

## Hormonal Regulation of Prolactin Cell Development in the Fetal Pituitary Gland of the Mouse

Kiyomoto Ogasawara, Haruo Nogami, Mumeko C. Tsuda, Jan-Åke Gustafsson, Kenneth S. Korach, Sonoko Ogawa, Toshio Harigaya, and Setsuji Hisano

Laboratory of Neuroendocrinology (K.O., H.N., S.H.), Institute of Basic Medical Sciences, and Laboratory of Behavioral Neuroendocrinology (M.C.T., S.O.), Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki 305-8575, Japan; Department of Biosciences and Nutrition (J.-Å.G.), Karolinska Institute, S-14186 Huddinge, Sweden; Laboratory of Reproductive and Developmental Toxicology (K.S.K.), National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709; and Laboratory of Functional Anatomy (T.H.), Faculty of Agriculture, Meiji University, Kanagawa 214-71, Japan

The developmental process of prolactin (PRL) cells in the fetal pituitary gland was studied in mice. Although PRL cells were hardly detectable in the pituitary gland of intact fetuses, a treatment with  $17\beta$ -estradiol ( $E_2$ ) *in vitro* induced a number of PRL cells that varied drastically in number depending on the stage of gestation with a peak at embryonic d 15. This effect was specific to  $E_2$  with epidermal growth factor, insulin, and forskolin failing to induce PRL cells. Although both estrogen receptor (ER) $\alpha$  and ER $\beta$  were expressed in the fetal pituitary gland, the results from ER knockout models showed that only ER $\alpha$  mediates  $E_2$  action on PRL cells. A few PRL cells were observed in ER $\alpha$ -deficient mice as well as in their control littermates, suggesting that estrogen is not required for the phenotype determination of PRL cells. Unexpectedly, the effect of  $E_2$  on the induction of PRL cells *in vitro* was diminished after embryonic d 15. Present results suggest that the exposure of fetal PRL cells to glucocorticoids (GCs) results in a reduction of sensitivity to  $E_2$ . The mechanism underlying the down-regulation of estrogen sensitivity by GCs was found not to be down-regulation of ER levels, induction of annexin 1, a GC-inducible inhibitor of PRL secretion, or a decrease in the number of PRL precursors by apoptosis. The effect of GCs appeared within 2 h and did not require a *de novo* protein synthesis. GCs are considered to be involved in the mechanisms of silencing pituitary PRL in gestation possibly through a novel mechanism. (*Endocrinology* 150: 1061–1068, 2009)

Early electron microscopic (1, 2), immunoelectron microscopic (3), and biochemical (4) studies failed in detecting prolactin (PRL) cells or PRL production in the fetal pituitary glands of mice. This has fostered the belief that PRL cells would not differentiate during gestation. However, it has been demonstrated by light microscopic immunohistochemistry that the PRL cells first appeared on embryonic d 16 (E16) (5). Intriguingly, PRL cells appeared at this stage transiently, and none were detectable thereafter until birth. Our previous study (6) confirmed the above finding, demonstrating the presence of a few PRL cells only in E15 and E16 intact pituitaries of the mouse fetus. Thus, the development of PRL cells appears to be suppressed after E16.

The regulation of the development of the fetal PRL cells is poorly understood. We found in our previous study that the injection of pregnant mice with diethylstilbestrol (DES), a synthetic estrogen with a very low affinity for  $\alpha$ -fetoprotein (7),

results in the premature development of PRL cells in the fetal pituitary gland within 24 h, suggesting that estrogen stimulated a differentiation of PRL cells from precursor cells and/or that estrogen stimulated PRL production in nascent PRL cells that have already differentiated during the embryonic period (6). The number of DES-induced PRL cells increased with age until E16, probably reflecting the increasing number of precursors or nascent PRL cells. Maternal estrogen treatment, thus, allowed us to detect fetal PRL cells; otherwise, they were undetectable. However, it was impossible to observe the development of PRL cells after E16, because the effect of DES declined dramatically, possibly due to the increase in circulating glucocorticoids (GCs) at this stage, which has been reported in rats (8, 9).

Although GCs are reported to stimulate PRL cell development in chicken embryos (10), GCs suppress the differentiation and development of PRL cells in mammals (6, 11). The mecha-

nism of GC action on fetal PRL cells has not been elucidated. In the promoter region of bovine and human PRL genes, the specific binding sites for GC receptor were demonstrated, and it is considered that GCs directly suppress the transcription of the PRL gene (12, 13). However, such elements have not been identified in rodent promoters. In the rat, the inhibitory effect of GCs on PRL secretion from adult pituitary cells has been reported, and this effect is believed to be partially mediated by annexin 1 (ANXA1), which is produced in several types of pituitary cells in response to GCs (14, 15). Whether this system functions also in the fetal pituitary gland has yet to be examined.

In this study, we further investigated the role of estrogen in the differentiation and development of PRL cells in the pituitary gland of the fetal mouse using the organ culture system, in which the actions of estrogen can be studied without direct interference from endogenous GCs. We also aimed at elucidating the mechanism responsible for the suppression of PRL cell development by GCs. The results suggest that estrogen is not directly implicated in the differentiation of PRL cells, although it is indispensable for their functional maturation. The fetal circulating GCs increase transiently in late gestation in mice, which suppresses estrogen action on PRL expression possibly through a novel mechanism.

## Materials and Methods

### Animals

Adult male and female mice of the ICR strain were obtained from CLEA Japan, Inc. (Tokyo, Japan). Mice deficient in estrogen receptor (ER) $\alpha$  [ER $\alpha$  knockout ( $\alpha$ ERKO)] (16) and  $\beta$  (BERKO) (17) were maintained at the University of Tsukuba by mating heterozygous mice, which were originally obtained from the National Institute of the Environmental Health Sciences (Research Triangle Park, NC). The animals were housed in a room maintained at 22 C and a 12-h light, 12-h dark lighting cycle (lights on at 0800 h) with free access to a standard diet and tap water. Timed pregnant mice were obtained by a single night mating, and the day of the vaginal plug was designated d 0 of pregnancy. The genotype was determined by PCR amplification of the fragments of ER genes from tail DNA or DNA extracted from fetal liver. The primers for amplifying wild-type ER $\alpha$  were 5'-CGGTC-TACGGCCAGTCGGGCATC-3' (forward) and 5'-CAGGCCTTACA-CAGCGCCACCC-3' (reverse), and the same reverse primer and Neo specific primer 5'-GCTGACCGCTTCTCGTGCTTTAC-3' (forward) were used for detecting the targeted allele. In BERKO, wild-type specific primers, 5'-TGGACTCACCACGTAGGCTC-3' (forward) and 5'-CATCCTTCA-CAGGACCAGACAC-3' (reverse), and a targeted allele-specific forward primer, 5'-GCAGCCTCTGTCCACATACAC-3', were used.

Our experiments were carried out with the approval of the Animal Experiment Committee of the University of Tsukuba.

### Organ culture

Pregnant mice were killed under ether anesthesia, and the fetuses were rapidly removed from the uterus. The whole pituitary gland was gently dissected away from the other tissues, put on a nylon mesh sheet, and then cultured for 2, 8, 24, or 48 h in a 24-well culture plate (Becton Dickinson Labware, Franklin Lakes, NJ) with 200  $\mu$ l/well serum-free DMEM/F12 (Invitrogen, Groningen, The Netherlands) containing antibiotics (Invitrogen). Dexamethasone (DEX), corticosterone (CS), and 17 $\beta$ -estradiol (E $_2$ ) were obtained from Sigma-Aldrich (St. Louis, MO). Puromycin was obtained from Calbiochem (San Diego, CA).

Tissues were then either stored frozen at  $-80$  C for RNA or protein analyses or fixed in a modified Bouin's solution (1:3 mixture of formalin

and saturated picric acid) at room temperature (25 C). Fixed tissues were embedded in paraffin and cut into 5- $\mu$ m-thick sections. In this study, the entire pituitary gland was cut into approximately 120 frontal serial sections. Every five consecutive sections were placed in order on four glass slides, so that each slide carried about six rows of consecutive sections.

### RNA extraction and RT-PCR

Total RNA was prepared by the method described previously (18). RNA concentration was determined by spectrophotometric analysis at 260 nm. The cDNA synthesis was carried out using murine leukemia virus reverse transcriptase and oligo d(T) $_{16}$  primer (Applied Biosystems, Foster City, CA) at 42 C for 15 min according to the manufacturer's protocol.

The PCR consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl $_2$ , 0.05 U/ $\mu$ l rTaq polymerase, and 0.2 mM each of dATP, dGTP, dCTP, and dTTP (all from Takara Shuzo Co., Ltd., Shiga, Japan). After incubation at 94 C for 1 min, a PCR was performed for 27 cycles (GAPDH and ER $\alpha$ ) or 36 cycles (ER $\beta$ ) at 94 C for 30 sec, 56 C for 30 sec, and 72 C for 1 min, followed by an additional incubation at 72 C for 5 min. The ER $\alpha$  cDNA fragment was amplified by primers 5'-GTCCAGCTACAAACCAATGC-3' (bases 856–875, GenBank accession no. NM007956) and 5'-CCTATCTGCTAGGTTG-TCA-3' (bases 1231–1250). The ER $\beta$  cDNA fragment was amplified by primers 5'-CCTTGGTGTGAAGCAAGATC-3' (bases 192–211, U81451) and 5'-GGATCCACTTGACCATTTC-3' (bases 514–533). The GAPDH mRNA was amplified by primers 5'-ACTCCACTCAGCGCAAATTC-3' (bases 194–213, M32599) and 5'-GTCATGAGCCCTTCCACAAT-3' (bases 548–567). All primer sets were intron spanning. The PCR products were separated in 2% agarose gel and stained with ethidium bromide.

### Quantitative RT-PCR

Real-time PCR was performed using a qPCR SuperMix UDG (Invitrogen) according to a protocol supplied by the manufacturer. A stock of cDNA generated by the reverse transcription of adult mouse pituitary was used to construct a standard curve in every assay. The PRL cDNA fragment was amplified by primers 5'-TGACTGCCAGACT-TCTCTCCG-3' (bases 162–182, NM011164) and 5'-GACCATA-AACTCACGGTCTTGC-3' (bases 270–291). The ER $\alpha$  cDNA fragment was amplified by primers 5'-GTCCAGCTACAAACCAATGC-3' (bases 856–875, NM007956) and 5'-AAGCACAAAGCGTCAGAGAGA-3' (bases 989–1008). Cyclophilin D mRNA, an internal control, was amplified by primers 5'-AGGTGGCGAAAGTATTTATG-3' (bases 363–382, NM026352) and 5'-ATCACAACAGTTCGGACTCC-3' (bases 484–503). Annexin1 mRNA was amplified by primers 5'-CGTTGT-CAGGACTTGAGTGT-3' (bases 645–664; NM010730) and 5'-CACTCTGCGAAGATGAGGAA-3' (bases 763–782). The amounts of mRNA were normalized for cyclophilin D mRNA.

### Immunohistochemistry and cell count

Sections were microwaved in 10 mM citrate solution (pH 6.0) for 5 min, incubated with rabbit antimouse PRL (1:10,000) serum overnight at 4 C, and incubated with the secondary antibodies followed by avidin-biotin-peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA); finally, the immunoreaction was visualized by incubating sections in PBS [0.02 M sodium phosphate buffer (pH 7.4)/0.154 M NaCl] containing 0.01% 3,3'-diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide. The PRL antiserum was raised against recombinant mouse PRL in rabbit; the specificity of the PRL antiserum has been described elsewhere (19, 20). For the cell count study, we made it a rule to select a third section of each row on a glass slide consisting of five consecutive sections, so that six to seven sections were selected as the representative sections of the gland. The number of stained cells with a nuclear profile was counted under a light microscope, and the area of the anterior pituitary gland was determined by Scion Image (Scion Corp., Frederick, MD).

## Polyacrylamide gel electrophoresis and Western blot analyses

The tissues were homogenized by sonication in 15  $\mu$ l PBS and combined with the same volume of 2 $\times$  loading buffer [122 mM Tris-HCl (pH 6.8), 3.6% sodium dodecyl sulfate, 12.6% glycerol, and 1.75% 2-mercaptoethanol]. The samples were denatured by incubation at 95 C for 5 min and applied to a 12.5% polyacrylamide gel. Separated proteins were blotted onto a nylon membrane (Immobilon P; Millipore, Billerica, MA) by electrophoresis. Blots were reacted overnight at 4 C with rabbit anti-mouse PRL (1:5000) diluted in buffer A [0.9% NaCl, 50 mM Tris-HCl (pH 7.5), 0.1% Tween 20, and 1% skim milk]. The blots were washed in buffer A without skim milk, followed by incubation in buffer A containing peroxidase-conjugated secondary antibody (1:8000; The Jackson Laboratory, Bar Harbor, ME). Antibody-protein complexes were visualized using an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ) and Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY).

## Determination of serum CS levels

For a determination of serum CS levels in the fetal period, trunk blood was collected from fetuses at E15–E18 and from newborn mice. Blood from four to five mice was pooled for each sample, and the serum CS

levels were determined using a CS RIA system and [1,2-<sup>3</sup>H]corticosterone from Teikoku Hormone Medical Co. Ltd. (Kanagawa, Japan).

## Statistical analyses

Data were analyzed for significance of difference by ANOVA unless otherwise stated. Multiple comparisons between experimental groups were conducted using the *post hoc* Tukey-Kramer test, and a difference of  $P < 0.05$  was considered significant.

## Results

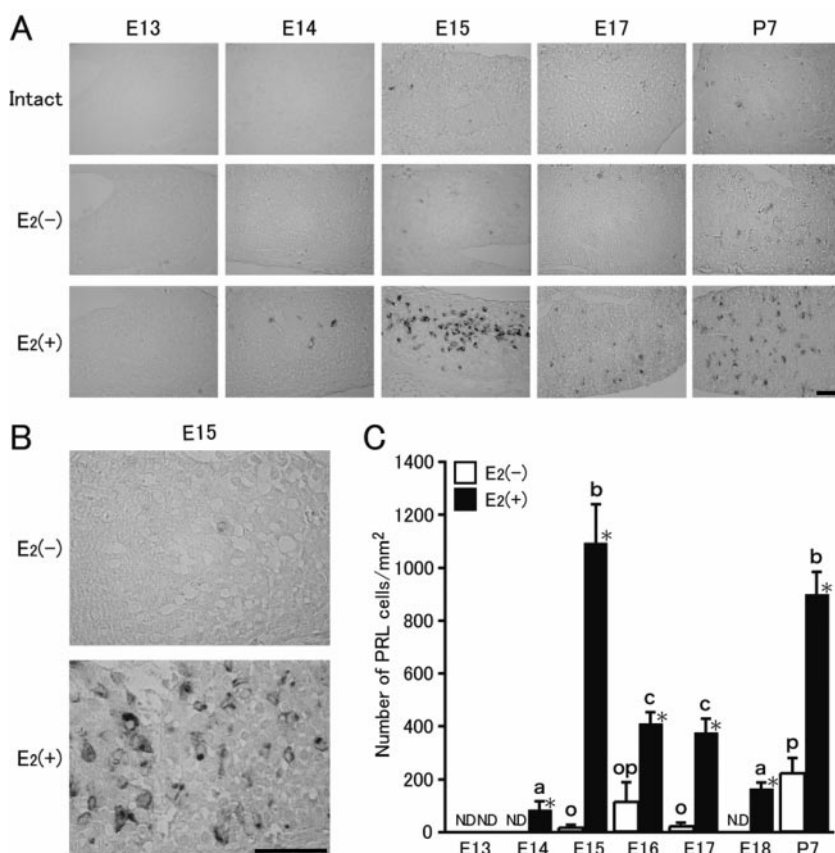
### E<sub>2</sub> stimulates PRL cell development *in vitro*

The pituitary sections from fetal and postnatal mice were stained immunocytochemically for PRL; the results are shown in Fig. 1. In the intact pituitaries, a few PRL cells were encountered only at E15 and at postnatal d 7 (P7), whereas no PRL cells were detected at any other stages examined. E<sub>2</sub> induced immunoreactive PRL cells in the pituitaries from fetuses at E14 or older in organ culture. PRL cells induced by E<sub>2</sub> showed weak PRL immunostaining in the periphery of the cytoplasm, and a strong staining was observed in the perinuclear area in some of them (Fig. 1B).

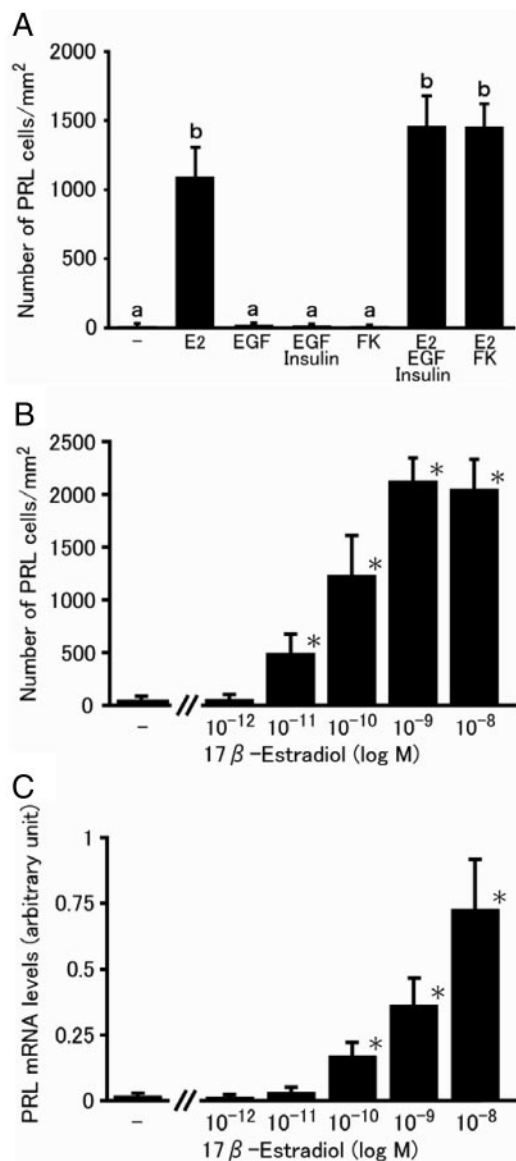
The number of PRL cells induced by E<sub>2</sub> increased with age until E15 but decreased in older fetuses to reach a nadir at E18. The effects of E<sub>2</sub> were again evident at P7 (Fig. 1). No PRL cells were observed in E13 pituitaries even after culture with E<sub>2</sub>. In the control experiment where fetal pituitaries were incubated with serum-free medium without E<sub>2</sub>, a few PRL cells appeared in the explant of E15–E17 pituitaries (Fig. 1C).

Because epidermal growth factor (EGF), insulin, and cAMP are reported to have a stimulatory effect on PRL gene expression in pituitary tumor cells (21–23), we examined whether these factors can induce PRL cells as well as E<sub>2</sub> using E15 pituitaries (Fig. 2A). Immunoreactive PRL cells did not increase in number after treatment with EGF, EGF plus insulin, or forskolin either alone or in conjunction with E<sub>2</sub>. Dose-response studies using pituitaries from E15 were carried out to determine the minimal effective dose of E<sub>2</sub> on PRL cell induction and PRL mRNA accumulation (Fig. 2, B and C). The effect of E<sub>2</sub> on PRL cell numbers was observed with 10 pM E<sub>2</sub> and increased in a dose-dependent manner to reach a plateau at 1 nM. The lowest dose of E<sub>2</sub> effective for augmentation of PRL mRNA levels was 100 pM.

The expression of ER subtypes was examined by RT-PCR, and as shown in Fig. 3A, mRNA for both subtypes was detected as early as E14. Next, we examined the role of ER $\alpha$  and ER $\beta$  in the induction of PRL cells in the explant of E15 pituitary glands from  $\alpha$ ERKO or  $\beta$ ERKO mice. In both  $\beta$ ERKO and its heterozygous littermates, a small number of PRL



**FIG. 1.** Ontogeny of PRL cells in the mouse pituitary gland and the effect of estrogen. A, Pituitary glands at the stages indicated were incubated overnight in serum-free DMEM/F12 with [E<sub>2</sub>(+)] or without [E<sub>2</sub>(-)] 10 nM E<sub>2</sub> and processed for immunocytochemical staining for PRL along with intact pituitaries that are not incubated. Scale bar, 40  $\mu$ m. B, Higher magnification of pituitary sections from E15 fetuses incubated overnight with [E<sub>2</sub>(+)] or without [E<sub>2</sub>(-)] E<sub>2</sub>. Scale bar, 40  $\mu$ m. C, Quantification of the number of PRL cells in the pituitary sections from those incubated with [E<sub>2</sub>(+)] or without E<sub>2</sub> [E<sub>2</sub>(-)]. In this and the following figures, the cell count was performed on five to six frontal sections per pituitary randomly selected from serial sections that span the rostral to caudal end of the gland (see *Materials and Methods* for details). Sex was not specified in the fetuses, and only female pituitaries were used at P7. Values are means  $\pm$  SEM; n = 4 for E14 (E<sub>2</sub>-), E15 (E<sub>2</sub>+), E16 (E<sub>2</sub>+), E17 (E<sub>2</sub>+), and 3 for others. Values with the same letter are not statistically significant. The difference between values was considered to be statistically significant when  $P$  value was below 0.05. \*,  $P < 0.05$  vs. E<sub>2</sub>(-) by Student's  $t$  test. ND, Not detected.

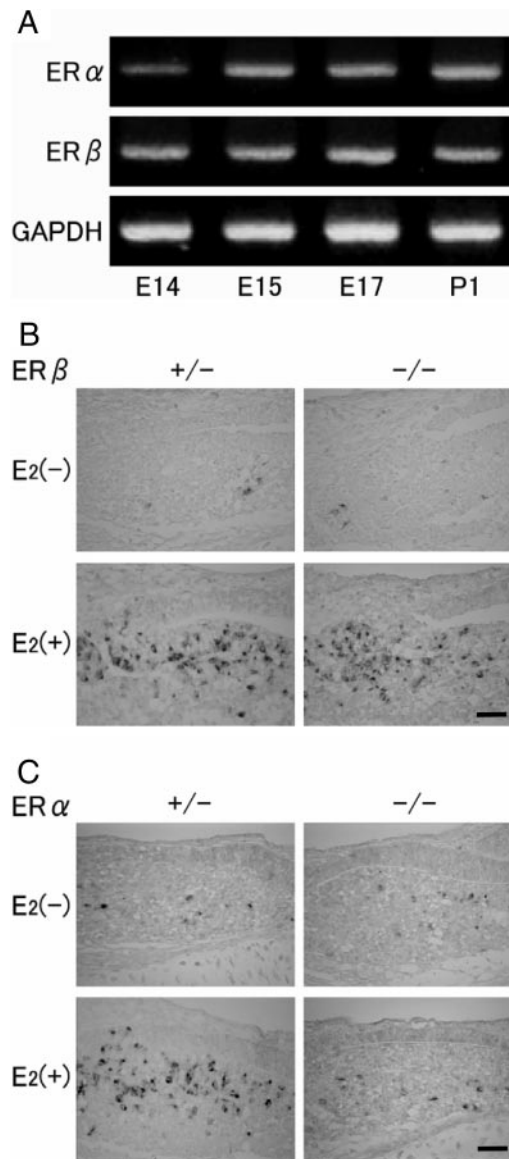


**FIG. 2.** A, Effects of forskolin (FK, 1  $\mu$ M), EGF (10 nM), and insulin (300 nM) on the development of PRL cells in the fetal mouse pituitary gland at E15. E15 pituitaries were incubated in a serum-free medium containing the substances indicated, and the number of PRL cells was counted. Values are means  $\pm$  SEM; n = 5. Values with the same letter are not statistically significant. The difference between values was considered to be statistically significant when  $P$  value was  $<0.05$ . B and C, Dose-response studies for the E<sub>2</sub> induction of PRL cells (B) or PRL mRNA (C) in E15 pituitaries. Values are means  $\pm$  SEM; n = 5–6. \*,  $P < 0.05$  vs. value of the culture without E<sub>2</sub> (-), by Student's  $t$  test.

cells were observed in the control culture, but they sharply increased after 24 h culture with 10 nM E<sub>2</sub>, suggesting that ER $\beta$  is not required for the induction of PRL cells in the fetal pituitary gland (Fig. 3B). On the contrary, E<sub>2</sub> failed in inducing PRL cells in  $\alpha$ ERKO (Fig. 3C), suggesting that only ER $\alpha$  is capable of inducing PRL cells in the fetal pituitary gland. Small numbers of PRL cells were observed in  $\alpha$ ERKO at E15 (Fig. 3C), suggesting that neither ER $\alpha$  nor ER $\beta$  is required for the phenotype determination of PRL cells.

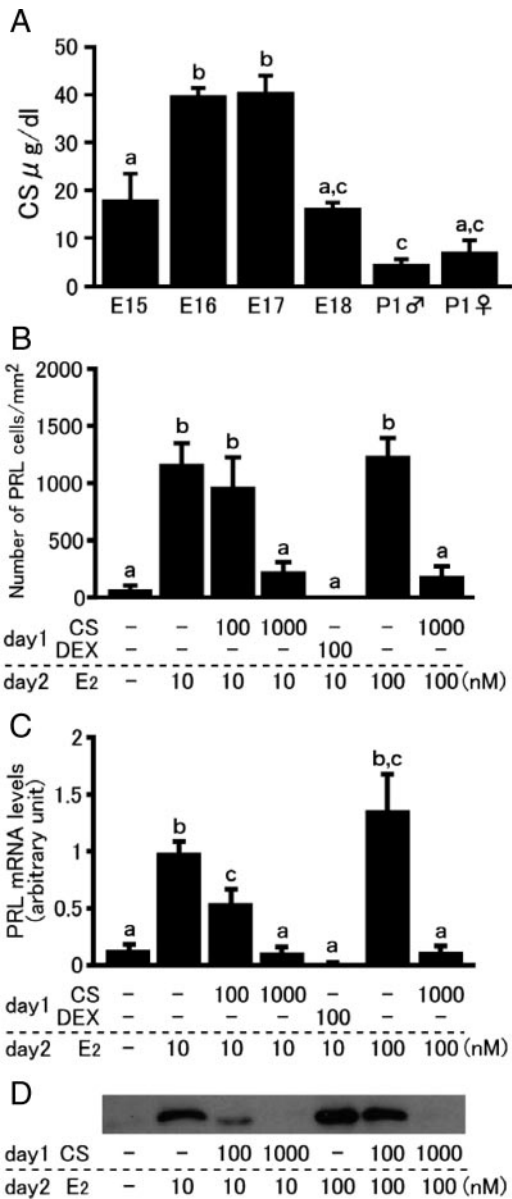
#### GCs inhibit estrogen action on PRL production

Although plasma concentration of CS is known to increase transiently in rats with a peak at E19 (8, 9), no information on



**FIG. 3.** Both ER $\alpha$  and ER $\beta$  were expressed in the fetal pituitary gland, but only ER $\alpha$  mediated estrogen action. A, Expression of two ER subtypes was examined by conventional RT-PCR. The mRNA for both subtypes was detected as early as E14 in mice. The mRNA for GAPDH was also amplified as an internal control. B and C, Pituitaries from  $\beta$ ERKO mice (-/-) and their heterozygote littermates (+/-) (B) or  $\alpha$ ERKO (-/-) and (+/-) (C) were incubated in a serum-free medium with [E<sub>2</sub>(+)] or without E<sub>2</sub> [E<sub>2</sub>(-)], and PRL cells were stained by immunocytochemistry. Scale bar, 40  $\mu$ m.

mice has been reported. We determined the serum concentration of CS in the fetal mouse at E15–P1. The result shown in Fig. 4A indicates that serum CS also increases transiently in mice, reaching a peak around E16 and E17 but decreasing rapidly on E18 to reach a level similar to that on E15. These data, taken together with Fig. 1A, shows that the number of PRL cells induced by E<sub>2</sub> *in vitro* decreased along with the increase in the fetal CS levels. Therefore, we postulated that the exposure of fetal PRL cells to high concentrations of GCs in late gestation decreased their sensitivity to estrogens even after the removal of GCs. This hypothesis was examined by the 2-d organ culture system using E15 pituitaries. When pituitaries were incubated in serum-free medium without hormones during the first 24 h followed by a 2-h



**FIG. 4.** A, Serum CS concentration in fetal and neonatal mice. Serum was prepared from trunk blood, and CS was determined by RIA. Sex was not specified in fetuses. Values are the means  $\pm$  SEM;  $n = 3$ . Values with the same letter are not statistically significant. Difference between values was considered statistically significant when  $P$  value was below 0.05. B–D, Pretreatment of GCs inhibits induction of PRL cells (B), PRL mRNA (C), and PRL protein (D) in E15 pituitaries. Pituitary glands from E15 fetuses were incubated for 24 h in a serum-free medium with or without the GCs indicated and followed by an additional 24-h incubation in a fresh serum-free medium containing 0, 10, or 100 nM  $E_2$ . After incubation, tissues were processed either for immunocytochemical detection of PRL cells and the cell count (B) or for PRL mRNA determination by real-time PCR (C). Values are the means  $\pm$  SEM;  $n = 3$  (B) or  $n = 5$  (C). Values with the same letter are not statistically significant. Difference between values was considered to be statistically significant when  $P$  value was below 0.05. D, Pituitaries were incubated as described in B and C, and cells were harvested for Western blot analysis for PRL expression. Protein extracted from two pituitaries was applied to one lane.

$E_2$  treatment, a number of PRL cells appeared, indicating that the pituitaries maintain sensitivity to  $E_2$  during a 48-h culture period (Fig. 4B). The number of  $E_2$ -induced PRL cells showed a marked decline after pretreatment with DEX (100 nM) or CS (100 or 1000 nM) (Fig. 4B). The PRL mRNA levels determined by real-

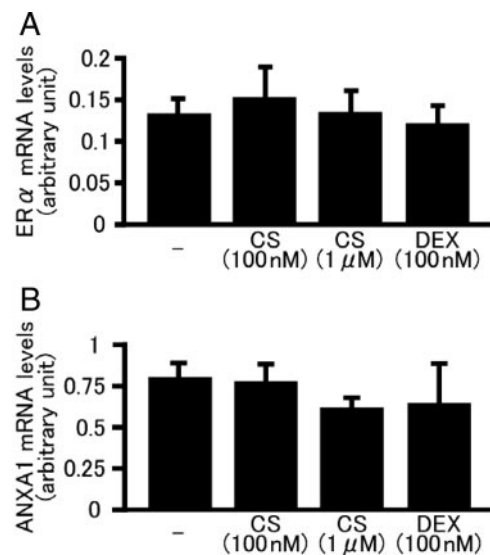
time PCR (Fig. 4C) and PRL protein levels determined by immunoblot (Fig. 4D) showed parallel changes with the PRL cell number. Experiments with higher doses of  $E_2$  (100 nM) yielded a similar result. These results indicated that the inhibitory effects of GCs appear to be sustained even after the removal of GCs. Several experiments were carried out to examine the mechanism of this sustained inhibitory effect of GCs.

**GCs do not affect ER $\alpha$  or ANXA1 expression in the pituitary gland of fetal mice**

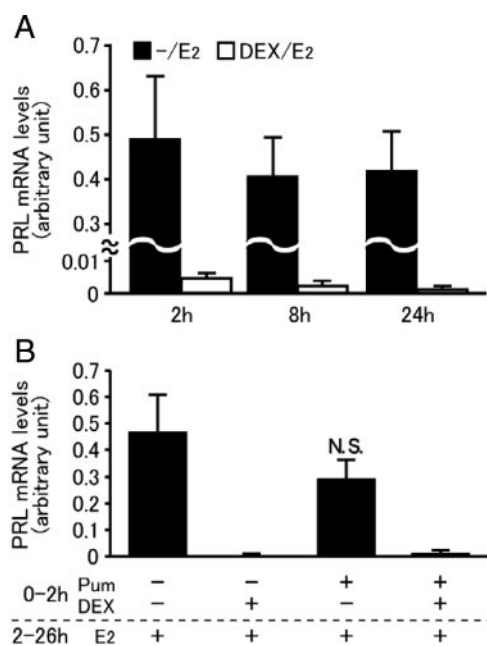
We began by postulating that the ER $\alpha$  level is decreased by GCs. To verify this hypothesis, E15 pituitaries were incubated with GCs, and the changes in ER $\alpha$  expression were determined. As shown in Fig. 5A, treatment with DEX or CS did not affect the ER $\alpha$  mRNA levels. ANXA1 is known to be induced in the anterior pituitary gland in response to GCs and suppresses PRL secretion (15). It is conceivable that the ANXA1 produced in the pituitary gland by preexposure to GCs mediates the inhibitory effects of GCs on the estrogen stimulation of PRL cells. By real-time PCR, we found that ANXA1 mRNA is expressed in pituitary glands as early as E15; however, no changes in the expression levels of ANXA1 were detected after the treatment with GCs (Fig. 5B).

**Inhibitory effect of GCs does not require ongoing protein synthesis**

Finally, we examined whether ongoing protein synthesis is required for GC action. Pituitaries from E15 fetuses were incubated with DEX for different periods before 24 h incubation with  $E_2$ . Surprisingly, only 2 h treatment almost completely suppressed the effect of  $E_2$  on PRL mRNA induction (Fig. 6A), suggesting that the *de novo* protein synthesis was not required for the inhibitory effects of DEX. In accordance with these data, it



**FIG. 5.** GCs do not affect expression of mRNA for ER $\alpha$  or ANXA1. Pituitary glands of E15 fetuses were incubated in a medium containing GCs as indicated, and the expression of ER $\alpha$  mRNA (A) and ANXA1 mRNA (B) was determined by real-time PCR. Values in A and B were the means  $\pm$  SEM;  $n = 3$  for all groups. No significant difference was observed.



**FIG. 6.** Effect of length of time of DEX treatment and implication of ongoing protein synthesis in the suppression of the levels of E<sub>2</sub>-inducible PRL mRNA. **A**, Pituitary glands from E15 fetuses were incubated for 2, 8, or 24 h in a serum-free medium with or without 100 nM DEX, followed by an additional 24-h incubation in a fresh serum-free medium containing 10 nM E<sub>2</sub>. After incubation, tissues were processed for PRL mRNA determination by real-time PCR. **B**, Pituitary glands were incubated for 2 h in a serum-free medium with 100 nM DEX, 100 μM puromycin (Pum), or a combination of DEX and Pum, followed by 24 h incubation with 10 nM E<sub>2</sub>. The expression of PRL mRNA was determined by real-time PCR. Values are the means ± SEM; n = 3 (A) or n = 4 (B). N.S., Not significant vs. control (Pum-, DEX-).

was revealed that the effects of 2 h DEX treatment were puromycin insensitive (Fig. 6B).

## Discussion

The present study demonstrated that estrogen induced PRL production in organ culture with a serum-free defined medium, showing that estrogen alone can stimulate PRL production and that the presence of any other humoral factors was not necessary for estrogen action. The effect of PRL cell induction was specific to E<sub>2</sub>, and no other stimulator of PRL production we tested exerted a similar effect. The results shown in Fig. 1 suggest that there are a number of cells in E14 or E15 pituitaries that can develop into PRL cells in response to E<sub>2</sub>. However, it was still obscure whether estrogen acts only to induce PRL expression in nascent PRL cells or is also implicated in the specification of precursors to nascent PRL cells. This issue was examined using ERKO mice.

Two distinct subtypes of estrogen receptors, ER $\alpha$  and ER $\beta$ , are expressed in a tissue-specific manner. In the anterior pituitary gland of adult mice, ER $\alpha$  is predominantly expressed, and ER $\beta$  expression is low or undetectable (24). A previous study (25) concluded that estrogen is not required for the specification of PRL cells, because those cells showed only a modest decrease in number in adult  $\alpha$ ERKO mice, and ER $\beta$  expression is not detectable in the adult pituitary gland. On the other hand, ER $\beta$  has

been found to be predominantly expressed in the pituitary gland of the fetal rat (26), with the expression level of ER $\alpha$  remaining very low until birth. We have also confirmed the expression of ER $\beta$  in the fetal mouse pituitary gland at E14 or older (Fig. 3A). Therefore, it is possible that estrogen is involved in PRL cell specification in the fetal pituitary gland via ER $\beta$ . However, the present results clearly showed that only ER $\alpha$  mediates the effects of estrogen on PRL cells (Fig. 3) in fetal pituitary glands. It has also been demonstrated that PRL cells were detectable in the explants from  $\alpha$ ERKO mice after incubation in serum-free medium without hormone as well as in those from their heterozygous littermates and  $\beta$ ERKO mice. Because ER $\beta$  does not mediate estrogen action on PRL cells, these results suggest that the specification of PRL cells does not require estrogen, being in agreement with the previous study (25). A current dose-response experiment showed that the minimum dose of E<sub>2</sub> which induced PRL cells to initiate PRL production was 10 pM (Fig. 2, B and C). The free E<sub>2</sub> in the fetal rat circulation is estimated to be approximately 2 pM at E19 (27). If this is also the case in mice, it is not likely that endogenous E<sub>2</sub> stimulates PRL cell specification or PRL production in the fetus, being consistent with the results from experiments with ERKO mice. However, it has been reported that the free E<sub>2</sub> level in rats reaches approximately 10 pM at birth (27), a dose that is shown to have stimulatory effects on PRL production in this study. This suggests that endogenous free E<sub>2</sub> may be able to stimulate PRL production at E18 or P1; however, PRL cells are still scarce during the perinatal period, probably due to the inhibitory effect of GCs as discussed below.

PRL cells have been reported to differentiate from GH cells based on the discovery of a marked decrease in the number of PRL cells in transgenic mice in which GH cells were genetically ablated (28). The present results, however, provide evidence that casts doubt on this hypothesis. When E15 pituitaries were incubated with estrogen, a number of PRL cells were induced, whereas these pituitaries contained no trace of the immunoreactive GH cells that first appeared on E16. This suggests that PRL cells induced by estrogen are not derived from preexisting GH cells, which is consistent with a recent study (29) showing that the majority of PRL cells in the adult mouse pituitary gland develop independently of the GH cell lineage as well as another study (30) demonstrating a different origin of GH and PRL cells in the chick embryo.

In our previous study, it was suggested that the effect of a maternal DES injection on the induction of PRL cells in the fetal pituitary gland was inhibited by endogenous GCs at E17 or older (6). In this study, we examined the effect of estrogen on the induction of PRL cells using an organ culture system eliminating the effects of GCs. Nevertheless, we obtained a similar result to the one in our previous *in vivo* study (6). The number of PRL cells induced by E<sub>2</sub> increased with age until E15 but unexpectedly showed a marked decline in the pituitary explants from fetuses at E16 or older (Fig. 1). These results suggest that even if GCs have an inhibitory effect on estrogen action, it would not be a direct inhibition of estrogen-induced PRL gene transcription as demonstrated in bovine or human PRL genes (12, 13). Because estrogen effects were declined along with the increase in serum CS levels as shown in Fig. 4A, we postulated that the exposure to

a high concentration of GCs *in utero* attenuates the sensitivity of fetal PRL cells to estrogen. This possibility was tested in this study using 48 h organ culture (Fig. 4), showing that the pretreatment of E15 pituitaries with GCs for 24 h clearly diminished the stimulatory effects of subsequent 24 h estrogen treatment on the induction of immunoreactive PRL cells as well as on the accumulation of PRL or PRL mRNA, suggesting that GCs exert indirect and sustained inhibition on the actions of estrogen. Several mechanisms seemed to be possible for this sustained inhibitory effect of GCs, which were verified experimentally in this study.

First, we hypothesized that the inhibitory effects of GCs on the estrogen induction of PRL cells depend upon the down-regulation of ER as observed in uterus (31) or the induction of an inhibitory factor such as ANXA1, which has been shown to be induced in pituitary gland by GCs and suppress the cAMP-driven PRL secretion from a subset of PRL cells in the rat (14, 15, 32). The data illustrated in Fig. 5 show that the overnight incubation with GCs does not affect the expression levels of ER $\alpha$  mRNA in E15 pituitaries, and unlike in adult pituitary gland of rats (32), GCs failed in inducing ANXA1 mRNA in the fetal pituitary gland. Thus, it appears that the effects of GCs are mediated neither by the down-regulation of ER nor by the induction of ANXA1. It is possible that GCs induce apoptosis of PRL cells. It has been shown that GCs induce apoptosis of T and B lymphocytes (33), and selective apoptosis of PRL cell in the anterior pituitary gland has been demonstrated in streptozotocin-induced diabetic or postlactating rats (34, 35). We tested this possibility using organ culture and terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) stain in this study, however, any increase in the number of TUNEL-positive cells could not be observed in response to CS (data not shown).

Finally, a possibility was examined that GCs induce a yet unknown substance that competes with estrogen action on PRL expression. As shown in Fig. 6, it was revealed that only 2 h incubation is highly effective for DEX to suppress almost completely the effects of estrogen, and the inhibition of protein synthesis during DEX treatment had little effect on DEX action. These results suggest that the inhibitory effects of GCs on PRL expression are not mediated by up-regulation of inhibitory factors. The mechanisms responsible for the sustained suppressive effect of GCs on estrogen action on fetal PRL cells currently remain to be elucidated. It is possible that the mechanism is a novel one responsible for the inhibition of PRL gene expression by GCs.

The present study revealed in part the developmental processes of PRL cells in the anterior pituitary gland of the fetal mouse. The nascent PRL cells differentiate from precursors through a yet unknown mechanism on E14 independent of estrogen action. Although a very small number of PRL cells develop to synthesize detectable amounts of PRL at E15 and E16, the PRL production is strongly suppressed in most of the nascent PRL cells during gestation by the increased circulating GCs as well as by a deficiency of estrogen due to the presence of circulating  $\alpha$ -fetoprotein. We consider that there must be a substantial number of PRL cells in the pituitary gland near term, but we have so far been unable to detect most of these cells even in an organ

culture where GCs are eliminated due to a long-lasting inhibitory effect of GCs on estrogen action. Even if the PRL cells might have developed in gestation, PRL secretion would not occur before birth. Although signaling through the PRL receptor is assumed to play diverse physiological roles during fetal development as suggested by the widespread distribution of PRL receptor in mice as well as in humans (36, 37), it is thought that the actual ligand for these receptors is not pituitary PRL but placental lactogen that is secreted from syncytiotrophoblasts of the placenta to both maternal and fetal compartments. The silencing of pituitary PRL may be required for preventing the overstimulation of PRL receptors in the fetus.

## Acknowledgments

Address all correspondence and requests for reprints to: Haruo Nogami, Ph.D., Laboratory of Neuroendocrinology, Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8575, Japan. E-mail: hnogami@md.tsukuba.ac.jp.

This work was supported by Twenty-First Century Center of Excellence Program (to H.N.).

Disclosure Statement: K.O., H.N., M.C.T., J.-Å.G., S.O., T.H., and S.H have nothing to disclose. K.S.K. receives royalties from the National Institutes of Health.

## References

1. Sano M, Sasaki F 1969 Embryonic development of the mouse anterior pituitary studied by light and electron microscopy. *Z Anat Entwickl Gesch* 129:195–222
2. Wilson DB, Christensen E 1980 Postnatal development of somatotrophs and mammatrophs in the pars distalis of the C57BL mouse. *Cell Tissue Res* 211:441–448
3. Harigaya T, Hoshino K 1985 Immunohistochemical study of postnatal development of prolactin-producing cells in C57BL mice. *Acta Histochem Cytochem* 18:343–351
4. Slabaugh MB, Lieberman ME, Rutledge JJ, Gorski J 1982 Ontogeny of growth hormone and prolactin gene expression in mice. *Endocrinology* 110:1489–1497
5. Dollé P, Castrillo J, Theill LE, Deerinck T, Ellisman M, Karin M 1990 Expression of GHF-1 protein in mouse pituitaries correlates both temporally and spatially with the onset of growth hormone gene activity. *Cell* 60:809–820
6. Matsubara M, Harigaya T, Nogami H 2001 Effects of diethylstilbestrol on the cytogenesis of prolactin cells in the pars distalis of the pituitary gland of the mouse. *Cell Tissue Res* 306:301–307
7. Stack G, Gorski J 1983 The ontogeny of estrogen responsiveness reexamined: the differential effectiveness of diethylstilbestrol and estradiol on uterine deoxyribonucleic acid synthesis in neonatal rats. *Endocrinology* 112:2142–2146
8. Dupouy JP, Coffigny H, Magre S 1974 Maternal and foetal corticosterone levels during late pregnancy in rats. *J Endocrinol* 65:347–352
9. Boudouresque F, Guillaume V, Grino M, Strbak V, Chautard T, Conte-Devolx B, Oliver C 1988 Maturation of the pituitary-adrenal function in rat fetuses. *Neuroendocrinology* 48:417–422
10. Fu X, Porter TE 2004 Glucocorticoid induction of lactotrophs and prolactin gene expression in chicken embryonic pituitary cells: a delayed response relative to stimulated growth hormone production. *Endocrinology* 145:1322–1330
11. Sato K, Watanabe YG 1998 Corticosteroids stimulate the differentiation of growth hormone cells but suppress that of prolactin cells in the fetal rat pituitary. *Arch Histol Cytol* 61:75–81
12. Sakai DD, Helms S, Carlstedt-Duke J, Gustafsson JA, Rottman FM, Yamamoto KR 1988 Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. *Genes Dev* 2:1144–1154
13. Berwaer M, Monget P, Peers B, Mathy-Hartert M, Bellefroid E, Davis JR, Belayew A, Martial JA 1991 Multihormonal regulation of the human prolactin gene expression from 5000 bp of its upstream sequence. *Mol Cell Endocrinol* 80:53–64

14. Christian HC, Taylor AD, Flower RJ, Morris JF, Buckingham JC 1997 Characterization and localization of lipocortin 1-binding sites on rat anterior pituitary cells by fluorescence-activated cell analysis/sorting and electron microscopy. *Endocrinology* 138:5341–5351
15. Taylor AD, Cowell AM, Flower RJ, Buckingham JC 1995 Dexamethasone suppresses the release of prolactin from the rat anterior pituitary gland by lipocortin 1 dependent and independent mechanisms. *Neuroendocrinology* 62:530–542
16. Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O 1993 Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc Natl Acad Sci USA* 90:11162–11166
17. Kregel JH, Hodgins JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA, Smithies O 1998 Generation and reproductive phenotypes of mice lacking estrogen receptor  $\beta$ . *Proc Natl Acad Sci USA* 95:15677–15682
18. Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159
19. Yamamoto M, Harigaya T, Ichikawa T, Hoshino K, Nakashima K 1992 Recombinant mouse prolactin: expression in *Escherichia coli*, purification and biological activity. *J Mol Endocrinol* 18:165–172
20. Harigaya T, Kitabayashi N, Yoshikawa K, Yamamoto M 1994 An enzyme immunoassay for the determination of serum prolactin in mice: developed by recombinant mouse prolactin. *Anim Sci Technol* 65:42–44
21. Maurer RA 1981 Transcriptional regulation of the prolactin gene by ergocryptine and cyclic AMP. *Nature* 294:94–97
22. Goda H, Sakai T, Kurosumi M, Inoue K 1998 Prolactin-producing cells differentiate from G0/G1-arrested somatotrophs *in vitro*: an analysis of cell cycle phases and mammotroph differentiation. *Endocr J* 45:725–735
23. Kakeya T, Takeuchi S, Takahashi S 2000 Epidermal growth factor, insulin, and estrogen stimulate development of prolactin-secreting cells in cultures of GH3 cells. *Cell Tissue Res* 299:237–243
24. Couse JF, Korach KS 1999 Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev* 20:358–417
25. Scully KM, Gleiberman AS, Lindzey J, Lubahn DB, Korach KS, Rosenfeld MG 1997 Role of estrogen receptor- $\alpha$  in the anterior pituitary gland. *Mol Endocrinol* 11:674–681
26. Nishihara E, Nagayama Y, Inoue S, Hiroi H, Muramatsu M, Yamashita S, Koji T 1999 Ontogenetic changes in the expression of estrogen receptor  $\alpha$  and  $\beta$  in rat pituitary gland detected by immunohistochemistry. *Endocrinology* 141:615–620
27. Montano MM, Welshons WV, vom Saal FS 1995 Free estradiol in serum and brain uptake of estradiol during fetal and neonatal sexual differentiation in female rats. *Biol Reprod* 53:1198–1207
28. Behringer RR, Mathews LS, Palmiter RD, Brinster RL 1988 Dwarf mice produced by genetic ablation of growth hormone-expressing cells. *Genes Dev* 2:453–461
29. Luque RM, Amargo G, Ishii S, Lobe C, Franks R, Kiyokawa H, Kineman RD 2007 Reporter expression, induced by a growth hormone promoter-driven Cre recombinase (rGHP-Cre) transgene, questions the developmental relationship between somatotropes and lactotrophs in the adult mouse pituitary gland. *Endocrinology* 148:1946–1953
30. Fu X, Nishimura S, Porter TE 2004 Evidence that lactotrophs do not differentiate directly from somatotrophs during chick embryonic development. *J Endocrinol* 183:417–425
31. Campbell PS 1978 The mechanism of the inhibition of uterotrophic responses by acute dexamethasone pretreatment. *Endocrinology* 103:716–723
32. Taylor AD, Philip JG, John CD, Cover PO, Morris JF, Flower RJ, Buckingham JC 2000 Annexin 1 (lipocortin 1) mediates the glucocorticoid inhibition of cyclic adenosine 3',5'-monophosphate-stimulated prolactin secretion. *Endocrinology* 141:2209–2219
33. Wyllie AH, Morris RG 1982 Hormone-induced cell death. Purification and properties of thymocytes undergoing apoptosis after glucocorticoid treatment. *Am J Pathol* 109:78–87
34. Jaubert A, Drutel G, Leste-Lasserre T, Ichas F, Bresson-Bepoldin L 2007 Tyrosine hydroxylase and dopamine transporter expression in lactotrophs from postlactating rats: involvement in dopamine-induced apoptosis. *Endocrinology* 148:2698–2707
35. Arroba AI, Frago LM, Argente J, Chowen JA 2005 Activation of caspase 8 in the pituitaries of streptozotocin-induced diabetic rats: implication in increased apoptosis of lactotrophs. *Endocrinology* 146:4417–4424
36. Freemark M 2001 Ontogenesis of prolactin receptors in the human fetus: roles in fetal development. *Biochem Soc Trans* 29:38–41
37. Tzeng SJ, Linzer DI 1997 Prolactin receptor expression in the developing mouse embryo. *Mol Reprod Dev* 48:45–52