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Structural and Ultrastructural Studies of GH, PRL and SMT Cells in Goat Fetus (*Capra hircus*) Using Immunocytochemical Methods

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Summary. **The first data based on immunolabeling techniques of goat fetus adenohypophysis show that the structure and ultrastructure of growth hormone (GH)-, prolactin (PRL)-, and GH- plus PRL-secreting cells (SMT cells) in fetuses aged 100 days differ from those in the adult. Both cell number and cell size are smaller in the fetus, and the percentage of dark cells decreases with development. The data do not support the hypothesis that SMT cells represent the common origin of GH- and PRL-cells.**

GÓMEZ et al. (1999) and SÁNCHEZ et al. (1994) used fluorescence to describe cell populations of the adenohypophysis in the adult goat, in addition to morphological and immunological techniques. Studies have shown that somatomammotrope or bihormonal cells (SMT cells) comprise about 1% of these cells, whereas prolactin cells (PRL cells) and growth hormone cells (GH cells) constitute about 51% and 42% of the total cell population, respectively. SMT cells usually comprise a very low percentage of total cells in mammals (THORPE et al., 1990; WATANABE and HARAGUCHI, 1994), although in the cow they may form up to 26% of all the cells (HASHIMOTO et al., 1987). SMT cells have been postulated to be a cell population different from PRL- and GH cells and unrelated to the physiological state of animal (HASHIMOTO et al., 1987; SÁNCHEZ et al., 1994), or an intermediate stage of interconversion between PRL- and GH cells (PORTER et al., 1991; BERNABÉ et al., 1997), or progenitor cells for PRL- and GH cells (MULEHAHEY and JAFFE, 1988). The last hypothesis predicts that SMT cells should be found in higher percentages in embryonic stages and young animals than in adults the finding by BERNABÉ et al. (1997) of a higher percentage of SMT cells in young sheep than in adult sheep supports this hypothesis.

Details about the differentiation of adenohypophyseal cell populations in the fetus are available for sheep (PARRY et al., 1979), humans (TACHIBANA et al., 1994), and rats (SÉTALO and NAKANE, 1976; WATANABE and HARAGUCHI, 1994; additional references in YAMAMURO et al., 1999). YAMAMURO et al. (1999) have recently shown the existence of bihormonal cells in the rat fetus using double immunofluorescence *in situ*. Using conventional techniques, SINGH and DHINGRA (1979) were only able to identify GH cells in goat fetuses aged 40 days, and PRL cells in goat fetuses aged 70 days. In the present paper we test current hypotheses on the occurence of SMT cells during embryonic development by analyzing the adenohypophysis in goat fetuses aged 100 days.

MATERIAL AND METHODS

Nine goat fetuses of the Murciano-Granadina breed aged 100 days were studied. The hypophysis was sagittally sectioned in two halves: one half was used for light microscopy, and the other for electron microscopy.

Light microscopy

The sagittal half of each gland was fixed in neutral formol 10% and embedded in poliwax (Difco). Sixty serial sagittal sections (4-5 μm thick) were mounted on slides. Double immunolabeling was done by combining indirect immunoalkaline phosphatase (AP) and Avidin-Biotin Comphex immunostaining (AP. ABC). After treatment with normal goat serum

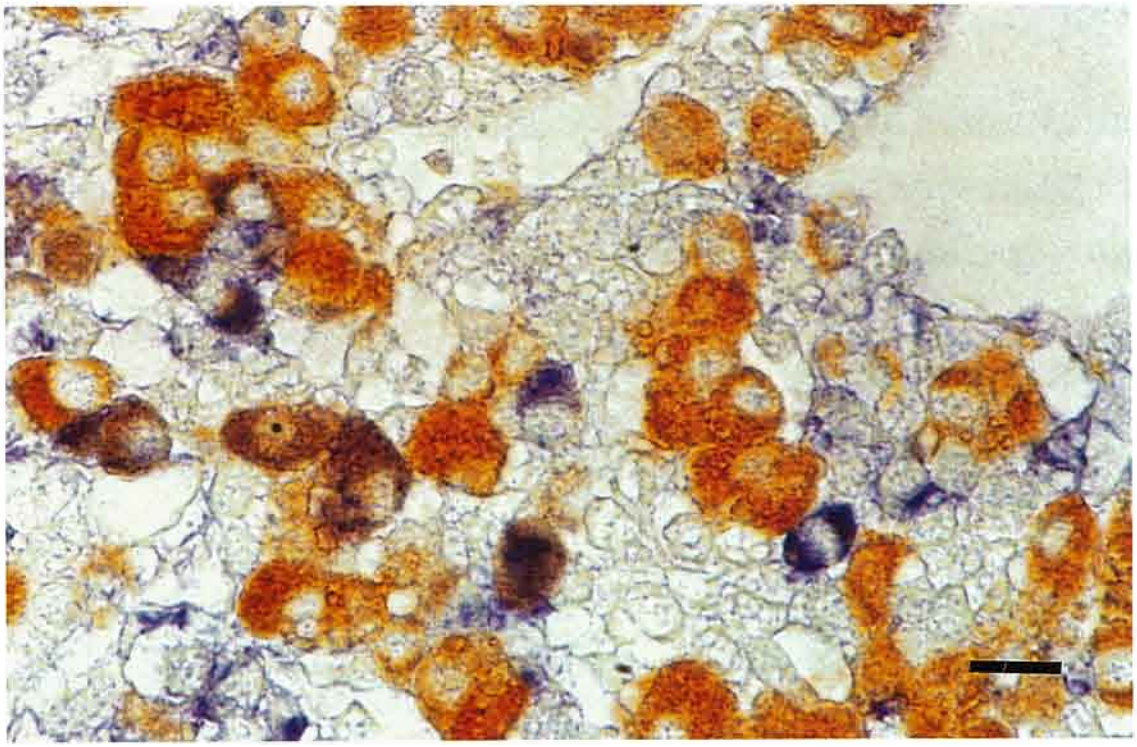

Fig 1. Double immunolabeling of goat fetus adenohypophysis pars distalis. PRL cells are colored brown with diaminobenzidine, and GH cells are stained blue (fast blue). SMT cells show dual stain. Bar=10 μm

(Sigma, EE. UU) at a 1/30 dilution, samples were incubated with rabbit anti-ovine GH (1 : 1000, Biogenesis, England) antiserum for 60 min at 32℃. Biotinylated swine anti rabbit IgG (Dako, Denmark) was used as the second antibody at a 1/250 dilution in TBS (0.05 M. pH 7.6) for 20 min. The slides were rinsed and incubated for 30 min with anti-biotin alkaline phosphatase complex (Sigma, EE. UU), diluted 1 : 100 in the same buffer. After rinsing with TBS, alkaline phosphatase was developed with naphthol-AS-MX-phosphate (0.1 M Tris buffer pH 8.2 containing 0.24 mg levamisol -Sigma - per liter and 1 mg fast blue salt - Sigma - per liter). Sections subsequently treated for 1 h at 60℃ with paraformaldehyde vapour were further incubated with rabbit anti-ovine PRL (1 : 1000, Biogenesis, England) antiserum for 60 min at 32℃. Swine anti-rabbit IgG (Sigma, EE. UU) was used as the second antibody at a 1 : 250 dilution. The reaction was continued with PAP soluble complex (Sigma, EE. UU) at a dilution of 1 : 100, and visualized with 3-3 diaminobenzidine tetrahydrochloride (DAB, Sigma. EE. UU). Slides were mounted with glycer gel (Dako, Denmark).

Electron microscopy

The remaining half of each gland was sectioned into 1 mm^3 blocks, fixed in 3% glutaraldehyde, postfixed with 1% OsO_4 in cacodylate buffer, dehydrated in graded alcohol and propylene oxide, and embedded in Epon. The immunocolloidal gold complex method was performed on one side of nickel grids. Ultrathin sections were etched by floatation on saturated aqueous sodium metaperiodate for 30 min at room temperature, and incubated at 30℃ with either rabbit anti-sheep GH serum at 1 : 2500 dilution in TBS or rabbit anti-sheep PRL serum (Biocell, Biogenesis, England) at a 1 : 2500 dilution. In double immunostainings, the grids were treated for 1 h at 60℃ with paraformadehyde vapour after the first staining according to WANG and LARSSON (1985). The sections were incubated at room temperature for 1 h with 10 or 20 nm colloidal gold goat anti-rabbit IgG (Biocell), diluted 1 : 40 as the secondary antibody. The grids were routinely contrasted with uranyl acetate and lead citrate,

To confirm the specificity of immunostaining, the following controls were done: 1) the specific antisera

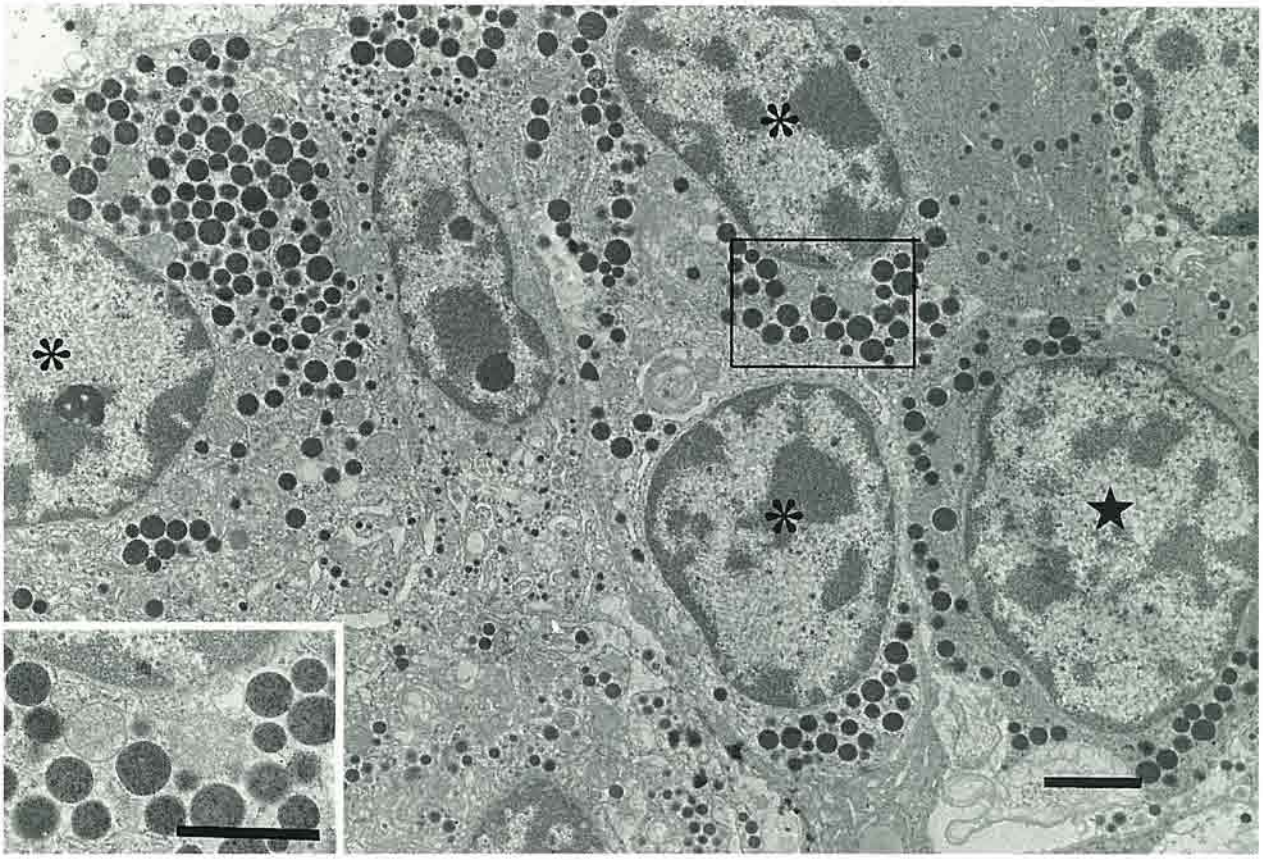

Fig 2. Double gold immunolabeling of goat fetus adenohypophysis pars distalis. *Asterisks* show light GH cells. *Star* shows dark GH cells. **Inset** shows immunolabeling with colloidal gold of 10 nm. Bars=1 μm

were substituted by normal rabbit serum or the buffer solution, and 2) the specific antiserum was preadsorbed with the corresponding hormone (0.1 ml anti-ovine GH 1 : 100 with 10 μg ovine GH or 0.1 ml anti-ovine PRL 1 : 1500 with 10 μg ovine PRL; Biogenesis, England). For light microscopy, controls for cross-reactions were done by adding only the second antibody after omitting the first. No immunostaining was observed in such cases.

Sampling for morphometric analysis was randomly carried out according to AHERNE and DUNNILL's formula (1982); the sample was considered satisfactory when the standard deviation was 5% smaller than the arithmetical mean.

The area and maximum diameter of each cell were calculated with an IMCO 10 Kontron image processor (Microm, Spain). Eight hundred immunoreagent cells of each cell type (GH-, PRL-, or SMT cells) were analyzed at 20 cells per field, 8 fields per slide, 5 slides per cell type. The percentage of positive cells (cell number) to anti-GH, anti-PRL or both sera was manually calculated by computing at random 8 fields of 17000 μm^2 per slide and 5 slides (separated 30 μm) per animal. Fifty electronmicrographs of each cell type (GH/PRL) were utilized per animal for calculating the mean diameter of 20 secretory granules per cell. Cells were divided into three types according to these parameters: type I when the mean diameter of the secretory granules was larger than 500 nm; type II when this parameter ranged from 300 to 500 nm, and type III when the mean diameter was smaller than 300 nm (GÓMEZ et al., 1999).

Data were analyzed by a one-way analysis of variance and applying Scheffe's test to determine the differences between means. The level of significance was taken as $P<0.01$.

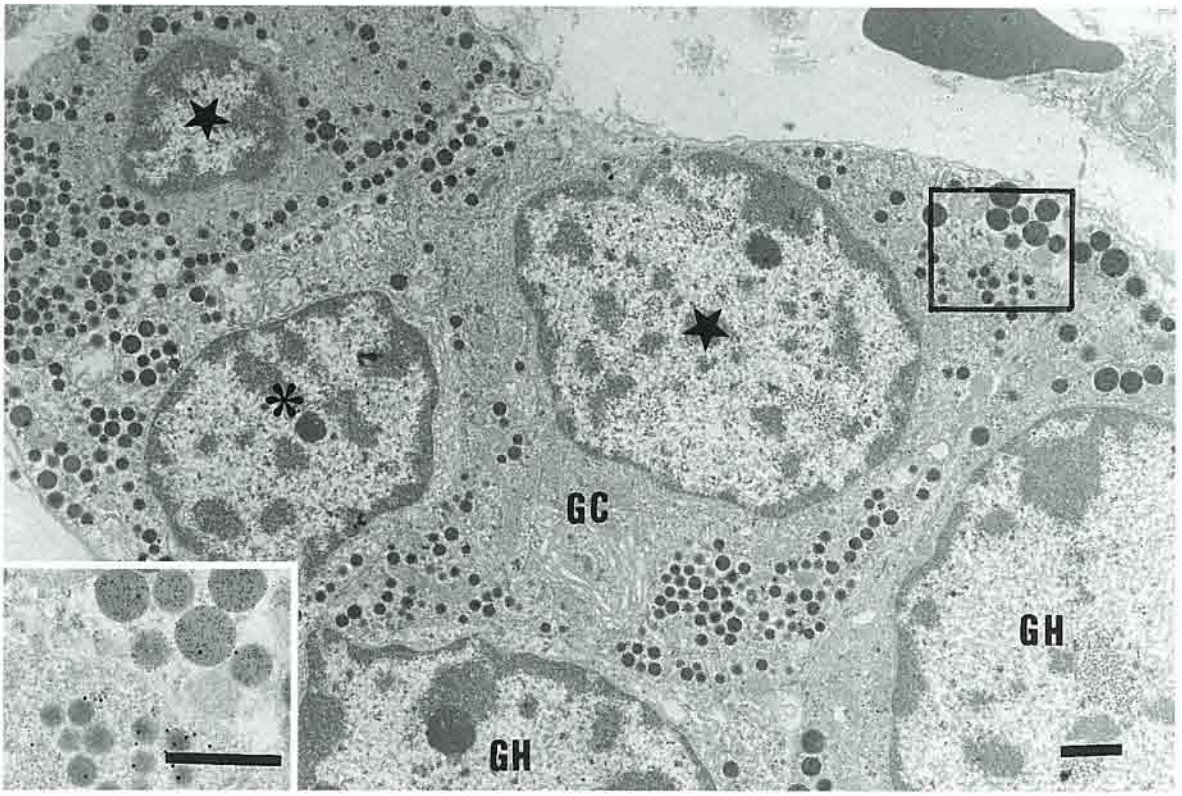

Fig 3. Double gold immunolabeling of goat fetus adenohypophysis pars distalis. Light PRL cell is indicated by an *asterisk*. Dark PRL cells (*stars*) show a moderate number of secretory granules and a small Golgi complex area (*GC*). There are also two dark GH cells (*GH*). **Inset** shows immunolabeling with colloidal gold of 20 nm for PRL- and colloidal gold of 10 nm for GH granules. Bars=1 μm

RESULTS

GH cells

GH cells stain blue with fast blue (Fig. 1). They are distributed througout the whole pars distalis, either in small clusters or isolated, and form larger aggregates only in the dorsal region. Morphometric data are shown in Table 1.

Electron microscopy shows GH cells ranging from few to abundantly electron-dense secretory granules distributed throughout the cytoplasm. The mean size of the secretory granules is 321 $\pm$ 0.06 nm (range 194–668 nm). Type II cells are predominant (Table 2).

Two cell types can be distinguished: dark cells (42%) with a higher number of free ribosomes and parallel long and narrow cisternae of the rough endoplasmic reticulum (RER) surrounding the nucleus, and light cells (58%) with abundant short and dilated cisternae of RER (Fig. 2).

Table 1. Mean diameter, cell number (% of immunopositive cells), and cellular area $\pm$ SD of GH-, PRL- and SMT-cells in the goat fetus.

Cell type	Diameter (μm)	Cell number (%)	Area (μm^2)
GH	8.54$\pm$0.76	15.21	24.56$\pm$4.93
PRL	9.30$\pm$0.10	22.59	29.31$\pm$6.33
SMT	10.03$\pm$0.71	1.59	34.03$\pm$6.18

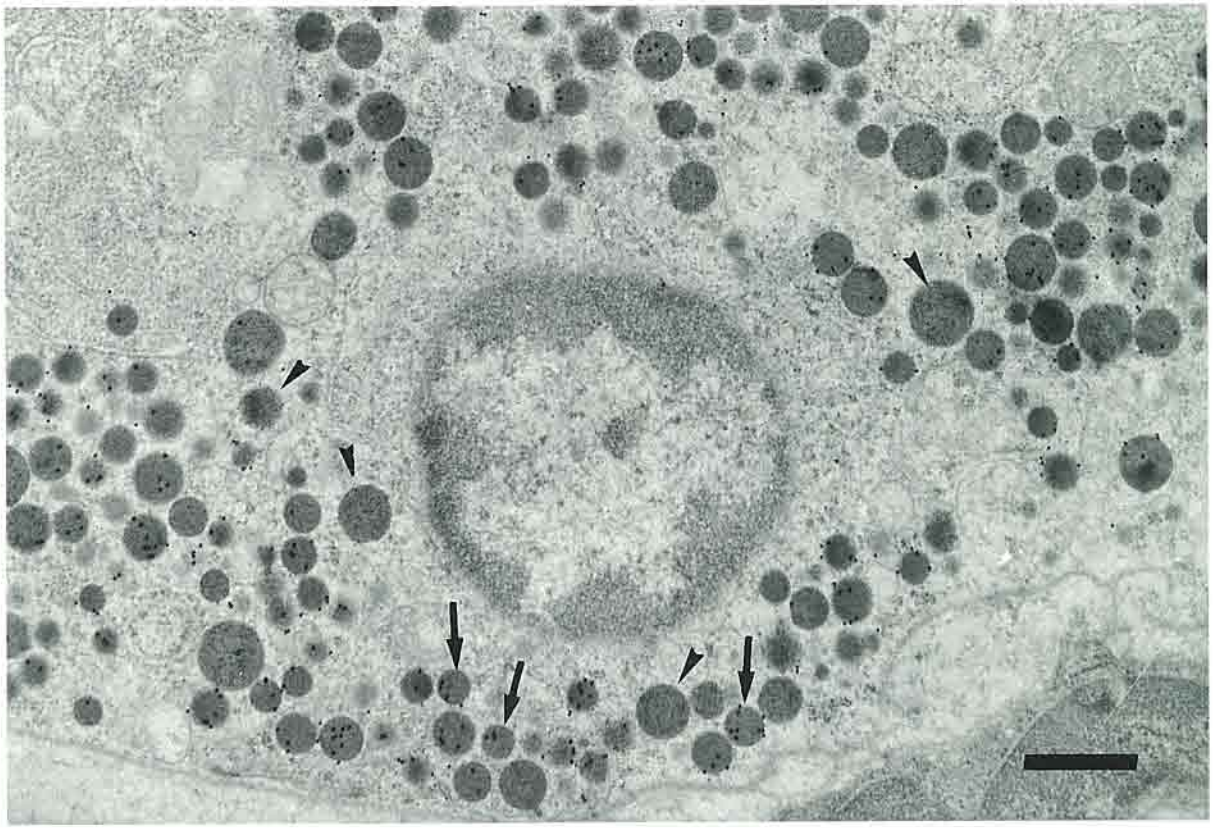

Fig 4. Double gold immunolabeling of goat fetus adenohypophysis pars distalis. The SMT cell shows secretory granules of GH (*arrowheads*) and PRL (*arrows*). Bar=1 μm

Table 2. Parameters of GH- and PRL cells in the goat fetus as grouped into types (type I was not found) according to granule size.

	Type II	Type III
GH %	65.8	34.2
D GH, nm	363±0.04	263±0.02
R GH, nm	300−668	194−298
PRL %	63.8	36.2
D PRL, nm	361±0.04	248±0.03
R PRL, nm	300−568	172−299

D: granule diameter±SD, R: range of granule size.

The Golgi complex is well developed in both cell types. The nucleus is oval and shows abundant euchromatin with one or two nucleoli. Mitochondria are particularly abundant in scarcely granulated cells. The relative abundance of secretory granules in both cell types is shown in Table 3.

PRL cells

PRL cells appear brownish when stained with diaminobenzidine (Fig. 1). They are more frequent in the ventral region where strings of cells can be seen. They show an oval or cylindrical shape. Data on morphometric parameters are shown in Table 1. Electron microscopy reveals these cells as electron-dense secretory granules regularly distributed throughout the cytoplasm (Fig. 3). The mean size of the secretory granules is 308±0.06 nm (range 172-568 nm). Type II cells are predominant (Table 2).

The dark (40%) and the light cell types (60%) can also be distinguished in PRL cells (Fig. 3). The

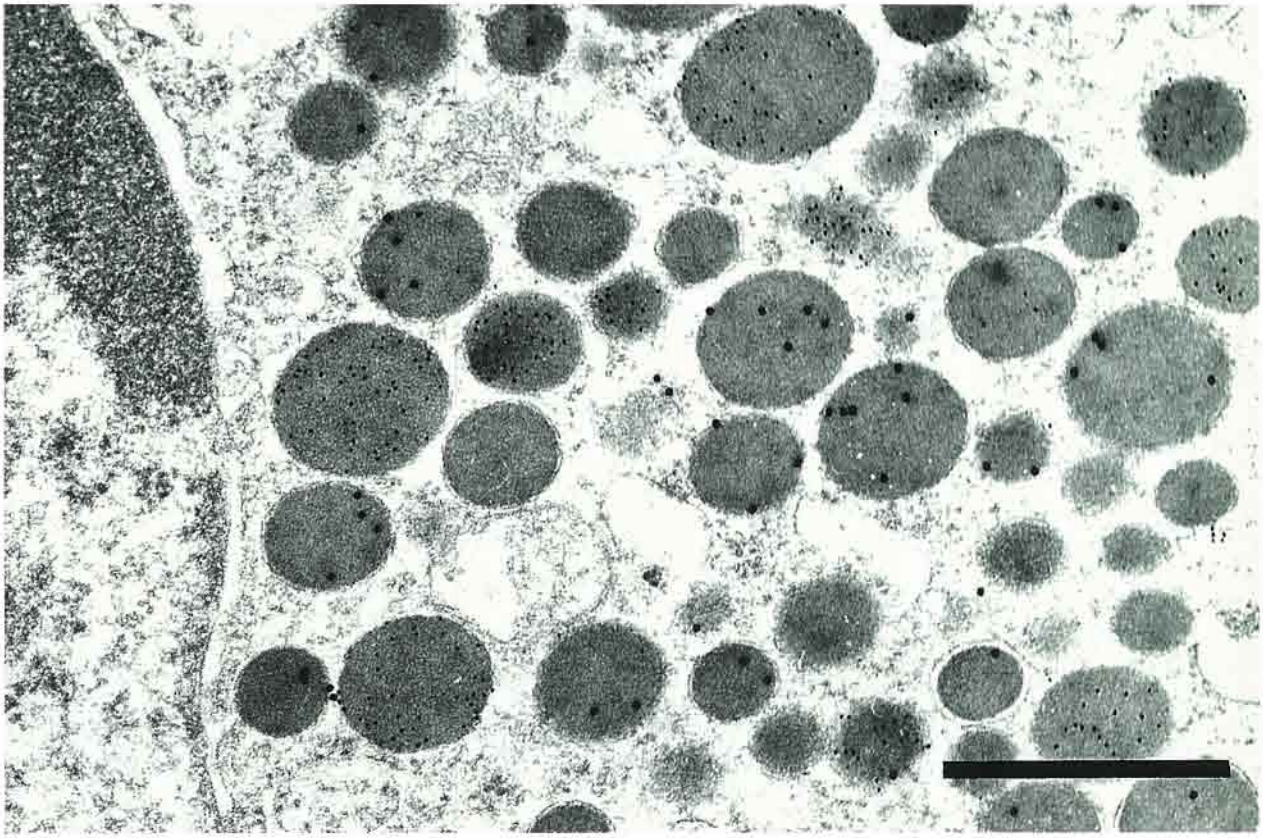

Fig 5. Double gold immunolabeling of goat fetus adenohypophysis pars distalis. Secretory granules contain either GH or PRL. Bar=1 μm

Table 3. Cell diameter and percentage of well/poorly granulated types of light and dark GH- and PRL cells in the goat fetus. Light cells are found in similar percentages in GH- (58%) and PRL (60%) cells.

	GH cells		PRL cells	
	Light cell	Dark cell	Light cell	Dark cell
well granulated	330 nm (77%)	310 nm (12%)	307 nm (75%)	264 nm (58%)
poorly granulated	310 nm (23%)	201 nm (88%)	289 nm (25%)	198 nm (42%)

relative abundance of secretory granules in both cell types is shown in Table 3.

SMT cells

Double staining with diaminobenzidine and fast blue identifies these cells (Fig. 1). They are found in the ventral region of the gland in isolation or in small clusters, and have an oval shape. Data on morphological parameters are shown in Table 1.
By electron microscopy, SMT cells appear with abundant electron-dense secretory granules regularly distributed throughout the cytoplasm. The RER shows narrow dilated cisternae (Fig. 4). There is only one kind of hormone within each secretory granule; those with GH have a mean diameter of 381 nm, whereas those with PRL are slightly smaller (mean diameter 366 nm) (Fig. 5).

DISCUSSION

In this paper we present the first data on the cellular composition of the adenohypophysis of 100-day-old goat fetuses. Using immunogold labeling, we demonstrate the presence of SMT cells, together with GH- and PRL cells.

The cells at this stage of development are clearly differentiated into typical dark and light cell populations. Differentiation of adenohypophyseal cells has usually been reported in the second half of embryonic development of the mammalian fetus, especially close to the end of the fetal period (see YAMAMURO et al., 1999 for references). GH cells are usually the first cells to differentiate, followed by PRL cells. SMT cells have been observed close to term (SÉTALO and NAKANE, 1976; PARRY et al., 1979; TACHIBANA et al., 1994; WATANABE and HARAGUCHI, 1994).

The high number of ribosomes, parallel disposition of RER cisternae, nuclear shape, and the low number of secretory granules are characteristic of dark PRL and GH cells of the fetus, since these features change in the adult (female, SÁNCHEZ et al., 1994; male, GÓMEZ et al., 1999). The dark cell probably represents an immature stage that develops into a light mature cell, as suggested by TACHIBANA et al. (1994) in the human fetus. Dark cells are not found in kid goats (Seva et al., 1998) or in adults (SÁNCHEZ et al., 1994; GÓMEZ et al., 1999). Granules of fetal GH cells are significantly larger than those of PRL cells, perhaps because of a higher demand for GH during embryonic development, and the earlier differentiation of GH cells.

Particularly interesting is the finding that the percentage of SMT cells is only slightly higher in the fetus than in the adult. This argues against the hypothesis of SMT cells being a precursor for both GH and PRL cells, since one would then expect to find a much higher number of SMT cells in the fetus and the young animal. The earlier differentiation of GH cells followed by PRL cells also argues against this hypothesis. However, the possibility can not be excluded that complete differentiation has taken place before 100 days of gestation. Additional studies are required to address this issue.

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