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Glucocorticoid-Inducible Glutamine Synthetase in GH Cells of Chick Embryos, *Gallus domesticus*: Ontogeny of Glutamine Synthetase, GH, and Pit-1 Protein in the Pituitary Gland

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It has been known that glutamine synthetase (GS, L-glutamate ammonia ligase) is an enzyme that catalyzes ATP-dependent condensation of glutamate and ammonia to form glutamine, and that GS and growth hormone (GH) are proteins that are induced by glucocorticoid-treatment during the development of animals. To understand the relationship between these glucocorticoid-inducible proteins, we studied pituitary GS and GH cells during the development of chick embryos, *Gallus domesticus*. GS cells were immunohistochemically identified in epithelia of Rathke's pouch as early as embryonic day 4 (E4) following corticosterone-treatment. The population of GS cells and GS activity gradually increased by E14, and then rose sharply after E16. Pituitary GS activity was precociously induced by corticosterone-treatment starting around E7. GH cells were first demonstrable in the pituitary gland on E11, and then increased until hatching. Corticosterone-treatment also caused premature induction of GH in the pituitary cells after E8, but not before. Dual-fluorescence immunohistochemistry showed a large population of GS cells in the cephalic lobe that was identical to the ACTH cells, and in the caudal lobe of the gland they were the same as the GH cells. Ontogeny of pituitary Pit-1 protein, an important transcription factor of GH, was also studied by immunohistochemistry. Pit-1 positive cells appeared at E6 and increased in number by E12 of development. The population of Pit-1 cells was, however, not affected by corticosterone-treatment. These result demonstrate that glucocorticoid receptors are present in pituitary GS cells even before day 8 when GH cells start to differentiate, and that a large population of GS cells may have some physiological roles in the gland. This is the first report showing that pituitary GS is a glucocorticoid-inducible marker protein to regulate pituitary functions.

Key words : corticosterone, glutamine synthetase, growth hormone, pit-1, pituitary gland

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Introduction

The presence of glutamine synthetase (GS, L-glutamate ammonia ligase, EC 6.3.1.2) in many diverse cell types suggests an important role for this amine. In mammals, high concentrations of GS are found in glial cells in the brain (D'Amelio *et al.,* 1990), in liver hepatocytes (Bennett *et al.,* 1987), pigment and Müller cells in the retina (Riepe and Norenburg, 1977), folliculo-stellate cells in the pituitary (Shirasawa and Yamanouchi, 1999), and the cytotrophoblast and mesenchymal layers of pla-

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cental villi (DeMarco *et al.,* 1997). It has been known that GS is precociously induced by glucocorticoid-treatment (Piddington and Moscona, 1967 ; Moscona and Moscona, 1979). However there is little information on GS in the pituitary gland during embryogenesis in the chicken.

Although there is a controversy on the development of GH cells during chick embryos, it is widely accepted that GH cells first appear between E11 and E12 and become a significant population by E16 (Józsa *et al.,* 1979 ; Malamed *et al.,* 1993 ; Hull *et al.,* 2000). A few reports showed an increased response to growth hormone (GH)-releasing hormone by E16 (Porter *et al.,* 1995 a ; Piper and Porter, 1997 ; Dean and Porter, 1999), and full differentiation of functional chicken GH cells gradually becomes evident from E16 through at least E20 of the 21-day incubation period (Dean *et al.,* 1997). GH cell differentiation in the pituitary was also induced *in ovo* by glucocorticoids (Bossis and Porter, 2000 ; Porter and Dean, 2001 ; Bossis and Porter, 2003 ; Fu *et al.,* 2004 ; Bossis *et al.,* 2004), and serum corticosterone levels corresponding to that seen on E16 were thought to be responsible for GH cell-differentiating activity *in vitro* (Morpugo *et al.,* 1997 ; Dean and Porter, 1999 ; Porter *et al.,* 2001). A role for the *in vitro* induction of GH cell differentiation by corticosterone is also supported by reports that an increase in circulating steroid levels occurs before GH cell differentiation on E16 (Tanabe *et al.,* 1983, 1986).

In recent years, a number of the studies have reported on pituitary-specific transcription factors involved in the development of the pituitary primordium, Rathke's pouch, and the proliferation and terminal differentiation of the five distinct adenohypophyseal cell types. These factors are highly expressed in the region that gives rise to the pituitary gland and include *Hesx1, Six3, Lhx3, Lhx4, Pitx1, Pit-1,* and *Prop-1* in mammals (Burrows *et al.,* 1999). The best characterized of the pituitary transcription factors is the POU homeodomain protein Pit-1/GHF-1 (Rhodes and Rosenfeld, 1996). Recently it was established that all cell types of the anterior pituitary contain Pit-1 mRNA transcripts, and that in rodents, protein expression is limited to thyrotrophs, somatotrophs, and lactotrophs (Ingraham *et al.,* 1988 ; Bodner *et al.,* 1988). The homeodomain protein Pit-1 is required not only for the establishment and maintenance of the differentiated phenotype but for cell proliferation as well (Castrillo *et al.,* 1991). Moreover, not only does Pit-1 bind to the promoter region of the GH and prolactin genes, it also autoregulates its own expression (Mangalam *et al.,* 1989). During ontogeny, Pit-1 transcripts are also observed in the chicken pituitary by RT-PCR (Van *et al.,* 2004 ; Nakamura *et al.,* 2004) or by immunohistochemistry (Chowdhury and Yoshimura, 2003 ; Fu and Porter, 2004). Pit-1 protein was detected in the glandular cells in the chicken pituitary (Chowdhury and Yoshimura, 2003), and was affected by glucocorticoid-treatement (Fu and Porter, 2004). In the present study we observed the relationships between Pit-1 cells and GS cells during the development of chicken embryos.

The purpose of the present study was to investigate the Pit-1 progenitors cells and the regulation of glucocorticoid-inducible somatotroph and GS cell differentiation during embryonic development using the chick embryo as a model system. The chicken embryo is a useful model for studying the influence of extracellular factors on pituitary cell differentiation due to its isolation from maternal influences and the relative ease with which the endocrine environment of individual embryos can be manipulated. The objective of the present study was to evaluate the effects of corticosterone and GH cell differentiation from their progenitor cells during early- to mid-embryogenesis *in ovo.*

Materials and Methods

Reagents

Corticosterone, Paraplast embedding media, poly-L-lysine and Nonidet P-40 were obtained from Sigma Chem. Co. (St. Louis, MO, U.S.A.), while the others reagents were acquired from Wako Pure Chemicals (Osaka, Japan). Corticosterone was dissolved as a 100 μg/ml stock in olive oil, and stored at 4℃ until injection into the yolk sac.

Animals and Tissue Preparation

Fertilized eggs of the chicken, *Gallus domesticus,* were purchased from the Kisen Hatchery (Wakayama, Japan), and chick embryos were used at all stages of development between day 3 and day 20 of incubation. Each was maintained at a temperature of 38℃ in a humidified incubator. The age of the embryos at the time of sacrifice was determined by

their incubation time, and their stages were checked by the criteria reported by Hamburger and Hamilton (1951). After making a pinhole on the shell, corticosterone in olive oil, or the vehicle was injected through the shell, and then a pinhole was sealed by grew. One hundred μl of the stock solution of corticosterone was injected through a pinhole into the yolk sac at a dose of 10μg/egg, and embryos were killed at 16h after the single injection of the steroid hormone. The dose and the incubation period could fully develop GS activities in the brain, neural retina, and liver (data not shown here). For immunohistochemical staining, the pituitary gland and tissues containing their primordia were fixed overnight at 4℃ in Bouin's solution without acetic acid. The tissues were then dehydrated in a graded ethanol series, and embedded in Paraplast embedding media. For measuring GS activity, the pituitaries were separated from the neural lobes under a stereoscopic microscope, quickly frozen in liquid nitrogen, and stored at −80℃ until GS activity was assayed. This study was carried out in accordance with Guide for Animal Experimentation, Yamagata University School of Medicine and Japanese Governmental Law (No. 105).

RT-PCR

Total RNAs were prepared by ISOGEN (Nippon Gene, Tokyo, Japan) from pituitary glands of 6- to 18-day-old embryos. Expression of mRNAs encoding receptors for glucocorticoid (GR) and estrogen (ERα), and β-actin as controls was determined by reverse transcriptase (RT)-polymerase chain reaction (PCR). Total RNA (1μg) was converted into cDNA by reverse transcription using poly $(dN)_6$ and poly $(dT)_{12-18}$ primers (Amersham Pharmacia Biotech, Buckinghamshire, UK) and Moloney murine leukemia virus reverse transcriptase (Life Technology Inc., Rockville, MD, U.S.A.) in total reaction volume of 50μl. The primer set of β-actin (forward : 5′-CACCATTGGCAATGAGAGGT-3′, reverse : 5′-GAAGCATTTGCGGTGGACAA-3′), GR (5′-GAGCTTGGAAAAGCCATCGTGA-3′, 5′-CCCAGAAATGTTGGCAGAAATCATCACG-3′), and ERα (5′-ACTGGACAGGAATCAAGGGA-3′, 5′-AGGTCGTAGAGCGGAACTAC-3′) were made using the sequences based on the nucleotide sequences in the chicken, and their product size were 382, 189 and 380, respectively. The amplification protocol consisted o 25 cycles of denaturation for 30 sec at 95℃, annealing for 30 sec at 55℃ and extension for 1 min a 72℃. The PCR products were electrophoresed on 2% agarose gel staining with ethidium bromide and visualized by ultraviolet illumination.

Immunohistochemistry

Serial sagittal sections at 4μm thickness were prepared using a microtome (Ultrotome V, LKB, Sweden), and mounted onto poly-L-lysine-coated slide glasses. The sections were immunocytochemically stained by the peroxidase- or alkaline phosphatase-labeled antibody method or by the avidin-biotin-peroxidase complex method (Hsu *et al.,* 1981), which was slightly modified by Shirasawa and Yoshimura (1982). Two double-staining methods were utilized to detect two different antigens in the sections. One detected GS, the pituitary hormones and Pit-1 protein. In this procedure, sections were reacted with mouse monoclonal antibody to GS and with rabbit polyclonal antibody to a pituitary hormone or Pit-1 protein, and then with Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR, U.S.A.). The sections were then observed under an UV-microscopy. The second method, designed to detect Pit-1 protein and pituitary hormones, used sections immunostained with the rabbit polyclonal antibody to chicken Pit-1 protein via the peroxidase-labeled antibody method. They were then colored dark blue using diaminobenzidine as a substrate in the presence of Ni^{2+}. After immersing the sections in an HCl solution to eliminate the first antibody, they were reacted with an antibody to one of the pituitary hormones, and then with an alkaline phosphatase-labeled antibody to rabbit IgG. Following this, each was colored using a Vector red substrate kit I (Vector Lab. Inc., Burlingame, CA, U.S.A.), and observed under a microscope. The following antibodies were employed for immunocytochemical procedures : mouse monoclonal antibody to ovine GS (at a concentration of 0.5μg/m*l*, Transduction Lab, Lexington, KY, U.S.A.), and rabbit polyclonal antibody to chicken LH, porcine $ACTH^{1-10}$, rat TSHβ, rat GH, or rat prolactin. The specificity of these antisera was reported elsewhere (Shirasawa *et al.,* 1996 ; Shirasawa and Yamanouchi, 1999). In the present study we employed two different antibodies to Pit-1 protein. One was an antibody to rat Pit-1 protein, a

gift from Dr. Rosenfeld M.D. (Howard Hughes Institute, La Jolla, CA, U.S.A.) and the other to chicken Pit-1. The latter was prepared as follows. The antigen was the Pit-1 peptide of C-terminal of chicken Pit-1 protein (amino acid position 273–292 ; KTSLHQNAFSSIIKEHHECR) conjugated to key-hole limpet hemocyanin (KLH) as the KLH-peptide (Sawady Liftech. Inc., Tokyo, Japan). Male Japanese white rabbits (Clea Japan, Tokyo, Japan) were injected subcutaneously with the KLH-peptide in emulsified Freund's complete adjuvant (Difco Lab., Detroit, MI, U.S.A.) and boosted 5 times every other week. A week after the last immunization, a blood sample was obtained and the antisera to chicken Pit-1 peptide separated by centrifugation. To detect Pit-1 protein in the sections, the tissue was place into a microwave oven to retrieve the antigen following a slight modification of the procedure reported by Vidal *et al.* (1998). Paraplast sections mounted onto poly-L-lysine-coated slide glasses were de-paraffinized in xylene, and then placed in 100% ethanol. After drying in air for 20 min at room temperature, they were first placed in a beaker containing boiling 10 mM citric acid solution, pH 6.0 for 5 min and then into a beaker containing ice water for over 20 min. Following this, they were washed twice with distilled water and PBS, and then process for immunostaining.

GS Assay

Frozen tissues were quickly thawed and homogenized in 10 mM sodium phosphate buffer, pH 7.2, containing 0.1% Nonidet P-40. After centrifuging at 10,000*g* for 15 min at 4℃, GS activity in the supernatants was measured by a spectrophotometer using the glutamine-γ-glutamyltransfer assay of Miller *et al.* (1978) with minor modifications as described elsewhere (Shirasawa and Yamanouchi, 1999). The assay mixture consisted of 100 μl of the supernatant, and final reagent concentrations of 100 mM L-glutamine, 50 mM imidazole-HCl (pH 6.8), 0.5 mM manganase chloride, 50 mM hydroxylamine-HCl, 25 mM potassium arsenate, and 0.2 mM disodium ADP. The reaction was initiated with 50 μl supernatant at 37℃ for 15–60 min and terminated by the addition of 100 μl of 0.37M $FeCl_3$ solution containing 0.3M trichloroacetic acid and 0.6M HCl. The precipitate was removed by centrifugation at 10,000*g* for 15 min, and γ-glutamyl hydroxamate was determined by comparing the absorbance at 505 nm to blanks including all reagents except ADP and potassium arsenate. One unit of GS activity was defined as the amount of activity catalyzing the formation of 1 μmole of γ-glutamyl hydroxamate in 15 min under assay conditions.

Cell Area and Statistical Analysis

The populations of immunostained GS, GH and Pit-1 cells were determined. Each group consisted of 5 embryos, and over 10 pictures of immunostained sections in each embryo were taken at a magnification of 50X by a 3CCD-camera (HV-C20 S, Nikon, Tokyo, Japan) equipped with a light microscope, and stored in a Macintosh computer. Because GH and GS cells have a tendency to form clusters in any given field, it became difficult to measure the exact number of immunostained cells by the system. To overcome this problem, the sum of the immunostained areas was employed to express the population of immunoreactive GH and GS cells. The pictures were analyzed by measuring the sum of the immunostained GH or GS cell areas, and by counting the number of immunostained nuclei of Pit-1 cell in a defined area (3×10^5 pixel^2 equivalents to 0.224 mm^2) using NIH Image software. The GH or GS cell population was then expressed in terms of mm^2/mm^2, and the Pit-1 cell population was expressed in terms of cells/mm^2. The data were analyzed using Bonferroni's procedure as a multiple comparison procedure, and p values were set at $<$ 0.05.

Results

Ontogenesis GS Cells and Effects of Corticosterone

During development of the chicken embryos, immunoreactive GS cells were not observed in the epithelia of Rathke's pouch and the brain wall at E 4 ; however, corticosterone-treatment caused induction of GS in both of these regions (Fig. 1 a). These cells increased in number with advancing embryonic age so by E7, they were mainly distributed in the cephalic lobe of the pituitary gland (Fig. 1 b). The GS immunoreaction products were present in the cell cytoplasm (Fig. 1 c). At E7, intensely stained GS cells were observed in the brain wall ; the pituitary stalk connected to the oral epithelium was only slightly stained with the GS antibody (Fig. 1 d). GS cells were rarely present in the pituitary gland at E10 (Fig. 1 e), while corticosterone-treatment induced many such cells to differentiate in the cephalic lobe

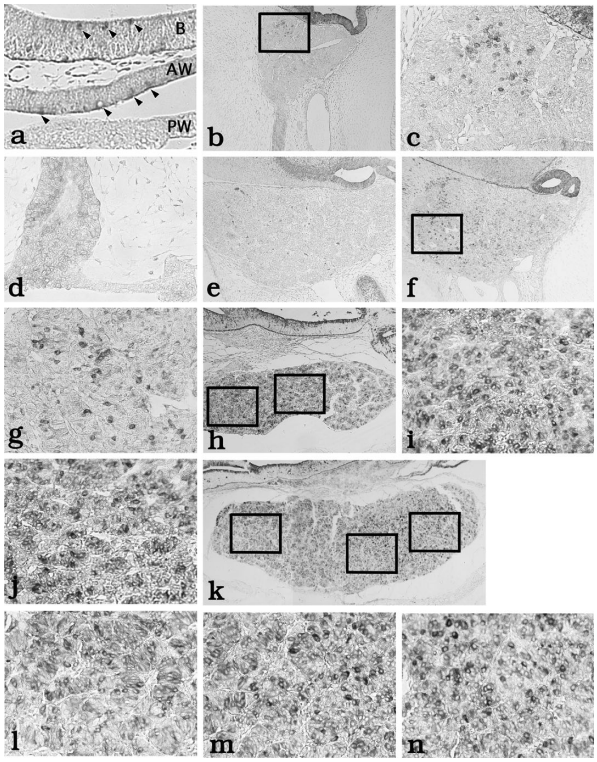

Fig. 1. **Immunoreactive GS cells in the pituitary glands.** Following corticosterone-treatment, GS cells are observed in the brain (B) and the anterior wall (AW), but not in the posterior wall (PW), of Rathke's pouch at E4 (a, arrowheads), and in the cephalic lobe of the pituitary gland at E7 (b). Immunoreactive GS is distributed in the cytoplasm of the pituitary cells (c ; enlargement of the area in b) and also in cells in the stalk of the gland at E7 (d). GS cells are rarely present at E10 (e) ; however, corticosterone induces GS cells in the cephalic lobe of the gland at E10 (f). Immunoractive GS is observed in the cytoplasm of oval, spherical and polygonal shaped cells (g ; enlargement of the area in f). At E 14, corticosterone induced many GS cells throughout the gland (h). The population of the GS cells in the cephalic lobe (i ; enlargement of the right-hand area in h) and the central part (j ; an enlargement of the left-hand area in h) is nearly the same. GS cells are observed in every part of the gland at E18 (k), and their shapes are polygonal and spherical at the cephalic portion (l), spherical and oval in the central region (m), and oval in the caudal part (n). X160 (a), X80 (a, c, d, g, I, j, l, m, n), X40 (b, e, f, h, k)

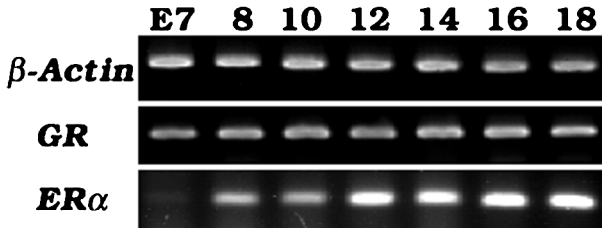

Fig. 2. **Expression o mRNAs encoding steroid receptors in the pituitary gland of chicken embryos.** During the development of embryos, pituitary gland showed a strong expression of glucocorticoid receptor (GR), and a weak expression of estrogen receptor alpha (ERα) at E7 in comparing to the expression of β-actin. The expression of GR and ERα continued by E18.

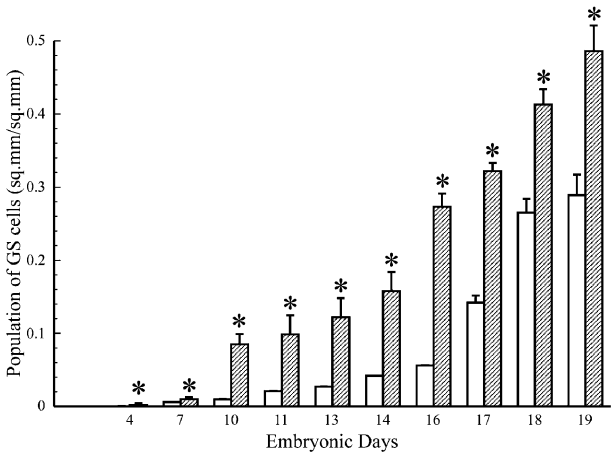

Fig. 3. **GS cell population during development.** The population of GS cells in the controls (open columns) is significantly lower ($p < 0.01$) than that in corticosterone-treated (stripped bars) embryos at all stages. Bars indicate the standard error, and asterisks mean the p values less than 0.001.

at the same embryonic stage (Fig. 1 f). Immunoractive GS was noted in the cytoplasm of oval, spherical and polygonal-shaped cells (Fig. 1 g). At E14, few GS cells were present in the gland, while corticosterone induced many to develop throughout the organ (Fig. 1 h). The population of the GS cells in the cephalic (Fig. 1 i) and central parts (Fig. 1) was nearly the same. GS cells were observed in every part of the gland at E18 (Fig. 1 k). They were polygonal and spherical in the cephalic part (Fig. 1 l), spherical and oval in the central part (Fig. 1 m), and oval in the caudal region (Fig. 1 n).

As shown in Fig. 2, the expression of mRNA encoding GR was detected in the pituitary gland of E7, and was continued by hatching. The expression of ERα mRNA started slightly at E7 and increased then after. This indicates that pituitary cells already have GR and have an ability to react to the exoge-

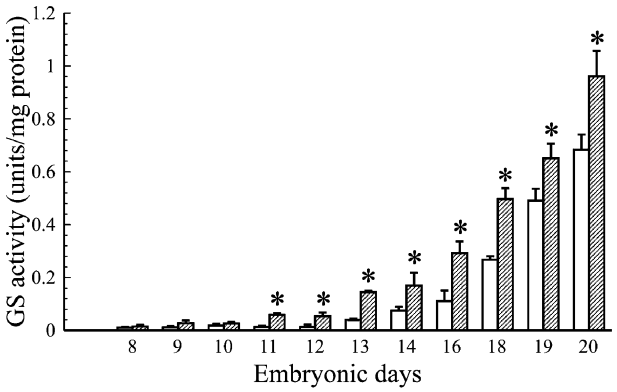

Fig. 4. **Pituitary GS activity during development.** The pituitary enzyme activity in the corticosterone-treated (stripped bars) embryos and in the controls (open columns) was measured using the glutamine-γ-glutamyltransfer assay. Precocious induction by the steroid-hormone is observed after E8, and it becomes significant at E11 and thereafter. Enzyme activities in the normal embryos did not reach the levels in the hormone-treated embryos even at E19. Bars indicate the standard error, and asterisks mean the p values less than 0.001.

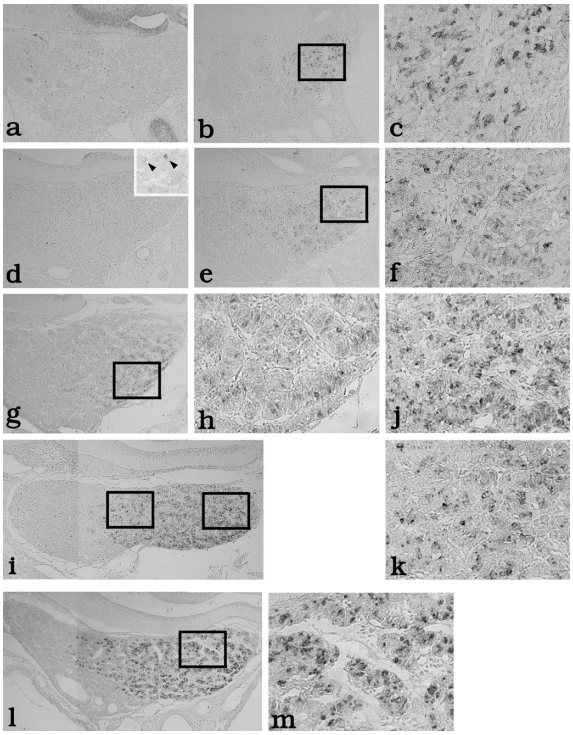

Fig. 5. **Immunoreactive GH cells in the pituitary.** The pituitary glands in control embryos at E10 (a), E 12 (d), E14 (g and h) and E18 (l and m), and those given corticosterone at E10 (b and c), E12 (e and f) and E14 (i-k) after staining with the GH antibody. Corticosterone-treated embryos show precocious induction of GH in the caudal lobe of the pituitary gland, and the immunoreaction products are distributed in the cell cytoplasm but not in the nucleus. Fig. 5c, f, h and m are insets of Fig. 5b, e, g and l, respectively, while Fig. 5j and k are insets at the central (right-hand box in Fig. 5i) and the caudal parts (left-hand box in Fig. 5i), respectively, of the pituitary gland. X40 (a, b, d, e, g, i and l), X80 (c, f, h, j, k and m)

nous glucocorticoids by E7.

The population of pituitary GS cells was determined during embryogenesis. These cells appeared in the cephalic part of the gland during the early stages following corticosterone treatment, and were distribute throughout the gland in the latter stages of development. Though the population of GS cells was higher in the cephalic rather than the caudal part, the population of GS cells was tentatively expressed as GS cell area in pituitary area, mm^2/mm^2 (Fig. 3). Pituitary GS cells appeared to be immunoreactive in the latter half of embryonic life in the untreated animals ; however, corticosterone-treatment caused precocious induction of GS in pituitary cells in the cephalic lobe during the first half of embryonic life. The population of GS cells increased sharply between E16 and E17. In the corticosterone-treated embryos, cell numbers were significantly larger ($p<0.01$) than in the control animals during the course of the development. Curiously, GS cells were not fully developed even at E 19.

The enzyme activity of pituitary GS was measured throughout development, and the results are shown in Fig. 4. Enzyme activity was at a low level by E12, and then increased gradually with advancing age. The increment of GS activity was well defined at E16-E18, and increased continuous until hatching. Corticosterone-treatment led to the precocious induction of enzyme activity during the early stages of embryogenesis and after E11 was significantly ($p<0.01$) higher than that observed in controls.

Ontogenesis GH Cells and Effects of Corticosterone

At E10, none of the pituitary cells was immunoreactive to the GH antibody (Fig. 5a), while a few were immunopositive by E11. In the corticosterone-treated embryos, premature induction of GH cells occurred in the caudal lobe of the pituitary

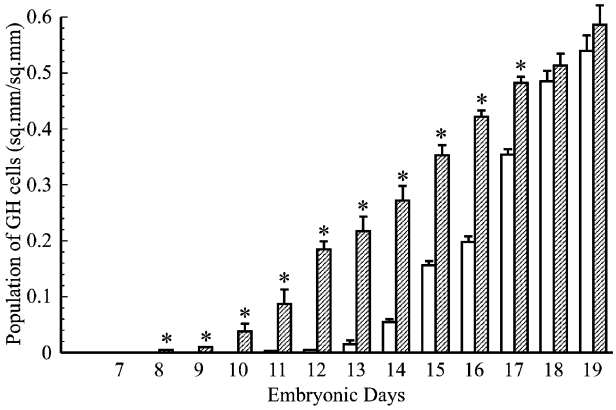

Fig. 6. **GH cell population during development.** The population of GH cells in the caudal lobe of control (open columns) embryos is compared to that in corticosterone-treated (stripped columns) animals during development. Administration of the steroid fails to cause precocious induction of GH in the progenitor cells before E7 but causes an increase in cell number starting on E8. The population of GH cells in hormone-treated embryos is significantly larger than that in control animals by E17 ; there are no differences between the two groups at E18 or E19. Bars indicate the standard error, and asterisks mean the p values less than 0.001.

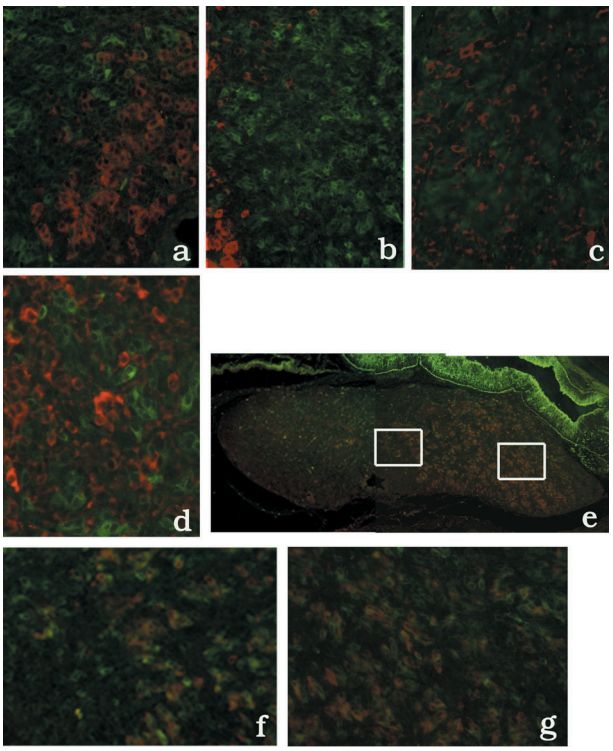

Fig. 7. **Relationship between GS cells and pituitary hormone-producing cells.** Pituitary tissues at E16 were double immunostained with GS antiserum (green) and with antiserum to pituitary hormones (red) — GS cells and ACTH (a-c), TSH (d), LH (e and f) or GH (g-j) cells. Many ACTH cells (a) and GH cells (h-j) are stained orange in color indicating that a large population of these two cells co-express both GS and either ACTH or GH. X80 (a, b, d, e, I, j), X130 (c, f), X40 (g), X260 (h)

gland (Fig. 5 b) ; the immunoreaction products were distributed in the cell cytoplasm but not in the nucleus (Fig. 5 c). A few GH cells were observed in the caudal lobe at E12 (Fig. 5 d) but their numbers increased following corticosterone administration (Figs. 5 e and f). GH cells became more numerous at E14 (Figs. 5 g and h) but were less prominent than in the E12, corticosterone-treated animals. A number of GH cells were observed occupying a large part of the caudal lobe in the corticosterone-treated embryos (Fig. 5 i). The population of GH cells in this region (Fig. 5 j) was larger than that found in the central part of the gland (Fig. 5 k). GH cells were rarely observed in the cephalic part in either the control or corticosterone-treated embryos. At E18, GH cells were well defined in both the caudal and central regions of the gland (Figs. 5 l and m).

The population of GH cells in the caudal lobe of the embryos given corticosterone was compared to that in the control animals throughout embryonic life (Fig. 6). Hormone treatment failed to cause the precocious induction of GH protein in the progenitor cells by E7 ; however, it was effective at E8. The result also showed that the population of GH cells in the corticosterone-treated embryos was significantly ($p < 0.01$) greater than that in control animals by E 17, and that there was no difference in the population of GH cells between the two groups at either E 18 or E19. Therefore, exogenous corticosterone-treatment was only effective on the GH cells before E17.

Relationships between GS Cells and Pituitary Hormone-producing Cells

The relationship between immunoreactive GS (green color) and pituitary hormone-producing cells (red color) was studied at E16 using double immunostaining with GS antiserum and antiserum to ACTH, TSH, LH, and GH. The ACTH cells were distributed in the cephalic lobe of the gland ; a large population was identical to the GS cells whose

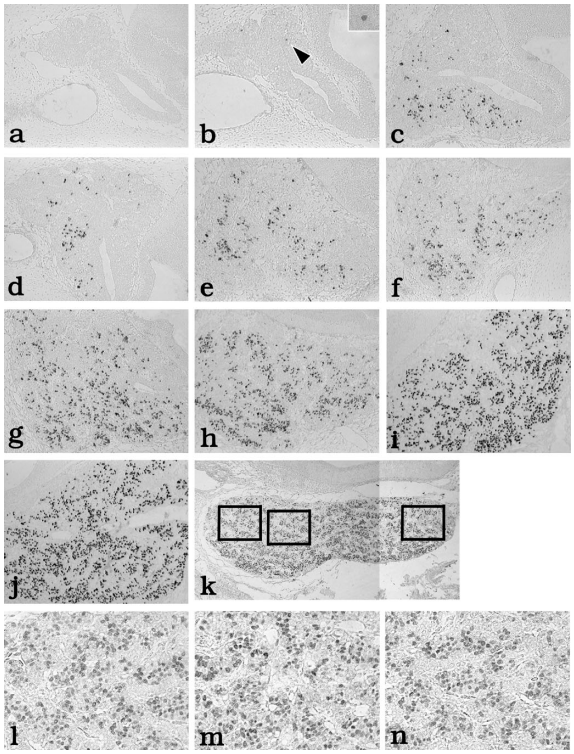

Fig. 8. **Pit-1 cell population during development.** None of the pituitary cells stained with the antibody to Pit-1 by E6 in the control embryos (a), while a few nuclei in the pituitary cells from the corticosterone-treated animals are immunostained (b, an arrowhead and inset). With advancing embryonic age the number of Pit-1 cells increased in control and corticosterone-treated embryos at E7 (c and d, respectively), E8 (e and f, respectively), E9 (g and h, respectively), and E10 (i and j, respectively). Immunoreactive Pit-1 cells are distributed in the cephalic and ventral parts at E6-E8, and then observed throughout the pituitary gland at E14 (k-n), in which Pit-1 cells are densely populated in the cephalic (l), central (m), and caudal lobes (n). X80 (a-j, l-n), X 160 (inset of b), X40 (k)

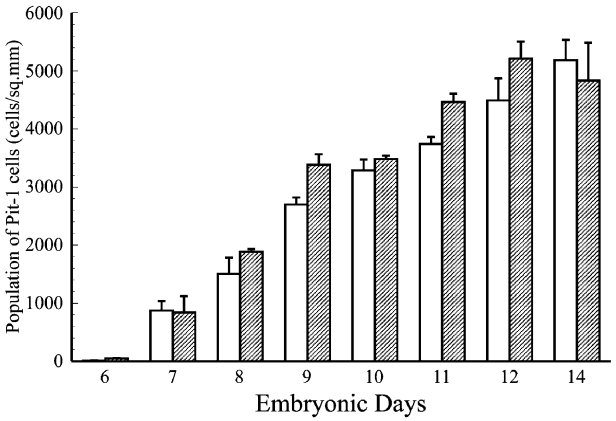

Fig. 9. **Pit-1 cell population during development.** Pit-1 cells are first detected at E6 and gradually increase in number to reach a plateau at E12. The administration of corticosterone (stripped columns) had no affect on the precocious induction of Pit-1 protein in the embryos regardless of age.

colors were orange (Fig. 7 a). In contrast, some ACTH cells located in the central part were not identical to the GS cells (Figs. 7 b and c). TSH and LH cells were localized in the caudal part of the hypophysis, and were independent from the GS cells (Figs. 7 d-f). GH cells were distributed in the caudal region (Fig. 7 g), where a large population was identical to the GS cells (Fig. 7 h) ; the GH cells in the caudal and central part of the gland were also immunoreactive to GS (Figs. 7 i and j, respectively). Thus, a large number of GS cells in the cephalic lobe were identical to the ACTH cells, and in the central and caudal lobes, they were identical to the GH cells. It is roughly estimated that at E16, 85, 15, 5, and 95% of ACTH, TSH, LH, and GH cells, respectively, corresponded to the GS cells. Prolactin cells were not fully differentiated at this time, and it is unclear whether they were identical to the GS cells. However, about 80% were identical to GS cells by E19 (data not shown).

Ontogenesis Pit-1 Cells and Effects of Corticosterone

Since a large population of GH cells was shown to be identical to the GS cells, expression of Pit-1, a transcription factor of GH, was studied during development of chicken embryos by immunostaining with antiserum to Pit-1 protein. By E5, none of the pituitary cells in either the control or corticosterone-treated embryos was stained with the antibody (Fig. 8 a). At E6, a few nuclei in the corticosterone-treated pituitary cells displayed immunostaining (Fig. 8 b), and their numbers increased with advancing embryonic age (Figs. 8 a-n). Immunoreactive Pit-1 cells were distributed in the cephalic and ventral regions at E6-E8, and were then observed throughout the pituitary gland with advancing age, being densely populated in the caudal lobe at E14. The antibody to rat Pit-1 protein revealed that reaction products were observed in the nuclei of pituitary cells but not in the cytoplasm as has been noted using the antibody to chicken Pit-1 (data not shown).

To observe the developmental change in the pop-

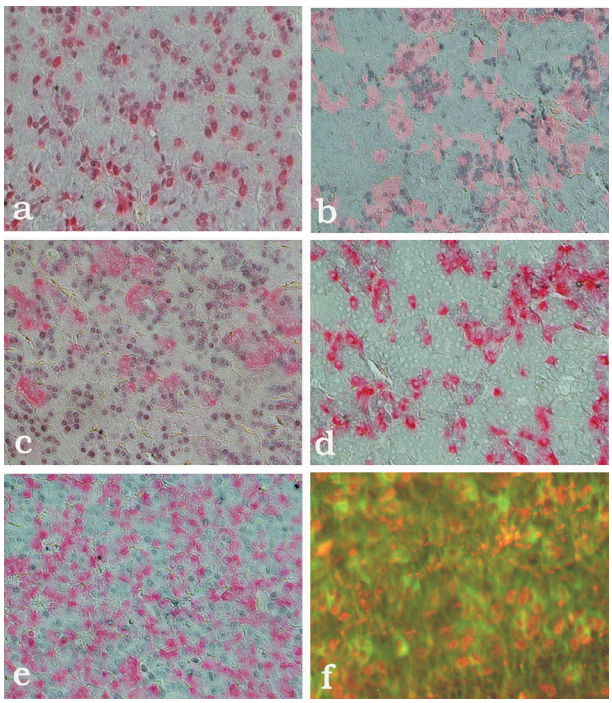

Fig. 10. **Relationship between Pit-1 cells and pituitary hormone-producing and GS cells.** Pituitary sections at E16 immunostained with the antibody to Pit-1 protein were additionally double stained with antibodies to GH (a), TSH (b), ACTH (c), LH (d and e), and GS (f). After staining with Pit-1 antibody and the peroxidase-labeled antibody method (dark blue), the sections were then immunostained with the alkaline phosphatase-labeled antibody method (pink) as described in the Materials and Method. Large numbers of Pit-1 positive nuclei also co-label with GH (a) and TSH cells (b). ACTH cells (c) and LH cells (d) located in the ventral portion of the gland (c) and in the cephalic (d) or caudal lobe (e) are not co-stained with the antibody to Pit-1. Pit-1 (red) and GS (green) are double stained as shown in Figure 6. Some of the nuclei of GS cells are also stained with the Pit-1 antibody (orange in f). X80

ulation of Pit-1 cells and the effect of corticosterone on Pit-1 cells, their population was determined by counting the number of immunostained nuclei in a defined area, and the result is shown in Fig. 9. Pit-1 cells increased gradually after E6 and reached a plateau at E12. Corticosterone did not cause precocious induction of Pit-1 protein in the embryos before E6, and the population of Pit-1 cells was not affected by the administration of the steroid hormone.

Relationships between Pit-1 Cells, Pituitary Hormone-Producing Cells, and GS Cells

Pituitary sections were double stained with Pit-1, pituitary hormones, and GS antiserum. As shown in Fig. 10 a, Pit-1 immunostaining was present in the nuclei of cells (dark blue), and a large number in the caudal lobe was identical to GH cells (red). However some Pit-1 positive nuclei did not co-stain for GH. Pit-1 cells formed clusters in the caudal lobe and were also immunoreactive to TSH antiserum (Fig. 10 b). Though there were many Pit-1 and ACTH cells at the ventral portion of the pituitary gland, they were independent of each other (Fig. 10 c). LH cells were distributed throughout the gland, but neither LH cells in the cephalic lobe (Fig. 10 d) nor those in the caudal lobe (Fig. 10 e) were immunostained with Pit-1 antibody. In contrast, GS cells at the caudal lobe displayed Pit-1 protein in their nuclei (Fig. 10 f). These results show that Pit-1 is expressed in GH, TSH and GS cells, and that Pit-1 positive GS cells in the caudal lobe also contain GH.

Discussion

Tissue-specific regulation of avian GS expression during development and its response to glucocorticoid hormones has been previously reported (Rudnick *et al.,* 1954 ; Patejunas and Young, 1987). These investigators observed high levels of GS activity and GS mRNA in the retina, brain and liver. Although brain GS showed a substantial upsurge late in development, hepatic GS activity rose around E5 and remained relatively constant thereafter. In the chicken retina, brain and liver, a dramatic developmental increase was observed in retinal GS (Moscona and Hubby, 1963). Retinal GS is a differentiation marker of Müller/glial cells and plays an important physiological role by recycling the neurotransmitter glutamate (Kennedy *et al.,* 1974 ; Kennedy and Voaden, 1974). The level of retinal GS is low until E15-E16 when it begins to rise sharply and reaches a high plateau a few days later (Vardimon *et al.,* 1986 ; Patejunas and Young, 1987). At these embryonic stages, glucocorticoid-inducible GH and GS in the pituitary gland start to abruptly increase as shown in Figures 1–5. This change is temporally correlated with the development of the adrenal cortex (Marie, 1981), with the appearance of circulating corticosteroid hormones

(Linser and Moscona, 1979), and with increased levels of corticosterone-binding globulin (Gasc and Martin, 1978). Moreover, injection of hydrocortisone into the yolk sacs of early embryos or in vitro incubation of early retinal tissue with hydrocortisone precociously induces production of retinal GS several days before E15 (Piddington and Moscona, 1967 ; Moscona and Moscona, 1979). However, it cannot be induced in retinas younger than E6-E7, and when induction occurs, it increases progressively with retina development as cell proliferation ceases and cell differentiation become more advanced (Vardimon *et al.,* 1986 ; Piddington and Moscona, 1967). These observations suggest a physiological role for the glucocorticoid hormone and indicate that the induction of GS in the chicken retina provides a model for the action of this steroid during development. These reports on retinal GS are in harmony with our results. Pituitary GS is the corticosterone-induced enzyme (Figs. 1, 3 and 4), and GH is also the corticosterone-induced hormone in chicken embryos (Figs. 5 and 6). The present results suggest that the induction of both GS and GH in the chicken pituitary provides a good model for the action of glucocorticoid hormones on pituitary cell differentiation during development.

Many researchers have studied the ontogeny of chicken pituitary GH cells. Immunoreactive GH cells were first detected in the caudal lobe of the adenohypophysis at E4.5 (Thommes *et al.,* 1987), E 8 (Mikami, 1986) or E11–12 (Józsa *et al.,* 1979 ; Malamed *et al.,* 1993 ; Bossis and Porter, 2000). Pituitary GH was noted at E15 by measuring its biological activity (Enemar, 1967), at E18–19 by radioimmunoassay (Harvey *et al.,* 1979 ; Gregory *et al.,* 1998 ; McCann-Levorose *et al.,* 1993), and at E 12-E16 by Northern blot and laser densitometry analyses (McCann-Levorose *et al.,* 1993 ; Kansaku *et al.,* 1994). The variations in the onset of GH secretion may be caused by the methods used to determine GH cell differentiation and maturation, and/or by the minimum detectable levels of each assay. Pit-1 cells were immunohistochemically detected at E6-E7, and their population increased with embryonic age (Fig. 8). It is widely accepted that Pit-1 is a marker of GH progenitor cells, and that GH cells differentiate after expressing the Pit-1 gene. In the present study a few GH cells were detected immunohistochemically at E11, but the results were not in agreement with the report that immunoreactive GH and prolactin cells are present in the pituitary gland at E4.5 (Thommes *et al.,* 1987) and E6 (Jozsa *et al.,* 1979), respectively. The differences in these findings can be explained in part by the dissimilarity of immunohisotchemical methods employed. After expressing Pit-1, there was a time lag of 4 days before GH expression occurs *in ovo.* Glucocorticoids are known to be an inducer of rat GH cells *in vivo* (Nogami *et al.,* 1997). Plasma corticosterone is at an undetectable level at E8, and then starts to increase gradually at E 10 reaching several times the E10 level on E16 (Tanabe *et al.,* 1983, 1986). Administration of glucocorticoids shortens the time lag for the precocious synthesis of GH, a result that is supported by *in vitro* studies (Porter *et al.,* 1995 b ; Morpurgo *et al.,* 1987).

Recently it has been established that all cell types in the anterior pituitary contain Pit-1 mRNA transcripts, but protein expression in rodents is limited to the TSH, GH, and prolactin cells (Ingraham *et al.,* 1988 ; Bodner *et al.,* 1988). In chicken embryos, ontogenesis of TSH cells has been studied, and immunoreactive TSH cells were detected at either E6.5 (Thommes *et al.,* 1983) or E7 (Mikami, 1986). Differentiation of TSH cells occurs at few days earlier or at the same time Pit-1 protein is expressed. LH cells differentiate from the posterior process of Rathke's epithelium at E4, a finding that is widely accepted by many investigators (Gasc and Sar, 1981 ; Mikami, 1986 ; Woods *et al.,* 1985). Immunoreactive ACTH molecules become detectable in the cephalic lobe at E7 (Józsa *et al.,* 1979), E 8 (Fellman *et al.,* 1975 ; Gasc and Sar, 1981) or E9 (Ferrand *et al.,* 1974). Since immunoreactive Pit-1 cells are different than LH cells or ACTH cells, Pit-1 expression is independent from these cell types (Fig. 10). A certain population of ACTH cells was identical to the GS cells, suggesting that some population of immunoreactive GS cells served as progenitors for the ACTH cells. GH protein is expressed in immunoreactive GS cells in the caudal lobe, suggesting that GS may have a role to controlling GH cell maturation and/or secretion.

Glucocorticoid induction of GH and GS was examined in cultured GH1 cells, and both proteins were induced by dexamethasone (Samuels *et al.,* 1978). In contrast to the GH response, induction of

GS by glucocorticoids was not influenced by triiodothyronine, which had no influence on nuclear-associated GC receptor levels. These investigators speculated that thyroid hormone control of glucocorticoid induction of GH may be a selective process, and the nuclear associated receptors for both thyroid hormone and glucocorticoid interrelate to control the GH response. L-glutamate and other acidic amino acids increased cytosolic Ca^{2+} concentrations in GH3 cells, and hormone secretion by these cells was regulated by oscillations of the cytosolic Ca^{2+} concentrations (Barabanov *et al.,* 1995). They suggested that glutamate might cooperate in the modulation of pituitary hormone secretion by an unconventional mechanism involving a high-affinity glutamate transporter. High concentrations of glutamate, however, are toxic to anterior pituitary cells (Shirasawa *et al.,* 1997 ; Caruso *et al.,* 2004), even though GH and prolactin cells have glutamate receptors (Caruso *et al.,* 2004). GS in GH cells may react to reduce glutamate toxicity by converting glutamate to a non-toxic form.

In mice, pituitary-dependent dwarfism (Ames, df ; Snell, dw ; Jackson, dwJ) was caused by the apparent failure of an initial Pit-1 lineage required for GH, PRL or TSH production (Li *et al.,* 1990 ; Sornson *et al.,* 1996). As in mice, there was a clear correlation between Pit-1 mRNA expression and hormone content in the developing human fetal adenohypophysis (Puy and Asa, 1996). Cells with GH, prolactin and/or TSH immunoreactivity had abundant Pit-1 mRNA, whereas those containing ACTH, FSH or LH were negative for Pit-1 mRNA by in situ hybridization. These investigators also showed that Pit-1 protein was detected in 70–90% of GH, PRL and TSH cells by immunohistochemistry, and that the correlation between Pit-1 mRNA detection and Pit-1 protein localization was consistent with a cell-type-specific pretranslational regulatory mechanism for Pit-1 expression. In the present study, Pit-1 protein was identified at E7, and GH immunoreactivity 4 days later. These results are consistent with reports in rodents (Dollé *et al.,* 1990 ; Simmons *et al.,* 1990) and humans (Puy and Asa, 1996) where Pit-1 appears a day or a week before the onset of GH cell differentiation. The population of Pit-1 cells was not affected by corticosterone-treatment during the course of embryogenesis, and the result is in accordance with the report showing that glucocorticoid-treatment does not affect Pit-1 or Pit-1 mRNA levels in rat pituitary glands (Nogami *et al.,* 1997).

In the present study, we provide evidence indicating that corticosteroid-inducible GS in chicken embryonic pituitary cells is related to GH cell differentiation. Somatotroph differentiation and GH gene expression are controlled by glucocorticoids involving glucocorticoid receptors. Developmental changes in the expression of the glucocorticoid receptor have been reported in the chicken retina (Gorovits *et al.,* 1994) and pituitary (Bossis *et al.,* 2004), which show that the receptor is expressed in the undifferentiated retina at E6 and in the pituitary at E8. These reports are consistent with our findings indicating that pituitary GS is precociously induced in the early stages of embryogenesis. Therefore, progenitors of somatotrophs must already possess high levels of glucocorticoid receptors before the onset of GH cell differentiation.

Finally, we demonstrate in the current study the ontogeny of GS, GH, and Pit-1 cells during chicken embryonic development. In a previous report, L-methionine sulphoximine, a reversible inhibitor of GS, reduced pituitary GS activity to 6% of that seen in controls (Shirasawa and Yamanouchi, 1996). Our preliminary studies showed that L-methionine sulphoximine treatment delayed GH cell development in the middle stages of chicken embryogenesis. These observations in combination with the dual-fluorescence immunohistochemical and the pharmacological studies indicate an involvement of GS in somatotroph differentiation and/or regulation of GH secretion.

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