

The role of IGF1 on the differentiation of prolactin secreting cells in the mouse anterior pituitary

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Abstract

IGF1 knockout (IGF1KO) mice show a reduced number of prolactin (PRL) producing cells (PRL cells); however, the role of IGF1 in PRL cell proliferation and differentiation in immature mice is unclear. In this study, ontogenic changes in the percentages of PRL cells, GH producing cells (GH cells), and 5-bromo-2'-deoxyuridine (BrdU)-labeled cells in the anterior pituitary of male IGF1KO mice during the postnatal period were investigated. The percentage of PRL cells in IGF1KO mice was significantly lower at day 20 compared with that in wild-type (WT) mice, while GH cells in IGF1KO mice were significantly increased from day 10. From days 5 to 20, the percentage of BrdU-labeled cells in WT and IGF1KO mice was similar. PRL cells and GH cells are thought to originate from the same progenitor cells,

therefore, PRL cells in IGF1KO mice are not able to differentiate because progenitor cells have already committed to be GH cells. However, IGF1, 17 β -estradiol (E₂), epidermal growth factor (EGF), or IGF1 plus E₂ treatments increased the PRL cell number in the pituitaries *in vitro* of 10-day-old WT and IGF1KO mice. This fact suggests that these factors are involved in PRL cell proliferation and differentiation. In addition, the increase of PRL cells in IGF1KO mice stimulated by E₂ or EGF was less than that of WT mice. Thus, IGF1 plays a crucial role in PRL cell proliferation and differentiation in mouse pituitaries by regulating the differentiation of progenitor cells and mediating the actions of E₂ and EGF.

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Introduction

Differentiation of anterior pituitary cells is thought to be controlled by various factors from the hypothalamus, peripheral hormones and/or growth factors (Schwardz 2000, Denef 2003). In fetal mice, hormone-producing cells except prolactin (PRL) producing cells (PRL cells) in the anterior pituitary can be detected by immunohistochemistry at embryonic day 18.5 (Watanabe & Haraguchi 1994, Stefaneanu *et al.* 1999). PRL cells are rarely observed in the fetal anterior pituitary (Ogasawara *et al.* 2009), and these cells begin to increase at postnatal day 3 (Taniguchi *et al.* 2001). Several factors involved in PRL cell proliferation and differentiation have been investigated using knockout (KO) techniques. In adult insulin-like growth factor 1 (IGF1) KO (IGF1KO) and estrogen receptor 1 (Esr1) KO mice, PRL cells are decreased in number (Stefaneanu *et al.* 1999, Pelletier *et al.* 2003). Therefore, IGF1 and estrogen are suggested to influence PRL cell proliferation and differentiation in postnatal life.

IGF1, a small peptide, plays an important role in the regulation, differentiation, and proliferation of various normal and neoplastic cells as autocrine and/or paracrine signals (Yokoyama *et al.* 1997). The mitogenic effect of IGF1 is

mediated via the IGF1 receptor which acts as a tyrosine kinase receptor (LeRoith *et al.* 1995). Circulating IGF1 produced in the liver acts as a mediator of GH action and regulates systemic growth (D'Ercole *et al.* 1984). In the regulation of GH synthesis and release, IGF1 specifically inhibits GH gene transcription and secretion by negative feedback mechanisms (Wallenius *et al.* 2001). Therefore, high blood GH levels are induced by severe IGF1 deficiency (Lembo *et al.* 1996). In fact, the *Gh* mRNA expression is increased in IGF1KO mice of both sexes (Stefaneanu *et al.* 1999).

Since PRL cells and GH-producing cells (GH cells) are thought to originate from the same progenitor cells which express the transcription factors *Pit-1* (listed as *Pou1f1* in the MGI Database) and *Prop1* that play important roles in the differentiation of PRL cells and GH cells (Slabaugh *et al.* 1981, Simmons *et al.* 1990). Insulin and IGF1-induce PRL synthesis in the MtT/S pituitary cells which produce large amount of GH with PIT-1 expression (Inoue & Sakai 1991). PRL cells are suggested to be transdifferentiated from somatotrophs in association with insulin or IGF1. IGF1 also stimulates the proliferation of PRL cells in mouse pituitaries *in vitro* (Oomizu *et al.* 1998). *Igf1* mRNA is detected only in GH cells of the mouse anterior pituitary, and immunoreactive IGF1 is

detected in corticotrophs and some of the GH cells in rats. However, IGF1 receptor is localized in GH cells and some corticotrophs in mice, and corticotrophs, gonadotrophs, GH cells, and some of PRL cells in rats (Honda *et al.* 1998, Eppler *et al.* 2007). Thus, IGF1 may act in a paracrine manner in PRL cells of anterior pituitary in rodents.

Several factors besides IGF1 which stimulate PRL production and release both *in vivo* and *in vitro* have been reported. Estrogen is a potent stimulator of PRL cell proliferation, PRL production and secretion (Lloyd *et al.* 1975, Raymond *et al.* 1978, Amara *et al.* 1987). Epidermal growth factor (EGF) also stimulates PRL production, and this effect may be mediated through ESR1 in mouse pituitaries (Ben-Jonathan *et al.* 2009). In addition, dopamine produced in the hypothalamus inhibits PRL production via dopaminergic D2 receptor (Drd2) in lactotrophs (Cristina *et al.* 2006). These factors may be necessary for PRL production; however, the involvement of IGF1 with these factors on PRL cells has not been investigated.

In this study, our aim was to clarify the role of IGF1 in PRL cell proliferation and differentiation in mouse pituitaries from neonate to adult, and analyze the ontogenic changes of PRL cells and GH cells in adult and immature homozygous male wild-type (WT) and IGF1KO mice. Expression of *Prl*, *Pit-1*, *Esr1*, *Egf*, and *Drd2* mRNAs in anterior pituitaries of WT and IGF1KO mice at days 10 and 20 was also examined by real-time RT-PCR. Finally, the effects of 17 β -estradiol (E₂), IGF1, and EGF on PRL production were examined in pituitaries from WT and IGF1KO mice at postnatal day 10 using an organ-culture system.

Materials and Methods

Animals

IGF1KO mice were obtained by mating of a mixed MF1 \times 129/Sv background that was heterozygous for the *Igf1* gene disruption, as previously described (Baker *et al.* 1996). The pups' genotypes were determined by multiplex PCR of DNA extracted from tails, homozygous WT, and homozygous IGF1KO males were used. They were housed under 12 h light:12 h darkness cycles with lights on at 0800 and controlled temperature (24 °C). Animals were given free access to mouse chow (MF, Oriental Yeast Co., Ltd, Tokyo, Japan) and tap water *ad libitum*, and were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All experiments were approved by the Institutional Animal Care Committee of the Yokohama City University.

Treatments

Pituitaries of 3- to 5-month-old WT and IGF1KO mice were collected. To examine the ontogenic changes of PRL in WT and IGF1KO mice, pituitaries were collected from 5-, 10-, 15- and 20-day-old WT and IGF1KO mice. Two hours before killing, WT and IGF1KO mice were given

a single injection of 1 mg/10 g body weight (BW) of 5-bromo-2'-deoxyuridine (BrdU, Sigma Chemical Co.) in saline.

PRL and GH immunohistochemistry

For PRL and GH immunohistochemistry, pituitaries were fixed in Bouin's solution overnight, then dehydrated and embedded in paraffin. Each 4- μ m thick section was mounted on silane (3-aminopropyl triethoxy-silane, Sigma Chemical Co.) coated glass slides. Sections were deparaffinized and hydrated through xylene and graded alcohol series. After washing in 0.1 M PBS (pH 7.4), sections were microwaved for 8 min in 1 M sodium citrate buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase was blocked by 1% (v/v) H₂O₂ in H₂O, and then the slides were washed in PBS. Sections were incubated with normal goat serum (VECTAS-TAIN Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA) for 20 min at room temperature to reduce nonspecific binding, then reacted with the rabbit polyclonal antibody to mouse PRL diluted with PBS (1:10 000, Biogenesis Ltd, Poole, UK) or to rat GH diluted with PBS (1:5000, Biogenesis Ltd) overnight at 4 °C. After washing in PBS, sections were incubated with biotinylated anti-rabbit IgG (1:500, Vector Laboratories Inc.) for 30 min. After washing in PBS, sections were incubated with ABC reagent (Vector Laboratories Inc.) for 30 min. Reaction products were visualized by using 3,3'-diaminobenzidine (DAB, Sigma Chemical Co). Negative control was prepared by incubation of sections with rabbit immunoglobulin fraction (DAKO Cytomation, Glostrup, Denmark) instead of the primary antibody. Finally, sections were counterstained with hematoxylin.

BrdU and PRL immunohistochemistry

For BrdU and PRL double-immunostaining, pituitaries were fixed in 10% (v/v) formalin neutral buffer solution (Wako Pure Chemical Industries, Ltd, Osaka, Japan) overnight as this fixative was better than Bouin's solution for the double staining of BrdU and PRL, dehydrated and embedded in paraffin. Sections of 4- μ m were mounted on silane coated glass slides. After deparaffinization, endogenous peroxidase was blocked by 0.3% (v/v) H₂O₂ in methanol for 30 min. For denaturation of DNA, sections were incubated in 2 M HCl for 20 min at room temperature, and immersed in 0.1 M borate buffer (pH 8.5) to neutralize pH. After washing in PBS, sections were digested with 0.1% (w/v) trypsin (Sigma Chemical Co.) in PBS for 30 min at 37 °C. After preincubation with 1% (w/v) BSA/PBS at room temperature to block nonspecific binding, sections were incubated with anti-BrdU antibody (1:15, Roche Molecular Biochemical) at room temperature for 60 min. Then, DAB with NiCl₂ reaction was carried out. After washing in PBS, sections were immunostained using anti-PRL antibody (Biogenesis Ltd) as described above and counterstained with methylgreen (Chroma-Gesellschaft Schmid, Kongen, Germany).

In situ hybridization

For *Gh* mRNA *in situ* hybridization, pituitaries were fixed in 4% (w/v) paraformaldehyde overnight at 4 °C, embedded in paraffin and sectioned at 4- μ m thickness. Sections were digested with 10 μ g/ml proteinase K (Wako Pure Chemical Industries, Ltd) for 15 min at 37 °C, and then post-fixed with 4% (w/v) paraformaldehyde in PBS, followed by an acetylation treatment.

The cDNA fragment encoding a part of the mouse *Gh* was generated from total RNA prepared from the WT mouse pituitary by RT-PCR with a pair of primers (forward 5' CTGGCTGCTGACACCTACAA 3' and reverse 5' GCTA-GAAGGCACAGCTGCTT 3'). These primers generate PCR products of 508 bp for the mouse *Gh*. The cDNA fragment was subcloned into pGEM-T Easy (Promega), sequenced and confirmed to be cDNA encoding a part of the mouse *Gh*. The probes were synthesized from subcloned plasmids using a PCR-digoxigenin (DIG) synthesis kit (Roche) with a pair of primers described above.

Sections were prehybridized with 4 \times SSC/40% (v/v) formamide at 37 °C, then hybridized in a solution containing 50% (v/v) formamide, 200 ng/ml tRNA, 10% (w/v) dextran sulfate, 1 \times Dehardt's solution, 10 mM Tris-HCl, 600 mM NaCl, 0.25% (w/v) SDS, 1 mM EDTA (pH 8.0), and DIG-labeled probe (1:50) at 37 °C overnight in a moist chamber. Slides were washed in 5 \times SSC for 5 min, and 2 \times SSC/50% (v/v) formamide for 1 h at 37 °C five times, then washed in 2 \times SSC twice. After preincubation with 1.5% (w/v) blocking reagent (DIG Luminescent Detection Kit, Roche) for 60 min, sections were incubated with the primary antibody diluted at 1:1000 for anti-DIG-AP, Fab fragments (Roche) for 2 h at room temperature. After washing in DIG buffer 1 (100 mM Tris-HCl, pH 7.5 and 150 mM NaCl) and DIG buffer 3 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 50 mM MgCl₂), hybridization signal was visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate in DIG buffer 3 for 15 h, then the reaction was stopped by Tris-EDTA buffer (10 mM Tris-HCl pH 7.6 and 1 mM EDTA). Finally, slides were post-fixed in 4% paraformaldehyde in PBS for 60 min at 4 °C and counter-stained with methylgreen (Chroma-Gesellschaft, Schmid GmbH).

Real-time RT-PCR

For real-time RT-PCR, anterior pituitaries were separated from posterior and intermediate lobes, and homogenized in TRIzol (Invitrogen Corp). Total RNA from anterior pituitaries was purified using DNase I (Roche) to remove genomic DNA and cleaned up with an RNeasy total RNA kit (Qiagen). The amount and integrity of RNA were assessed by measurement of optical density (OD) at 260 and 280 nm. RNA (1 μ g) was reverse transcribed into first strand cDNA using SuperScript II reverse transcriptase (Invitrogen Corp.) using oligo dT primer (Invitrogen Corp). Real-time PCR was carried out with a Smart Cycler II System with a total reaction volume of 25 μ l consisting of 12.5 μ l SYBR Green Master Mix (Takara Bio Inc., Otsu, Japan) by the standard curve method. Primer sequences are indicated in Table 1. Cyclophilin was chosen as an internal standard to control variability in amplification due to differences in starting mRNA concentration. Pituitaries from 3 to 5 mice were pooled for each point and three independent experiments were carried out for each study.

Organ culture

Three-to-five pituitaries of 10-day-old WT and IGF1KO mice were placed on a 24-well culture plate with 200 μ l serum-free DMEM/Nutrient Mixture F12 (1:1, phenol red free, Invitrogen Corp.) with or without 10⁻⁹ M E₂ (Sigma Chemical Co.), 20 ng/ml IGF1 (Sigma Chemical Co.) or 50 ng/ml EGF (Sigma Chemical Co.) and 1 ng/ml BSA (Sigma Chemical Co.) and incubated at 37 °C in a humidified 5% CO₂ atmosphere in air for 5 days. Serum-free DMEM/Nutrient Mixture F12 (phenol red free, 50 μ l) was added in a well on day 3 of culture. After 5 days in culture, pituitaries were fixed in Bouin's solution and immunostained for PRL cells and GH cells as described above.

To investigate cell proliferation, pituitaries of WT mice were incubated with or without IGF1, E₂, or EGF for 24 h, and then culture medium containing 100 mg/ml BrdU was added to each treatment and incubated for 24 h. After a total of 48 h culture, each pituitary was fixed with 10% (v/v) formalin neutral buffer solution and PRL/BrdU double immunohistochemistry was performed as described above.

Table 1 Primer sequences used for real-time RT-PCR

Gene	Forward sequence (5' → 3')	Reverse sequence (5' → 3')
<i>Prl</i>	TCAAGAAGCCCCGAATACA	TCCCATTCCTTTGGCTTCA
<i>Pit-1</i>	TGAGCAGGTCCGAGCTTTGTGA	TCCGTTTCCTCTTCCTTCGT
<i>Esr1</i>	CCTAGCTCAGCTGCTCCTTCTCATTCTT	GGCACACGTTCTTGCAATTC
<i>Drd2</i>	CCCAGAGAGGACCCGGTATAG	CTGGTTTGGCAGGACTGTCA
<i>Egf</i>	ATGTCCTGGACAAACGGCTCT	ACAAACTGTCCGTGCTTGA
<i>Cyclophilin</i>	CCATCCAGCCATTCAGTCTTG	AGGTCTGGCATCTTGTCCAT

Cell count and data analysis

For PRL/BrdU and GH immunohistochemistry and *in situ* hybridization, five individual pituitaries were used. GH, PRL, and BrdU immunoreactive cells were counted under a light microscope with an $\times 40$ objective lens. Positive cells in 200 anterior pituitary cells were counted in six randomly chosen areas from transversal horizontal sections. Three different sections from each pituitary were chosen to avoid counting the same cells.

For multiple comparisons, treatment groups were compared using ANOVA followed by appropriate *post hoc* tests. Two-tailed Student's *t*-test after application of *F* test was used for comparison of two mean values. A statistically significant difference was defined as $P < 0.05$.

Results

PRL and GH immunoreactive cells in the anterior pituitary of WT and IGF1KO adult mice

PRL and GH immunoreactivities were observed in the cytoplasm of anterior pituitary cells of 3- to 5-month-old WT and IGF1KO mice, respectively (Fig. 1A–D). Negative control incubating with rabbit immunoglobulin fraction showed no staining (Fig. 1E). These cells formed clusters

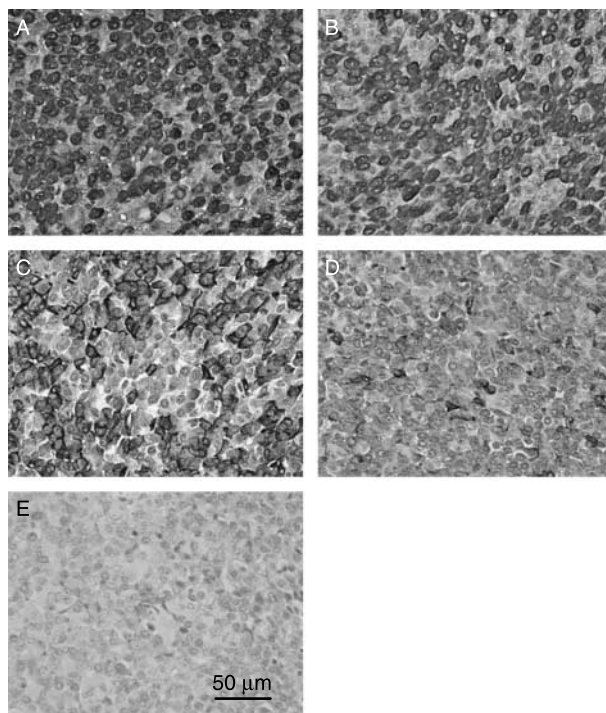


Figure 1 Immunohistochemical staining of GH in the pituitaries of 3- to 5-month-old WT (A) and IGF1KO (B) mice and prolactin (PRL) in the pituitaries of 3- to 5-month-old WT (C) and IGF1KO (D) mice. Negative staining for PRL and GH immunostaining (E).

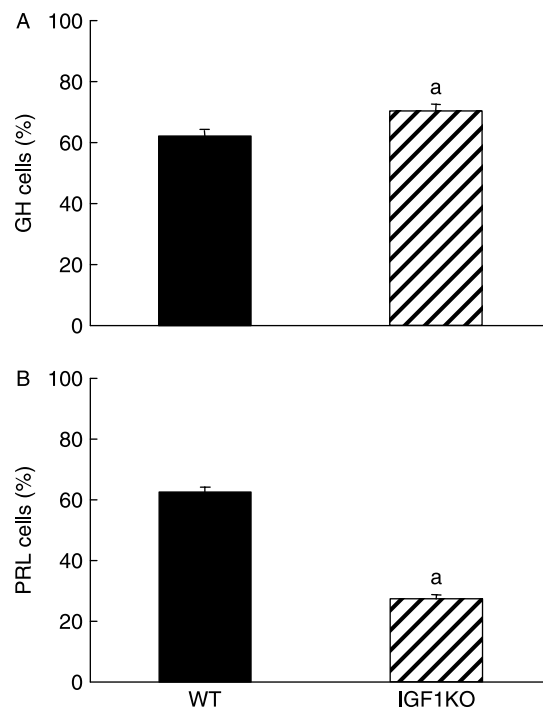


Figure 2 The percentages of GH positive cells (A) and PRL positive cells (B) in the anterior pituitaries of adult (3–5 months old) WT and IGF1KO mice. ^aSignificantly different from that in WT mice, $P < 0.05$ (Student's *t*-test).

and were distributed randomly in the anterior pituitaries in both WT and IGF1KO mice. The percentage of GH cells in pituitaries of IGF1KO mice was significantly higher as compared with that in WT mice (Fig. 2A). In contrast, the percentage of PRL cells in IGF1KO mice was significantly lower than that in WT mice (Fig. 2B). Some PRL cells in the anterior pituitaries both in WT and IGF1KO mice also showed positive staining for GH (somatolactotrophs, data not shown).

Gh mRNA expressing cells in the anterior pituitary of adult WT and IGF1KO adult mice

Stefaneanu *et al.* (1999) already demonstrated that *Prl* mRNA expressing cells were reduced in IGF1KO mice and the IGF1 administration to adult IGF1KO mice had no effect on the intensity or *Prl* RNA signal. Therefore, in this study, only *Gh* mRNA expressing cells were analyzed. *Gh* mRNA expressing cells were detected in the anterior lobe but not in the intermediate and posterior lobes of pituitaries of adult WT and IGF1KO mice, and no signal was detected in the negative controls in the anterior lobe by *in situ* hybridization (Fig. 3A and B). *Gh* mRNA expressing cells in IGF1KO mice were distinguishable, and the percentage of *Gh* mRNA expressing cells was significantly higher compared with that in WT mice (Fig. 3C).

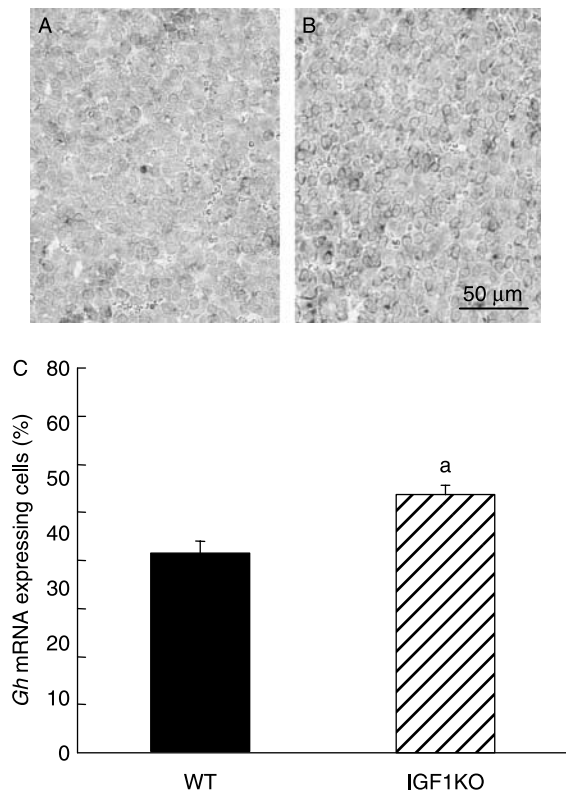


Figure 3 *In situ* hybridization of *Gh* mRNA in the pituitaries of WT (A) and IGF1KO (B) mice and the percentage of *Gh* mRNA expressing cells of adult (3–5 months old) WT and IGF1KO mice (C). ^aSignificantly different from that in WT mice, $P < 0.05$ (Student's *t*-test).

BrdU and PRL double immunostaining in 5- to 20-day-old WT and IGF1KO mice

In the anterior pituitary of WT and IGF1KO mice, BrdU-labeled cells were observed between days 5 and 20 (Fig. 4A–D). The percentage of BrdU-labeled cells in WT mice was high at day 5 and it decreased from days 10 to 20 (Fig. 4E). PRL cells in WT mice conversely increased with age (Fig. 4E). In the anterior pituitaries of IGF1KO mice, the percentage of BrdU-labeled cells was similar to that in WT mice at days 5–20 (Fig. 4E). In IGF1KO mice, the percentage of PRL cells increased with age as seen in WT mice; however, the percentage of PRL cells in IGF1KO mice at day 20 was significantly lower than that in the age-matched WT mice (Fig. 4E). Furthermore, a few PRL and BrdU double positive cells were found in WT and IGF1KO mice at days 5–20, although, there was no difference in the percentages of double positive cells between WT and IGF1KO mice (data not shown).

GH immunostaining in 10- to 20-day-old WT and IGF1KO mice

In postnatal mice, GH immunoreactive cells were clearly distinguishable (Fig. 5A and B). The percentage of GH cells in IGF1KO mice had significantly increased as compared with that in WT mice through days 10–20 (Fig. 5C).

Real-time RT-PCR

To clarify the changes in expression of factors involved in PRL cell proliferation and differentiation in anterior pituitaries, mRNA expression of *Prl*, *Pit-1*, *Esr1*, *Egf*, and *Drd2* was analyzed in WT and IGF1KO mice at days 10 and 20. mRNA expression of these genes showed no difference between WT and IGF1KO mice at days 10 and 20 (Fig. 6).

PRL and GH cells in organ-cultured WT and IGF1KO mouse pituitaries

Stefaneanu *et al.* (1999) report that PRL cell number and serum PRL levels in adult IGF1KO mice are not changed by IGF1 injections; therefore, we investigated the effects of IGF1, E₂, and EGF on PRL production in pituitaries of WT and IGF1KO mice at day 10 using an organ culture system. After 5 days in culture with control medium, the percentage of PRL cells was not different between WT and IGF1KO

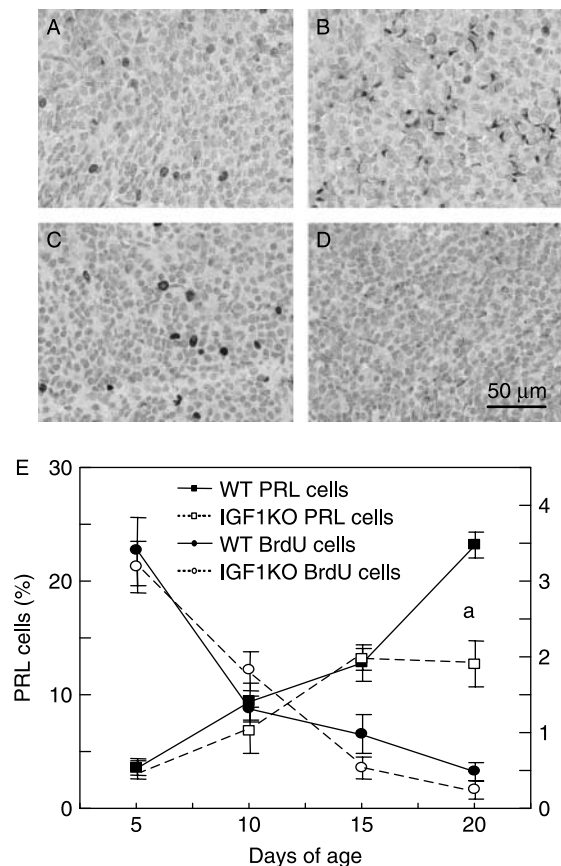


Figure 4 Immunohistochemical double staining of PRL and BrdU in WT (A and B) and IGF1KO (C and D) mice at day 10 (A and C) and day 20 (B and D). The percentages of PRL-stained cells (filled squares (WT) and open squares (IGF1KO)) and BrdU-labeled cells (filled circles (WT) and open circles (IGF1KO)) in the anterior pituitaries of mice at days 5–20 (E). ^aSignificantly different from that in age-matched WT mice. $P < 0.05$ (Student's *t*-test).

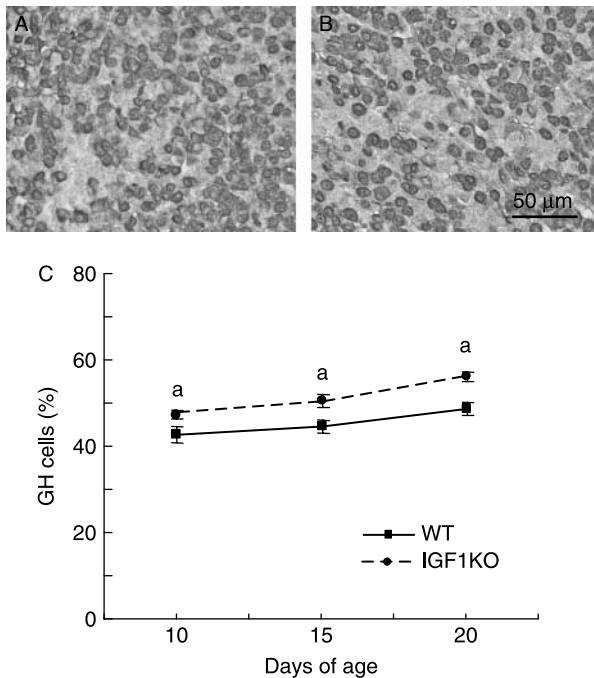


Figure 5 Immunohistochemical staining of GH in WT (A) and IGF1KO (B) mice at day 10. The percentage of GH cells (squares (WT) and circles (IGF1KO)) in the anterior pituitaries of WT and IGF1KO mice (C) at days 10, 15, and 20. ^aSignificantly different from that in the age-matched WT mice. $P < 0.05$ (Student's *t*-test).

mouse pituitaries (Fig. 7A and B). When pituitaries of WT and IGF1KO mice were cultured with IGF1, E₂, or EGF, the percentages of PRL cells were significantly increased as compared with those in controls (Figs 7A, B, E, F, I, J, M, N and 8). A combination of IGF1 and E₂ treatment also caused a significant increase as compared with that in controls, but it was not an additive effect on pituitaries from both WT and

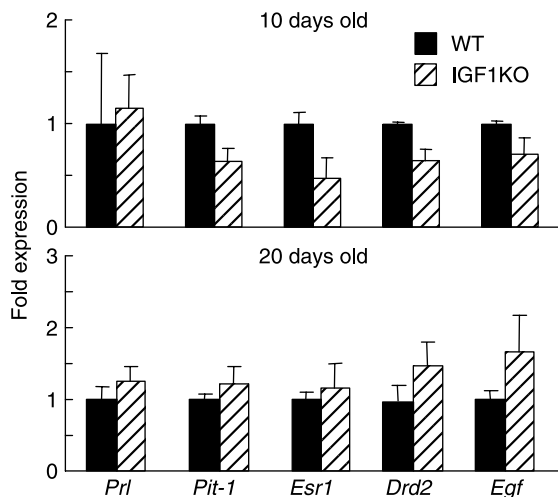


Figure 6 Changes in the mRNA expression of genes related to PRL production in WT and IGF1KO mice at days 10 and 20.

IGF1KO mice (Figs 7A, B, Q, R and 8). Interestingly, when pituitaries of IGF1KO mice were cultured with E₂ or EGF, the percentage of PRL cells was significantly lower than that in WT mice (Fig. 8).

The percentage of GH cells in pituitaries of WT mice was not different from those in IGF1KO mice after 5 days in culture (Figs 7C, D, G, H, K, L, O, P, S, T and 8).

To elucidate whether an increase of PRL cell number was due to cell proliferation of PRL cells, BrdU-labeled cells were counted in pituitaries of WT mice after 2 days in culture. The number of BrdU-labeled cells was similar among treatments (data not shown).

Discussion

IGF1 has a crucial role in the increase in PRL cell number because these cells are reduced in female IGF1KO mice from day 10 to adult (Saitoh *et al.* 2005). In this study, the percentage of PRL cells was significantly lower in male IGF1KO mice at day 20 as compared with age-matched WT mice, but the percentage of BrdU-labeled cells was not different between WT and IGF1KO mice. Thus, some of the anterior pituitary cells in immature IGF1KO mice may not be able to differentiate into PRL cells in both sexes. PRL cells in the anterior pituitary proliferate and differentiate from late fetal ages to postnatal periods (Taniguchi *et al.* 2001). In this study, the percentage of BrdU-labeled cells was high at day 5 and decreased; however, PRL cells increased in number with age, suggesting that PRL is not synthesized during mitosis of progenitor cells, and differentiated cells begin to produce PRL from days 15 to 20. A similar phenomenon is also reported in rat anterior pituitaries (Taniguchi *et al.* 2001). However, BrdU- and PRL-labeled cells were also found during neonatal period, indicating that some differentiated PRL cells have the potential to proliferate.

GH cells and PRL cells are thought to originate from common progenitor cells. The progenitor cells express *Pit-1* and *Prop-1*, which are essential for differentiation of PRL cells (Slabaugh *et al.* 1981, Simmons *et al.* 1990). PRL cells are also believed to be differentiated from GH-producing cells or somatolactotrophs that express GH and PRL. Borrelli *et al.* (1989) show that transgenic mice expressing *HSV1-TK* gene under the rat *Gh* promoter treated with FIAU results in the complete loss of somatotrophs and lactotrophs. Behringer *et al.* (1988) also show a severe decrease in lactotrophs in the anterior pituitary of *rGh-DT-A* transgenic mice which targeted diphtheria toxin-mediated destruction of GH producing cells. These results indicate that PRL cells arise from GH cells. However, in the adult *rGhp-Cre* transgenic mice, only 10% of lactotrophs are derived from somatotrophs (Luque *et al.* 2007). In this study, GH cells in IGF1KO mice were already increased at day 10 because of the absence of the IGF1 negative feedback loop that suppressed GH production (Wallenius *et al.* 2001). An increase of GH cells may be due to the mass differentiation of progenitor cells to GH cells,

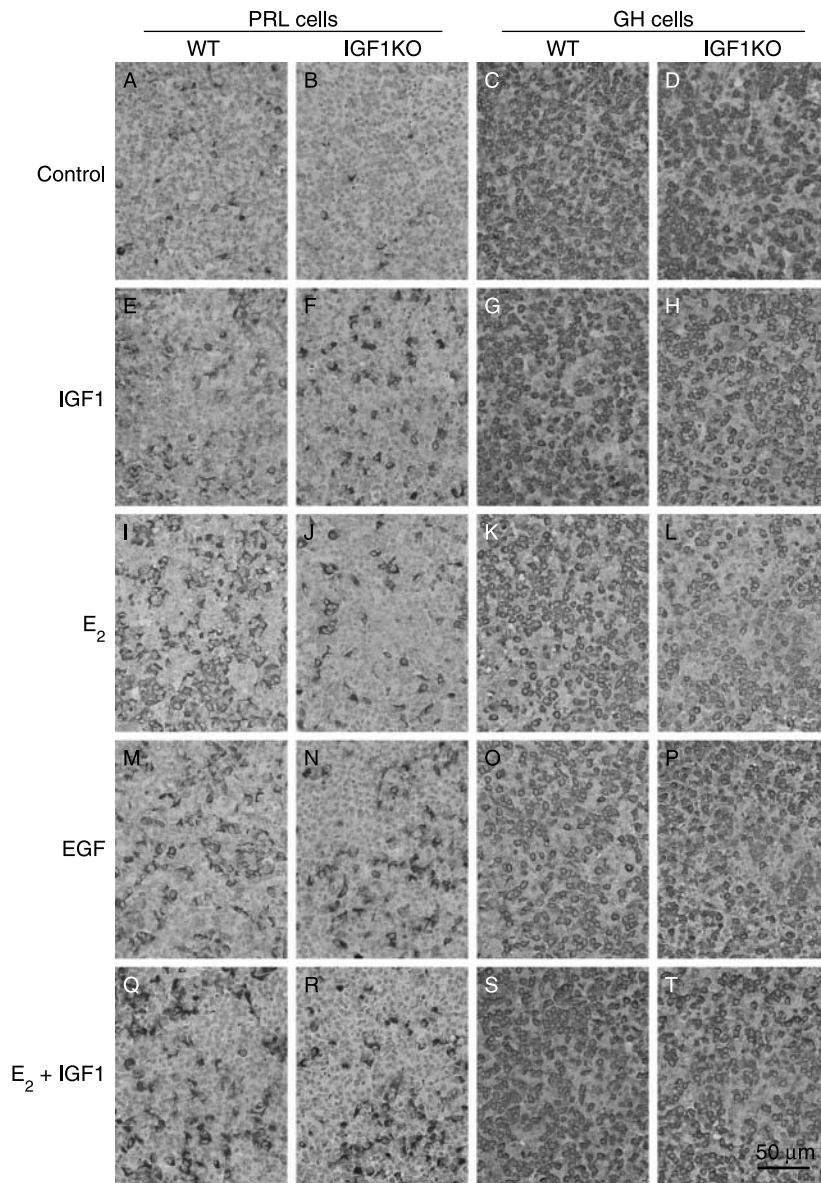


Figure 7 Immunohistochemical staining of PRL in the organ-cultured anterior pituitaries from WT (A, E, I, M, and Q) and IGF1KO (B, F, J, N, and R) mice, and GH in the organ-cultured anterior pituitaries from WT (C, G, K, O, and S) and IGF1KO (D, H, L, P, and T) mice. Control (A–D), IGF1 (E–H), E₂ (I–L), EGF (M–P), and IGF1 + E₂ (Q–T) treatments. Control, BSA; IGF1, 20 ng/ml insulin-like growth factor 1; E₂, 10⁻⁹ M 17β-estradiol; EGF, 50 ng/ml epidermal growth factor.

therefore, it may result in suppression of PRL cell differentiation in IGF1KO mice because PRL cells and GH cells are derived from the same origin. IGF1 may regulate the balance of GH cells and PRL cells during the immature period.

Several factors may be related to PRL cell proliferation and/or differentiation, including E₂, IGF1, and EGF. E₂ stimulates PRL cell proliferation (Wiklund *et al.* 1981, Amara *et al.* 1987) and E₂ also induces transdifferentiation of PRL

cells from GH cells via somatolactotrophs (Behringer *et al.* 1988, Borrelli *et al.* 1989, Goda *et al.* 1998). Furthermore, EGF plays a role in cell proliferation and differentiation in the mouse anterior pituitary (Schwardz 2000). EGF induces the expression of dopamine receptor and transdifferentiation into lactotroph-like cells in the GH3 cell line (Missale *et al.* 1991) and PRL cell differentiation in the MtT/S cell line (Goda *et al.* 1998). Ben-Jonathan *et al.* (2009) show that the ability of EGF to stimulate *Prl* gene expression and release is mediated

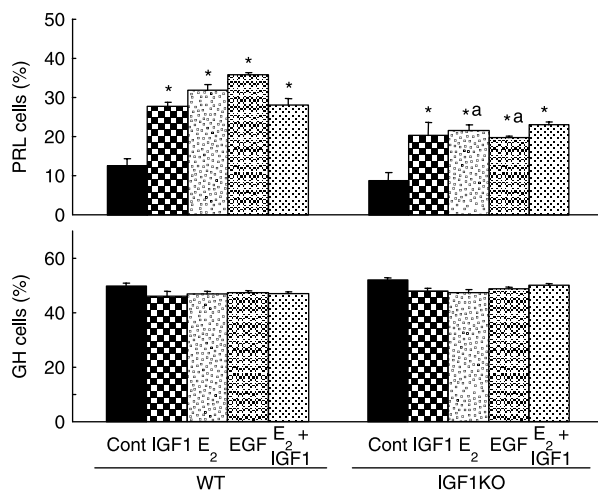


Figure 8 The percentages of PRL and GH cells in organ-cultured anterior pituitaries treated with BSA control, IGF1, E₂, EGF or IGF1 + E₂. *Significantly different from control treatment, $P < 0.05$. ^aSignificantly different from the same treatment of WT pituitaries, $P < 0.05$ (post hoc test).

by Erk1/2-induced phosphorylation of *Esr1* in GH3 cells, which express both *Esr1* and *Esr2* (Mitchner *et al.* 1999). Thus, E₂ and EGF can share a common pathway in the control of *Prl* gene expression. IGF1 also converts GH cells into PRL cells in the MtT/S cells (Inoue & Sakai 1991). Hypothalamic dopamine inhibits PRL production and cell proliferation (Freeman *et al.* 2000, Ben-Jonathan & Hnasko 2001). In this study, the differentiation of PRL cells was stimulated by IGF1, E₂ or EGF in the anterior pituitary of IGF1KO mice *in vitro*. In addition, the percentage of PRL cells in IGF1KO mice stimulated by E₂ or EGF was significantly lower than that in the WT mice, suggesting that IGF1 mediates E₂ or EGF action partially. In addition, *Esr1*, *Egf*, and *Drd2* mRNA were not different between WT and IGF1KO mice at days 10 and 20, in spite of a decrease in PRL cell number at day 20. This fact suggests that deletion of IGF1 does not affect the expression of these genes.

Pit-1 mRNA level was not changed in pituitaries from both WT and IGF1KO mice, suggesting that IGF1KO mice may have the same population of progenitor cells. Interestingly, the *Prl* mRNA level in IGF1KO mice was similar to that in WT mice at days 10 or 20. However, *Prl* mRNA expressing cells in adult IGF1KO mice are decreased (Stefaneanu *et al.* 1999), suggesting that IGF1 is responsible for PRL protein synthesis rather than PRL transcription in immature male IGF1KO mice.

IGF1 injection to IGF1KO mice shows no effect on the percentage of *Prl* mRNA expressing cells (Stefaneanu *et al.* 1999), which is in conflict with the result in this study. The discrepancy between these studies may be due to differences in the age of the mice used. In the anterior pituitary of day 10 mice, PRL cells are undergoing differentiation and progenitor cells may still exist, while percentages of PRL cells in adult

mice are maximally increased. Therefore, IGF1 can stimulate the differentiation of these progenitor cells in WT and IGF1KO mice at day 10 but not in the adult.

A combination of IGF1 and E₂ increased the percentage of PRL cells, but it was not an additive effect. Several reports show that E₂ inhibits IGF1 action which induces proliferation of PRL cells in primary culture of rat pituitary (Kawashima *et al.* 2002, Gutiérrez *et al.* 2005). However, an antagonistic effect of estrogen was not observed in this study, it may be due to the use of pituitaries from the developmental period of mice. Arroba *et al.* (2005) show that E₂ utilizes IGF1 receptor to stimulate PRL cells and activates MAPK cascade. This may be one of the reasons that an additive increase was not observed in E₂ and IGF1 treatments because of the sharing the same signaling pathway. Another possibility is the limit of progenitor cells in number, the percentage of PRL cells can reach a plateau even after a combination of IGF1 and E₂ treatment. In addition, the percentage of PRL cells in our data was higher than that in the previous report (Stefaneanu *et al.* 1999), therefore, it is necessary to confirm the percentage of cells producing both GH and PRL in the next study.

GH cells in the pituitaries of WT and IGF1KO mice were increased after 5 days in culture with the control medium. Since *Gh* gene expression is suppressed by IGF1 at the pituitary level (Goodyer *et al.* 1984, Yamashita & Melmed 1986), the hypothalamic level (Abe *et al.* 1983, Tannenbaum *et al.* 1983) and somatostatin from the hypothalamus (Sugihara *et al.* 1999, Morishita *et al.* 2003), GH cells can be increased when the pituitary is released from suppression. However, addition of IGF1 did not decrease GH cell number *in vitro*, suggesting that IGF1 suppresses GH synthesis through the hypothalamus or somatostatin at day 10. Similarly, PRL cells did not increase in control medium, indicating that PRL cell differentiation is not inhibited by the factors from the hypothalamus and stimulative factors induce differentiation from progenitor cells to PRL cells at this age.

In conclusion, IGF1 is essential for PRL cell differentiation, but not proliferation. In IGF1KO mice, the number of PRL cells is low due to the increase of GH cells from the same progenitor cells. IGF1 is a mediator of E₂ and EGF signaling in pituitary cells. Thus, IGF1 plays a crucial role for PRL cell development by regulating differentiation of progenitor cells and mediating actions of E₂ and EGF.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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