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Induction of mammotroph development by a combination of epidermal growth factor, insulin, and estradiol-17 β in rat pituitary tumor GH3 cells

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ABSTRACT—Several reports have indicated that prolactin-secreting cells (PRL cells) are generated from growth hormone-secreting cells (GH cells). We have shown that treatment with a combination of epidermal growth factor (EGF), insulin, and estradiol-17 β (E_2) induces the appearance of PRL cells in pituitary tumor GH3 cells. The aim of the present study was to clarify the involvement of mitosis in the cytogenesis of PRL cells in rat pituitary and GH3 cells. The effects of the treatment with EGF, insulin and E_2 on DNA-replication were studied by detecting the uptake of bromodeoxyuridine (BrdU) into the nucleus. In cultured rat pituitary cells, BrdU-labeled PRL cells were observed irrespective of the hormone treatment. In GH3 cells, BrdU-labeled GH cells and mammosomatotrophs (MS cells) were detected; BrdU-labeled PRL cells were not detected, however, when GH3 cells were treated with BrdU for 3 hr and then immediately examined for BrdU-labeling. BrdU-labeled PRL cells were found only when GH3 cells treated with BrdU were allowed to grow for another 3 days. This finding suggests that during the additional 3-day culture, BrdU-labeled PRL cells were generated from BrdU-labeled cells other than PRL cells. These results indicate that PRL cells are transdifferentiated from GH cells or MS cells in GH3 cells by a combined treatment with EGF, insulin and E_2 , while PRL cells in rat pituitaries are able to proliferate in response to the hormone treatment. Thus, there may be two pathways for cytogenesis of PRL cells: the transdifferentiation of GH cells or MS cells, and a self-duplication of PRL cells.

Key words: GH3 cells, pituitary, mammosomatotroph, mammotroph, somatotroph

INTRODUCTION

Prolactin-secreting cells (PRL cells) are generated from preexisting growth hormone-secreting cells (GH cells) in the initial cytogenesis of pituitary cells during the fetal period in mice (Behringer *et al.*, 1988; Borrelli *et al.*, 1989). We have recently demonstrated that PRL cells differentiate from GH cells through mammosomatotrophs (MS cells), which synthesize and secrete both GH and PRL in response to a combined treatment of epidermal growth factor (EGF), insulin and estradiol-17 β (E_2) in rat pituitary tumor GH3 cells (Kakeya *et al.*, 2000). In contrast, the proliferation of PRL cells is a well-known event in the rat and mouse pituitary gland (Takahashi, 1995). The highest mitotic activity of PRL cells is observed at estrus in the female rat pituitary (Takahashi *et al.*, 1984; Oishi *et al.*, 1993). As estrogen treatment stimulates the proliferation of PRL cells (Lloyd *et al.*, 1975;

Takahashi and Kawashima, 1987; Oomizu *et al.*, 2000), the high proliferating activity of PRL cells is caused by the increase in estrogen secretion during the proestrous and estrous days. Thus, it is evident that PRL cells are generated from preexisting PRL cells through mitosis under estrogenic stimulation (self-duplication) *in vivo*. The above findings indicate that there are two pathways for the cytogenesis of PRL cells: the transdifferentiation of GH cells into PRL cells, and the self-duplication of preexisting PRL cells. However, it remains unclear whether the former pathway operating in GH3 cells requires a mitotic process. The present study attempted to clarify the involvement of mitosis of pituitary cells in the cytogenesis of PRL cells in GH3 cells.

MATERIALS AND METHODS

Cell cultures

Pituitary cells

Two-month-old male rats of the Sprague-Dawley strain obtained from CLEA Japan (Osaka, Japan) were used. Anterior pituitary cells were dispersed by trypsin/DNase digestion following the method of Oomizu *et al.* (1998). Cell yield was estimated by

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counting the cells with a haemocytometer. The viability of cells was checked using a trypan blue exclusion test. The dispersed cells were seeded at a density of 3×10^5 cells/ml in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DME/F12, Sigma Chemical Co., St. Louis, MO) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL, Life Technologies, Inc., Rockville, MD,) on poly-L-lysine (Sigma Chemical Co.)-coated glass coverslips (diameter 13 mm, Matsunami Glass Ind., Osaka, Japan) in a 24-well plate (Becton Dickinson, Lincoln Park, NJ) for 3 days before the hormone treatment. After a 3-day culture, the pituitary cells were maintained in serum-free DME/F12 medium supplemented with hydrocortisone (100 μ g/l), 3,5,3'-triiodothyronine (400 ng/l), transferrin (10 mg/l), bovine glucagon (10 ng/l), EGF (10 ng/l), parathyroid hormone (200 ng/l), and sodium selenite (5 μ g/l). After the culture under the serum-free condition for 2 days, the pituitary cells were treated with a combination of EGF (10 nM), insulin (300 nM), and E_2 (1 nM) for 6 hr or 3 days. These concentrations were determined following the previous reports (Scammell *et al.*, 1986; Kakeya *et al.*, 2000). EGF, insulin and E_2 were obtained from Sigma Chemical Co. Experiments were performed under the Guideline of Animal Experimentation, Faculty of Science, Okayama University.

GH3 cells

GH3 cells, obtained from the Japan Health Sciences Foundation, Osaka, Japan, were maintained in DME/F12 medium supplemented with 5% (v/v) FBS at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. GH3 cells were cultured in a 24-well plate at a density of 5×10^4 cells/ml. EGF (10 nM), insulin (300 nM), E_2 (1 nM) or a combination of EGF, insulin and E_2 were added to the culture medium for 6 days or 9 days. Each group was consisted of three wells, and the medium was changed every 2 days. Independent experiments were performed three times.

Detection of DNA-synthesizing cells

DNA-synthesizing cells were identified by monitoring the uptake of 5-bromo-2'-deoxyuridine (BrdU; Amersham, Buckinghamshire, UK), a thymidine analogue, into the cell nucleus following the previous report (Kakeya *et al.*, 2000). BrdU solution from a cell proliferation kit (Amersham) was added into the culture medium (3 mg/ml) for 3 hr on day 6 or day 9 of the culture. The half of the pituitary cells and GH3 cells treated with BrdU on day 6 were cultured for another 3 days. All cells were rinsed with 0.01M phosphate-buffered saline (PBS; pH 7.4) and fixed in Bouin's solution for 2 hr at room temperature. The cells were dehydrated through a graded series of ethanol concentrations, left in xylene for 5 min, and then rehydrated. Nonspecific immunoreactivity was blocked by incubating the glass coverslips with 1% low-fat dried milk (w/v) in PBS for 30 min at room temperature. The cells were then incubated with monoclonal antibodies raised against BrdU (Amersham) for 1 hr at room temperature in a humidified box. The cells were washed three times with PBS. They were then incubated with peroxidase-conjugated anti-mouse immunoglobulin G (IgG) as a second antibody for 30 min at room temperature. BrdU immunoreactivity was detected using 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co.) following the method of Hsu and Soban (1982). After the BrdU detection, the coverslips were incubated in 0.3% H₂O₂-methanol to inactivate the peroxidase of the second antibodies (peroxidase-conjugated anti-mouse IgG) for the following immunostaining.

Dual-detection of GH and PRL

Dual-detection of GH and PRL in rat pituitary cells was performed with two primary antisera generated from animals of different species. The pituitary cells were first incubated in antiserum against rat PRL (1:4000, NIDDK-anti-rPRL-IC-5, NIH, Bethesda, MD) in a humidified box for 1 hr at room temperature. Detection of PRL was carried out using Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, CA) and DAB as a chromogen. Subsequent

detection of GH was carried out using monkey anti-GH antiserum (1:500, NIDDK-anti-rGH-IC-1, NIH) and tetramethylrhodamine isothiocyanate (TRITC)-labeled goat anti-monkey immunoglobulin (IgG) (E-Y Laboratories, San Mateo, CA).

Dual immunofluorescent detection of GH and PRL in GH3 cells was performed two primary antisera used for the rat pituitary cells. The cells were first incubated with monkey antiserum against rat GH (1:500, NIDDK-anti-rGH-IC-1) in a humidified box for 1 hr at room temperature, rinsed with PBS three times, and then incubated in rabbit antiserum against rat PRL (1:1000, NIDDK-anti-rPRL-IC-5). The immunostained coverslips were then washed three times with PBS, incubated in TRITC-labeled goat anti-monkey IgG and then fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG

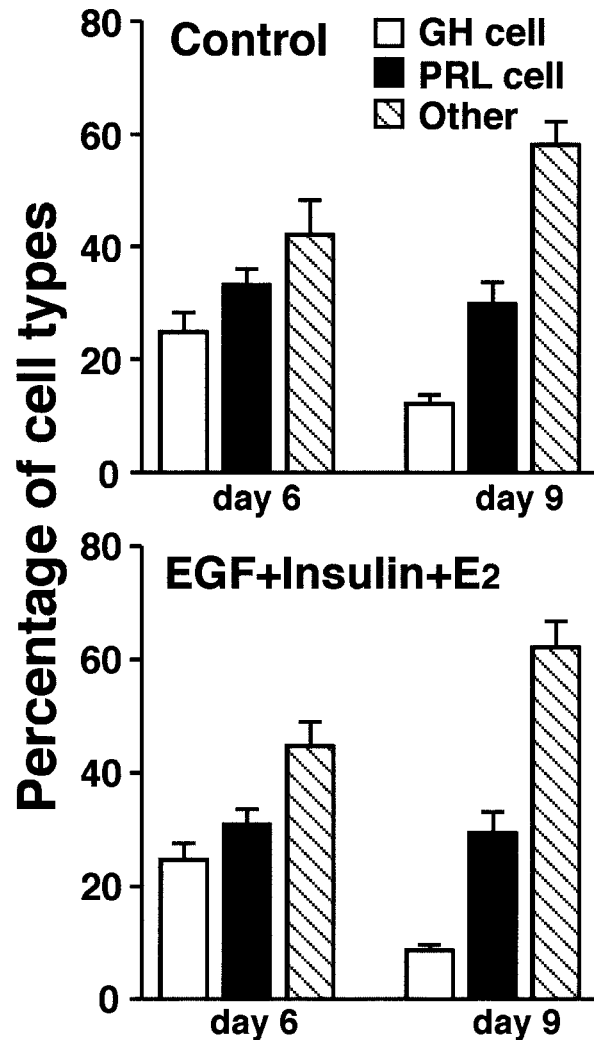


Fig. 1. Percentages of GH, PRL, and other cells (Other) that secrete neither GH nor PRL in adult male rat pituitary cells. Pituitary cells were cultured in the control medium or in medium supplemented with a combination of EGF, insulin and E_2 for 1 day (day 6) or 3 days (day 9). MS cells were not detected under the present condition. The proportions of GH, PRL, and other cells differed between the control and hormone-treated groups on day 9 ($p < 0.01$). In both control and hormone-treated groups, the proportions of GH cells, PRL cells and other cells differed between day 6 and day 9 (control, $p < 0.05$; the hormone-treated, $p < 0.01$). Data are expressed as the mean \pm standard errors of the means (SEM) of three independent experiments.

(E-Y Laboratories) in a humidified box for 30 min at room temperature.

Immunofluorescence of each hormone and light field images of BrdU-labeled cells were observed with a laser scanning confocal fluorescent microscope (MRC-600, Bio-Rad Laboratories, Richmond, CA). The fields were scanned across the coverslip to avoid overlap. Each group consisted of three coverslips, and more than 500 cells were observed from each coverslip. The percentage of BrdU-labeled hormone-synthesizing cells was determined by counting the number of those cells against the total cells observed.

The specificity of the antisera for immunocytochemistry was evaluated as follows: (1) incubation of normal rabbit serum instead of each antiserum; (2) preabsorption of each antiserum with corresponding purified antigens (NIDDK rGH I-5 or rPRL I-5, NIH, 50 µg/ml diluted antiserum) 24 hr before the immunostaining. No immun-

ostaining was observed in these controls.

Statistics

Statistical significance of the difference between the means was assessed with one-way analysis of variance (ANOVA). Three independent experiments were performed in each study. The relative number of GH, MS, and PRL cells was analyzed by the method of $R \times C$ test of independence using G-test (Sokal and Rohlf, 1981). Values of the mean and standard errors were calculated from the three independent experiments.

RESULTS

Percentages of GH, PRL, MS and the other cells

The cultured pituitary cell population under the serum-free condition was heterogeneous, consisting of various types of hormone-secreting cells, with GH and PRL cells accounting for approximately one-third of the total pituitary cells (Fig. 1). Treatment with a combination of EGF, insulin, and E_2 on pituitary cells induced a slight change in the proportion of GH, PRL, and other cells that secrete neither GH

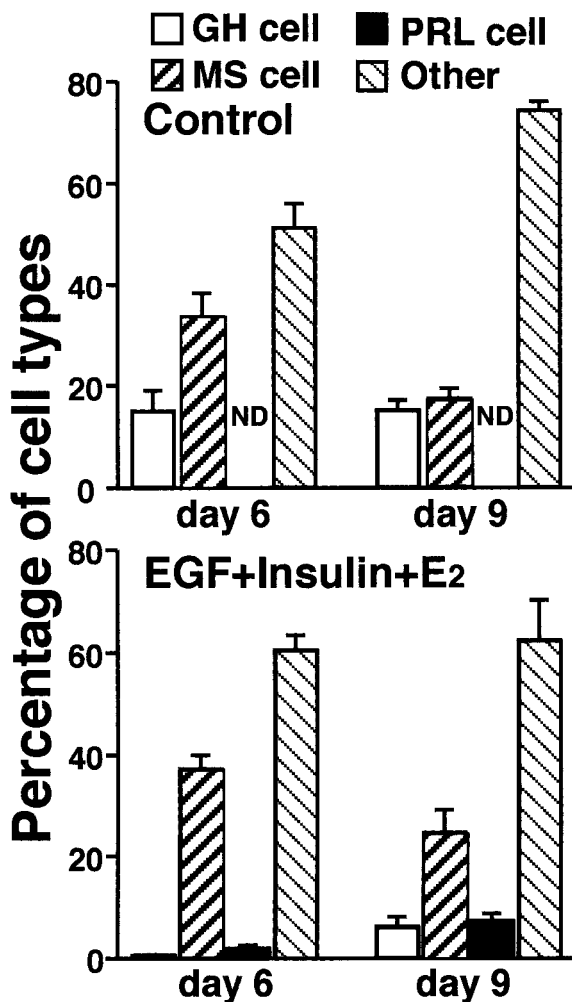


Fig. 2. Percentages of GH, MS, and PRL cells in GH3 cells. GH3 cells were cultured in the control medium or in medium supplemented with a combination of EGF, insulin and E_2 for 6 days or 9 days. The hormone treatment induced the appearance of PRL cells. The proportions of GH, MS, PRL, and other cells differed between the control and hormone-treated groups on day 6 ($p < 0.001$) and day 9 ($p < 0.01$), respectively. In both the control and hormone-treated groups, the proportions of GH, PRL, and other cells differed between day 6 and day 9 ($p < 0.05$, in both groups). Data are expressed as the mean \pm SEM of three independent experiments. ND: not detected.

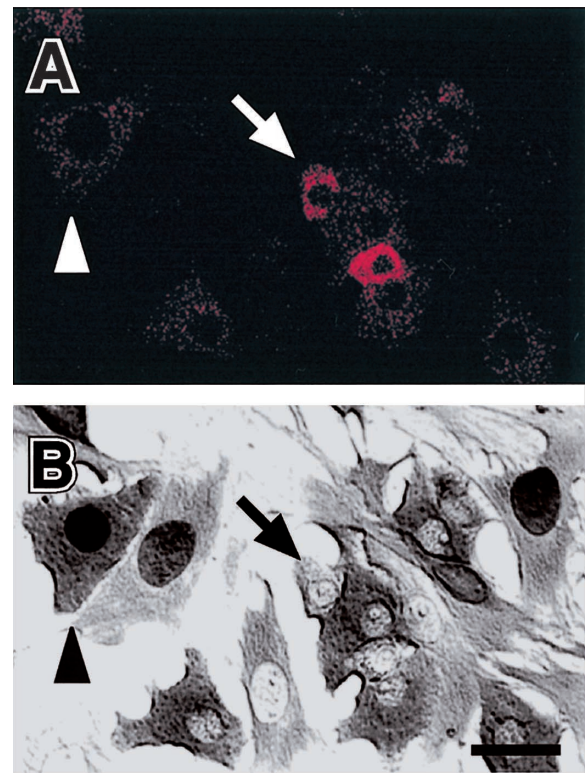


Fig. 3. Typical view of triple-immunostaining for BrdU, PRL and GH in cultured rat pituitary cells. Pituitary cells were cultured in medium supplemented with a combination of EGF, insulin and E_2 for 3 days. Pituitary cells were stained with anti-rat GH antiserum by fluorescence immunocytochemistry (A), and with anti-rat PRL antiserum and anti-BrdU antiserum by conventional immunocytochemistry (B). A and B are the same field of a culture plate. Immunoreactive BrdU was detected in a nucleus, and immunoreactive GH and PRL were detected in the cytoplasm. An arrow indicates GH cell and an arrowhead indicates BrdU-labeled PRL cell. MS cells in pituitary cells were not detected in the present study. Bar=20 µm

nor PRL relative to the control on day 9 (Fig. 1, $p < 0.01$). During a 3-day culture, the proportions of GH, PRL, and other cells varied in the control and hormone-treatment groups, regardless of treatment (control, $p < 0.05$; the hormone-treated, $p < 0.01$), and the percentages of GH cells declined in both groups.

GH3 cells consist of GH and MS cells, which secrete both GH and PRL, but PRL cells were not detected under the control culture condition (5% FBS supplemented). Treatment with a combination of EGF, insulin, and E_2 stimulated the appearance of PRL cells in GH3 cells and altered the proportion of GH, MS, and PRL cells relative to the control on days 6 and 9 of culture (Fig. 2, $p < 0.001$ and $p < 0.01$,

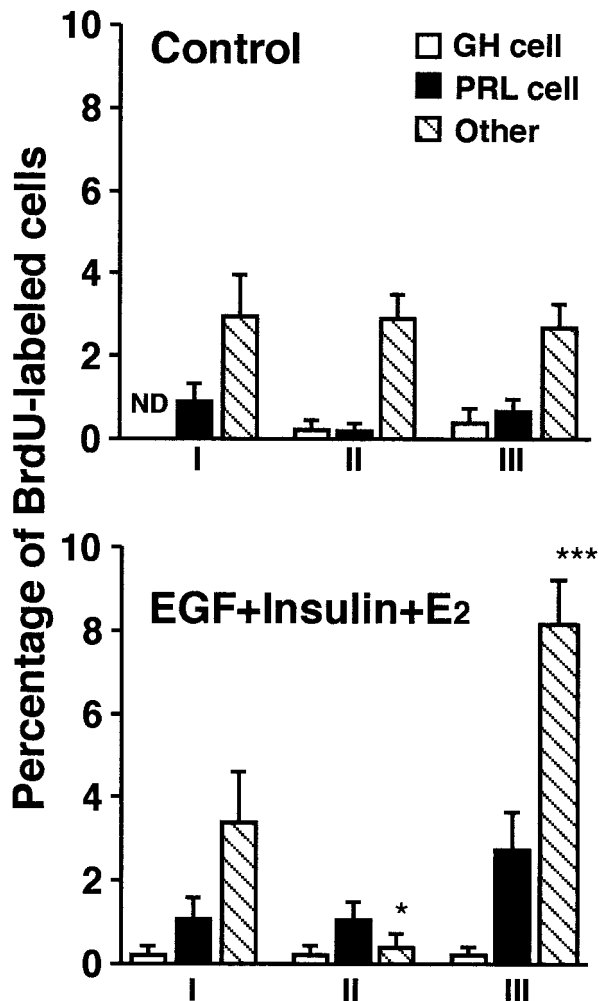


Fig. 4. Percentages of BrdU-labeled cells in pituitary cells. Pituitary cells were cultured in the control medium or in medium supplemented with a combination of EGF, insulin and E_2 on day 6 of the culture. Pulse labeling of BrdU for 3 hr was performed on day 6 or day 9 of the culture, and immediately after the labeling the pituitary cells were fixed, and then BrdU-labeled cells were immunocytochemically detected (Groups I and III). The other group of pituitary cells treated with BrdU on day 6 were cultured, and was analyzed for BrdU-labeling on day 9 (Group II). Data are expressed as the mean \pm SEM of three independent experiments. * $P < 0.05$, *** $P < 0.001$, compared with the control of the same group.

respectively). During a 3-day culture, the proportions of GH, MS, PRL, and other cells varied in the control and hormone-treatment groups ($p < 0.05$, in both groups).

DNA-replication in pituitary cells and GH3 cells

Pituitary cells

DNA-synthesizing pituitary cells, immunocytochemically identified using the BrdU-labeling method, were observed in

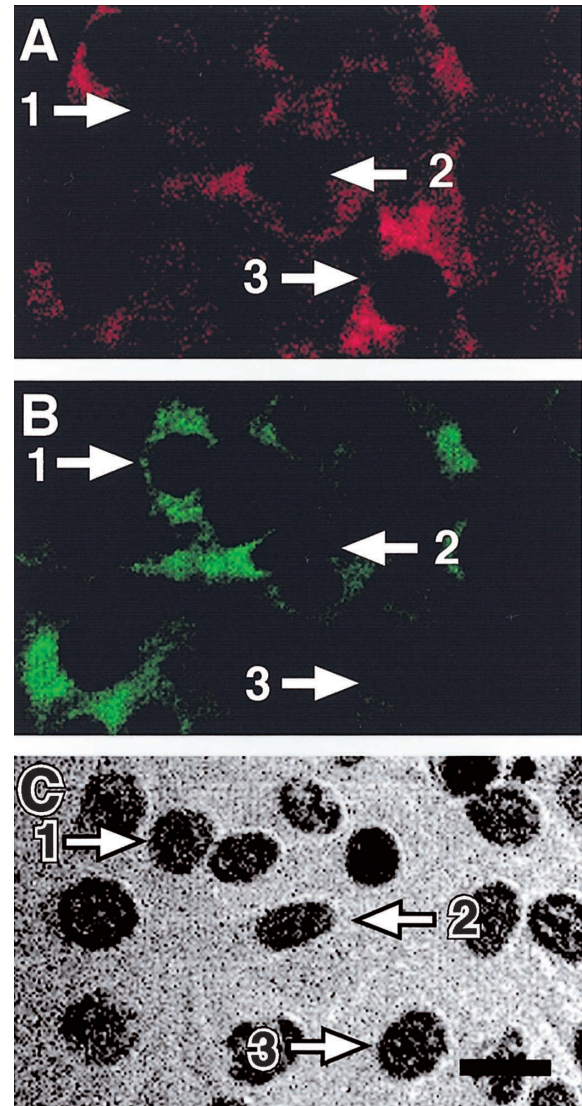


Fig. 5. Typical view of triple-immunostaining for BrdU, PRL and GH in GH3 cells. GH3 cells which were cultured in medium supplemented with a combination of EGF, insulin and E_2 , were treated with BrdU for 3 hr on day 6, and were allowed to grow for another 3 days. On day 9 of the culture, GH3 cells were fixed, and were stained with anti-rat GH antiserum (A), anti-rat PRL antiserum (B), and anti-BrdU antiserum (C). Three photographs are the same field of a culture plate. Photographs (A and B) are immunofluorescent images, and photograph (C) is a bright-field image taken by confocal microscopy. Immunoreactive BrdU was visualized by DAB coloration and detected in a nucleus. The cells designated by arrows (#1, #2 and #3) were labeled with BrdU (C). The #1 cell is a PRL cell, and the #3 cell is a GH cell. The #2 cell, which contained immunoreactive GH (A) and PRL (B) in the cytoplasm, is an MS cell. Bar=10 μ m

GH and PRL cells (Fig. 3), indicating that pituitary GH cells and PRL cells have the ability to proliferate under the culture conditions employed. Pituitary cells were treated with BrdU solution for 3 hr on day 6 or day 9 of the culture, and were examined immediately after the treatment. BrdU-labeled cells were immunocytochemically detected (Fig. 4, Group I and III). The other group of pituitary cells that had been treated with BrdU on day 6 was cultured until day 9 (Fig. 4, Group II). BrdU-labeled GH cells and PRL cells were detected regardless of the hormone treatment on days 6 and 9. The additional 3-day culture of GH3 cells after being treated with a combination of EGF, insulin, and E₂ did not affect the percentage of BrdU-labeled PRL cells.

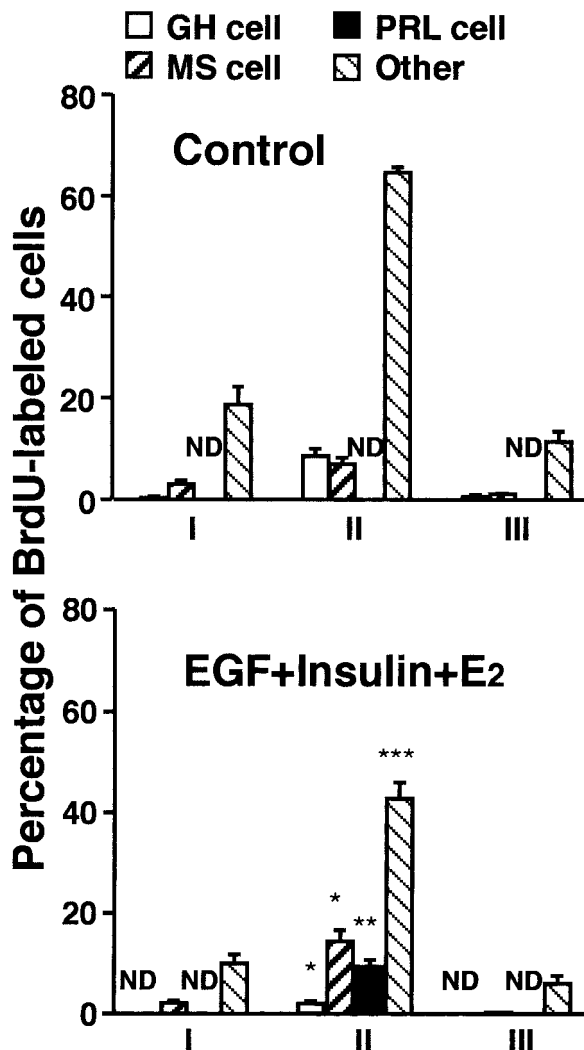


Fig. 6. Percentages of BrdU-labeled cells in GH3 cells. GH3 cells were cultured in the control medium or in medium supplemented with a combination of EGF, insulin and E₂. GH3 cells were treated with BrdU for 3 hr on day 6 or day 9 of the culture, and then immediately analyzed for BrdU-labeling (Groups I and III). The other group of GH3 cells that had been treated with BrdU on day 6 was analyzed for BrdU-labeling on day 9 (Group II). Data are expressed as the mean±SEM of three independent experiments. ND: not detected. * P<0.05, ** P<0.01, *** P<0.001; compared with the control of the same group.

GH3 cells

Pulse BrdU-labeling for 3 hr was performed as described in the pituitary cells. Fig. 5 shows typical views of BrdU-labeled PRL (#1 cell), GH (#3 cell), and MS cells (#2 cell) in GH3 cells treated with a combination of EGF, insulin, and E₂. Fig. 6 illustrates the percentage of BrdU-labeled GH3 cells. BrdU-labeled GH cells and MS cells were detected in both the control and hormone-treated groups, but BrdU-labeled PRL cells were not detected, when BrdU-labeling was performed on days 6 and 9 (Groups I and III). In contrast, when GH3 cells treated with BrdU on day 6 were cultured for another 3 days (Group II), BrdU-labeled PRL cells were detected, and the percentages of BrdU-labeled MS cells and PRL cells increased relative to those of GH3 cells in the control group (p<0.05, p<0.01, respectively), while those of BrdU-labeled GH cells decreased (P<0.05).

DISCUSSION

Transdifferentiation of GH cells into PRL cells had been previously suggested in several reports (Hoeffler *et al.*, 1985; Frawley and Boockfor, 1991; Takahashi, 1995). Initial differentiation of PRL cells from GH cells is a post-mitotic process in the immature mouse pituitary gland (Behringer *et al.*, 1988; Borrelli *et al.*, 1989). Similarly, we have suggested that the transdifferentiation of GH cells into PRL cells can be induced by treatment of GH3 cells with a combination of EGF, insulin, and E₂ (Takeya *et al.*, 2000). However, it remains unclear whether this appearance of PRL cells is a postmitotic event, as reported in the case of mouse pituitary PRL cells. Therefore, we attempted to clarify the involvement of cell division in the process of PRL cell differentiation observed in GH3 cells. BrdU-labeling was performed to study the DNA-synthesizing cells. The result of the present study demonstrate that PRL cells of GH3 cells cannot be immediately labeled with BrdU, but that BrdU-labeled PRL cells will appear with an additional 3 day-culture. This finding indicates that PRL cells of GH3 cells lose their ability to synthesize DNA and likely their ability to undergo mitosis. Goda *et al.* (1998) have reported that in the pituitary tumor MtT/S cell line, PRL cells fail to replicate their DNA, which is in agreement with the present study. Despite the inability of the PRL cells to replicate their DNA, BrdU-labeled PRL cells were detected after the additional culture, suggesting that BrdU-labeled GH3 cells other than the PRL cells were transdifferentiated into PRL-only secretors in response to the treatment with a combination of EGF, insulin, and E₂.

GH3 cell-populations usually consist of GH and MS cells under the culture conditions employed with the growth of GH3 cells being stimulated (in the medium containing 5% FBS); in contrast, EGF treatment with insulin and/or E₂ stimulates the appearance of PRL cells (Takeya *et al.*, 2000). The appearance of BrdU-labeled PRL cells after the additional 3-day incubation in the present study indicates that PRL cells were generated from preexisting GH cells and/or MS cells that had been labeled with BrdU. Therefore, it is

highly probable that the preexisting GH or MS cells directly transdifferentiate into PRL cells without cell division. MS cells are thought to be transitional cells in the process of cytogenesis of PRL cells from GH cells.

Roh *et al.* (2001) have used a transgenic mouse system targeting the expression of a dominant negative EGF receptor to find that EGF receptor signaling is necessary for the differentiation and/or maintenance of GH and PRL cells in the early cytogenesis of both types of pituitary cells. EGF treatment stimulates PRL gene-transcription and, in contrast, decreases GH gene-transcription in GH3 cells (Kakeya *et al.*, 2000). These findings substantiate the notion that EGF together with insulin and/or E₂ is involved in the regulation of PRL cell development. GH gene expression in MS cells may be terminated and PRL gene expression may be started by EGF, and finally, PRL cells (PRL only-secreters) will be generated by treatment with a combination of EGF, insulin, and E₂.

It is well known that PRL cells have the ability to replicate DNA and to divide in rat and mouse pituitary glands *in vivo* and *in vitro* in response to estrogen and various growth factors (Takahashi *et al.*, 1984; Oishi *et al.*, 1993; Oomizu *et al.*, 2000). In contrast, it is evident from the present study that PRL cells of GH3 cells are unable to replicate DNA molecules. The reason for this discrepancy remains unclear. It is possible, however, that there is a difference in the heterogeneity of cell populations between GH3 and normal pituitary cells, although the major difference between these two cells resides in the cell-immortality. GH3 cells consist of homogeneous cells and are probably derived from a single cell type. In contrast, normal pituitary cells consist of various cell types. Several reports have indicated that an intrapituitary control system, particularly cell-to-cell interactions, is important to the regulation of pituitary function (Schwartz and Cherny, 1992; Denef, 1994; Renner *et al.*, 1996; Vankelecom and Denef, 1997; Schwartz, 2000). Growth factors, which are synthesized in pituitary cells, exert a regulatory function on pituitary cells in a paracrine manner. Therefore, cell division in PRL cells may require various paracrine factors produced in pituitary cells other than PRL cells. Our recent study demonstrated that TGF- α is necessary for estrogen-induced proliferation of pituitary cells (Oomizu *et al.*, 2000). Another interesting finding concerning paracrine factors has been reported by Hentges *et al.* (2000). They found that basic fibroblast growth factor, produced in folliculo-stellate cells, mediates TGF- β -induced proliferation of PRL cells. From these findings, it is probable that some factors necessary to DNA-replication and proliferation of PRL cells are deficient in GH3 cells, resulting in the inability of PRL cells to self-duplicate.

Dopamine is well known to inhibit PRL synthesis and release in PRL cells of the normal pituitary glands through its receptors (Caron *et al.*, 1978). GH3 cells do not express dopamine receptors (Cronin *et al.*, 1980). Hence, dopamine fails to inhibit PRL synthesis and release in GH3 cells maintained under normal culture conditions. EGF treatment

induces the expression of dopamine receptors in GH3 cells (Missalle *et al.*, 1990), suggesting that dopamine-inhibition of PRL secretion begins to operate. Those previous studies together with the present results indicate that EGF plays a number of roles in the maturation process of PRL cells such as in the stimulation of PRL synthesis, formation of secretory granules (Scammell *et al.*, 1986), and induction of dopamine receptors.

Mouihate and Lestage (1995) have reported that estrogen stimulates EGF synthesis in the rat pituitary. EGF and its receptors are expressed in the mouse pituitary (Honda *et al.*, 2000). EGF released from pituitary cells may act on pituitary cells themselves by a paracrine or autocrine mechanism. Zhang *et al.* (1993) have found that both EGF and E₂ stimulate PRL mRNA synthesis and PRL secretion. Judging from these previous reports, it seems possible that estrogen action and EGF action are closely related to the development of MS and PRL cells. As such, the regulatory mechanism concerning EGF production in GH3 cells needs to be studied.

We have recently found in mouse pituitary cells that insulin or IGF-I stimulates the proliferation of PRL cells *in vitro*, and IGF-I action that is more potent than insulin action (Oomizu *et al.*, 1998). We also demonstrated by *in situ* hybridization and immunocytochemistry that IGF-I and IGF-I receptors are expressed in mouse GH cells, indicating that pituitary IGF-I acts on GH cells in a paracrine or autocrine manner (Honda *et al.*, 1998). Furthermore, GH3 cells express IGF-I protein (Fagin *et al.*, 1987) and IGF-I receptors (Honda *et al.*, 1998). From these findings, it can be assumed that the insulin action shown in the present study may be mediated through IGF-I receptors located on GH3 cells, as insulin at high concentration is known to act on IGF-I receptors (Houslay and Wakelam, 1988).

In conclusion, GH3 cells consist primarily of GH and MS cells in a 5% FBS-supplemented condition. EGF, insulin, and E₂ are required for the development of PRL cells. MS cells of GH3 cells are thought to be transitional cells in the process of the transdifferentiation of GH cells into PRL cells. In normal pituitary glands PRL cells proliferate and increase in number in response to estrogen (Takahashi *et al.*, 1984). Thus, there may be at least two pathways for the growth of PRL cells; transdifferentiation of GH cells into PRL cells, and cell division of preexisting PRL cells.

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