

# Effects of Follicle-Stimulating Hormone on Intermediate Filaments and Cell Division of Sertoli Cells of Fetal Rat Testis in Culture

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**ABSTRACT.** The present study was aimed to examine the effects of follicle-stimulating hormone (FSH) on cell division of Sertoli cells from rat fetal testes and on the kinetics of 2 kinds of intermediate filaments, cytokeratin and vimentin, which comprise the cytoskeleton of Sertoli cells. Testes from rat fetuses of different ages (from day 15 to day 17 of gestation) were cultured for 48 hr, with or without added FSH. In 15-day testes, FSH influenced neither cell division of Sertoli cells nor kinetics of intermediate filaments. In 16-day testes, FSH promoted cell division of Sertoli cells and kinetic differentiation of intermediate filaments distributed toward the lumen of the seminiferous tubules. These findings suggest that 16-day testes in culture can respond to FSH in a fashion that cell division of Sertoli cells is promoted and that intermediate filaments increase in number and change in intracellular distribution. It is concluded that FSH influences both proliferation and morphological differentiation of Sertoli cells. — **KEY WORDS:** cytokeratin, fetal testis, FSH, vimentin.

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Many studies have been made on differentiation and development of rat testes during the fetal period. In the formation of seminiferous tubule in the rat [20] and mouse [3], several mesenchyme-derived cells initially surround primordial germ cells to create a tube-like shape in the gonadal primordium. These mesenchyme-derived cells are considered to differentiate into Sertoli cells thereafter [7, 8].

It has been reported that intermediate filaments, cytokeratin and vimentin, are simultaneously found in Sertoli cells during the fetal period [15]. Cytokeratin disappears on 14 days after birth. Rat Sertoli cells show active cell division and proliferation during the fetal period, but that the activity does not persist for more than 21 days after birth [13].

In the adult rat, follicle-stimulating hormone (FSH) regulates Sertoli cell functions [11]. FSH influences transient shape changes in cultured Sertoli cells [16]. It is known that Sertoli cells possess FSH receptors on the surface of the cellular membrane [9, 10, 12], and that they synthesize and secrete androgen-binding protein in response to FSH [10, 19]. In the fetal rat, FSH can promote cell division of Sertoli cells on day 19 of gestation [14]. However, it remains to be clarified how FSH is associated with proliferation, differentiation and development of fetal Sertoli cells.

The present study was carried out to examine how FSH influences Sertoli cells in cultures of fetal rat testes in terms of the activity of cell division and the kinetics of cytoskeleton, intermediate filaments.

## MATERIALS AND METHODS

Wistar rats were given a commercial diet (Labo MR Breeder) and water, both *ad libitum*. Females were placed with males overnight and were examined the next morning

for the presence of sperm in the vaginal smear. The day on which sperm was detected was counted as day 0 of gestation.

Fetuses of 15–17 days of age were used for culture of their testes. These fetuses were removed from maternal uteri under aseptic conditions and transferred to sterile petri dishes containing Hanks' balanced salt solution (HBSS) with penicillin (100 units/ml) and streptomycin (100 µg/ml). Their testes were removed and rinsed with HBSS. Each testis was placed on the surface of a Millipore filter in the medium (Medium RPMI 1640; Gibco-BRL, U.S.A.) containing 0.2% sodium bicarbonate. Three series of culture were prepared. One was added with no FSH as control (group C). The second was added with 0.5 µg/ml FSH (Sigma, 1 mg equivalent to 1 unit shown by Steelman and Pohley [18]) as group FSH 0.5. The third was added with 5 µg/ml FSH as group FSH 5. The Millipore filter rested on a stainless-steel grid in a culture dish (Nunc, Roskilde, Denmark). All the cultures were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% O<sub>2</sub> for 48 hr. All of the above procedures were performed under sterile conditions.

After incubation, the cultures were immediately frozen in liquid nitrogen. Frozen sections (8 µm) were air-dried for a few min, fixed in acetone at -20°C for 10 min and allowed to be air-dried. The indirect immunofluorescence with amplification by the biotin-streptavidin system was used. Sections were incubated with monoclonal antibody to cytokeratin (clone Lu5, Boehringer Mannheim GmbH, Germany) or vimentin (clone V 9, Biotess International, U.S.A.) and visualized with rhodamine X isothiocyanate (XRITC) or fluorescein isothiocyanate (FITC), respectively.

To measure the proliferative activity of Sertoli cells in cultured testes, in another series of experiments, 10 µM 5-bromo-2'-deoxyuridine (BrdU) was added to the culture medium after 24 hr-incubation and the incubation was continued for 2 hr. Then, each explant was transferred

again to a medium without BrdU and incubated for 22 hr. After incubation, the cultures were fixed in Bouin's fluid, dehydrated in a graded series of ethanol, embedded in Paraplast, and sectioned serially at 4  $\mu$ m. To reveal BrdU immunochemical localization, DNA was denatured in 4 N HCl for 30 min and digested with 0.1% trypsin for 20 min. Sections were incubated with monoclonal anti-BrdU (clone Bu20a, Daco, Denmark) and subjected to the ABC method. To estimate the cell division index, the number of BrdU-positive cells per 2,000 Sertoli cells was determined.

Data were analyzed with Duncan's new multiple range test. A *p* less than 0.05 was considered to be statistically significant.

## RESULTS

When 15-day testes were cultured for 48 hr, immunoreaction with cytokeratin was strongly positive in the basal side of each Sertoli cell as an oval mass in all groups, control, FSH 0.5 and FSH 5. However, neither cells in the interstitial tissue nor primordial germ cells in the seminiferous tubule reacted (Fig. 1a). Immunoreaction with vimentin was also only slightly positive in the basal side of Sertoli cells in all groups, together with strong reaction in the interstitial cells but no reaction in primordial germ cells (Fig. 1b).

When 16-day testes were cultured for 48 hr, immunoreaction with cytokeratin was markedly positive in the basal side of Sertoli cells in all groups (Figs. 2a, b). In

some Sertoli cells, this reaction was also noted in the perinuclear region, the reaction being stronger in groups FSH 0.5 and FSH 5 (Fig. 2b) than that in group C (Fig. 2a). In addition, in some cases in groups FSH 0.5 and FSH 5, the cytokeratin positivity in Sertoli cells appeared to extend toward the lumen of the seminiferous tubule (Fig. 2b). Immunoreaction with vimentin was slightly positive in the basal side of Sertoli cells in group C, where some cells showed positive reaction in the perinuclear region as well (Fig. 2c). Such perinuclear reaction was stronger in groups FSH 0.5 and FSH 5 (Fig. 2d) than in group C (Fig. 2c). The cases showing extension of vimentin positivity toward the lumen of the seminiferous tubules appeared less than those for cytokeratin.

When 17-day testes were cultured for 48 hr, immunoreaction with cytokeratin was markedly positive in the perinuclear region of Sertoli cells in all groups. Cytokeratin positive reaction extending toward the lumen of the seminiferous tubules were encountered more often (Fig. 3a). In all groups, Sertoli cells showed strong reactivity with vimentin in the perinuclear region, with only a few vimentin extensions towards the lumen of the seminiferous tubule (Fig. 3b).

Overall results showed that the stimulative effect of FSH on the cellular distribution of cytokeratin and vimentin was not different between the two different doses added throughout this experimental duration.

The cell division indices of BrdU-positive Sertoli cells are shown in Table 1. In 15-day testes, the cell division

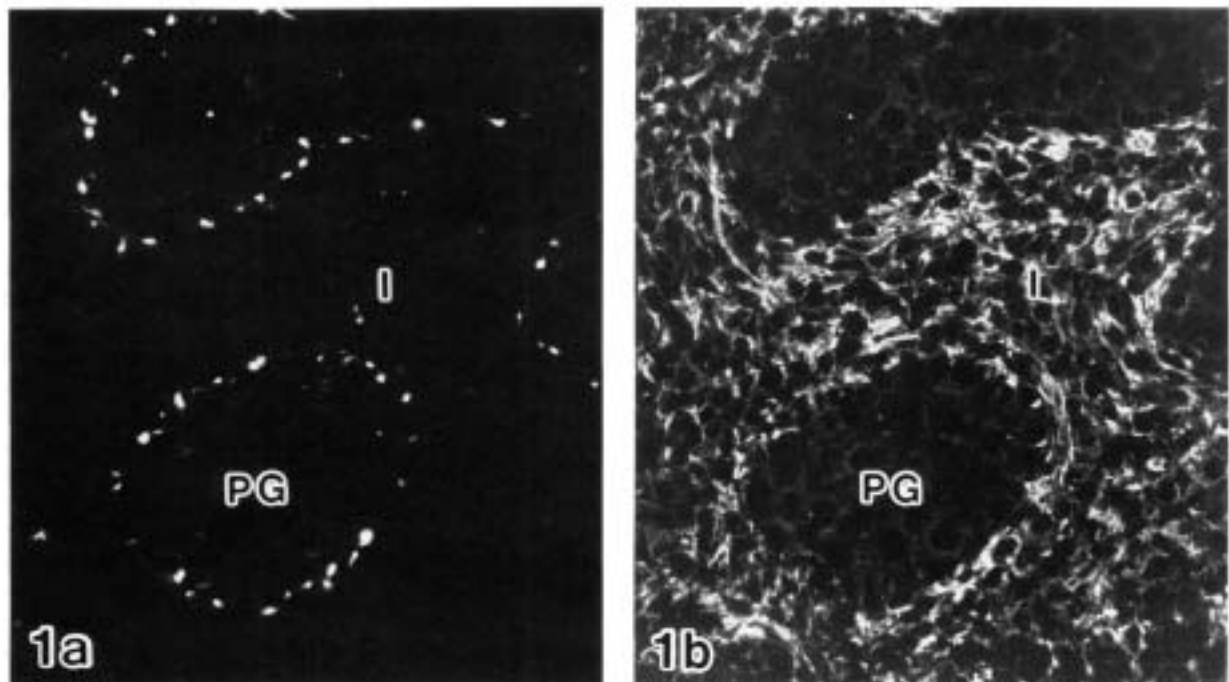


Fig. 1. Double immunoreaction in the same section of 48 hr culture of a 15-day testis in group C showing localization of cytokeratin (a) and vimentin (b). Immunoreaction with cytokeratin is strong in the basal side of Sertoli cells. Both primordial germ cells (PG) and interstitial tissues (I) are negative for cytokeratin (a). Immunoreaction with vimentin is weak in the basal region of Sertoli cells. Primordial germ cells (PG) are negative and interstitial tissues (I) are strongly positive for vimentin.  $\times 370$ .

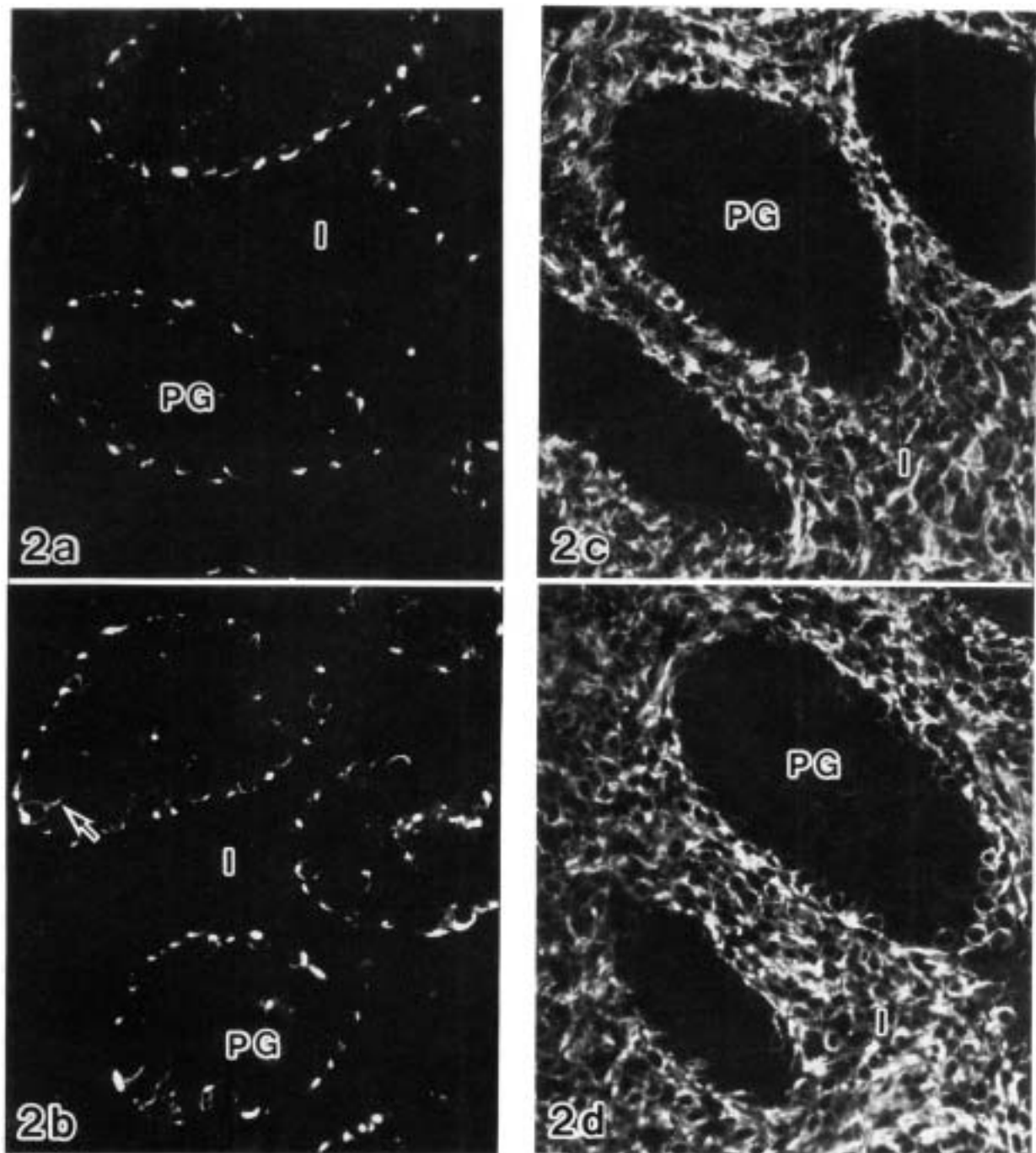


Fig. 2. Sections of 48 hr cultures of 16-day testes showing localization of cytokeratin (a, b) and vimentin (c, d). (a, c: group C, b, d: group FSH 0.5). Sertoli cells show stronger reactivity with cytokeratin in group FSH (b) than that in group C (a). Cytokeratin filaments sometimes extend from the basal to the apical side of the tubules (arrows). Vimentin filaments exist in the perinuclear region in Sertoli cells, more numerous in group FSH (d) than in group C (c). I: interstitial tissues, PG: primordial germ cells.  $\times 370$ .

index did not differ among the 3 groups. However, in 16-day testes, the index in the two FSH groups was significantly larger than that in group C. In 17-day testes the index did not differ between group C and group FSH 0.5. However, the index in group FSH 5 was significantly larger than that

in group C.

#### DISCUSSION

In the fetal rat, FSH is found in plasma on day 16 of

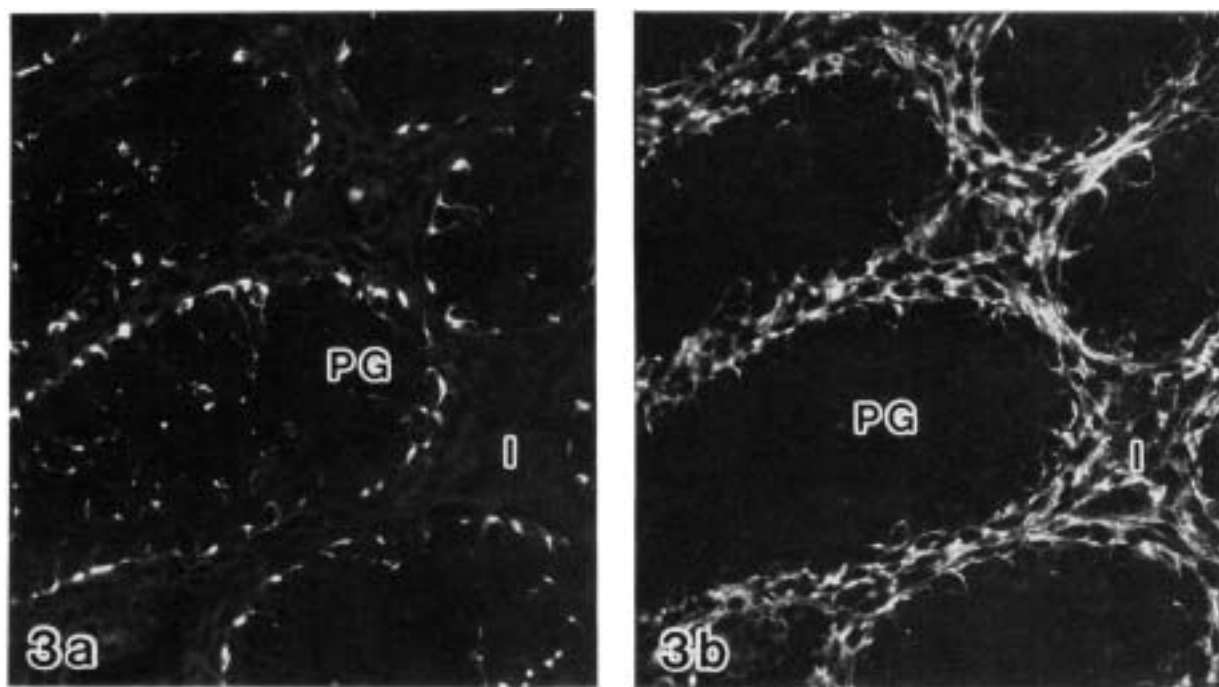


Fig. 3. Double immunoreaction in the same section of a 17-day testis in group C showing localization of cyokeratin (a) and vimentin (b). Many Sertoli cells show strong reactivity with cyokeratin from the basal to the apical side of the tubules (a). Many vimentin filaments are observed in the perinuclear region of Sertoli cells, sometimes being oriented parallel to the long axis of the cells (b). I: interstitial tissues, PG: primordial germ cells.  $\times 370$ .

Table 1. Cell division index of Sertoli cells of 15- to 17-day testes in FSH 0.5, FSH 5, and control groups

Age of testis in days	Group	No. of samples	Cell division index (%)
15	Control	10	$22.3 \pm 0.8$
	FSH 0.5	10	$22.4 \pm 0.7$
	FSH 5	10	$22.6 \pm 0.7$
16	Control	10	$24.4 \pm 0.3$
	FSH 0.5	9	$29.3 \pm 0.6^*$
	FSH 5	9	$29.7 \pm 0.6^*$
17	Control	9	$30.1 \pm 0.4$
	FSH 0.5	9	$30.6 \pm 0.6$
	FSH 5	8	$33.8 \pm 0.6^*$

Cell division index: the number of BrdU positive cells per 2,000 Sertoli cells. \*: Significantly different from the control group ( $P < 0.05$ ).

gestation. FSH levels rapidly decrease on day 17, followed by a gradual increase thereafter [2]. Furthermore, it has been reported that FSH receptors in the fetal testis appear on day 17.5 of gestation, and that the level of FSH receptors remains low until day 19.5, followed by a rapid increase thereafter [21]. It has also been demonstrated that FSH stimulates Sertoli cells of fetal and neonatal rat testes to promote cell division [6, 14].

In this experiment, FSH treatment promoted cell division in Sertoli cells from fetal 16-day testes cultured, consistent with the result by Orth [14]. However, in 17-day testes, lower dose of FSH in this experiment failed to show any

increase in cell division index as compared with higher dose. The reason for this is unknown at present, but it may be that FSH receptors in 17-day testes are relatively too developed to be stimulated any more with low level of FSH.

Several investigators have reported the kinetics of intermediate filaments during the developmental process of the seminiferous tubule in rat testes. When somatic cells of the urogenital ridge differentiate into precursor Sertoli cells, an intermediate filament, desmin, is substituted for cyokeratin, a main composer of the cytoskeleton of epithelial cells. Fröjdman *et al.* [4, 5] suggested that the appearance of cyokeratin in precursor Sertoli cells is associated with the cellular polarity acquired by extending the cytoplasm from the basement side to the lumen of the seminiferous tubule. Vimentin also appears in the basal area of Sertoli cells on day 12 of gestation and seems to extend the cytoplasm of Sertoli cells toward the lumen of the seminiferous tubule during the end of gestation. After birth, the extension further continues to allow vimentin to be main cytoskeleton of Sertoli cells [15]. Therefore, intermediate filaments may play an important role in the morphological changes and cellular polarity of Sertoli cells. The disappearance or existence of intermediate filaments, that is, polymerization and depolymerization of filaments, may be reversibly regulated in accordance with the developmental process of Sertoli cells and environmental conditions.

In this experiment, when 15-day testes were cultured,

there were a large number of cytokeratin filaments detected in the basal cytoplasm of Sertoli cells. However, in 16-day testes cultured, the detection site of cytokeratin extended to the perinuclear region. In 17-day testes cultured, cytokeratin appeared to form a cone toward the lumen of the seminiferous tubule. Furthermore, localization of vimentin was initially found in the basal cytoplasm of Sertoli cells, and then in the perinuclear region. During this period, Sertoli cells were initially cube- or column-shaped, and gradually became cone-shaped toward the lumen of the seminiferous tubule, acquiring polarity. Thus, it seems that these intermediate filaments contribute to morphological differentiation of Sertoli cells before birth. Furthermore, in 16-day testes cultured with FSH, localization of cytokeratin in Sertoli cells became similar to that in 17-day testes cultured without FSH. Therefore, it can be said that Sertoli cells in 16-day testes are already sensitive to FSH, or otherwise they have acquired sensitivity while cultured for 48 hr.

It has been shown that cAMP-dependent protein kinase (PKA) is activated when postnatal Sertoli cells are cultured with FSH and cAMP, and that vimentin in Sertoli cells is phosphorylated by activated PKA, and thus phosphorylated vimentin induces cell shape changes [17]. This may be the case in the present study on fetal Sertoli cells. On the other hand, phosphorylation of cytokeratin by PKA in Sertoli cells has not yet been reported, although demonstrated in other cells such as hepatic cells [1, 22]. It may be assumed that phosphorylation of cytokeratin also occurs in Sertoli cells, promoting changes in the intracellular distribution of cytokeratin to cause cell shape changes in Sertoli cells as well.

In conclusion, the present results suggest that FSH simultaneously facilitates not only proliferation of Sertoli cells but also morphological differentiation of Sertoli cells.

## REFERENCES

- Ando, S., Tokui, T., Yano, T. and Inagaki, M. 1996. Keratin 8 phosphorylation *in vitro* by cAMP-dependent protein kinase occurs within the amino- and carboxyl-terminal end domains. *Biochem. Biophys. Res. Commun.* 221: 67–71.
- Chowdhury, M. and Steinberger, E. 1976. Pituitary and plasma levels of gonadotrophins in foetal and newborn male and female rats. *J. Endocrinol.* 69: 381–384.
- Eguchi, Y. and Hashimoto, Y. 1961. Histological development of the testis of the mouse during embryonic stages. *Bull. Univ. Osaka Pref.* 11: 77–83.
- Fröjdman, K., Paranko, J., Kuopio, T. and Pelliniemi, L. J. 1989. Structural proteins in sexual differentiation of embryonic gonads. *Int. J. Dev. Biol.* 33: 99–103.
- Fröjdman, K., Paranko, J., Virtanen, I. and Pelliniemi, L. J. 1992. Intermediate filaments and epithelial differentiation of male rat embryonic gonad. *Differentiation* 50: 113–123.
- Griswold, M. D., Solari, A., Tung, P. S. and Fritz, I. B. 1977. Stimulation by follicle-stimulating hormone of DNA synthesis and of mitosis in cultured Sertoli cells prepared from testes of immature rats. *Mol. Cell. Endocrinol.* 7: 151–165.
- Jost, A. 1972. Données Préliminaires sur les stades initiaux de la différenciation du testicule chez le rat. *Arch. Anat. Micro.* 61: 415–438.
- Magre, S. and Jost, A. 1980. The initial phases of testicular organogenesis in the rat. *Arch. Anat. Micro.* 69: 297–318.
- Means, A. R. and Vaitukaitis, J. 1972. Peptide hormone "Receptors": Specific binding of  $^3\text{H}$ -FSH to testis. *Endocrinology* 90: 39–46.
- Means, A. R., Fakunding, J. L., Huckins, C., Tindall, D. J. and Vitale, R. 1976. Follicle-stimulating hormone, the Sertoli cell, and spermatogenesis. *Recent. Prog. Horm. Res.* 32: 477–527.
- Means, A. R., Dedman, J. R., Tash, J. S., Tindall, D. J., Van Sickle, M. and Welsh, M. J. 1980. Regulation of the testis Sertoli cell by follicle stimulating hormone. *Annu. Rev. Physiol.* 42: 59–70.
- Orth, J. and Christensen, A. K. 1977. Localization of  $^{125}\text{I}$ -labeled FSH in the testes of hypophysectomized rats by autoradiography at the light and electron microscope levels. *Endocrinology* 101: 262–278.
- Orth, J. M. 1982. Proliferation of Sertoli cells in fetal and postnatal rats: A quantitative autoradiographic study. *Anat. Rec.* 203: 485–492.
- Orth, J. M. 1984. The role of follicle-stimulating hormone in controlling Sertoli cell proliferation in testes of fetal rats. *Endocrinology* 115: 1248–1255.
- Paranko, J., Kallajoki, M., Pelliniemi, L. J., Lehto, V.-P. and Virtanen, I. 1986. Transient coexpression of cytokeratin and vimentin in differentiating rat Sertoli cells. *Dev. Biol.* 117: 35–44.
- Spruill, W. A., White, M. G., Steiner, A. L., Tres, L. L. and Kierszenbaum, A. L. 1981. Temporal sequence of cell shape changes in cultured rat Sertoli cells after experimental elevation of intercellular cAMP. *Exp. Cell Res.* 131: 131–148.
- Spruill, W. A., Steiner, A. L., Tres, L. L. and Kierszenbaum, A. L. 1983. Follicle-stimulating hormone-dependent phosphorylation of vimentin in cultures of rat Sertoli cells. *Proc. Natl. Acad. Sci. U.S.A.* 80: 993–997.
- Steelman, S. L. and Pohley, F. M. 1953. Assay of the follicle stimulating hormone based on the augmentation with human chorionic gonadotropin. *Endocrinology* 53: 604–616.
- Tindall, D. J., Vitale, R. and Means, A. R. 1975. Androgen binding protein as a biochemical marker of formation of the blood-testis barrier. *Endocrinology* 97: 636–648.
- Torrey, T. W. 1945. The development of the urinogenital system of the albino rat. II. The gonads. *Am. J. Anat.* 76: 375–397.
- Warren, D. W., Huhtaniemi, I. T., Tapanainen, J., Dufau, M. L. and Catt, K. J. 1984. Ontogeny of gonadotropin receptors in the fetal and neonatal rat testis. *Endocrinology* 114: 470–476.
- Yano, T., Tokui, T., Nishi, Y., Nishizawa, K., Shibata, M., Kikuchi, K., Tsuiki, S., Yamauchi, T. and Inagaki, M. 1991. Phosphorylation of keratin intermediate filaments by protein kinase C, by calmodulin-dependent protein kinase and by cAMP-dependent protein kinase. *Eur. J. Biochem.* 197: 281–290.