Journal of Andrology, Vol. 22, No. 6, November/December 2001

A Century of Research on Mammalian Male Germ Cell Meiotic Differentiation In Vitro

Review

CHRISTOPHE STAUB

From the Department of Veterinary Anatomy and Public Health, Center for Environmental and Rural Health, Texas A&M University, College Station, Texas.

Many germ cell studies over the last century have focused on testicular cell culture (Wolff and Haffen, 1965; Russell and Steinberger, 1989; Kierszenbaum, 1994). The first germ cell culture systems were directed at studying gonadal embryogenesis and male germ cell differentiation. Today's culture systems are powerful tools that bring about new knowledge and applications to the study of spermatogenesis. The culture of Sertoli cells is mentioned only as a coculture process with germ cells. The main focus of this review is the differentiation of male germ cells, and more precisely, the meiotic step of that differentiation.

The various studies can be divided into 3 main categories depending on the historical advancement (early attempts at gametogenesis in vitro, intermediate progress, and latest refinements). Examples highlighting the strong and weak points of each period are summarized in this review.

Early Attempts at Gametogenesis In Vitro

Tissue culture—Champy (1920) first described differentiation of male germ cells in vitro in his cultures of small parts of testis from adult rabbits in blood plasma. But this basic method appeared to be inadequate for the survival of cultured germ cells. Indeed, although somatic and undifferentiated germ cells survived for 1 week at best, differentiating germ cells (spermatocytes and spermatids) degenerated within 2 to 7 days. Yet, Champy observed spermatogonial mitosis in the first week and newly formed leptotene primary spermatocytes after 9 days. It was the first report of male germ cells having entered meiotic prophase in vitro. Michailow (1937) reported that seminiferous tubules from immature rabbits could survive 70 days in culture. Again, it seemed that only undifferentiated germ cells were able to survive in vitro. Gaillard and Varossieau (1938) confirmed this result with cultures of rat testicular fragments in rat plasma. Testicular fragments from immature animals (28-day-old rats) survived better when they were cultured in plasma from adult animals than from that of immature animals. For differentiation, however, their results were more ambiguous. Indeed, it is difficult to determine on the basis of morphological criteria only, whether the spermatids they observed were produced in vitro or whether they simply survived in culture after being produced in vivo.

Organ Culture: Undifferentiated Gonads—In 1952, Wolff reported that undifferentiated mouse gonads (10–11.5 days postcoitum) could differentiate in vitro either into ovaries or testes after 4 to 6 days of culture using the maturation medium devised by Wolff and Haffen (1951). The undifferentiated gonads became sterile ovaries or testes. The medium ensured the good development of the somatic tissue of the gonads, but not the survival and differentiation of germ cells.

Asayama and Furusawa (1960, 1961), using mouse gonads just before and after sexual differentiation, placed the gonads in culture for 21 to 44 hours. The results showed that horse serum exhibited a delayed effect on gonadal sexual differentiation of gonads. Magre and Jost (1984) confirmed the negative effect of serum on the development of seminiferous tubules. On the contrary, a medium made of Tyrodes buffer supplemented with glucose enhanced the differentiation of gonads in these short-term cultures, but no germ cell differentiation was noted (Asayama and Furusawa, 1960).

Organ Culture: Differentiated Gonads—Martinovitch produced spectacular results as early as 1937. He cultured gonads taken from rats and mice just after birth in a medium devised by Fell and Robison (1929). Martinovitch observed the long-lasting survival of gonadal tissue in a system that allowed the testicular architecture to be maintained, and this resulted in the differentiation of male germ cells from immature animals. Pachytene primary spermatocytes were formed after 11 days of culture from newborn mouse testes.

Borghese and Venini (1956) confirmed parts of the results of Wolff (1952) and Martinovitch (1937) by using gonads from mouse embryos aged 13 to 18 days postcoi-

Correspondence to: Dr Christophe Staub, Department of Veterinary Anatomy and Public Health, Center for Environmental and Rural Health, Texas A&M University, College Station, TX 77843 (e-mail: cstaub@cvm.tamu.edu).

Received for publication January 8, 2001; accepted for publication March 20, 2001.

tum. They showed that fetal gonads can keep developing in vitro. At the time when the gonads were removed, the primary testes contained Sertoli cells and spermatogonial germ cells in close contact to one another. After a few days of culture, the germ cells moved away from the Sertoli cells, toward the apical part of the seminiferous tubules.

Summary of Methods and Success—At least 2 methods have been presented. 1) At the beginning of the century, the tissue culture system allowed gonocytes to survive for the long term and germ cells to enter meiotic prophase. 2) The organ culture system gave better results. With undifferentiated gonads, it was proven that sexual differentiation could occur in vitro. However, in this model, few germ cells survived and none differentiated. With differentiated gonads, seminiferous tubules were able to survive several weeks in culture. Germ cells continued their differentiation to reach the developmental steps of pachytene primary spermatocytes in the experiments of Martinovitch (1937), who seems to have had particularly good conditions for that time.

Intermediate Progress

At the beginning of the 1960s, Anna and Emil Steinberger, together with William Perloff, began studying the long-term survival of testicular fragments in vitro (Steinberger A et al, 1964). They made changes in culture parameters such as temperature, incubation atmosphere, pH, or the various nutriments of a chemically defined medium that replaced the blood plasma that had been used before (Champy, 1920; Strangeways and Fell, 1926; Martinovitch, 1937; Michailow, 1937; Gaillard and Varossieau, 1938; Dux, 1939; Trowell, 1959; Lostroh, 1960). The necessity of developing a long-term culture system was underlined in the work by Clermont and his group on the timing of spermatogenesis (Clermont et al, 1959). Their methodology was also inspired from the organ culture techniques of Trowell (1959), following the work of Fell and Robison (1929), who developed the first organ culture system.

Trowell (1959) cultured small organ fragments on stainless steel grids covered with a paper towel soaked in culture medium and sometimes agar. He obtained good results with the culture of deferent ducts or various glands (pituitary, pineal, parathyroid glands, and prostate) that kept a remarkable histologic aspect for 9 days. However, his results were not as good with small testicular fragments or seminiferous tubules from 4-week-old rats. After 3 days, most tubules had degenerated. In surviving tubules, the cells appeared normal with a usual rate of mitotic and meiotic figures. By changing the gas phase (ie, by culturing with 5% CO_2 in air rather than in oxygen), Trowell (1959) got more tubules to survive 3 days. He found that contrary to other cultured organs, the testis was very sensitive to oxidative stress. Trowell had mentioned ''oxygen poisoning'' as early as 1959. Not a single living cell was found after 6 days of culture in 5% CO_2 in oxygen or in air.

The first improvement to this technique, culture technique, was attempted by the Steinberger team. They reported that 37°C, which had been generally used for mammal tissue culture, was not suitable for testis tissue (Steinberger and Nelson, 1955; Steinberger and Dixon, 1959) because it reproduced the pathological conditions of cryptorchidism. The regular intratestis temperature in vivo varies from 32°C to 35°C, depending on outside temperature and scrotal epithelial humidity. The second major improvement was the use of pyruvate as an energy substrate for the germ cells because only simple sugars such as glucose or mannose, or dioses such as galactose, fructose, or saccharose had been used until then (Wolff and Haffen, 1965). It is known today that during a short time in meiotic prophase, germ cells are unable to metabolize glucose. Hence, these germ cells need another energy substrate (Jutte et al, 1981, 1982). The deadly effect of oxygen on germ cells mentioned by Trowell (1959) was confirmed by Steinberger A et al (1964). In an atmosphere with 5% CO_2 in oxygen instead of air, they noticed that necrosis patches appeared less frequently in seminiferous tubules. However, the number of mitotic figures of type A spermatogonia decreased and primary spermatocytes degenerated earlier, which is the expression of oxidative stress.

Successful experimental conditions used by the Steinberger team include small testicular fragments cultured in a chemically defined medium supplemented with sodium pyruvate, 10% fetal calf serum, and various amino acids (see Tables 1 and 2) on stainless steel grids covered with an agar film. The depth of the medium was just high enough to wet the lower surface of the agar. The testicular fragments were incubated at 31°C in a water-saturated atmosphere of 5% CO_2 in air. Testicular fragments from prepuberal rats (14 days postpartum [dpp]) cultured in these conditions kept testicular structures in culture for 6 months (Steinberger A et al, 1964). After this time, the Sertoli cells were still in good shape, and these authors still observed germ cell mitosis. A spermatogonia were present for several weeks but were difficult to identify after 3 weeks. The primary spermatocytes survived about 4 weeks. No spermatid formation was observed. It was shown that the spermatogonia that survived in culture retained competency and were still able to differentiate in vivo when transplanted into host rats (Steinberger and Steinberger, 1967).

These scientists would soon obtain their first significant results in germ cell differentiation (Steinberger E et al, 1964). They studied in vitro the effect of gonadotrope hormones (follicle-stimulating hormone [FSH] and hu-

man chorionic gonadotropin [hCG]) and vitamins (A, C, and E) on spermatogenesis using testicular cultures from prepuberal Long-Evans rats (4 dpp). At that age, only 2 different cell types are present in the seminiferous tubules: Sertoli cells and A spermatogonia. Steinberger used techniques that allowed maintenance of the morphologic structure of the testis for a long time and that kept primitive germ cells alive. Despite the changes on the supporting cells (maturation), neither FSH nor hCG could initiate the in vitro differentiation of germ cells from gonocytes. However, germ cell differentiation occurred until the pachytene primary spermatocyte stage in 2 weeks when vitamins A, C, and E were added to the culture medium. The positive effect of these vitamins and of glutamine on the differentiation of germ cells in culture was confirmed 2 years later (Steinberger and Steinberger, 1966). The authors showed that gonadotropins were not essential to the initiation of spermatogenesis. After 3 weeks of culture, testicular tissue was degenerative. After 4 weeks, most primary spermatocytes had disappeared, and no postmeiotic cells were observed. After 6 weeks, a new wave of B spermatogonia appeared in some tubules.

These results were confirmed in cultures from rats at 12 dpp or from adult rats using tritiated thymidine as a marker to follow the differentiation of germ cells in vitro (Steinberger and Steinberger, 1965). In these experiments, the researchers used the same medium without gonadotropins or vitamins, but the glutamine concentration was doubled. They used the tritiated thymidine method to detect cell DNA synthesis and autoradiography to detect cells that had incorporated the radioactivity as used previously by Oakberg (1956) on the mouse and Clermont (1959) on the rat in order to measure the duration of the epithelial cycle. Animals were injected with a tritiated thymidine solution, which is incorporated during DNA synthesis by the cells preparing for division. The spermatogonia and preleptotene primary spermatocytes are the only germ cells duplicating their DNA for mitosis or before entering meiosis. The differentiation of the cells that have incorporated the tritiated thymidine can be followed by autoradiography (Oakberg, 1956; Clermont et al, 1959); in vivo, by regularly killing the animals after the injection, or in vitro by gradually terminating some of the cultured testicular fragments at different times.

In order to show that a differentiation occurs in vitro and that a new cell type appears in culture without labeling with tritiated thymidine, examples of the differentiated cells must not be present at the start of the culture. That is why researchers had to work on very young animals (ie, very immature testes in which germ cells and somatic cells were immature). By using tritiated thymidine, these authors could work on older animals or even adults in which spermatogenesis is complete. They could distinguish between precocious cells that incorporate radioactive thymidine at the beginning of meiotic prophase and differentiate until the pachytene primary spermatocyte stage in culture (stained cells) and primary spermatocytes that were present at the start and simply survived without differentiating (unstained cells). Following the progress of labeled cells also provided information on the timing of differentiation of germ cells in vitro (Steinberger and Steinberger, 1965). In culture of tissues taken from animals 3 hours after injection of the isotope, the authors observed that the most advanced labeled germ cells were spermatogonia and primary preleptotene spermatocytes. After 3 to 7 days of culture, the labeled cells were leptotene and zygotene primary spermatocytes and after 2 to 3 weeks, pachytene primary spermatocytes. No labeled round spermatid was found even after 6 weeks. To make sure that the obstacle was not simply the length of the culture period, they injected adult animals with tritiated thymidine, but this time they waited for 12 days before culture. When the animals were killed and the cultures began, the most advanced labeled cells were pachytene primary spermatocytes. According to the timing of differentiation of male germ cells in vivo (Clermont et al, 1959), the differentiation of pachytene primary spermatocytes into round spermatids must take less than 2 weeks. However, no postmeiotic cells were identified after 2 weeks of culture.

In short, the differentiation in vitro of some testicular cells was possible (from the preleptotene primary spermatocyte stage to the pachytene stage), but it was limited. Similar results were obtained in humans (Steinberger, 1967); survival of primitive A spermatogonia and Sertoli cells for several weeks, differentiation of primary spermatocytes from the preleptotene stage to the pachytene stage (proven by tritiated thymidine), degeneration of primary spermatocytes after 4 weeks, and degeneration of round spermatids in the first days of culture.

The work of Anna and Emil Steinberger and their team resulted in spectacular progress in testicular culture and it is still of great significance today for biology researchers who want to reproduce spermatogenesis in vitro. Their system was not perfect, but it could keep a testis working for a long period. It was necessary to better understand the needs of the germ cells in culture, and they made important advances in this field. An important part of the meiotic differentiation of germ cells was performed in culture, which brought precise data on its timing, and which corresponds to the kinetics of the differentiation of germ cells in their natural environment (Steinberger and Steinberger, 1965). Their results, first obtained with rats, were confirmed with human testicular biopsies (Steinberger, 1967).

Table 1. *Composition of the chemically-defined media used in the different studies*

Type of culture/ Authors	Date	Media	Carbohydrates	Amino acids	Nucleic acids	Complex fluids	Hormones	Proteins
Tissue culture/organ culture								
Steinberger et al	1964	EMEM	Pyruvate 1 mM	Nonessentials 0.1 mM	…	FCS 10%	…	…
Steinberger and Steinberger	1965	EMEM	Pyruvate 1 mM	Nonessentials 0.1 mM Glutamine 0.2 mM	…	FCS 10%	…	…
Ghatnekar et al	1974	Parker 199	Fructose 0.2 mM	…	…	FCS 15% Coconut milk 10%	FSH 1-10-100 μg/mL LH 1-10-100 μg/mL Testosterone 4×10^{-6} M	…
Coculture Sertoli/germ cells on impermeable supports								
Dietrich et al	1983	EMEM (modified)	Glucose 1 g/L	Glutamine 2.7 mM	…	…	…	…
Tres and Kierszenbaum	1983	EMEM	Pyruvate 1 mM	Nonessentials 0.1 mM Glutamine 4 mM	…	FCS 10%	FSH 0.5–5 μg/mL hCG 6.5 μU/mL Testosterone 10^{-7} M Insulin 5 μg/mL	Transferrin 5 μg/mL
Nagao	1989	F12-L15	…	…	…	FCS 10%	Adrenaline 1 μg/mL Noradrenaline 1 μg/mL	…
Le Maguerresse-Battistoni et al	1991	F12-DMEM	…	…	…	—	FSH 0.25 μg/mL Testosterone 10^{-7} M Insulin 10 μg/mL	Transferrin 5 μg/mL
Culture of seminiferous tubules on impermeable supports								
Ellingson and Yao	1970	EMEM	Pyruvate 1 mM	Nonessentials 0.1 mM Glutamine 2–4 mM	…	FCS 20%	…	…
Palombi et al	1979	EMEM	…	Nonessentials 0.1 mM Glutamine 4 mM	cAMP 0.1 mM	…	FSH 5 μg/mL	…
Parvinen et al	1983	F-12-DMEM	Glucose 2.25 g/L	…	…	…	FSH 0.2 μg/mL	Transferrin 5 μg/mL
Toppari and Parvinen	1985						LH 0.1 μg/mL Testosterone 10^{-7} M Insulin 10 μg/mL Hydrocortisone 10^{-8} M	Ceruloplasmin 1 U/mL
Toebosch et al	1989	DMEM	…	Glutamine 2 mM	…	…	FSH 0.1 μg/mL Testosterone 7×10^{-7} M Insulin 5 μg/mL	…

Table 1. *Continued*

Type of culture Authors	Date	Media	Carbohydrates	Amino acids	Nucleic acids	Complex fluids	Hormones	Proteins
Coculture Sertoli/germ cells on permeable supports								
Tres et al	1986	EMEM	Pyruvate 1 mM	Glutamine 2 mM	…	…	FSH 0.5µg/mL	Transferrin 5 µg/mL
							hCG 133 µU/mL	
Tres et al	1991						Testosterone 10^{-7} M	
Tres et al	1992						Dihydrotestosterone 10^{-7} M	
							Insulin 5 µg/mL	
Weiss et al	1997	F12-DMEM	Pyruvate 1 mM	…	…	…	FSH 1 ng/mL	Transferrin 10 µg/mL
							Testosterone 10^{-7} M	
							Insulin 10 µg/mL	
Culture of seminiferous tubules on permeable supports								
Hue et al	1998	F12-DMEM	Pyruvate 1 mM	…	…	…	FSH 1 ng/mL	Transferrin 10 µg/mL
Staub et al	2000						Testosterone 10^{-7} M	
							Insulin 10 µg/mL	

Latest Refinements

Tissue/Organ Culture—In 1974, directly inspired by the culture techniques of the Steinberger group a few years before, Lima-de-Faria's team published interesting results in humans (Ghatnekar et al, 1974). They cultured small testicular cubes using testicular biopsies from 16 men aged 27 to 73 years. The small organ bits were first placed in a culture medium containing tritiated thymidine in order to label cells in the S phase of the cell cycle and to follow the germ cell differentiation at the beginning of meiotic prophase. The fragments were then cultured at 36°C for 14 to 25 days. The culture plates were slanted and rotated so that the organ was only intermittently soaked in the medium. The medium was supplemented in fructose, fetal calf serum, deproteinized coconut milk, FSH, LH, and testosterone (see Tables 1 and 2 for details). The FSH and LH concentrations vary according to the experiments. In these conditions and after 14 days of culture, germ cells that had incorporated tritiated thymidine before culture were identified at telophase II stage. These cells would have almost entirely passed meiosis in vitro! The authors suggested that success was due to 2 main characteristics of the culture. There was a positive effect of deproteinized coconut milk; its positive effect on the differentiation in vitro of germ cells of some plants has been known since 1955 (Sparrow et al, 1955). More recently, several cytokines involved in the growth of plants have been identified and isolated (Van Overbeek et al, 1941; Dua and Chandra, 1993). Coconut water is also used in the preparation of numerous spermatozoa diluents for its antioxidative quality, which is important in the conservation of semen (Chairussyuhur et al, 1993; Nuñes, 1998). The circulation of culture was also an important characteristic because it prevents dessication and reduces the necrosis resulting from anoxia, a major drawback of soaked organ culture systems (Trowell, 1959).

However, some details make those results suspect. Indeed, the results were presented for only 5 out of the 16 men of the study, and there was only 1 case of the labeled germ cells reaching the telophase II stage. The differentiation from preleptotene to telophase II step of development would have occurred in 14 days. This does not correspond to the timing established by Clermont (1963), even though the authors indicate that it does. Finally, they acknowledged the existence of an important DNA synthesis for humans, contrary to rodents, during meiotic prophase (Lima-de-Faria et al, 1966, 1968). Hence, we must wonder how far germ cells have gone in vitro in this culture system. For instance, DNA repair at the young pachytene stage would explain the existence of labeled cells at telophase II stage after only 2 weeks of culture.

Interesting results were reported by Aizawa and Nishimune (1979). Using the medium and the culture system

Table 2. *Composition of the chemically-defined media used in the different studies*

Type of culture/ Authors	Date	Growth factors	Vitamins	Antibiotics	Fungicides	pH	Temperature	Gas phase
Tissue culture, organ culture								
Steinberger et al	1964	···	Retinol 40 μM Tocopherol 120 μg/mL Ascorbic acid 50 μg/mL	Penicillin 100 U/mL	Amphotericin B 5 μg/mL	7.0–7.2	31°C	5% CO_2 in air
Steinberger and Steinberger	1965	···	···	Penicillin 100 U/mL	Amphotericin B 5 μg/mL	7.0–7.2	31°C	5% CO_2 in air
Ghatnekar et al	1974	···	···	Penicillin 100 U/mL Streptomycin 100 U/mL	···	7.2	36°C	
Coculture Sertoli/germ cells on impermeable supports								
Dietrich et al	1983	···	···	Penicillin 100 U/mL Streptomycin 1 μg/mL	Fungizone 0.25 μg/mL to 1 μg/mL	7.25–7.3	20°C	air
Tres and Kierszenbaum	1983	EGF 3 ng/mL	Retinol 10 μM	···	···	···	32°C	
Nagao	1989	···	···	Penicillin 100 U/mL Streptomycin 100 μg/mL	···	7.3	32.5°C	5% CO_2 in air
Le Maguerresse-Battistoni et al	1991	TGFα 10 ng/mL	Retinoic acid 0.33 μM	Gentamycin 4 μg/mL	···	···	32.5°C	
Culture of seminiferous tubules on impermeable supports								
Ellingson and Yao	1970	···	···	Penicillin 100 U/mL Streptomycin 100 μg/mL	···	6.8–7.2	31°C	
Palombi et al	1979	···	···	Penicillin 100 U/mL Streptomycin 100 μg/mL	Fungizone 5 μg/mL	7.0–7.2	32°C	5% CO_2 in air
Parvinen et al	1983	EGF 2.5 ng/mL	···	Gentamycin 50 μg/mL	···	7.4	32°C	5% CO_2 in air
Toppari and Parvinen	1985							
Toebosch et al	1989	···	Retinol 0.35 μM	Penicillin 100 U/mL Streptomycin 100 μg/mL	Fungizone 0.6 μg/mL	···	32°C	5% CO_2 in air
Coculture Sertoli/germ cells on permeable supports								
Tres et al	1986	EGF 10 ng/mL	Retinol 5 μM	Penicillin 100 U/mL Streptomycin 100 μg/mL	···	···	32°C	5% CO_2 in air
Tres et al	1991							
Tres et al	1992							

Table 2. *Continued*

Type of culture Authors	Date	Growth factors	Vitamins	Antibiotics	Fungicides	pH	Temperature	Gas phase
Weiss et al	1997	…	Retinol 0.33 μM Retinoic acid 0.33 μM Tocopherol 10 μg/mL Ascorbic acid 17.6 μg/mL	Gentamycin 20 μg/mL	Nystatin 10^5 U/mL	7.35–7.4	32°C	5% CO_2 in air
Culture of seminiferous tubules on permeable supports								
Hue et al	1998	…	Retinol 0.33 μM	Gentamycin 20 μg/mL	Nystatin 10^5 U/mL	7.35–7.4	32°C	5% CO_2 in air
Staub et al	2000		Retinoic acid 0.33 μM Tocopherol 10 μg/mL Ascorbic acid 17.6 μg/mL					

developed by the Steinbergers in 1967, Aizawa and Nishimune cultured small testicular fragments of cryptorchid adult mice. They also used tritiated thymidine to study the mitotic activity of type A spermatogonia, the only type of germ cell present at the beginning of the experiment in their model. Germ cell differentiation occurred in vitro and type B spermatogonia, preleptotene, leptotene, and pachytene primary spermatocytes were observed after 6, 9, 12, and 15 days of culture, respectively. That corresponds to the kinetics of the differentiation of germ cells in vivo. No germ cells progressed further than this stage.

Later, Aizawa and Nishimune also used the same culture system to show the synergistic effect of FSH and retinoids (retinol, retinal, retinol acetate, and retinoic acid) on the proliferation and differentiation of type A spermatogonia (Haneji et al, 1984). Again, despite the positive effect of these compounds on germ cell differentiation, no germ cells achieved meiosis in vitro and postmeiotic cells were never produced.

More recently, Boitani and her team (1993) have confirmed the previous results and shown how useful organ culture systems could be for the study of the initiation of spermatogenesis and spermatogonial divisions. The experiments were performed according to the Steinberger team technique (Steinberger A et al, 1964) with 9-day-old Wistar rats. In 3 weeks, with 200 ng/mL ovine FSH added to the culture media, the A spermatogonia differentiated into pachytene primary spermatocytes.

The organ culture system has proved to be an excellent model for the study of the mechanisms that regulate spermatogonial divisions and the germ cells entering meiotic prophase (Steinberger E et al, 1964; Aizawa and Nishimune, 1979; Boitani et al, 1993). Its major advantage is that it respects the architecture of seminiferous tubules and the interactions between somatic and germ cells that are so important for advancement to subsequent steps of spermatogenesis. However, the relatively short life expectancy of differentiated germ cells in such a system makes it inappropriate for the completion of the entire meiotic part of spermatogenesis in culture.

Cultures on Impermeable Supports

Cocultures of Sertoli and Germ Cells—For the first time, in 1982 researchers in Holland presented a culture system that allowed mouse male germ cells to survive 12 days and to go through the 2 meiotic divisions in vitro (Dietrich et al, 1982, 1983). In order to avoid using radioactivity to follow the differentiation of germ cells, the animals were treated with hydroxyurea. This treatment destroyed the DNA-synthesizing cells (S-phase cells; ie, spermatogonia and preleptotene primary spermatocytes) and resulted in a blank in the spermatogenic line. After the treatment, spermatogenesis normally resumed with the

surviving stem cells. So, 8 days after the treatment, the ''blank'' had moved and the authors noted the absence of germ cells from type B spermatogonia to the preleptone primary spermatocyte stage. This allowed them to identify the different meiotic steps by following the ''repopulating front'' of the seminiferous tubules.

Dietrich et al cultured suspensions of testicular cells (Sertoli and germ cells) from the mice treated with hydroxyurea and without pachytene to diplotene primary spermatocytes (Dietrich et al, 1982, 1983). After 7 to 10 days of culture in a completely synthetic medium (see Tables 1 and 2), the apparition of pachytene followed by diplotene primary spermatocytes and finally round spermatids proved germ cell differentiation and meiotic division in vitro. The germ cell differentiation in vitro had occurred with the same timing as differentiation in vivo. This was the first time that satisfying results had been obtained with a system in which the seeded cells were completely dissociated. Two particular features should be considered. The culture medium was slightly hypertonic, and the Na^+:K^+ ratio was different from that of plasma. This is very likely to act positively upon the survival of the cells normally located within the blood-testis barrier in vivo, their differentiation, or both (ie, primary and secondary spermatocytes and spermatids; Setchell, 1970, 1978). Further, the incubation temperature was 20°C, which is unusual for mammalian germ cell culture, but it did not seem to have a negative effect.

At the beginning of the 1980s, several teams contributed to making drastic changes in testicular cell culture. Tres and Kierszenbaum (1983) indicated that germ cells could differentiate only in close contact with Sertoli cells. Indeed, they knew that Sertoli cells secreted various nutrients that could be useful for germ cell development. Sertoli cells also provide the necessary microenvironment for germ cell differentiation by maintaining the blood-testis barrier in vivo. Without them, pachytene primary spermatocytes survived no more than 24 hours in a chemically defined medium supplemented with lactate and pyruvate (Jutte et al, 1985).

Kierszenbaum and Tres (1981) designed a Sertoli/germ cell coculture method respecting the interactions between these cells. The cultures were made with 20- to 35-day-old prepuberal rats (Tres and Kierszenbaum, 1983). They used Eagle minimal essential medium (EMEM) culture medium (see Tables 1 and 2 for composition). During the 15-day experiment, they alternated a high FSH concentration for 6 hours with a low concentration for 18 hours in the culture medium. The S-phase cells were allowed to incorporate tritiated thymidine for 24 hours after 2 to 4 days of culture. In these conditions, the most differentiated germ cells that had incorporated the tritiated thymidine were at the preleptotene primary spermatocyte stage 1 hour after the addition of the radioactive element. These cells were identified by silver grains on their surface after autoradiography. Radioactivity was found in leptotene followed by zygotene, and then pachytene primary spermatocytes, respectively, 1, 2, and 3 to 4 days after adding the tritiated thymidine. After 11 days, late pachytene primary spermatocytes contained the radioactive thymidine, which proved the differentiation of these cells from the preleptotene stage in culture.

The first meiotic division figures were observed 6 days after the beginning of a culture from 35-day-old rats (Tres and Kierszenbaum, 1983) and suggested that primary spermatocytes that had already entered meiotic prophase could pass the reductional meiotic division in vitro. In conclusion, these authors have discovered two crucial characteristics of successful culture. These are that germ cells need Sertoli cells, and that it is necessary to keep the cell associations existing in vivo between the different somatic and germ cells.

Nagao (1989) had a radically different but interesting approach to culturing dissociated germ cells without somatic cells. This contradicted the results of Tres and Kierszenbaum (1983) that coculture was needed. Nagao used 14-day-old prepuberal rats and separated the various types of cells according to the Romrell technique (Romrell et al, 1976). Briefly, this consisted of 2 enzymatic digestions (collagenase and trypsin) followed by several rinsings, centrifugations, and filtrations. Before the culture, the wells were coated with collagen. The dissociated cells were then seeded in a culture medium containing adrenaline and noradrenaline (see Tables 1 and 2). According to the authors, these surrenalian factors allowed the germ cells to survive 2 weeks and to differentiate. The flow cytometric analysis indicated an increase in the number of tetraploid cells in the first week. Indeed, after 7 days of culture, 90% of the cells were primary spermatocytes in meiotic prophase. After 10 days, the first spermatids were identified, and the apparition of these cells corresponded to the presence of the first round spermatids in vivo in 24-day-old rats. In short, the germ cells already engaged in meiotic prophase taken from 14-day-old prepuberal rats were able to complete meiosis in vitro according to the same timing as occurs in vivo. Nagao used a complex technique of germ cell isolation followed by a culture system that was very simple but probably inappropriate for germ cells that had been weakened by multiple purification steps. The fact that he started with a large population of primary spermatocytes, whose expected survival would be improved by adding surrenalian hormones, seems to have overcome these drawbacks of no coculture with Sertoli cells. Unfortunately, this work has not been continued, and the protective role of adrenaline and noradrenaline has never been confirmed.

The Jégou team confirmed that dissociated germ cells could complete the end of the meiotic step of spermato-

genesis in vitro (Le Magueresse-Battistoni et al, 1991). They used the work of Mather and Phillips (1984) on primary culture of testicular somatic cells. Elutriated pachytene primary spermatocytes from testes of adult rats were seeded on a layer of Sertoli cells from 20-day-old rats. During the 7-day experiment, the cells were cultured at 32°C in F12-Dulbecco modified essential medium (DMEM) supplemented with insulin, transferrin, retinoic acid, transforming growth factor-α, FSH, and testosterone (see Tables 1 and 2). In these conditions, pachytene spermatocytes were able to complete meiosis in vitro. Indeed, a population of haploid cells was identified by flow cytometry after 4 days of culture. The expression of the gene coding for protamine 1, specific for postmeiotic germ cells, and Northern blot analysis after 1 week confirmed the completion of meiosis in vitro. Hormones and other elements added to the culture medium did not seem to improve the rate of cells passing through meiosis. They would, however, have had a positive effect on germ cell viability in long-term cultures.

The Tesarik and Mendoza team published surprising results (Tesarik et al, 1998). They cultured testicular biopsy samples from men with azoospermia and an obstruction of the genital tract. These biopsies were treated with a lengthy collagenase and elastase enzymatic digestion to produce dissociated cells. The cells were cultured at 30°C for 24 to 48 hours in a medium supplemented or not supplemented with recombinant FSH. In these conditions, the authors declared that cells at the end of meiotic prophase completed meiosis in vitro. Indeed, they observed pairs of secondary spermatocytes and round spermatids, which could indicate that cell division might have occurred. However, they failed to distinguish in vitro differentiated cells from spermatids that simply survived 24 hours in culture. The latter were so numerous that they represented more than 40% of the total cells at the beginning of the experiment. The researchers also reported that in 24 hours some spermatids went through almost the entire spermiogenesis in vitro, whereas this step takes 20 to 22 days in vivo (Heller and Clermont, 1964). This interpreted result of differentiated cells in culture was different from that published so far on the timing of germ cell differentiation and surprised the authors. They explained that, in their culture, there were none of the in vivo obstacles, which could lead to hasty development in spermiogenesis. It also should be noted that the majority of the spermatids seen were abnormal.

There was much controversy around these results from all researchers working on the testis. However, the rapid development of spermatids in vitro was supported recently (Cremades et al, 1999). Using testicular biopsies from men with azoospermia, spermatozoa were reported to be produced in 5 days of culture, from round spermatids cultured on immortalized cells. This process should have taken 20 to 22 days as it does in vivo (Heller and Clermont, 1964).

We have just seen that culture systems of dissociated cells seeded on an impermeable support allow male germ cells that have already entered prophase to complete meiosis in vitro. However, the cultures last no more than 2 weeks, which is not long enough in the majority of mammals for the reproduction in vitro of the entire meiotic step of spermatogenesis. Indeed, when germ cells have no contact with neighboring cells, they quickly lose their morphological characteristics, which explains the short duration of cultures. Yet, such systems have other advantages. First, the methods used to prepare the cells are simple and easy to repeat. Second, the systems allow the use of somatic and germ cells of different ages for the same coculture. They also make it possible to start from homogenous populations of germ cells (at the same stage) and to study their progress in culture. Finally, it is easy to compare what is seeded and what is obtained at the end of the experiment. As far as spermatogenesis is concerned, rates of mitotic and meiotic division can be determined.

Systems Using Immortalized Cells—In 1993, the Cuzin team reported that male germ cells could go through meiotic divisions in vitro in a culture system that included immortalized Sertoli cells (Rassoulzadegan et al, 1993). The Sertoli cells came from transgenic mice, and they expressed a viral oncoprotein with immortalizing but not transforming properties. They had the same morphological characteristics as Sertoli cells and also expressed the *WT1* and *Steel* genes. Testicular cells from mice aged 9 dpp were isolated by mechanical dissociation and cultured at 32°C on the immortalized Sertoli cells. After 8 days, tetrads of round spermatids were detected by Feulgen coloration and flow cytometry analysis. These postmeiotic cells expressed the gene of protamine-1. The other results published must be questioned because the flow cytometric analysis was performed on cultures from testicular cells from adult animals containing a lot of spermatids. According to the study, the spermatid population would increase 43-fold in 5 days. Finally, one must wonder about the existence of elongated spermatids containing tritiated thymidine if its incorporation was made 7 days earlier. Contrary to what the authors suggested, these results do not correspond to the timing of differentiation of germ cells established for mice by Oakberg (1956).

One year later, a promising study was published by the Millán team (Hofmann et al, 1994). Preleptotene primary spermatocytes from 6-week-old mice were immortalized by cotransfection of the gene coding for a viral oncoprotein and a variation of the p53 gene that is sensitive to temperature. At 32°C, the transformed germ cells turn from the proliferation phase to a differentiation phase, when the p53 gene is expressed. The transfected cells

were first cultured at 37°C. After 10 passages, the flow cytometry analysis indicated the presence of diploid and tetraploid cells. After 17 passages (ie, 85 days after the beginning of culture), a population of haploid cells was noted. This population represented 28% of cells after 30 passages. Finally, the percentage of cells with an acrosomic granule increased from 3% at 37°C to 10% after 4 passages at 32°C.

These results were refuted 2 years later by the same team (Wolkowich et al, 1996), when subsequent study on the same culture line gave different results. These cells expressed neither the genes coding for the lactate dehydrogenase (LDH-C4) and acrosin, premeiotic markers, nor the genes coding for protamin-2 and the SP-10 protein (spermatid-stage acrosomal protein) postmeiotic markers. The flow cytometry results were not confirmed and no haploid population was detected. It was concluded that those immortalized germ cells could not differentiate into round spermatids.

Cultures of Seminiferous Tubules—In 1970, Ellingson and Yao (1970) cultured seminiferous tubule fragments from Chinese hamsters aged 1 to 3.5 months. During the cultures, the tubules were covered with a cellophane dialysis membrane in order to simulate the role of the basal membrane. It separated the tubules from all the elements of the incubation medium except those smaller than 4 nm (see Tables 1 and 2). In these conditions, the spermatogonia survived 2 to 3 weeks. Considerable mitosis activity was observed in this time span. Although their life expectancy is shorter, a few primary spermatocytes went through meiotic divisions in the first 72 hours. This time span was much too short for germ cells to go through the entire meiosis in vitro.

As a remedy, some teams tried to devise systems allowing differentiated germ cells to survive longer in vitro. That is how Eddy and Kahri (1976) developed the first seminiferous tubule culture system. After a single mechanical dissociation, 1- to 2-mm fragments of seminiferous tubules were cultured at 32°C or 37°C in a water-saturated atmosphere of air with 5% CO_2. The cultures were generally made with seminiferous tubules from 10- to 14-day-old rats but also with animals aged more than 35 days. These experiments lasted up to 4 weeks, and the organization and quality of the cultured cells were followed by light and electron microscopy. These techniques made it easy to reference the beginning of the culture. The 2 types of somatic cells (Sertoli and peritubular cells) and the 2 types of germ cells (spermatogonia and primary spermatocytes) could be followed during their evolution in vitro. In these conditions, the authors observed that the organization of the seminiferous tubules in 3 dimensions soon changed to 2 dimensions. The somatic cells colonized the surfaces touching the seeded fragments, taking the germ cells to their surface at the same time. The joint migration of Sertoli and germ cells was explained by the presence of desmosome junctions between these 2 cell types. A mass of germ cells were observed at the surface of Sertoli cells but no peritubular cells. That type of reorganization is a characteristic feature of the cultures of small fragments of seminiferous tubules that allow the migration of somatic and germ cells. Specific adhering junctions were identified between Sertoli cells after their reorganization, but the authors did not mention that the blood-testis barrier was present in these areas. This is evidence that spermatogonia and primary spermatocytes that normally live in different environments are able to survive in the same surroundings. The cytoplasmic bridges between germ cells were also maintained in culture. Their published photographs are indeed remarkable considering they were produced only from short-term cultures (2 to 6 days). Apparently, Eddy and Kahri did not study germ cell differentiation, but nonetheless, they devised a system allowing primary spermatocytes to survive for 4 weeks. This paved the way for the study of the differentiation of these cells in culture.

In 1979, the Stefanini team confirmed the previous results with a quite different culture system of seminiferous tubules (Palombi et al, 1979). They took their inspiration from successes of the Dorrington group for cell preparation (Dorrington and Armstrong, 1975) and from the Steinberger group (Steinberger and Steinberger, 1966) for the incubation medium. Testes from 20-day-old rats were decapsulated and the tubules were incubated at 32°C in a water-saturated atmosphere of 5% CO_2 in air. The medium was changed once after 24 hours of culture, then every 6 days. FSH or dibutyryl cAMP was added at the first change of medium (see Tables 1 and 2). In these conditions, Sertoli cells migrated to form a single layer and the germ cells looked morphologically normal and maintained their contacts with the somatic cells (desmosome-type junctions) for 3 weeks. It is difficult to explain the exact role of these rather unusual junctions. However, they allowed the survival of a large population of primary spermatocytes, reputedly fragile in culture, possibly by helping to maintain cytoplasmic bridges and adhering junctions between the germ cells.

In 1983, Parvinen and his colleagues, by using a culture system of seminiferous tubules, showed that it was possible to reproduce the end of meiosis as well as the beginning of spermiogenesis in vitro. The originality of their method lay in the sorting of the seminiferous tubules before seeding. This technique of transillumination-assisted microdissection of seminiferous tubules produced a reputation for the Finnish school (Parvinen, 1982; Parvinen and Ruokonen, 1982). It consists, simply, of placing the seminiferous tubules on a white light and observing them by transparence. Every stage of the seminiferous epithelium cycle can be recognized according to the type,

number, and location of the germ cells it contains. This technique made it possible to isolate tubule fragments at clearly defined stages and to culture them. This was interesting because it wiped out a major drawback of the culture of seminiferous tubules. No longer was it necessary to use testicular tissues or organs from prepuberal animals to eliminate germ cell populations that included all the developmental steps of the spermatogenic cycle.

Seminiferous tubule fragments in stages XII to XIII from adult rats were sorted using this method to eliminate the presence of round spermatids in the selected tubule population. The tubule fragments were then placed in an incubator to be cultured at 32°C in a water-saturated atmosphere containing 5% CO_2 in air, for 1 to 6 days (see Tables 1 and 2). The tubule fragments were observed by transillumination, then by light and electron microscopy. After 2 days of culture, most of the seeded primary spermatocytes (stages XII to XIII) had gone through the 2 meiotic divisions in vitro (Parvinen et al, 1983). After 6 days of incubation, the epithelium of the cultured tubule fragments developed the morphological and biochemical features of a stage V tubule. Indeed, the newly formed round spermatids had an acrosomic system typical of postmeiotic germ cells in stage V of spermiogenesis. There was an increase in the quantity of secreted CP2 and CP3 proteins (cyclic proteins 2 and 3) revealed by bidimensional electrophoresis. These proteins are present in larger number in stage IV through VII seminiferous tubules in vivo. It should be noted that the in vitro differentiation of the germinal epithelium occurred without FSH or testosterone. These results were confirmed 2 years later with the use of tritiated thymidine. Toppari and Parvinen (1985) undoubtedly showed that male germ cells are able to go through their meiotic divisions in vitro in a relatively simple culture system and without the help of hormones or growth factors. For the first time, meiosis rate could be estimated. Another advantage of the technique was the possibility of following the differentiation of germ cells of any stage, without having to use radioactivity. With the addition of a quicker quantification method such as flow cytometry, they now had an ideal tool with which to study spermatogenesis (Toppari et al, 1986).

More recently, the Dutch team of Toebosch, together with Grootegoed, suggested a new interesting approach (Toebosch et al, 1989). For the first time, open seminiferous tubules were used in 72-hour experiments that were more like cell survival than culture. Seminiferous tubules from 26-day-old prepuberal Wistar rats were isolated by enzymatic digestion. The tubule fragments were then seeded at 32°C in a water-saturated atmosphere of air with 5% CO_2 and terminated at 24, 48, and 72 hours of culture (see Tables 1 and 2). Those cell samples were then dissociated and analyzed by flow cytometry. In these conditions and despite the presence of postmeiotic cells at the beginning of culture, the percentage of spermatids increased significantly from 2.8% to 6.2% in 3 days. Primary spermatocytes at the end of meiotic prophase had undergone the 2 meiotic divisions in vitro. The increase was not linked to the addition of FSH, insulin, retinol, or testosterone. These elements seemed, however, to improve the survival of the primary spermatocytes when added to the medium. According to the authors, the decrease in the number of primary spermatocytes in 3 days of experiment was due to their completion of meiosis.

In conclusion, seminiferous tubule culture offers the same advantages as tissue or organ culture but without the drawbacks. The culture of seminiferous tubules, isolated by microdissection or careful enzymatic digestion, allows the culture of germ cells without depriving them of their somatic environment. The architecture of the seminiferous tissue is maintained. The preservation in vitro of the communications existing in vivo between the different cell categories is necessary to reproduce spermatogenesis in an experimental system. These cell interactions seem to improve the survival of germ cells in culture. In such a system, it is possible for germ cells from immature or prepuberal animals to survive 1 month in vitro (Eddy and Kahri, 1976; Palombi et al, 1979), but nothing has been said about their ability to differentiate in this lapse of time. The fact that the tubules are seeded open may still improve the life expectancy of the cultured cells, simply by making the exchange of metabolites between the culture medium and a maximum of cells easier (Toebosch et al, 1989). The better circulation of the medium is indeed a good way to avoid necrosis due to tissue anoxia. There are two more advantages to the Parvinen and Toppari model. Transillumination-assisted microdissection allows to start from a homogenous population of seminiferous tubule fragments, which is extremely useful in the study of a synchronized process, such as spermatogenesis. It also allows evaluation of the meiosis rate of germ cells. The differentiation of germ cells can be followed in vitro without using radioactivity because the tubule fragments are sorted, but it is necessary to work with adult animals because the criteria for identification require that all the stages of spermatogenesis be represented until spermiation. Unfortunately, the experiments conducted by this team were much too short to reproduce the entire meiotic step of spermatogenesis in vitro.

Cultures on Permeable Supports

Cocultures of Sertoli and Germ Cells—In the 1970s, researchers began to understand the role of the blood-testis barrier. This series of tight junctions between the Sertoli cells is the structural basis of the compartmentalization of the seminiferous epithelium. The meiotic and postmeiotic steps of spermatogenesis occur in the adluminal com-

partment (Dym and Fawcett, 1970) in a privileged environment (Setchell, 1970, 1978). As culture systems had been unable to reproduce that compartmentalization thus far, researchers wondered about the possibility of reproducing male gametogenesis in vitro. Indeed, it had been proven that the organization of the ''tight-gap'' complexes, which are necessary for maintaining the blood-testis barrier, was changed in culture. In fact, the importance of the morphological changes in the junctions between Sertoli cells increases with the time of culture (Bigliardi and Vegni Talluri, 1976).

That is why the Kierszenbaum team attempted to devise a culture system in which the polarity of the testicular cells would be kept in vitro (Ueda et al, 1988). Without changing the way they prepared testicular cells, they tried two different permeable supports. These included a nylon support covered with extracts from extracellular matrix (matrigel) with 100-μm pores or a filter with 0.45-μm pores, placed in a perfusion system. The Sertoli cells, still associated with spermatogonia and primary spermatocytes and prepared from 20 to 22-day-old rats, were seeded on these supports, with or without precultures of peritubular cells (see Tables 1 and 2). The best results were obtained on the 0.45-μm filter (ie, without extracellular matrix). On this kind of support, the Sertoli cells organized into a polarized, cubic epithelium. At the basal level, numerous Sertoli cell projections covered the microporous support. The germ cells were observed at the apical level. Besides, vectorial secretions of Sertoli cell proteins were spotted in vitro. The S70 protein and the S45-S35 heterodimer were temporary and cyclically secreted during spermatogenesis and would play a part during spermiogenesis. Through this method, by culturing for 12 hours peritubular cells that organize into a double layer on that same support before seeding the Sertoli and germ cells, it is possible to reproduce in vitro the organization of the different testicular cell categories as it exists in vivo in the seminiferous tubules.

Another improvement took place a few years later (Tres et al, 1992). The aim was to combine the physical and chemical advantages of both supports previously used. Indeed, the microporous filter is good for the cells but its opacity makes it difficult to follow and observe the cultures. In contrast, matrigel is transparent, but it has a negative effect on the viability of the spermatogenic cells in coculture with Sertoli cells. That is why Tres et al made a third permeable support from the polymers making up the extracellular matrix (ie, collagen and glycosaminoglycans). On this transparent support, the typical cell organization of the testis was preserved. According to the authors, spermatogonia proliferated and entered meiotic prophase, primary spermatocytes differentiated and reached the stage of meiotic divisions, and round spermatids became elongated spermatids with a correctly organized acrosome and a motile flagella in vitro. Because that team's aim was to devise a culture system of polarized testicular cells, they gave little evidence regarding germ cell differentiation in vitro. No primary spermatocytes were seen to go through the 2 meiotic divisions in vitro (Tres et al, 1991).

More recently, the Durand team confirmed the positive role of systems that use the polarity of Sertoli cells in culture on germ cell development (Weiss et al, 1997). Pachytene primary spermatocytes and round spermatids, obtained from adult rats by centrifugal elutriation, were seeded on Sertoli primary cultures of Sertoli cells isolated from 20-day-old rats. The experiments were performed in bicameral chambers. During the culture, only the medium in the basal compartment was changed every other day, which allowed the Sertoli cells, little by little, to become conditioned to the medium of the apical compartment. Sertoli cells and germ cells were cocultured for 2 weeks in a chemically defined, serum-free medium (see Tables 1 and 2) in a water-saturated atmosphere of 5% CO_2 in air. In these conditions, when pachytene primary spermatocytes and Sertoli cells were cocultured, a population of haploid cells (round spermatids) was noted 48 hours after the beginning of the experiment. Using 5-bromo-2′-deoxyuridine (BrdU) as a thymidine analogue that is incorporated during the S-phase of cells preparing for cell division (spermatogonia and preleptotene primary spermatocytes), the authors were able to follow the differentiation of germ cells in vitro. They showed that BrdU-labeled pachytene primary spermatocytes of stages V through VIII are able to differentiate into round spermatids (Weiss et al, 1997). The results were confirmed by reverse transcription-polymerase chain reaction and a clever system that allowed the comparison of the expression of genes expressed during meiosis (phosphoprotein 19 [p19] and testis-specific histone 2B [TH_2B]), and after meiosis (transition proteins 1 and 2 [TP1 and TP2]).

Cultures of Seminiferous Tubules—When comparing the results of all the teams presented in this review (Table 3), it is clear that many advancements have been made, but none of the systems described are adequate for the reproduction in vitro of the entire meiotic step of spermatogenesis. It seems that the main obstacle is the survival of germ cells in culture. However, the culture of fragments from seminiferous tubules on a permeable support combines the advantages of other models. As in organ culture, culture of seminiferous tubules seed germ cells without depriving them of the Sertolian environment that is necessary to their survival and differentiation. Hence, germ cells keep their morphological characteristics, as well as their contacts with somatic cells and other germ cells. Seeding open fragments of seminiferous tubules allows the cells to be well irrigated, even at the center of the fragments, and avoids the apparition of large

Table 3. *Main results on mammalian male germ cell meiotic differentiation in vitro**

Authors	Date	Animals	Culture system	Method used to follow germ cell differentiation	In vitro meiotic differentiation
Champy	1920	Adult rabbits	Tissue culture	···	Spermatogonia→ leptotene spermatocytes
Martinovitch	1937	Newborn mice	Organ culture	···	Gonocytes→ pachytene spermatocytes
Gaillard and Varossieau	1938	Prepubertal rats (28 dpp)	Tissue culture	···	Meiotic divisions?
Trowell	1959	Prepubertal rats (28 dpp)	Tissue culture	···	Mitotic and meiotic divisions
Steinberger et al	1964	Prepubertal rats (4 dpp)	Tissue culture	···	A spermatogonia→ pachytene spermatocytes
Steinberger and Steinberger	1965	Prepubertal rats (12 dpp)	Tissue culture	Tritiated thymidine	Preleptotene → pachytene spermatocytes
Steinberger	1967	Adult men	Tissue culture	Tritiated thymidine	Preleptotene → pachytene spermatocytes
Ellingson and Yao	1970	Chinese hamster (1–3.5 months)	Seminiferous tubules Impermeable support	···	Meiotic divisions
Ghatnekar et al	1974	Adult men	Tissue culture	Tritiated thymidine	Preleptotene spermatocytes → telophase II?
Aizawa and Nishimune	1979	Adult cryptorchid mice	Tissue culture	Tritiated thymidine	A spermatogonia → pachytene spermatocytes
Dietrich et al	1982	Adult mice	Sertoli/germ cells coculture Impermeable support	Hydroxyurea	Zygotene spermatocytes → round spermatids
Tres and Kierszenbaum	1983	Prepubertal rats (20–35 dpp)	Sertoli/germ cells coculture Impermeable support	Tritiated thymidine	Preleptotene → pachytene spermatocytes First meiotic division
Parvinen et al	1983	Adult rats	Seminiferous tubules Impermeable support	···	Late primary spermatocytes (stage XII–XIII) → round spermatids (stage 5)
Toppari and Parvinen	1985	Adult rats	Seminiferous tubules Impermeable support	Tritiated thymidine	Preleptotene → pachytene spermatocytes Pachytene spermatocytes → round spermatids
Nagao	1989	Prepubertal rats (14 dpp)	Germ cells culture Impermeable support	···	Meiotic divisions
Toebosch et al	1989	Prepubertal rats (26 dpp)	Seminiferous tubules Impermeable support	···	Meiotic divisions
Le Maguerresse-Battistoni et al	1991	Prepubertal rats (20 dpp) and adult rats	Sertoli/germ cells coculture Impermeable support	···	Pachytene spermatocytes→ round spermatids
Tres et al	1992	Prepubertal rats (20–35 dpp)	Sertoli/germ cells coculture Permeable support	···	Spermatogonia→ meiotic prophase Primary → secondary spermatocytes
Boitani et al	1993	Prepubertal rats (9 dpp)	Tissue culture	···	A spermatogonia→ pachytene spermatocytes
Rassoulzadegan et al	1993	Prepubertal mice (9 dpp)	Immortalized Sertoli cells	Tritiated thymidine	Meiotic divisions
Weiss et al	1997	Prepubertal rats (20 dpp)	Sertoli/germ cells coculture Permeable support	Bromodeoxyuridine	Pachytene spermatocytes (stage V–VIII) → round spermatids
Hue et al	1998	Prepubertal rats (25 dpp)	Seminiferous tubules Permeable support	Bromodeoxyuridine	Pachytene spermatocytes (stage IV–VI) → round spermatids
Tesarik et al	1998	Adult men	Sertoli/germ cells coculture Impermeable support	···	Meiotic divisions
Staub et al	2000	Prepubertal rats (20 dpp)	Seminiferous tubules Permeable support	Bromodeoxyuridine	Leptotene spermatocytes → round spermatids

* dpp indicates days postpartum

necrosis areas due to tissue anoxia (Toebosch et al, 1989). It also allows somatic and germ cells to migrate out of the tubules to settle in an organized fashion in the free zones of the culture support, as Eddy and Kahri (1976) described it. As seen in the works of the Kierszenbaum team (Ueda et al, 1988), a permeable culture support, if appropriate, allows the partial reproduction of the compartmentalization existing in the seminiferous tubule. The cells organize themselves into a pseudopolarized system similar to that of the seminiferous epithelium in vivo.

This last system, which best reproduces compartmentalization of the seminiferous tubule without dissociating the cocultured somatic and germ cells, has been successfully used recently by the Durand team in which a part (Hue et al, 1998), then the entire (Staub et al, 2000) meiotic stage of spermatogenesis was reproduced in vitro. In those experiments, small fragments of seminiferous tubules, obtained by enzymatic digestion of testes from 20- to 25-day-old rats, were seeded in bicameral chambers in a chemically defined, serum-free medium. In the first 2 days, 0.2% of fetal calf serum was added to the supplemented medium (see Tables 1 and 2) in order to help the tubules stick to the permeable support. The cultures lasted for 4 weeks at 32°C in a water-saturated atmosphere of 5% CO_2 in air. Again, only the medium of the basal compartment was changed every other day. The medium in the apical compartment was gradually conditioned by the different cell populations as shown by an increase of the transferrin concentration using radioimmunoassay. In these conditions, starting from a system without postmeiotic cells, the authors observed the apparition of a population of haploid cells (round spermatids) after 1 week of culture. This population remained throughout the entire experiment of 4 weeks. Moreover, round spermatids were identified by electron microscopy after 3 weeks of culture. The study of gene expression, p19/TP1 and TH_2B/TP2 (Hue et al, 1998) or TP1 and TP2 (Staub et al, 2000), confirmed the previous results and showed that round spermatids produced in vitro were able to express genes in culture. The use of BrdU showed that leptotene primary spermatocytes were able to differentiate into round spermatids after 3 weeks of culture (Staub et al, 2000). Contrary to what Tesarik et al (2000) have recently published, the timing of differentiation of male germ cells in vitro in the 2 systems mentioned above seemed to correspond to that of male germ cells in vivo. Finally, BrdU-labeled haploid germ cells were isolated by flow cytometry coupled with immunocytochemistry detection. The authors demonstrated here, for the first time, that the entire meiotic part of spermatogenesis could be achieved in vitro with mammalian male germ cells.

Conclusion

Due to the collective efforts and success of the different laboratories, the entire meiotic part of spermatogenesis has been achieved in vitro. These works have already allowed and will further help to better understand meiosis. Indeed, the possibility of inhibiting the expression of genes (by using antisense mRNAs) or the action of proteins (by using antibodies against a protein or its receptor) during long-term culture reproducing male gametogenesis in vitro should help to better understand the regulation of the meiotic step of spermatogenesis. In addition, the ability to produce in vitro round spermatids that have reached the stage of differentiation necessary for in vitro fertilization would open new possibilities for the treatment of male sterility. It will then be necessary to adapt the culture techniques for other species than rodents.

Acknowledgments

I thank S. Carreau, P. Durand, A. Gérard, R. Habert, M.T. Hochereau de Reviers, D. Hue, J.C. Nicolle, and M.C. Viguier-Martinez for reading and correcting the French version of the manuscript. I profoundly thank my wife Gaëlle for editing and translating it into English. I also thank L. Johnson and A. Steinberger for discussing this review, and R.S. Heck and S.L. Van De Wiele for reading and correcting it.

References

Aizawa S, Nishimune Y. In vitro differentiation of type A spermatogonia in mouse cryptorchid testis. *J Reprod Fertil.* 1979;56:99–104.

Asayama S, Furusawa M. Culture in vitro of prospective gonads and gonad primordia of mouse embryo. *Zool Magazine.* 1960;69:280–284.

Asayama S, Furusawa M. Sex differentiation of primordial gonads of the mouse embryo after cultivation in vitro. *Jpn J Exp Morphol.* 1961; 15:34.

Bigliardi E, Vegni Talluri M. Ultrastructural details of Sertoli cell junctional complexes in vivo and their modifications in tissue culture. *Cell Tissue Res.* 1976;172:29–38.

Boitani C, Politi MG, Menna T. Spermatogonial cell proliferation in organ culture of immature rat testis. *Biol Reprod.* 1993;48:761–767.

Borghese E, Venini MA. Culture in vitro de gonades embryonnaires de Mus musculus. *Symp Genetica.* 1956;5:69–83.

Chairussyuhur A, Sánchez-Partida LG, Maddocks S, Setchell BP. Quail yolk and coconut extract in diluents for storage of ram semen at 30°C and 5°C. Proceedings of the 25th Annual Conference of the Australian Society for Reproductive Biology; 1993.

Champy C. Quelques résultats de la méthode de culture des tissus. *Arch Zool Exp Gen.* 1920;60:461–500.

Clermont Y. The cycle of the seminiferous epithelium in man. *Am J Anat.* 1963;112:35–51.

Clermont Y, Leblond CP, Messier B. Durée du cycle de l'épithelium séminal du rat. *Arch Anat Microsc Morphol Exp.* 1959;48:37–56.

Cremades N, Bernabeu R, Barros A, Sousa M. In vitro maturation of round spermatids using co-culture on Vero cells. *Hum Reprod.* 1999; 14:1287–1293.

Dietrich AJJ, Oud JL, Vink ACG. Male germ cells in vitro. Differentiation throughout meiosis in cultures of testicular cell suspensions of the mouse. Second European Testis Workshop. Molecular and Cellular Endocrinology of the Testis; 1982.

Dietrich AJJ, Scholten R, Vink ACG, Oud JL. Testicular cell suspensions of the mouse in vitro. *Andrologia.* 1983;15:236–246.

Dorrington JH, Armstrong DT. Follicle-stimulating hormone stimulates

estradiol-17β synthesis in cultured Sertoli cells. *Proc Natl Acad Sci USA.* 1975;72:2677–2681.

Dua IS, Chandra M. The identification and isolation of plant growth regulating substances from liquid endosperm of *Cocos nucifera.* In: Nair MK, Khan HH, Gopalasundaram P, Bhaskararad EVY, eds. *Advances in Coconut Research and Development.* New Delhi, India: 1993.

Dux C. Recherches sur les cultures de testicule hors de l'organisme. Action de l'hormone gonadotrope. *Arch Anat Microsc (Paris).* 1939;35: 391.

Dym M, Fawcett DW. The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. *Biol Reprod.* 1970;3:308–326.

Eddy EM, Kahri AI. Cell associations and surface features in cultures of juvenile rat seminiferous tubules. *Anat Rec.* 1976;185:333–358.

Ellingson DJ, Yao KTS. Growth and observations of Chinese hamster seminiferous epithelium in vitro. *J Cell Sci.* 1970;6:195–205.

Fell HB, Robison R. The growth, development and phosphatase activity of embryonic avian femora and limb buds cultivated in vitro. *Biochem J.* 1929;23:767.

Gaillard PJ, Varossieau WW. The structure of explants from different stages of development of the testis on cultivation in media obtained from individuals of different stages. *Acta Neerl Morphol.* 1938;1:313.

Ghatnekar R, Lima-de-Faria A, Rubin S, Menander K. Development of human male meiosis in vitro. *Hereditas.* 1974;78:265–272.

Haneji T, Maekawa M, Nishimune Y. Vitamin A and follicle-stimulating hormone synergistically induce differentiation of type A spermatogonia in adult cryptorchid testes in vitro. *Endocrinology.* 1984;114: 801–805.

Heller GC, Clermont Y. Kinetics of the germinal epithelium in man. *Recent Prog Horm Res.* 1964;20:545–575.

Hofmann MC, Hess RA, Goldberg E, Millán JL. Immortalized germ cells undergo meiosis in vitro. *Proc Natl Acad Sci USA.* 1994;91:5533–5537.

Hue D, Staub C, Perrard-Sappori MH, Weiss M, Nicolle JC, Vigier M, Durand P. Meiotic differentiation of germinal cell in three-week cultures of whole cell population from rat seminiferous tubules. *Biol Reprod.* 1998;59:379–387.

Jutte NHPM, Grootegoed JA, Rommerts FFG, van der Molen HJ. Exogenous lactate is essential for metabolic activities in isolated rat spermatocytes and spermatids. *J Reprod Fertil.* 1981;62:399–405.

Jutte NHPM, Jansen R, Grootegoed JA, Rommerts FFG, Clausen OPF, van der Molen HJ. Regulation of survival of rat pachytene spermatocytes by lactate supply from Sertoli cells. *J Reprod Fertil.* 1982;65: 431–438.

Jutte NHPM, Jansen R, Grootegoed JA, Rommerts FFG, van der Molen HJ. Protein synthesis by isolated pachytene spermatocytes in the absence of Sertoli cells. *J Exp Zool.* 1985;233:285–290.

Kierszenbaum AL. Mammalian spermatogenesis in vivo and in vitro: a partnership of spermatogenic and somatic cell lineages. *Endocr Rev.* 1994;15:116–134.

Kierszenbaum AL, Tres LL. The structural and junctional cycle of Sertoli cells in culture. In: Jagiello G, Vogel HJ, eds. *Bioregulators of Reproduction.* New York, NY: Academic Press; 1981:207–228.

Le Magueresse-Battistoni B, Gérard N, Jégou B. Pachytene spermatocytes can achieve meiotic process in vitro. *Biochem Biophys Res Commun.* 1991;179:1115–1121.

Lima-de-Faria A, German J, Ghatnekar M, McGovern J, Anderson L. DNA synthesis in the meiotic chromosomes of man. A preliminary report. *Hereditas.* 1966;56:398–399.

Lima-de-Faria A, German J, Ghatnekar M, McGovern J, Anderson L. In vitro labelling of human meiotic chromosomes with H_3-thymidine. *Hereditas.* 1968;60:249–261.

Lostroh AJ. In vitro response of mouse testis to human chorionic gonadotropin. *Proc Soc Exp Biol NY.* 1960;103:25.

Magre S, Jost A. Dissociation between testicular organogenesis and endocrine cytodifferentiation of Sertoli cells. *Proc Natl Acad Sci USA.* 1984;81:7831–7834.

Martinovitch PN. Development in vitro of the mammalian gonad. *Nature.* 1937;139:413.

Mather JP, Phillips DM. Primary culture of testicular somatic cells. In: Barnes D, Sibarsku D, Sato GH, eds. *Methods in Molecular and Cell Biology. Methods for Serum-Free Culture of Cells of the Endocrine System.* Vol. 2. New York, NY: AR Liss; 1984:29–45.

Michailow W. Experimentell-histologische Untersuchungen über die Elemente der Hodenkanälchen. *Z Zellforsch.* 1937;26:174.

Nagao Y. Viability of meiotic prophase spermatocytes of rats is facilitated in primary culture of dispersed testicular cells on collagen gel by supplementing epinephrine or norepinephrine: evidence that meiotic prophase spermatocytes complete meiotic divisions in vitro. *In Vitro Cell Dev Biol.* 1989;25:1088–1098.

Nuñes JF. Utilization of the coconut water as extender for the semen of domestic animals and man. *Rev Bras Reprod Anim.* 1998;22:109–112.

Oakberg EF. Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. *Am J Anat.* 1956; 99:507–516.

Palombi F, Ziparo E, Rommerts FFG, Grootegoed JA, Antonini M, Stefanini M. Morphological characteristics of male germ cells of rats in contact with Sertoli cells in vitro. *J Reprod Fertil.* 1979;57:325–330.

Parvinen M. Regulation of the seminiferous epithelium. *Endocr Rev.* 1982;3:404–417.

Parvinen M, Ruokonen A. Endogenous steroids in rat seminiferous tubules. Comparison of different stages of the epithelial cycle isolated by transillumination-assisted microdissection. *J Androl.* 1982;3:211–220.

Parvinen M, Wright WW, Phillips DM, Mather NA, Musto NA, Bardin CW. Spermatogenesis in vitro: completion of meiosis and early spermiogenesis. *Endocrinology.* 1983;112:1150–1152.

Rassoulzadegan M, Paquis-Flucklinger V, Bertino B, et al. Transmeiotic differentiation of male germ cells in culture. *Cell.* 1993;75:997–1006.

Romrell LJ, Bellve AR, Fawcett DW. Separation of mouse spermatogenic cells by sedimentation velocity. *Dev Biol.* 1976;49:119–131.

Russell LD, Steinberger A. Sertoli cells in culture: views from the perspectives of an In Vivoist and an In Vitroist. *Biol Reprod.* 1989;41: 571–577.

Setchell BP. Testicular blood supply, lymphatic drainage, and secretion of fluid. In: Johnson AD, Gomes WR, Vandemark NL, eds. *The Testis. Development, Anatomy, and Physiology.* Vol 1. New York, NY: Academic Press; 1970:101–239.

Setchell BP. *The Mammalian Testis.* London, United Kingdom: Elec Books Limited; 1978.

Sparrow AH, Pond V, Kojan S. Microsporogenesis in excised anthers of *Trillium erectum* grown on sterile media. *Am J Botany.* 1955;42:384–394.

Staub C, Hue D, Nicolle JC, Perrard-Sappori MH, Segretain D, Durand P. The whole meiotic process can occur in vitro in untransformed rat spermatogenic cells. *Exp Cell Res.* 2000;260:85–95.

Steinberger A, Steinberger E. Differentiation of rat seminiferous epithelium in organ culture. *J Reprod Fertil.* 1965;9:243–248.

Steinberger A, Steinberger E. Stimulatory effect of vitamins and glutamine on the differentiation of germ cells in rat testes organ culture grown in chemically defined media. *Exp Cell Res.* 1966;44:429–435.

Steinberger A, Steinberger E. Factors affecting spermatogenesis in organ cultures of mammalian testes. *J Reprod Fertil.* 1967;(suppl 2):117–124.

Steinberger A, Steinberger E, Perloff WH. Mammalian testes in organ culture. *Exp Cell Res.* 1964;36:19–27.

Steinberger E. Maintenance of adult human testicular tissue in culture. *Anat Rec.* 1967;157:327–328.

Steinberger E, Dixon WJ. Some observations on the effect of heat on the testicular germinal epithelium. *Fertil Steril.* 1959;10:578–595.

Steinberger E, Nelson WO. The effect of hypophysectomy, cryptorchidism, estrogen and androgen upon the level of hyaluronidase in the rat testis. *Endocrinology.* 1955;56:429–444.

Steinberger E, Steinberger A, Perloff WH. Initiation of spermatogenesis in vitro. *Endocrinology.* 1964;74:788–792.

Strangeways TSP, Fell HB. Experimental studies on the differentiation of embryonic tissues growing in vivo and in vitro. I. The development of the undifferentiated limb bud. *Proc R Soc Biol Sci Ser B.* 1926; 99:340.

Tesarik J, Greco E, Rienzi L, Ubaldi P, Guido M, Cohen-Bacrie P, Mendoza C. Differentiation of spermatogenic cells during in vitro culture of testicular biopsy samples from patients with obstructive azoospermia: effect of recombinant follicle stimulating hormone. *Hum Reprod.* 1998;13:2772–2781.

Tesarik J, Mendoza C, Greco E. In-vitro maturation of immature human male germ cells. *Mol Cell Endocrinol.* 2000;166:45–50.

Toebosch AMW, Brussée R, Verkerk A, Grootegoed JA. Quantitative evaluation of the maintenance and development of spermatocytes and round spermatids in cultured tubule fragments from immature rat testis. *Int J Androl.* 1989;12:360–374.

Toppari J, Mali P, Eerola E. Rat spermatogenesis in vitro traced by quantitative flow cytometry. *J Histochem Cytochem.* 1986;34:1029–1035.

Toppari J, Parvinen M. In vitro differentiation of rat seminiferous tubular segments from defined stages of the epithelial cycle. Morphologic and immunolocalization analysis. *J Androl.* 1985;6:334–343.

Tres LL, Cahn F, Kierszenbaum AL. Rat Sertoli-spermatogenic cell cocultures established on collagen-glycosaminoglycan crosslinked copolymers. *J Tissue Cult Methods.* 1992;14:265–270.

Tres LL, Kierszenbaum AL. Viability of rat spermatogenic cells in vitro is facilitated by their coculture with Sertoli cells in serum-free hormone-supplemented medium. *Proc Natl Acad Sci USA.* 1983;80: 3377–3381.

Tres LL, Smith FF, Kierszenbaum AL. Spermatogenesis in vitro: methodological advances and cellular functional parameters. Serono Symposia. New York, NY: Raven Press; 1991;71:115–125.

Trowell OA. The culture of mature organs in a synthetic medium. *Exp Cell Res.* 1959;16:118–147.

Ueda H, Tres LL, Kierszenbaum AL. Culture patterns and sorting of rat Sertoli cell secretory proteins. *J Cell Sci.* 1988;89:175–188.

Van Overbeek J, Conklin ME, Blakeslee AF. Factors in coconut milk essential for growth and development of very young *Datura* embryos. *Science.* 1941;94:350–351.

Weiss M, Vigier M, Hue D, Perrard-Sappori MH, Marret C, Avallet O, Durand P. Pre- and post-meiotic expression of male germ cell specific genes throughout 2-week cocultures of rat germinal and Sertoli cells. *Biol Reprod.* 1997;57:68–76.

Wolff E. Sur la différenciation sexuelle des gonades de souris explantées in vitro. Paris: Séance de l'Académie des Sciences; 1952:234.

Wolff E, Haffen K. Sur la culture in vitro des glandes génitales des embryons d'oiseau: obtention de la différenciation sexuelle normale et de l'intersexualité expérimentale des gonades explantées. Paris: Séance de l'Académie des Sciences; 1951:233.

Wolff E, Haffen K. Germ cells and gonads. In: Willmer EN, ed. *Cells and Tissues in Culture. Methods, Biology and Physiology.* Vol 2. London, United Kingdom: Academic Press; 1965:697–743.

Wolkowicz MJ, Coonrod SM, Reddi PP, Millán JL, Hofmann MC, Herr JC. Refinement of the differentiated phenotype of the spermatogenic cell line GC-2spd(ts). *Biol Reprod.* 1996;55:923–932.