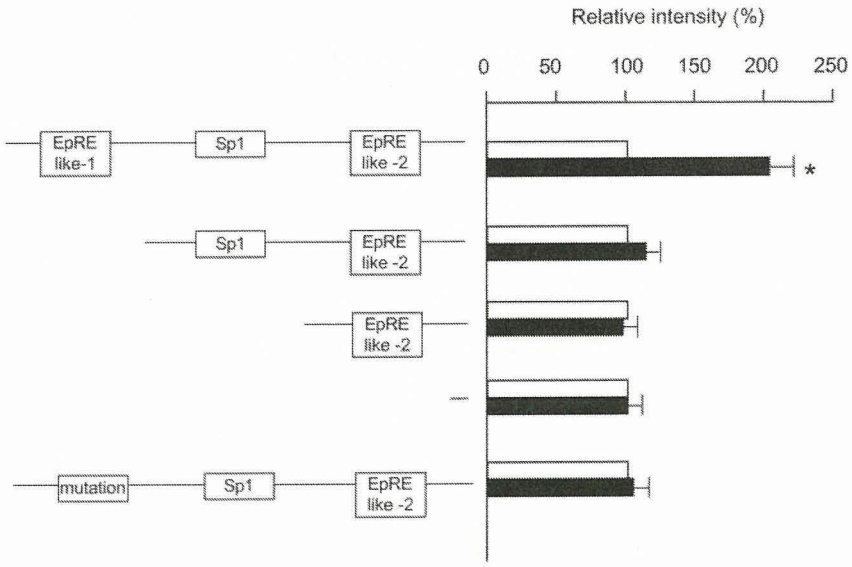
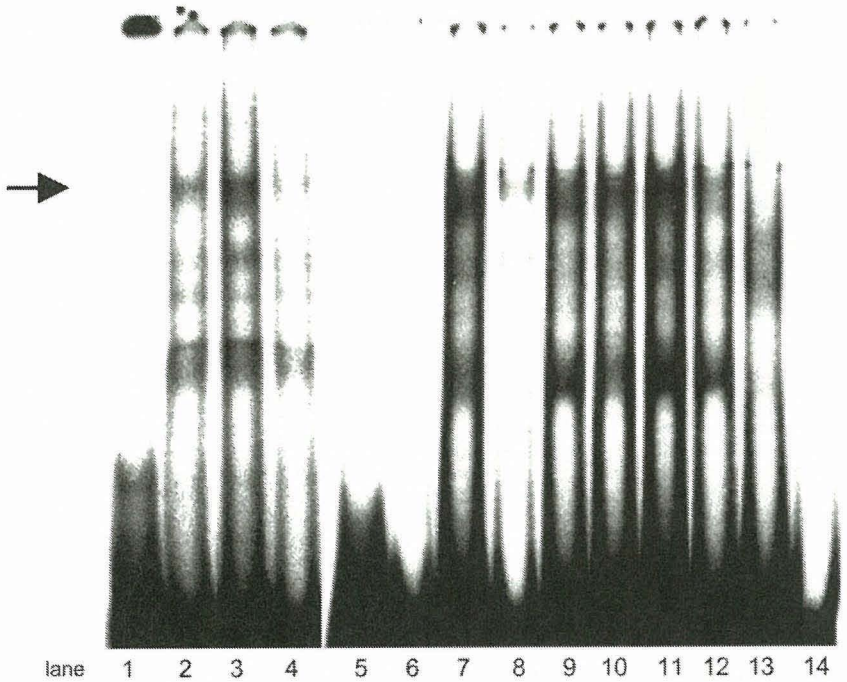


FIGURE 7. **The EpRE-like element is important for the E_2-dependent induction of the GRX promoter in H9c2 cells.** *Left*, schematic representation of luciferase vector constructs for the human GRX promoter. Each luciferase vector construct was generated as described under "Materials and Methods." *Right*, luciferase activity of the vector constructs for the human GRX gene promoter in H9c2 cells. The cells were transiently transfected with the GRX promoter-luciferase gene fusion plasmids. After the transfection, luciferase activity was assayed with cellular extracts as described under "Materials and Methods." Each value represents the mean of at least three independent experiments, and the S.D. was always within 10% of the mean. *, $p < 0.05$ compared with control.



lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14
probe	+	+	+	+	+	-	+	-	+	+	+	+	+	-
mutated probe	-	-	-	-	-	+	-	+	-	-	-	-	-	+
untreated NE	-	+	-	-	-	-	-	-	-	-	-	-	-	-
E_2 NE	-	-	+	+	-	-	+	+	+	+	+	+	+	+
antibody	-	-	-	-	-	-	-	-	c-Fos	c-Jun	Nrf2	ERβ	-	-
competitor	-	-	-	+	-	-	-	-	-	-	-	-	+	+

FIGURE 8. **The EpRE-like 1 element is responsive to E_2 in electrophoretic mobility shift assays.** H9c2 cells were incubated with 100 nM E_2 for 18 h, and the nuclear extracts were prepared as described under "Materials and Methods." ^{32}P-Labeled oligonucleotides specific to EpRE-like elements 1 and 2 of the GRX gene promoter were prepared and incubated with each nuclear extract and then subjected to a 5% nondenatured PAGE. In *lanes 1*, *5*, and *6*, the nuclear extract (*NE*) was free. In *lanes 6*, *8*, and *14*, ^{32}P-labeled mutant oligonucleotides were used. In *lanes 9–12*, *+Ab*, specific antibodies were added to the reaction mixture during the binding reaction for the supershift assay. *Arrowhead*, protein-DNA complex.

Important Role of ERβ in Other Cells—To further confirm the role of ERβ in protection against oxidative stress through redox regulation of Akt, we employed human breast cancer cells, SK-BR-3 and MDA-MB-231 cells. As shown in Fig. 9*A*, an RT-PCR analysis revealed that these cells mainly expressed ERβ mRNA. A stimulatory effect of E_2 on the activity of Akt was observed in these cells (Fig. 9, *B* and *C*). However, ICI182,780 abolished the protective effect of E_2 (Fig. 9, *B* and *C*). E_2 induced the expression of GRX (Fig. 9, *D* and *E*). The results suggested that the cytoprotective effect of E_2 is mediated through redox regulation of Akt activity in ERβ-expressing cells.

DISCUSSION

ERβ-mediated Cytoprotection against Oxidative Stress—Estrogenic hormones are required for the growth and differentiation of female

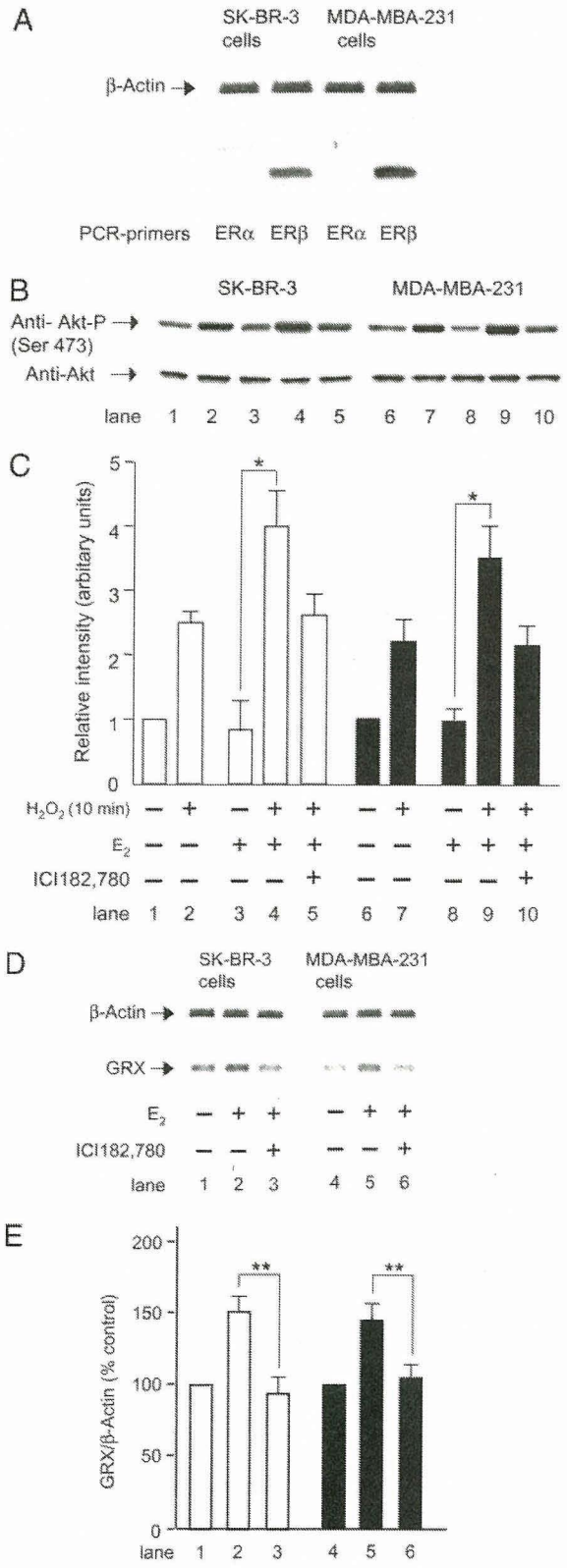


FIGURE 9. **Protective effect of E_2 in other cell lines.** The effect of E_2 was studied using ERβ-expressing human breast cancer SK-BR-3 cells and MDA-MB-231 cells. *A*, expression of ERs. The gene expression of ERs was estimated by RT-PCR analysis as described in the legend to Fig. 1. *B*, phosphorylation of Akt. The effect of E_2 on the phosphorylation of Akt under oxidative stress was estimated by immunoblot analysis using specific antibodies as described under "Materials and Methods." *C*, band intensity was estimated densitometrically, and the phosphorylation rates are expressed as the relative intensity of phosphorylated Akt to total Akt (*Akt-p/Akt*). The data are the mean ± S.D. of three independent analyses (*B* and *D*). *D*, gene expression of GRX. The effect of E_2 on levels of the GRX was estimated as described under "Materials and Methods." *E*, band intensity was estimated densitometrically and expressed as the intensity of GRX/β-actin. Each datum is a mean ± S.D. of three independent analyses. *, $p < 0.05$ compared with cells with H_2O_2 without E_2; **, $p < 0.05$ compared with cells with E_2 without ICI182,780.

reproductive tissues, contribute to male fertility, and play a role in maintaining cardiovascular, skeletal, and neural cell functions (9). Estrogen has been widely used to regulate fertility, relieve postmenopausal symptoms, and decrease the incidence and recurrence of mammary tumors. The ERs were the first members of the nuclear receptor family to be identified. ERα has been well characterized and plays a major role in E_2-mediated genomic actions in both reproductive and nonreproductive tissues. ERα-mediated cytoprotection against oxidative stress-induced cell damage has been reported in neurological cells (33, 34) and breast cancer cells (35). On the other hand, the role of ERβ is not well understood. A report using microarray analyses showed that most of the genes regulated by ERβ are distinct from those regulated by ERα in response to E_2 and selective estrogen receptor modulators (36). ERβ regulates plasminogen activator inhibitor-1 in endothelial cells, and a clinical evaluation of ERβ was suggested as a prognostic or predictive factor of drug resistance in breast cancer (37). These results suggest a significant role for ERβ in the regulation of cellular function, although the function of ERβ and its precise mechanism are still unclear (3). Thus, this is the first report aimed at the significant role of ERβ-mediated signals of E_2 in redox regulation in response to oxidative stress.

Involvement of Akt in the Cytoprotection of E2 Mediated by ERβ—The importance of Akt has been suggested in the cytoprotective effect of E_2 against oxidative stress. This effect of E_2 was rapid and nongenomic in neurological cells (38), vascular endothelial cells (39), and ovarian cancer cells (40). On the other hand, Stoica *et al.* (41) reported that ERα-mediated signals up-regulated the expression of Akt in ERα-positive MCF-7 breast cancer cells. They also demonstrated that Akt-mediated signals up-regulated the expression of ERα in these cells, suggesting that Akt plays a central role in the growth and survival of breast cancer cells; however, the mechanism by which Akt is activated by E_2 was not fully characterized.

In the present study, we were interested in the possible involvement of Akt signals in the ERβ-mediated anti-apoptotic effect against oxidative stress. We employed H9c2 cells that apparently express only ERβ (Fig. 1). We found that 1) H_2O_2-induced apoptosis was prevented when the cells were incubated with E_2 for over 18 h; 2) the anti-oxidative effect of E_2 was mediated by a genomic pathway through ERβ; and 3) E_2 retained the level of phosphorylated Akt in response to H_2O_2 via the GSH/GRX system.

Role of the GSH/GRX System in ERβ-mediated Akt Signals—We reported previously a role for the GRX/GSH system in the regulation of Akt phosphorylation (19). Akt is a Ser/Thr protein kinase with anti-apoptotic and oncogenic activities. Akt is activated through a growth factor receptor-mediated activation of the phosphatidylinositol 3-kinase pathway (21). The unphosphorylated form of Akt is virtually inactive, and phosphorylation at Thr^{308} and Ser^{473} stimulates its activity. Inactivation of Akt also occurs via dephosphorylation of the two phosphorylation sites by PP2A (23, 24). The activation of Akt contributes to the survival of H_2O_2-treated cells (25). H_2O_2 induces oxidation of Akt at Cys^{297} and Cys^{311}, and the oxidized form of Akt can be dephosphorylated by PP2A (19). PP2A is a major Ser/Thr phosphatase implicated in the regulation of many cellular processes, including the regulation of different signal transduction pathways, cell cycle progression, DNA replication, gene transcription, and protein translation (42). Yasukawa *et al.* have reported that Akt is also inactivated by *S*-nitrosylation at Cys^{224} in NO donor-treated cells (43). Furthermore, we recently reported that the phosphorylation of Akt is down-regulated by cytoplasmic calcium (32).

Calcium induced the expression of the PP2A catalytic subunit mediated by cAMP via the cAMP-response element. In the present study, the activity and the expression of the anti-PP2A catalytic C subunit did not change upon treatment with E_2 in H9c2 cells (Fig. 4*H*), suggesting that the modulation of calcium levels may not be involved. Inactivation by ROS of protein phosphatases, such as protein-tyrosine phosphatase 1B (44), mitogen-activated protein kinase (MAPK) phosphatases (45), and PP2A (46), has been reported. In the present study, the activity of PP2A was not changed by H_2O_2 (Fig. 4*H*), suggesting that inactivation of PP2A by ROS is not involved. The redox state of Akt is regulated by GSH/GRX (19). Oxidation of Akt at Cys^{297} and Cys^{311} facilitates the association of PP2A, leading to the dephosphorylation of Akt. However, the activity of Akt is not affected by the oxidation. In the present study, oxidation of Akt was observed in the medium with 0.5% fetal calf serum in the absence of H_2O_2, and after the treatment with H_2O_2, the oxidation of Akt continued for 60 min. In such conditions, E_2 maintained Akt in the reduced form (Fig. 5). This suggested that E_2 potentiates the functions of the GSH/GRX system. The GSH/GRX system regulates many signals, such as ASK-1, NF1, PTP1B, protein kinase C, and protein kinase A (49). The present study indicates for the first time that ERβ-mediated signaling via E2 up-regulates the activity of the GSH/GRX system to stimulate Akt and protects cells against oxidative stress.

Up-Regulation of γ-GCS and GRX by E_2—The ERα-mediated expression of antioxidants in response to oxidative stress has been reported. Genomic effects on the expression of antioxidant enzymes reported were Mn-SOD (4, 6), Cu,Zn-SOD (6), COX-1 (47), and COX-2 (5). Induction of GRX expression by E_2 was reported in bovine aortic endothelial cells (48) and in female mice (34). These reports suggested a potential contribution of TRX and GRX to the protection of cells against oxidative stress. As to ERβ, the expression of γ-GCS induced by E_2 was reported to be mediated by ERβ (7) in breast epithelial cell lines.

In the present study, we found that the expression of both GRX and γ-GCS is up-regulated by E_2 in ERβ-expressing cells. Data on the induction of the γ-GCS heavy subunit (Fig. 6, *B* and *C*) together with an increase in the level of GSH obtained here (Fig. 6*A*) is consistent with such a contribution. Furthermore, E_2 up-regulated the expression of GRX (Fig. 6, *D* and *E*). Elevated levels of both GSH and GRX were necessary to retain the reduced form of Akt. BSO abolished the effect of E_2 on the phosphorylation of Akt (Fig. 5*B*), and cadmium also abolished the effect of E_2 (Fig. 5*C*). The up-regulation of γ-GCS as well as GRX expression by E_2 was abolished by ICI182,780 (Fig. 6, *A–E*), suggesting involvement of the ERβ-mediated genomic effect of E_2. The possible role of ERβ in the cytoprotection against oxidative stress was supported by the results obtained using other ERβ-expressing cells (Fig. 9). Although involvement of ERα in the cytoprotective effect of E_2 cannot be ruled out in these cells, it is suggested that the GRX/GSH system is involved in the cytoprotective and genomic effects of E_2 on the redox state of Akt, a pathway that is mediated, at least in part, by ERβ. This mechanism may also play an antiapoptotic role in cancer cells during carcinogenesis or chemotherapy. A difference in the distribution of ERα and ERβ was reported (3, 27, 50, 51). ERα and ERβ differ in the distribution in tissue cells and how they regulate cell proliferation and apoptosis, which may provide some insight into the tissue-specific functions and interplay between the two receptors.

The role of the GSH/GRX redox system in the antiapoptotic effect of E_2 was studied further. In the present study, we found that the induction of GRX expression by E_2 is mediated by an EpRE-like 1 element (Figs. 7 and 8). The human GRX promoter employed here possessed no apparent ERE or EpRE but had two EpRE-like sites. Interestingly, one of these sites, EpRE-like 1, bound to ERβ and promoted the transcriptional activity of GRX. Transcription of the GRX gene was increased by E_2 but decreased by anti-ERβ antibody. However, EpRE-like 1 did not bind to Nrf-2 or AP-1. This element may be a novel kind of ERE. In summary, E_2 has a cytoprotective effect against oxidative stress in H9c2 cells expressing ERβ. The genomic effect of E_2 on the GSH/GRX redox system potentiates Akt activity, a mechanism that may also play an antiapoptotic role in cancer cells during carcinogenesis or chemotherapy.

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ORIGINAL PAPER

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Diethylstilbestrol increases the density of prolactin cells in male mouse pituitary by inducing proliferation of prolactin cells and transdifferentiation of gonadotropic cells

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Abstract Diethylstilbestrol (DES) has been implicated in mammalian abnormalities. We examined the effects of DES on follicle-stimulating hormone (FSH), luteinizing hormone (LH), and prolactin (PRL) cells in the pituitaries of male mice treated with various doses of DES for 20 days. DES reduced the density of FSH and LH cells in a dose-dependent manner, but increased that of PRL cells. When the expression of estrogen receptor (ER) α and β was assessed, an induction of ERβ by DES was found predominantly in PRL cells. However, since these effects were abolished in ERα knockout mice, DES appears to act primarily through ERα. When the expression of Ki-67 and Pit-1 in PRL cells was examined at various time-points after DES treatment, some PRL cells became Ki-67 positive at 10–15 days, and Pit-1-positive cells were increased at 5–15 days. Furthermore, some FSH and LH cells became Pit-1 positive, and co-localized with PRL at 5–10 days. Our results indicate that DES increases PRL cells by inducing proliferation of PRL cells and transdifferentiation of FSH/LH cells to PRL cells.

Keywords Diethylstilbestrol · Pituitary · Prolactin · Estrogen receptors · Pit-1

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Introduction

Diethylstilbestrol (DES), a stilbene estrogen analogue acting as an agonist of estrogen, causes structural abnormalities in reproductive organs, infertility, and neoplasia in mammals (McLachlan et al. 1980). In the pituitary, it is known that treatment with DES causes pituitary hyperplasia followed by the development of prolactinomas (Walker and Kurth 1993), and DES increased the prolactin (PRL) cell population in rodent pituitaries (Cauwenberge et al. 2001; Matsubara et al. 2001). However, the precise mechanism of DES effect on pituitary cells remains unclear.

Estrogens play various roles in the pituitary, affecting the states of proliferation of certain cell populations (Hashi et al. 1996) and their hormone production (Scully et al. 1997). The effects of estrogens are mediated through estrogen receptors (ERs), ligand-dependent transcription regulatory factors (Osborne et al. 2000). ERs bind to estrogen responsive element (ERE) in the promoter region of some genes, such as follicle-stimulating hormone (FSH), luteinizing hormone (LH), and PRL (Maurer and Notides 1987; Shupnik and Rosenzweig 1991), and regulate their expression (Osborne et al. 2000). Currently, ER is categorized into two subtypes, ERα and β. The classical ER, ERα, is distributed in various reproductive organs as well as pituitary (McClellan et al. 1984; Greene et al. 1984; Koji and Brenner 1993; Friend et al. 1994). Furthermore, ERβ, a newly identified ER, was cloned in the rat prostate and ovary (Kuiper et al. 1996). Much attention has been

recently paid to the analysis of expression of both ERs in various tissues, including reproductive organs (Pelletier and El-Alfy 2000; Tsurusaki et al. 2003), and intestine (Kawano et al. 2004). In the rodent pituitary, ERα is distributed in many anterior pituitary cell types, including FSH, LH, and PRL cells (Mitchner et al. 1998), and plays a critical role in transcription of these genes (Scully et al. 1997). On the other hand, there is still a controversy about the localization and characterization of ERβ (Mitchner et al. 1998; Nishihara et al. 2000). Since ERs bind to ERE as homodimers or heterodimers (Petterson et al. 1997), a different pattern of gene regulation might be raised.

The expression of PRL gene is also regulated by a pituitary-specific transcription factor, referred to as Pit-1 (Ingraham et al. 1988; Crewshaw et al. 1989). Since Pit-1 can bind to PRL, growth hormone (GH), and thyroid-stimulating hormone (TSH) gene promoters, it is responsible for the transcriptional activation of genes for these hormones (Fox et al.1990; Lloyd and Osamura 1997). In mouse PRL gene promoter, Pit-1 binds to four sites within the distal enhancer and further four sites within the proximal region, thereafter activates the cell-type specific expression of PRL gene (Crewshaw et al. 1989). Therefore, the cellular analysis of Pit-1 expression would provide useful information on the promotion of PRL cell differentiation. However, our knowledge of the affection of estrogens on the PRL cell differentiation is still largely limited.

In the present study, we first investigated the effect of DES on the expression of sex-related pituitary hormones and its correlation with ERs in wild-type and ERα knockout (ERαKO) male mice. For this purpose, we quantitatively measured the effects of in vivo treatment of various doses of DES on the density of pituitary cells positive for these hormones and ERs. Second, to gain further insight into the kinetics of PRL cells, we examined the expression of Ki-67, which is regarded as a marker of the proliferative activity, and Pit-1 at various time-points after DES treatment by immunohistochemistry and Southwestern histochemistry, respectively. The results indicated that DES actions are mediated through ERα, and that DES increased the density of PRL cells by enhancing their proliferation and induction of transdifferentiation of FSH/LH cells to PRL cells.

Materials and methods

Chemicals and biochemicals

DES was purchased from ICN Biomedical (Aurora, OH, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents were purchased from Daiichi Pure Chemicals (Tokyo, Japan). Immobilon, polyvinylidene difluoride membrane, was purchased from Millipore (Bedford, MA, USA). Lima bean trypsin inhibitor was purchased from Worthington Biochemical (Freehold, NJ, USA). The protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Tris[hydroxymethyl]aminomethane (Tris), sodium molybdate, phenylmethylsulfonylfluoride (PMSF), bovine serum albumin (BSA; minimum 98%, γ-globulin free), 2-mercaptoethanol, Triton X-100, Brij 35, yeast transfer RNA, and salmon testis DNA were purchased from Sigma Chemical (St Louis, MO, USA). Paraformaldehyde (PFA) was purchased from Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) and 3,3′-diaminobenzidine–4 HCl (DAB) were purchased from Dojin Chemicals (Kumamoto, Japan). 4-Cl-1-naphthol was from Tokyo Kasei Kogyo (Tokyo, Japan). All other reagents used in this study were purchased from Wako Pure Chemicals (Osaka, Japan) and were of analytical grade.

Antibodies

Specific rabbit polyclonal antisera against mouse FSH (1:400), LH (1:1,000), PRL (1:800), GH (1:1,600), and TSH (1:400) were purchased from Biogenesis (Bournemouth, UK). Each antiserum did not cross-react with other pituitary hormones including adreno-corticotropic hormone (ACTH), according to the data provided by the company and previous study (Nishihara et al. 2000). A mouse monoclonal antibody against ERα (ER88; 1:160, 0.7 μg/ml) was purchased from BioGenex (San Ramon, CA, USA). For the detection of ERβ, a rabbit polyclonal antiserum against rat ERβ (1:400) was generated as described previously (Nishihara et al. 2000). A mouse monoclonal antibody against Ki-67 (MIB-5; 1:50, 4.0 μg/ml) was purchased from Immunotech (Marseille, France), and a mouse monoclonal antibody against β-actin (AC-15; 1:6,400, 0.45 μg/ml) was purchased from Sigma. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was purchased from Chemicon International (Temecula, CA, USA). HRP-conjugated goat anti-rabbit IgG was purchased from MBL (Nagoya, Japan). HRP-conjugated mouse monoclonal anti-thymine-thymine (T–T) IgG (1:80) was from Kyowa Medex (Tokyo, Japan). Normal goat IgG and normal mouse IgG were purchased from Sigma. Normal rabbit IgG was purchased from DAKO (Glostrup, Denmark).

Animals

Adult male and female ICR (Crj: CD-1), male C57BL/6J, and male ERαKO mice (6 weeks old) were used in the present study. ICR and C57BL/6J mice were purchased from Charles River Japan (Kanagawa, Japan), and ERαKO mice from a background of C57BL/6J were purchased from Taconic (Germantown, NY, USA). The experimental protocols (# 0112100012 and 0202200048) were approved by the Biochemical Research Center, Center for Frontier Life Sciences, Nagasaki University. Three mice were housed per cage in an air-conditioned and light-controlled room at the animal facility of

Nagasaki University. In the beginning of experiments, the male mice were injected subcutaneously with DES [1 ng, 10 ng, 100 ng, 1 μg, 10 μg, 100 μg, 1 mg, or 20 mg/kg body weight (BW)] dissolved in corn oil containing 5% ethanol or with the vehicle alone as a control, every 5 days as described previously (Libbus and Schuetz 1980; Kondo et al. 2002) ($n=3$ in each group). In fact, the maximum injection dose of DES was determined based on the previous studies in mice (Marselos and Tomatis 1992; Walker and Kurth 1993). In physiological conditions, the blood level of estradiol was 10–60 pg/ml in adult male mammals (Wada et al. 1996), and so the doses of DES around 10–100 ng/kg BW would be regarded as physiological conditions in male animals. In the present study, we used a wide range of doses of physiological and pharmacological DES. Later, we concentrated on the analysis of specimens treated with 100 ng, 100 μg, and 20 mg/kg BW DES, in which significant differences in the density of positive cells for various pituitary hormones, as compared to the control group, were observed. On day 20, all animals were sacrificed, and their pituitary glands were removed. Normal female ICR mice at the estrous stage ($n=5$) were also used as positive controls for ERs. The estrous cycle phase was estimated in each mouse by examination of vaginal cell smears. In the experiment designed to investigate time-course changes in cell kinetics of pituitaries of DES-treated mice, male ICR mice were treated with 20 mg/kg BW DES or the vehicle alone for 0, 5, 10, 15, and 20 days ($n=3$ for each time-point).

Tissue preparation

Pituitaries, uteri, ovaries, and livers were divided into several pieces. Some pieces of the tissues were quickly frozen, stored at –80°C and later used for Western blotting. The other pieces were fixed in 4% PFA in phosphate-buffered saline (PBS; pH 7.2) at room temperature (RT) for about 20 h and embedded in paraffin using a standard procedure.

Western blot analysis of ERα and ERβ

Tissues were homogenized in a lysis buffer containing 50 mM Tris–HCl buffer (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.1 mM PMSF, 20 mM sodium molybdate, and 50 μg/ml lima bean trypsin inhibitor, as described previously (Nishihara et al. 2000; Kawano et al. 2004). After centrifugation of the homogenate at 13,000× g for 10 min at 4°C, the supernatant was collected and stored at −80°C. The protein concentration in each preparation was determined using a kit from Bio-Rad Laboratories according to the method of Bradford (1976), using BSA as a standard. An aliquot (30 μg) of sample lysates was mixed with the loading buffer (200 mM Tris–HCl, pH 8.0, 0.5 M sucrose, 5 mM EDTA, 0.01% bromophenol blue, 2.5% SDS, and 10% 2-mercaptoethanol), boiled for 5 min, and separated by SDS-PAGE with a 4–20% gradient gel according to the method of Laemmli (1970), and electrophoretically transferred onto polyvinylidene difluoride membranes. After blocking with 10% nonfat dry milk in Tris-buffered saline (TBS) for 1 h, blots were incubated overnight at 4°C with mouse anti-ERα (1:80) or rabbit anti-ERβ (1:200) in TBS/0.05% Triton X-100 buffer. The membranes were washed three times for 10 min each time with TBS/0.05% Triton X-100 buffer. Each membrane was reacted with HRP-goat anti-mouse IgG (1:400) or anti-rabbit IgG (1:200) in 10% nonfat dry milk in TBS for 1 h at RT and was again washed six times, 15 min each, with TBS/0.05% Triton X-100 buffer. The bands were visualized with DAB, Ni, Co, and H_2O_2 according to the method of Adams (1981). To confirm that the applied protein amounts were the same in each lane, the membranes were further reacted with mouse anti-β-actin (1:6,400) for 1 h, and with HRP-goat anti-mouse IgG (1:1,600). These were visualized as described above.

Immunohistochemistry for ERα, ERβ, FSH, LH, PRL, GH, TSH, and Ki-67

Immunohistochemical staining was performed using the methods described previously (Kawano et al. 2004; An et al. 2005). Paraffin-embedded tissues were cut into 5-μm thick sections and placed onto silane-coated glass slides. For ERα, ERβ, and Ki-67, the sections were deparaffinized with toluene, and rehydrated with serial ethanol solutions, and then autoclaved at 120°C for 15 min in 10 mM citrate buffer (pH 6.0) (Ehara et al. 1995). After inhibition of endogenous peroxidase activity with 0.3% H_2O_2 in methanol for 15 min at RT, they were preincubated with 500 μg/ml normal goat IgG and 1% BSA in PBS for 1 h at RT to block nonspecific binding of antibodies. Sections were then reacted with the primary antibodies overnight at RT. After washing with 0.075% Brij 35 in PBS, they were reacted with HRP-goat anti-mouse IgG (1:100) or HRP-goat anti-rabbit IgG (1:200) for 1 h at RT. After washing in 0.075% Brij 35 in PBS, the sites of HRP were visualized with DAB, Ni, Co, and H_2O_2. For FSH, LH, PRL, GH, and TSH detection, sections were similarly processed as described above, except for omitting the autoclave step. Sections were reacted with the primary antibodies for 1 h at RT. After washing, they were incubated with HRP-goat anti-rabbit IgG (1:100) for 1 h at RT. The sites of HRP were visualized with DAB and H_2O_2. As a negative control, normal mouse or rabbit IgG was used at the same dilution instead of the primary antibodies in every run.

Preparation of oligo-DNA probes for Pit-1

We synthesized double-stranded oligo-DNA containing the consensus sequence of Pit-1 binding domain (Pit-1

(+): 5′-CCCTATACATTTATTCATGGC-3′, Pit-1 (-): 5′-GCCATGAATAAATGTATAGGG-3′) (−89 to −69) of mouse GH gene promoter, which can bind to and activate mouse PRL gene promoter (Ingraham et al. 1988; Izumi et al. 2005). For a negative control probe, we synthesized the mutated Pit-1 (mPit-1 (+): 5′-CCCTATACATTTAggtggtGC-3′, mPit-1 (–): 5′-GCaccaccTAAATGTATAGGG-3′), with a six-base mutation indicated by the small letters (Ingraham et al. 1988; Izumi et al. 2005). Ingraham et al. (1998) revealed that the mutated Pit-1 oligo-DNA, which was mutated only one consensus Pit-1 element, did not compete for binding with Pit-1 probe, suggesting that the mutated Pit-1 probe could not bind to Pit-1. These oligo-DNAs were added with three repeats of adenine-thymine-thymine at the 3′-end for T–T dimers (Koji et al. 1994; Koji and Nakane 1996). We conducted a computer-assisted search (GenBank nucleic acid sequence database release 142) of the above Pit-1 oligo-DNA sequences and found 100% homology with these sequences. These oligo-DNAs were haptenized by introducing T–T dimers by UV irradiation using a dose of 12,000 J/m^2, as described previously (Koji et al. 1994; Koji and Nakane 1996).

Southwestern histochemistry for Pit-1

Southwestern histochemistry for Pit-1 of mouse pituitaries was performed, as described previously (Koji et al. 1994). Briefly, paraffin sections were deparaffinized, rehydrated, and autoclaved. Slides were immersed in 50 mM Tris–HCl buffer (pH 7.4) containing 5% nonfat dry milk, 150 mM NaCl, and 1 mM EDTA (TMSE) for 1 h at RT. They were incubated with TMSE solution, including T–T dimerized probe DNA at 2.0 μg/ml overnight at RT. Sections were washed in TMSE solution twice, 0.075% Brij 35 in PBS three times, and immersed in 500 μg/ml normal mouse IgG, 100 μg/ml salmon testis DNA, 100 μg/ml yeast transfer RNA, and 5% BSA in PBS for 1 h at RT. They were reacted overnight with HRP-mouse anti-T–T antibody (1:80) at RT. After washing in 0.075% Brij 35 in PBS, they were visualized with DAB, Ni, Co, and H_2O_2. To confirm the specificity of Pit-1 signals, mutated Pit-1 and non-probe were used as a negative control.

Simultaneous detection of PRL and Ki-67, identification of cell type of Pit-1-positive cells, and simultaneous detection of PRL and gonadotropins or GH.

For simultaneous detection of PRL and Ki-67, we performed double staining, as described previously (Kawano et al. 2004; An et al. 2005). Briefly, the sections were stained with anti-PRL antiserum, and HRP sites were visualized with DAB and H_2O_2. Then the sections were immersed in PBS and autoclaved. The slides were immersed in 0.1 M glycine–HCl buffer (pH 2.2) three times for 30 min each. After washing with Milli-Q water once and with PBS three times, the sections were reacted with anti-Ki-67 antibody. HRP sites were visualized with 4-Cl-1-naphthol and H_2O_2 solution. To identify Pit-1-positive cells, we also performed double staining for PRL, FSH, or LH and Pit-1 processed similarly, as described above. PRL, FSH, or LH signal was first visualized with DAB and H_2O_2, and Pit-1 signal was visualized with 4-Cl-1-naphthol and H_2O_2 solution. For simultaneous detection of PRL and FSH, LH, or GH, sections were similarly processed, except for omitting the autoclave step. PRL signal was first visualized with DAB and H_2O_2, and FSH, LH, or GH signal was visualized with 4-Cl-1-naphthol and H_2O_2 solution.

Quantitative analysis

The results of immunohistochemistry were graded as positive or negative, compared with that of negative control. For each section, the numbers of cell nuclei were counted in more than 2,000 nuclei in randomly selected fields at 400× magnification. Cell counts were performed in a blind fashion by three individuals. The number of positive cells was expressed as the percentage of cells with positive nuclei per total number of counted nuclei. The counting error from the cell size could be reduced by counting the cell nuclei (Davies et al. 1974). In the case of Pit-1-positive cells, red color was given to the positive cells by a computer-assisted image analyzer (DAB analysis system; Carl Zeiss, Göttingen, Germany) before counting. Positive cells were evaluated based on the staining density over the level of staining with the mutated probe. The signal intensity of pituitary hormones was measured by an image analyzer (DAB analysis system). We calculated the optical density of each cell in the pituitary as the sum of the gray values of all pixels corresponding to the cell.

Statistical analysis

All data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the unpaired Student's *t*-test. A *P* value less than 0.05 denoted the presence of a statistically significant difference. All analyses were performed with a statistical software package (StatView, version 5.0; Abacus Concepts, Berkeley, CA).

Results

Effects of DES on FSH, LH, and PRL expression in pituitary glands

We examined the effects of DES on the expression of FSH, LH, and PRL in male mouse pituitaries by immunohistochemstry. These hormones were detected in the cytoplasm of anterior pituitary cells (Fig. 1). Quantitative analysis revealed that DES reduced the population density of FSH and LH cells in a dose-dependent manner, but increased that of PRL cells (Table 1). The

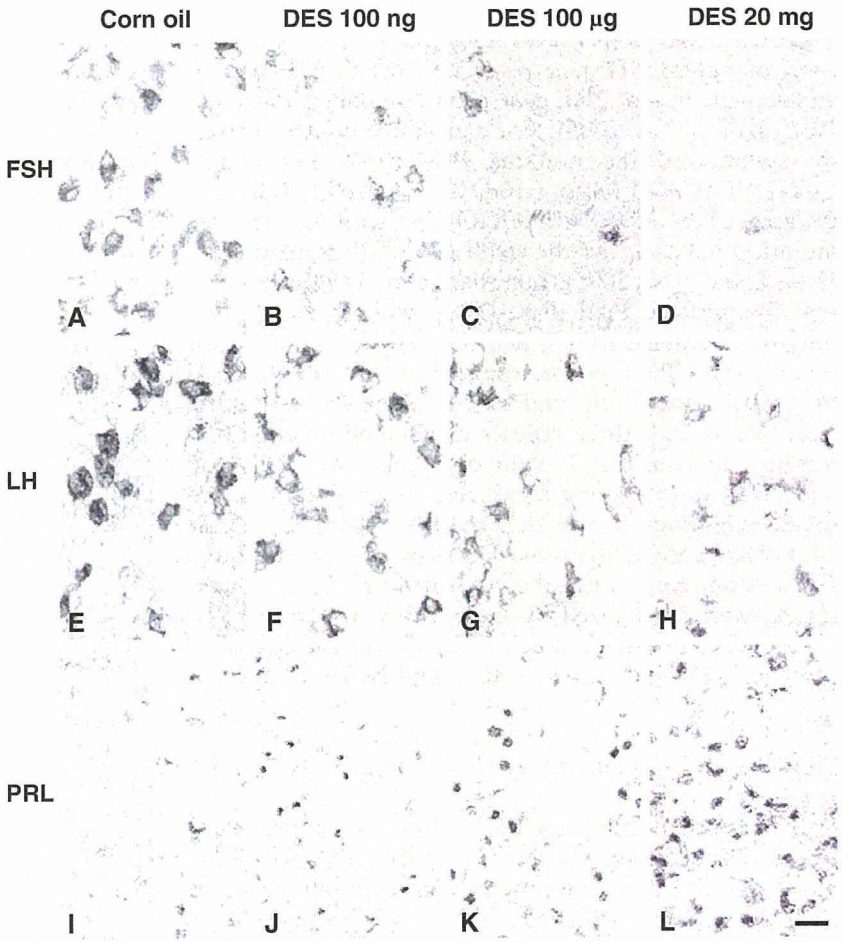

Fig. 1 Immunohistochemical localization of FSH (**a–d**), LH (**e–h**), and PRL (**i–l**) cells in paraffin sections of male mouse pituitaries treated with corn oil or DES at 100 ng, 100 µg, or 20 mg/kg BW. Magnification, 400×. Scale bar, 20 µm

percentage of PRL-positive cells at 20 mg/kg BW DES was four times higher than the value of control group injected with corn oil (Table 1). In parallel with the changes in the cell density, we confirmed similar DES effects on the signal intensities of the same hormones by image analysis (Fig. 2). The sections reacted with normal rabbit IgG at the same dilution instead of the specific antibody as a negative control showed no staining (data not shown).

Effects of DES on ERα and β expression in pituitary glands

First, we performed Western blot analysis of pituitary tissue extracts from wild-type male mice, and liver tissue extracts from ERαKO male mice to confirm the expression of ERα and β. We used extracts from the uterus and ovaries of normal female mice at the estrous stage as positive controls for ERα and β, respectively. A

Table 1 Percentage of positive cells in male mouse pituitaries

Positive cells	Treatment (dose/kg BW)			
	Corn oil	DES 100 ng	DES 100 µg	DES 20 mg
FSH	13.7 ± 0.4	6.3 ± 0.2*	4.2 ± 0.3**	1.9 ± 0.5**
LH	12.6 ± 0.5	10.4 ± 0.4	6.2 ± 1.2*	4.5 ± 0.8**
PRL	10.6 ± 0.4	12.2 ± 0.6	20.8 ± 1.2**	40.5 ± 2.5**
ERα	44.6 ± 1.3	37.3 ± 0.6*	25.4 ± 1.1**	17.6 ± 0.8**
ERβ	0.2 ± 0.5	0.3 ± 0.6	8.0 ± 0.7*	15.1 ± 0.5**
ERα alone in PRL[a]	80.2 ± 2.0	ND	ND	27.3 ± 1.3
ERβ alone in PRL[b]	ND	ND	ND	20.2 ± 0.9
ERα and β in PRL[c]	ND	ND	ND	36.4 ± 1.5
PRL in ERβ[d]	ND	ND	ND	89.6 ± 1.2

Data represent mean ± SD (%). *ND* not determined. *$P < 0.05$, **$P < 0.01$, compared with corn oil
[a]ERα alone-positive cells in PRL cells
[b]ERβ alone-positive cells in PRL cells
[c]ERα and β-positive cells in PRL cells
[d]PRL cells in ERβ-positive cells

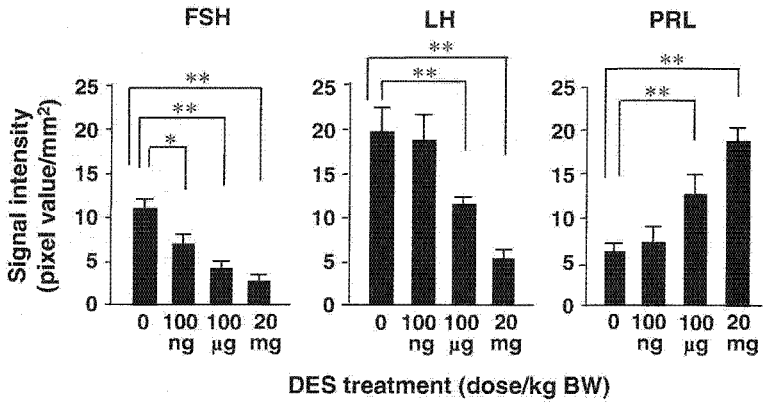

Fig. 2 Quantitative analysis of the signal intensity of FSH (*left panel*), LH (*middle panel*), and PRL (*right panel*). Data represent mean ± SD. $*P < 0.05$, $**P < 0.01$

66-kDa band for ERα was detected in the control pituitary, but no band for ERβ was detected (Fig. 3). The pituitary extracts from mice treated with DES at 20 mg/kg BW showed weaker 66-kDa ERα band and the appearance of a 55-kDa band for ERβ. In the liver extracts from ERαKO mice treated with corn oil, the 66-kDa band for ERα was not detected. In addition, the 42-kDa β-actin band confirmed the loading of equivalent amounts of protein.

In the next step, we analyzed the effect of DES treatment on the population of ERα- and β-positive cells in the pituitary by immunohistochemistry. ERα was detected in the nuclei of many pituitary cells in the control, whereas DES markedly decreased the percentage of ERα-positive cells (Fig. 4a–d; Table 1). In contrast, ERβ signal was detected in the nuclei of some anterior lobe cells at DES doses higher than 100 µg/kg BW, although it was not detected in the control or at lower DES doses (Fig. 4e–h; Table 1). The sections reacted with normal mouse or rabbit IgG as a negative control showed no staining (data not shown).

To clarify the correlation between ER expression and PRL cell counts, we performed immunohistochemical analysis of ERs and PRL by the combined use of adjacent and mirror sections. Most of the PRL cells were positive for ERα alone in the control (Table 1), while PRL cells positive for ERβ alone, or both ERα and β were induced in mice treated with DES at 20 mg/kg BW (Fig. 5; Table 1). In addition, ERβ expression was predominantly in PRL cells (Table 1).

Effect of DES on Ki-67 labeling of PRL cells in pituitary glands

To investigate the underlying mechanism of the increase in the percentage of PRL cells, we examined the expression of Ki-67 (a marker for the proliferative activity) by immunohistochemistry at 0, 5, 10, 15, and 20 days after treatment with 20 mg/kg BW DES. Then we also performed double staining for Ki-67 and PRL in the same sections. Ki-67 was detected in the nuclei of only a few anterior pituitary cells at 0 day (Fig. 6a), and Ki-67-positive cells were increased by DES (Fig. 6b). The percentage of Ki-67-positive PRL cells was significantly increased at 10–15 days after DES treatment (Fig. 6c; Table 2).

Effect of DES on Pit-1 expression in pituitary glands

Since Pit-1 expression is regarded as a marker of enhanced PRL cell differentiation (Ingraham et al. 1988; Crewshaw et al. 1989), we next examined the expression of Pit-1 by Southwestern histochemistry at 0, 5, 10, 15 and 20 days after DES treatment. As shown in Fig. 7, Pit-1 was specifically detected in the nuclei of anterior pituitary cells, and the DES treatment increased Pit-1-positive cells. The sections reacted with mutated Pit-1 probe at the same concentration, or without probes as a negative control, showed no signals. As can be seen in Fig. 8a–c, double-positive cells of Pit-1 and PRL were increased by DES treatment. Quantitative analysis re-

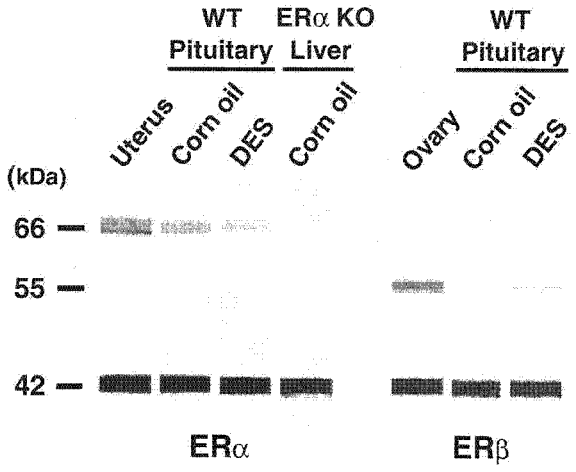

Fig. 3 Western blot analysis of ERα and ERβ. Thirty micrograms of the extracts from pituitaries of wildtype (WT) mice and livers of ERα knockout mice (ERαKO) treated with corn oil or 20 mg/kg BW DES (and uterus and ovary at estrous as a positive control) were subjected to SDS-PAGE. Western blotting was performed using monoclonal antibody ER 88 for ERα (66-kDa) and a polyclonal antibody for ERβ (55-kDa). The membranes were further reacted with monoclonal antibody for β-actin (42-kDa) as a loading control

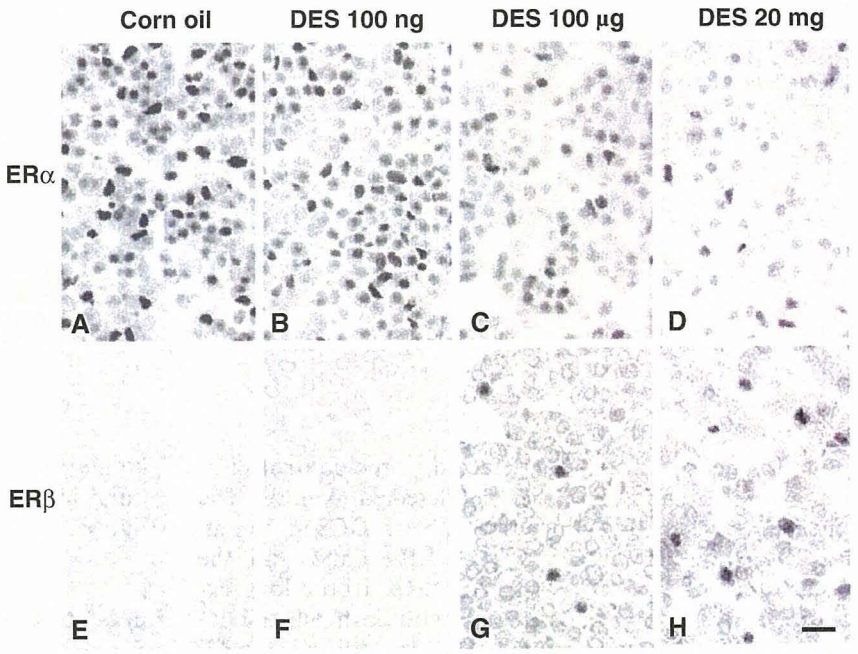

Fig. 4 Immunohistochemical localization of ERα (**a–d**) and ERβ (**e–h**) in male mouse pituitaries treated with corn oil or DES at 100 ng, 100 μg, or 20 mg/kg BW. Magnification, 400×. Scale bar, 20 μm

vealed that the percentage of Pit-1-positive PRL cells was significantly increased at 5–15 days (Table 2). DES treatment did not significantly change the percentage of GH and TSH cells in the pituitary, but reduced those of FSH and LH cells (Table 2). Double-positive cells of Pit-1 and FSH or LH were found at 5–10 days, although such cells were not found at 0 day (Fig. 8d–i; Table 2).

Appearance of PRL- and FSH-, or LH-double positive cells by DES treatment

To identify the type of cells that differentiate to PRL cells, we examined the co-expression of PRL and gonadotropins or GH. As shown in Fig. 9a–f, double positive cells of PRL and FSH or LH were found at 5 days after DES treatment. Quantitative analysis revealed that the percentage of PRL-positive FSH cells and PRL-positive LH cells was significantly increased at 5–10 days (Table 3). On the other hand, only a few PRL- and GH-double positive cells were detected (about 2%) through the experiment (Fig. 9g–i; Table 3).

Effects of DES on expression of sex-related hormones and ERβ in pituitaries of ERαKO mice

To gain insight into the roles of ERα and β in the effect of DES on the expression of sex-related hormones, we conducted the same set of experiments on the pituitaries of ERαKO mice. DES had no effect on the population density of cells positive for these hormones in ERαKO mice (Fig. 10). While ERβ-positive cells were detected in the pituitaries of corn oil-treated ERαKO mice, their density did not change by DES treatment. In addition, there were no significant differences in the expression of these hormones and ERs between the pituitaries of ICR and C57BL/6 wild-type mice (data not shown).

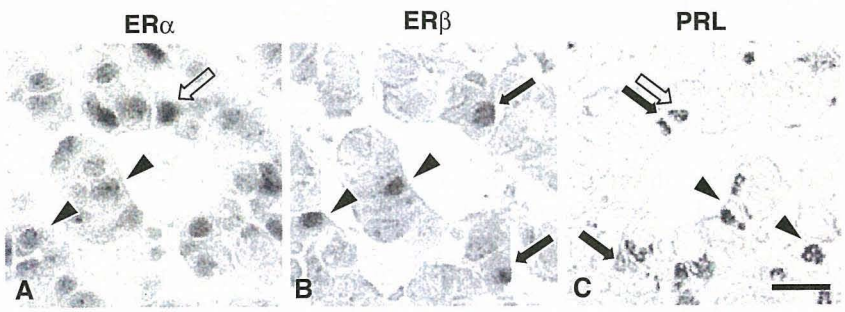

Fig. 5 Co-localization of ERs and PRL in pituitaries of mice treated with DES at 20 mg/kg BW. Pituitary sections were immunostained for ERα (**a**), ERβ (**b**), and PRL (**c**) by the combined use of adjacent and mirror sections treated with DES. *Open arrows* indicate PRL cells positive for ERα alone, *solid arrows* indicate PRL cells positive for ERβ alone, and *arrowheads* indicate PRL cells positive for both receptors. Magnification, 400×. Scale bar, 20 μm

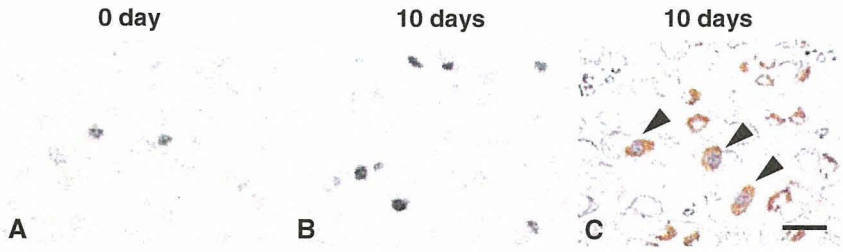

Fig. 6 Expression of Ki-67 in pituitaries of mice treated with 20 mg/kg BW DES. **a** and **b** Immunohistochemical localization of Ki-67 at 0 day (**a**) and 10 days (**b**) after DES treatment. **c** Double staining for Ki-67 and PRL in pituitaries of DES-treated mice at 10 days. Ki-67-positive cells are stained *blue*, and PRL-positive cells are *brown*. *Arrowheads* indicate double-positive cells. Magnification, 400×. Scale bar, 20 μm

Discussion

In the present study, we examined the effects of DES on the population of FSH-, LH-, and PRL-positive cells in male mouse pituitary, and found that DES reduced the density of FSH and LH cells in a dose-dependent manner, but conversely increased that of PRL cells. Interestingly, DES induced ERβ expression predominantly in PRL cells. Since these effects of DES were almost abolished in ERαKO mice, it is conceivable that ERα mediates the major action of DES. Although the role of ERβ expression in the PRL cell kinetics is unknown, our results strongly indicate that the increase of PRL cells by DES depends upon both enhanced proliferation of PRL cells and induction of transdifferentiation of FSH/LH cells to PRL cells.

It was surprising that ERβ-positive cells were increased while ERα-positive cells were decreased in parallel with the increase in the density of PRL cells after DES treatment. A change in the major population of ER subtypes is known to occur around the day of birth in the rat pituitary (Nishihara et al. 2000); ERβ rather than ERα is predominantly expressed in the fetal pituitary,

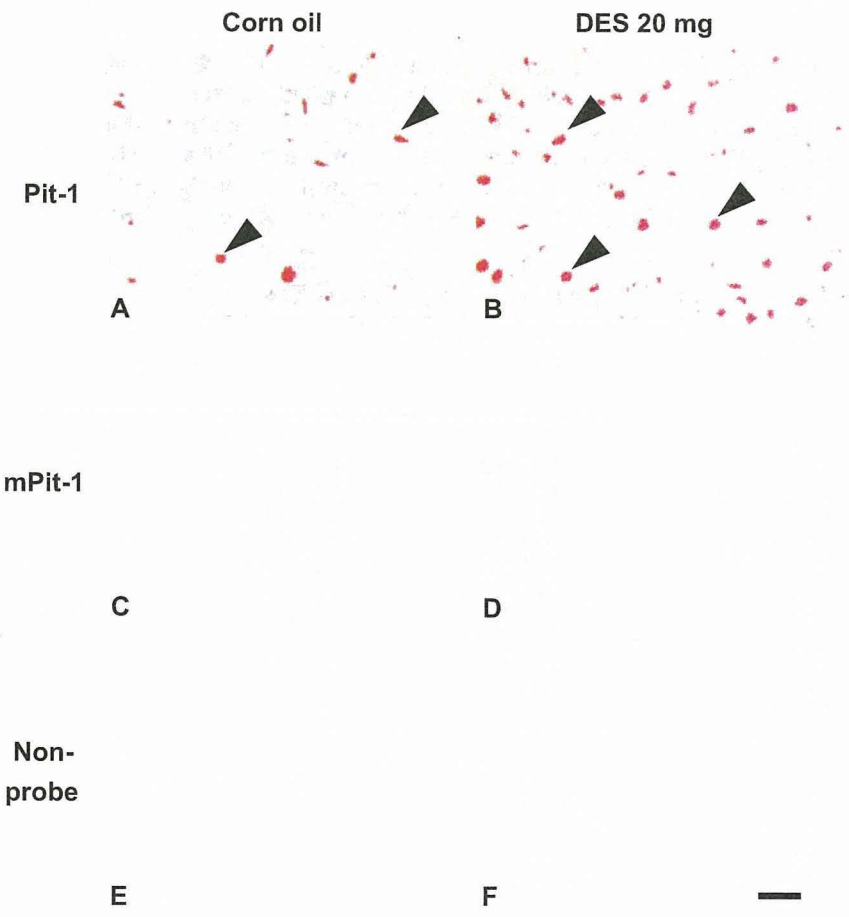

Fig. 7 Localization of Pit-1 in mouse pituitary by Southwestern histochemistry after 15-day-treatment with corn oil (**a**, **c**, **e**) or DES 20 mg/kg BW (**b**, **d**, **f**). The pituitary sections were reacted with T–T dimerized Pit-1 probe (**a**, **b**), T–T dimerized mutated Pit-1 (mPit-1) probe (**c**, **d**), or without probe (**e**, **f**). The *red color* represents positive cells as determined by image analysis (**a**–**f**). *Arrowheads* indicate positive cells. Magnification, 400×. Scale bar, 20 μm

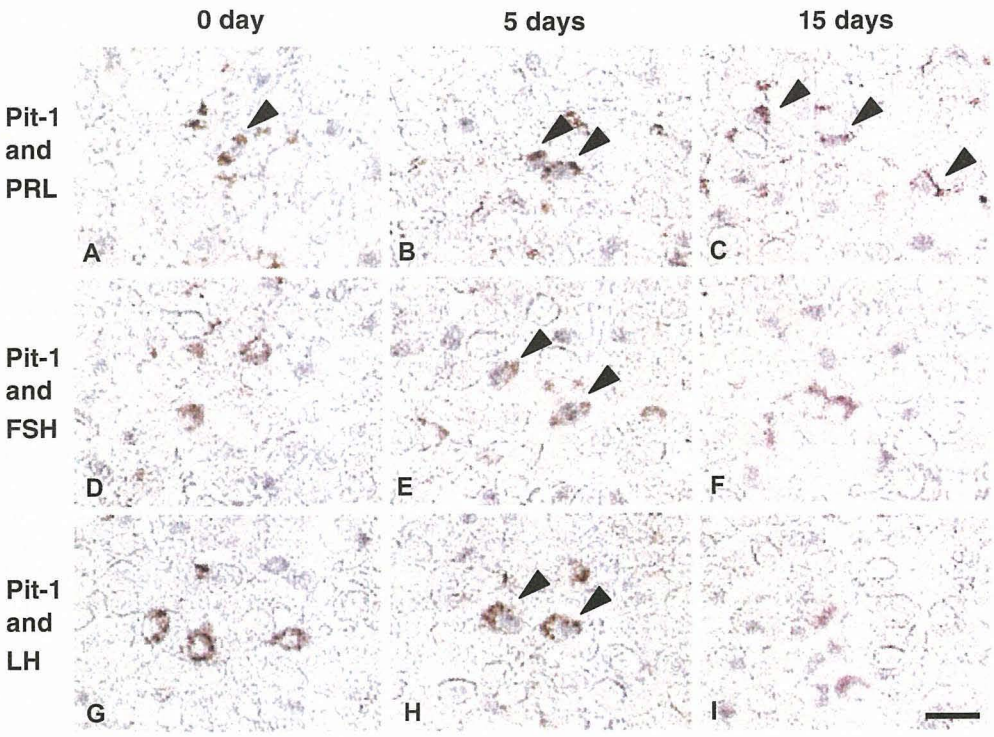

Fig. 8 Co-localization of Pit-1 and PRL or gonadotropins in pituitaries of mice treated with 20 mg/kg BW DES. Double staining for Pit-1 and PRL (**a**–**c**), Pit-1 and FSH (**d**–**f**), Pit-1 and LH (**g**–**i**) in pituitaries of mice at 0 day (**a**, **d**, **g**), 5 days (**b**, **e**, **h**), or 15 days (**c**, **f**, **i**) after DES treatment. Pit-1-positive cells are stained *blue*, and PRL-, FSH-, or LH-positive cells are *brown*. *Arrowheads* indicate double-positive cells. Magnification, 400×. Scale bar, 20 μm

but after birth the expression changes from ERβ to ERα, and ERβ almost disappears in adult mouse pituitary (Schreihofer et al. 2002). In accord, our study revealed the lack of ERβ-positive cells in the control group, but they appeared after treatment with a high-dose DES. In addition, the ERβ expression was almost limited to PRL cells. Considering that the DES effects were abolished in ERαKO mice, which maintain ERβ expression, DES seems to affect the pituitary at least primarily through ERα. However, the change of ER subtypes in PRL cells by DES might be related to the enhanced expression of protooncogene, such as c-*fos* (Allen et al. 1997). Since the DES-ERβ complex may function as a negative regulator of genes controlled by activating protein-1 (AP-1) element, which was activated by binding of Fos/Jun protein complex (Paech et al. 1997), the switching of ER subtype from ERα to ERβ may suggest that the pituitary becomes more resistant to DES exposure.

Recently, similar effects of DES on the population of FSH, LH, and PRL cells have been reported in male

Table 2 Percentage of positive cells in mouse pituitaries treated with DES 20 mg/kg BW

Positive cells	DES treatment (days)				
	0	5	10	15	20
PRL	11.2 ± 0.5	19.4 ± 1.7*	32.1 ± 1.0**	41.8 ± 1.5**	41.1 ± 0.5**
Ki-67	1.3 ± 0.2	4.1 ± 0.2	10.0 ± 0.3*	8.8 ± 0.3*	4.9 ± 0.2
Ki-67 in PRL[a]	1.2 ± 0.7	4.4 ± 1.6	16.4 ± 1.2**	15.7 ± 1.2**	5.8 ± 1.3
Pit-1	6.5 ± 0.8	19.4 ± 0.3**	26.8 ± 0.4**	37.1 ± 1.7**	10.8 ± 1.4
Pit-1 in PRL[b]	16.5 ± 0.5	33.4 ± 1.6**	61.0 ± 1.0**	76.7 ± 2.4**	20.2 ± 2.1
PRL in Pit-1[c]	24.1 ± 0.6	32.5 ± 2.5*	52.2 ± 2.1**	74.5 ± 0.9**	67.2 ± 1.1**
GH	44.8 ± 1.0	44.7 ± 1.1	43.8 ± 1.5	42.8 ± 1.0	42.7 ± 0.7
TSH	9.0 ± 0.5	8.6 ± 0.2	9.1 ± 0.2	8.7 ± 0.6	8.9 ± 0.4
FSH	11.4 ± 1.2	9.7 ± 0.3	5.5 ± 0.4*	2.9 ± 0.3**	2.5 ± 0.5**
Pit-1 in FSH[d]	0.2 ± 0.1	33.2 ± 1.0**	29.1 ± 0.9**	1.9 ± 0.2	0.4 ± 0.2
LH	10.6 ± 1.1	8.4 ± 0.3	6.4 ± 0.7*	4.0 ± 0.7**	3.6 ± 0.4**
Pit-1 in LH[e]	0.4 ± 0.3	31.6 ± 0.5**	27.9 ± 0.7**	1.3 ± 0.2	0.3 ± 0.1

Data represent mean ± SD (%). The percentage of Ki-67- or Pit-1-positive cells in the pituitaries of corn oil-treated mice was 1.2 ± 0.3, 6.2 ± 0.8%, respectively, and no changes were noted during this period. *$P < 0.05$, **$P < 0.01$, compared with 0 day

[a]Ki-67-positive cells in PRL cells
[b]Pit-1-positive cells in PRL cells
[c]PRL cells in Pit-1-positive cells
[d]Pit-1-positive cells in FSH cells
[e]Pit-1-positive cells in LH cells

Table 3 Percentage of positive cells in mouse pituitaries treated with DES 20 mg/kg BW

Positive cells	DES treatment (days)				
	0	5	10	15	20
PRL in FSH[a]	0.3±0.3	25.0±4.0*	26.3±3.2*	0.7±0.5	0.3±0.2
PRL in LH[b]	0.6±0.5	26.4±3.1*	28.3±1.5*	1.0±0.4	0.4±0.2
PRL in GH[c]	1.7±0.6	1.8±0.5	1.6±0.4	1.7±0.2	1.6±0.3

Data represent mean ± SD (%). *$P < 0.01$, compared with 0 day
[a]PRL-positive cells in FSH cells
[b]PRL-positive cells in LH cells
[c]PRL-positive cells in GH cells

Syrian hamster pituitary (Cauwenberge et al. 2001). The DES-induced reduction of FSH and LH cells could be explained as an estrogen function of DES, through the negative feedback mechanism (Scully et al. 1997). Estrogens are also known to stimulate PRL synthesis and secretion (Scully et al. 1997), and PRL cell proliferation (Hashi et al. 1996). Therefore, the effects of DES on FSH, LH, and PRL cells in the present study may be largely ascribed to the strong estrogen function of DES.

In the present study, we examined the expression of functional Pit-1 by Southwestern histochemistry. In the control pituitary, only 16.5% of PRL cells were Pit-1 positive. If Pit-1 is a prerequisite for the transcription of mRNA for PRL, all PRL cells should be positive for Pit-1. Therefore, it may be suggested that PRL cells, once fully differentiated, would not require the continuous presence of Pit-1 at an enough amount to be detected by Southwestern histochemistry. In contrast, the percentage of Pit-1-positive PRL cells was significantly increased at 5–15 days after DES treatment. Since Pit-1 expression is regarded as a marker of enhanced PRL cell differentiation, DES may promote the differentiation of PRL cells, particularly at the early stage of DES treatment.

Our study also revealed that the DES treatment did not significantly change the population density of GH and TSH cells among pituitary cells, but decreased that of FSH and LH cells. Considering that double-positive cells for Pit-1 and FSH or LH were detected at 5–10 days, and also that double-positive cells for PRL and FSH or LH were found at the same time, these results strongly indicate that the transdifferentiation of FSH/LH cells to PRL cells was activated by DES. Actually, in the differentiation of pituitary cells, two pathways may be possible; differentiation of stem cells into mature cells or transdifferentiation of certain type of cells to another cell type. The present results seem to support, at least, the latter case. In this context, it should be noted that bihormonal cells, which might be an intermediate stage of transdifferentiation (Horvath et al. 1990), have been

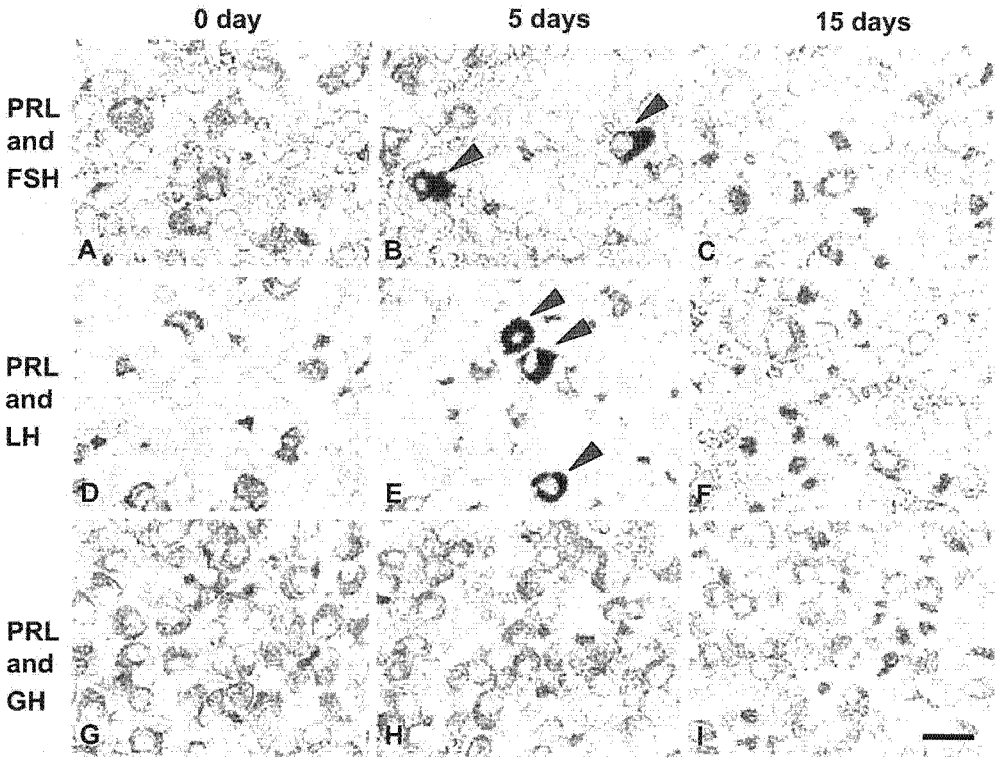

Fig. 9 Co-localization of PRL and gonadotropins or GH in pituitaries of mice treated with DES 20 mg/kg BW. Double staining for PRL and FSH (**a–c**), PRL and LH (**d–f**), and PRL and GH (**g–i**) in pituitaries of mice at 0 day (**a**, **d**, **g**), 5 days (**b**, **e**, **h**), or 15 days (**c**, **f**, **i**) after DES treatment. PRL-positive cells are stained *brown*, and FSH-, LH-, or GH-positive cells are *blue*. *Arrowheads* indicate double-positive cells. Magnification, 400×. Scale bar, 20 μm

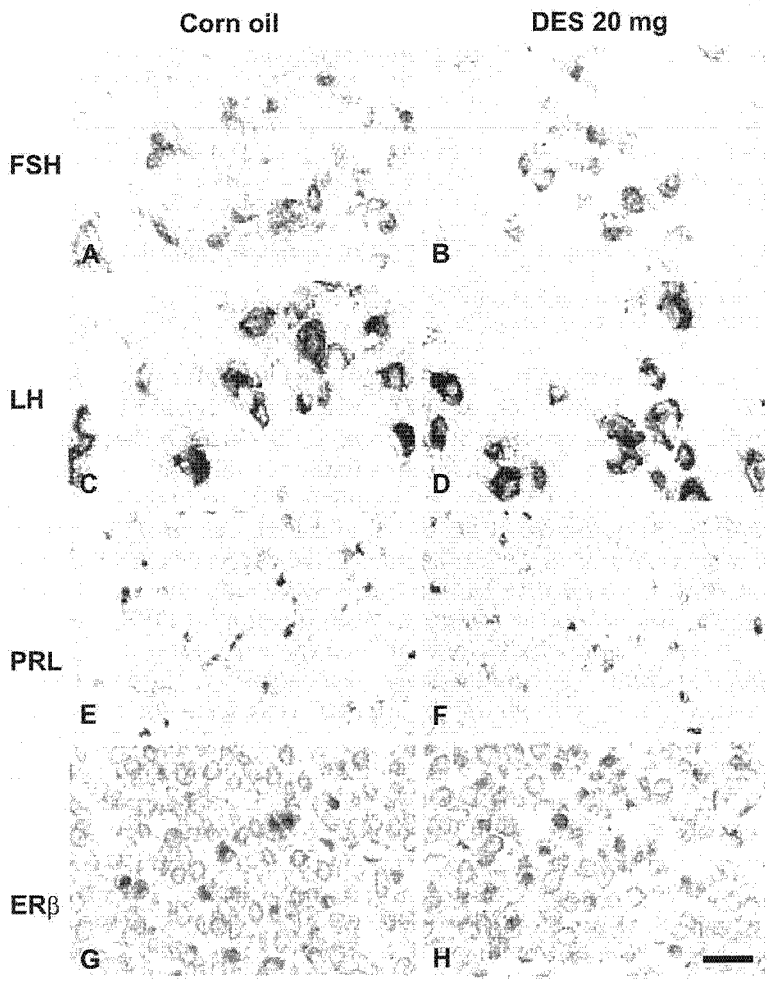

Fig. 10 Immunohistochemical localization of FSH (**a**, **b**), LH (**c**, **d**), PRL (**e**, **f**), and ERβ (**g**, **h**) in pituitaries of ERα knockout mice treated with corn oil (**a**, **c**, **e**, and **g**) or DES 20 mg/kg BW (**b**, **d**, **f**, and **h**). Magnification, 400×. Scale bar, 20 μm

observed in the pituitaries of mammals (Childs et al. 1994; Goth et al. 1996), and pituitary adenomas (Senovilla et al. 2004). In fact, that long-term estrogen treatment induced mammosomatotrophs, which are interconvertible between PRL cells and GH cells (Goth et al. 1996), though this may not be in the present case, because only a few double-positive cells for PRL and GH were detected in the DES-treated pituitary. Moreover, co-expression of Pit-1 and FSH or LH was also observed in pregnant rats (Vidal et al. 1998) and prolactinomas induced by estrogen (Mukdsi et al. 2004). Since it is known that PRL and GH cells belong to the Pit-1 lineage, whereas FSH and LH cells belong to another lineage (Hauspie et al. 2003), these results may indicate that the specification of the different lineages in the pituitary is made less stringent by DES or estrogens, and then transdifferentiation of FSH/LH cells to PRL cells might be induced. In addition, although the expression of Pit-1 was stimulated by DES in this study, we could not find either ERE or AP-1 sequence in the Pit-1 gene and its promoter region by computer-based searching (data not shown). Therefore, DES-induced Pit-1 expression might occur through an indirect pathway, and is an important subject that needs to be clarified in the future.

In conclusion, the present study indicated that DES affects the population density of FSH, LH, and PRL cells through ERα in male mouse pituitary, and also induces the expression of ERβ predominantly in PRL cells. Moreover, our results indicate that DES increases the population density of PRL cells by inducing proliferation of PRL cells and transdifferentiation of FSH/LH cells to PRL cells. Further studies are needed to elucidate the differential roles of ERα and ERβ in the regulation of PRL cell kinetics under the influence of DES.

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A ubiquitin E3 ligase Efp is up-regulated by interferons and conjugated with ISG15

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Abstract

Interferon (IFN) regulates various target genes that mediate important roles in immune responses such as antiviral state. Protein ISG15-conjugation (ISGylation) is implicated in the IFN-induced antiviral response. Here we demonstrate that Efp mRNA as well as protein expression could be up-regulated by Type I IFN in HeLa cells and HepG2 cells. Luciferase assay reveals that the first intron of Efp gene contains a functional interferon-stimulated response element (ISRE) and electrophoretic mobility shift assay shows that the ISRE binds to signal transducer and activator of transcription 1 (STAT1). Chromatin immunoprecipitation assays have shown that the ISRE recruits STAT1 *in vivo* IFN-dependently. Moreover, we demonstrate that Efp protein could be conjugated with not only ubiquitin but also ISG15. These data suggest that Efp is an IFN-responsive gene that potentially mediates IFN actions, involved in ISGylation and ubiquitination of proteins including Efp itself.

Keywords: Estrogen-responsive finger protein (Efp); Interferon; ISG15; Interferon-stimulated response element (ISRE); Signal transducer and activator of transcription 1 (STAT1)

Interferons (IFNs) have an important role in immune system to defend against viral infections. Type I IFN (IFN-α and -β) is produced by virus infected cells and then binds to receptors on the cell surface to activate a transcription factor complex, interferon-stimulated gene factor-3 (ISGF3), which is composed of signal transducer and activator of transcription 1 (STAT1), STAT2, and interferon regulatory factor 9 (IRF9) [1]. ISGF3 binds to IFN-stimulated response elements (ISREs) and induces expression of various target genes that function in antiviral state [2].

The interferon-stimulated gene 15 (ISG15) has been originally identified as type I IFN-stimulated gene encoding a 15-kDa protein [3]. ISG15 protein contains two conserved ubiquitin-like domains and is conjugated to target proteins, or ISGylated together with E1, E2, and E3 enzymes through the system similar to ubiquitination [4]. Based on the findings that ISG15 expression and protein ISGylation were highly induced upon viral infection [5,6], the ISGylation system is considered as a novel pathway that transduces the antiviral response in IFN-stimulated cells. Indeed, it was reported that ISG15 can inhibit release of human immunodeficiency virus type 1 (HIV-1) virions [7] and attenuate Sindbis virus infection [8]. However, since ISG15 knockout mice showed that ISG15 had no effect on infection of certain viruses [9], the precise mechanism and regulation of ISGylation remains to be resolved.

We previously identified and demonstrated that estrogen-responsive finger protein Efp is an ubiquitin E3 ligase that ubiquitinates the 14-3-3σ protein, a negative regulator of the cell cycle progression [10,11]. Recently, Efp has been found as an ISG15 E3 ligase [12]. Efp belongs to the protein

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family of tripartite motif (TRIM), which is composed of RING, B-box, and coiled coil domains. Recent evidence suggests that TRIM family proteins have antiviral properties. TRIM19/promyelocytic leukemia (PML), which has been identified as a fusion protein with retinoic acid receptor α (RARα) in promyelocytic leukemia, is well documented to have a potential role against virus infections [13,14]. TRIM5 is a host cell factor responsible for the antiretroviral activities, previously described as Lv1 and Ref1 [15,16]. Taken together, these findings lead us to the notion that the TRIM family represents a new and widespread class of antiviral proteins involved in innate immunity [17].

Here we demonstrate that Efp expression is enhanced by IFNs. Luciferase assay reveals that Efp promoter/enhancer is activated by IFNs through a functional ISRE that is located in the first intron. This ISRE binds with one of the ISGF3 components STAT1 *in vitro* and recruits STAT1 *in vivo* IFN-dependently. Moreover, we have shown that Efp could be conjugated with not only ubiquitin, but also ISG15.

Materials and methods

Cell culture. HeLa, HepG2, and 293T cells were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37 °C in 5% CO_2 and a humidified atmosphere.

Quantitative PCR. HeLa cells and HepG2 cells were treated with 500 U/ml IFN-α, IFN-β, IFN-γ (PeproTech EC) or vehicle (PBS) for 6 and 24 h in the presence or absence of cycloheximide (CHX) (1 μg/ml) (Nacalai Tesque, Kyoto, JAPAN). Real-time quantitative RT-PCR (qPCR) was performed as described previously [18], using 36B4 for normalization. The sequences of primers are as follows: Efp forward primer, 5′-GTGACCACGGCTTTGTCATCT-3′; Efp reverse primer, 5′-TAAAGTCCACCCTGAACTTATACATCAG-3′; ISG15 forward primer, 5′-TGGCGGGCAACGAATT-3′; ISG15 reverse primer, 5′-GG GTGATCTGCGCCTTCA-3′; UBE1L forward primer, 5′-TGATGTT TGAGAAGGATGATGACA-3′; UBE1L reverse primer, 5′-CCGGTGG AATCCCGTAGTT-3′; UBCH8 forward primer, 5′-GGAACCTGTCCA GCGATGAT-3′; UBCH8 reverse primer, 5′-TCAGGTGGTAGGGAG GTTGGT-3′; 36B4 forward primer, 5′-CCACGCTGCTGAACATGCT-3′; 36B4 reverse primer, 5′-GATGCTGCCATTGTCGAACA-3′.

Cloning of promoter region of Efp gene containing ISRE, luciferase assay, and chloramphenicol acetyl transferase (CAT) assay. An 1559 bp length of a putative ISRE-containing genomic DNA fragment (−173 to +1386 bp, where the translation initiation site stands for nucleotide +1) [19] and its 1415 bp deletion mutant lacking the ISRE sequence were amplified by PCR and cloned into pGL3-basic vector (Efp-Luc and EfpΔISRE-Luc), respectively. Luciferase assay was performed using HeLa cells transfected with 0.1 μg of each luciferase reporter together with 0.02 μg pRL-TK (Promega) as described previously [20]. Twelve hours after transfection, cells were treated with 500 U/ml IFN-β or vehicle for 24 h and luciferase activities were determined. Data are expressed as means ± SD of three independent experiments performed in triplicate. CAT assay was performed in HeLa cells treated with 500 U/ml IFN-β or vehicle, using reporter plasmids, pCAThe-173 and pCAThe-1625, as previously described [19].

Electrophoretic mobility shift assay (EMSA). EMSA was performed as described previously [19]. Nuclear extract from HeLa cells treated with IFN-β for 24 h was prepared using NP-40 lysis buffer. Annealed oligonucleotides 5′-GATTTAGTTTCATTTTACTGTTGA-3′ corresponding to the ISRE in Efp intron 1 were labeled using [γ-^{32}P]ATP (Amersham Biosciences) using a MEGA label kit (Takara Bio, Tokyo, JAPAN). Ten micrograms of nuclear protein, a ^{32}P-labeled double-stranded probe (20,000 counts/min), and 3 μl of 5× binding buffer [19] were mixed in a total volume of 15 μl. In competition assays, 10-fold or 50-fold amounts of unlabeled oligonucleotides of ISRE or mutated ISRE (GATTTAGTGGAATTTTA CTGTTGA) were added simultaneously to the radio-labeled probe. For neutralization experiments, anti-human STAT1 antibody (E-23, Santa Cruz Biotechnology) or non-immune IgG was incubated with the nuclear extracts for 20 min before the addition of the probes.

Western blotting. Western blotting was performed as described previously [20]. Lysates from HeLa cells and HepG2 cells treated with 500 U/ml IFN-α, IFN-β or IFN-γ for 24 or 36 h were resolved by 10% denaturing SDS–polyacrylamide gel electrophoresis. Blotted membranes were incubated with Efp antibody [11], followed by a reaction with anti-rabbit Ig (Amersham Biosciences) and the enhanced chemiluminescence system (Amersham Biosciences).

Chromatin immunoprecipitation assay (ChIP). ChIP assay was performed as described previously [18]. HeLa cells were treated with 500 U/ml IFN-β or vehicle for 24 h. Lysates corresponding to 2×10^7 cells were incubated with 2 μg of anti-STAT1 antibody (E-23, Santa Cruz Biotechnology) or acetylated histone H3 (Upstate). Precipitated DNA fragments were quantified by quantitative real-time PCR. The sequences of ISRE and GAPDH primers are as follows: ISRE forward primer, 5′-TC CCTCACGAAGTCCTGGTGGCA-3′, ISRE reverse primer, 5′-AGGT CAGGCTAAGTTCATTTCTC-3′, GAPDH forward primer, 5′-GGTG GTCTCCTCTGACTTCAACA-3′, GAPDH reverse primer, 5′-GTGG TCGTTGAGGGCAATG-3′. Fold enrichment of STAT1 or acetylated H3 in IFN-β-treated cells was quantified by $\Delta\Delta C_T$ method [18].

Immunoprecipitation. cDNAs of human ISG15, ubiquitin, and Efp were amplified by PCR with specific primers and subcloned into pcDNA3 (Invitrogen) or pCI-NEO (Promega) to generate mammalian expression plasmids. To analyze ISGylation and ubiquitination of Efp protein, 293T cells were transiently transfected with S-tagged Efp together with FLAG-tagged ubiquitin or ISG15. After cultured for 48 h, cells were lysed in 1% Triton X-100 buffer (50 mM Hepes, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). For immunoprecipitation, cell lysate was mixed with anti-FLAG M2 affinity gel (Sigma) and FLAG-tagged proteins were eluted with 3× FLAG peptide after washing. The elutant was immunodetected by anti-S monoclonal antibody (Novagen).

Results

Induction of Efp mRNA and protein by IFNs

To examine whether Efp expression is modulated by IFNs, HeLa, and HepG2 cells were treated with IFNs or vehicle and Efp mRNA expression was quantified using qPCR (Fig. 1A). The results showed that type I IFN (IFN-α and -β) up-regulated Efp mRNA levels by 4- to 8-fold over vehicle treatment after 6 and 24 h in HeLa cells whereas type II IFN (IFN-γ) did not. Similarly, Efp mRNA was induced by type I IFN but not by IFN-γ in HepG2 cells. These IFN-induced mRNA expression could also be observed by the addition of cycloheximide, indicating that IFN could up-regulate Efp mRNA expression independent of *de novo* protein synthesis. Next, we assessed endogenous Efp protein expression in HeLa and HepG2 cells by Western blot analysis. The up-regulation of Efp protein was observed at 24 and 36 h after treatment with IFN-α and β in both HeLa and HepG2 cells (Fig. 1B), consistent with the IFN-induced expression of Efp mRNA.

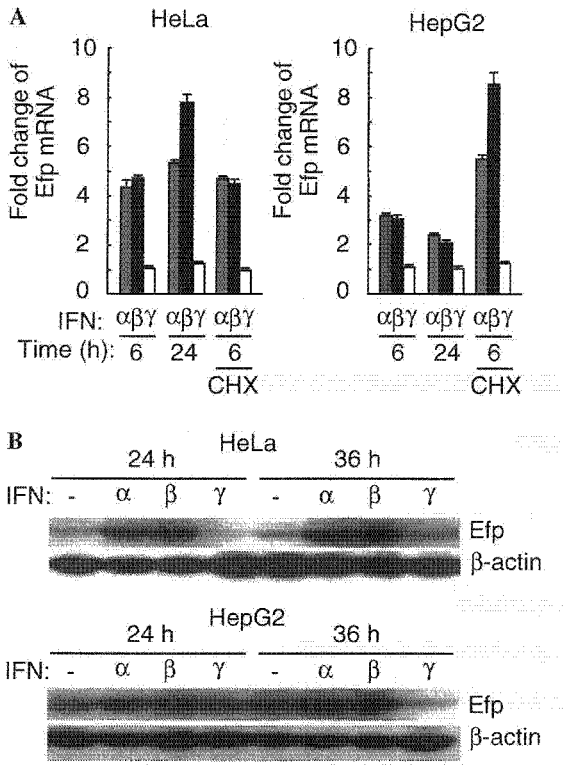

Fig. 1. IFN-induced expression of Efp. (A) IFN-induced expression of Efp mRNA. HeLa and HepG2 cells were treated with the indicated IFNs (500 U/ml each) or the vehicle (PBS) for the indicated times in the presence or the absence of cycloheximide (1 μg/ml). Real-time quantitative PCR (qPCR) was performed using cDNAs generated from total RNA. Experiments were repeated three times and the results are represented as means ± SD. (B) IFN-induced expression of Efp protein. HeLa cells and HepG2 cells were treated with IFNs or vehicle for 24 and 36 h. Cell lysates were immunodetected by anti-Efp antibody [11].

Functional ISRE in the first intron of human Efp gene

It is known that type I IFN stimulates gene transcription through IFN-stimulated response elements (ISRE, Fig. 2A) [21]. Computational analysis was performed to search for ISREs in the Efp gene locus and its 5-kb upstream or downstream regions. Only one putative ISRE sequence was found in the first intron of the gene (Fig. 2A). We have previously shown that the 5′-flanking region of the Efp gene had a basal transcriptional promoter activity; this 5′-region of Efp contains an E-box element that could bind USF-1 at positions −110 to −105 bp, and another positive regulatory region containing two GC-boxes locates just upstream to the E-box [19]. We checked IFN responsiveness of two CAT reporter constructs pCAThe-173 and pCAThe-1625, which possesses the basal promoter region and 1625-bp 5′-flanking region of Efp gene, respectively. IFN-β treatment scarcely changed the transcription activities of pCAThe-173 and pCAThe-1625 compared with vehicle treatment (1.06 ± 0.10- and 1.12 ± 0.19-fold, respectively). These results suggest that this 5'-upstream region without the ISRE sequence could not respond to IFN-β. Then, we generated two luciferase reporter plasmids, Efp-Luc and EfpΔISRE-Luc. Efp-Luc contained the 1559-bp genomic DNA fragment of Efp gene at positions −173 to +1386 bp, while EfpΔISRE-Luc included a 1415-bp fragment lacking the ISRE sequence from Efp-Luc (Fig. 2B). Luciferase activity of Efp-Luc but not EfpΔISRE-Luc in HeLa cells was increased by INF-β treatment. The IFN responsiveness of the ISRE sequence was further examined using SV40 promoter-driven luciferase vectors containing the intact ISRE or its mutated sequence (Fig. 2C). Our data indicate that this bioinformatically identified ISRE is indeed functional and would contribute to the IFN responsiveness of human Efp promoter in HeLa cells.

STAT1 binds to the ISRE of Efp intron 1

To examine the possible trans-acting factors that bind to the ISRE, we performed electrophoretic mobility shift assay. Nuclear extracts of HeLa cells treated with or without IFN-β were incubated with a ^{32}P-labeled ISRE oligonucleotide in the presence or absence of competitive oligonucleotides (Fig. 3A). A single DNA–protein complex was detected and the amount of this complex was clearly increased when the cells were stimulated with IFN-β. The DNA–protein interaction was competed by a 10- and 50-fold excess of unlabeled ISRE oligonucleotide over the radiolabeled probe, whereas no competition of DNA–protein binding was observed by the addition of the mutated ISRE oligonucleotide. Since it is known that type I IFN activates STAT1 and STAT2, which are components of the ISRE-recognizing ISGF3 complex [2], we investigated whether STAT1 modulates the signal of DNA–protein complex (Fig. 3B). The addition of anti-STAT1 antibody to IFN-treated nuclear extracts diminished the signals of DNA–protein complex in a dose-dependent manner. Because control IgG treatment had no effect on the complex signal, the data indicate that STAT1 would play a role in the complex formation that binds to the ISRE in Efp intron 1. Next, to examine *in vivo* binding of STAT1 to the ISRE of Efp intron 1, we performed ChIP assays using specific antibodies against STAT1 or acetylated histone H3 (acH3) in HeLa cells treated with IFN-β or vehicle (Fig. 3C). The result showed that IFN-β increased STAT1 recruitment on this ISRE by approximately 6-fold compared to vehicle. It is also notable that acH3 was enriched at the ISRE after IFN-β treatment, suggesting that IFN-β may accelerate chromatin acetylation around this locus. These results indicate that STAT1 is an IFN-responsive trans-acting factor of ISRE in Efp intron 1.

Efp protein modification mediated by IFN-induced ISGylation components

Next, we studied the regulation of ISGylation components, ISG15, UBE1L (E1 enzyme), and UBCH8 (E2 enzyme) in HeLa cells. These expressions were measured by qPCR using cDNAs from HeLa cells treated with either