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
RXRα	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

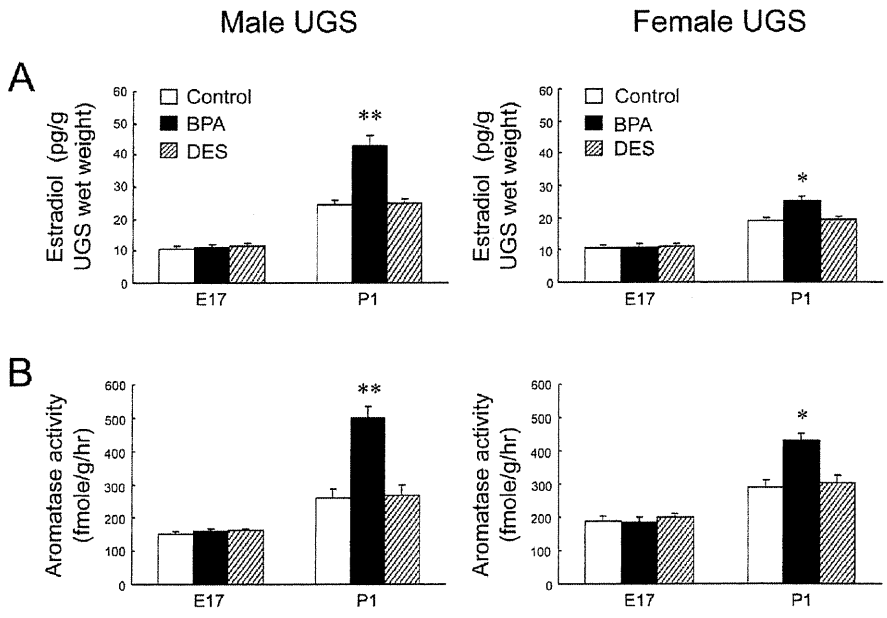

FIG. 1. BPA-specific increases of E_2 levels and CYP19A1 (aromatase) activity in mouse UGS. E_2 levels (**A**) and CYP19A1 (aromatase) activity (**B**) were measured in the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar) at E17 and P1. $*P < 0.01$, $**P 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

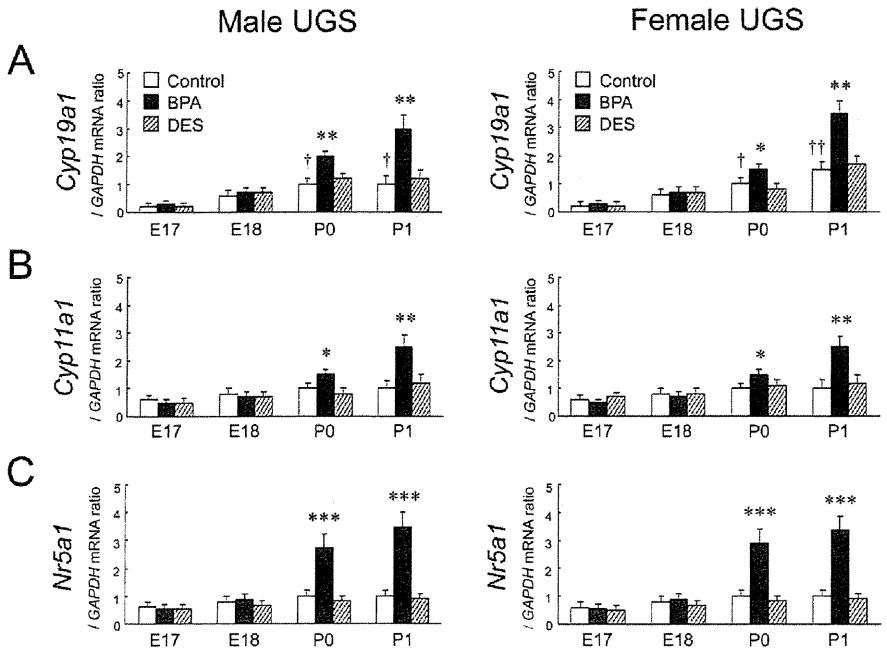

FIG. 2. BPA-specific up-regulation of steroidogenic enzyme and sex-determining gene mRNA in mouse UGS. The relative mRNA expressions of *Cyp19a1* (**A**), *Cyp11a1* (**B**), and *Nr5a1* (**C**) were determined in the untreated control (open bar), BPA-treated UGA (closed bar), and DES-treated UGS (slashed 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

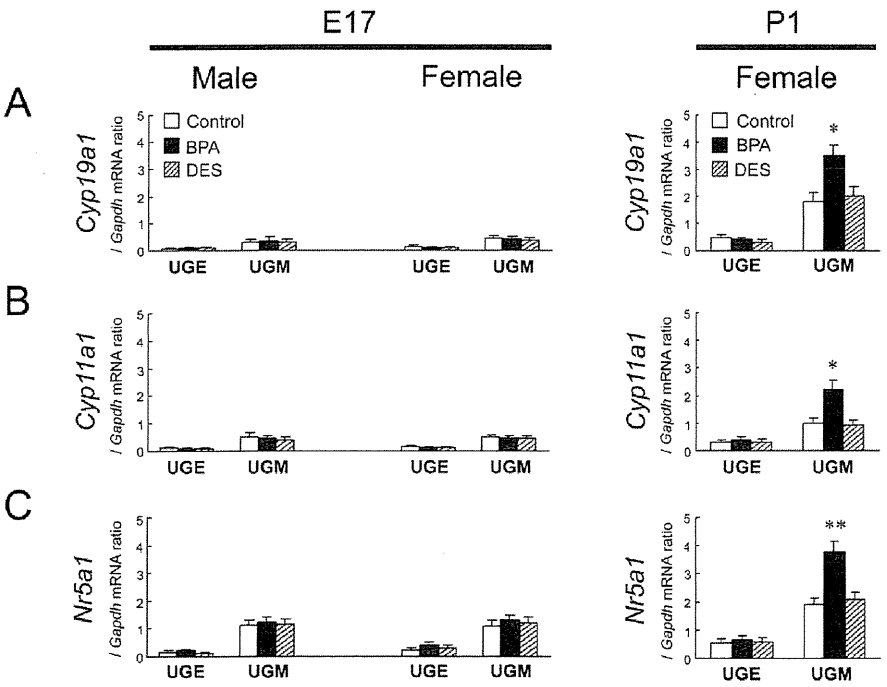

FIG. 3. Restricted BPA-specific up-regulation of steroidogenic enzyme and sex-determining gene mRNA in UGE and UGM. The relative mRNA expressions of *Cyp19a1* (**A**), *Cyp11a1* (**B**), and *Nr5a1* (**C**) were determined for UGE and UGM of the untreated control (open bar), BPA-treated UGS (closed bar),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

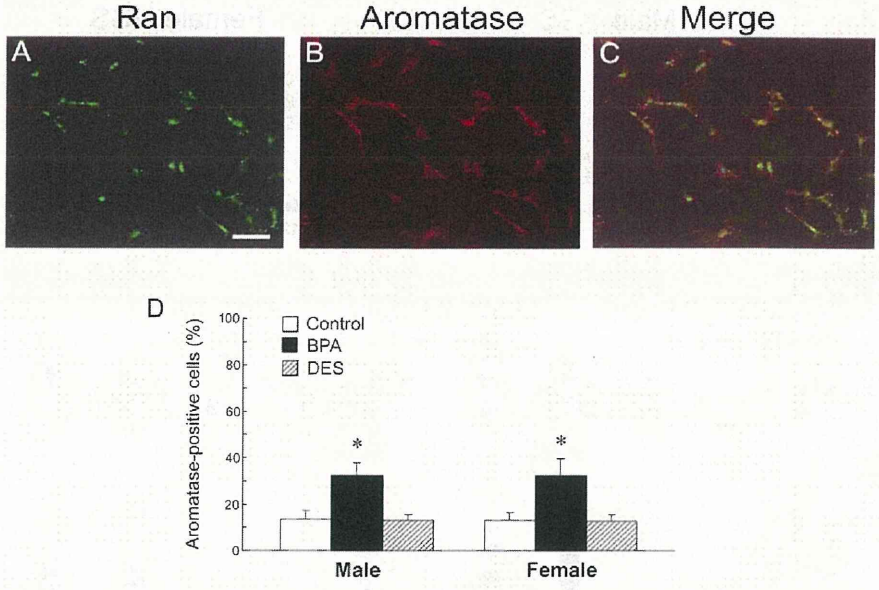

FIG. 4. BPA-specific increases of aromatase-expressing cells in primary cultured UGM. **A–C**) Fluorescence signals were detected for the CYP19A1 (aromatase) protein in primary cultured UGM. The nuclei were identified by Ran staining. Bar = 100 μm, magnification ×400. **D**) The number of aromatase-positive 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

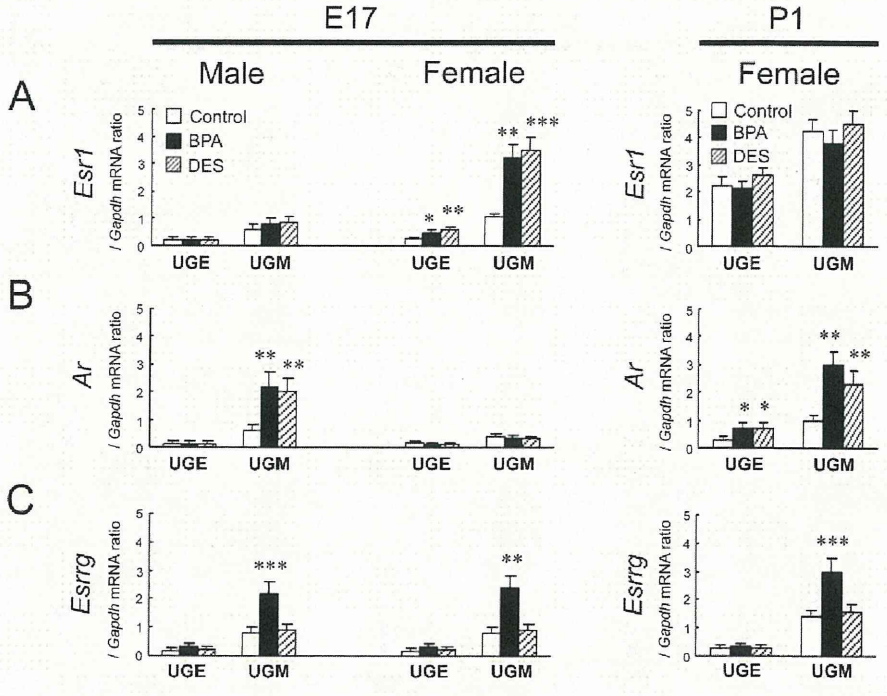

FIG. 5. Restricted BPA-specific up-regulation of *Esrrg* mRNA in UGE and UGM. The relative mRNA expressions of *Esr1* (**A**), *Ar* (**B**), and *Esrrg* (**C**) were determined in UGE and UGM of the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar) at E17 and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

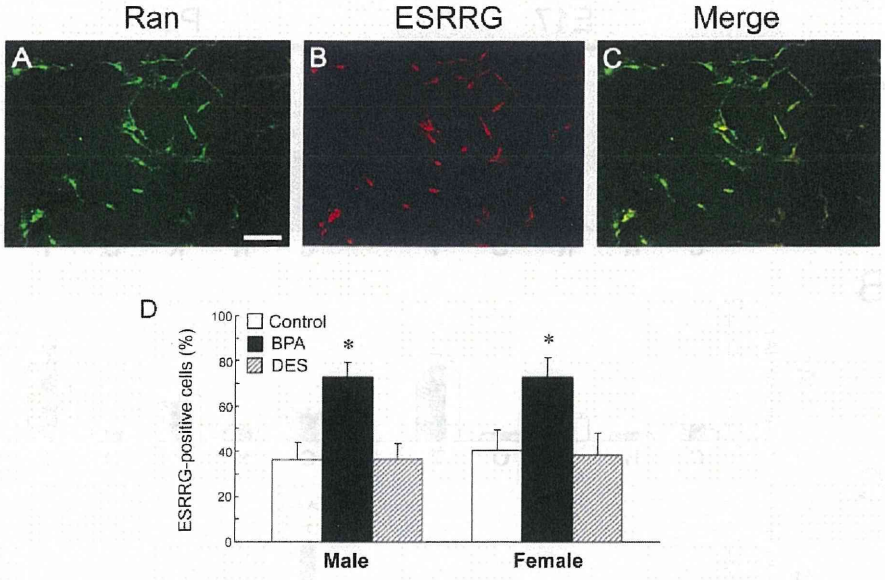

FIG. 6. BPA-specific increases of ESRRG-expressing cells in primary cultured UGM. **A–C**) Fluorescence signals were detected for the ESRRG protein in primary cultured UGM. The nuclei were identified by Ran staining. Bar = 100 μm, magnification ×400. **D**) The number of ESRRG-positive cells was counted 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

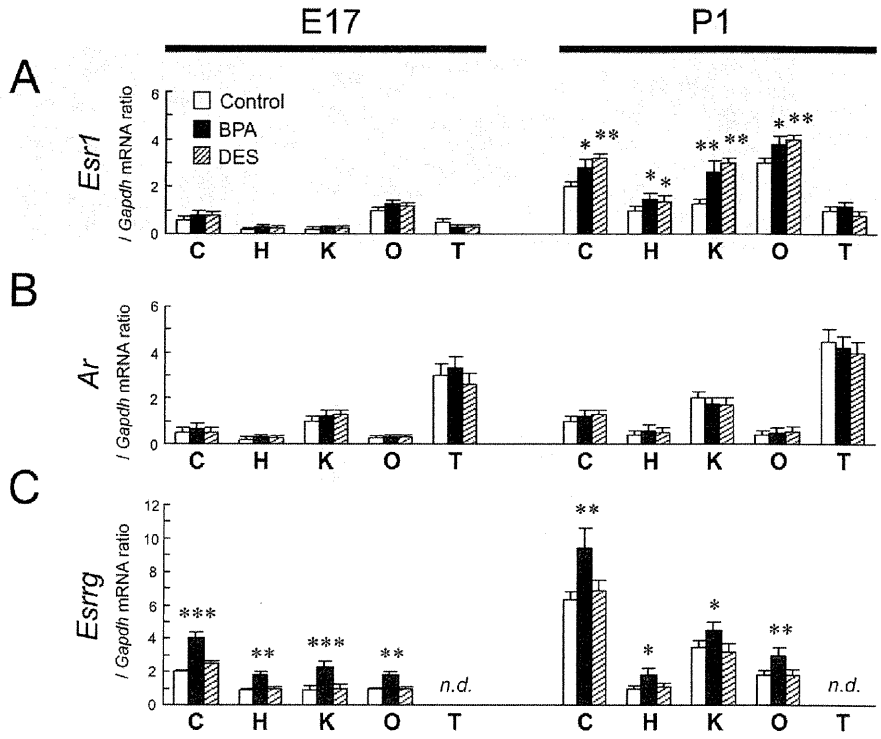

FIG. 7. BPA-specific up-regulation of *Esrrg* mRNA in sex steroid hormone-related organs. The relative mRNA expressions of *Esr1* (**A**), *Ar* (**B**), and *Esrrg* (**C**) were determined in sex steroid hormone-related organs of the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated 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,

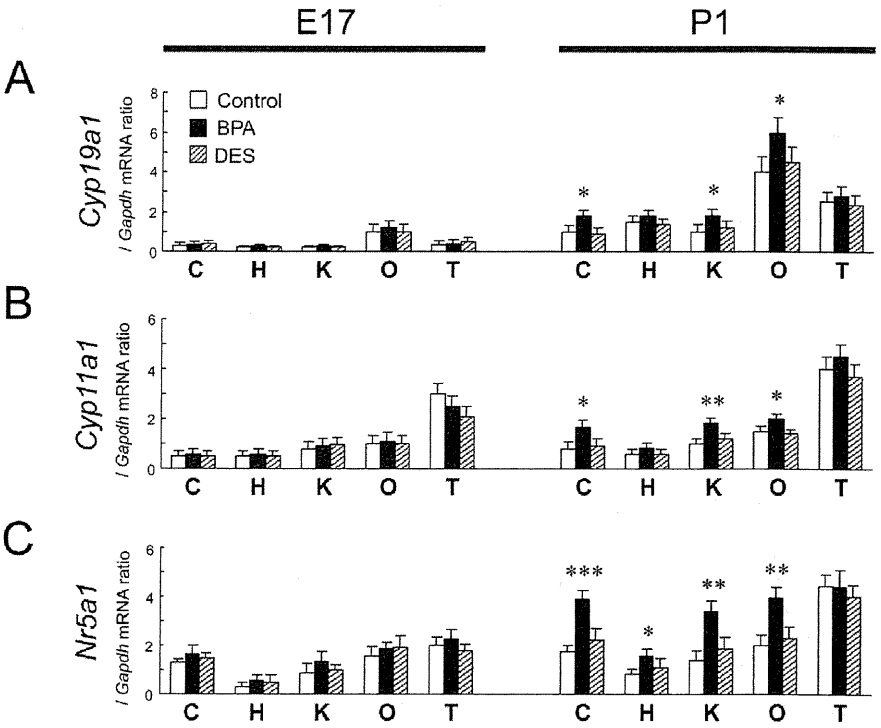

FIG. 8. BPA-specific up-regulation of steroidogenic enzyme and sex-determining gene mRNA in sex steroid hormone-related organs. The relative mRNA expressions of *Cyp19a1* (**A**), *Cyp11a1* (**B**), and *Nr5a1* (**C**) were determined in sex steroid hormone-related organs of the untreated control (open 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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Social engineering for virtual 'big science' in systems biology

Hiroaki Kitano, Samik Ghosh & Yukiko Matsuoka

A new type of big science is emerging that involves knowledge integration and collaboration among small sciences. Because open collaboration involves participants with diverse motivations and interests, social dynamics have a critical role in making the project successful. Thus, proper 'social engineering' will have greater role in scientific project planning and management in the future.

Scientific projects with large amounts of funding to achieve a defined mission are often referred to as 'big science'. Successful big science projects should have a clearly defined goal, a possible means to achieve it and a strong sociological rationale to justify public funding of such endeavors. Similarly, the type of project that can be widely supported beyond the scientific community depends on societal needs at the time.

Although most biology has been and continues to be small science, the Human Genome Project and other genome projects are considered big science in biology. A defining feature of these projects is a large-scale engineering effort designed in support of a specific scientific aim. Projects involving particle colliders and genome sequencing are essentially equipment-driven data-acquisition projects, and such projects will continue to provide new findings through equipment advances.

There is a related desire to obtain a comprehensive understanding of specific cellular systems and biological processes through high-throughput methods. Emergence of systems biology as mainstream biology is accelerating this tendency, because it often requires measurements and analysis of various large-scale and multifaceted data. At the same time, new knowledge critical for in-depth and precise understanding of systems is often derived from small science. This means that a new type of big science is needed that consolidates data and knowledge not only from large-scale projects, but also from discoveries by small science.

It is therefore inevitable that a 'virtual' big science will form, connecting large numbers of researchers around the globe to attain large-scale knowledge integration in an emergent manner. The implication is that such an initiative must have widely acceptable objectives, leadership and proper sociological design to make it sustainable.

There is an impossible number of problems that have yet to be resolved in the biomedical field, and some of these would benefit from being included in big science projects, either in a deliberate and organized or in a more emergent manner. For example, in the numerous cases of diseases for which effective cures are not available but are being proposed and developed, problems remain. Indeed, the cost of drug discovery is so high that it puts severe pressure on the public medical system and impedes access to drugs for underprivileged segments, which in turn prevents development of drugs for rare diseases or those that are prevalent in the poorest areas of the world.

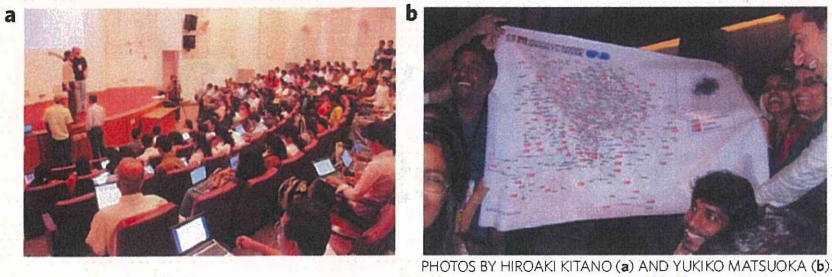
The rising cost of drug discovery affects all segments of society. With increased understanding of individual genomic variations and their impacts on drug efficacy and side effects, we can envision an era of personalized medicine in which patients are selected on the basis of genetic and biochemical differences that underlie different responses to drugs. This would help patients minimize side effects and help health-care systems eliminate considerable misdirected cost. At the same time, it may also mean substantial revenue reduction for pharmaceutical companies. Research and development (R&D) as an industry, and thereby the ability to find possible cures for orphan diseases, may not be sustainable unless drastic reductions in R&D costs are achieved.

Cost and access to medical services is a critical factor for the base-of-the-pyramid segment of the population. For example, tuberculosis is still a major killer in developing countries, as 9.27 million new infections were reported in 2007 globally, with a significant percentage of them being multidrug resistant and some being extensively drug resistant[1]. Yet only a handful of drug-discovery projects exist because of the mismatch between the investment of R&D and the ability of countries where tuberculosis is prevalent to afford treatment. Unless cost-effective drug development can be achieved, those who suffer from neglected diseases will not be saved. Developing technologies to substantially mitigate these problems is socially valuable.

Knowledge integration at all levels

One of the fundamental causes of low productivity in drug discovery is a lack of in-depth understanding of the complexity of biological systems and a means of predicting potential outcomes of candidate compounds when used in cells, model animals and patients (http://www.fda.gov/downloads/ScienceResearch/SpecialTopics/CriticalPathInitiative/CriticalPathOpportunitiesReports/ucm113411.pdf and http://www.pwc.com/gx/en/pharma-life-sciences/pharma-2020/pharma-2020-vision-path.jhtml). Proper introduction of a system- and network-oriented approach to drug discovery, with prediction capabilities even at the cellular level, is expected to rectify the situation by providing a better understanding of the biology that underlies diseases and, ultimately, by enabling us to use precise computational models of cells, organs and patients. Already, several systems biology efforts are under way and being planned.

Development of precise biological models requires integration of knowledge and data at all levels, from genomics and proteomics to imaging and physiology. Various data from high-throughput experiments provide us with genome-wide characteristics, but small-sciences data must be incorporated to gain an understanding of the detailed mechanisms. For both financial and sociological reasons, no single large-scale project can address a systems-level problem truly comprehensively. For instance, even if a large-scale project is



PHOTOS BY HIROAKI KITANO (**a**) AND YUKIKO MATSUOKA (**b**).

Figure 1 | Large projects require many enthusiastic and fulfilled participants. (**a**) A scene from a Connect2Decode session, a portion of the OSDD project funded by CSIR, India. More than 200 participants gather for a week for the final assembly of the pathway. Each student is given a laptop and input pathway-interaction data from publications. A detailed workflow of the project is in **Box 1**. (**b**) The map of *Mycobacterium tuberculosis* metabolic pathways on a poster presented by joyous students. The upper right box of the poster shows the entire dense 'hair-ball' network comprising more than 1,000 reactions involved in an entire metabolic network. The main component of the poster represents a part of the network in the central metabolic cycle.

fully funded, it is impractical to assume that sufficiently large numbers of researchers will be willing to focus on a single species and biological problem and put their own established systems aside.

Existing resources such as pathway databases, developed increasingly by manual curation of publications in which curators read all relevant papers one by one for precise computational model developments are also not a viable solution. Pathway databases do not necessarily cover all relevant molecules and interactions, nor are they necessarily accurate. The current gold standard for such databases is manually curated models carefully built from the literature by small groups of people hired by the project, who curate every associated study for a small subsystem[2]. This has been termed 'deep curation' and is exemplified by a series of comprehensive molecular-interaction maps[3,4]. However, the deep curation of large-scale network maps from the literature is extremely labor-intensive and stressful work. Also, it is very difficult at a sociological level to motivate manual curators to continuously update maps and models to keep up with new discoveries over many years. Automated literature mining has been extensively pursued, but replacing manual curators is decades away. At the same time, quality control is dependent upon the individual groups, and updating and correcting errors can be slowed by this centralization. The solution to this problem affects the productivity and practicality of computational approaches for drug discovery.

Is WikiBiology a solution?

One approach toward building a comprehensive and rich data resource is to follow the success of Wikipedia, which is continuously updated and covers every possible subject. There is increasing interest in a Wikipedia-like approach, also called a 'Web 2.0' or 'community-based' approach, in biology. There are several attempts to create Wikipedia-like resources such as WikiGenes and WikiPathways[5], as well as open-access approaches such as Science Commons, which aims to promote extensive sharing of knowledge through a community-driven, bottom-up strategy. Whereas there is often a core group that receives funding to create an initial seed of these types of Web sites, the wiki-like approach fundamentally relies on voluntary contributions from community members.

One of the remarkable features of Web 2.0, such as Wikipedia and Google Earth Community, is the collective contribution of knowledge and experiences to a globally shared Web space. Although considerable resources must be diverted for quality control and prevention of spam[6], services such as Wikipedia have been extremely effective and have grown to be indispensable resources. The question remains, however, whether such a model can be applied to biology successfully.

Motivational and sociological factors are critical to the success of the community-driven system. Skeptical views exist on whether biologists are willing to spend time to provide feedback on community efforts. Indeed, there is no mechanism to reward contribution in any formal way. Contributing to a wiki-based biology site is certainly not a factor in hiring and promotion, nor is it considered an honor within the scientific community[7]. In the extreme case, disclosing one's knowledge may help to speed up a competitor's research. It should be noted that this does not mean that large-scale collaboration for pathway curation is not feasible. In fact, there are efforts to construct consensus pathway maps as a community activity. An example of such a project was the reconstruction of the yeast metabolic network[8]. A series of genome annotation projects fall into the same category. In these projects, members are well defined, and they gain the spoils of authorship on published works. The social dynamics therefore differ and are in fact quite the opposite of those in the Web 2.0 approach.

We should carefully look at reasons why projects such as Wikipedia and Linux have soared and keep flying. In the case of Linux, a hacker culture supported open sourcing and sharing of knowledge, as signified by the Free Software Foundation, founded by Richard Stallman, where contribution to the community at large was the pride of the hackers. At the same time, there was a practical need to develop open-source operating systems as opposed to closed commercial systems. Among the efforts for open-source operating systems exemplified by FreeBSD, Linux survived mainly because it happened at the right moment and had more applications and publications than other initiatives. In the absence of Linux, FreeBSD or other initiatives would have filled this space. Wikipedia essentially inherited a similar culture. Having goals that are widely shared, are exciting and provide a sense of participation has been the key factor driving the community-based initiative. Whether these motivations are sustainable over time is yet to be seen, although certainly they were effective in getting these projects to maturity.

Unfortunately, such a hacker culture does not exist in biology today. At least, it is not a mainstream idea. Short of a formalized recognition system, any chance of a successful Web 2.0 approach will require a cultural shift in the community. Assignment of microimpact factors, or microattribution, for contribution to such an initiative should be considered. However, for such a system to be truly effective, such indices must be a core part of the merit system[9]. The citation index, for example, is simple and widely used as a measure of scientific influence. For microattribution to be accepted in the merit system, it has to acquire universality and attain the same "currency" status as the citation index in merit evaluation. Receiving microattributions in exchange for contributions should still be considered a weak motivating factor. In the most successful projects, people are driven when the vision, passion and dedication of the project are aligned with individual aspirations.

Emergent collaborations in engineering

It is useful to learn from successful emergent collaboration projects in other fields. One of the most successful of these projects in robotics and artificial intelligence is RoboCup[9], an initiative started in the mid-1990s with the aim of developing a series of high-impact technologies. A landmark goal was set that states, "by mid-21st century, a team of fully autonomous humanoid robot soccer players shall win the soccer game, comply with the official rule of the FIFA, against the winner of the most recent World Cup" (http://www.robocup.org/). Over the last 15 years, the project successfully attracted more than 4,000 researchers for scientific and technological research and hundreds of thousands of students from elementary to undergraduate levels for educational practices from at least 35 countries annually. There is no central research funding, so each researcher acquires his or her own funding. Extensive collaborations are taking place without top-down coordination. The governing body, the RoboCup Federation, only organizes the annual convention, publishes proceedings and approves regional activities, akin to an academic society. However, what is different from academic societies is that all the federation's activities are ultimately focused on achieving a defined goal rather than general promotion of the field. At the same time, it is not a traditional project, because there is no principal investigator (PI) to govern it as a whole, and the management team of the RoboCup Federation is democratically elected every year from the community. This seems to promote a sense of ownership of the project, and it thereby avoids becoming an orphan.

This emergent collaboration is sustainable for the following reasons: the goal is wildly exciting, widely shared and understood even by nonscientists; it is clear that the goal cannot be achieved by a single group or country; collaboration to promote the technology benefits everyone involved; and participants have the satisfaction of knowing that some spin-off activities have emerged that contribute to major humanitarian efforts, such as RoboCup Rescue, which was involved in the rescue operation at the World Trade Center on 9/11 (ref. 10) and more recently in the earthquake in Japan on 11 March. In addition, contributions to RoboCup offer professional opportunities beyond participation in the project itself, and many contributions will be published as well.

With regard to knowledge sharing, a principle is imposed in emergent collaborations that technical details must be disclosed at the end of the annual world convention. In some cases, facilitating a multidirectional open flow of knowledge involves mandatory disclosure of technical details and source code as a precondition for obtaining access to the rich source-code repository. This feature distinguishes this type of large-scale emergent collaboration from a wiki-type project. Proper means of collaboration would be different based on the interest of potential stakeholders, and it is critical to choose the right level of incentive mechanisms, commitment and benefits for each interested party[11]. RoboCup is one of the rare projects in which vision, social appeal, passion and ego fulfillment have been well aligned with the professional and personal aspirations of the participants.

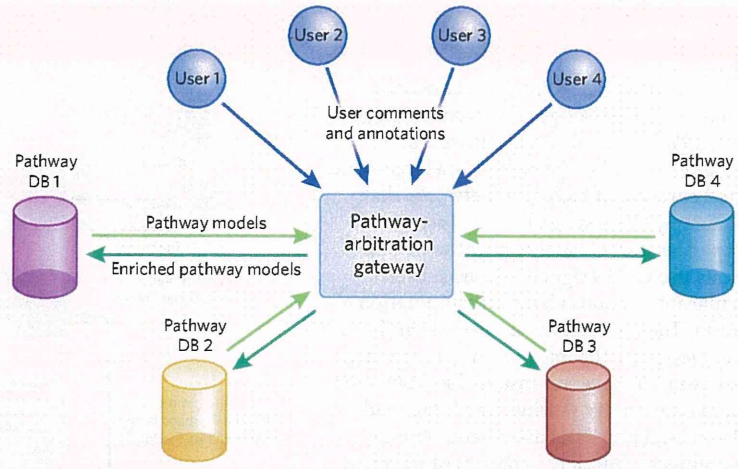


Figure 2 | Conceptual diagram of an open-flow model of knowledge sharing and integration. In the open-flow model, contributors benefit as well. Each pathway-database provider may contribute his or her pathway information to enrich and correct pathways placed on the open-flow gateway or an arbitration site, then may also obtain information enriched through other pathway databases (DB) as well as individual contributors. This model is essentially a combination of community-based pathway annotation, Creative Commons Attribution-ShareAlike and RoboCup-like social engineering.

Toward the open-flow paradigm of knowledge aggregation

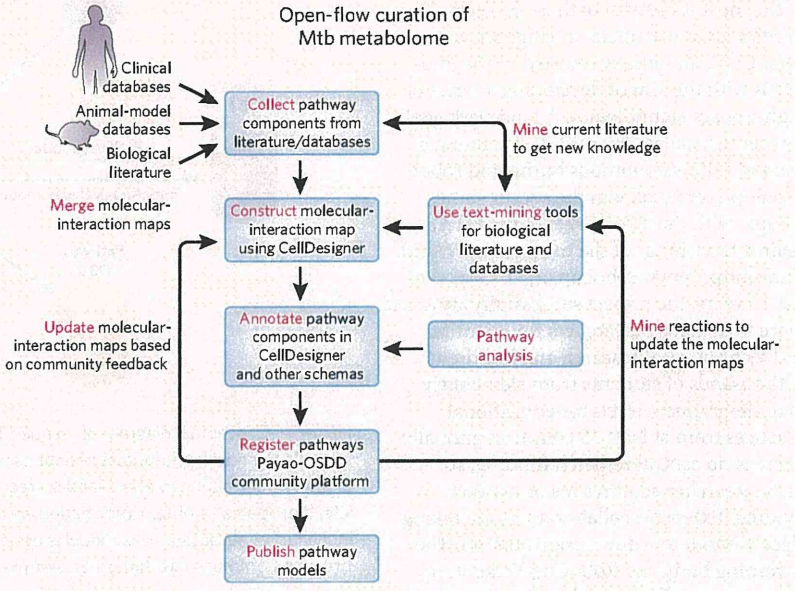
The field of drug discovery for neglected diseases could meet the criteria for a successful emergent collaboration project. It is a good cause, is socially appealing, needs collective efforts and can impart a sense of pride to participants for their contributions. A recent attempt is the Open Source Drug Discovery (OSDD) project initiated by the Council of Scientific and Industrial Research (CSIR), a branch of the Indian government[12]. OSDD is aimed specifically at drug discovery for tuberculosis through open collaboration. As an initial phase of the program, major funding within India was allocated for genome annotation, network reconstruction and a set of initial screens. More than 830 researchers and students are participating in the project, with 200 students tasked with curating the literature to construct a comprehensive and detailed metabolic map of *Mycobacterium tuberculosis* (**Fig. 1**). Unlike past efforts for collective pathway reconstruction and curation, such as the yeast metabolic map initiative, any researcher or student can join the effort on a volunteer basis, with a core principal investigator team having received funding from CSIR to drive the project in a sustainable manner. Thus, this project is distinctively open ended in terms of both quantity and quality of participation.

This project satisfies some of the criteria for a successful emerging project, such as having a clear and appealing goal and an alignment with participant motivations. A distributed and collective pathway reconstruction that was performed with the OSDD project was a true social experiment. With this experiment, a large-scale distributive reconstruction of biological networks was shown to be possible with the proper software platform, a well-defined workflow (**Box 1**), and project management when the objectives of the project were designed to motivate potential participants.

Unresolved issues include quality assurance over time and the need for continuous maintenance and updating of the map. Although not quite a strategy, the hope is that some of the participants will continue to work on these issues, but owing to the open-ended and emergent nature of the project, this cannot be expected or guaranteed. An alternative to the continued work by the participants is a call to the wider community of scientists for verification in a wiki-based challenge. Because of the

Box 1 | Distributed pathway reconstruction workflow

A project-wide distributed reconstruction has been tested using CellDesigner network editing software[18], a Payao-based community curation system[19], and a workflow (at right) for a large-scale distributed curation of biological networks that was provided by the Systems Biology Institute, Tokyo, Japan, as part of an agreement with CSIR. Participants use CellDesigner to draw subsets of molecular interactions along with annotation information. The interactions are merged and relaid out in CellDesigner. The interaction data are then sent to a Payao-based server installed in Tokyo for storage and community sharing. The integrated molecular-interaction database is accessible to all team members. Multiple iterations of pathway construction and integration take place. During this process, members can add notes and comments on the interaction map. After a few months of distributed curation sessions, everyone from the Indian side and Tokyo side got together in Delhi for a week for the final assembly of the entire network. Mtb, *Mycobacterium tuberculosis*.



social significance of finding an effective cure for drug-resistant tuberculosis, such a proposal may attract people who are willing to contribute even without personal or professional benefit. Medical practitioners, retired scientists and those with scientific backgrounds who are not directly engaged in science as their profession may be willing to participate in such a challenge. It is critical, therefore, to design the project in the context of proper social dynamics so that potential contributors become and remain motivated. Of course, this may still be wishful thinking and therefore may fail to generate sufficient contributions.

A more deliberate strategy is to apply the open-flow approach among pathway-database providers. As with the code-access principle in RoboCup, the basic tuberculosis map could be made accessible to pathway-database providers under an appropriate licensing scheme such as the Attribution-ShareAlike licensing in Creative Commons. This allows pathway-database providers to integrate the tuberculosis map into their database, but any corrections and enrichment they make are to be shared with the community. Creating a central gateway to various pathway databases would allow for the integration of currently fragmented knowledge. An integrated collection of pathway databases would also allow for a centralized forum for feedback to the original map providers. Such bidirectional and open exchange of information among the community and data-resource providers is certainly critical in maintaining quality data (**Fig. 2**). In the long run, it may solve the intrinsic trade-off between quality and coverage inherent to current pathway databases. The flagship project for tuberculosis can be used as a proof of concept, and similar efforts such as the GlaxoSmithKline deposition of malaria-related compounds to ChEMBL may provide the other case of open collaboration[13].

It should be noted that such widespread and large collaborations are possible now because of the development of various standards and software that complies with those standards. Systems Biology Markup Language (SBML)[14], Systems Biology Graphical Notation (SBGN)[15] and BioPAX[16] all ensure a certain level of interoperability. However, technology alone cannot make things work, particularly when projects necessarily involve large numbers of parties with varying motivations, career aspirations and opinions. Broader social consideration can be a major key for success when launching increasingly complex projects[17]. Social engineering will be recognized as an indispensable part of research activity in the coming years for large-scale and complex big science, because it is the people who do science, not technology or machines. ■

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Tissue Specific subnetworks and characteristics of publicly available human protein interaction databases

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ABSTRACT

Motivation: Protein-protein interaction (PPI) databases are widely used tools to study cellular pathways and networks, however there are several databases available that still do not account for cell type-specific differences. Here, we evaluated the characteristics of six interaction databases, incorporated tissue-specific gene expression information and finally, investigated if the most popular proteins of scientific literature are involved in good quality interactions.

Results: We found that the evaluated databases are comparable in terms of node connectivity (i.e., proteins with few interaction partners also have few interaction partners in other databases), but may differ in the identity of interaction partners. We also observed that the incorporation of tissue specific expression information significantly altered the interaction landscape and finally, we demonstrated that many of the most intensively studied proteins are engaged in interactions associated with low confidence scores. In summary, interaction databases are valuable research tools but may lead to different predictions on interactions or pathways. The accuracy of predictions can be improved by incorporating datasets on organ- and cell type-specific gene expression, and by obtaining additional interaction evidence for the most 'popular' proteins.

Availability: Supplementary information is available at the Bioinformatics Journal website.

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1 INTRODUCTION

Traditionally, studies that assess the cellular metabolism, disease and cancer development, pathogens infections, or drug-protein interaction have focused on single genes or proteins. While such studies have created large amounts of data, they typically do not account for the multiple interactions that regulate cellular networks. Recently, high-throughput approaches including yeast two-hybrid screens (Rual, et al., 2005), immunoprecipitation studies followed by mass-spectrometry analysis (Ewing, et al., 2007), transcriptomics (Wilhelm, et al., 2008) and metabolomics studies (Braaksma, et al., 2011) have become important research tools to identify protein-protein interaction partners (Krogan, et al., 2006) or cellular factors that are up- or down-regulated in response to specific stimuli (Bhattacharya, et al., 2004). With the availability of the resulting large datasets, the challenge now lies in the generation of comprehensive and robust interactome maps, ideally capturing all protein-protein interactions within a cell and between cells at any given moment in time.

The human proteome is estimated to encompass 130,000–650,000 protein-protein interactions (Stumpf, et al., 2008; Venkatesan, et al., 2009). Of those, only a subset has been described at this point, establishing protein-protein interaction (PPI) databases that provide valuable information about the reactions occurring at the proteome level. Previous studies analyzed and compared some of these databases (Mathivanan, et al., 2006; Ramirez, et al., 2007; von Mering, et al., 2002); however, these analyses were based on the significantly smaller data sets available at the time of the analysis, and included only

subsets of currently popular PPI databases. Therefore, we analyzed the following four popular PPI databases (Table 1): HPRD (Human Protein Reference Database, (Prasad, et al., 2009)); MINT (Molecular INTeraction, (Ceol, et al., 2010)); INTACT (Aranda, et al., 2010), and BioGRID (Biological General Repository for Interaction Datasets, (Breitkreutz, et al., 2008)). In addition, we also included in the comparison a recently published database named HIPPIE (Human Integrated Protein Protein Interaction reference - http://cbdm.mdc-berlin.de/tools/hippie/) (M.S. et al. submitted). It is assembled through the compilation of several PPI sources, including the previously mentioned databases. Lastly, for the sections of this study not involving network topological characteristics, we also included the STRING database (Search Tool for the Retrieval of INteracting Genes/Proteins) (Jensen, et al., 2009), a popular resource that in addition to protein interactions, also contains protein associations from several pathway databases. MINT, HPRD, BIOGRID and INTACT are manually curated and have thousands of interactions submitted by the community; thus, since they offer original interactions used by other databases, we refer to these four databases as 'primary resources'. HIPPIE and STRING are composed of interactions taken from primary databases and other sources; hence, we refer to HIPPIE and STRING as 'derived databases'. In addition, for the purpose of this study we removed all predicted functional associations present in STRING.

Here, we focused on the human subset of interaction databases, and as an improvement over most current analyses, we demonstrated the usefulness of organ or cell type-specific subnetworks. We analyzed these databases for their basic features including protein coverage, number of interactions and neighborhood characteristics (i.e. we compared the number and identity of interactions partners, and asked whether proteins that are a hub in one database occupy a similar position in other databases). Finally, using three databases that assign confidence scores to its interactions, we demonstrated that there is a lack of interaction data with high confidence scores for many intensively studied proteins. Additional experimental evidence for those interactions - either confirming or refuting - would significantly increase the robustness of current PPI databases.

2 METHODS

2.1 Databases

The databases were obtained from their respective websites in the following versions or latest updates: HPRD Release 9; HIPPIE 1.1; STRING 8.3; MINT 15.December.2010; INTACT 21.April.2011; BIOGRID 3.1.76. Before initiating the analysis, the following pre-processing steps were carried out: (A) We removed all redundant interactions, keeping just the interaction with the highest score. (B) For all protein entries, their database-specific identification tags were converted to a common nomenclature (Entrez Gene IDs). Proteins that did not have a matching ID in Entrez Gene were discarded. Approximately 10% of interactions had to be removed from each database.

In the STRING database we performed additional pre-processing step: we removed all interactions involving non-human proteins, left only interactions with experimental evidence or obtained from pathway and other interaction databases (i.e. removed interactions derived from co-expression, genomic neighborhood, text-mining and other predictive techniques).

2.2 Network and Statistical Analysis

All interaction databases were converted to an undirected graph and further analyzed using R (version 2.10.1) and the iGraph library (version 0.5.4). From this library we used routines to find the degree, betweenness, diameter, shortest path, immediate neighbors and clustering coefficient. The other statistical tests (Welch, Kolmogorov-Smirnov, Wilcoxon, z-score) were performed using R with 0.95 confidence interval. Pathway and Gene Ontology enrichment analysis were performed with DAVID (Huang, et al., 2008) and ConsensusPath DB (Kamburov, et al., 2011) using the default parameters values. For the enrichment analysis tests, we used the list of proteins present in the tissue-specific subnetworks as background.

2.3 Popular Genes

The file gene2pubmed from the NCBI public FTP site contains a table with Pubmed IDs and the genes present in this each abstract (sorted by species). This file was used to rank the human genes according to the number of abstracts in which they appear and to select the 10% most popular genes (2,911 entries). The file was obtained on April 22nd 2010.

2.4 Gene expression data

We obtained an Affymetrix dataset containing the transcription levels of 84 human tissues and cell lines. This dataset is publicly available for query and download from the BioGPS project (Su, et al., 2004; Wu, et al., 2009).

We obtained the normalized expression data (pre-processed using GCRMA - GeneChip Robust Multiarray Averaging (Gentleman, et al., 2004)) and divided our analysis in the following steps: first, we defined that each probe must have an absolute intensity greater than 50 for at least one condition, thus removing any probe not being moderately or strongly expressed in at least one tissue (the original datasets have specific no background level). After this cut-off, 16,704 probes remained from the original dataset of 44,775 probes. Second, we performed the Kolmogorov-Smirnov test to evaluate the normality of each probe's intensity distribution, keeping probes with suitably normal distributions. Only 211 probes had a p-value greater than 0.1 and were excluded from further analysis. With 16,493 probes remaining, we converted their Affy_ID to Entrez Gene IDs and in this conversion 3,537 probes had no matching ID. In the end, our dataset consisted of 12,956 probes that mapped to 9,176 different genes. Finally, we calculated the z-score for each probe across all tissues. Using a Z-score cut-off of 0.1 (p-value 0.46), we determined which genes were moderately to highly expressed in each tissue.

2.5 Protein Degree Categorization

We classified the proteins into three categories (high-, middle- and low-degree) according to their number of interactions. To define the appropriate ranges, we ranked the proteins in decreasing order according to their number of interactions. With this list, we used a procedure which selected two random numbers: the first in the interval [80, 98] (we refer to it as *value1*) and the second in the interval [60, *value1*] (we call it *value2*). Subsequently, we considered high-degree proteins as those that occupied a position among the top *value1*% of the ranked list. Middle-degree proteins were those that occupied a position in the interval [*value2*, *value1*]% of the ranked list and finally, the low-degree proteins were on the [1, value2]% of the list. For a visual explanation of the procedure, please refer to Supplementary Figure 1. To verify the robustness of the results, this

procedure was repeated 100 times for each pair of databases being compared and the mean and standard deviations determined. We used this procedure instead of defining a fixed number of neighbors that a protein should have to belong to each category. The differences in the network sizes would cause the results to be unfairly dependent on the ranges selected.

3 RESULTS

3.1 Database Features

Table 1 compares the features of the six databases included in the analysis. The number of proteins (nodes) in these databases ranges from ~5,200 to ~12,000. STRING and HIPPIE contain the largest numbers of proteins since they include data from several other databases in addition to their own unique data.

For all databases except STRING, the total number of interactions ranges from ~12,500 to ~73,000 (Table 1). MINT has relatively few proteins and interactions, all of which are covered by one or several of the other databases. By contrast, over 140,000 interactions are reported in STRING, which comes close to the number of estimated interactions in the human proteome (Stumpf, et al., 2008; Venkatesan, et al., 2009). We found that 4,361 proteins and 5,589 protein-protein interactions were reported in at least two different databases, with the largest overlap between STRING and HIPPIE (Supplementary Table 1). Only 1,453 proteins and 1,619 protein-protein interactions are reported in all six databases. These interactions are reported in primary resource databases and are likely to stem from the same portion of literature that was manually curated by the authors(Turinsky, et al., 2010).

Table 1. Database characteristics

	HPRD	HIPPIE	STRING*	MINT	INTACT	BIOGRID
Proteins	9,117	11,835	10,546	5,206	8,310	9,057
Interactions	36,239	72,916	144,099	12,579	33,299	37,469
Average degree[1]	8	12	-	4.83	8.01	8.27
Average betweenness	13,528	15,840	-	8,009	11,909	13,639
Diameter[3]	14	13	-	12	13	12
Average Path Length[4]	4.25	3.79	-	4.43	3.96	4.21
Clustering Coefficient[5]	0.05	0.05	-	0.03	0.03	0.06

[1]Average degree describes the average number of interactions; [2]Average betweenness describes the 'centrality' of a factor in a network; [3]Diameter describes the maximal distance between the two most distant nodes in a network; [4]Average path length describes the average number of steps that connect any two components; [5]Clustering coefficient describes the tendency of nodes to interact among each other forming groups. * STRING is not a PPI database, thus we did not compute the features that are commonly used for network structure analysis.

Next, we compared the average degree and betweenness of the proteins in each in database. The average degree (average number of interactions per protein) ranges between 5-12, with HIPPIE showing the highest average number of neighbors for each protein (Supplementary Figure 2A shows the distributions of degree and betweenness in each database). Betweenness, in a broader sense describes the significance of a node (i.e. a protein in a PPI network) for the flow of information between different points in the network. It is calculated as follows:

$$B(v) = \sum \frac{s_{ij}(v)}{s_{ij}}, \qquad with\ i \neq j, v \neq i\ and\ v \neq j \qquad (1)$$

where s_{ij} is the number of shortest paths between the nodes i and j and $s_{ij}(v)$ is the fraction of those shortest paths passing through node v. High betweenness thus indicates that the respective protein has a 'central' position in the network, and that the perturbation of this protein may significantly affect the flow of information through the network. The average betweenness of the analyzed databases are similar (Table 1), with the exception of MINT, which has a slightly lower value. This was expected for all networks since they have similar structure, observed in their clustering coefficients, average degree and path lengths. The majority of proteins in all databases have medium to high betweenness values (defined here as 4.5 to 10.5 on a natural logarithm scale; see Supplementary Figure 2B), even though the number of interaction partners may be limited for these proteins. This suggests that even proteins with few interaction partners occupy important intermediate positions in a network (Joy, et al., 2005).

Finally, several measures of the overall network structure were compared for each database. The 'diameter' of a network defines the *maximal* distance between the two most distant nodes in the network while the average path length (APL) is the mean distance between all protein pairs in the network. As summarized in Table 1, the diameters and APLs of each network are comparable.

These findings collectively show that the databases have a similar network structure, although primary (MINT, INTACT, HPRD) and the derived database (HIPPIE) have a considerable difference in the number of interactions.

3.2 Conserved Topological characteristics between databases

After characterizing the basic features of the databases selected for this study, we next assessed their topological characteristics. 'Topology' describes the arrangements in which nodes are connected to each other in a database. Important topological parameters are the number and the identity of interaction partners. Such information is critical for the identification of hubs, which are often targeted for the identification of possible lethal genes (Albert, et al., 2000; Coulomb, et al., 2005; Jeong, et al., 2001), the development of novel drugs (Hase, et al., 2009; Yildirim, et al., 2007), or network disruption (Quayle, et al., 2007).

To this end, we adopted a strategy used for drug target identification and protein essentiality studies in which proteins are grouped into one of three categories based on the number of interactions (Han, et al., 2004; Hase, et al., 2009; Patil and Nakamura, 2006). We ranked the proteins according to their number of interactions and classified them as high-, middle- or low-degree proteins (see Methods). STRING was excluded from this analysis because it comprises not only protein interactions but also other types of non-physical, protein associations derived from pathway databases, in addition to co-expression of genes and genomic neighborhood.

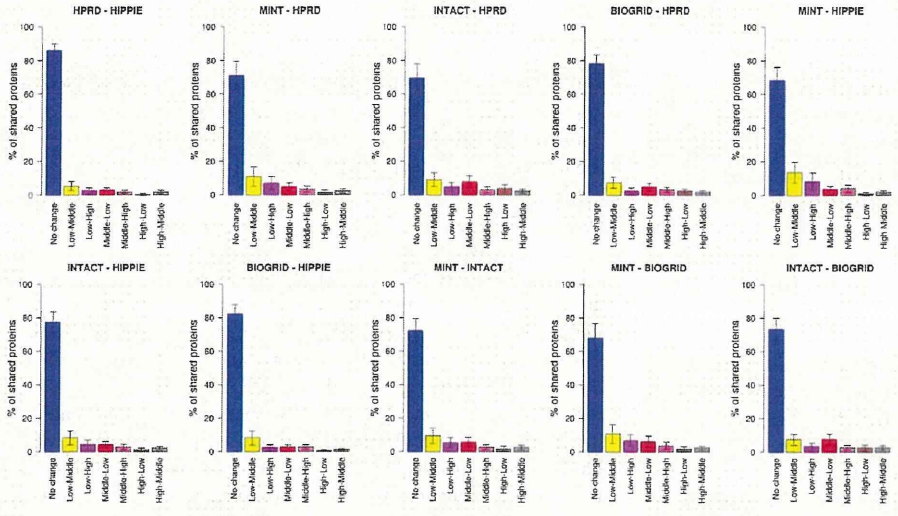

Fig. 1. Proteins were grouped into three categories: low-, middle- and high degree (see Methods). Then, we assessed the percentages of proteins that fall into the same (or different) categories in pair-wise comparisons of two databases. For most comparisons, 60-80% of proteins fall into the same category in both databases compared.

After categorizing all proteins, we assessed the percentages of proteins that fall into the same or different categories in pair-wise database comparisons. Figure 1 shows that 60-80% of the proteins shared between two databases fall into the same category in both databases. This shows that although the databases differ in the number of proteins and interactions, their shared proteins still have similar connectivity levels.

In our pair-wise comparisons, we matched the smaller database (with fewer interactions; e.g. HPRD) against the larger database (with more interactions; e.g. HIPPIE) (Figure 1). As a result, most proteins that fall into different categories between the databases shift into a higher degree category (e.g., the protein shifts from 'low degree' to 'middle degree'). However, we observed that when INTACT is matched against HPRD and BIOGRID, around ~10% of the proteins that are in the 'middle degree' category in the smaller database (i.e. INTACT) shift to the 'low degree' category in the larger database (i.e. HPRD or BIOGRID) (Figure 1). Most likely, this is a consequence of the different experimental datasets used in the different databases and we observed that those proteins show enrichment for translational elongation and RNA processing Gene Ontology categories (p-value < 0.01).

Notably, very few proteins changed between the 'high degree' and 'low degree' categories (or vice versa) when comparing databases (Figure 1). This further supports our notion that the five databases included in this analysis are in fairly good agreement regarding the connectivity of the proteins.

The only exception is the comparison of MINT with HIPPIE and other larger databases, with almost 10% of the proteins falling into the 'low degree' category in MINT, but into the 'high degree' category in HIPPIE. We attribute this finding to the different sizes of databases, with MINT and HIPPIE representing the smallest and largest datasets analyzed (both in terms of numbers of proteins and interactions, Table 1).

3.3 Neighborhood characteristics of datasets

The topological characteristics of a protein in a database are not only defined by the *number* of interaction partners, but, perhaps even more importantly, by the *identity* of interaction partners. We therefore assessed whether proteins have similar or different interaction partners in the databases analyzed. For our analysis, we focused on the 'shared' proteins, i.e., those listed in the two databases being compared. For these proteins, we identified their interaction partners in each of the databases, and then compared the interaction partners between the databases (Figure 2; see also Supplementary Figure 3 for the absolute numbers).

As expected, the highest percentage of shared neighbors was detected for the comparison of 'derived resources (STRING and HIPPIE) to 'primary resources (MINT, BIOGRID and INTACT). However, for comparisons that do not involve the HIPPIE database, no more than 40% of interaction partners are shared. As described earlier, STRING

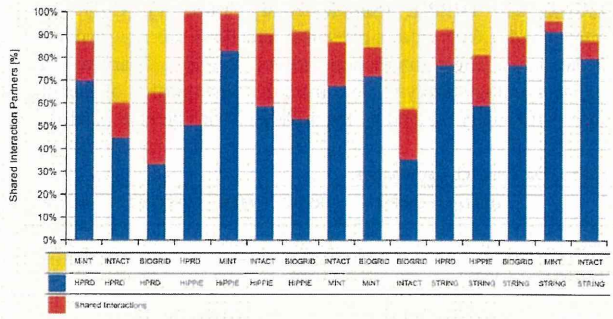

Fig. 2. Shared and exclusive interaction partners in a pair-wise comparison of PPI databases. For proteins shared between two databases, we identified their interaction partners in each of the databases, and then compared the interaction partners. Yellow and blue represent the indicated databases. Shown in red are the interaction partners predicted in both databases.

comprises not only protein interactions but also other functional associations originating, for example, from pathway databases (Jensen, et al., 2009; von Mering, et al., 2005). This results in a large number of interactions that are not covered by the other databases and transforms the interactions of the other databases into a subset of those reported by STRING.

Collectively, our analysis revealed considerable differences in predicted interaction partners between the databases. These differences likely stem from differences in the size of databases, algorithms used, and differences in the portion of the literature used by primary database curators. Researchers should take these issues into account when attempting to identify critical interaction partners of their protein(s) of interest.