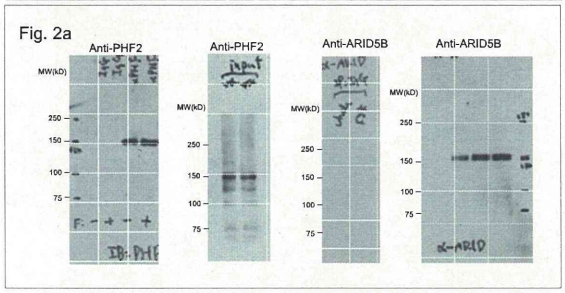
Figure S8 Western blot data for quantification. **a**, For *in vitro* demethylation assay and quantification in Fig. 1c-d, three independent experiments were performed, and histone methylation marks were analyzed by Western blotting as shown in Fig. 1d. The other two sets of data for quantification are shown. **b-c,** Quantification analysis for PKA-mediated phosphorylation of PHF2. Assay was performed as in Fig. 2d, and the other data for quantification in Fig. 2d (left, n=4 and right, n=3) were shown. **d-e**, Quantification analysis for PKA-dependent PHF2 demethylase activity. Three independent assays were performed as in Fig. 3b-c and 3e-f, and the other two sets of data for quantification (n=3) were shown. **f**, Quantification analysis for demethylation of ARID5B by PHF2 *in vitro*. Three independent assay was performed as in Fig. 5e, and the other two sets of data for quantification in the lower panel of Fig. 5e (n=3) were shown.

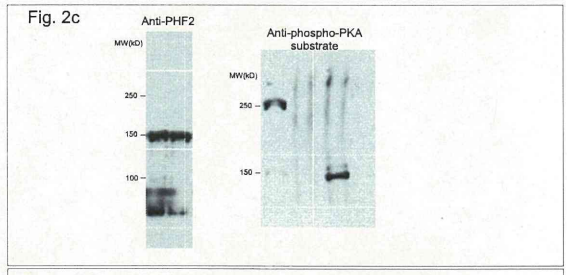


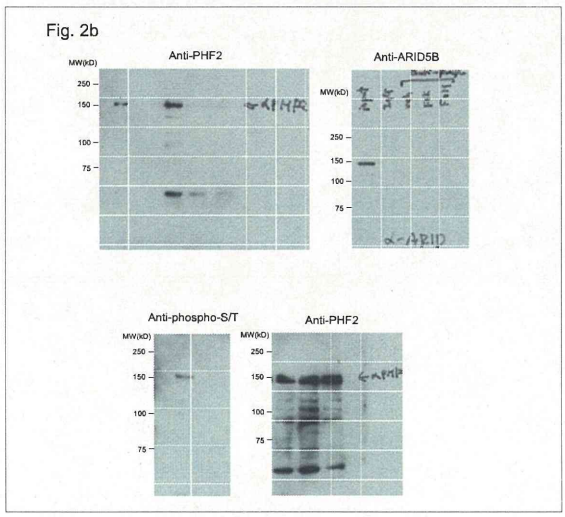












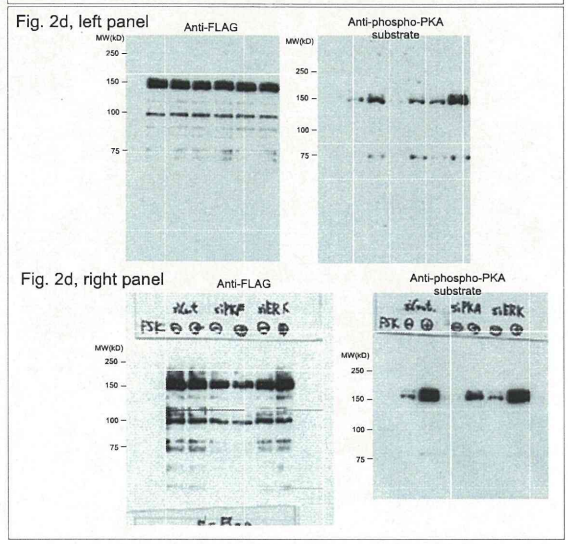


Figure S9 Full scans

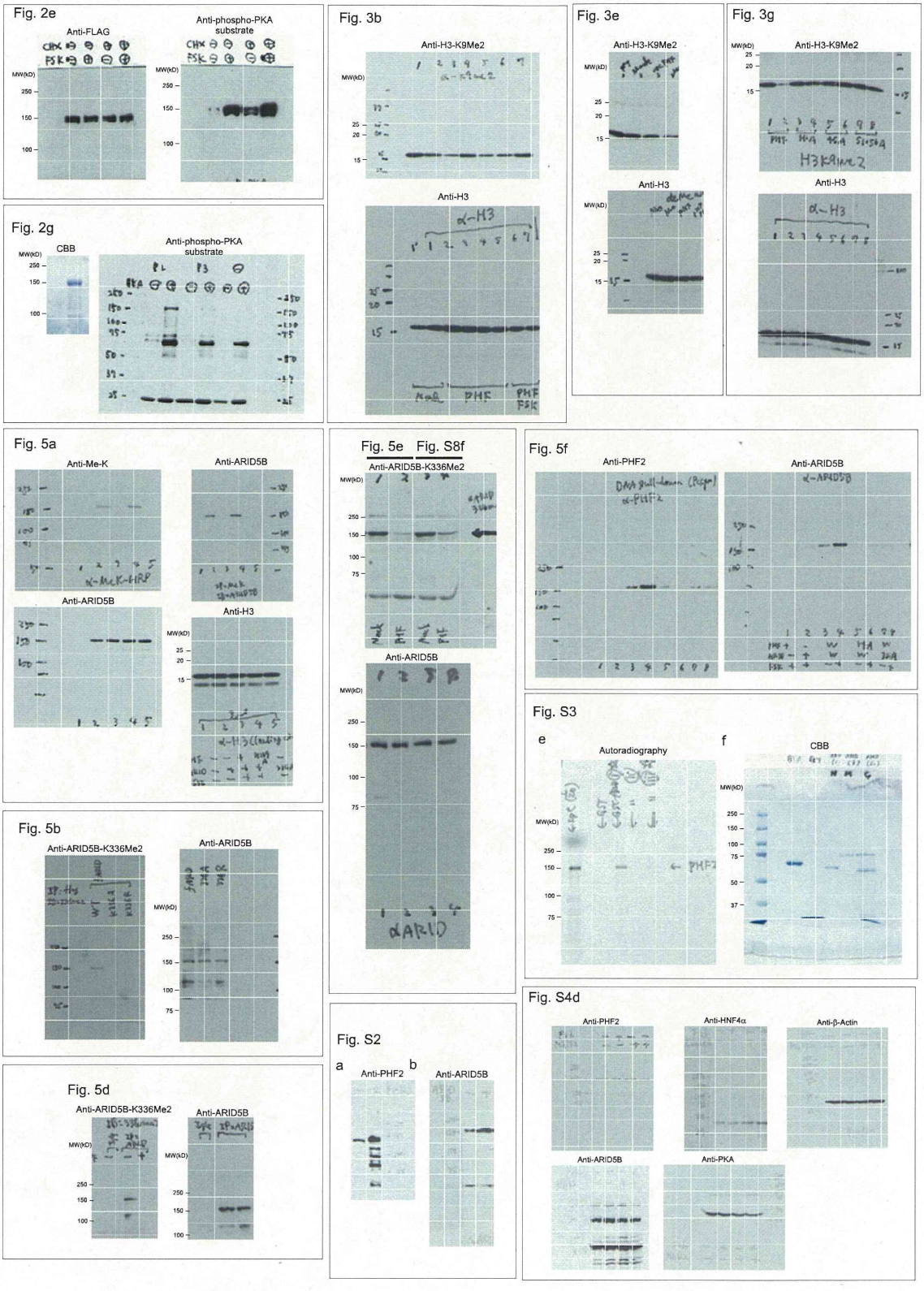


Figure S9 continued

Supplementary Table 1

Lists of antibodies, primers, and siRNA sequences used in this study.

Antibodies for immunoprecipitation

Antibody	Manufacture	Catalag No.	Lot No.
HNF4	Santa Cruz	H-171	K0204
FXR	Santa Cruz	H-130	K2805
FLAG M2	Sigma	F1804	088K6018
PHF2	Our laboratory		
ARID5B	Our laboratory		

Antibodies for Western blotting

Antibody	manufacture	Catalog No.	Lot No.	dilution
PHF2	Our laboratory			1/1000
ARID5B	Our laboratory			1/1000
FXR	Santa Cruz	sc1204	B052	1/500
RXR α	Santa Cruz	D-20	L210	1/1000
methyl-lysine	Abcam	ab23367	458685	1/300
FLAG	Sigma	F-7425	069K4767	1/1000
HA	Immunology Consultants Laboratory	RHGT-45A-4	22	1/1000

Antibodies for histone modification

Antibody	manufacture	Catalog No.	Lot No.	dilution
H3K9Me3	Abcam	ab6001	641998	1/1000
H3K9Me2	Abcam	1220	764743	1/1000
	Upstate	07-441	DAM1463717	1/1000
H3K9Me1	Upstate	07-450	DAM1394811	1/1000
H3K27Me3	Upstate	07-449	DAM1421462	1/1000
H3K27Me2	Upstate	07-452	24461	1/1000
H3K27Me1	Upstate	07-448	24439	1/1000
H3K4Me3	Upstate	07-473	131172	1/1000
H3K4Me2	Upstate	07-030,	DAM15170816	1/1000
H3K36Me3	Abcam	9050	826245	1/1000
H3K36Me2	Upstate	07-369	22475	1/1000
H3K79Me2	Abcam	ab3594	62690	1/1000
H4K20Me1	Upstate	07-748	30587	1/1000
H3	Abcam	ab1791	940500	1/2000

Primers for ChIP

mouse *Pepck* promoter	Fw	5'- TGTGCAGCCAGCAACATATGAA -3'
	Rv	5'- TGCAGGCTCTTGCCTTAATTGTC -3'
mouse *G6Pase* promoter	Fw	5'- GTCAAGCAGTGTGCCCAAGTTAATA -3'
	Rv	5'- CCCAGCCCTGATCTTTGGAC -3'
mouse *Gapdh* promoter	Fw	5'- CCTGCTTATCCAGTCCTAGCTCA -3'
	Rv	5'- AAATGAGGCGGGTCCAAAG -3'

Primers for Realtime RT-qPCR

mouse *Pepck*	Fw	5'- GTGTTTGTAGGAGCAGCCATGAGA -3'
	Rv	5'- GCCAGGTATTTGCCGAAGTTGTAG-3'
mouse *G6Pase*	Fw	5'- GGATCCTGGGACAGACACACAA-3'
	Rv	5'- TGTCAACACCTCTGGCCTCAC -3'
mouse *Hnf4a*	Fw	5'- CCGGGTGTCAGGAACAGTTG -3'
	Rv	5'- TGCAGGACAGTCTGAGCCATC -3'
mouse *Gapdh*	Fw	5'-AAATGGTGAAGGTCGGTGTG -3'
	Rv	5'-TGAAGGGGTCGTTGATGG -3'

Sequences of siRNA

mouse PHF2	#1	5'-CAGCAAACCUGACUCGUUAUU -3'
	#2	5'-GCAAAGGCUUGGAAAGAUCUU -3'
mouse ARID5B	#1	5'-CCAAUCAUUUGACAUGUUCUU -3'
	#2	5'-UCACAUGGGCGCAUUCUGAUU -3'
human PHF2	#1	5'- GCAAGCGCCUGACGUCAAG -3'
	#2	5'- AGGAGUUUGUGGACUAUUA -3'
human ARID5B	#1	5'- UAACGGACCAGUUUGCAUU -3'
	#2	5'- GCAGUCAACCCUAAACAGU -3'
mouse HNF4α	#1	5'- GAAGGAAGCUGUCCAAAAU -3'
	#2	5'- AGAGGUCCAUGGUGUUUAA -3'

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Endocrine Disrupter Bisphenol A Increases In Situ Estrogen Production in the Mouse Urogenital Sinus[1]

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ABSTRACT

The balance between androgens and estrogens is very important in the development of the prostate, and even small changes in estrogen levels, including those of estrogen-mimicking chemicals, can lead to serious changes. Bisphenol A (BPA), an endocrine-disrupting chemical, is a well-known, ubiquitous, estrogenic chemical. To investigate the effects of fetal exposure to low-dose BPA on the development of the prostate, we examined alterations of the in situ sex steroid hormonal environment in the mouse urogenital sinus (UGS). In the BPA-treated UGS, estradiol (E_2) levels and CYP19A1 (cytochrome P450 aromatase) activity were significantly increased compared with those of the untreated and diethylstilbestrol (DES)-treated UGS. The mRNAs of steroidogenic enzymes, *Cyp19a1* and *Cyp11a1*, and the sex-determining gene, *Nr5a1*, were up-regulated specifically in the BPA-treated group. The up-regulation of mRNAs was observed in the mesenchymal component of the UGS as well as in the cerebellum, heart, kidney, and ovary but not in the testis. The number of aromatase-expressing mesenchymal cells in the BPA-treated UGS was approximately twice that in the untreated and DES-treated UGS. The up-regulation of *Esrrg* mRNA was observed in organs for which mRNAs of steroidogenic enzymes were also up-regulated. We demonstrate here that fetal exposure to low-dose BPA has the unique action of increasing in situ E_2 levels and CYP19A1 (aromatase) activity in the mouse UGS. Our data suggest that BPA might interact with in situ steroidogenesis by altering tissue components, such as the accumulation of aromatase-expressing mesenchymal cells, in particular organs.

aromatase, bisphenol A, developmental biology, embryo, estradiol/estrogen receptor, in situ estrogen production, male reproductive tract, prostate, steroidogenic enzyme, urogenital sinus

INTRODUCTION

Endocrine-disrupting chemicals (EDCs) have been implicated in the alteration of fetal development of urogenital organs as well as the reproductive and endocrine systems in humans and other species [1]. The fetal development of urogenital organs is induced by endogenous hormonal messages that originate in fetal and maternal hormone systems. Fetal exposure to EDCs disrupts the interactions between endogenous hormones and their receptors, causing adverse effects later in life [2]. In the prostate, both androgens and estrogens play a significant role in development and differentiation as well as in the maintenance of adult homeostasis [3]. Therefore, even small changes in estrogen levels, including those of estrogen-mimicking chemicals, can lead to changes in prostate development and differentiation.

Bisphenol A (BPA), one of the EDCs, is a well-known, ubiquitous, estrogenic chemical used in the manufacture of polycarbonate plastics, as a lining in metal food and drink cans, and in dental sealants [4]. The concern with BPA originates from its detection in maternal and fetal plasma as well as the placenta [5, 6]. Thus, fetal exposure to BPA is implicated in fetal toxicity as well as in subsequent growth of the infant. Histopathologically, fetal exposure to low-dose BPA (10 μg kg^{-1} day^{-1}) has been shown to increase cell proliferation of urogenital sinus epithelium (UGE) in the primary prostatic ducts of CD1 mice [7]. Recently, our group reported that fetal exposure to low-dose BPA (20 μg kg^{-1} day^{-1}) specifically increased the number of basal epithelial cells in the adult prostate of BALB/c mice and also induced permanent cytokeratin 10 expression in such cells similar to the effects of synthetic estrogen diethylstilbestrol (DES; 0.2 μg kg^{-1} day^{-1}) [8]. Epigenetically, neonatal exposure of male rats to low-dose BPA (10 μg kg^{-1} day^{-1}) elicited critical molecular changes during prostate development and also increased prostatic gland susceptibility to precancerous neoplastic lesions and hormonal carcinogenesis [9]. Toxicological studies of BPA at less than 50 μg kg^{-1} day^{-1} in rodent fetuses and offspring have demonstrated alterations of mammary gland development, open-field behavior, and reproductive functioning [10–12].

Some EDCs are reported to alter the in situ sex steroid hormonal environment in the reproductive system. The triazine herbicide atrazine binds directly to adrenal-4-binding protein/steroidogenic factor-1 (official symbol NR5A1) and increases CYP19A1 (cytochrome P450 aromatase) expression and, ultimately, estradiol (E_2) production in human genital cancer cell lines [13]. The aryl hydrocarbon (dioxin) also increases CYP19A1 (aromatase) expression mediated by its receptor in mouse ovaries [14]. In contrast, the phosphorothioate insecticide profenofos increases the expression of steroidogenic genes

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and testosterone levels in rat testes [15]. Recently reported adverse effects of BPA on in situ steroidogenesis include increased testosterone levels in mouse Leydig cells and decreased E_2 levels in porcine ovarian granulosa cells [16, 17]. Thus, BPA may have the potential not only to mimic estrogenic action but also to alter in situ steroidogenesis in the prostate as well as other reproductive organs.

To investigate the effects of fetal exposure to low-dose BPA on in situ steroidogenesis in the developing prostate, we first measured sex steroid hormone levels and CYP19A1 (aromatase) activity in the BPA-treated mouse urogenital sinus (UGS), from which the prostate develops embryologically. Subsequently, we examined the alterations of steroidogenic enzyme gene expression to confirm the alterations of the in situ sex steroid hormonal environment in the BPA-treated mouse UGS. Finally, we identified the BPA-specific biological effects for in situ steroidogenesis during fetal prostate development.

MATERIALS AND METHODS

Animals

In the present study, 36 pregnant female C57BL/6 mice were purchased on the 12th day of gestation from Japan SLC, where the breeding strategy was to mate three female C57BL/6 mice (age, 10 wk) with one male overnight and separate them the next morning (plug date denoted as Day 0). All animals were housed individually in chip-bedded polyolefin cages in a room with controlled temperature (23 ± 1°C) and humidity (45 to 65%) on a 12L:12D photoperiod. Mice were fed a low-phytoestrogen diet (NIH-07PLD; Oriental Yeast Co.) and tap water ad libitum.

Chemicals

For the present study, both BPA and DES with a purity of 99% or greater were purchased from Nacalai Tesque and Wako Pure Chemical Industries, respectively.

Fetal Exposure to Chemicals

We randomly assigned 36 pregnant female C57BL/6 mice to three different treatment groups: BPA (20 μg kg^{-1} day^{-1}, n = 12) or DES (0.2 μg kg^{-1} day^{-1}, n = 12), both of which were dissolved in tocopherol-stripped corn oil (MP Biomedical, Inc.), administered by oral gavages on Embryonic Day (E) 13 to E16 and the control group, in which pregnant mice were fed tocopherol-stripped corn oil (2 ml/kg, n = 12). Previously, our group reported that this protocol of fetal exposure to BPA and DES resulted in similar histopathological changes of adult prostate—that is, increased basal epithelial cell number and induction of cytokeratin 10, a classic marker associated with squamous differentiation, in such cells [8]. Our dose level of BPA for the present study was also based on reported results suggesting that BPA is less than 100-fold less potent than DES. The Mie University's Committee on Animal Investigation approved the experimental protocol.

Termination and UGS Dissection

Between E17 and Postnatal Day (P) 1, all animals were terminated by an overdose of isoflurane followed by cervical dislocation. For each of the three groups, from 15 to 18 fetuses (both male and female) from three pregnant mice were collected at E17, E18, P0, and P1. The bladder and urethra were removed and dissected to isolate the UGS, and then the five or six UGS obtained were pooled as one sample. Thus, the 15–18 UGS were divided into three samples at each time point. The UGS, cerebellum, heart, kidney, testis, and ovary were collected in RNAlater (Applied Biosystems).

To isolate pure UGS, other tissues, such as the bladder, urethra, wolffian duct, seminal vesicle, and mullerian duct, were removed from both the male and female urogenital tracts. The histopathology of the mouse UGS was then examined by hematoxylin-and-eosin staining.

Measurements of In Situ E_2 Levels and CYP19A1 (Aromatase) Activity in UGS

The E_2 levels and CYP19A1 (aromatase) activity in UGS were determined by liquid chromatography-tandem mass spectrometry [18] and a tritiated water release assay [19], respectively, which were made available by Aska Pharma Medical. Briefly, the organs were homogenized, and the extracts were applied to a C18 Amprep solid-phase column (Amersham Biosciences) to remove contaminating fats. The E_2 was then separated using a normal-phase high-performance liquid chromatography system (Jasco) with a silica gel column (Cosmosil 5SI; Nacalai Tesque), and 100 pg of isotope-labeled [$^{13}C_4$]E_2 were added to extracts. The evaporated extracts were reacted with 5% pentafluorobenzyl bromide/acetonitrile, under KOH/ethanol, for 1 h at 55°C. After evaporation, the products were reacted with 100 ml of picolinic acid solution (2% picolinic acid, 2% 2-dimethylaminopyridinc, and 1% 2-methyl-6-nitrobenzoic acid in tetrahydrofuran) and 20 ml of triethylamine for 0.5 h at room temperature. The reaction products were dissolved in 1% acetic acid and then purified using a Bond Elute C18 column (Varian). The products were measured with a reverse-phase liquid chromatograph (Agilent 1100; Agilent Technologies) coupled with an API 5000 triple-stage quadrupole mass spectrometer (Applied Biosystems) in the positive-ion mode. This device monitored the *m/z* 558 to *m/z* 339 (E_2) and *m/z* 562 to *m/z* 343 ([$^{13}C_4$]E_2) transitions.

The tritiated water release assay was used for the measurement of CYP19A1 (aromatase) activity. This method measures the production of ^3H_2O, which forms as a result of aromatization of the substrate [1b-^{3}H]androst-4-ene-3,17-dione (New England Nuclear). Serum-free medium containing [1b-^{3}H]androst-4-ene-3,17-dione solution (54 nM) was prepared, of which 0.5 ml was added to each sample. After incubation for 1 h, the samples were placed on ice, and 200 μl of culture medium were withdrawn. The medium was extracted with 500 μl of chloroform, vortexed, and then centrifuged for 1 min at 9000 × *g*. A 100-μl aliquot of the aqueous phase was mixed with 100 μl of a 5% (wt/vol) charcoal/0.5% (wt/vol) dextran T-70 suspension, vortexed, and then incubated at room temperature for 10 min. Then, after centrifugation of the solution for 5 min at 9000 × *g*, a 150-μl aliquot was removed for measurement of radioactivity by liquid scintillation.

RNA Extraction and cDNA Preparation

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Inc.) in accordance with the manufacturer's instructions. The RNA concentration was then determined spectrophotometrically by a multidetection microplate reader (Dainippon Sumitomo Pharma Co.). From 50 ng of total RNA, cDNA was reverse transcribed using oligo(dT) and Superscript II RNase H-reverse transcriptase (Invitrogen) as previously described [8].

Analysis of Gene Expression Profile

For determining gene expression profiles of the male UGS, GeneChip analysis with the Percellome method was performed [20]. Briefly, organs were prepared using RLT buffer (Qiagen, Inc.). Total RNA was extracted using RNeasy Mini Kit. First-strand cDNA was synthesized by incubating 5 mg of total RNA with a T7 oligo(dT) primer (Invitrogen) according to the manufacturer's protocol. The dsDNA was mixed with T7 RNA polymerase (Enzo Biochem, Inc.). During the in vitro transcription, generated cRNAs were labeled with biotin-16-UTP and biotin-11-CTP (Enzo Biochem, Inc.). The purified cRNA was fragmented at 300–500 bp into the target solution. Hybridization was performed with the GeneChip Mouse Genome 430 Version 2.0 (Affymetrix, Inc.) at 45°C for 18 h after staining with streptavidin-R-phycoerythrin conjugates (Molecular Probes, Invitrogen). The reacted arrays were then scanned as digital image files, and the scanned data were analyzed with GeneChip Operating Software (Affymetrix, Inc.). The expression data were converted to copy numbers of mRNA per cell by the Percellome method, quality controlled, and analyzed using Percellome software [20].

Real-Time PCR Analysis

Real-time PCR was carried out in the iCycler iQ Detection System (Bio-Rad Laboratories) with iQ SYBR-Green Supermix reagents (Bio-Rad Laboratories) as previously described [8]. The PCR amplification reaction was performed with specific primers as shown in Table 1. After PCR, melting-curve analysis was performed to verify specificity and identity of the PCR products. All data were analyzed with the iCycler iQ Optical System Software Version 3.0A (Bio-Rad Laboratories). All PCR data were normalized to *Gapdh* mRNA.

Preparation of Primary Cultured Mesenchymal Cells from UGS

The UGS were dissected from the fetuses and separated into UGE and urogenital sinus mesenchyme (UGM) by tryptic digestion and mechanical separation as previously described [21]. UGM were cultured in RPMI-1640

TABLE 1. Sequences of oligonucleotide primers used for the real-time PCR analyses.

Gene	Primer[a]
Gapdh	F: 5′-AAATGGTGAAGGTCGGTGTG-3′
	R: 5′-TGAAGGGGTCGTTGATGG-3′
Cyp19a1	F: 5′-GCCCAATGAATTTACCCTCGAA-3′
	R: 5′-AAGCCAAAAGGCTGAAAGTACCT-3′
Cyp11a1	F: 5′-TCGACTCCTCAGAACTAAGACCTG-3′
	R: 5′-GTACCCTGGTGTCCTTTATAGCCT-3′
Nr5a1	F: 5′-CCTGGGCTGGCTACCTCTATC-3′
	R: 5′-CGAACTAGAGCCAGAGGAGGAC-3′
Esr1	F: 5′-GCACAGGATGCTAGCCTTGTCTC-3′
	R: 5′-AATTGTCACCAGCTTGCAGGTTC-3′
Ar	F: 5′-GGCGGTCCTTCACTAATGTCAACT-3′
	R: 5′-CTGACTTGTGCATGCGGTACTCAT-3′
Esrrg	F: 5′-CCGAGAGTTGGTGGTTATCATTGG-3′
	R: 5′-GGAAGACCCTCGCCGTGC-3′

[a] F, forward; R, reverse.

with 5% fetal bovine serum and plated out on four-well glass slides (BD Falcon). After several days, cells were fixed in methanol and processed for immunocytochemical analysis.

Immunocytochemical Staining

The sections were first incubated for 15 min in 0.01 M PBS. After inhibition of endogenous peroxidases (10 min in 0.6% H_2O_2 diluted in 0.01 M PBS plus 0.2% Triton X-100 [PBST]) and saturation (2 h in a 5% normal goat serum solution), sections were incubated overnight at 4°C in a polyclonal affinity-purified antiaromatase antibody or estrogen-related receptor gamma (ESRRG) antibody raised in rabbit against quail recombinant aromatase or ESRRG diluted 1:500 in 0.01 M PBST. The next day, the sections were immersed for 2 h at room temperature in a biotin-conjugated goat anti-rabbit immunoglobulin G (DakoCytomation, Inc.) diluted 1:400 in PBST and then for 2 h in a streptavidin-fluorescein complex (Rhodamine; DakoCytomation, Inc.) diluted 1:50 in PBST. Between each step, sections were extensively rinsed in PBST. The sections were mounted onto microscope slides, coverslipped with a gelatin-based mounting medium, and stored in the dark at 4°C. For double-labeling immunofluorescence, Alexa Fluor 488- or 594-conjugated secondary antibodies were used. Rabbit polyclonal anti-aromatase antibody was kindly provided by Prof. Nobuhiro Harada (Department of Biochemistry, Fujita Health University School of Medicine, Aichi, Japan) [22]. The rabbit polyclonal anti-ESRRG antibody used in the present study was established and characterized as previously reported [23]. The mouse monoclonal anti-Ran antibody (Santa Cruz Biotechnology, Inc.) was used to detect nucleus in cells. Ran, also called TC4, is the small RAS-related protein that is localized in the nucleus.

Statistical Analysis

Results are expressed as the mean ± SD. Differences among the three groups were determined using Student *t*-test with Dunnett multiple comparison. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

BPA-Specific Increases of E_2 Levels and CYP19A1 (Aromatase) Activity in Mouse UGS

The pregnant mice were exposed to low-dose BPA during the onset of prostatic budding (E13–E16), and the UGS of fetuses were collected during bud elongation (E17–P1). In analyses of in situ sex steroid hormonal environment, E_2 levels and CYP19A1 (aromatase) activity were significantly increased only at P1 in BPA-treated UGS, not at P1 in the DES-treated UGS (Fig. 1). At E17 and P1, both the E_2 levels and CYP19A1 (aromatase) activity in untreated male UGS were not significantly different compared with those in untreated female UGS.

BPA-Specific Up-Regulation of Steroidogenic Enzyme and Sex-Determining Gene mRNA in Mouse UGS

To investigate the BPA-specific gene alterations related to increases of the E_2 levels and aromatase activity, we performed preliminary GeneChip analysis with the Percellome method in the BPA- or DES-treated male UGS at E17 and P1. The results showed BPA-specific mRNA up-regulation of steroidogenic enzymes, such as *Cyp11a1*, *Cyp11b1*, and *Cyp17a1*, and sex-determining factors, such as *Nr5a1*, *Nr0b1*, *Gata4*, and *Amhr2* (data not shown). Furthermore, quantitative PCR analysis confirmed the mRNA up-regulation of *Cyp19a1*, *Cyp11a1*, and *Nr5a1* only in the BPA-treated neonatal (P0 and P1) UGS, not in the DES-treated neonatal UGS (Fig. 2). No difference in mRNA expression levels was found between E17 and P1 when comparing the untreated male UGS to that of the female. In

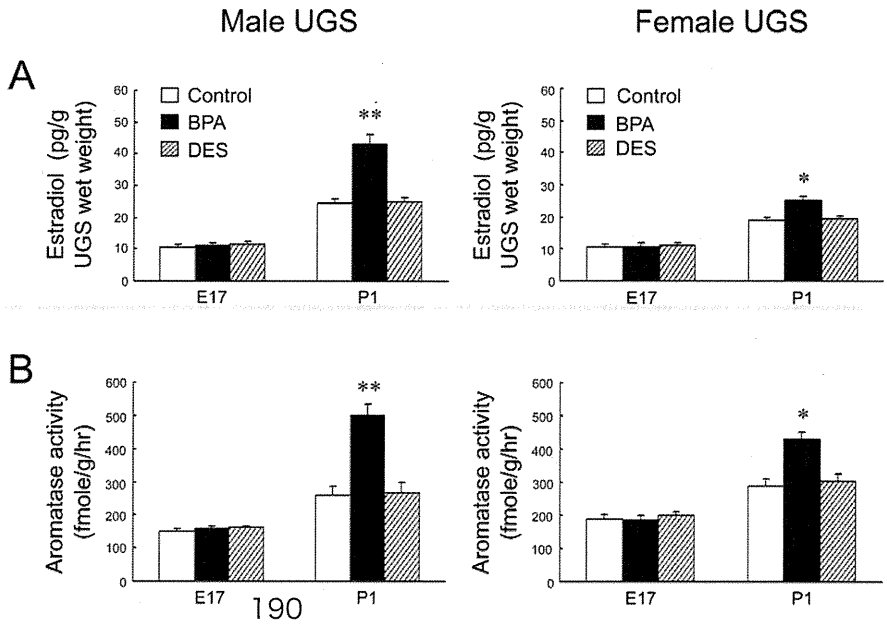

FIG. 1. BPA-specific increases of E_2 levels and CYP19A1 (aromatase) activity in mouse UGS. E_2 levels (**A**) and CYP19A1 (aromatase) activity (**B**) were measured in the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar) at E17 and P1. *$P < 0.01$, **$P < 0.001$ vs. control.

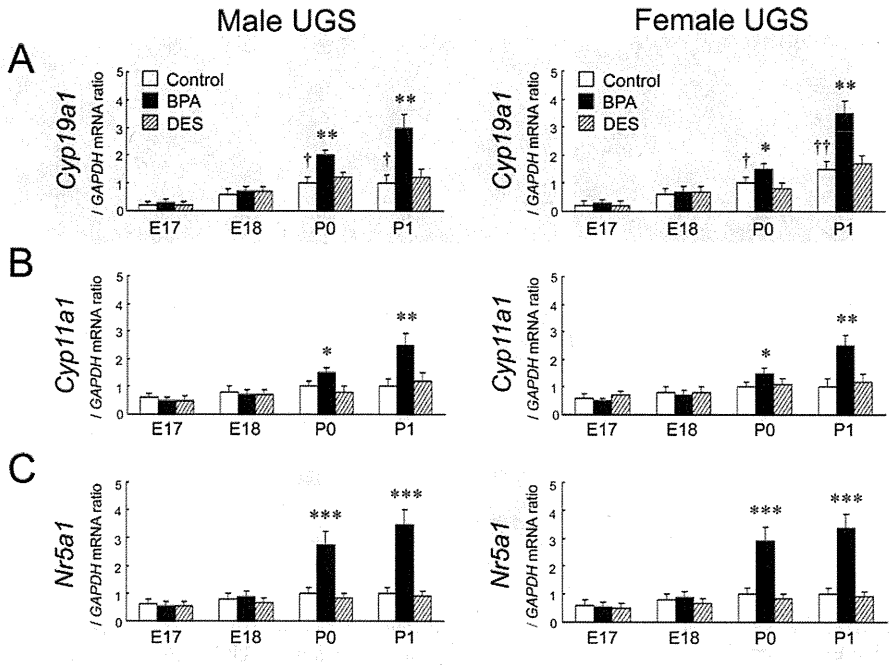

FIG. 2. BPA-specific up-regulation of steroidogenic enzyme and sex-determining gene mRNA in mouse UGS. The relative mRNA expressions of *Cyp19a1* (**A**), *Cyp11a1* (**B**), and *Nr5a1* (**C**) were determined in the untreated control (open bar), BPA-treated UGA (closed bar), and DES-treated UGS (slashed bar) between E17 and P1. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control at each time point; $^{\dagger}P < 0.01$, $^{\dagger\dagger}P < 0.001$ vs. control at E17.

untreated male and female UGS, the mRNA of *Cyp19a1* was gradually increased between E17 and P1.

Restricted BPA-Specific Up-Regulation of Steroidogenic Enzyme and Sex-Determining Gene mRNA in UGE and UGM

In male fetuses at P1, it was not feasible to separate UGE and UGM components within the male UGS because of the formation of prostatic buds. In the female at P1, the up-regulation of *Cyp19a1*, *Cyp11a1*, and *Nr5a1* mRNA was observed only in UGM, not in UGE, of the BPA-treated group (Fig. 3). In both male and female UGE, expressions of such mRNAs were quite low and not up-regulated, even in the BPA-treated group. At E17, no difference in mRNA expression levels was found when comparing the untreated male UGM with that of the female.

BPA-Specific Increases of Aromatase-Expressing Cells in Primary Cultured UGM

In both the male and female, P1 UGM was primary cultured in vitro. Representative pictures of aromatase-positive cells are shown in Figure 4, A–C. The aromatase-positive staining was

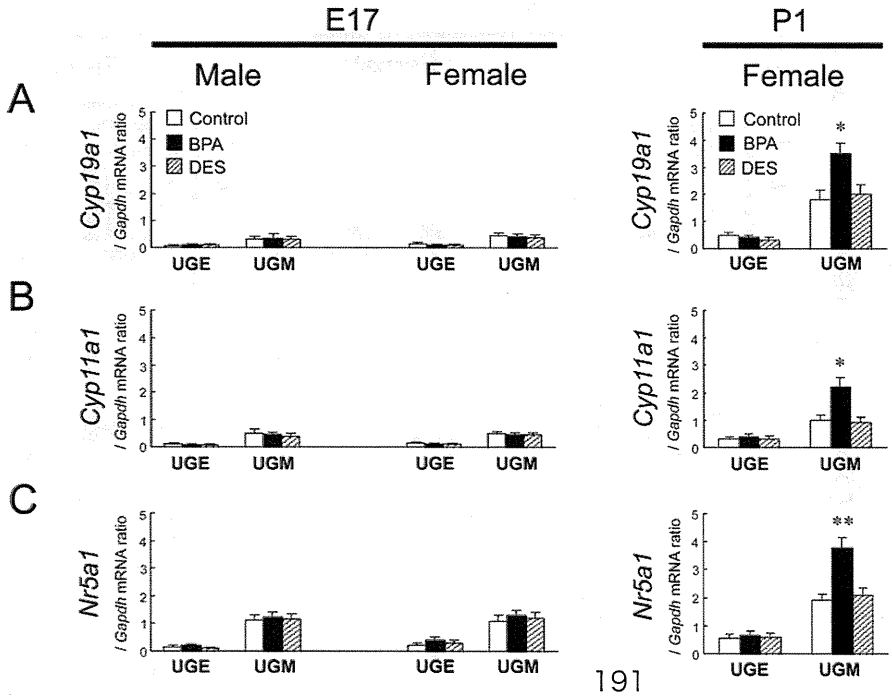

FIG. 3. Restricted BPA-specific up-regulation of steroidogenic enzyme and sex-determining gene mRNA in UGE and UGM. The relative mRNA expressions of *Cyp19a1* (**A**), *Cyp11a1* (**B**), and *Nr5a1* (**C**) were determined for UGE and UGM of the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar) at E17 and P1. $^{*}P < 0.01$, $^{**}P < 0.001$ vs. control.

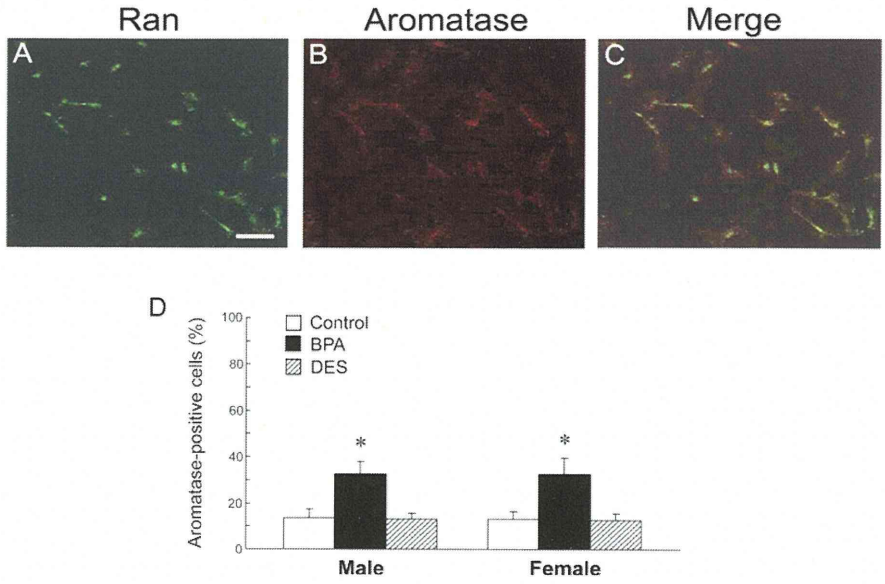

FIG. 4. BPA-specific increases of aromatase-expressing cells in primary cultured UGM. **A–C**) Fluorescence signals were detected for the CYP19A1 (aromatase) protein in primary cultured UGM. The nuclei were identified by Ran staining. Bar = 100 μm, magnification ×400. **D**) The number of aromatase-positive cells was counted in primary cultured UGM of the untreated control open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar), and the percentage of aromatase-positive cells was calculated from at least 10 areas. $*P < 0.01$ vs. control.

observed in the cytoplasm of cultured UGM. The rate of positivity (i.e., the percentage of cells that expressed CYP19A1 [aromatase] protein), was approximately 10% in the untreated and the DES-treated groups, whereas it was as high as approximately 30% in the BPA-treated group (Fig. 4D). No difference in the rate of positivity of CYP19A1 (aromatase) was found when comparing the untreated male UGM to that of the female.

Restricted BPA-Specific Up-Regulation of Esrrg *mRNA in UGE and UGM*

In E17 female UGM, the mRNA expression of *Esr1* was up-regulated by both BPA and DES treatment (Fig. 5A). At E17, however, the mRNA expression of *Ar* was up-regulated by both BPA and DES treatment in the male UGS (Fig. 5B). At P1, mRNA expression of *Ar* was up-regulated by both BPA and DES treatment in the female UGS (Fig. 5B). In both the male and female, the up-regulation of *Esrrg* mRNA was observed at E17 and restricted in UGM, but not in UGE, of the BPA-treated group (Fig. 5C). In both the male and female UGE, the expression of *Esrrg* mRNA was quite low and not up-regulated, even in the BPA-treated group. At E17, no difference in mRNA expression levels was found when comparing the untreated male UGS with that of the female.

BPA-Specific Increases of ESRRG-Expressing Cells in Primary Cultured UGM

In both the male and female, E17 UGM was primary cultured in vitro. Representative pictures of ESRRG-positive

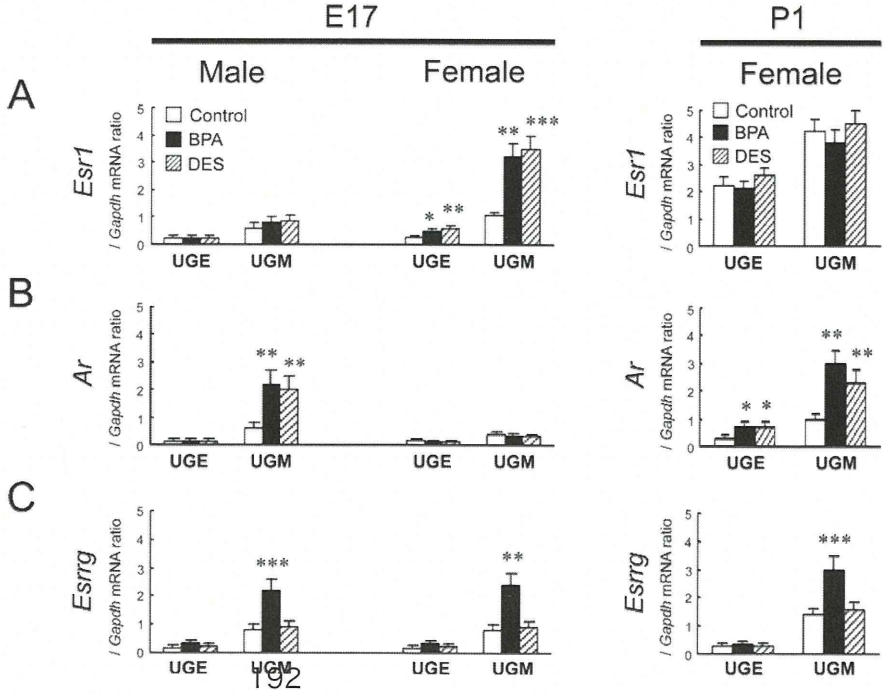

FIG. 5. Restricted BPA-specific up-regulation of *Esrrg* mRNA in UGE and UGM. The relative mRNA expressions of *Esr1* (**A**), *Ar* (**B**), and *Esrrg* (**C**) were determined in UGE and UGM of the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar) at E17 and P1. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. control.

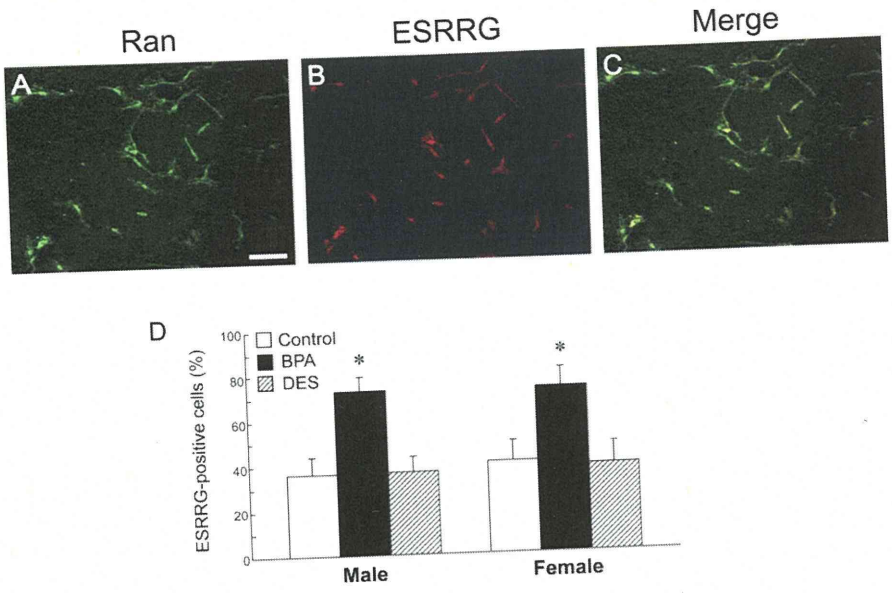

FIG. 6. BPA-specific increases of ESRRG-expressing cells in primary cultured UGM. **A–C**) Fluorescence signals were detected for the ESRRG protein in primary cultured UGM. The nuclei were identified by Ran staining. Bar = 100 μm, magnification ×400. **D**) The number of ESRRG-positive cells was counted in primary cultured UGM of the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar), and the percentage of ESRRG-positive cells was calculated from at least 10 areas. $*P < 0.01$ vs. control.

cells are shown in Figure 6, A–C. The ESRRG-positive staining was observed in both the nucleus and the cytoplasm of cultured UGM. The number of ESRRG-positive UGM was significantly increased only in the BPA-treated group and showed a 2.2-fold increase in males and a 1.6-fold increase in females (Fig. 6D). No difference was found in the rate of positivity of ESRRG when comparing the untreated male UGM with that of the female.

BPA-Specific Up-Regulation of Esrrg *and Steroidogenic Enzyme mRNA in Sex Hormone-Related Organs*

To investigate the BPA-specific up-regulation of in situ steroidogenesis in other organs, we first examined the changes in *Esrrg* mRNA expression in sex hormone-related organs, such as the cerebellum, heart, kidney, ovary, and testis. At P1, the mRNA expression of *Esr1* in the cerebellum, heart, kidney, and ovary, but not in the testis, was up-regulated by both BPA and DES treatment (Fig. 7A). However, no significant difference in *Ar* mRNA expression was observed in all organs examined (Fig. 7B). In the untreated group, the mRNA expression of *Esrrg* was not detected in the testis at E17 and P1 (Fig. 7C). The up-regulation of *Esrrg* mRNA was observed at E17 and restricted to the cerebellum, heart, kidney, and ovary (Fig. 7C). The BPA-specific up-regulation of *Cyp19a1*, *Cyp11a1*, and *Nr5a1* mRNA was observed only at P1 in the cerebellum, heart, kidney, and ovary, but not in the testis (Fig. 8).

DISCUSSION

Concern about the effects of EDCs such as BPA on human health has been increasing [24]. Although the majority of EDCs have the potential to alter functioning of the reproductive and endocrine system, the actual mechanism responsible for such alterations has not been identified thoroughly. BPA is of concern because its chemical structure resembles that of DES. Several studies have reported that BPA can mimic estrogen action, such as induction of vaginal cornification, uterine vascular permeability, growth and differentiation of the mammary gland, and synaptic plasticity in the hippocampus [25–28]. In the prostate, alterations in normal development can produce permanent changes that persist throughout adulthood and may increase the risk of disease in later life [9]. Thus, our objective was to investigate the biological effects of low-dose BPA on the initial development of primary ducts in the fetal prostate.

During prostatic development, alteration of sex steroid hormone synthesis may be responsible for prostatic anomalies associated with fetal exposure to EDCs. In the present study, fetal exposure to low-dose BPA increased E_2 levels in P1 UGS of both the male and female, whereas DES-induced changes were not detected. This alteration was also correlated with increased activity of CYP19A1 (aromatase) in UGS at P1, suggesting the unique action of BPA for in situ steroidogenesis in UGS. The BPA-specific increase of E_2 levels in UGS at P1 was correlated with the following: mRNA up-regulation of steroidogenic enzymes, such as *Cyp19a1* and *Cyp11a1*, and an increased number of aromatase-expressing UGM. The enzyme CYP19A1 (aromatase) is responsible for in situ E_2 production and the crucial testosterone/E_2 balance necessary for normal embryonic and fetal development, even in males. The data presented here shows that the up-regulation of *Cyp19a1* mRNA in BPA-treated UGM was comparable to changes in both in situ E_2 production and CYP19A1 (aromatase) activity.

In the present study, we demonstrated that the BPA-specific increase in steroidogenic enzyme mRNA and aromatase-expressing cell number were observed in both the male and female UGM. During embryonic development, the mesenchymal component is involved in the induction and organogenesis of various organs, including the prostate, mammary gland, lung, kidney, and pancreas. It has been well established that subpopulations of the mesenchymal component are a source of potent molecules that regulate epithelial growth and differentiation [29]. In the prostate, androgen-responsive signals derived from UGM permissively and instructively induce UGE to form primary ducts of the prostate [30].

Comparison between the neonatal male and female UGS shows a similarity in the condensed mesenchyme of the ventral areas—that is, the ventral prostate mesenchyme (VPM) in the male and the ventral mesenchymal pad (VMP) in the female [31]. In the male, a defined VPM is specifically associated with ductal branching morphogenesis and cytodifferentiation of the ventral prostate. Females do not usually form a prostate. In a

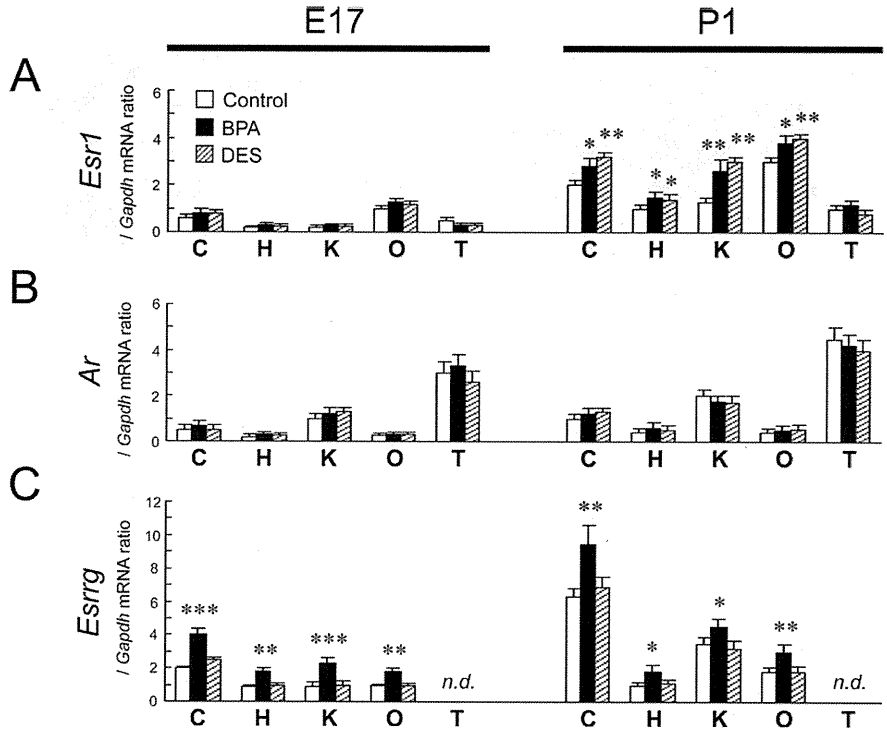

FIG. 7. BPA-specific up-regulation of *Esrrg* mRNA in sex steroid hormone-related organs. The relative mRNA expressions of *Esr1* (**A**), *Ar* (**B**), and *Esrrg* (**C**) were determined in sex steroid hormone-related organs of the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar) at E17 and P1. C, cerebellum; H, heart; K, kidney; O, ovary; T, testis; *n.d.*, not detected. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control.

tissue recombination model, the female VMP induces prostate development in response to androgens [32], suggesting that cells within the female VMP have prostatic-inductive activity. Moreover, an earlier tissue recombination study showed that the ability of the female UGS to respond to androgens in forming prostate was gradually lost between P1 and P5 [33]. These results suggest strongly that androgen-responsive regulatory molecules are expressed constitutively even in the female VMP. Although the female VMP forms in the absence of androgens, androgen receptor (AR) expression was observed in the neonatal female VMP in a pattern similar to that observed in the male VPM [34]. Therefore, the BPA-specific increase in E_2 levels might interact with the intracellular AR signaling in both the male VPM and the female VMP. However, to our knowledge,

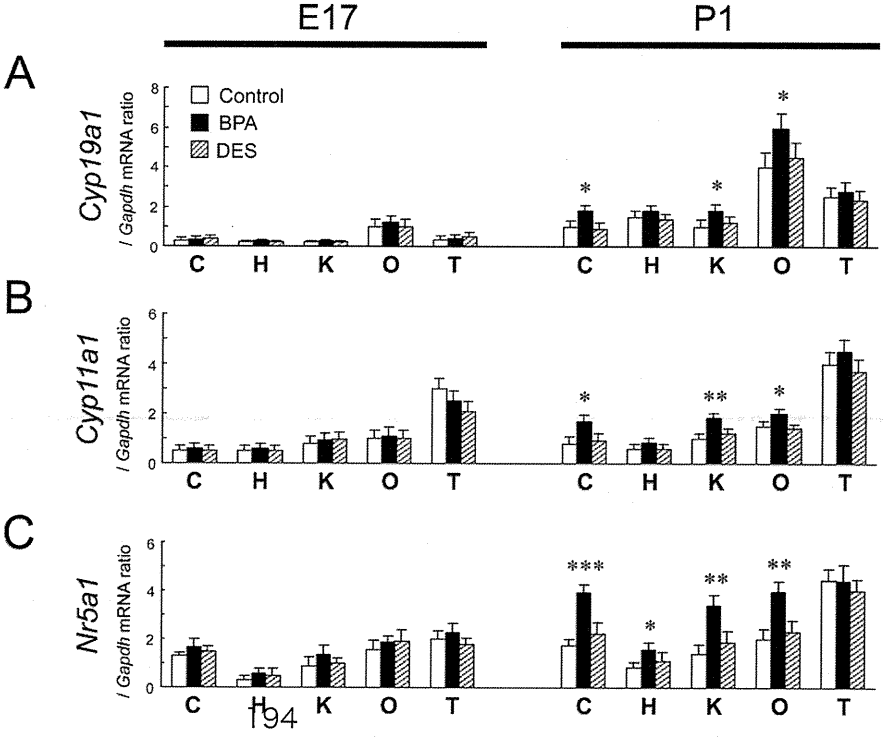

FIG. 8. BPA-specific up-regulation of steroidogenic enzyme and sex-determining gene mRNA in sex steroid hormone-related organs. The relative mRNA expressions of *Cyp19a1* (**A**), *Cyp11a1* (**B**), and *Nr5a1* (**C**) were determined in sex steroid hormone-related organs of the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar) at E17 and P1. C, cerebellum; H, heart; K, kidney; O, ovary; T, testis. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control.

the morphological changes in neonatal female UGS have not yet been investigated.

Our results suggest that BPA has a stimulatory effect on in situ steroidogenesis in P1 UGS of both the male and female at low-dose exposure levels. Recently, ESRRG has been reported to bind strongly with BPA [35]. Susens et al. [36] have reported that expression of ESRRG in the mouse is organ-specific: ESRRG is expressed in the brain, heart, kidney, and skeletal muscle but not in the lung, spleen, and testis. In the present study, the up-regulation of *Cyp19a1* and *Cyp11a1* mRNA by BPA treatment was detected only in organs expressing *Esrrg* mRNA. These data suggest that the possibility of a stimulatory effect on in situ steroidogenesis by fetal exposure to low-dose BPA may be a concern not only in UGS but also in organs expressing ESRRG, such as the brain, heart, kidney, and ovary. It is important to note that Takeda et al. [23] have recently reported that ESRRG was detected in the human testis, suggesting that the distribution of ESRRG differs slightly between mice and humans.

In the present study, the BPA-specific up-regulation of steroidogenic enzyme mRNA in UGS, cerebellum, heart, kidney, and ovary was observed only during the neonatal period (i.e., P0 and P1) and not during the prenatal period (i.e., E17 and E18). During pregnancy in rodents, large amounts of estrogens produced in the maternal ovaries are continuously delivered to the fetus through the placenta. After birth, however, the fetus may be released from the maternal, high-estrogen environment. Thus, one possibility is that the maternal, high-estrogen environment in pregnancy may protect the fetus from the effect of BPA on in situ steroidogenesis during the prenatal period. However, we did not investigate the effects of neonatal BPA treatment on in situ steroidogenesis.

The EDC-induced alterations of the in situ estrogen environment depend on each compound. In addition to atrazine and dioxin, the organotin compound tributyltin also increases E_2 production in human placental choriocarcinoma cells [37]. Tributyltin has been demonstrated to induce the superimposition of male sex organs, such as a penis and/or a vas deferens, over female sex organs, which is a phenomenon known as imposex [38]. These studies suggest strongly that EDCs might affect fetal development not only by mimicking the actions of sex steroid hormones but also by alteration of in situ steroidogenesis.

In the prostate, AR expressed in mesenchyme is required for directing growth and branching morphogenesis of epithelia, presumably by induction of growth factors [39]. In the present study, fetal exposure to BPA or DES increased *Ar* mRNA expression in E17 UGM of the male, whereas *Esr1* mRNA expression was up-regulated in E17 UGM of the female. Recently, Richter et al. [40] have reported that in vitro BPA treatment stimulates *Ar* and *Esr1* mRNA expression in mesenchymal cells isolated from fetal mouse prostate. Thus, our results support the idea that BPA-induced cell proliferation of the primary prostatic ducts may be caused by inducing *Ar* mRNA expression in the male UGM. In contrast, the induction of *Esr1* mRNA expression by BPA or DES may create a positive-feedback loop in the female UGM. Further investigation and morphological analysis will be necessary to confirm the effects of up-regulated ESR1 in the female UGS.

In conclusion, we have shown the unique action of BPA in the mouse UGS. Specifically, we have demonstrated that the increases in E_2 levels and CYP19A1 (aromatase) activity were observed in the BPA-treated UGS but not in the DES-treated UGS. Ricke et al. [41] have recently reported that stromal hormone imbalance, a potential source of local E_2 production, may be responsible for prostatic disease, such as benign prostatic hyperplasia and prostate cancer. The data in the present study give rise to the concept that the development and differentiation of UGS in mouse fetuses is very sensitive to fetal exposure to low-dose BPA via the mother. Further investigation of various aspects of BPA-specific action is necessary to fully understand the role of BPA as an EDC.

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Short Communication

A simplified PCR assay for fast and easy mycoplasma mastitis screening in dairy cattle

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A simplified polymerase chain reaction (PCR) assay was developed for fast and easy screening of mycoplasma mastitis in dairy cattle. Species of major mycoplasma strains [*Mycoplasma* (*M.*) *bovis, M. arginini, M. bovigenitalium, M. californicum, M. bovirhinis, M. alkalescens* and *M. canadense*] in cultured milk samples were detected by this simplified PCR-based method as well as a standard PCR technique. The minimum concentration limit for detecting *mycoplasma* by the simplified PCR was estimated to be about 2.5×10^3 cfu/mL and was similar to that of the standard PCR. We compared the specificity and sensitivity of the simplified PCR to those of a culture method. Out of 1,685 milk samples cultured in mycoplasma broth, the simplified PCR detected *Mycoplasma* DNA in 152 that were also positive according to the culture assay. The sensitivity and specificity of the simplified PCR were 98.7% and 99.7%, respectively, for detecting mycoplasma in those cultures. The results obtained by the simplified PCR were consistent with ones from standard PCR. This newly developed simplified PCR, which does not require DNA purification, can analyze about 300 cultured samples within 3 h. The results from our study suggest that the simplified PCR can be used for mycoplasma mastitis screening in large-scale dairy farms.

Keywords: bovine, mastitis, mycoplasma, simplified PCR

Mycoplasma are highly contagious pathogens and intramammary infection by these species is a serious problem in dairy herds [7]. Clinical cases of mycoplasma mastitis exhibit severe clinical symptoms including fever along with swelling and induration of the udder [3]. Abnormal milk with flaky sediments in watery or serous fluid is also observed in the infected quarter. Since the cure rate of clinical mastitis caused by *Mycoplasma* spp. is very low because of their resistance to antibiotic therapy, *Mycoplasma*-infected cows must be culled in an emergency to prevent an outbreak of mycoplasma mastitis in dairy herds [5,6,8]. Polymerase chain reaction (PCR) is an accurate method for diagnosing Mycoplasma infection [2,4,9,10]. PCR has been widely accepted as a reliable method for detecting mycoplasma strains in milk samples. However, DNA extraction from a large number of milk samples is labor-intensive for laboratories in veterinary hospitals. Fast and easy screening for mycoplasma mastitis using a simplified PCR would make it possible to isolate infected cows from herds and prevent outbreaks mycoplasma mastitis on commercial dairy farms. This study describes the development of a simplified PCR assay for fast and easy mycoplasma mastitis screening on commercial dairy farms.

The following bacteria strains were used: *Mycoplasma* (*M.*) *bovis* (ATCC 25523), *M. arginini* (ATCC 23838), *M. bovigenitalium* (ATCC 19852), *M. californicum* (ATCC 33461), *M. bovirhinis* (ATCC 27748), *M. alkalescens* (ATCC 29103), and *M. canadense* (ATCC 29418). Additionally, each species of two Mycoplasma strains isolated from cases of naturally occurring mycoplasma mastitis were used for this study. All strains were grown in mycoplasma culture broth (Kanto Kagaku, Japan) at 37°C for 72 h.

Simplified PCR was performed in a total reaction volume of 20 μL containing 10 μL of 2 × Ampdirect Plus (Shimadzu, Japan), 0.5 U of Nova taq TM Hot Start DNA polymerase (Novagen, UK), 5 pmol of a mycoplasma universal primer set (MycoAce; Nihon Dobutsu Tokusyu Shindan, Japan),

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and 5 μL of each mycoplasma suspension (10^3, 10^4, and 10^5 cfu/mL), which were equal to 2.5×10^2, 2.5×10^3, and 2.5×10^4 cfu/mL. The DNA extraction and purification is unnecessary for this PCR method. PCR was performed in an iCycler PCR System (Biorad, USA). Conditions for the simplified PCR were as follows: initial denaturation at 95°C for 10 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 45 sec, and extension at 72°C for 1 min. The PCR products were separated by electrophoresis on 1.5% (w/v) agarose gels, stained with ethidium bromide, and visualized with a UV transilluminator.

To compare the performance of the simplified PCR with that of standard PCR, we performed a standard PCR assay as previously described [2]. Briefly, DNA from a Mycoplasma suspension was extracted using a DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions. Standard PCR was performed in a total reaction volume of 20 μL containing 10 × buffer (GE Healthcare, UK), 0.5 U Taq DNA polymerase (GE Healthcare, UK), 4 mM dNTPs, 5 pmol mycoplasma universal primer set, and 5 μL of DNA template which was equal to 2.5×10^2, 2.5×10^3, and 2.5×10^4 cfu/mL of *Mycoplasma*. Conditions of the PCR and electrophoresis were the same as those for the simplified PCR described above.

To evaluate the usefulness of the simplified PCR on commercial dairy farms, we compared the sensitivity and specificity of the simplified PCR to that of a culture method. A total of 1,683 quarter milk samples from lactating cows were randomly collected from 18 commercial dairy farms. A total of 159 milk samples were collected from quarters with clinical symptoms such as swelling, induration, and flare. We confirmed that 202 milk samples were collected from quarters that showed no clinical symptoms but had high somatic cell counts ($> 400 \times 10^3$ cfu/mL). One hundred μL of milk sample were used to inoculate 2.9 mL of mycoplasma broth (Kanto Kagaku, Japan) and incubated at 37°C for 72 h. One hundred μL of the broth culture were then plated on a *Mycoplasma* agar plate (Kanto Kagaku, Japan) and incubated in 5% CO_2 at 37°C for 14 to 28 days to produce typical *Mycoplasma* colonies [7]. Each broth culture was analyzed using the simplified and standard PCR as described above.

In this study, we compared the detection rates for seven major *Mycoplasma* spp. of the simplified and standard PCR assays. All ATCC strains were clearly detected by both methods (Fig. 1). Seven species of two mycoplasma strains isolated from animals with mycoplasma mastitis were also detected by both simplified and standard PCR (data not shown). Gene sequences of PCR-amplified products showed 99% homology with documented sequences in an established gene bank (intergenic spacer region). The minimum detection limit for Mycoplasma by simplified PCR was estimated to be 2.5×10^3 cfu/mL (Fig. 2). The results obtained from the

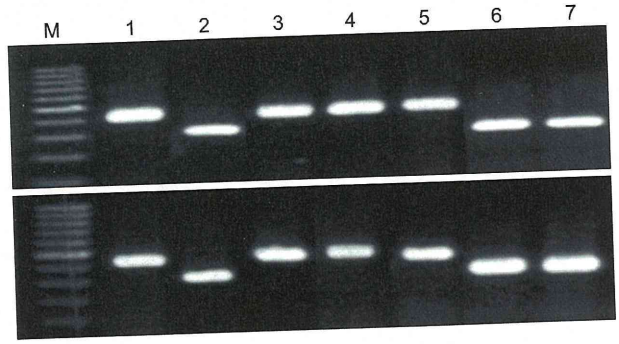

Fig. 1. Detection of major mycoplasma mastitis pathogens by simplified (upper) and standard (lower) PCR. M. marker, 1: *Mycoplasma* (*M.*) *bovis* (ATCC 25523), 2: *M. arginini* (ATCC 23838), 3: *M. bovigenitalium* (ATCC 19852), 4: *M. californicum* (ATCC 33461), 5: *M. bovirhinis* (ATCC 27748), 6: *M. alkalescens* (ATCC 29103), 7: *M. canadense* (ATCC 29418).

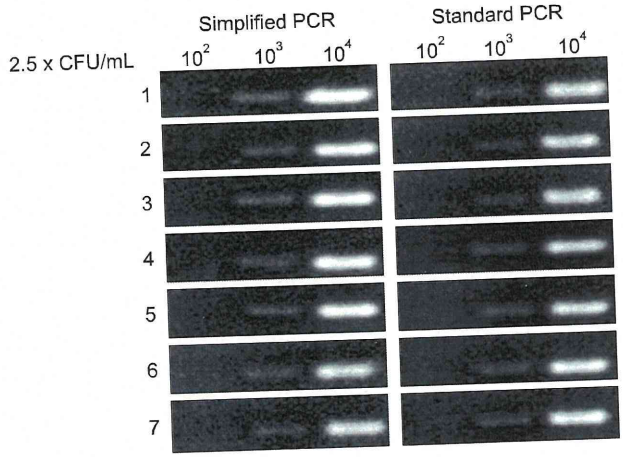

Fig. 2. Minimum limits of detection for major mycoplasma mastitis pathogens by simplified PCR and standard PCR. 1: *M. bovis* (ATCC 25523), 2: *M. arginini* (ATCC 23838), 3: *M. bovigenitalium* (ATCC 19852), 4: *M. californicum* (ATCC 33461), 5: *M. bovirhinis* (ATCC 27748), 6: *M. alkalescens* (ATCC 29103), 7: *M. canadense* (ATCC 29418).

simplified PCR agreed well with those from standard PCR using purified DNA from broth cultures. Our results showed that simplified PCR for detecting *Mycoplasma* spp. permits gene amplification without any DNA preparation and performs as well as standard PCR. In this study, we used Amdirect Plus for the simplified PCR to detect *Mycoplasma* spp. in broth cultures. Ampdirect Plus is a commercially available reagent used for preparing PCR samples without DNA extraction and purification [1]. We have confirmed that the use of Ampdirect Plus reduces labor and time for detecting *Mycoplasma* spp. in samples.

Out of the 1,685 milk samples cultured in mycoplasma broth, simplified PCR detected Mycoplasma DNA in 152 samples that were also positive according to the culture

assay. The concentration of Mycoplasma in these broth cultures was more than 1×10^4 cfu/mL. Four samples were found to be negative by culture and positive by simplified PCR. We speculated that the amplification of DNA from non-viable Mycoplasma in the broth cultures caused the differences between the simplified PCR and culture method results. Two samples were found to be negative by the simplified PCR and positive by culture assay. We confirmed that the concentrations of Mycoplasma in these cultured broths were 2×10^2 and 3×10^2 cfu/mL, which are less than the minimum detection limit of the simplified PCR. Both milk samples were obtained from cows with no clinical symptoms and normal somatic cell counts. Our results suggested that the number of Mycoplasma in the broth cultures of a few milk samples was insufficient for simplified PCR. These samples were not further investigated. The sensitivity and specificity of the simplified PCR method were 98.7% and 99.7%, respectively, of those of the culture assay. Results from the simplified PCR assay completely concurred with those obtained by standard PCR. It has been reported that the sensitivity and specificity of standard PCR for detecting Mycoplasma are 96.2% and 99.1%, respectively, of those of the culture method [2]. In the present study, we succeeded in establishing a simplified PCR method that is able to provide the results identical to those obtained using conventional culture and standard PCR methods.

Standard PCR techniques are labor-intensive and time-consuming, making this method impractical for assaying a large number of milk samples from a commercial dairy farm. This method requires many steps that are quite lengthy; processing about 300 samples requires at least 4 ~ 5 h for DNA template preparation and 3 h for completing the PCR cycle. In contrast, 300 samples to be examined within 3 h using the simplified PCR, which does not require several of these laborious steps including Mycoplasma DNA template isolation and purification. Fast and easy screening for mycoplasma mastitis using a simplified PCR assay would enable the quick isolation of infected cows from herds. Our newly developed simplified PCR assay for detecting *Mycoplasma* spp. is a useful method that can be used to help control and prevent mycoplasma mastitis outbreaks on commercial dairy farms.

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Short Communications

Prevalence of *Mycoplasma* species in bulk tank milk in Japan

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Mycoplasma species are highly contagious pathogens and their ability to cause intramammary infection is a serious problem on dairy farms (Nicholas and Ayling 2003). Since the cure rate of clinical mastitis caused by *Mycoplasma* species is very low due to difficulties in antibiotic therapy, *Mycoplasma*-infected cows on farms must be culled in an emergency to prevent outbreaks of *Mycoplasma* mastitis (Nicholas and Ayling 2003). Bovine *Mycoplasma* mastitis was first reported in 1962 (Hale and others 1962). However, little is known about the prevalence of *Mycoplasma* mastitis on dairy farms in Japan. In this study, the prevalence of *Mycoplasma* species in bulk tank milk from dairy farms in Japan was investigated.

A total of 1241 commercial dairy farms (n=45 to 1125 cows/farm) were randomly selected for bulk tank milk screening. The samples were collected from April to September 2010. Each bulk tank contained milk from two days of production. Milk samples were aseptically collected into 50 ml tubes. One hundred microlitres of milk samples were inoculated into 3.0 ml of *Mycoplasma* broth (Kanto Kagaku; Tokyo) and then incubated at 37°C for 72 hours. One hundred microlitres of *Mycoplasma*-enriched culture broth was plated on a *Mycoplasma* agar plate (Kanto Kagaku; Tokyo) and incubated in 5 per cent CO_2 at 37°C for one month for the development of typical *Mycoplasma* colonies (Nicholas and Ayling 2003). PCR for the detection of *Mycoplasma* species in cultured broth was performed according to the method described by Higuchi and others (2011). Identification of major *Mycoplasma* species including *M bovis*, *M arginini*, *M bovigenitalium*, *M californicum*, *M bovirhinis*, *M alkalescens* and *M canadense* was performed by DNA sequence analysis.

Mycoplasma species were isolated from bulk tank milk obtained from 16 (1.29 per cent) of 1241 farms screened in this study. These results are similar to the results of a previous study carried out in the USA, which showed that the prevalence of *Mycoplasma*-positive samples from bulk tank milk ranged from 1.8 to 5.8 per cent (Kirk and others 1997). Seven species of *Mycoplasma*, *M bovis* (0.56 per cent, n=7), *M californicum* (0.24 per cent, n=3), *M canadense* (0.24 per cent, n=3), *M arginini* (0.16 per cent, n=2), *M bovigenitalium* (0.08 per cent, n=1), *M alkalescens* (0.08 per cent, n=1) and *M bovirhinis* (0.08 per cent, n=1), were isolated from the bulk tank milk samples collected in this study. Intramammary infection with *M bovis* is difficult to treat, and culling of infected cows and loss of milk production can lead to significant economic loss for a dairy farm (Nicholas and Ayling 2003). It is recommended that *Mycoplasma*-infected cows on farms be segregated from healthy animals as soon as they are identified to reduce the risk of spread in an outbreak (Bicknell and others 1983). Mastitis due to *M bovis* has been studied by epidemiological investigations of bulk tank milk in France (Arcangioli and others 2011), New Zealand (McDonald and others 2009), Australia (Jelinek and others 1993), Belgium (Passchyn and others 2011) and the USA (Jasper and others 1979), with the prevalence varying from 0 (New Zealand) to 4 per cent (California, USA). This short communication is the first to report on the prevalence of *M bovis* in bulk tank milk of commercial dairy farms in Japan.

It was confirmed that outbreaks of *Mycoplasma* mastitis on two dairy farms (farm 5, 34.78 per cent; farm 16, 24.76 and 2.38 per cent) were caused by *M bovis*. All of the infected cows showed severe clinical symptoms, including decreased milk production, and swelling and induration of the udder. Similar clinical cases were observed on other *M bovis*-infected farms (farms 10 to 13 and 15) and *M californicum*-infected farms (farms 3, 4 and 14). However, except for a slight increase in somatic cell counts in the milk samples (3.3-4.1x10^5/ml), clear clinical symptoms were not detected in the udders of cows infected with *M arginini*, *M alkalescens*, *M canadense*, *M bovigenitalium* and *M bovirhinis*. These results suggested that periodic inspection of bulk tank milk to detect *Mycoplasma* species, especially *M bovis* and *M californicum*, is

TABLE 1 Prevalence of *Mycoplasma* species in bulk tank milk of 16 commercial dairy farms in Japan

Herd size		Bulk tank milk			Cows		
Farm Number	(cows/herd)	Culture	PCR	*Mycoplasma* species	Number of infected cows	(%)	*Mycoplasma* species
1	50	+	+	*M bovigenitalium*	1	2.00	*M bovigenitalium*
2	59	+	+	*M arginini*	1	1.69	*M arginini*
3	60	+	+	*M californicum*	5	8.33	*M californicum*
4	64	+	+	*M californicum*	5	7.81	*M californicum*
5	69	+	+	*M bovis*	24	34.78	*M bovis*
6	98	+	+	*M alkalescens*	1	1.02	*M alkalescens*
7	102	+	+	*M canadense*	3	2.94	*M canadense*
8	105	+	+	*M bovirhinis*	2	1.90	*M bovirhinis*
9	112	+	+	*M canadense*	2	1.79	*M canadense*
10	120	+	+	*M bovis*	4	3.33	*M bovis*
11	162	+	+	*M bovis*	4	2.47	*M bovis*
12	166	+	+	*M bovis*	3	1.81	*M bovis*
13	180	+	+	*M bovis*	11	6.11	*M bovis*
14	258	+	+	*M californicum*	18	6.98	*M californicum*
15	358	+	+	*M bovis*	15	4.19	Only *M bovis*
		+	+	*M arginini*	5	1.40	Only *M arginini*
					3	0.84	*M bovis*+*M arginini*
16	420	+	+	*M bovis*	104	24.76	Only *M bovis*
		+	+	*M canadense*	15	3.57	Only *M canadense*
					10	2.38	*M bovis*+*M canadense*

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