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Estrogen Receptor ESR1 Is Indispensable for the Induction of Persistent Vaginal Change by Neonatal 5alpha-Dihydrotestosterone Exposure in Mice[1]

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ABSTRACT

?2 ?3 **Development of the reproductive organs can be strongly affected by the hormonal environment. In the mouse, exposure to estrogens and androgens during the critical developmental period induces estrogen-independent cell proliferation and differentiation in the adult vaginal epithelium, which often results in cancerous lesions later in life. In the present study, we assessed the contributions of estrogen receptor 1 (alpha) (ESR1) to the developmental effects of the nonaromatizable androgen 5alpha-dihydrotestosterone (DHT) on female mouse vagina and external genitalia. The vagina of *Esr1*$^{-/-}$ mice treated neonatally with DHT showed atrophic epithelium, whereas the vaginal epithelium of *Esr1*$^{+/+}$ mice was stratified and keratinized even after ovariectomy. In addition, neonatal treatment with DHT led to persistent phosphorylation of ESR1 in the vaginae of 60-day-old ovariectomized mice. We infer from these data that ESR1 is obligatory for the induction and maintenance of persistent vaginal epithelial changes induced by neonatal administration of DHT. Neonatal DHT treatment also induced hypospadias in both *Esr1*$^{-/-}$ and *Esr1*$^{+/+}$ mice. In contrast, DHT-induced formation of an os penis-like large bone in the clitoris was found in *Esr1*$^{-/-}$ mice but not in *Esr1*$^{+/-}$ or *Esr1*$^{+/+}$ mice. These results shed light on mechanisms of the induction of developmental effects elicited by sex steroid hormones on the developing animals.**

developmental effect, 5α-dihydrotestosterone (DHT), estradiol, estrogen receptor 1 (α) (ESR1), external genitalia, female reproductive tract, mouse, penis, testosterone, vagina

INTRODUCTION

Sex hormones exert developmental effects on humans, laboratory animals, and wildlife. The synthetic estrogen diethylstilbestrol (DES) was routinely prescribed to pregnant women for the prevention of miscarriages from the 1940s to1970s. In the early1970s, DES was found to induce vaginal clear-cell adenocarcinoma and various malformations in the uterus and vagina of young women exposed in utero [1]. It has been hypothesized that in utero DES exposure increases the subsequent incidence of breast cancer, squamous neoplasia of the cervix and vagina, and vaginal clear-cell adenocarcinoma [2–4]. As in humans, female mice exposed to natural or synthetic estrogens during the critical period develop estrogen-independent persistent cell proliferation and differentiation of the vaginal epithelium, accompanied by hypospadias [5–8]. This rodent model has been used to explore the mechanisms underlying reproductive abnormalities induced by developmental DES exposure in humans. However, the molecular mechanism of estrogen-independent vaginal changes induced by perinatal estrogen exposure remains poorly understood.

The developmental actions of estrogens can be mediated through estrogen receptor (ESR)-dependent pathways and/or ESR-independent pathways. These include the induction of DNA adducts, microsatellite instability, sequential mutation, and single-strand breaks seen in both in vitro and in vivo systems [9]. Because ESR 1 (α) (*Esr1*)-deficient mice do not exhibit stimulatory changes in the reproductive tracts by neonatal DES exposure, developmental effects of estrogens on female reproductive tracts are dependent on ESR1.

Intriguingly, androgens such as testosterone and 5α-dihydrotestosterone (DHT) also induce irreversible changes in female reproductive tracts and external genitalia [10, 11]. It remains controversial whether the effects of androgens on the developing female reproductive organs are mediated through ESR, androgen receptor (AR), or other epigenetic effects. In this study, we investigated the effects of DHT on the developing female reproductive organs in the mouse, particularly the vagina and external genitalia. We found that ESR1 has a critical role in mediating the effects of neonatal DHT exposure in the mouse vagina. We also found that neonatal DHT exposure induced os penis-like large bones in the clitoris in *Esr1*$^{-/-}$ mice but not *Esr1*$^{+/+}$ and *Esr1*$^{+/-}$ mice. Fetal to neonatal stages showing active morphogenesis and development are more sensitive to sex steroids than are adults. Although perinatal sex hormone exposure results in various adverse changes, we understand little about the signaling events induced by treatment with various hormones during the perinatal period. The present results provide the first evidence to date that androgens can induce developmental effects on reproductive organs mediated through ESR1.

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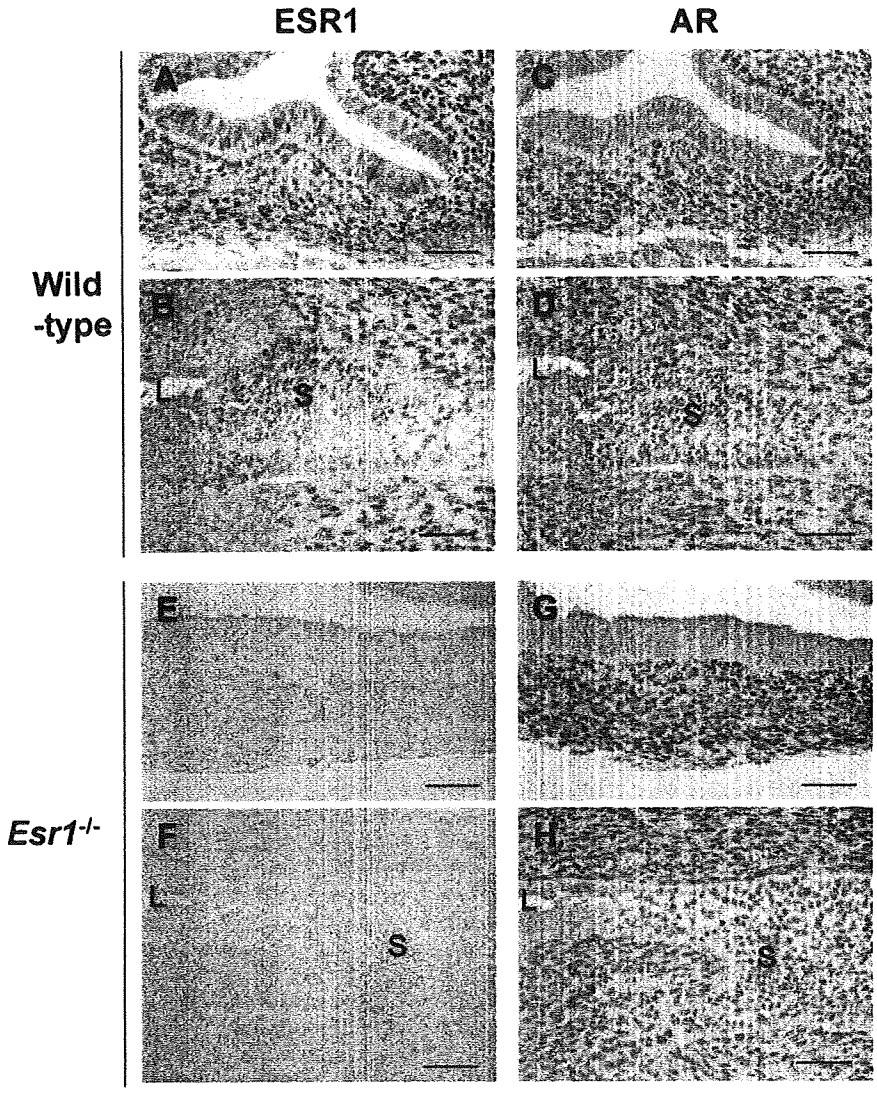

FIG. 1. Expression pattern of ESR1 and AR in the female reproductive organs. Immunohistochemical localization of ESR1 (**A**, **B**, **E**, and **F**) and AR (**C**, **D**, **G**, and **H**) in the müllerian duct-derived vagina (**A**, **C**, **E**, and **G**) and urogenital sinus-derived distal vagina (**B**, **D**, **F**, and **H**) at the day of birth. ESR1 is not detected in $Er1^{-/-}$ mouse reproductive organs (**E** and **F**). Expression pattern of AR is similar in both $Esr1^{+/+}$ and $Esr1^{-/-}$ mice (**G** and **H**). L, vaginal lumen; S, solid cord; bar = 50 μm.

MATERIALS AND METHODS

Animals and Treatments

Female C57BL/6J (CLEA, Tokyo, Japan) and *Esr1* knockout (KO) mice (C57BL/6J background [12]) were maintained under 12L:12D at 23–25°C and fed laboratory chow (CA-1; CLEA) and tap water ad libitum. All procedures and protocols were approved by the institutional animal care and use committee at the National Institute for Basic Biology.

Female newborn mice were given five daily s.c. injections of 50 μg of DHT (Sigma, St. Louis, MO) per gram of body weight per day dissolved in sesame oil or vehicle alone beginning from Day 0 (the day of birth). Gross morphology of the external genitalia was recorded using a digital camera. Mice were ovariectomized at Day 46 and killed at Day 60. For histological analysis, tissues were fixed in Bouin solution, embedded in paraffin, and sectioned at 8 μm. Sections were stained with hematoxylin-eosin.

Immunohistochemistry

Tissues fixed in 10% formalin neutral buffered solution were embedded in paraffin and sectioned at 4 μm. Deparaffinized sections were incubated in 0.3% H_2O_2 in methanol for 30 min to eliminate endogenous peroxidases. After washing with PBS, the sections were stained with the DAKO (Carpinteria, CA) LSAB kit according to the manufacturer's supplied protocol. Rabbit polyclonal antibodies against ESR1 and AR were obtained from Novocastra Laboratories (Tyne, England) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. For negative controls, normal rabbit immunoglobulin fraction (DAKO) at the same dilution of each antibody was used (data not shown).

Immunoblotting

The samples were prepared as described previously [13]. Protein contents were determined using the Bradford assay (Protein Assay reagent; BioRad, Hercules, CA). Anti-ESR1 was obtained from Santa Cruz Biotechnology, and anti-phospho-ESR1 (Ser 118 and Ser 167 to Ser 122 and Ser 171 of mouse sequences) antibodies were obtained from Cell Signaling Technology (Beverly, MA). The numbering of amino acid residues in this article is according to the human protein sequences, and the manufacturer's product name and specificity of these phosphospecific antibodies against mouse proteins are described in the product information. Signals were detected using the ECL kit (GE Healthcare, Piscataway, NJ).

Transactivation Assays

Full-length mouse ESR1 and AR were constructed by PCR amplification of the entire protein coding region. The PCR product was cut by restriction enzyme, gel purified, and ligated into pcDNA3.1 vector (Invitrogen, Carlsbad, CA). Transactivation assays were carried out as described previously [14, 15]. HEK293 (for ESR1) or HepG2 (for AR) cells were cultured in 24-well plates at 5×10^4 cells/well in phenol red-free Dulbecco modified Eagle medium for HEK293 or phenol red-free modified Eagle medium for HepG2 (Sigma) and were supplemented with 10% charcoal/dextran-treated fetal bovine serum

TABLE 1. Organ weight and reproductive organ findings in *Esr1* KO mice with neonatal exposure to DHT.

Genotype and treatment	Body weight (BW; g)[a]	Organ weight (mg/20 g BW)		No. of mice showing vaginal epithelial		No. of mice	
		Uterus[a]	Vagina[a]	Stratification[b]	Keratinization	With cleft clitoris	Formed bone exposing outer clitoris
$Esr1^{+/+}$-DHT	20.0 ± 0.5	18.7 ± 1.1	44.0 ± 6.0	6/7	6/7	7/7	0/7
$Esr1^{+/-}$-DHT	19.1 ± 0.5	22.4 ± 1.8	30.8 ± 4.2	1/5[d]	1/5[d]	5/5	0/5
$Esr1^{-/-}$-DHT	19.8 ± 0.6	20.4 ± 1.9	13.2 ± 0.8[c]	0/7[d]	0/7[d]	7/7	7/7[d]

[a] All data are represented as mean ± SEM.
[b] More than 4 layers of epithelium, at least in part, is considered as stratification because oil-treated OVX mice showed 1–3 layers of epithelium.
[c] Statistical difference vs. *Esr1*+/+-DHT group by Student *t*-test or Welch *t*-test followed by *F*-test ($P < 0.05$).
[d] Statistical difference vs. *Esr1*+/+-DHT group by Fisher exact probability test ($P < 0.05$).

(Hyclone, South Logan, UT). After 24 h, the cells were transfected with 400 ng of pGV2-MMTV for AR or pGL3-Basic-4xERE tk-Luc for ESR, 100 ng of pRL-TK (Promega, Madison WI), and 400 ng of pcDNA3.1-ESR1 or pcDNA3.1-AR using Fugene 6 transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. After 20 h of incubation, steroid hormones (DHT, DES, and 17β-estradiol [E_2]; Sigma) were introduced to the media. After an additional 24 h, the cells were collected, and the luciferase activity of the cells was measured by a chemiluminescence assay using the Dual-Luciferase Reporter Assay System (Promega). Promoter activity was calculated as firefly (*Photinus pyralis*) luciferase activity/sea pansy (*Renilla reniformis*) luciferase activity. All transfections were performed at least three times using triplicate sample points in each experiment.

RESULTS

AR and ESR1 Expression in Neonatal Mice

The vaginal epithelium in neonatally DHT-treated ovariectomized adult mice (C57BL/6J) was stratified and keratinized as previously reported [11]. Hypospadias with cleft clitoris and formation a tiny bone in the clitoris were induced in the present study by neonatal DHT exposure as described previously [11]. To examine whether DHT action was mediated through AR or ESR1, we investigated the localizations of AR and ESR1 in female mice at the day of birth.

In general, both receptors were strongly immunostained in vaginae at the day of birth. ESR1 was expressed in the müllerian duct-derived vaginal epithelial cells and stromal cells (Fig. 1A) and was faintly expressed in cells of the sinus cord and distal vaginal rudiment derived from urogenital sinus (Fig. 1B). In contrast to ESR1, AR was not detected in müllerian duct-derived vaginal epithelial cells but was instead expressed in stromal cells (Fig. 1C). Androgen receptor was detected in urogenital sinus-derived distal vaginal epithelial cells showing solid cord and in the mesenchymal cells (Fig. 1D).

Vaginal Histology and External Morphology of Esr1 *KO Mice Given Neonatal DHT Exposure*

Although AR was not expressed in the müllerian duct-derived vaginal epithelial cells, normal vaginal epithelial morphogenesis requires vaginal stromal cells [16]. In addition, stromal components contribute to developmental effects on the uterus and vagina [17]. Thus, we could not rule out a possible role for AR in mediating the DHT-induced persistent vaginal changes. To determine whether ESR was required for DHT action in the neonatal vagina, we utilized *Esr1* KO mice. ESR1 was not detected in the $Esr1^{-/-}$ mice (Fig. 1, E and F). In contrast, AR expression in the $Esr1^{-/-}$ female reproductive organs was similar to that of $Esr1^{+/+}$ mice (Fig. 1, G and H), indicating that AR expression is independent of ESR1.

The effects of neonatal DHT exposure on organ weights, vaginal histology, and external genitalia are summarized in Table 1. The vaginal epithelium of ovariectomized $Er1^{+/+}$ mice exposed neonatally to DHT ($Esr1^{+/+}$-DHT) showed ovary-independent proliferation consisting of seven to 12 layers of cells with superficial keratinization (Fig. 2A). In contrast, atrophic vaginal epithelium was found in ovariectomized 60-day-old $Esr1^{+/+}$ mice exposed neonatally to oil

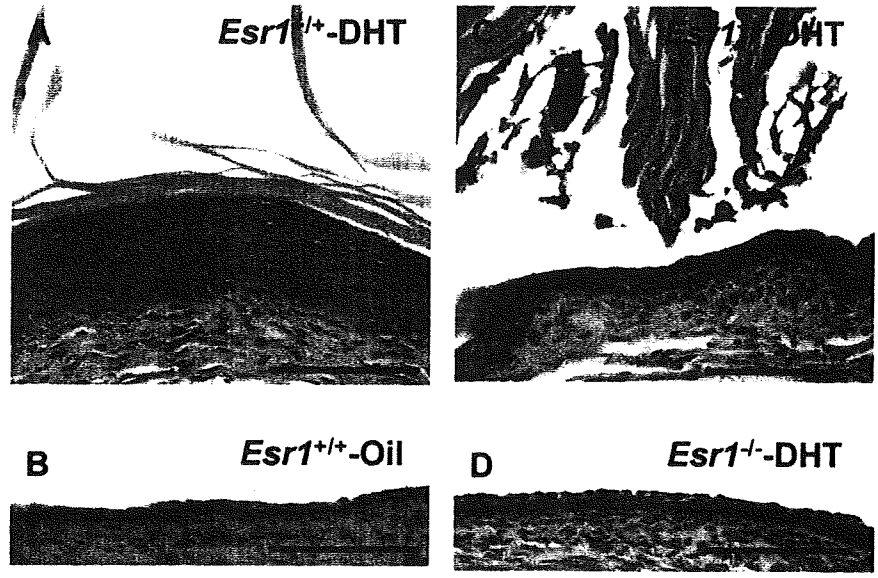

FIG. 2. Developmental effects of DHT on the vagina of *Esr1* KO mice. Vaginae of $Esr1^{+/+}$-DHT mouse (**A**), ovariectomized $Esr1^{+/+}$ mouse treated neonatally with oil vehicle alone (**B**), $Esr1^{+/-}$-DHT mouse (**C**), and $Esr1^{-/-}$-DHT mouse (**D**). Sections were stained with hematoxylin-eosin. Bar = 100 μm.

vehicle alone (Fig. 2B). In four of five ovariectomized *Esr1*$^{+/-}$ mice treated neonatally with DHT (*Esr1*$^{+/-}$-DHT), the vaginal epithelium was composed of two to four layers of cells. However, keratin and detached epithelial cells were observed in the vaginal lumen (Fig. 2C), indicating that these *Esr1*$^{+/-}$-DHT mice showed an ovary-dependent vaginal epithelial phenotype. The elements in its alternating layers indicate multiple ovarian cycles in the *Esr1*$^{+/-}$-DHT mice. This phenotype was also seen when mice were treated neonatally with a relatively low concentration of estrogen [18]. The remaining *Esr1*$^{+/-}$-DHT mouse showed ovary-independent vaginal epithelial stratification and superficial keratinization. The ovariectomized *Esr1*$^{-/-}$ mice treated neonatally with DHT (*Esr1*$^{-/-}$-DHT) all had a vaginal epithelium with one to two layers of cells and never showed mucus and keratin in the lumen (Fig. 2D). Taken together, these results indicated that DHT-induced vaginal epithelial changes were mainly mediated through ESR1. In support of this result, we found using a reporter gene assay that high-dose DHT can indeed stimulate the transcriptional activity of ESR1 (Fig. 3A). In contrast, transcriptional activity of mouse AR was not induced by DES (Fig. 3B).

The morphology of external genitalia showed no differences between intact *Esr1*$^{+/+}$ and *Esr1*$^{-/-}$ mice (Fig. 4, A and B [other data not shown]). However, all mice from the *Esr1*$^{+/+}$, *Esr1*$^{+/-}$, and *Esr1*$^{-/-}$ groups treated neonatally with DHT exhibited hypospadias and formation of a common urethral-vaginal canal, accompanied by a wide cleft clitoris independent of their genotypes (Fig. 4, C–F). Intriguingly, only *Esr1*$^{-/-}$-DHT mice (and not *Esr1*$^{+/+}$-DHT or *Esr1*$^{+/-}$-DHT mice) formed an os penis-like bony structure that extruded from the clitoris (Table 1 and Fig. 4, E and F).

Neonatal DHT Exposure Induces Phosphorylation of ESR1 in Mouse Vagina

ESR1 was persistently phosphorylated in the vagina of ovariectomized mice treated neonatally with DES, leading to ovary-independent epithelial cell proliferation and keratinization [13]. We tested whether neonatal DHT exposure induces phosphorylation of ESR1 in later life using anti-phospho-ESR1 antibodies. As shown in Figure 4, neonatal DHT exposure induced phosphorylation of ESR1 in vaginae from ovariectomized mice. Therefore, ESR1 is obligatory for both induction and maintenance of persistent vaginal epithelial cell proliferation and differentiation caused by neonatal DHT exposure, which is similar to results from neonatal DES exposure [13].

DISCUSSION

Androgens mediate various aspects of physiological functions not only in males but also in females. However, the physiological and developmental roles of androgen action in immature female reproductive organs have not been clarified. Both ESR1 and AR are expressed in female embryos and neonates [19–21]. The present study also showed that AR and ESR1 were expressed in the epithelium of female reproductive tracts and their surrounding mesenchyme in neonates. Developing female reproductive organs in neonates are affected by hormones and hormone-like chemicals and can induce various adverse effects [22]. Clarification of the signaling pathway of steroid hormones is thus important for understanding molecular events in reproductive tracts during animal development. In the present study, we analyzed the developmental effects of DHT on the female reproductive organs using *Esr1* KO mice.

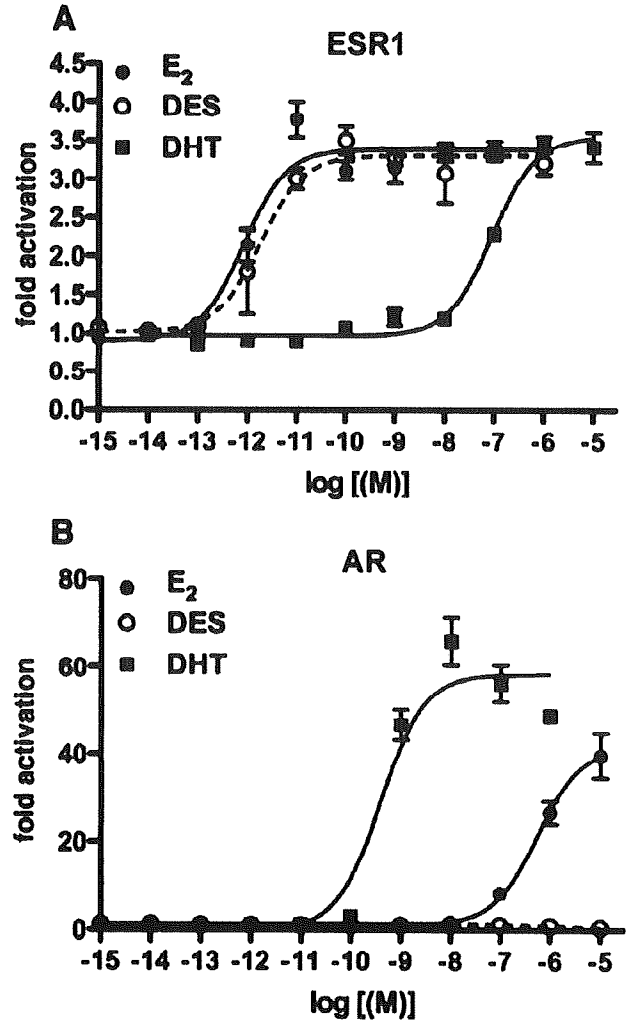

FIG. 3. Dose-response profiles of ESR1 and AR activation by E_2, DES, and DHT. Transcriptional activities of mouse ESR1 for E_2, DES, and DHT (**A**). HEK293 cells were transiently transfected with the ERE-containing vector together with a mouse ESR1 expression vector. Cells were incubated with increasing concentrations of E_2/DES (10^{-15} M to 10^{-6} M) and DHT (10^{-14} M to 10^{-5} M). Transcriptional activities of mouse AR for E_2, DES, and DHT (**B**). HepG2 cells were transiently transfected with the MMTV-luciferase vector together with a mouse AR expression vector. Cells were incubated with increasing concentrations of E_2/DES (10^{-15} M to 10^{-5} M) or DHT (10^{-15} M to 10^{-6} M). Data are expressed as a ratio of steroid:vehicle (dimethyl sulfoxide). Each column represents the mean of triplicate determinations, and vertical bars represent the mean ± SE.

The normal development and differentiation of reproductive tract components derived from the müllerian duct and urogenital sinus are dependent on the hormonal environment during critical periods of morphogenesis. Laboratory and epidemiological findings showed that developmental exposure within the critical period to sex steroids, particularly estrogens, induced various abnormalities in reproductive organs and often led to cancers later in life [23]. In utero DES exposure induces vaginal clear-cell adenocarcinoma in young women [1], as well as various developmental abnormalities in the reproductive tracts later in life, including squamous neoplasia of the cervix and vagina and vaginal clear-cell adenocarcinoma [3]. To help in understanding the DES syndrome, a laboratory rodent model has been characterized. Mice treated perinatally with DES

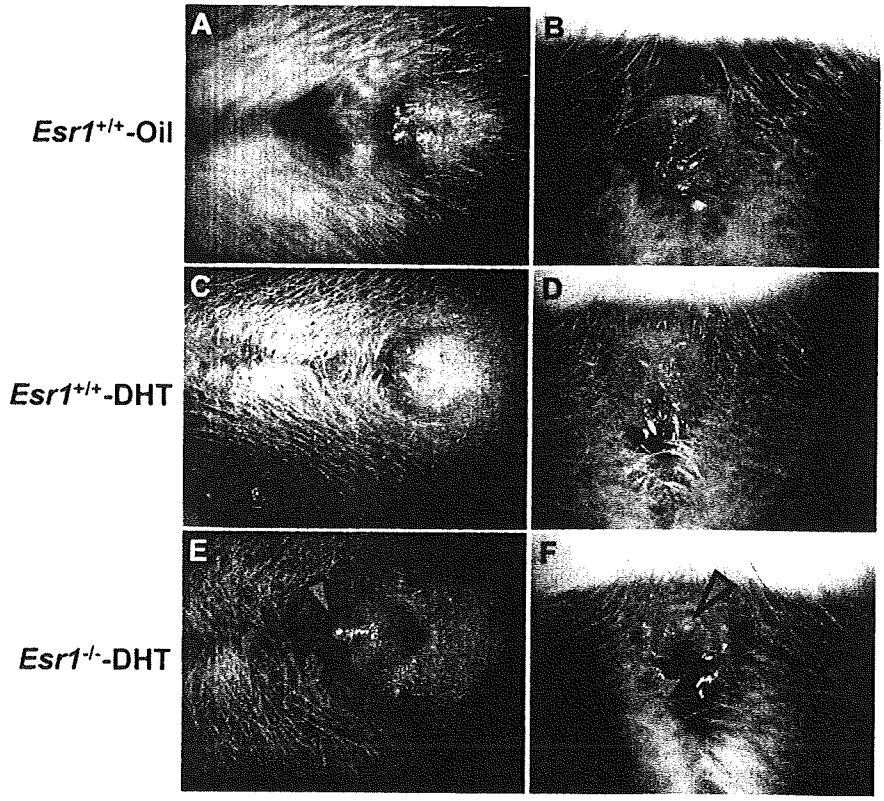

FIG. 4. Developmental effects of DHT on the external genitalia. External genitalia of 60-day-old $Esr1^{+/+}$ mouse treated neonatally with oil vehicle alone (**A** and **B**), $Esr1^{+/+}$-DHT mouse (**C** and **D**), and $Esr1^{-/-}$-DHT mouse (**E** and **F**). $Esr1^{+/+}$-DHT and $Esr1^{-/-}$-DHT mice show cleft clitoris (arrows). Note the bone formation in $Esr1^{-/-}$-DHT mice showing extrusion from the clitoris (arrowheads).

exhibit estrogen-independent proliferation and keratinization in the vaginal epithelium and develop cancers later in life [11, 18, 24]. ESR1 has an essential role in the induction of estrogen-independent vaginal changes caused by neonatal DES exposure, as such exposure did not result in morphological changes in $Esr1^{-/-}$ mice [25]. Intriguingly, neonatal treatment with androgens also induced irreversible effects in female reproductive tracts and external genitalia as reviewed previously [26]. Indeed, high concentrations of both testosterone and DHT induced a uterotrophic effect in ovariectomized rats [27]. Because DHT is a nonaromatizable androgen, it would be expected to act primarily through AR. However, a study [28] of *Ar* KO mice showed that AR is dispensable for uterine function. Thus, it has not been clarified how DHT induces developmental effects in female reproductive organs. To test the necessity of ESR for the developmental effects of DHT, we used *Esr1* KO mice. We found that $Esr1^{+/+}$-DHT mice showed ovary-independent vaginal epithelial cell proliferation, stratification, and keratinization, while $Esr1^{-/-}$-DHT mice showed atrophic epithelium with neither stratification nor keratinization. These results indicate that persistent vaginal changes by neonatal DHT treatment are mediated through ESR1.

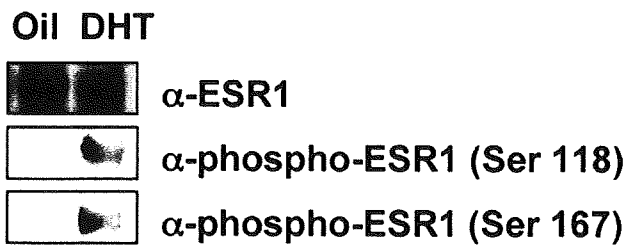

FIG. 5. Activation status of ESR1 in the mouse vagina. Phosphorylation of ESR1 is detected by anti-phospho-ESR1 antibodies. The samples are from vaginae of 60-day-old ovariectomized mice treated neonatally with oil vehicle alone and DHT.

In support of this idea, we found that high concentrations of DHT can activate the ESR, whereas DES cannot activate the AR. Despite that the relative binding and transcriptional ability of DHT to ESR is 0.001% that of estradiol [29, 30], DHT action through ESR1 mimics the action of estrogens in the developing vagina. This is potentially of concern because much of the evidence regarding effects of hormonally active agents has dealt with altered sexual development [22], and most hormonally active agents known to date are more likely to bind to ESR than to AR [31, 32]. Nevertheless, the estrogen-like chemical bisphenol-A, which binds poorly to ESR, induces estrogen-independent vaginal epithelial cell proliferation and keratinization [33]. Recently, additional nongenomic effects of sex steroids have been established. Androgen receptor has been found to interact with the intercellular tyrosine kinase SRC (c-Src) and can function cooperatively with ESR1 and following signal transduction activation [34, 35]. In the present study, we could not exclude the possible involvement of AR in DHT-treated mouse vagina. Further analysis is necessary to determine whether DHT directly binds to and activates ESR1 in vivo.

Neonatal DES exposure induced persistent phosphorylation of ESR1 even in the absence of estrogen ligands, and this phosphorylation maintains the estrogen-independent cell proliferation and differentiation of the vaginal epithelium [13]. Such estrogen-independent ESR1 phosphorylation was also detected in the neonatally DHT-treated vagina. Thus, the effects of neonatal DHT exposure are similar to those resulting from neonatal DES treatment in adult mice. This result shows that ESR1 is essential for not only induction but also

maintenance of persistent vaginal changes caused by neonatal DHT exposure.

The involvement of ESR1 has been reported in disorders of male rat penis induced by estrogen [36, 37]; however, the developmental effects of female mouse external genitalia elicited by androgens have been unelucidated. Development of external genitalia is dependent on sex steroid hormones during the perinatal stage [38–40]. The induction of penis-like enlargements and formation of bone tissue in the clitoris (os clitoris) by neonatal administration of androgens has been observed in female mice and rats [11, 41]. Os clitoris formation was not induced by estrogens [11, 41], establishing it as an androgen-dependent phenomenon. Indeed, os penis formation in male mice is dependent on androgens during the early period [42]. In the present study, the formation of a tiny bone in the clitoris was induced by neonatal DHT exposure in *Esr1*$^{+/+}$ and *Esr1*$^{+/-}$ mice as in C57BL/Tw mice [10, 11]. Intriguingly, only *Esr1*$^{-/-}$ mice treated neonatally with DHT formed a large os clitoris that was similar to the os penis in which three bones are present. It cannot be explained by a high serum testosterone level in the female *Esr1* KO mice [43] because oil-treated *Esr1*$^{-/-}$ control mice did not show such large os clitoris formation. The difference in response to DHT in these mice may be due to AR expression levels in mesenchymal cells of the clitoris. In support of this idea, AR expression in the bone of *Esr1*$^{-/-}$ mice is 2-fold higher than that of normal mice [44]. In contrast to os penis formation, hypospadias and cleft clitoris occurred in all animals that received neonatal DHT exposure. Female hypospadias is characterized by clefting of the clitoris and mislocalization of vaginal and urethral openings. The formation of hypospadias results from an imbalance between cell proliferation and apoptosis in the urogenital sinus in response to sex steroid hormone exposure [7]. Thus, DHT exerts the developmental effects in the urogenital sinus without ESR1 activation.

In summary, ESR1 was indispensable for the induction of persistent vaginal epithelial cell proliferation and differentiation in response to neonatal administration of DHT. Persistent phosphorylation of ESR1 reveals an essential role of ESR1 for the maintenance of developmental effects of the vagina. Neonatal treatment of female mice with sex steroid hormones and estrogenic chemicals induced various morphological and functional changes [26, 33]; therefore, it is important to clarify the mediator that induces such effects. Studies [25, 45] have shown that the developmental effects in mouse reproductive organs elicited by estrogen are mediated through ESR1, but the present study is the first to date to provide evidence that androgen-induced developmental effects require ESR1 activation. Further analysis is needed to understand the developmental effects of sex steroid hormones and hormonally active agents and the functions of ESR and AR in developing reproductive organs.

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Why Public Health Agencies Cannot Depend on Good Laboratory Practices as a Criterion for Selecting Data: The Case of Bisphenol A

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Background: In their safety evaluations of bisphenol A (BPA), the U.S. Food and Drug Administration (FDA) and a counterpart in Europe, the European Food Safety Authority (EFSA), have given special prominence to two industry-funded studies that adhered to standards defined by Good Laboratory Practices (GLP). These same agencies have given much less weight in risk assessments to a large number of independently replicated non-GLP studies conducted with government funding by the leading experts in various fields of science from around the world.

Objectives: We reviewed differences between industry-funded GLP studies of BPA conducted by commercial laboratories for regulatory purposes and non-GLP studies conducted in academic and government laboratories to identify hazards and molecular mechanisms mediating adverse effects. We examined the methods and results in the GLP studies that were pivotal in the draft decision of the U.S. FDA declaring BPA safe in relation to findings from studies that were competitive for U.S. National Institutes of Health (NIH) funding, peer-reviewed for publication in leading journals, subject to independent replication, but rejected by the U.S. FDA for regulatory purposes.

Discussion: Although the U.S. FDA and EFSA have deemed two industry-funded GLP studies of BPA to be superior to hundreds of studies funded by the U.S. NIH and NIH counterparts in other countries, the GLP studies on which the agencies based their decisions have serious conceptual and methodologic flaws. In addition, the U.S. FDA and EFSA have mistakenly assumed that GLP yields valid and reliable scientific findings (i.e., "good science"). Their rationale for favoring GLP studies over hundreds of publically funded studies ignores the central factor in determining the reliability and validity of scientific findings, namely, independent replication, and use of the most appropriate and sensitive state-of-the-art assays, neither of which is an expectation of industry-funded GLP research.

Conclusions: Public health decisions should be based on studies using appropriate protocols with appropriate controls and the most sensitive assays, not GLP. Relevant NIH-funded research using state-of-the-art techniques should play a prominent role in safety evaluations of chemicals.

Key words: bisphenol A, endocrine disruptors, FDA, Food and Drug Administration, GLP, good laboratory practices, low-dose, nonmonotonic, positive control. *Environ Health Perspect* 117:309–315 (2009). doi:10.1289/ehp.0800173 available via *http://dx.doi.org/* [Online 22 October 2008]

Regulatory agencies in the United States and the European Union (EU) have justified the decision to declare the estrogenic chemical bisphenol A (BPA) safe at current levels of human exposure based on a few studies conducted using Good Laboratory Practices (GLP). In contrast, these agencies have rejected for consideration in their risk assessment of BPA hundreds of laboratory animal and mechanistic cell culture studies conducted by academic and government scientists reporting harm at very low doses of BPA. These studies were rejected primarily because they were not conducted using GLP. We suggest that decisions based on this logic are misguided and will result in continued risk to public health from exposure to BPA, as well as other manmade chemicals.

GLP is a federal rule for conducting research on the health effects or safety testing of drugs or chemicals submitted by private research companies for regulatory purposes. The GLP outlines basic guidelines for conducting scientific research, including the care and feeding of laboratory animals, standards for facility maintenance, calibration and care of equipment, personnel requirements, inspections, study protocols, and collection and storage of raw data (Goldman 1988). These regulations were developed in response to widespread misconduct by private research companies; this misconduct was possible because their data usually do not go through the rigorous, multistage scientific review that is normal for academic data funded by federal agencies and published in the peer-reviewed literature. The lack of these safeguards from academic science had enabled fraud. The U.S. Food and Drug

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Administration (U.S. FDA) first issued rules for GLP in 1978 after a 2-year federal investigation into sloppy laboratory practices of a number of private research companies (Lublin 1978; Markowitz and Rosner 2002). What began as serious concerns about poor quality research expanded into a criminal investigation of Industrial Bio-Test (IBT), one of the largest private laboratories at the time and a subsidiary of Nalco Chemical Company. In response to the federal investigation, the U.S. Environmental Protection Agency (EPA) demanded that 235 chemical companies re-examine the > 4,000 tests conducted by the laboratory. In 1983, three men from IBT were found guilty of deliberating doctoring data and were sentenced to prison (Lublin 1978; Markowitz and Rosner 2002). The fraudulent practices of IBT brought into question 15% of the pesticides approved for use in the United States. That same year, the U.S. EPA issued similar GLP rules for regulatory testing.

Both the U.S. FDA (2008a) and European Food Safety Authority (ESFA 2006) have recently published documents demonstrating that their decision to continue to declare BPA safe at current exposure levels was based primarily on the results of a few industry-funded studies that followed GLP guidelines. These decisions stand in stark contrast to the decisions concerning the potential risks to human health reached by a panel of 38 experts at a U.S. National Institutes of Health (NIH)-sponsored conference, who published The Chapel Hill Consensus Statement (vom Saal et al. 2007), as well as five review articles (Crain et al. 2007; Keri et al. 2007; Richter et al. 2007a; Vandenberg et al. 2007a; Wetherill et al. 2007). These peer-reviewed articles covered approximately 700 articles concerning BPA and represented a comprehensive review of the literature as of the end of 2006. In addition, the U.S. FDA draft decision contradicted the conclusions reached by the National Toxicology Program (NTP), which had spent 2 years investigating this question (NTP 2008). An important role of the NTP is to advise the U.S. FDA about the science relating to toxic chemicals in food, but in an unusual move, the U.S. FDA chose to release its draft report before the release of the final report on BPA by the NTP and without indicating who at the U.S. FDA was involved in preparing the draft report (U.S. FDA 2008b). At a hearing on 16 September 2008 regarding the draft report on BPA, the U.S. FDA announced that their goal was to have a subcommittee of the U.S. FDA Science Board complete a review of the draft decision by the end of October 2008. This would presumably also involve review by the subcommittee members of the approximately 1,000 articles relating to BPA.

We believe that the methods employed in chemical industry–sponsored GLP studies are incapable of detecting low-dose endocrine-disrupting effects of BPA and other hormonally active chemicals. Detecting endocrine-disrupting effects at low doses of chemicals such as BPA requires sophisticated and modern assays and analyses that have been developed in advanced, usually federally funded laboratories over the past decade. This is especially apparent when one examines what is now known about functional effects of BPA on a wide range of end points (Richter et al. 2007a; Welshons et al. 2006; Wetherill et al. 2007). These end points include those mediated by recently discovered estrogen response pathways initiated in human and animal cell membranes (nonclassical or alternative estrogen response mechanisms), which multiple laboratories have shown to be equally sensitive to BPA and estradiol in terms of activating effects in human and animal cells at low picomolar through low nanomolar concentrations (Alonso-Magdalena et al. 2008; Wetherill et al. 2007; Wozniak et al. 2005; Zsarnovszky et al. 2005).

The effects of BPA documented in these studies include a diverse array for which there are no data from GLP studies because the end points have not been examined: altered metabolism related to metabolic syndrome (Alonso-Magdalena et al. 2005, 2006, 2008; Ropero et al. 2008); altered adiponectin secretion (Hugo et al. 2008), which is a condition predicting heart disease and type 2 diabetes (Lang et al. 2008); altered epigenetic programming leading to precancerous lesions of the prostate (Ho et al. 2006); differential growth patterns in the developing prostate (Timms et al. 2005); abnormal growth, gene expression, and precancerous lesions of the mammary glands (Soto et al. 2008); and adverse effects on the female reproductive system, including uterine fibroids, paraovarian cysts, and chromosomal abnormalities in oocytes (Newbold et al. 2007; Susiarjo et al. 2007). There is also a large literature on neuroanatomic, neurochemical, and behavioral abnormalities caused by low doses of BPA (Leranth et al. 2008; Richter et al. 2007a), which also are not capable of being detected by current GLP studies conducted for regulatory purposes because of their out-of-date assays.

The approaches used by academic and government scientists to study the potential health hazards of BPA contrast sharply with those still used by the chemical industry that are relied on by regulatory agencies in the United States and Europe, including the two studies identified by both the U.S. FDA and European Food Safety Authority (EFSA) as central to the decision to declare BPA safe at current human exposure levels (Tyl et al. 2002, 2008a). By using outdated and insensitive assays that were supposed to have been replaced by a new battery of screens and tests by 2000 [as mandated by the U.S. Congress in 1996 in the Food Quality Protection Act (1996), but which has, as yet, still not occurred], these studies conducted using GLP fail to find any adverse effects.

Reliability and Validity

Reliability and validity are separate issues, although in the experimental research described here, validity and reliability basically refer to research that is credible. Golafshani (2003) noted that "reliability" refers to the extent to which results are consistent over time and are an accurate representation of the total population under study. Of central importance is that the results of a study must be reproduced under a similar methodology to be considered to be reliable. "Validity" refers to whether the research measures what it was intended to measure, and valid findings are considered to be true. In other words, reliability is determined by whether the results are replicable, whereas validity is assessed by whether the methods used result in finding the truth as a result of the investigator actually measuring what the study intended to measure.

Use of GLP in Regulatory Decision Making

Despite strong evidence of aberrations caused by low doses of BPA in animals exposed during fetal and neonatal life in studies conducted by the world's leading academic and government experts in the fields of endocrine disruption, endocrinology, neurobiology, reproductive biology, genetics, and metabolism, a relatively small number of studies reporting no adverse effects at low doses of BPA have continued to be promoted by the chemical industry and used by regulatory agencies (e.g., Ashby et al. 1999; Cagen et al. 1999; Tyl et al. 2002, 2008a). According to the U.S. FDA, these are accepted because they used GLP (U.S. EPA 2008), with the implication that studies not employing GLP are not reliable or valid (U.S. FDA 2008a).

GLP does not guarantee reliability or validity of scientific results. Unfortunately, although GLP creates the semblance of reliable and valid science, it actually offers no such guarantee. GLP specifies nothing about the quality of the research design, the skills of the technicians, the sensitivity of the assays, or whether the methods employed are current or out-of-date. (All of the above are central issues in the review of a grant proposal by an NIH panel.) GLP simply indicates that the laboratory technicians/scientists performing experiments follow highly detailed U.S. EPA requirements [or in the EU, Organization for Economic Co-operation and Development (OECD) requirements] for record keeping, including details of the conduct of the

experiment and archiving relevant biological and chemical materials (U.S. EPA 2008).

These record-keeping procedures in GLP were instituted because of widespread misconduct being committed by commercial testing laboratories (described above). These fraudulent results were possible because contract laboratory studies used in the regulatory process are rarely subject to the checks and balances that peer-reviewed, replicated scientific findings undergo. Without that acid test of reliability (replication by other independent scientists), other procedures were needed. Hence GLP was implemented, despite its severe limitations.

NIH-funded research subject to more stringent reviews than GLP. Although few NIH-funded investigators adhere to GLP-mandated record keeping, the procedures of GLP are actually surpassed by the procedures required for NIH-funded science published in peer-reviewed journals. NIH-funded studies pass through three phases of peer review that are far more challenging than GLP requirements. First, the principal scientists must have demonstrated competence to conduct the research, and experimental methods, assays, and laboratory environment must involve use of state-of-the-art techniques to be competitive for NIH funding. Second, results are published in peer-reviewed journals, with detailed evaluations by independent experts examining all aspects of the study. And third, the findings are challenged by independent efforts to replicate; for example, the initial findings concerning the stimulating effects of estrogenic chemicals on the mouse prostate (Nagel et al. 1997; vom Saal et al. 1997) were independently replicated and extended by Gupta (2000), which led to an editorial identifying "initial results confirmed" (Sheehan 2000).

Typically, within a laboratory, interesting findings are also followed by subsequent publications extending the prior findings; examples include the findings of BPA effects on β cells in the mouse pancreas (Alonso-Magdalena et al. 2005, 2006, 2008) and the effects of estrogenic chemicals and drugs on the developing mouse prostate that followed earlier findings (described above) from this same group (Timms et al. 2005; Richter et al. 2007b). In particular, independent replication by competent, respected scientists is the main criterion of acceptance of the findings as having been demonstrated to be reliable and having been validated by virtue of coming to the same conclusion using a variety of sophisticated techniques in multiple publications.

An important criticism of the approach taken by the U.S. FDA in its assessment of the now approximately 1,000 articles on BPA is that it appears to have made no attempt to connect the dots between replicated studies; instead, the U.S. FDA appears to have assessed each study without regard to whether it had been confirmed by other studies.

Thus, collectively, many phases used to verify the reliability and validity of NIH-funded published research have been completely ignored by the U.S. FDA, whereas industry-funded GLP research is rarely, if ever, subject to these central requirements and yet is accepted by regulatory agencies as reliable and valid.

The U.S. FDA's misguided gold standard. In this light, the U.S. FDA's reliance upon GLP as the gold standard is scientifically misguided. Furthermore, U.S. FDA administrators are ignoring published critiques of the GLP studies it considers reliable and valid, such as the study by Tyl et al. (2002) and two coordinated studies conducted at the same time by Ashby et al. (1999) and Cagen et al. (1999). Each was an industry-funded study conducted using GLP. Each was harshly criticized in peer-reviewed publications by academic scientists and government panels [Center for the Evaluation of Risks to Human Reproduction (CERHR) 2007; NTP 2001; vom Saal and Hughes 2005; vom Saal and Welshons 2006]. Yet, the U.S. FDA and EFSA panels still assert that these studies represent the gold standard in toxicologic research.

Specifically, the studies of Cagen et al. (1999) and Ashby et al. (1999) were recently rejected by the NTP CERHR panel on BPA as unusable for consideration in its evaluation of the health hazards posed by BPA (CERHR 2007). Both the Ashby et al. (1999) and Cagen et al. (1999) studies reported finding no effect of their positive control [the estrogenic drug diethylstilbestrol (DES)] on any outcome, although these failures were not acknowledged by the authors in either article. In experimental science, the failure of a positive control to show an effect indicates the experiment failed, which is the conclusion reached by the CERHR panel (CERHR 2007).

The Tyl et al. 2002 study, which the U.S. FDA still accepts as a major study for determination of the safety of BPA (U.S. FDA 2008a, 2008b), was criticized by an NTP panel that met in 2000 to examine the low-dose issue (NTP 2001), as well as in subsequent publications (vom Saal and Hughes 2005; vom Saal and Welshons 2006), for using an insensitive rat (the CD-SD rat) that requires extremely high doses (≥ 50 μg/kg/day) of the potent estrogenic drug ethinylestradiol to show effects such as those examined in the study by Tyl et al. (2002). This dose of ethinylestradiol is > 100 times higher than the approximately 0.3 μg/kg/day used by women in oral contraceptives. The fact that Tyl et al. (2002) adhered to GLP did not protect them from using insensitive animals. This led the NTP (2001) to state:

> Because of clear species and strain differences in sensitivity, animal model selection should be based on responsiveness to endocrine-active agents of concern (i.e., responsive to positive controls), not on convenience and familiarity.

Thus, when reviewed by other scientists, three prior major GLP studies of BPA have been found to be so flawed as to be useless for guiding regulatory agencies in decision making. A new GLP study has now been published by Tyl et al. (2008a). Close examination of this study also reveals fatal flaws which render it useless for regulatory purposes, even though it conforms to GLP.

Examples of Flaws Ignored by the U.S. FDA and EFSA in a Recent GLP Study of BPA

In summary, the flaws in Tyl et al. (2008a) are as follows:

- The high dose required for the positive control (estradiol) to cause an effect means the system used by Tyl et al. (2008a), at least in her laboratory, is relatively insensitive to exogenous estrogens and thus inappropriate for studying low-dose effects of estrogenic compounds such as BPA. The lack of response to low doses of estradiol or BPA in the Tyl laboratory is puzzling, in that the strain of mice used in these experiments (the CD-1 mouse) has been reported in > 20 other peer-reviewed publications to show adverse effects in response to very low doses of BPA (vom Saal 2008), as well as many other studies showing low-dose effects in response to the natural hormone estradiol, the estrogenic drugs ethinylestradiol and DES, and to other estrogenic chemicals.
- Tyl et al. (2008a) used insensitive, out-of-date protocols and assays that are incapable of finding many of the adverse effects reported by more sophisticated studies conducted by independent NIH-funded scientists as well as scientists funded by government agencies in other countries.
- In the specific case of testing for changes in prostate weight, Tyl et al. (2008a) reported an abnormally high prostate weight for control animals that exceeds by > 70% the prostate weights reported by other studies for animals of the same strain and similar age (e.g., Gupta 2000; Ruhlen et al. 2008). This suggests that the dissection procedures for the prostate in the Tyl laboratory included other nonprostatic tissues in the weight measurements, rendering them unusable for studying weight changes in the prostate in response to BPA or estradiol; neither chemical showed any effect on the selected end points, which directly contradicts other findings concerning opposite effects of low and high doses of estrogen on the prostate (Putz et al. 2001; Timms et al. 2005; vom Saal et al. 1997).

Aberrant insensitivity of CD-1 mouse to estrogens. Tyl et al. (2008a) used estradiol as a positive control. It was fed to female mice before and during pregnancy and lactation at 80–220 μg/kg/day; after weaning, estradiol was fed to offspring at doses of 80–100 μg/kg/day. Estradiol was used as a positive control because BPA is a man-made endocrine-disrupting estrogenic chemical.

Many published findings reporting effects of very low doses of positive control estrogens and BPA in CD-1 mice demonstrate that the CD-1 mouse was somehow rendered insensitive in the test system used by Tyl et al. (2008a). The fact that a dose of 100–200 μg/kg/day estradiol was necessary to show an effect of the positive control predicts that Tyl et al. (2008a) should not detect effects of BPA < 10–100 mg/kg/day, far above the low-dose range relevant to human exposures that was supposedly of interest.

For nuclear estrogen receptor–mediated effects via regulation of gene activity (nuclear estrogen receptors are transcription factors whose activity is regulated by binding to estrogen), prior studies have typically shown a 1,000-fold lower activity for BPA relative to estradiol or potent estrogenic drugs, including DES and ethinylestradiol. For example, Richter et al. (2007b) reported an increase in androgen receptor gene activity to estradiol at 1 pM (0.28 pg/mL) in fetal CD-1 mouse prostatic mesenchyme cells in primary culture, and the same response was found for BPA at 1,000 pM (228 pg/mL); the *in vitro* response to estradiol was predicted by the response of the prostate to increasing free serum estradiol from 0.2 to 0.3 pg/mL in male mouse fetuses via estradiol administration to the mother (vom Saal et al. 1997). Other research showed that a significant effect on development of the male reproductive system in CF-1 mice occurred at a maternal dose of 0.002 μg/kg/day ethinylestradiol (Thayer et al. 2001), similar to effects observed with 2–20 μg/kg/day BPA (vom Saal et al. 1998). The research of Honma et al. (2002) showed accelerated puberty in CD-1 (ICR) mice at a DES dose of 0.02 μg/kg/day (the positive control), and the same response to BPA occurred at 20 μg/kg/day, again revealing a 1,000-fold difference between the positive control estrogen and BPA.

There are many other examples of findings where a higher dose of BPA was required to cause the same effect as the positive control estrogen (estradiol, ethinylestradiol, or DES) in studies where the effects were mediated by the classical nuclear estrogen receptors, in contrast to the more recently discovered rapid signaling estrogen response system where BPA and these positive control estrogens have equal potency, as described above. In summary, CD-1 mice have been used by a large number of academic and government investigators and have been reported in peer-reviewed publications to be sensitive to positive control estrogens within the range of human sensitivity based on *in vivo* and *in vitro* studies via the classical estrogen receptor α–mediated response mechanism. The CD-1 mouse is the animal model that has been used by the U.S. National Institute of Environmental Health Sciences (NIEHS) for decades, because it is considered the best animal model for predicting the effects of developmental exposure to estrogen in humans (Newbold 1995; Newbold et al. 2007).

The failure of traditional toxicologic studies conducted by Tyl et al. (2008a, 2008b) to detect the wide range of adverse effects of even relatively high doses of BPA or of low doses of estradiol that have been reported in numerous studies by academic and government scientists provides evidence that the GLP protocols established long ago by regulatory agencies to determine the toxicity of chemicals are inappropriate for detecting the endocrine-disrupting activities of chemicals such as BPA. Indeed, this was the premise of the congressional mandate in the Food Quality Protection Act (1996) for the U.S. EPA to establish a new set of assays for endocrine-disrupting chemicals, although this process has been systematically delayed and is > 8 years behind the congressionally mandated date of 2000 to have these new assays validated.

Citing Tyl et al. (2008a), the EFSA report on BPA (EFSA 2006) stated that "the positive control substance, 17β-estradiol, resulted in reproductive and developmental toxicity." This report failed to acknowledge that only a very high dose of the positive control was sufficient to elicit effects and that this meant that the experiments conducted in the Tyl laboratory were for some reason very insensitive to any estrogen and thus inappropriate for use in a study to examine low-dose estrogenic effects of BPA.

Based on the preliminary report released by the U.S. FDA regarding BPA (U.S. FDA 2008a), it appears that the U.S. FDA has followed the lead of the EFSA in its lack of understanding of the importance of the dose of the positive control estrogen required to cause adverse effects. The consequence is that the U.S. FDA has relied primarily on the study of Tyl et al. (2008a, 2008b), with the result that the U.S. FDA has assured Americans that BPA is safe at current human exposure levels.

Several factors might account for the insensitivity of the CD-1 mouse in the Tyl et al. studies (2008a, 2008b) conducted at Research Triangle Institute (RTI), a testing facility that conducted these (as well as previous) studies funded by the American Chemistry Council. One possibility is that the diet used in these studies may have interfered with the results. The feed used by Tyl et al. (2008a) in this experiment (Purina 5002) has been shown by others to interfere with responses to exogenous estrogenic chemicals, blocking adverse effects documented on other diets. For example, a number of years ago, Thigpen et al. (2003) at the NIEHS recommended against the use of Purina 5002 in studies of endocrine-disrupting chemicals. Tyl et al. (2008a) measured some specific phytoestrogens in Purina 5002 feed by chemical analysis; however, in a report on NIH-sponsored meetings on this subject, Heindel and vom Saal (2008) pointed out that this is an insufficient control for total dietary estrogenic contaminants that can disrupt studies involving the effects of estrogenic chemicals.

A second possibility is that there are strain differences in sensitivity developed in the CD-1 mouse sold by the various Charles River Laboratories located in different regions. We consider this unlikely, because most laboratories regularly replace their CD-1 mouse breeder stock from Charles River Laboratories, and practices there make it unlikely that the sensitivity of this outbred stock to estrogens has changed dramatically over a very short period of time. Also, because RTI, where the Tyl studies were conducted, is very near the laboratories of the NIEHS, it is likely that the CD-1 mice used by these two programs were purchased from the same breeding facility.

Use of insensitive, out-of-date protocols and assays. Another serious concern about the two recent studies by Tyl et al. (2008a, 2008b) is the experimental approach used, thus raising questions about the validity of the studies. The study design used by Tyl et al. (2008a, 2008b) has been superseded by advances in both experimental design and analytical tools developed by NIH-funded scientists (and their counterparts in Europe and Asia) since the mid-1990s. The methods used by Tyl et al., primarily wet weight changes of tissues, gross histologic changes, and developmental landmarks such as vaginal opening, were established procedures by the 1950s. Thus, a major limitation of the Tyl studies is the failure to measure more meaningful and sensitive end points in order to detect the effects of low-dose BPA exposure, which are often not macroscopic in nature. Indeed, in 2001, the director of the reproductive division of the National Health and Environmental Effects Research Laboratory at the U.S. EPA stated that the inconclusive results concerning effects of BPA on reproductive toxicology can only be solved by understanding the mechanisms (Triendl 2001). With current GLP standards it is not possible to study mechanisms because they still rely on out-of-date assays.

As one example of a comparison between the approach by Tyl et al. (2008a) and independent government-funded academic scientists, extensive research has been conducted by Soto et al. (2008) and by other independent academic and government scientists

describing effects of exposure of female mice and rats to very low doses of BPA during perinatal development on the mammary glands (Jenkins et al. 2009). Although Tyl et al. (2008a) reported no low-dose effects of BPA on the mammary glands using conventional histologic analysis, there have been consistent findings of adverse effects of low doses of BPA from studies that used more sophisticated and sensitive analysis of whole mounted mammary glands to facilitate detection of microscopic lesions, coupled with immunostaining for regulatory proteins as well as techniques for determination of aberrant gene expression associated with progression to cancer. These peer-reviewed studies have reported detecting changes during embryonic development of mammary glands as well as abnormalities detected during adolescence through adulthood that are indicative of mammary gland cancer as well as other developmental abnormalities (Colerangle and Roy 1997; Durando et al. 2007; Jenkins et al. 2009; LaPensee et al. 2008; Markey et al. 2001, 2005; Moral et al. 2008; Munoz-de-Toro et al. 2005; Murray et al. 2007; Nikaido et al. 2004; Vandenberg et al. 2006, 2007b; Wadia et al. 2007).

Similar to the findings for the mammary gland, Ogura et al. (2007) reported that if tissues were analyzed by conventional histologic methods (staining with hematoxalin and eosin), prenatal exposure to low doses of BPA or DES showed no effects on prostate development, whereas if the sections were analyzed using antibodies that identified basal cells and basal cell squamous metaplasia, then significant effects were revealed. Squamous metaplasia of basal cells indicates abnormal proliferation and function of the prostate stem cell population that is thought to transform into neoplastic cells; Ho et al. (2006) reported that neonatal exposure to very low doses of BPA caused 100% of male rats to develop high-grade prostatic intraepithelial neoplastic lesions later in life. All of these studies were rejected by the U.S. FDA as not adequate for making regulatory decisions about the safety of BPA. Instead, the U.S. FDA relied upon Tyl et al. (2008a), even though the study used techniques that Ogura et al. (2007) showed lacked the sensitivity of 21st century experimental approaches.

Although findings regarding changes in brain structure, brain chemistry, and behavior represent the largest portion of the literature on low-dose BPA, Tyl et al. (2008a) did not examine any neurobehavioral end points. The NTP (2008) and the NIEHS conference consensus reports (vom Saal et al. 2007) both indicated concern about neurobehavioral effects of low doses of BPA. Thus, the absence of studies that included neurobehavioral end points is a glaring omission of Tyl et al. (2008a, 2008b).

Flawed prostate dissection. Data presented by Tyl et al. (2008a) raise questions about the adequacy of techniques used in their BPA studies. Specifically, Tyl et al. (2008a) reported that the prostate in 3.5-month-old control male CD-1 mice weighed > 70 mg [see Table 3 in Tyl et al. (2008a) for data on F_1 retained males]. This average control weight contrasts sharply with those reported from other laboratories. Specifically, the weight of the prostate in 2- to 3-month-old CD-1 mice using the dissection technique based on both Ruhlen et al. (2008) and Gupta (2000) and at the NIEHS (Newbold RR, personal communication) is about 40 mg. Several studies have reported that prenatal exposure to very low doses of BPA and positive control estrogens increased prostate size, prostatic androgen receptors, and prostate androgen receptor gene activity (Gupta 2000; Richter et al. 2007b; Thayer et al. 2001; Timms et al. 2005; vom Saal et al. 1997), but the enlarged prostate of experimental animals exposed to BPA in these laboratories weighed less than the prostates in the control animals of Tyl et al. (2008a). This raises serious questions about the procedures and/or animals used by Tyl et al. The weight of prostate reported by Tyl et al. (2008a) suggests that the technique used for dissecting the prostate resulted in nonprostatic tissue being weighed along with prostate. The seminal vesicle, coagulating gland, and dorsolateral prostate all merge together where the ejaculatory ducts enter the urethra, and there are also fat deposits on the prostate. This poses a challenge for those without proper training in distinguishing these different tissues during dissection in mice.

Alternatively, as male rodents age, they are prone to develop prostatitis. Although this inflammatory disease leads to an increase in prostate size and could thus account for the very large prostate weights reported by Tyl et al. (2008a), anyone familiar with the appearance of prostatitis would detect this abnormality upon histologic examination, which Tyl et al (2008a) supposedly conducted. Also, prostatitis is rare in young-adult mice or rats (Cowin et al. 2008), and the size of the prostates in the Tyl et al. (2008a) study were similar to those for middle-aged and old male mice.

The findings regarding effects of BPA on the prostate presented by Tyl et al. (2008a) are thus suspect and cannot be used as evidence that other earlier studies (Gupta 2000; Timms et al. 2005; vom Saal et al. 1997) are not replicable. Given these problems in prostate weight measurements, it is not surprising that even very high doses of BPA or estradiol reported by Tyl et al. (2008a) had no effect on the prostate, in sharp contrast to other studies that showed stimulation of the prostate at low doses of estrogen and inhibition at high doses (Putz et al. 2001; Timms et al. 2005).

In addition to the problem associated with the high prostate weight reported by Tyl et al. (2008a), in a separate measurement the authors combined the anterior prostate (coagulating gland) and seminal vesicle, presenting these two organs as one combined outcome measure. This is wrong and misleading. The coagulating glands emerge as the anterior ducts of the prostate from the dorsocranial region of the urogenital sinus, whereas the seminal vesicles bud from the proximal region of the Wolffian ducts. Elevated estrogen is associated with an increase in prostate size associated with an increase in prostate androgen receptors, whereas a decrease in seminal vesicle size is associated with a reduction in 5α-reductase, an enzyme that converts testosterone to the more potent androgen 5α-dihydrotestosterone (Nonneman et al. 1992). Low doses of BPA have been shown to decrease the size of organs that differentiate from the embryonic Wolffian ducts (epididymides and seminal vesicles) while increasing the size of regions of the prostate that develop from the urogenital sinus (vom Saal et al. 1998). Combining these different organs (it is technically not difficult to separate them) was thus inappropriate because they develop from different embryonic tissues that show markedly different responses to estrogenic chemicals during development. In fact, Ogura et al. (2007) reported that the anterior prostate (coagulating glands) showed the greatest expression of ER-α, and also showed the most pronounced indication of basal cell squamous metaplasia in response to developmental exposure to low doses of DES and BPA relative to other regions of the prostate.

Conclusions

Because the control data of Tyl et al. (2008a) were not consistent with the prior published literature for prostate weight of young-adult CD-1 male mice and because their methods were inappropriate for revealing an extensive body of adverse effects detected using more sophisticated approaches, we deem the findings by Tyl et al. to be invalid. Hundreds of studies show adverse effects of BPA in animals, with many conducted at concentrations equivalent to current human levels of BPA exposure; thus, it is unlikely that academic scientists would bother to replicate the outdated approaches used by Tyl et al. (2008a, 2008b). This lack of replication is typical of GLP studies, which tend to involve unnecessarily large numbers of animals [Tyl et al. (2002) used > 8,000 rats], and reliability appears to be accepted because of the numbers of animals that were used. Although using excessive numbers of animals is accepted as good science by the U.S. FDA, the use of arbitrarily large numbers of animals per group (> 20 animals per treatment group is common) actually violates guidelines in the NIH *Guide for the*

Care and Use of Laboratory Animals (Institute of Laboratory Animal Research 1996) that govern research conducted by academic and government scientists. For research with animals to be approved by any university animal care and use committee, group sizes must be based on power analysis conducted using historic data. Based on this criterion in the NIH Guide, all of the studies by Tyl et al. were significantly over powered and thus in direct violation of federal guidelines for conducting animal research, a fact about which U.S. FDA regulators seem unaware.

Each of the four main industry-funded GLP studies of BPA (Ashby et al. 1999; Cagen et al. 1999; Tyl et al. 2008a, 2008b) is flawed and not appropriate for use in setting health standards. Clearly, meeting GLP standards is not a guarantee of reliable or valid science. It is of great concern that the U.S. and EU regulatory communities are willing to accept these industry-funded, antiquated, and flawed studies as proof of the safety of BPA while rejecting as invalid for regulatory purposes the findings from a very large number of academic and government investigators using 21st-century scientific approaches. The basis for these decisions by U.S. and EU regulatory agencies should be thoroughly investigated, particularly since the NTP (2008) concluded that BPA exposure to human infants was in the range shown to cause harm in experimental animals and since both the Canadian Ministry of Health and the Ministry of the Environment recently concluded that BPA was a toxic chemical (Environment Canada 2008).

Problems inherent with reliance on GLP as the standard for choosing data are compounded by the process used by federal agencies to determine membership on science advisory panels. Leading experts qualified by specific experience on the chemical or end points under consideration are often specifically excluded from membership. For example, the U.S. FDA's BPA review panel was identified as an expert panel, when in fact the panel was composed largely of scientists lacking any experience in research with BPA. This process, which appears to consider almost any scientist knowledgeable about a chemical to create bias, makes it vastly more difficult for the panel to integrate scientific data from the relevant literature, especially since, as with BPA, there are almost 1,000 relevant studies and the review panel is provided with very little time to become knowledgeable about the details. It means that the depth of knowledge present on this and similarly constituted government regulatory agency panels is unlikely to be sufficient to subject draft assessments to the scrutiny that peer review by experts normally entails. Combined with reliance on GLP data, this process has a high potential to yield flawed assessments that jeopardize public health.

We are not suggesting that GLP should be abandoned as a requirement for industry-funded studies. We object, however, to regulatory agencies implying that GLP indicates that industry-funded GLP research is somehow superior to NIH-funded studies that are not conducted using GLP. This argument demonstrates a lack of understanding of the profound difference between the use of replication as a mechanism to assess reliability and the methods used to assess validity for peer-reviewed published academic studies, whereas GLP was instituted with the expectation that this type of verification would not occur.

Public health decisions should be based on studies using appropriate protocols and the most sensitive assays. They should not be based on criteria that include or exclude data depending on whether or not the studies use GLP. Simply meeting GLP requirements is insufficient to guarantee scientific reliability and validity.

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Coactivation of Estrogen Receptor β by Gonadotropin-Induced Cofactor GIOT-4[▽]

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Estrogen exerts its diverse effects through two subtypes of estrogen receptors (ER), ERα and ERβ. Each subtype has its own distinct function and expression pattern in its target tissues. Little, however, is known about the transcriptional regulatory mechanism of ERβ in the major ERβ-expressing tissues. Using biochemical methods, we identified and described a novel ERβ coactivator. This protein, designated GIOT-4, was biochemically purified from 293F cells. It coactivated ERβ in ovarian granulosa cells. GIOT-4 expression was induced by stimulation with follicle-stimulating hormone (FSH). GIOT-4 recruited an SWI/SNF-type complex in a ligand-independent manner to ERβ as an ER subtype-specific physical bridging factor and induced subsequent histone modifications in the ERβ target gene promoters in a human ovarian granulosa cell line (KGN). Indeed, two ERβ-specific target genes were upregulated by FSH at a specific stage of a normal ovulatory cycle in intact mice. These findings imply the presence of a novel regulatory convergence between the gonadotropin signaling cascade and ERβ-mediated transcription in the ovary.

Estrogen plays important roles in many target organs, including the female reproductive organs, the central nervous system, and bone. Estrogen exerts its diverse biological actions through binding to and activating one of two nuclear estrogen receptor (ER) subtypes (ERα or ERβ) (12, 22, 35, 40). ERs are members of the nuclear receptor (NR) gene superfamily. ERs, bound to and activated by estrogen, bind to specific DNA sequences called estrogen-responsive elements (ERE) to induce target genes (14, 21).

Like the other NR members, the ER requires the cooperation of distinct classes of coregulators and multiprotein coregulator complexes in order to initiate estrogen-mediated chromatin reorganization (16, 46). These complexes appear to modify the chromatin configuration in a highly regulated manner by controlling nucleosomal rearrangement and enzyme-catalyzed modifications of histone tails. By altering chromatin structure, the coregulator complexes facilitate bridging between NRs and basal transcription factors, along with RNA polymerase II, thereby controlling transcription. As for the nucleosomal rearrangement, two major classes of chromatin-modifying complexes that coregulate NRs have been well-characterized. One class is the histone-modifying complexes, including discrete subfamilies of transcription coregulatory complexes (2, 29, 36). The best-characterized NR coregulator complexes possess either histone acetylase or histone deacetylase activities. Recently, histone methylases/demethylases have also been shown to be significant NR coregulators. The other class of coregulator complexes is ATP-dependent chromatin-remodeling complexes. These complexes use ATP hydrolysis to rearrange nucleosomal arrays in a noncovalent manner to facilitate or prevent the access of NRs to nucleosomal DNA (5, 17, 33). These ATP-dependent chromatin-remodeling complexes have been classified into three subfamilies based on the major catalytic components possessing DNA-dependent ATPase activity. BRG1/Brm is a core component of the SWI/SNF-type complexes, SNF2h is a major component of imitation SWI-type complexes, and Mi2 is a core component of NuRD-type complexes. Recently, several distinct complexes with spatiotemporally specific functions have been identified. Generally, these complexes have components that confer specificity for certain transcription factors, including NRs (11, 18, 26).

ERβ and ERα have different distributions and biological functions in the target tissues. ERα is expressed in the breast, uterus, and bone, while ERβ is expressed predominantly in the prostate, central nervous system, and intestinal tissues (23, 24, 31, 50, 62). Even within a single tissue, the expression pattern of each subtype is cell type specific. In the ovary, clear expression of ERβ is detectable in granulosa cells but ERα is more abundant in theca cells than in granulosa cells (41). Reflecting the different subtype distribution patterns, ERβ knockout (KO) and ERα KO mice show different phenotypes. ERα KO mice are infertile and have a hypotrophic uterus and anovulatory, hemorrhagic ovaries (23, 32). In contrast, ERβ KO mice are subfertile, with reduced ovulation (34). Further analysis revealed previously that ERβ is essential for granulosa cell differentiation (9).

The ovary is an ER target tissue whose function and development are under control by ER-mediated estrogen actions. The ovulatory cycles are also regulated by hormones and cytokines through the hypothalamic-pituitary-ovarian axis. Gonadotropin-releasing hormone, produced in the hypothalamus,

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stimulates the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary. FSH then induces follicle maturation from preantral follicles to antral follicles via cyclic AMP signals (25). Though a number of hormones and cytokines coordinate to promote ovarian development and support ovarian function (13, 43–45), the molecular mechanisms underlying these complicated events in the ovary remain largely unknown.

In this study, we found that treatment with an FSH analogue (pregnant mare serum gonadotropin [PMSG]) stimulated ERβ function via protein kinase A (PKA) signaling in a human granulosa tumor cell line (KGN). We biochemically identified a novel coactivator of ERβ, designated GIOT-4. GIOT-4 expression was induced by PMSG in KGN cells and in the mouse ovary. GIOT-4 recruited an SWI/SNF complex to ERβ for further nucleosomal reorganization for gene activation. This GIOT-4-induced recruitment of SWI/SNF complex components was detected in the endogenous promoters of genes encoding aromatase and the activin βA precursor, both of which are essential for folliculogenesis. Thus, GIOT-4 is a novel ERβ coactivator that participates in a chromatin-remodeling complex to mediate gonadotropin actions in the ovary.

MATERIALS AND METHODS

Plasmids. Glutathione *S*-transferase (GST)–ERβ AB domain and GST–ERβ DEF domain constructs were prepared as described previously (30). FLAG-tagged full-length cDNAs for ERα and ERβ were inserted into the pcDNA3 vector (Invitrogen, Carlsbad, CA). Full-length cDNA for Myc-tagged GIOT-4 (GenBank accession no. AB021644) was cloned from 293F cells and inserted into the pcDNA3 vector. SRC-1 and BRG1 expression vectors were prepared as described previously (26). A BRG1 mutant construct (designated K798R) was made by site-directed mutagenesis as described previously (27, 57).

Reagents. Rabbit polyclonal anti-human GIOT-4 antibodies against human GIOT-4 and mouse GIOT-4 were made by Operon (Huntsville, AL). The following commercially available antibodies were used: anti-FLAG (Sigma, St Louis, MO); anti-BRG1 (catalog no. sc-17796 for immunofluorescence and catalog no. sc-10768 for immunoprecipitation and Western blotting), anti-ERα (catalog no. sc-543), anti-INI1 (catalog no. sc-13058), anti-SRC-1 (catalog no. sc-8995), and anti-TRRAP (catalog no. sc-5405) (all from Santa Cruz Biotechnology, Santa Cruz, CA); anti-ERβ (catalog no. ab16813) and anti-BAF57 (catalog no. ab14764) (Abcam, Cambridge, United Kingdom); and anti-Myc (catalog no. 05724), anti-histone H4 (catalog no. 06-866), H3K9me3 (catalog no. 8898-100), and H3K9me2 (catalog no. 207-212) (Upstate Biotechnology, Lake Placid, NY). PMSG reagent and human chorionic gonadotropin (hCG) reagent were purchased from Teikokuzouki Co. Ltd. (Tokyo, Japan), and estradiol (E2) reagent and H89, a PKA inhibitor, were purchased from Sigma. Small interfering RNAs (siRNAs) for ERβ (catalog no. L-003402-00), GIOT-4 (catalog no. L-020805-01), and BAF57 (catalog no. L-017522-00) and the nonspecific control (catalog no. D-001810-01) were purchased as an ON-TARGET*plus* SMART pool from Dharmacon Inc. (Lafayette, CO).

Cell culture. 293F cells were maintained in Dulbecco's modified Eagle medium (Gibco BRL, Gaithersburg, MD) supplemented with antibiotics and 10% fetal bovine serum. KGN cells were maintained in Dulbecco's modified Eagle medium–Ham F-12 medium supplemented with antibiotics and 10% fetal bovine serum (8). For 72 h before transfection, the cells were cultured in phenol red-free medium with 10% charcoal-stripped serum.

Purification and characterization of the ERβ-interacting complex. The procedure to convert an adherent culture of 293 cells into a suspension culture of 293F cells was described previously (27, 54). Nuclear extracts from 293F cells, transformed with FLAG-ERβ in a suspension culture, were loaded onto an anti-FLAG M2 affinity resin column and washed extensively with washing buffer (20 mM Tris-HCl [pH 8.0], 300 mM KCl, 0.2 mM EDTA, 0.05% NP-40, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). Bound proteins were eluted from the column by incubation with 300 μg/ml FLAG peptide in washing buffer for 30 min at 4°C. For fractionation on glycerol gradients, eluates were layered on top of 13 ml of linear 10 to 40% glycerol gradients in washing buffer and centrifuged for 16 h at 4°C and 40,000 rpm in an SW40 rotor (Beckman Coulter, Fullerton, CA). After the collection of each fraction, Western blotting analysis of fractions 1 through 12 was performed with anti-ERβ antibody. Protein standards used were ovalbumin (44 kDa), beta globulin (158 kDa), and thyroglobulin (670 kDa). Each sample was applied to a NuPAGE bis-Tris 4 to 12% gradient gel (Invitrogen) (26, 27, 54).

RT-PCR and qRT-PCR. Total RNAs from KGN cells, 293F cells, and mouse ovary tissue were extracted using TRIzol reagent (Invitrogen), and cDNA was synthesized as described previously (27, 48). PCR was performed as previously described (27, 48). PCR products were visualized on 2% agarose–Tris-acetate-EDTA gels. Real-time quantitative reverse transcription-PCR (qRT-PCR) was performed using Sybr premix *Ex Taq* (TaKaRa Bio Inc., Tokyo, Japan) with the Dice real-time system TP800 thermal cycler (TaKaRa), and normalization and calculation steps were performed as reported previously (40, 54).

Specific primers for PCR were as follows: human GIOT-4 gene (GenBank accession no. NM_021030), 5′-CTGTGTGGGTGTCGAGAGCAAATG-3′ and 5′-TGCCACTGTCATGGCTCAGCAATG-3′; mouse GIOT-4 gene (GenBank accession no. NM_145624), 5′-GGGCAGCACATCTTAGAAGC-3′ and 5′-TTGCCAAAGCTGTTTCTCCT-3′; human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, 5′-CGAGATCCCTCCAAAATCAA-3′ and 5′-GTCTTCTGGGTGGCAGTGAT-3′; and mouse GAPDH gene, 5′-GGGTGTGAACCACGAGAAAT-3′ and 5′-ACACATTGGGGGTAGGAACA-3′. Specific primers for qRT-PCR for human aromatase or activin βA were purchased from TaKaRa Bio Inc.

Immunoprecipitation and Western blotting. Immunoprecipitation was performed by following our standard protocol (26, 42). For 293F cells, 3×10^7 cells were transfected with the FLAG-ERβ-expressing vector and the Myc–GIOT-4-expressing vector. Twenty-four hours after transfection, cells were harvested with 10 nM E2 for 1 h before collection. For KGN cells, 3×10^6 cells were transfected with GIOT-4 siRNA or control siRNA and harvested, with or without 10 nM E2 and 500 mIU/ml PMSG. Anti-FLAG M2 affinity resin or anti-Myc affinity resin (Sigma) was used for the precipitation for 293F cells. ERβ antibody and protein A agarose were used for the KGN cells. The washed agarose was subjected to Western blotting.

GST pull-down assay. The GST pull-down assay was performed by following our standard protocol (26, 42). GST fusion proteins were expressed in *Escherichia coli* and bound to glutathione-Sepharose 4B beads (GE Healthcare, Buckinghamshire, England). In vitro-translated proteins were prepared by in vitro translation using the T7 promoter of the pcDNA3 vector. Proteins were labeled using [^{35}S]methionine (GE Healthcare), and in vitro translation was carried out using the TNT-coupled rabbit reticulocyte lysate system (Promega, Madison, WI). The in vitro-translated proteins were incubated with beads for 1 h at 4°C with or without 1 μM E2 (6).

Luciferase assay. Luciferase assays involving the ERs were performed by following our standard protocols (30, 42). For KGN cells, 80% confluent cells were transfected with plasmids and siRNA by using Lipofectamine 2000 reagent (Invitrogen). A 500-mIU/ml concentration of PMSG, 1,000-mIU/ml hCG, or 10 nM E2 was added 3 h after transfection, and the cells were incubated for 18 h at 37°C. Values were reported as the means ± standard deviations (SD) of results from at least three independent experiments. For the RNA interference (RNAi) experiment, KGN cells were transfected with 20 nM siRNA together with the DNA.

Chromatin immunoprecipitation (ChIP) assay. Samples of soluble chromatin from KGN cells treated with or without ligands (500 mIU/ml PMSG and 10 nM E2) and from mouse ovarian tissue were prepared with an immunoprecipitation assay kit (Upstate Biotechnology) and were immunoprecipitated with antibodies against the proteins indicated below. Specific primer pairs were designed to amplify the promoter region of the human aromatase gene; 5′-TTTGGCAATGACCAGAAATG-3′ and 5′-AAGGACAACGGGACTCTGTG-3′), the human activin βA precursor gene; 5′-TGGGTCAGGGGTGAGTTTAG-3′ and 5′-GTGTGGCTTAAGCAGGTTCC-3′), the mouse aromatase gene; 5′-GGTACGGGAGCCTTTTCCTG-3′ and 5′-TGTGGCTCCTGTCACTTGGA-3′), or the mouse activin βA precursor gene; 5′-CCACAGGCTTTACTGGCTCAC-3′ and 5′-TTCGGGTCCCTTCTGTTTTG-3′) from genomic DNA. PCR products were visualized on 2% agarose–Tris-acetate-EDTA gels (18, 54, 61).

Immunohistochemistry. All mice were maintained according to the protocol approved by the animal care and use committee of the University of Tokyo. Immature 21-day-old female mice (CD-1) were injected intraperitoneally (i.p.) with 3.25 IU of PMSG or vehicle (saline) between 1300 and 1400 h. After 48 h, some of the animals were injected i.p. with 5 IU of hCG at 16 h before sampling (40). Anesthetized mice were perfused with 4% paraformaldehyde, and ovaries were sectioned after paraffin embedding. Immunohistochemistry was performed as described previously (48). Antigen retrieval was performed by incubating the

slides in citric acid buffer (pH 6.0) at 95°C for 20 min. The sections were incubated with a mixture of mouse anti-BRG1 (1:100) and either rabbit anti-GIOT-4 (1:50) or anti-ERβ (1:50) at 4°C for 24 h. The sections were then incubated with a mixture of donkey anti-mouse immunoglobulin G labeled with Cy3 and anti-rabbit immunoglobulin G labeled with fluorescein isothiocyanate or Cy5 at room temperature for 1 h. Confocal microscopy was carried out with a Zeiss 510 confocal laser scanning system.

RESULTS

FSH analogue treatment superactivates ERβ transcriptional activity through PKA signaling in KGN cells. Estrogen and gonadotropins are key hormones during folliculogenesis. Though their individual signaling pathways are well-described, cross talk between their signaling pathways has not been studied. To address this issue, we first tested possible gonadotropin effects on ER transactivation function. We used a luciferase assay that employed a luciferase vector plasmid with a consensus ERE in the promoter in KGN cells (Fig. 1A). We applied two commonly used gonadotropin compounds, PMSG and hCG, to provide FSH and LH stimulation, respectively. In this assay, clear activation of human ERα and ERβ transactivation function by E2 was expectedly seen. PMSG stimulated ERβ, but not ERα, in the presence or absence of E2. Such stimulation was not seen for hCG. A PKA inhibitor (H89) that blocks the downstream signaling cascade of gonadotropins (47, 49) inhibited this stimulation of ligand-bound ERβ by PMSG (Fig. 1A). In KGN cells, endogenous ERβ, but not ERα, was expressed at significant levels (Fig. 1B) as previously reported (8). The expression of the known E2 target genes, including those for aromatase and the activin βA precursor (1, 49, 51, 58), was also detectable. Reflecting the observed hormonal actions, the additive induction of these genes by two hormones was detected. However, the knockdown of ERβ by RNAi (Fig. 1B) resulted in the loss of the hormonal effect on gene expression (Fig. 1C).

Identification of GIOT-4 as a novel ERβ-interacting protein. ERβ is regulated by a different mechanism from that of ERα (7, 28, 30, 31, 53, 55). This finding suggests that ERβ requires subtype-specific coregulators. To test this idea, we generated a stable transformant expressing FLAG-tagged ERβ in 293F cells in a suspension culture (Fig. 2A). We biochemically purified ERβ interactants from nuclear extracts by a standard column step purification as we have reported previously (Fig. 2B) (26, 54). The fractions, after elution with FLAG peptide off FLAG M2 resin, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2C). SRC-1 interacted with ERβ in a ligand-dependent manner (Fig. 2C, lower panels), confirming our biochemical approach for identifying ERβ coregulators. By matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) analysis of the ERβ interactants, one interactant, designated GIOT-4, was identified in both the ligand-negative and -positive fractions (Fig. 2C). GIOT-4 has been recognized as a member of a Cys_2-His_2 (C2H2)-type zinc finger protein family, and the protein structure of GIOT-4 is closely related to those of GIOT-1 and GIOT-2 (38, 60). However, its physiological roles in gene regulation have remained to be studied (38, 52, 60). To examine whether GIOT-4 was a complex component, we then fractionated the ERβ interactants on a glycerol density gradient (Fig. 2D). GIOT-4 was

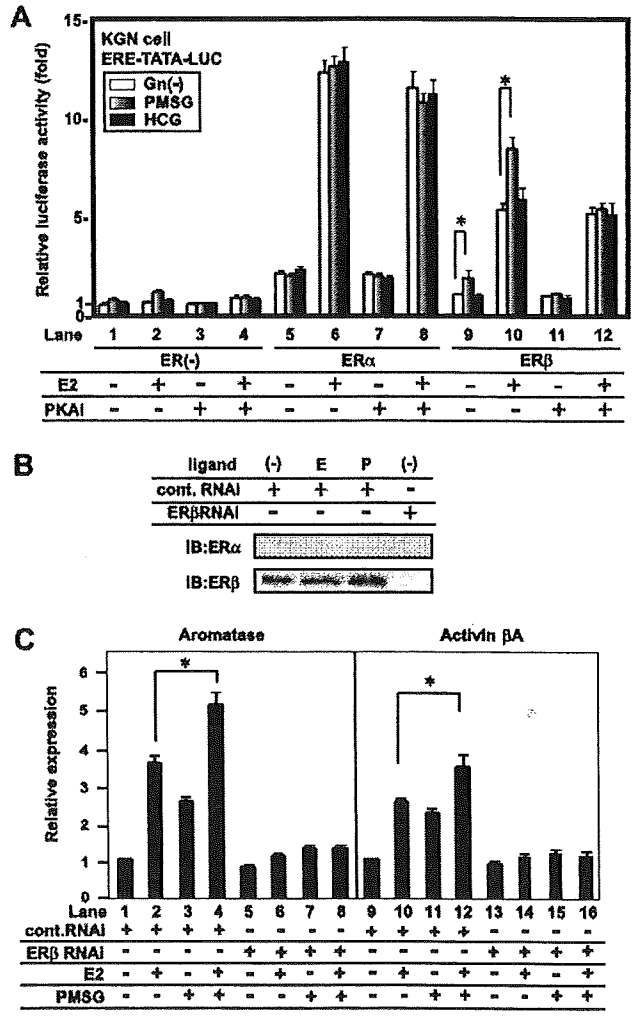

FIG. 1. An FSH analogue superactivates ERβ transcriptional activity via PKA signaling in KGN cells. (A) PMSG potentiates the transcriptional activity of ERβ but not ERα in KGN cells. KGN cells were transfected with ERs or a control vector. Without exogenous ERs, 10 nM E2 and 500m IU/ml PMSG, but not 1,000 mIU/ml hCG, increased transcriptional activity (lanes 1 and 2). Similar results were also obtained following transfection with ERβ. H89, a PKA inhibitor, suppressed the gonadotropin effect (lanes 1 to 4 and 9 to 12). Data are expressed as the mean ± SD of results from six independent experiments. Asterisks represent the findings of the statistical analysis, which showed that the results observed were significant ($P < 0.05$). ERE-TATA-LUC, construct containing ERE, TATA, and luciferase reporter sequences; Gn, gonadotropin; PKAi, PKA inhibitor H89; +, present; −, absent. (B) Expression of ERs in KGN cells. ERβ, but not ERα, was detected in KGN cells by Western blot analysis. The expression level did not change with 10 nM E2 (E) or PMSG (P) stimulation. siRNA for ERβ suppressed the expression of ERβ. Cont., control; IB, immunoblotting. (C) Transcriptional regulation of ERβ target genes, those encoding aromatase and the activin βA precursor, by E2 and PMSG. KGN cells were treated with 10 nM E2 and 500 mIU/ml PMSG for 8 h, and total RNA was extracted. When ERβ RNAi was used, KGN cells were transfected with siRNA 12 h before stimulation. Real-time PCR analysis revealed that E2 and PMSG increased mRNA levels of target genes. The hormonal responses in gene expression were inhibited by ERβ RNAi. Data are expressed as the mean ± SD of results from six independent experiments. Asterisks represent the findings of the statistical analysis, which showed that the results observed were significant ($P < 0.05$).

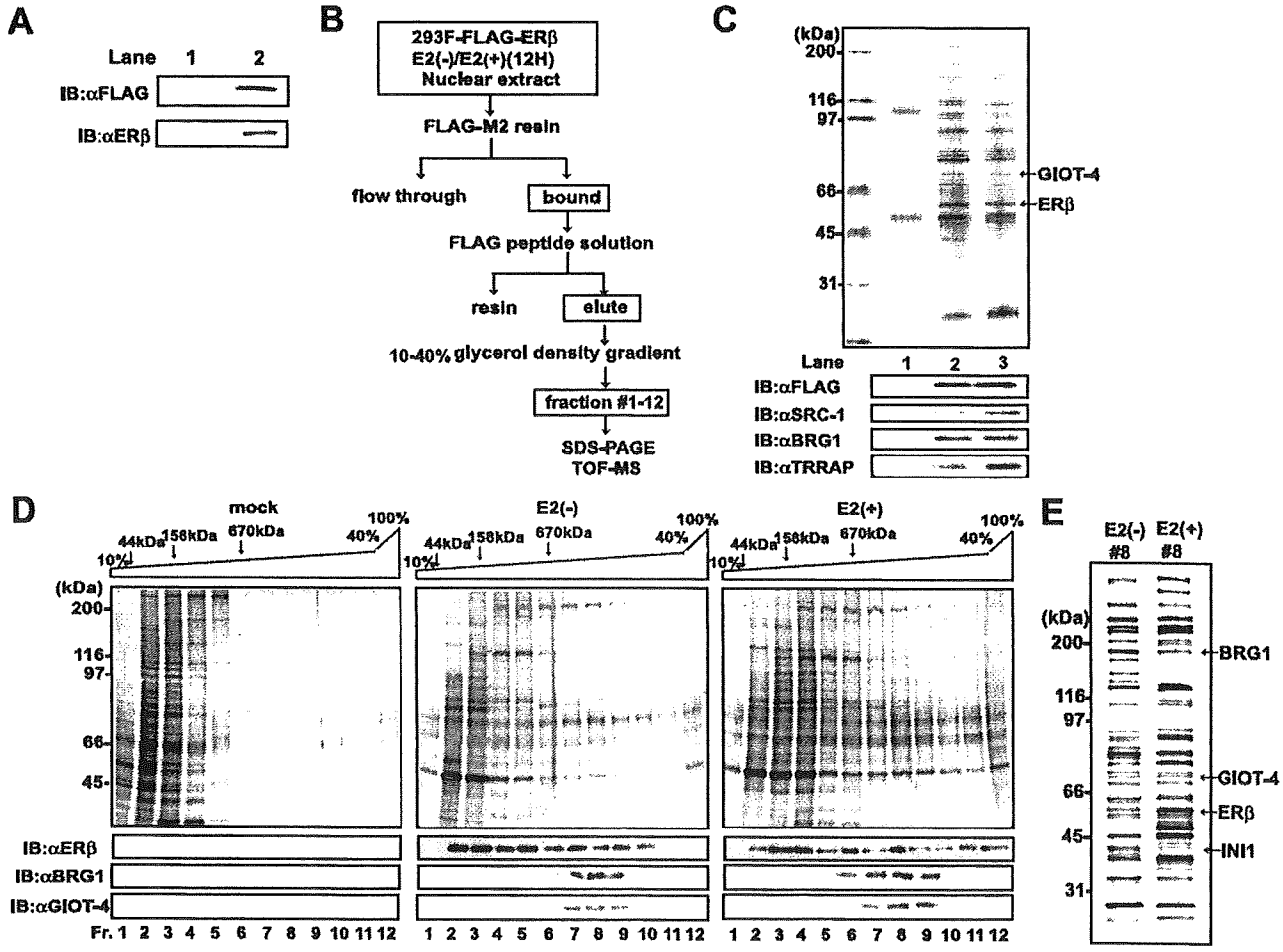

FIG. 2. Purification of ERβ interactants and identification of GIOT-4 as a novel ERβ-interacting protein. (A) Establishment of 293F cell lines stably expressing FLAG-tagged ERβ. FLAG-ERβ protein was detected in whole-cell lysates by Western blot analysis. Lanes: 1, mock-transfected 293F cells; 2, 293F cells stably expressing FLAG-ERβ. IB, immunoblotting; αFLAG and αERβ, anti-FLAG and anti-ERβ antibodies. (B) Purification scheme for ERβ-interacting complexes. The cells were collected 12 h (12H) after the initiation of E2 stimulation. (C) Identification of the ERβ interactants. Nuclear extracts were loaded onto FLAG-M2 resin, and bound proteins were eluted with FLAG peptide solution. Each eluted solution was subjected to SDS-PAGE, followed by silver staining, the results of which are shown in the upper panel. ERβ and GIOT-4 were identified by MALDI-TOF MS analysis. Each solution was immunoblotted with the antibodies indicated in the lower panels. The known cofactors were detected in lanes 2 and 3. (D) Proteins interacting with FLAG-ERβ in stable transformant 293F cells were fractionated by molecular mass on a 10 to 40% glycerol density gradient following FLAG affinity purification. Each fraction was subjected to SDS-PAGE. The upper panels show the results of silver staining. The lower panels show Western blots with ERβ-containing fractions (fr.). BRG1 and GIOT-4 were present in the same fractions. −, absent; +, present. (E) Results of SDS-PAGE and silver staining of ERβ/GIOT-4-containing fractions (#8). ERβ, GIOT-4, BRG1, and INI1 were identified by MALDI-TOF MS analysis.

detected in the fractions containing complexes of more than 670 kDa (Fig. 2D and E). Interestingly, in the same fractions, we identified BRG1 and INI1, both of which are components of mammalian SWI/SNF complexes (Fig. 2E). By Western blot analysis of the fractions, BRG1 was also seen together with GIOT-4 in the same fractions (Fig. 2D, lower panels), raising the possibility that GIOT-4 associates with SWI/SNF complex components interacting with ERβ.

GIOT-4 physically interacts with ERβ as an SWI/SNF-type complex component. To confirm if ERβ, GIOT-4, and SWI/SNF complex components indeed form a complex, we performed the coimmunoprecipitation of ERs and GIOT-4 from 293F cells (Fig. 3A). ERβ, but not ERα, was coimmunoprecipitated with GIOT-4 in a ligand-independent manner. This result was further confirmed by coimmunoprecipitation with endogenously expressed ERs from MCF7 cells (Fig. 3B). Additionally, in the immunoprecipitates containing ERβ, BRG1 and other SWI/SNF components, INI1 and BAF57 (20), were detected. In a GST pull-down assay, GIOT-4 physically interacted with the DEF region of ERβ (Fig. 3D). GIOT-4 and other GIOT family proteins are known to harbor a Kruppel-associated box (KRAB) domain and C2H2-type zinc finger motifs (Fig. 3C). As a GIOT deletion mutant protein (GIOT-4 del.) failed to associate with ERβ (Fig. 3D), the C-terminal C2H2 domain may be a domain for ERβ interaction.

The expression of GIOT-4 in KGN cells is PMSG dependent. Since the expression of GIOT-1, one of the GIOT family

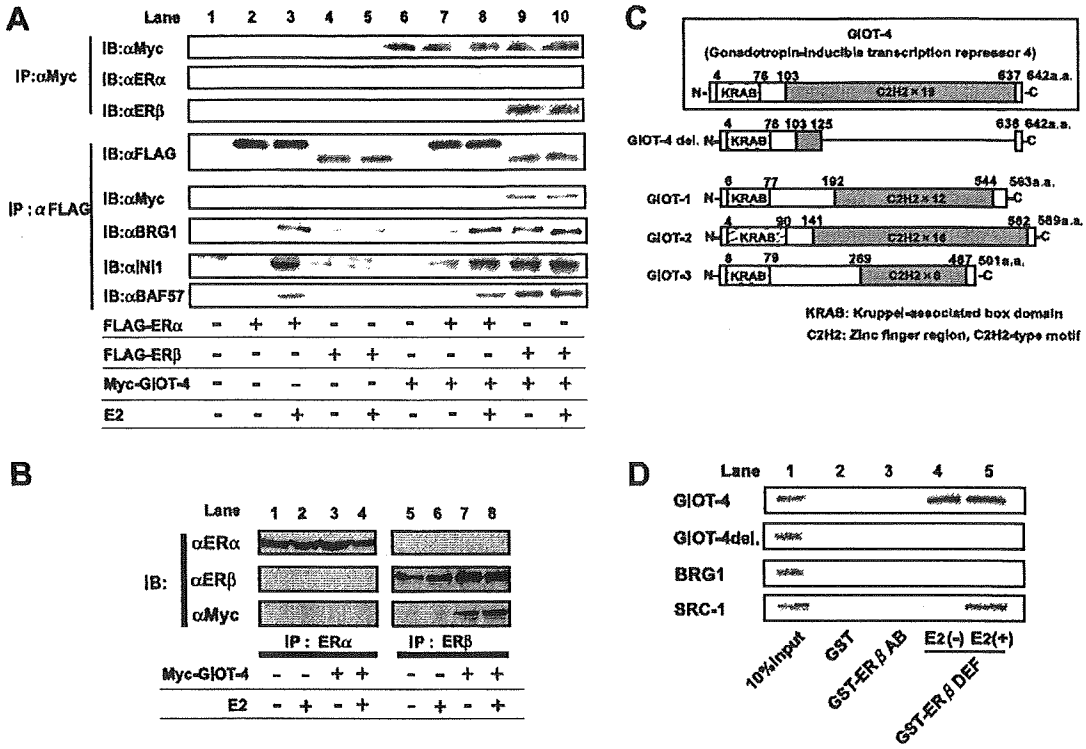

FIG. 3. GIOT-4 specifically interacts with ERβ in vivo and in vitro. (A) 293F cells were transfected with FLAG-tagged ERα or ERβ and Myc-tagged GIOT-4 as indicated below the panels. Coimmunoprecipitation assays were performed as indicated. ERβ, but not ERα, was immunoprecipitated with GIOT-4 in a ligand-independent manner. When cells were cotransfected GIOT-4 (lanes 6 to 10), BRG1, INI1, or BAF57 was precipitated with ERβ. IP, immunoprecipitation; IB, immunoblotting; αMyc, αFLAG, αERα, αERβ, αBRG1, αINI1, and αBAF57, anti-Myc, anti-FLAG, anti-ERα, anti-ERβ, anti-BRG1, anti-INI1, and anti-BAF57 antibodies; +, present; −, absent. (B) ER subtype-specific association with GIOT-4. Myc-tagged GIOT-4 was detected in the immunoprecipitate from ERβ antibody but not in the immunoprecipitate from ERα antibody. (C) Schematic illustration of GIOT family proteins. They have in common a KRAB domain in the N-terminal region and C2H2-type zinc finger motifs in the C-terminal region. GIOT-4 del. is a mutant protein in which most of the C-terminal region of GIOT-4 is deleted. a.a., amino acids. (D) GIOT-4 physically interacts with the DEF region of ERβ in a ligand-independent manner. GIOT-4 del. and BRG1 did not interact with ERβ. SRC-1 is shown as a positive control for the ligand-dependent interaction with ERβ. The GST pull-down assay was performed as explained in Materials and Methods.

members (Fig. 3B), was inducible by gonadotropin (38, 52, 60), we examined whether the expression of GIOT-4 protein was also regulated by PMSG. The expression of the endogenous GIOT-4 gene in KGN cells was indeed induced by treatment with PMSG, but not E2, at both the mRNA and protein levels (Fig. 4A and B). However, such PMSG-dependent regulation was not seen in 293F cells, which do not express gonadotropin receptors (Fig. 4A). Moreover, in accordance with these observations, the association of GIOT-4 with ERβ was induced by PMSG (Fig. 4C). Additionally, GIOT-4 RNAi, but not BAF57 RNAi, was found to inhibit the recruitment of BRG1 onto ERβ following PMSG treatment (Fig. 4C). These results suggest that PMSG stimulation upregulates GIOT-4 and thereby induces the recruitment of the SWI/SNF complex to ERβ in a mode different from that of recruitment to ERα (4, 7, 20).

The transcriptional activity of ERβ is stimulated by the expression of GIOT-4 induced by PMSG treatment. Next, we examined whether GIOT-4 indeed activated the transcriptional function of ERβ through the recruitment of an SWI/SNF complex in KGN cells. In 293F cells, GIOT-4 and BRG1 cooperatively coactivated ERβ (Fig. 5A). In KGN cells, BRG1 hyperactivated the transcriptional property of ERβ in the presence of PMSG (Fig. 5B, compare lanes 1 and 2). This coactivation of BRG1 by PMSG was not seen when GIOT-4 was knocked down by RNAi (Fig. 5B, compare lanes 1 and 3 and lanes 2 and 4) or when cells were transfected with a BRG1 mutant form (K798R) lacking the ATPase activity (57) (Fig. 5A, compare lanes 2 and 5 and lanes 3 and 6, and B, compare lanes 2 and 5). These results suggest that the coactivation of ERβ by GIOT-4 is mediated through the ATPase activity of the BRG1-containing complex (56).

GIOT-4 promotes histone modifications adjacent to the EREs in ERβ target gene promoters. We then confirmed that an SWI/SNF-type complex was associated with GIOT-4 and recruited to ERβ in the endogenous target gene promoters. For the ChIP assay, we chose the promoters for the aromatase and activin βA precursor genes. Both of these genes were expressed and were transcriptionally regulated by PMSG as well as E2 in KGN cells (Fig. 1C). As their promoters expectedly contained putative ERE-like sequences (Fig. 6A, upper panels), we first tested whether these putative ERE-like sequences indeed served as EREs by overexpressing ERβ and GIOT-4 in the presence or absence of E2

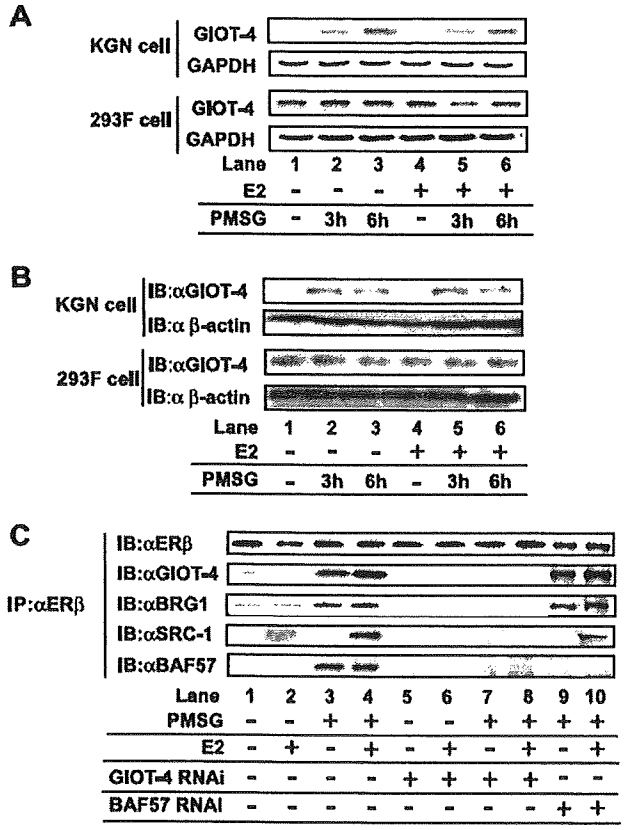

FIG. 4. The expression of GIOT-4 was induced by PMSG stimulation. (A) Semiquantitative RT-PCR analysis showing an increase in GIOT-4 mRNA in KGN cells but not in 293F cells following treatment with 500 mIU/ml PMSG. Cells were treated with (+) or without − 10 nM E2 for 6 h and 500 mIU/ml PMSG for the indicated times. (B) Western blot analysis showing the PMSG-dependent increases in GIOT-4 protein in KGN cells and 293F cells. Cells were stimulated as described in the legend to panel A. IB, immunoblotting; αGIOT-4 and α β-actin, anti-GIOT-4 and anti-β-actin antibodies. (C) PMSG-induced GIOT-4 recruits BRG1 onto endogenously expressed ERβ in a ligand-independent manner. KGN cells were transfected with 20 nM control siRNA, GIOT-4 siRNA, or BAF57 siRNA and stimulated by 10 nM E2 and 500 mIU/ml PMSG 12 h after siRNA transfection. IP, immunoprecipitation.

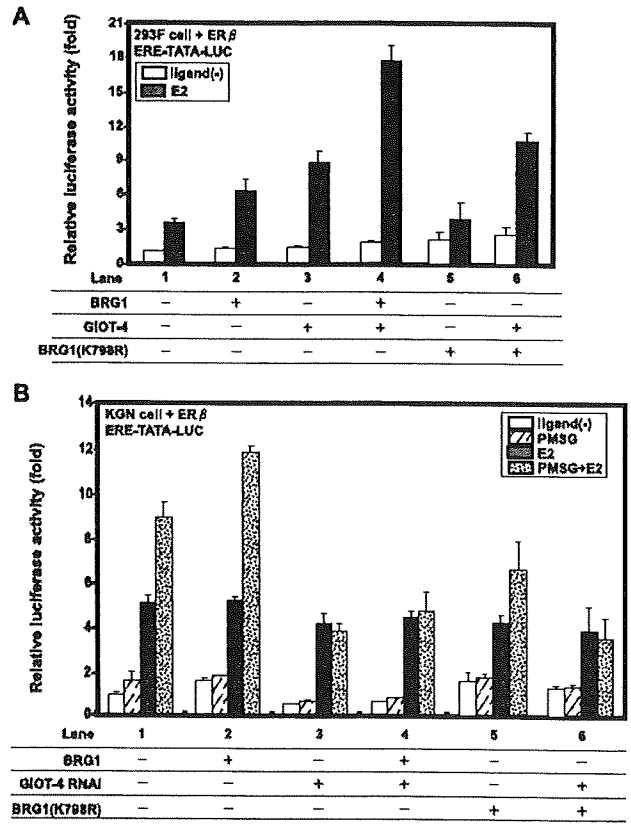

FIG. 5. GIOT-4 and BRG1 cooperatively potentiate the transcriptional activity of ERβ through ATP-dependent chromatin remodeling. (A) Exogenous BRG1 and GIOT-4 cooperatively activate the ERβ transcriptional property. 293F cells were transfected with ERβ, BRG1, a BRG1 mutant form (K798R), and GIOT-4 as indicated. The transcriptional activity of ERβ was highest following the cotransfection of cells with ERβ and both BRG1 and GIOT-4 (lane 4). E2 concentration, 10 nM. ERE-TATA-LUC, construct containing ERE, TATA, and luciferase reporter sequences; ligand(−), without ligand; +, present, −, absent. (B) Endogenous GIOT-4, induced by PMSG, activates the ERβ transcriptional property with the recruitment of BRG1. KGN cells were transfected as described in the legend to panel A. The coactivation of BRG1 and PMSG was inhibited by transfection with GIOT-4 siRNA (lanes 3 and 4) or the replacement of BRG1 with the BRG1 mutant form (lanes 5 and 6). Concentrations: E2, 10 nM; PMSG, 500 mIU/ml.

(Fig. 6A, lower panels). Clear E2-dependent recruitment of ERβ onto both promoters was detected, suggesting that these two genes were the direct target genes of ERβ. Endogenous ERβ was also detected on the putative EREs in an E2-dependent manner (Fig. 6B). Both PMSG treatment and the overexpression of GIOT-4 resulted in the recruitment of the SWI/SNF components BRG1 and INI1 and of GIOT-4 accompanied by ERβ (Fig. 6). Consistently, the demethylation of H3K9 (19), as well as the hyperacetylation of histone H4 adjacent to the ERβ binding sites in the aromatase gene promoter, was induced by PMSG treatment and was undetectable after the knockdown of either ERβ or GIOT-4 by RNAi. BAF57 RNAi had no effect on this observation (Fig. 6B). Thus, it is likely that the PMSG-induced expression of GIOT-4 triggers subsequent histone modifications of the adjacent chromatin domain of the ERβ binding site in certain ERβ target gene promoters. The histone modifications may account for the induction of the target genes following the treatment of KGN cells with PMSG or E2 (Fig. 1C).

PMSG-induced expression of ERβ target genes during murine folliculogenesis. As ERβ function appears to be indispensable for normal folliculogenesis, we tested if the observed FSH-induced regulation of ERβ target genes occurred during folliculogenesis in the intact ovary. To model the murine ovulatory cycle, we injected mice at the diestrous phase with PMSG and hCG and collected ovarian samples from immature ovaries (9, 15) (Fig. 7A).

By an immunofluorescence analysis, the expression of GIOT-4 in growing follicle granulosa cells at all developmental stages was detected in a PMSG-dependent manner. The colocalization of ERβ and GIOT-4 with Brg1 in the primary, sec-