The effects of FSH and of testosterone on the completion of meiosis and the very early steps of spermiogenesis of the rat: an *in vitro study*

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Abstract

The role of FSH and of testosterone in spermatogenesis has been a matter of controversy. In the present study, we addressed the involvement of these hormones in the regulation of the completion of meiosis of male rats under *in vitro* conditions. In the first series of experiments, middle/late pachytene spermatocytes were cocultured with Sertoli cells for 2 weeks in the absence or presence of FSH and/or testosterone. Treatment with both FSH and testosterone reduced slightly the percentage of apoptotic germinal cells in the cultures. Moreover, the number of round spermatids formed *in vitro* was enhanced by FSH or testosterone when compared with control cultures. Neither hormone influenced the half-life of round spermatids under the present culture conditions. The amounts of TP1 mRNAs in FSH- or FSH plus testosterone-treated cultures were higher than those of controls. In another series of experiments, round spermatids were incubated for 24 h in media conditioned by Sertoli cells cultured in the absence or presence of FSH and/or testosterone. TP1 mRNA contents of round spermatids incubated in media from Sertoli cells cultured in the presence of FSH and/or testosterone were two- to threefold higher than those of spermatids incubated in media from Sertoli cells cultured without hormones. These results indicate that FSH and testosterone have positive and somewhat overlapping effects on the meiotic divisions and the post-meiotic expression of a germ cell-specific gene, effects which cannot be related solely to their ability to reduce germinal cell apoptosis. Use of this culture system should help to test the effect of any hormone or factor on those steps in order to understand better their regulation.

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Introduction

The role of follicle-stimulating hormone (FSH) and testosterone in spermatogenesis has been the subject of numerous studies and controversies for several decades (Sharpe 1994, McLachlan *et al.* 2002). Indeed, what would seem to be a relatively simple question to answer has remained unclear. The elucidation of the role of each hormone *in vivo* requires perturbation of the system by a variety of experimental conditions: hypophysectomy (Ahmad *et al.* 1975, Russell *et al.* 1987), treatment with a gonadotropin-releasing hormone (GnRH) antagonist (Sinha Hikim & Swerdloff 1993, Billig *et al.* 1995), immunoneutralization of gonadotropin (Marathe *et al.* 1995, Shetty *et al.* 1996) or GnRH (McLachlan *et al.* 1995), treatment with ethane dimethanesulfonate, a cytotoxic compound for Leydig cells (Henriksen *et al.* 1995) or steroid implants (McLachlan *et al.* 1994), or use of specific animal models such as the hypogonadal mouse (O'Shaughnessy *et al.* 1992) or mice rendered deficient in FSHβ (Kumar *et al.* 1997) or FSH receptor (Dierich *et al.* 1998) by knockout (KO) strategies. However, it has become clear that it is difficult *in vivo* to perturb testosterone production independently of gonadotropin secretion and reciprocally (Sinha Hikim & Swerdloff 1993, McLachlan *et al.* 1994). Moreover, obviously, the number of germ cell types at any stage of the seminiferous epithelium is dependent on the germ cell populations present at the preceding stages, a fact which may complicate, or even prevent, the interpretation of the data when some germ cell type

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numbers have become very low. Furthermore, the above studies examined the number and the state (healthy, degenerating or apoptotic) of germinal cells, but none dealt with the effect of testosterone and/or FSH on the expression of germ cell-specific genes. We have established (Weiss *et al.* 1997) a culture system that allows the study, in a chemically defined medium, of the expression of male germ cell-specific genes during the meiotic process. In the present study, we have used this culture system to address the specific roles of FSH and testosterone in the transformation of pachytene spermatocytes (PS) into round spermatids (RS) and in the expression of genes specific to PS or RS.

Materials and methods

Isolation and coculture of rat Sertoli cells and pachytene spermatocytes (PS)

Sertoli cells and PS were isolated as previously described (Weiss *et al.* 1997). Briefly, Sertoli cells were isolated from 20-day-old Sprague-Dawley rats by enzymatic digestion and plated in bicameral chambers (area 1 cm^2; polyester membrane, pores 0·4 μm diameter) (Falcon, Becton-Dickinson, Meylan, France) at a density of about 3×10^5 cells/cm^2. Cells were then cultured for 3 days in HEPES-buffered F12/DMEM supplemented with insulin (10 μg/ml), transferrin (10 μg/ml), vitamin C (10^{-4} M), vitamin E (10 μg/ml), retinoic acid ($3{\cdot}3 \times 10^{-7}$ M), retinol ($3{\cdot}3 \times 10^{-7}$ M), pyruvate (1 mM) (all products from Sigma, La Verpillière, France), 0·2% fetal calf serum (Life Technologies, Cergy-Pontoise, France) in the absence or presence of 10^{-7} M testosterone (Sigma) and/or 1 ng/ml ovine NIH FSH-20, obtained through NIDDK and Dr A.F. Parlow (lot no. AFP-7028D).

On day 3 of culture (referred to as day 0 of coculture), PS obtained from adult Sprague-Dawley rats by centrifugal elutriation (Onoda *et al.* 1991) were seeded (3×10^5 cells/cm^2) on Sertoli cells. The purity of the PS fraction was assessed by flow cytometry (see below) (94 ± 3% of the cells were 4C cells, 3 ± 2% were 2C cells and 1 ± 0·5% were 1C cells, $n=5$). The staging of the PS seeded was established as follows. In the first experiment, the large and the small diameters of the nuclei of the PS were measured at every stage of the seminiferous epithelium on stained histologic sections of testes from adult rats; then, the value obtained after addition of the two diameters was expressed as a percentage of the values obtained for diplotene spermatocytes (stage XIII), which is the largest type of spermatocyte. Thus, three classes of PS were defined: early PS (stages XIV–IV), the sum of the two nucleus diameters (14·0–15·7 μm), which ranged from 59% to 66% of the value obtained for diplotene spermatocytes; middle PS (stages V–IX) (16·5–20·9 μm) (69–88%) and late spermatocytes (stages X–XIII) (21·6–23·8 μm) (91–100%) (Perrard *et al.* 2003). In the second experiment, total germ cell preparations from adult rats were cytospun on glass slides and stained with hematoxylin/eosin. The two diameters of the spermatocytes observed (range 19·0–32·0 μm) were measured and expressed, as above, as a percentage of the value obtained for the largest spermatocytes found in these preparations (early PS 19·0–21·1 μm; middle PS 22·0–28·2 μm; late PS 29·0–32·0 μm). In the third experiment, elutriated spermatocytes were cytospun and stained, and their diameters were measured; the values obtained were expressed as a percentage of the largest values found in total germ cell preparations (second experiment). Thus, it was found that 13 ± 1% of elutriated PS were early PS, 61 ± 5% were middle PS and 26 ± 4% were late PS ($n=3$). In some experiments, adult rats were injected with 50 mg/kg 5-bromodeoxyuridine (BrdU, Sigma) 13 or 14 days before they were killed in order to label PS of stages V–VIII or VII–XII respectively (Weiss *et al.* 1997). Cocultures were carried out for 2 weeks in the above medium without serum at 33 °C in a humidified atmosphere of 95% air:5% CO_2. The medium in the basal compartment was changed every second day. At the end of the culture period, the medium in the apical compartment was centrifuged, and the tiny cell pellet, if any, was added to the cell layer before RNA preparation, or pooled with the cells detached from the culture wells for cell sorting or identification of apoptotic cells (see below).

Isolation of round spermatids (RS) and coculture with Sertoli cells

RS from adult (90–120-day-old) Sprague-Dawley rats were isolated as previously described (Weiss *et al.* 1997), and then seeded ($3{\cdot}4 \times 10^5$cells/cm^2) on Sertoli cells and cocultured for 4 days in the absence or presence of 10^{-7} M testosterone

and/or 1 ng/ml ovine FSH as above. The purity of the RS fraction was assessed by flow cytometry (see below) (81 ± 2% of cells were 1C cells, 5 ± 1% were 2C cells and 10 ± 2% were 4C cells, $n=3$).

Preparation of Sertoli cell conditioned medium (SCCM)

Rat Sertoli cells were plated in tissue-culture flasks at a density of $0{\cdot}2 \times 10^6$ cells/cm^2 and were cultured for 3 days in the above medium supplemented with 0·2% fetal calf serum in the absence or presence of FSH and/or testosterone or 10^{-9} M ACTH. On day 3, the medium was replaced by serum-free medium supplemented or not with hormones. On day 5, culture medium was recovered, centrifuged to eliminate cell debris and stored at –20 °C until used. Four SCCM were prepared: control (no FSH, no testosterone (F-/T-)), FSH (1 ng/ml) (F+/T-), testosterone 10^{-7} M (F-/T+) and a combination of both hormones (F+/T+).

Preparation of conditioned media from adrenocortical cells

Rat adrenocortical cells were isolated by the procedure described previously for bovine adrenocortical cells (Penhoat *et al.* 1994) and plated in tissue-culture flasks at a density of $0{\cdot}2 \times 10^6$ cells/cm^2 in Hepes-buffered F12/DMEM supplemented as for Sertoli cells (see above) and containing 1% fetal calf serum. On the day after, the medium was replaced by serum-free medium supplemented or not with 10^{-9} M ACTH or FSH plus testosterone. On day 3, the culture medium was recovered, centrifuged and conserved at –20 °C until used. Adrenocortical cells were chosen as they could be cultured in the same medium as Sertoli cells.

Incubation of RS in conditioned medium

RS isolated from 32-day-old Sprague-Dawley rats by centrifugal elutriation were incubated ($1{\cdot}0 \times 10^6$ cells/1·5 ml) for 24 h in the different conditioned medium half-diluted with fresh culture medium. Of cells present in RS preparations, 55 ± 2% ($n=7$) were 1C cells, 27 ± 1% were 2C cells and 8 ± 2% were 4C cells. In these experiments, 32-day-old animals were used, since, at that age, TP1 expression is not yet maximal (Marret *et al.* 1998).

RNA preparation and analysis

At selected days of the cocultures, or after incubation of RS, total RNA was extracted by the method of Chomczynski and Sacchi (1987).

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA extracted from cocultures of Sertoli cells–PS was analyzed by RT-PCR. Sequences corresponding to TP1 mRNAs and TH2B mRNAs were amplified by RT-PCR in the presence of [α-33P] dATP (ICN, Orsay, France), as previously described (Weiss *et al.* 1997, Hue *et al.* 1998).

Northern blot

The amounts of TP1 mRNAs in RS fractions were determined by Northern blot. Samples (6 μg of total RNA) were separated by electrophoresis through a 1% agarose gel containing 10% formaldehyde. RNA was then transferred to a Hybond-N membrane (Amersham, Les Ulis, France) and cross-linked to the membrane by baking at 80 °C for 2 h and by irradiation for 13 s with u.v. light. Prehybridization and hybridization were performed at 42 °C for 2 h and overnight respectively. The rat cDNA probe for TP1 (175 bp) was obtained after amplifying the TP1 sequence by RT-PCR. Labeling of this probe with [α-32P] dCTP (Amersham) was performed with a Megaprime DNA labeling system (Amersham). The labeled probe was added to hybridization solutions at a concentration of 1×10^6 d.p.m./ml. The blots were then washed twice in 2 × SSC (1 × SSC = 150 mM NaCl and 15 mM sodium citrate, pH 7·4), SDS 0·1% for 15 min at room temperature; twice in 1 × SSC, SDS 0·1% for 15 min at 65 °C; twice in 0·5 × SSC, SDS 0·1% for 15 min at 65 °C and twice in 0·2 × SSC, SDS 0·1% for 15 min at 65 °C, and then exposed to photographic film. The relative intensities of hybridization signals were quantified with a scanning densitometer (SAMBA 2005; Alcatel TITN, Meylan, France). Portions of membrane corresponding to 28S rRNA were hybridized with a radiolabeled 28S oligonucleotide probe (Barbu & Dautry 1989). All values for TP1 mRNAs levels were normalized relative to levels of 28S to correct for potential differences in the amounts of loaded RNA.

Identification of apoptotic cells by annexin V-binding assay

Cells were isolated at selected days of culture by IX trypsin-EDTA (Life Technologies) for 3–5 min. Then, soybean trypsin inhibitor (Sigma) was added to stop trypsin action, and the cells were placed in a humidified atmosphere of 95% air:5% CO_2 for 1 h at 33 °C. The cells were then centrifuged, and cell pellets were washed twice. The analysis of phosphatidylserine on the outer leaflet of apoptotic cell membrane was performed with annexin-V-fluos (Roche Diagnostics, Meylan, France) and propidium iodide (Sigma) to differentiate necrotic cells from apoptotic cells, according the instructions of the manufacturer. Then, cells were fixed in 4% paraformaldehyde for 10 min, at 4 °C and washed twice in 1 × PBS.

Immunocytochemical reaction against vimentin

To distinguish between somatic cells and germ cells, an immunocytochemical reaction against vimentin (solely expressed by somatic cells in the testis) was performed as follows. The cells were deposed on 3-aminopropyltriethoxysilane pre-treated slides and permeabilized with Triton X-100 0·03% in PBS. The cells were then incubated with 3% hydroxide peroxide (Gifrer and Barbezat, Décines, France) for 5 min in a humidified dark chamber at room temperature, rinsed with PBS, and then incubated for 10 min with a mouse monoclonal antibody (clone v9), which recognizes vimentin filaments (at 1:1000 dilution in antibody diluent). After washing in PBS, the cells were incubated with a second biotinylated antibody (Multilink-biotin anti-goat, mouse and rabbit antibody) for 10 min (at 1:150 dilution in antibody diluent). Then, the cells were washed for 10 min in PBS. The staining reaction was performed with streptavidin-biotinylated horseradish peroxidase (StrepABC Complex/HRP) and diaminobenzidine as a chromogen (all products from Dako, Trappes, France).

Quantification of apoptotic germ cells

Slides were examined under light and fluorescence microscopy with an Axioscope microscope (Carl Zeiss, Oberkochen, Germany). Two measures were performed: first, germ cells were identified (at least 500 cells were counted, in duplicate samples) by light microscopy (germ cells were vimentin negative); second, the number of apoptotic germ cells was determined by fluorescence microscopy. Necrosis represented 3–5% of positive annexin-V-fluos cells, and no variations were observed during cocultures. The validity of this method was assessed by treating cocultures with different concentrations of H_2O_2, which induced a dose-dependent increase in the number of apoptotic germinal cells (data not shown).

Identification of apoptotic cells by TUNEL assay

Cells were isolated at selected days of culture by trypsin-EDTA as above and fixed in cold ethanol 70%. Cells were permeabilized with 0·25% Triton X100 in PBS for 20 min on ice. The cells were exposed to an antivimentin antibody (see above) at 1:500 dilution. After three washes in PBS, the cells were exposed to fluorescein (FITC)-conjugated rabbit antimouse immunoglobulins (Ig) (IgG, Dako) used at a dilution of 1:60 in PBS for 1 h at 4 °C. TUNEL assay was performed with the *In Situ* Cell Death Detection Kit, TMR Red (Roche Diagnostics), according to the instructions of the manufacturer. Before analysis, Hoechst 33342 (Sigma) was added to the suspensions of labeled cells at a final concentration of 20 μg/ml. Two controls were included: 1. cells incubated with mouse IgG1 negative control (Dako) and FITC-conjugated secondary antibody; 2. cells incubated with label solution without terminal transferase. Cells were then analyzed by flow cytometry (see below).

Flow cytometry

Flow cytometry analysis of both germ cells and somatic cells was performed as described by Godet *et al.* (2000, 2004).

Immunocytochemical studies on cultured cells

Cells were rinsed with PBS and fixed with Bouin's fixative at room temperature. After 5 washes with PBS, immunocytochemical reactions against a 90-kDa protein specific to the acrosome (Tanii *et al.* 1994) or BrdU were performed as previously described in detail (Weiss *et al.* 1997). The morphologic identification of the different types of

Table 1 Percentage of apoptotic germinal cells in cocultures of PS and Sertoli cells under different hormonal conditions

a) Annexin V binding assay

	Treatments			
	FSH−/Testo−$^{(a)}$	FSH+/Testo−$^{(a,b)}$	FSH−/Testo+$^{(a,b)}$	FSH+/Testo+$^{(b)}$
Days of coculture				
2	11±2^{a}	10±3^{a}	11±3^{a}	8±1^{a}
5	15±4^{a}	13±1^{a}	13±3^{a}	12±3^{a}
8	21±2^{a}	18±2^{ab}	18±3^{ab}	15±1^{b}
11	26±2^{a}	22±4^{a}	23±5^{a}	17±3^{b}
15	24±1^{a}	26±2^{a}	23±2^{ab}	22±1^{b}

b) TUNEL assay

	Treatments			
	FSH−/Testo−$^{(a)}$	FSH+/Testo−$^{(a)}$	FSH−/Testo+$^{(a)}$	FSH+/Testo+$^{(a)}$
Days of coculture				
2	3·8±0·8^{a}	3·5±1·0^{a}	3·6±0·8^{a}	2·8±0·4^{a}
5	4·6±1·7^{a}	4·4±1·4^{a}	3·3±0·7^{a}	3·7±0·9^{a}
8	4·7±0·6^{a}	4·6±1·4^{a}	4·4±1·0^{a}	4·8±1·0^{a}
11	6·0±2·6^{a}	5·4±1·4^{a}	6·2±2·3^{a}	5·1±1·9^{a}
15	8·5±3·7^{a}	7·6±2·3^{a}	8·5±5·2^{a}	8·6±2·1^{a}

PS were cultured in the absence (FSH−/Testo−) or presence of FSH (FSH+/Testo−) or testosterone (FSH−/Testo+) or both (FSH+/Testo+). The percentage of apoptotic germ cells was determined at selected days of culture as described in the Materials and methods section. Each value is the mean±S.E.M. of three different experiments. Treatments with different superscripts are significantly different ($P<0·05$); moreover, within lines, values with different superscripts are significantly different.

germ cells was performed according to the criteria described by Russell *et al.* (1990).

Statistical analysis

Two-way analysis of variance followed by the Bonferroni/Dunn *a posteriori* tests or paired Student's *t*-test was used. When required, values were logarithmically transformed to eliminate heterogeneity of variance. Differences were considered significant at $P<0·05$.

Results

Effects of FSH and/or testosterone on apoptosis of germ cells

When PS were cocultured with Sertoli cells, the total number of cells (somatic cells plus germ cells) decreased during the 2 weeks of the experiment in such a way that on day 15, the percentage of cells, compared with day 1, was 69 ± 6% (mean ± S.E.M. for six different experiments) irrespective of the absence or presence of either FSH or testosterone in the culture medium. Likewise, neither hormone influenced the viability of the cocultures, which decreased slightly between days 1 (87 ± 2%) and 15 (72 ± 1%). This latter result was completed by determining the percentage of apoptotic cells in the cultures by two methods. As shown in Table 1a and b, the proportion of apoptotic germinal cells increased steadily during culture by about threefold by either method ($P<0·05$). Treatment with both FSH and testosterone reduced slightly the percentage of apoptotic germinal cells in the cultures when compared with cells cultured in the absence of hormones ($P<0·05$) only when assessed by annexin V binding. The percentages of apoptotic germinal cells in cultures treated with either FSH or testosterone were different neither from control cultures nor from cultures treated with both hormones. When germ cells were analyzed according to their ploidy, the proportion of apoptotic cells, determined by TUNEL assay,

increased roughly twofold, during the culture period, in the 1C- and 2C-cell populations, whereas it increased threefold in the 4C-cell population (all $P<0{\cdot}05$). However, neither hormone treatment modified significantly the percentage of apoptotic cells in either population (data not shown). The proportion of somatic apoptotic cells was always low (less than 2%; data not shown).

Effects of FSH and/or testosterone on the number of *in vitro* differentiated RS

The number of somatic cells remained roughly constant during the first 4 days of culture, and then it increased 1·7-fold under either culture condition (Fig. 1a). The number of PS cocultured with Sertoli cells decreased during the 2-week culture period, irrespective of the absence or presence of hormone in the culture medium (Fig. 1b). Some of the PS differentiated into RS, as illustrated on Fig. 2, which presents cytologic evidence that PS (either labeled or unlabeled with BrdU) completed the two meiotic divisions throughout the culture period. When FSH was present in the culture medium, either alone or together with testosterone, the number of *in vitro* formed RS was higher than that in control wells (range 1·8–5·4-fold between days 3 and 8 of culture) (Fig. 1c). Likewise, when testosterone alone was added to the culture medium, the number of RS was 1·2–2-fold higher than in controls on those days (Fig. 1c). Such an effect of FSH and/or testosterone was observed in six different experiments, as shown in Table 2a, which reports the percentages of 1C-, 2C- or 4C-germ cell populations in short-term cocultures of PS and Sertoli cells performed under different hormonal conditions. The proportion of 4C cells was slightly but significantly ($P<0$ .05) higher in the absence of hormone than under all other conditions, whereas no difference among the different treatments was observed for the 2C cell populations. By contrast, the percentage of 1C cells was higher than that of controls, when FSH and/or testosterone were/was present in the culture medium. This resulted in higher meiotic indexes in hormone-treated cocultures than in controls (Table 2b). Taken together, these results suggest that both the first and second meiotic divisions are positively influenced by the presence of FSH or testosterone.

The 2C germ cell population is composed of a mix of secondary spermatocytes and of doublets of

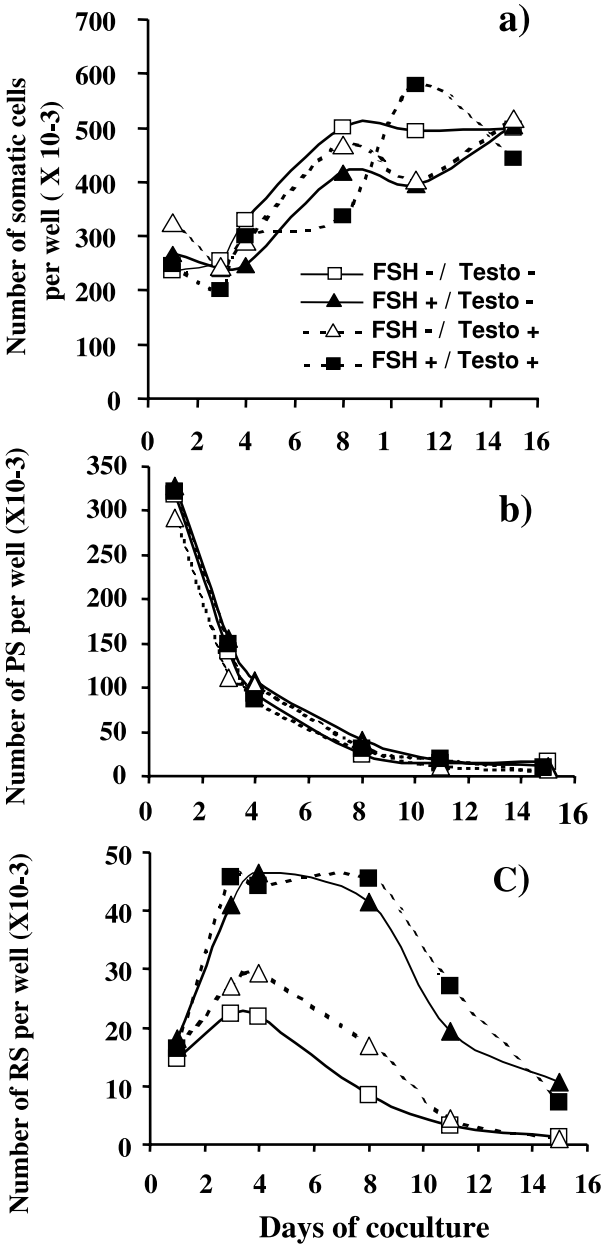

Figure 1 Changes in the number of somatic cells (a) pachytene spermatocytes (PS) (b) and of round spermatids (RS) (c) in a representative coculture of PS and Sertoli cells under different hormonal conditions (FSH-/Testo- control; FSH+/Testo- in presence of FSH alone; FSH-/Testo+ in presence of testosterone alone; FSH+/Testo+ in presence of both hormones).

RS (Godet *et al.* 2000), and the number of secondary spermatocytes results from an equilibrium between the two meiotic divisions. Hence, small differences in the number of secondary

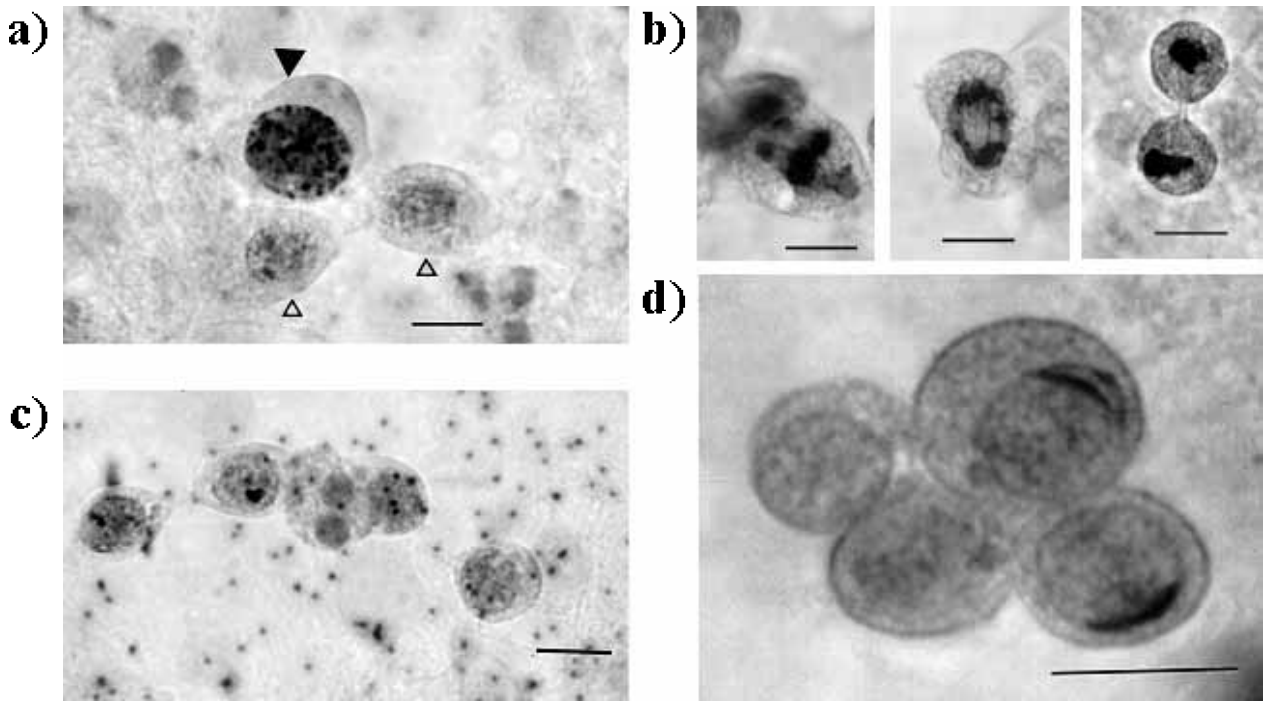

Figure 2 Cytologic and immunocytochemical analysis of the differentiation of PS cultured on Sertoli cells. (a) One large BrdU-labeled PS (▼) and two BrdU-labeled secondary spermatocytes (Δ) on day 5 of culture. (b) Meiotic metaphasis (left), anaphasis (middle) and telophasis (right) of BrdU-labeled spermatocytes on day 5 of culture. (c) Four BrdU-labeled RS on day 9 of culture. (d) Immunocytochemical staining of the acrosomal granules of RS (stages 5–6) on day 11 of culture. All bars represent 10 μm.

spermatocytes may not be detected by analysis of total cells. Therefore, in additional experiments, BrdU-labeled PS of stages VII–XII were cocultured with Sertoli cells in the absence or presence of hormones; then, the number of BrdU-labeled primary and secondary spermatocytes and that of BrdU-labeled RS were assessed by microscopic examination of the culture wells on day 3 of culture. The data presented in Table 3 show that, indeed, in the presence of both hormones, the number of secondary spermatocytes and of RS was significantly higher than in control cultures.

Effects of FSH and/or testosterone on the survival of RS cultured with Sertoli cells

The higher number of RS in hormone-treated cultures could be due to an enhancement of the completion of meiosis of seeded PS but also to improved survival of RS. Therefore, in the next experiments, purified RS were cocultured with Sertoli cells for 4 days, in either the absence or presence of FSH and/or testosterone, and the number of RS was determined every day. As expected (Weiss *et al.* 1997), there was a steady decrease in the haploid population (data not shown) during the culture period. More important, neither hormone, either alone or in combination, influenced the half-life of RS under the present culture conditions (Table 4).

Effects of FSH and/or testosterone on the amounts of mRNAs specific for PS or RS in cocultures of PS and Sertoli cells

Next, we studied the changes in the levels of mRNAs encoding for the testis-specific histone TH2B (specific to PS) and the mRNAs encoding for the transition protein TP1 (specific to RS) (Marret *et al.* 1998) in cells cultured under different hormonal conditions. The amount of radioactivity incorporated in each PCR product within the exponential phase of the reaction, at every day of culture studied, was expressed as a percentage of the radioactivity incorporated in the corresponding product on day 0 of the experiment (Weiss *et al.* 1997, Hue *et al.* 1998) (Fig. 3). The steady decrease

Table 2 *In vitro* effects of FSH and/or testosterone on a) the proportions of germ cells of different ploidy; b) the calculated meiotic index

a) Percentage of total germ cells

	IC cells	2C cells	4C cells
Treatments			
FSH–/Testo–	16·8±3·0[a]	24·7±2·1[a]	56·8±1·6[a]
FSH+/Testo–	23·4±3·0[b,c]	27·5±3·2[a]	47·2±3·3[b]
FSH–/Testo+	21·3±4·0[b]	26·3±3·3[a]	50·2±2·4[b]
FSH+/Testo+	27·7±5·4[c]	24·7±3·3[a]	46·2±3·4[b]

b) Calculated meiotic index

Treatments	
FSH–/Testo–	0·78±0·25[a]
FSH+/Testo–	1·27±0·28[c]
FSH–/Testo+	0·99±0·28[b]
FSH+/Testo+	1·27±0·35[c]

PS were cocultured with Sertoli cells for 3–5 days in the absence (FSH–/Testo–) or presence of FSH (FSH+/Testo–) or testosterone (FSH–/Testo+) or both (FSH+/Testo+). DNA flow cytometry analysis of germ cells was performed as described in the Materials and methods section. Values are the mean±S.E.M. of six different experiments. Within columns, values with different superscripts are significantly different ($P<0·05$).
The meiotic index was calculated by dividing the number of RS formed *in vitro* by the number of late PS seeded (26±4% of total PS seeded: see Materials and methods section).

($P<0·05$) of the amount of TH2B mRNAs throughout the culture was similar to that observed in a previous work (Weiss *et al.* 1997), and neither hormone influenced significantly the variations of these levels (Fig. 3a). By contrast, the amount of TP1 mRNAs increased four- to fivefold when both FSH and testosterone were present in the culture medium ($P<0·05$ on days 8, 11 and 15 vs day 0) (Fig. 3b). Moreover, the amount of TP1 mRNAs on day 15 was higher ($P<.0·05$) than that of control cells when FSH was present in the culture medium either alone or in the presence of testosterone. When testosterone alone was present, the amount of TP1 mRNAs on day 15 was similar to that observed in the presence of FSH alone, but the higher variability of the value made the difference with controls not significant.

Table 4 Half-lives of round spermatids cocultured with Sertoli cells under different hormonal conditions

	Half-life (days)
Hormonal treatment	
FSH–/Testo–	1·6±0·4
FSH+/Testo–	1·7±0·3
FSH–/Testo+	1·5±0·3
FSH+/Testo+	1·6±0·2

RS were cocultured with Sertoli cells for 4 days in the absence or presence of FSH and/or testosterone. The number of remaining RS was determined by flow cytometry on every day, allowing the determination of their half-life in culture. Each value is the mean±S.E.M. of four different experiments. No significant difference between the values was observed.

Effects of different SCCM on TP1-mRNA contents of RS

The enhanced amounts of the mRNAs encoding for TP1 in hormone-treated cocultures of PS and

Table 3 Numbers of BrdU-labeled PS, of BrdU-labeled secondary spermatocytes and BrdU-labeled RS on day 3 of cocultures of BrdU-labeled PS of stages VII–XII and Sertoli cells under different hormonal conditions

	Number of PS/mm	Number of secondary spermatocytes/mm	Number of RS/mm
Treatments			
FSH–/Testo–	27·3±4·4[a]	1·5±0·4[a]	1·4±0·4[a]
FSH+/Testo–	24·5±5·2[a]	2·0±0·8[ab]	2·1±0·8[ab]
FSH–/Testo+	30·8±5·7[a]	2·4±0·8[b]	2·0±0·8[ab]
FSH+/Testo+	27·0±5·0[a]	2·5±0·3[b]	3·0±0·8[b]

BrdU-labeled PS of stages VII–XII were cocultured with Sertoli cells for 3 days in the absence (FSH–/Testo–) or presence of FSH (FSH+/Testo–) or testosterone (FSH–/Testo+) or both (FSH+/Testo+). On day 3, the number of BrdU-labeled PS and secondary spermatocytes and of BrdU-labeled RS was determined by microscopic examination of the culture wells and expressed as the number of each cell type per mm of well and on the width of the microscope field objective ×100). At least 500 BrdU-labeled spermatocytes and/or RS were counted in each condition.
Values with different superscripts are significantly different ($P<0·05$).

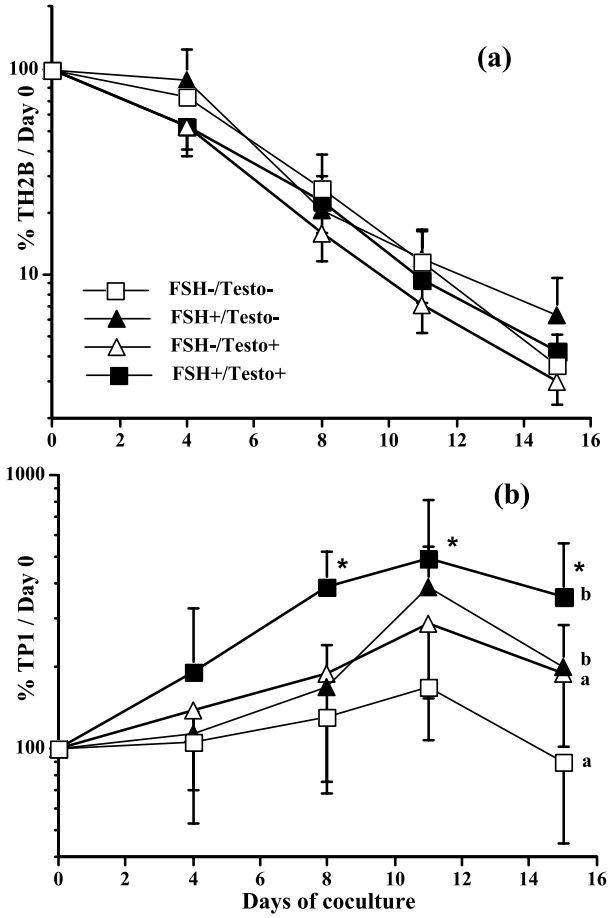

Figure 3 Changes in the amount of radioactivity incorporated in TH2B (a) or in TP1 (b) mRNA-related PCR products in cocultures of PS and Sertoli cells under different hormonal conditions. The results are the mean±S.E.M. from triplicate cultures in three different experiments and are expressed as a percentage of the radioactivity incorporated in these respective PCR products on day 0 of coculture. The number of c.p.m. at 19–20 cycles of PCR amplification corresponding to 100% was 39 000±6700 for TH2B and 14 600±2100 for TP1. Note the logarithmic scale of the y-axis. On day 15, values with different superscripts are significantly different ($P<0{\cdot}05$). Values labeled with asterisks are significantly different from day 0 ($P<0{\cdot}05$).

Sertoli cells could be due to the higher proportion of RS formed in these cultures, and/or to an enhancement in the transcription of this gene by the hormones. In an attempt to clarify this point, in another series of experiments, RS prepared by elutriation from 32-day-old rat testes were incubated for 24 h in serum-free conditioned media from Sertoli cells cultured in the absence or presence of FSH and/or testosterone (see Materials and methods section). Then, the amounts of TP1 mRNAs were measured. The viability of RS decreased during the 24-h incubation period from $97 \pm 1\%$ to $80 \pm 2\%$, irrespective of the presence or absence of hormones in the conditioned media. When RS were incubated with medium from Sertoli cells cultured without hormone, their contents in TP1 mRNAs tended to be lower than those of freshly isolated RS (Fig. 4). By contrast, when RS were incubated in medium from Sertoli cells cultured in the presence of either FSH or testosterone, their TP1 mRNA contents were not different from those of freshly isolated RS, but were two- to threefold higher than those of RS maintained in medium from Sertoli cells cultured without hormone (all $P<0{\cdot}05$). As for RS incubated in medium from Sertoli cells cultured in the presence of both FSH and testosterone, their content in TP1 mRNAs was even higher than that of freshly isolated RS. These effects appear to be specific to SCCM, since they were not observed when media were conditioned by adrenocortical cells (Table 5).

Discussion

The cytologic monitoring of the fate of PS seeded on Sertoli cell layers clearly demonstrates that some of them can go through the two meiotic divisions under the present culture conditions. Besides, several teams have now demonstrated that meiosis can proceed *in vitro* when mammalian spermatogenic cells are cocultured with Sertoli cells (Parvinen *et al.* 1983, Weiss *et al.* 1997, Hue *et al.* 1998, Staub *et al.* 2000, Lee *et al.* 2001, Sousa *et al.* 2002, Godet *et al.* 2004). In rodents, the kinetics of the meiotic process is similar *in vivo* and during the first week of culture (Parvinen *et al.* 1983, Perrard *et al.* 2003), but the efficiency of the meiosis events is somewhat lower than *in vivo*; this can be explained partly by a higher proportion of apoptotic 1C cells in culture and a bottleneck at the transition from middle to late PS which would lead to the phagocytosis of PS by the Sertoli cells (Perrard *et al.* 2003). Nevertheless, it has been shown recently that RS developed *in vitro* can produce normal offspring in the mouse (Marh *et al.* 2003). Taken together, these results support the use of such culture systems to address some aspects of the meiotic process (Weiss *et al.* 1997, Godet *et al.* 2004).

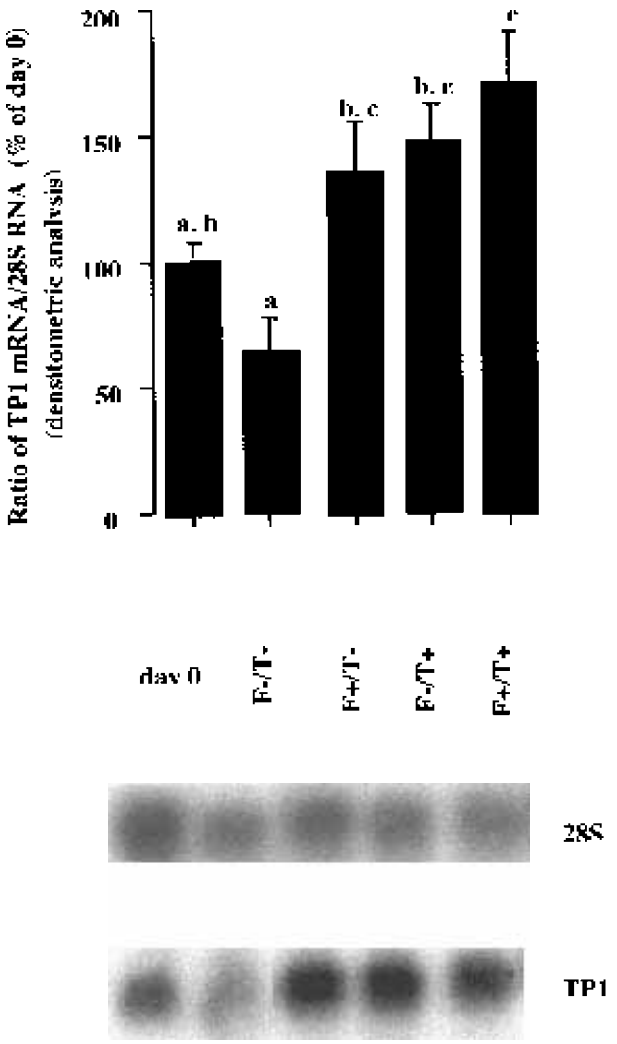

Figure 4 Effects of different Sertoli cell conditioned media (SCCM) on the TP1 mRNA/28S RNA ratio of RS. Top: elutriated RS were incubated for 24 h in serum-free conditioned medium from Sertoli cells cultured in the absence (F-/T-) or presence of FSH (F+/T-) or testosterone (F-/T+) or both (F+/T+); RNA was analyzed by Northern blotting (day 0: freshly elutriated RS). Each point is the mean±S.E.M. from six experiments. Values with different superscripts are significantly different ($P<0{\cdot}05$). Bottom: representative Northern blot of TP1 transcripts of freshly isolated (day 0) RS or of RS incubated in different SCCM for 24 h.

We have shown in the present study that, *in vitro*, both FSH and testosterone influence positively the completion of the meiotic process of spermatogenic cells, as well as the levels of TP1 mRNAs specifically expressed in RS. The concentration of FSH we used was shown, in preliminary experiments, to stimulate maximally the changes in the p19/TP1 and TH2B/TP2 ratios which occur when PS are cocultured with Sertoli cells for a 2-week period (Weiss *et al.* 1997, and unpublished results). Furthermore, this concentration of FSH is close to that used by Henriksen *et al.* (1996) (2 ng/ml of FSH from another source), giving a near maximal effect in experiments of incubation of seminiferous tubular segments. As for testosterone, a 'physiologic' concentration (Maddocks & Sharpe 1989) was chosen.

Neither the total (germinal and somatic) number of cells nor their viability was significantly altered by either hormonal treatment, whereas FSH in combination with testosterone decreased slightly the proportion of apoptotic germinal cells as determined by annexin V-binding assay. This former result is indeed explained by the fact that very few, if any, apoptotic somatic cells have ever been observed under the present culture conditions. Therefore, the 'dilution' of germinal cells with somatic cells made the small differences observed in total cell viability nonsignificant. Two different methods were used to detect apoptotic cells in the present study. The appearance of phosphatidyl serine on the outer leaflet of cell membrane detected by annexin V binding is an early event in the apoptotic process, while DNA fragmentation (TUNEL assay) is a latter event. The proportions of apoptotic germ cells, determined in the present study, by these two methods were rather low, at least during the first week of culture. This appears to contradict the somewhat large decrease in PS during the early culture period. No definite explanation can be given at the present time, but it might be that the number of degenerating cells was underestimated due to the rapid phagocytosis of apoptotic cells, as has been shown in other tissues (Raff *et al.* 1993). This assumption seems rather likely, as Sertoli cells are highly phagocytic (Byers *et al.* 1993). This property of Sertoli cells might also explain, at least partly, the lower percentages of apoptotic germinal cells measured by TUNEL assay than by annexin V binding. These results fit quite well with the proportions of apoptotic germ cells determined by measuring the level of activated caspase 3 in freshly isolated germ cell preparations or in cultured seminiferous tubules (Perrard *et al.* 2003). Many *in vivo* studies have shown that both FSH and testosterone decrease apoptosis of both PS and RS of the rat (Billig *et al.* 1995, Brinckworth *et al.* 1995,

Table 5 Effects of media conditioned by different types of cells on the contents in TP1 mRNA of round spermatids

	Sertoli cell conditioned medium	Adrenocortical cell conditioned medium
Control	100±16	108±12
FSH (1 ng/ml) +testosterone (10^{-7} M)	225±40*	97±9
ACTH (10^{-9} M)	97±3	100±19

Sertoli cells or adrenocortical cells were cultured in the absence or presence of FSH+ testosterone or ACTH, and conditioned media were prepared as described in the Materials and methods section. Elutriated round spermatids were incubated for 24 h in these conditioned media. RNA were analyzed by Northern blotting; TP1 mRNA levels were normalized to levels of 28S RNA. Values are the mean±S.E.M. of at least three experiments and are expressed as a percentage of the value obtained with media conditioned by Sertoli cells cultured in the absence of hormone.
*$P<0{\cdot}05$ vs the corresponding control.

Henriksen *et al.* 1995, Marathe *et al.* 1995, El Shennawy *et al.* 1998), but some of them did not produce statistically significant results (Saito *et al.* 2000). Moreover, Henriksen *et al.* (1995) suggested an increase in apoptosis of PS of stage XII under testosterone stimulation. The difference in the proportion of apoptotic germinal cells in control compared with FSH plus testosterone-treated cultured cells was rather small when compared with the results of some *in vivo* data (Billig *et al.* 1995, Brinckworth *et al.* 1995, Henriksen *et al.* 1995, Marathe *et al.* 1995, El Shennawy *et al.* 1998); however, its amplitude was in the same range as that observed by Henriksen *et al.* (1996) when seminiferous tubular fragments from stages V-VIII were incubated for 3 days in the absence or presence of FSH. Hence, the present results indicate that the overall effect of treatment of the cocultures with FSH and/or testosterone cannot be related solely to the effect of these hormones on germ cell apoptosis. Indeed, the enhancement of the RS population *in vitro* by FSH was observed not only on the total number of RS per well (Fig. 1) but also when 1C cells were expressed as a proportion of the total population of germ cells (Table 2). This assumption is substantiated by the higher content of FSH-treated cultures in TP1 mRNAs (specific to the haploid state) without any significant modification by the hormones of the amounts of TH2B mRNAs (specific to pachytene spermatocytes). The rather small decrease of TH2B mRNAs between days 0 and 4, that is, at a time when the number of PS decreased more abruptly, can be explained by a maturation of PS in culture. Indeed, the proportion of middle PS, which express TH2B at the highest level (Marret *et al.* 1998), increases 1·5-fold during the first days of coculture, whereas that of early PS decreases 7-fold, with no change in the proportion of late PS (M.H. Perrard, unpublished results). The delay between the occurrence in PS-Sertoli cell cocultures of a 1C population, on one hand, and the increase in the amount of TP1 mRNA, on the other hand, can be explained on the basis that expression of TP1 appears to be more important from steps 5–6 of spermiogenesis, that is, 4–5 days after completion of meiosis (Marret *et al.* 1998). Since the PS seeded on Sertoli cells were mostly middle to late PS and neither hormone tested modified the half-life of RS in our cultures, it seems reasonable to conclude that FSH has a positive effect on the completion of the meiotic divisions. Testosterone also appears to be involved in the regulation of this step. Testosterone-treated cultures were usually intermediary between control and FSH-treated or FSH plus testosterone-treated cells, and a combination of testosterone and FSH was often more potent than FSH alone. These results extend *in vivo* data on the hormonal regulation of spermatogenesis in the adult or pubertal hypophysectomized rat (El Shennawy *et al.* 1998, França *et al.* 1998, Russell *et al.* 1998) and after long-term suppression of testosterone or both testosterone and FSH in the adult rat (Meachem *et al.* 1997, 1998, review in McLachlan *et al.* 2002). Indeed, these authors reported a role on cell viability for both FSH and testosterone at early stages of the meiotic cycle and suggested an overlapping and synergistic/additive effect of these

hormones in this step. In addition, Parvinen and Ventela (1999) reported that the preferential action of FSH was associated with stages that involve meiotic divisions and early spermiogenesis. Nevertheless, even cultures maintained in the absence of these hormones maintained some capacity to support the meiotic divisions of middle/late pachytene spermatocytes. Since, as opposed to *in vivo* experiments, such a result cannot be explained by 'residual' concentrations of hormones, it suggests that most of the endocrine message for meiosis is acquired by the germinal cells around steps VI–VIII of the spermatogenic cycle (Toppari & Parvinen 1985, Perrard *et al.* 2003) and/or that other local factor(s) originating from Sertoli cells can compensate for the absence of these hormones (see below).

Further support for an overlapping and additive effect of FSH and testosterone on some steps of spermatogenesis was gained from a different experimental approach in which RS were incubated with SCCM. The contents in TP1 mRNAs of RS incubated with medium from Sertoli cells cultured with either hormone were similar to, and higher than, those of spermatids incubated in control medium, but not different from those of freshly isolated round spermatids. Hence, it cannot be ascertained from these results whether such differences were due to an enhancement of transcription of these genes under the influence of hormone dependent-Sertoli cell-derived factors and/or to an inhibition of the degradation of these mRNAs. By contrast, the combination of FSH and testosterone resulted in an enhancement of the transcription of TP1. Indeed, the contents in TP1 mRNAs of spermatids incubated in medium from Sertoli cells cultured with both hormones were higher than those of spermatids incubated in control media but also from those of spermatids immediately after their isolation.

Since the Sertoli cells used in the present experiments were isolated from early pubertal animals and such cells have been shown to exhibit a different responsiveness to FSH and testosterone from Sertoli cells of adult animals (Griswold 1993, Sar *et al.* 1993), the question of how the effects observed in the present work relate to the physiologic situation in the adult could be raised. No definite answer can be given since coculture of adult Sertoli cells with PS cannot be maintained beyond a few days (M. Vigier & Ph. Durand, unpublished data). However, it has been shown that in the hypophysectomized rat, FSH and/or testosterone prevent(s) germ cell loss during meiosis, in both pubertal (Russell *et al.* 1998) and adult (El Shennawy *et al.* 1998) animals. Moreover, we have observed that from day 4 of culture onward, Sertoli cells from 20-day-old rats respond to FSH, in terms of cAMP production, like short-term-cultured Sertoli cells from mature (Clermont & Perey 1957) 45-day-old animals (M. Vigier & Ph. Durand, unpublished data). Besides, the present results fit rather well with a recent report using hypogonadal mice expressing transgenic FSH, alone or in combination with testosterone (Haywood *et al.* 2003). Indeed, these authors showed that FSH actions combine synergistically with androgens to have a marked effect on both meiotic and postmeiotic germ cell maturation.

It might be argued that the effects observed in culture are relatively minor. However, in view of the *in vivo* data reported in the literature, it appears that the amplitude of the short-term effects of either FSH or testosterone on the narrow step of spermatogenesis studied in the present study, is rather similar *in vivo* (Sinha Hikim & Swerdloff 1995, El Shennawy *et al.* 1998, Meachem *et al.* 1999, O'Donnell *et al.* 1999, Saito *et al.* 2000) and *in vitro* (present results). In addition, it is becoming clearer that hormones and intratesticular regulatory factors may compensate, at least in part, for the absence of some hormones or factors, including FSH (Kumar *et al.* 1997, Dierich *et al.* 1998, Abel *et al.* 2000) and androgen (Yeh *et al.* 2002, Chang *et al.* 2004, De Gendt *et al.* 2004, Holdcraft & Braun 2004), or luteinizing hormone (Zhang *et al.* 2003) receptors. Thus, it is likely that synergism and/or redundancy between regulatory molecules is a characteristic of the spermatogenic process, on which depends species survival. Hence, it is not unexpected that addition or removal of some hormone or factor, at least for a short time, either *in vivo* or *in vitro*, may have only a limited, although significant, effect on a given step of spermatogenesis.

It seems important to recall that even KO experiments for FSH (Kumar *et al.* 1997, Dierich *et al.* 1998, Abel *et al.* 2000) and androgen receptors in Sertoli cells (Chang *et al.* 2004, De Gendt *et al.* 2004, Holdcraft & Braun 2004) have produced somewhat different results. This might indicate

accumulative disorders throughout development in the different mouse models. Culture systems are not affected by such drawbacks, although indeed they remain *in vitro* models. Hence putting together results from KO experiments and *in vitro* data should help to approach again the physiologic role of these hormones in spermatogenesis.

In conclusion, these studies, performed *in vitro*, show that FSH and testosterone have positive and somewhat overlapping effects on the rat meiotic divisions and the postmeiotic expression of a germ cell-specific gene, which cannot be related solely to their ability to reduce germ cell apoptosis. Finally, use of this culture system should help to test the effect of any hormone or factor on those steps in order to understand better their regulation.

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