Cell Tissue Res (2005) 322: 279–287
DOI 10.1007/s00441-005-0021-3

REGULAR ARTICLE

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Developmental changes of Islet-1 and its co-localization with pituitary hormones in the pituitary gland of chick embryo by immunohistochemistry

Received: 31 December 2004 / Accepted: 17 May 2005 / Published online: 27 July 2005
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Abstract Although Islet-1 expression in the pituitary gland of early mouse embryo has been previously described, there are no reports concerning the correlation of Islet-1 expression with lineage restrictions in cell types at the later stages of pituitary development. The role of Islet-1 in chickens is also unknown. The purpose of this study was to follow, by using immunohistochemistry, the ontogeny of pituitary Islet-1 and the various cell types that contain Islet-1 throughout chick embryo development. A few Islet-1-immunopositive (Islet-1$^+$) cells were first detected in the pituitary primordium in two out of six embryos at embryonic day 5.5 (E5.5), most of the Islet-1$^+$ cells being ventrally located. As development progressed, many more Islet-1$^+$ cells were observed throughout the pars distalis. The relative percentage of Islet-1$^+$ cells amongst the total Rathke's pouch cells was 4.4% at E6.5. This increased significantly, reaching 11.1% by E10.5, followed by no significant change until hatching. Dual immunohistochemistry showed that adrenocorticotrophs, somatotrophs and lactotrophs did not express Islet-1. The cellular types expressing Islet-1 included luteinizing-hormone-positive (LH$^+$) gonadotrophs and thyroid-stimulating-hormone-positive (TSH$^+$) thyrotrophs. The cells co-expressing LH and Islet-1 were initially detected at E6.5, the proportion of LH$^+$ cells possessing Islet-1 being about 4%; this increased to 63% at E14.5, followed by no significant changes until hatching. TSH and Islet-1 co-localized cells were first observed at E10.5, with about 37% TSH$^+$ cell expressing Islet-1; this increased to about 50% by E16.5, after which there was no evident change until hatching. These results suggest that Islet-1 is involved in determining the cell lineages, proliferation, differentiation and maintenance of hormone-secreting functions of pituitary gonadotrophs and thyrotrophs of chick embryo.

Keywords Islet-1 · Pituitary hormones · Immunohistochemistry · Chick embryo

Introduction

The pituitary gland, or hypophysis, is one of the most important endocrine glands in the vertebrate and consists of a neurohypophysis derived from the infundibular process of the brain floor and an adenohypophysis derived from Rathkes' pouch (RP) of the stomodeal ectoderm. It comprises a pars distalis, a pars tuberalis and a pars intermedia in the majority of higher vertebrates (Mullis 2000). However, avian species lack the pars intermedia, whereas the pars distalis includes only a well-defined cephalic lobe and a caudal lobe (Freeman 1974; Mikami 1983). The cephalic lobe contains corticotrophs that secrete adrenocorticotropic hormone (ACTH), lactotrophs that secrete prolactin (PRL), thyrotrophs that secrete thyroid-stimulating hormone (TSH), gonadotrophs that secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The caudal lobe consists of somatotrophs that secrete growth hormone (GH) and gonadotrophs that secrete LH and FSH (Freeman 1974; Mikami 1983). These cell types emerge from a common primordium in a precise spatial and temporal pattern in which a number of molecular signal and transcription factors are involved in pituitary organogenesis (Mullis 2000)

J. Liu and Y. He contributed equally to this article. This work was supported by grants from Beijing Natural Science Foundation (6042013) and the Natural Science Foundation of China (30471264, 30325034).

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and in the progressive differentiation of distinct cell phenotypes during embryonic development (Pfaff et al. 1996; Ahlgren et al. 1997; Cui and Goldstein 2000a; Avivi et al. 2002).

LIM homeodomain transcription factors contain a homeodomain and two LIM domains of cystine-rich motifs and are known to play crucial roles in the control of cell fate specification, survival, differentiation and axonal projection patterns in vertebrates and invertebrates (Karlsson et al. 1990). Expression of the LIM-homeodomain factors, Lhx3/P-Lim and Lhx4, are restricted to RP. Lhx3 plays critical roles in the direct regulation of target genes for the terminal differentiation of mammalian pituitary cell types (Sheng et al. 1996, 1997; Tremblay et al. 1998). Islet-1 is a member of the homeodomain-containing protein family that processes an amino-terminal pair of zinc-binding LIM domains (Pfaff et al. 1996) and is expressed in polypeptide hormone-producing cells of the endocrine system (Dong et al. 1991; Thor et al. 1991), except those of the central and peripheral nervous system (Avivi and Goldstein 1999; Shiga and Oppenheim 1999; Cui and Goldstein 2000a; Avivi et al. 2002) and inner ear of the chick embryo (Li et al. 2004). In addition, Islet-1 has recently been shown to be required for the development of pancreatic endocrine cells and to be co-expressed in the mammalian RP with Lhx3, Pitx1, Pitx2 and Rpx. This co-expression occurs throughout the oral ectoderm in early phases of pituitary development (Ericson et al. 1998; Dasen and Rosenfeld 1999; Mullis 2000). Little information is available concerning Islet-1 expression in the late development of the pituitary gland and no reports have been published describing the ontogeny of Islet-1 in the developing pituitary of chick embryo as yet. The aims of the present study have been to determine the ontogeny of Islet-1 and the cell types expressing Islet-1 in developing pituitary gland in chick embryo by immunohistochemistry methods.

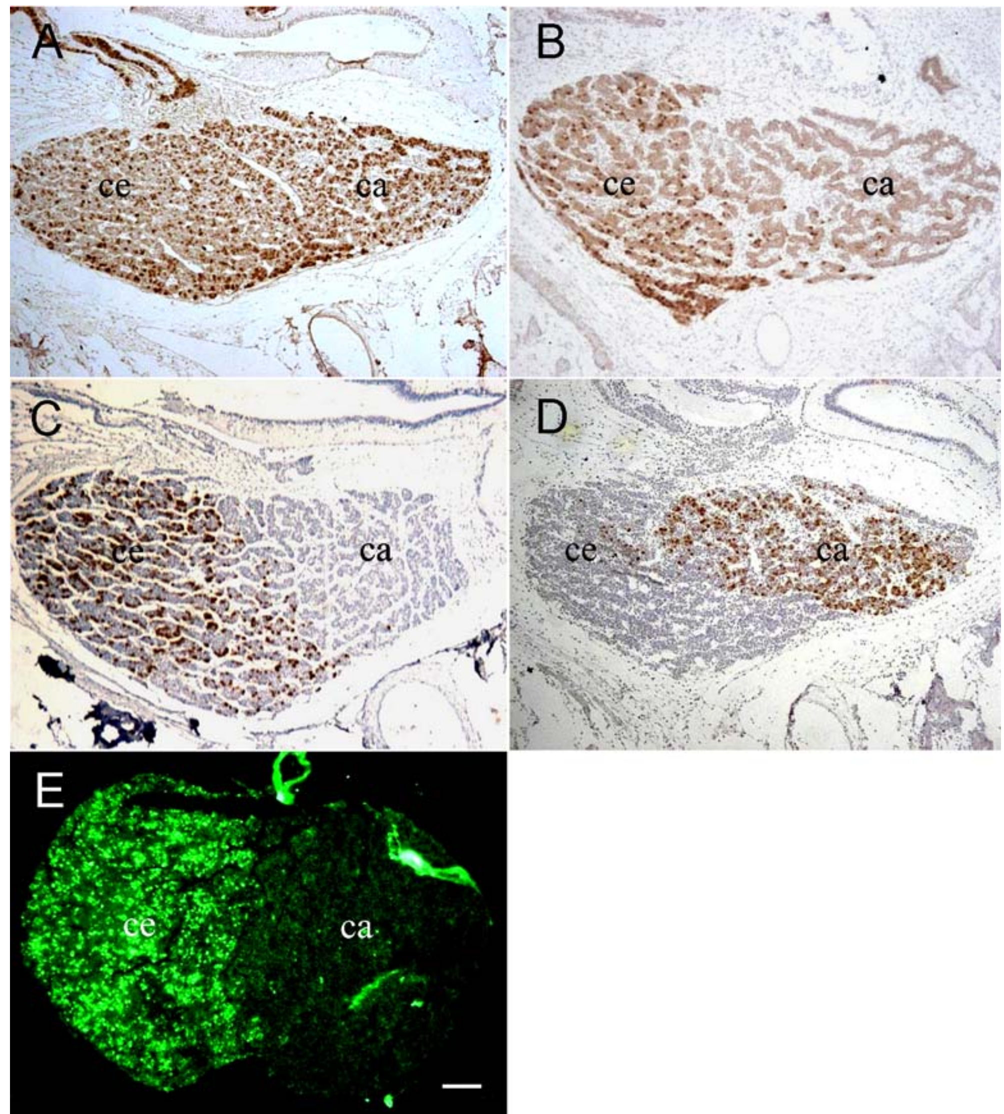


Fig. 1 Distributions of pituitary-hormone-immunopositive cells in the anterior pituitary gland of chick embryos (*ce* cephalic lobe, *ca* caudal lobe). **a** LH^+ cells are distributed in all areas of the pars distalis at E16.5. **b** TSH^+ cells are mainly located in the cephalic lobe of the pars distalis at E16.5. **c** $ACTH^+$ cells are detected in the cephalic lobe of the pars distalis at E16.5. **d** GH^+ cells are distributed in the caudal lobe of the pars distalis at E16.5. **e** PRL^+ cells are present in the cephalic lobe of the pars distalis at E18.5. *Bar* 100 μm

Materials and methods

Animals and tissue collection

The use of animals in this study and its experimental design were approved by the Chinese Association for Laboratory Animal Sciences. White Leghorn chick embryos were used. Fertilized chicken eggs were incubated at 37.5°C and embryos were collected at embryonic day 3.5 (E3.5; stage 21), E4.5 (stage 25), E5.5 (stage 27), E6.5 (stage 29), E8.5 (stage 35), E10.5 (stage 36), E12.5 (stage 38), E14.5 (stage 40), E16.5 (stage 42) and E18.5 (stage 44) of incubation (staging was according to Hamburger and Hamilton 1951). Six embryos at every stage were anaesthetized by cooling in 4°C phosphate-buffered saline (PBS) for 1 min (Cui and Goldstein 2000b) and then decapitated. In addition, six newly hatched chickens were anaesthetized by using sodium phenobarbitol (1.5 mg in 0.2 ml 0.9% NaCl for each bird) by intramuscular injection and decapitated after 30 s. The whole heads of embryos aged E3.5–E12.5 and the surgically dissected pituitaries of embryos aged E12.5–E18.5 and of newly hatched chickens were fixed overnight in 4% paraformaldehyde in PBS at 4°C. The tissues were

Table 1 Average cell numbers of Islet-1$^+$ and total cells in the RP and anterior pituitary gland (*NH* newly hatched)

Embryonic age	Islet-1$^+$ cells/embryo	Total cells/embryo
E6.5	516±62	11,923±752
E8.5	1,382±121	12,489±623
E10.5	1,360±97	11,232±768
E12.5	2,374±334	19,502±745
E14.5	2,953±401	27,081±1,356
E16.5	2,403±152	21,351±2,008
E18.5	2,638±171	22,504±1,530
NH	2,949±188	24,320±1,459

Six embryos at each age were examined. The cells were counted from four randomly selected fields in each section under a 40× objective and from eight sections from each embryo. Values are expressed as means±SEM.

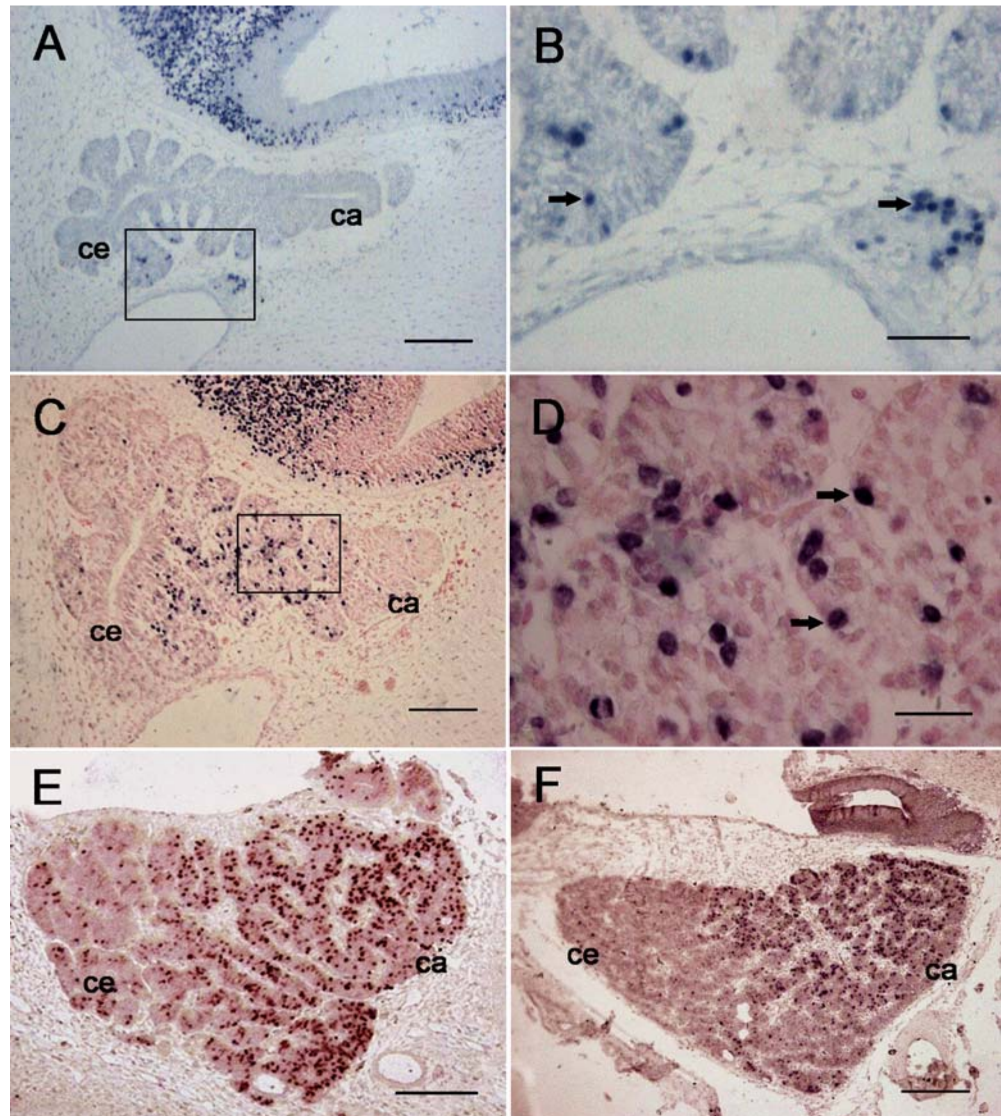


Fig. 2 Photomicrographs from parasagittal paraffin sections of the RP and anterior pituitary gland of chick embryo immunostained for Islet-1. Islet-1 is located in the nuclei throughout the development of chick embryos (*ce* cephalic lobe of pars distalis, *ca* caudal lobe of pars distalis, *arrows* Islet-1$^+$ cells). **a** Expression of Islet-1 in the RP at E5.5. *Bar* 100 μm. **b** *Boxed area* of **a** at higher magnification. *Bar* 30 μm. **c** Sagittal sections of RP at E6.5 demonstrating the presence of Islet-1. *Bar* 100 μm. **d** *Boxed area* of **c** at higher magnification. *Bar* 20 μm. **e**, **f** Expression of Islet-1 in the anterior pituitary gland at E10.5 and E14.5, respectively. *Bar* 100 μm

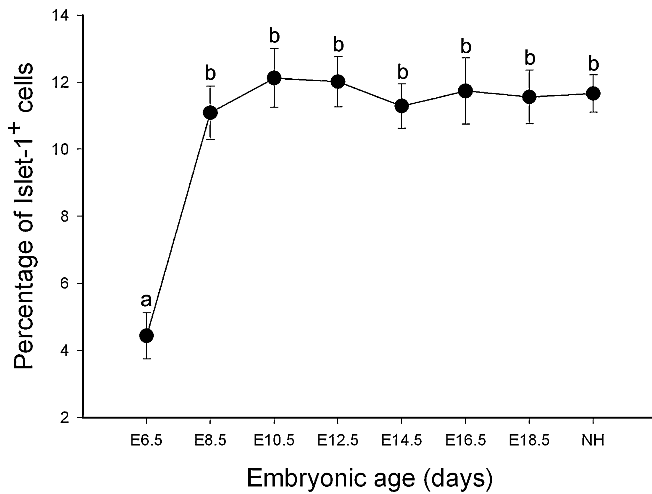


Fig. 3 Changes in percentages for Islet-1$^+$ cells in the RP and anterior pituitary gland during the development of chick embryo. Values are expressed as means±SEM (*n*=6; *NH* newly hatched, *a, b* significant differences at $P<0.05$, ANOVA)

embedded in paraffin and serial sections (5 μm thick) of the whole pituitary or head were cut at sagittal levels.

Immunohistochemistry

Islet-1 immunohistochemical localization was performed with a monoclonal antibody against chicken Islet-1 (40.2D6, Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA), as used to detect Islet-1 expression in our previous studies (Avivi and Goldstein 1999; Cui and Goldstein 2000a; Avivi et al. 2002). Serial sections were dewaxed and rehydrated and antigen retrieval was performed by microwaving the sections (3×4 min at full power) in 0.01 M sodium citrate buffer (pH 6.0). Endogenous peroxidase was blocked by incubating the sections in 3% H_2O_2 in methanol for 30 min. The sections were treated with 10% normal sheep serum in PBS, incubated with the primary antibody (1:30) overnight at 4°C in a humid box, then in sheep anti-mouse immunoglobulin (1:50; Scottish Antibody Product Unit, UK) for 2 h, followed by mouse alkaline phosphatase anti-alkaline phosphatase (1:50, DAKO, Demark) for 2 h, and visualized with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium (Sigma, St. Louis, Mo., USA). Finally, the sections were counterstained with nuclear fast red (Labvision, Fremont, Calif., USA), dehydrated and mounted by conventional methods.

For the dual labelling of Islet-1 with pituitary hormones, the sections were treated for Islet-1 detection as above. The sections were then incubated for 12 h at 4°C with one of the following polyclonal antibodies: rabbit anti-chicken LH (cLH, kindly supplied by Dr. T. Matozaki, Gunma University, Japan; 1:1,000), which was utilized to detected chicken LH (Shirasawa et al. 1996; Kameda et al. 2000; Liu and Cui 2005), rabbit anti-chicken GH (cGH, NIDDK, 1:300; Liu and Cui 2005); rabbit anti-rat TSH (NIDDK, 1:500; Murphy and Harvey 2001; Liu and Cui 2005); and rabbit anti-human ACTH (NIDDK, 1: 500; Liu and Cui 2005). The sections were then incubated with biotinylated swine anti-rabbit IgG (1:300, DAKO) for 2 h at room temperature. Subsequently, ABC complex (Vector Laboratories, Burlingame, Calif., USA) was added for 1 h and peroxidase activity was detected by using diaminobenzidine (Sigma) for 2 min.

For Islet-1 and PRL double-labelling, Islet-1 immunoreactivity was first detected by using the above procedures. The sections were then incubated for 12 h at 4°C with sheep anti-chicken PRL antiserum (cPRL, kindly supplied by Professor P.J. Sharp, UK; 1:1,000; Liu and Cui 2005), followed by fluorescein-isothiocyanate-conjugated swine anti-sheep IgG (1:50, Scottish Antibody Product Unit, UK) for 2 h, and embedded in 75% glycerol in PBS.

The controls for both single and dual staining included the use of non-immunized rabbit, mouse or sheep serum and the omission of the second antibodies. No positive staining was observed in these experiments. Positive controls included immunohistochemical staining with each pituitary hormone to test the specificity of the antibodies. The results showed that LH-positive (LH$^+$) cells were distributed in all areas of the pars distalis (Fig. 1a), that TSH$^+$, ACTH$^+$ and PRL$^+$ cells were mainly located in the cephalic lobe of the pars distalis (Fig. 1b, c, e) and that GH$^+$ cells were detected in the caudal lobe of the pars distalis (Fig. 1d).

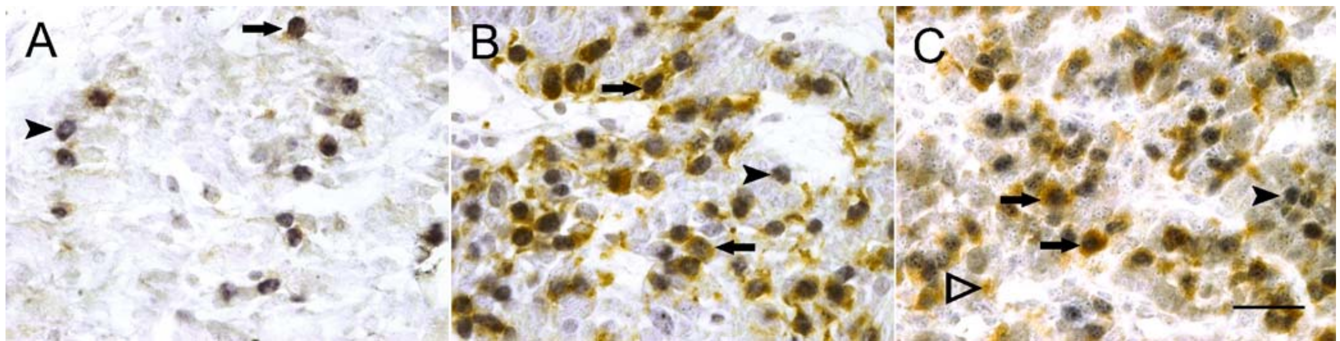


Fig. 4 Micrographs of double-staining for Islet-1 and LH in the RP and anterior pituitary gland of chick embryo (*arrows* hormone-producing cells also expressing Islet-1$^+$, *arrowheads* Islet-1 single-staining cells, *open triangle* LH single-staining). **a** Co-localization of Islet-1 and LH in RP at E6.5. **b**, **c** Co-localization of Islet-1 and LH at E16.5 and E18.5, respectively. *Bar* 20 μm

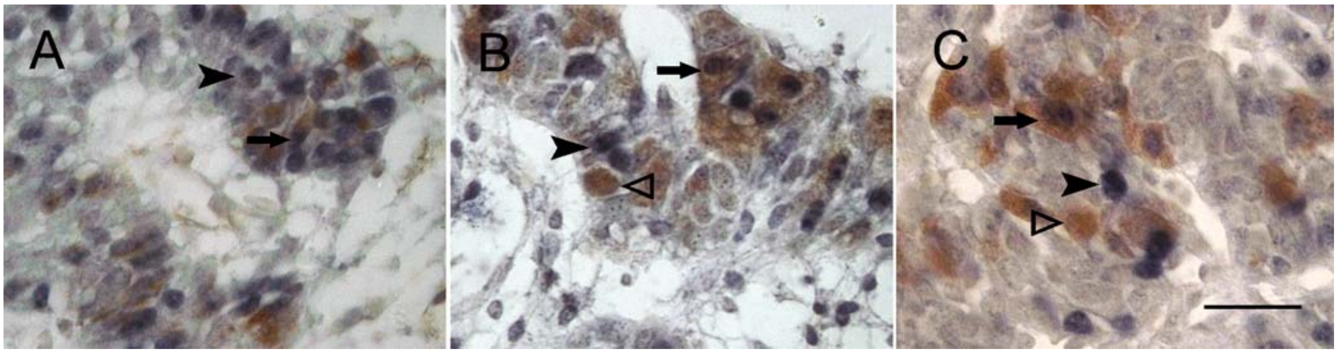


Fig. 5 Micrographs of double-staining for Islet-1 and TSH in the anterior pituitary gland of chick embryo (*arrows* hormone-producing cells also expressing Islet-1^{+}, *arrowheads* Islet-1 single-staining cells, *open triangles* TSH single-staining cells). Co-localization of Islet-1 and TSH in the cephalic lobe of the pars distalis at E10.5 (**a**), E16.5 (**b**) and E18.5 (**c**). *Bar* 20 μm

Data analysis

We counted cells by using image analysis software (Leica QWin) to determine the percentage of Islet-1^{+} cells as described in a previous report (Hausmann et al. 1998) and the Leica QWin product manual (Leica Microsystems, Cambridge, UK). For each embryo, four fields were selected randomly from every fifth section and eight sections were examined. Briefly, the sections were photographed with the Leica DFC 320 (Leica Microsystems) in the grey mode under a 40× objective and the micrographs were opened by Leica QWin (Leica Microsystems). Following Grey Detect (Adjust: White; Value: 160), cell counting was performed by the Interactive Measurements program at the grey level (a grey level higher than 160 was considered as a positive result) on the photos and expressed as a percentage of Islet-1^{+} cells with respect to the total cell number. The average cell numbers of Islet-1^{+} and total cells in the RP at E6.5 and in the anterior pituitary gland from E8.5 to the newly hatched stage were calculated. For Islet-1/LH and Islet-1/TSH double-stained sections, the total Islet-1^{+} cell numbers, total LH^{+} and TSH^{+} cell numbers and Islet-1/LH and Islet-1/TSH double-labelled cell numbers were counted as above under a 100× objective and the average cell numbers were calculated. In the Islet-1/GH and Islet-1/ACTH double-labelled sections, no double-labelled cells were observed at the different stages and so we did not perform cell counting. PRL^{+} dual-labelled sections were photographed by using a Leica DFC 320 digital camera (Leica Microsystems) equipped with appropriate filter combinations (Leica, I3-513808) for fluorescence and bright-field microscopy. We did not find Islet-1 and PRL dual-labelled cells and hence cell counting was not performed. All of the cell counting was performed by a single investigator blinded to the age of the embryo. All values are presented as the mean±SEM. Statistical differences were assessed by a one-way analysis of variance (ANOVA) and $P<0.05$ was considered significant.

Results

We first analyzed the Islet-1 staining pattern in the pituitary of chick embryos during embryonic development. Immunohistochemistry revealed that Islet-1 staining was localized to the nuclei of the pituitary cells during all developmental stages. At E3.5 and E4.5, Islet-1^{+} cells were observed in the areas around the RP but not in areas directly within the RP (data not shown). At E5.5, only a few Islet-1^{+} cells were observed in the ventral region of the

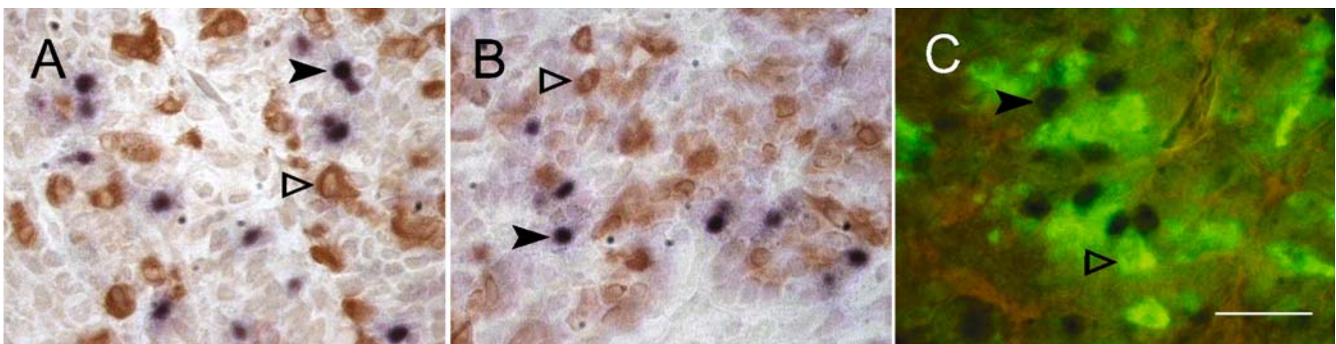


Fig. 6 Dual-labelling immunohistochemistry for Islet-1 and ACTH (**a**), GH (**b**) and PRL (**c**) in the anterior pituitary gland of chick embryo (*arrowheads* Islet-1 single-stained cells, *open triangles* single-staining for pituitary hormones). **a** Cephalic lobe of the pars distalis at E18.5. **b** Caudal lobe of the pars distalis at E18.5. **c** Cephalic lobe of the pars distalis at E18.5. *Bar* 20 μm

Table 2 Average of the total single-stained LH$^+$ cell numbers/embryo, total single-stained Islet-1$^+$ cell numbers/embryo and total double-stained LH$^+$-Islet-1 cell numbers/embryo after dual immunostaining for LH and Islet-1, and total single-stained TSH$^+$ cell numbers/embryo, total single-stained Islet-1$^+$ cell numbers/embryo and total double-stained TSH$^+$-Islet-1 cell numbers/embryo after dual immunostaining for TSH and Islet-1 (*NH* newly hatched)

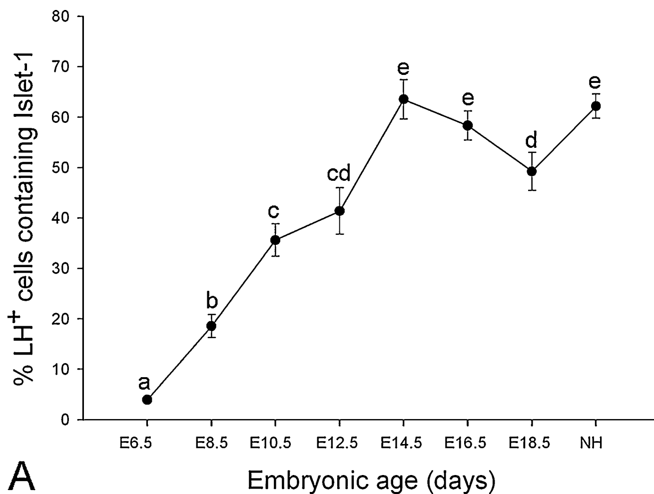
Immunostaining	E6.5	E8.5	E10.5	E12.5	E14.5	E16.5	E18.5	NH
LH$^+$/embryo	276±29	390±65	488±52	746±72	818±86	853±101	761±92	754±82
Islet-1$^+$/embryo	156±16	286±57	340±50	564±78	802±76	960±105	866±123	1,011±113
LH$^+$-Islet-1$^+$/embryo	22±4	72±8	167±18	337±16	528±57	520±54	379±42	465±57
TSH$^+$/embryo	–	–	66±6	82±23	139±24	160±52	213±44	193±35
Islet-1$^+$/embryo	–	–	404±43	593±62	878±82	953±76	909±105	1,036±92
TSH$^+$-Islet-1$^+$/embryo	–	–	23±5	36±19	71±14	87±20	118±32	113±18

Six embryos at each age were examined. Cells were counted from four randomly selected fields in each section under a 100× objective and from eight sections for each embryo from each pituitary hormone. Values are expressed as means±SEM.

RP in two out of six embryos (Fig. 2a,b). Islet-1$^+$ cells were consistently detected in the RP at E6.5 (Fig. 2c,d). By E8.5, the RP had broken away from the roof of the mouth and had developed into anterior pituitary gland. As embryonic development progressed, many more Islet-1$^+$ cells were observed throughout the pars distalis (Fig. 2e,f), although more Islet-1$^+$ cells were detected in the caudal lobe of the pars distalis than in the remaining areas (Fig. 2e,f). This distribution pattern persisted until hatching. Cell counts and the change in the percentage of Islet-1$^+$ cells with respect to the total RP and anterior pituitary gland cell number are shown in Table 1 and Fig. 3. At E6.5, the relative percentage of Islet-1$^+$ cells with respect to the total RP cells was 4.4%. This value then increased significantly ($P<0.01$) and reached 11.1% by E10.5, followed by no significant changes until hatching.

Dual immunohistochemical results are shown in Figs. 4, 5 and 6, with Islet-1 immunoreactivity as blue nuclear staining, immunostaining for pituitary hormones (LH, TSH, GH and ACTH) in brown, and PRL staining as green cytoplasmic fluorescence. These results indicated that the cell types expressing Islet-1 included LH$^+$ gonadotrophs (Fig. 4a–c) and TSH$^+$ thyrotrophs (Fig. 5a–c). Corticotrophs, somatotrophs and lactotrophs did not express Islet-1 (Fig. 6a–c). In addition, TSH immunoreactivity was also observed in the brain region of chick embryo at E8.5 and E10.5, although we did not detect this immunostaining in the later stages of the development (data not shown).

Cells co-expressing LH and Islet-1 were first consistently detected at E6.5 (Fig. 4a). The distribution pattern of the cells co-expressing LH and Islet-1 was similar to that observed after Islet-1 single-staining. Cell counts following dual-staining for LH and Islet-1 are shown in Table 2 and the proportions of LH$^+$ cells possessing Islet-1 are shown in Fig. 7a. At E6.5, the proportion of LH$^+$ cells possessing Islet-1 was about 4%. This proportion steadily rose, reaching about 63% by E14.5. No further significant change was seen until hatching, although there was a significant decline from days 16.5–18.5 of incubation. The proportion of Islet-1 cells possessing LH is shown in Fig. 7b. At E6.5, about 17.5% of Islet-1$^+$ cells expressed LH; this value sharply increased and reached 68% by E16.5 but was followed by a constant decline and decreased to about 44% at hatching.



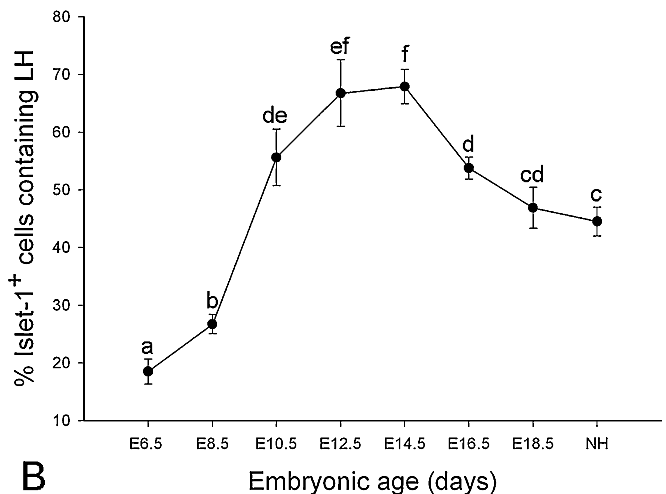
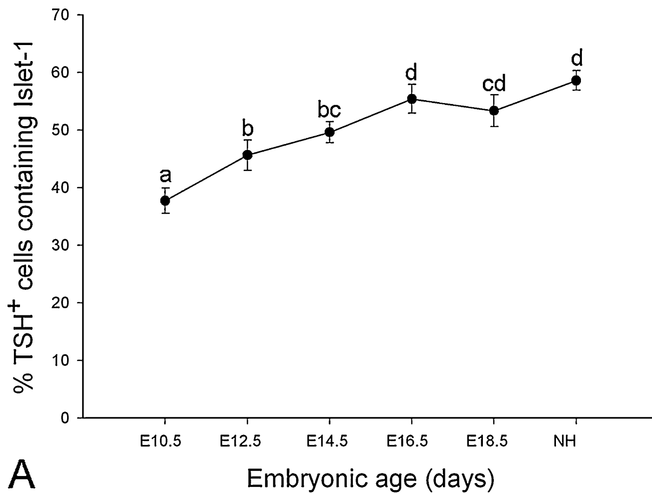


Fig. 7 **a** Percentage of dual-labelled Islet-1-LH cells with respect to LH$^+$ cells during the development of chick embryo. **b** Percentage of dual-labelled Islet-1-LH cells with respect to Islet-1$^+$ cells during the development of chick embryos. Values are expressed as means± SEM ($n=6$; *NH* newly hatched, *a–f* significant differences at $P<0.05$, ANOVA)



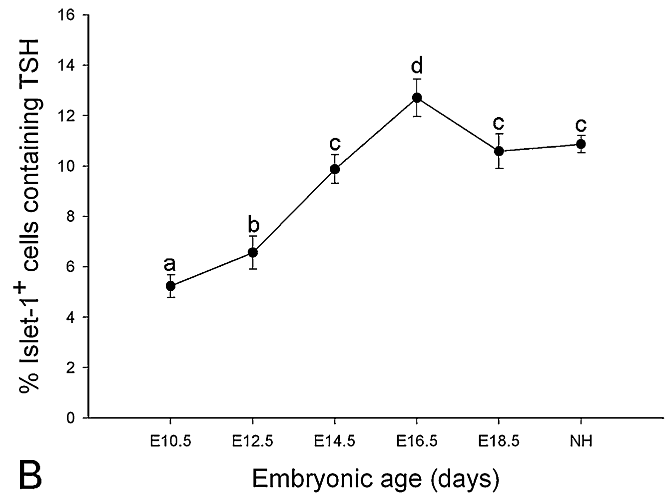


Fig. 8 **a** Percentage of dual-labelling Islet-1-TSH cells with respect to TSH^+ cells during the development of chick embryo. **b** Percentage of dual-labelling Islet-1-TSH cells with respect to Islet-1^+ cells during the development of chick embryos. Values are expressed as means±SEM (n=6, *NH* newly hatched, *a–d* significant differences at $P<0.05$, ANOVA)

TSH and Islet-1 double-staining results are shown in Fig. 5a–c. TSH and Islet-1 double-stained cells were first detected at E10.5 (Fig. 5a) and were located only in the cephalic lobe of the pars distalis. As the development of chick embryo progressed, the number of cells co-expressing TSH and Islet-1 increased, and this distribution pattern persisted until hatching. The proportions of TSH^+ cells containing Islet-1 are shown in Fig. 8a. At E10.5, about 37% of TSH^+ cells expressed Islet-1, after which the percentage of TSH-immunopositive cells that expressed Islet-1 continued to rise, reaching 59% at hatching. Cell counts of the dual-staining for TSH and Islet-1 are shown in Table 2 and the proportion of Islet-1^+ cells containing TSH is shown in Fig. 8b. At E10.5, about 5.5% of Islet-1^+ cells expressed TSH; this value then significantly increased to 12.5% by E16.5, followed by no significant changes until hatching.

Discussion

This is the first description of the ontogeny and developmental changes of pituitary Islet-1 throughout chick embryonic development by immunohistochemistry, although Islet-1 expression has previously been detected in the early stages of mouse pituitary development at both the mRNA and protein levels (Ericson et al. 1998; Takuma et al. 1998). The present results demonstrate that Islet-1 is differentially expressed during the chick embryonic development, the cell types expressing Islet-1 in the pituitary gland including the gonadotrophs and thyrotrophs.

Previous reports have demonstrated Islet-1 expression in the nervous system of chick embryo from E3 (stage 18–19) and hence Islet-1 can be thought of as an early marker of neuronal differentiation (Pfaff et al. 1996; Cui and Goldstein 2000a; Avivi et al. 2002). The results of the present study show that Islet-1 expression in the RP begins at E5.5 (stage 27–28), which is about 2 days later than in the nervous system. In addition, Islet-1^+ cells have not been detected in the area of the diencephalon floor near the RP, where the neurohypophysis develops. In the subsequent development stages, few Islet-1^+ cells have been observed in the neurohypophysis, although Islet-1 is intensively expressed in the adenohypophysis. These findings suggest that Islet-1 is mainly expressed in adenohypophysial development of the embryonic pituitary gland.

Islet-1 is temporally expressed in all of the oral roof ectoderm cells in the early stages of mouse embryo development and is subsequently absent (Ericson et al. 1998). In the chick embryo, Islet-1^+ cell number is low in the area of the oral ectoderm, which will develop into the RP, in the early phases of the chick embryo (from E3.5 to E5.5). This suggests that the pattern of Islet-1 expression in the RP of chick embryo is substantially different from that in mouse embryo, although we have not detected Islet-1 mRNA expression in the present study. However, from E6.5, Islet-1^+ cells have been consistently detected in the RP, and many more Islet-1^+ cells are ventrally located and gradually distributed throughout the adenohypophysis as embryonic development progresses. This is similar to that in mouse and suggests that Islet-1 expression plays crucial roles in the determination of the oral ectoderm as it differentiates into the organ-specific cell lineages, namely the α-glycoprotein subunit (α-GSU)-specific cells, including thyrotrophs (Ericson et al. 1998) and gonadotrophs.

The present results have shown that Islet-1 is differently expressed throughout the embryonic development. From E5.5 to E10.5, the number of Islet-1^+ cells in the pituitary of chick embryo increases significantly. During E5.5 to E10.5, the pituitary-hormone-producing cells of the chicken pituitary are mostly in the phase of proliferation and differentiation, with the exception of the somatotrophs, which differentiate after E10.5 (Barabanov 1987; Scanes et al. 1987). In later developmental stages of chick embryo, the proportion of Islet-1 cells with respect to the total number of pituitary cells does not change significantly.

However, the total pituitary cell number keeps increasing, thus implying that the net Islet-1$^+$ cell number also rises. Over this period, the hormone-secreting functions of the pituitary gland of chick embryo become mature (Barabanov 1987). Therefore, the change of Islet-1 expression during pituitary development corresponds to the time of proliferation and differentiation of pituitary endocrine cells and indicates a potential role for Islet-1 in the regulation of pituitary development.

The co-localization results of Islet-1 with the pituitary hormones confirm that Islet-1 immunoreactivity is limited to the gonadotrophs and thyrotrophs in the pituitary gland of chick embryo. Similar to the pituitary gonadotrophs of mammals, avian pituitary gonadotrophs consist of LH-secreting cells and FSH secreting-cells (Freeman 1974; Mikami 1983), although these two cell types reside in separate cell populations (Proudman et al. 1999; Puebla-Osorio et al. 2002). FSH and Islet-1 double-staining has not been performed because of the lack of a specific antibody against chicken FSH. The proportions of Islet-1 cells that express LH is not more than 68%, and only a small portion (less than 12%) of Islet-1$^+$ cells express TSH throughout embryonic development. Perhaps the remaining 20% of Islet-1$^+$ cells express FSH.

The LH and Islet-1 dual-staining results have shown that the proportion of LH$^+$ cells containing Islet-1 sharply increases from E6.5 to E14.5, in parallel to the increase of the proportion of Islet-1$^+$ cells containing LH. In the later stages of the chick embryonic development, the proportions of LH$^+$ cells containing Islet-1 and Islet-1$^+$ cells containing LH decline, which correlates with changes in the plasma LH level and the pituitary LH$^+$ cell number of the developing chick embryo (Woods and Thommes 1984; Woods et al. 1989). These data imply that LH$^+$ cells are the dominant pituitary cell type expressing Islet-1 and that Islet-1 may be involved in the differentiation, proliferation and secretory function of LH$^+$ gonadotrophs during the development of chick embryo, although the related mechanisms need to be further elucidated.

The thyrotrophs are another type of pituitary cells expressing Islet-1 in chick embryo. This is in agreement with reports in mouse that LIM-homeobox transcription proteins, including Islet-1, are involved in determining the cell lineage of pituitary thyrotrophs and gonadotrophs (Ericson et al. 1998), all of which share α-GSU (Mikami 1983). However, in contrast to the mouse (Ericson et al. 1998), the ontogeny of TSH cells in chickens occurs much later than that of LH-immunopositive gonadotrophs in chick embryo. TSH-β^+ and Islet-1$^+$ cells have been detected by E10.5. The proportion of Islet-1$^+$ cells expressing TSH continues to rise until hatching, which is consistent with the changes in TSH$^+$ cell number (Thommes et al. 1983). Therefore, Islet-1 expression might be involved in cellular differentiation and proliferation and might affect the secretory function of the pituitary thyrotrophs after the middle stage of the development of chick embryo.

In conclusion, our data demonstrate that Islet-1$^+$ cells in the anterior pituitary gland significantly increase from E5.5 to E8.5 during chick embryo development but do not significantly change further until hatching. The dominant cell type expressing Islet-1 in the pituitary gland is the gonadotroph. In addition, a small proportion of thyrotrophs also express Islet-1. The changes in the proportions of Islet-1$^+$ cells containing LH or TSH correspond to the changes in the numbers of LH- and TSH-immunopositive cells. This suggests that Islet-1 expression is involved in regulating the development of the pituitary gland and the maturation of the hormone-secreting functions of the pituitary gland during the development of chick embryos, although the related mechanisms still need to be elucidated.

Acknowledgements We are grateful to Professor A.S. McNeilly (MRC Reproductive Sciences Unit, Centre for Reproductive Biology, University of Edinburgh, Edinburgh, UK) for his critical reading of and comments on this manuscript. We thank Professor P.J. Sharp (Roslin Institute, UK) for the kind gift of the polyclonal anti-chicken PRL primary antibody and Dr. T. Matozaki (Gunma University, Japan) for the kind gift of the polyclonal anti-chicken LH primary antibody. Anti-chicken GH, anti-rat TSH and anti-human ACTH primary antibodies were obtained through the National Hormone and Peptide Program (NHPP), National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and Dr. Parlow (NHPP). The 40.2D6 Islet-1 antibody was developed by Thomas Jessel and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, University of Iowa, Iowa City, USA.

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