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NEW RESEARCH HORIZON *Review*

New horizons for *in vitro* spermatogenesis? An update on novel three-dimensional culture systems as tools for meiotic and post-meiotic differentiation of testicular germ cells

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ABSTRACT: Culture and differentiation of male germ cells has been performed for various purposes in the past. To date, none of the studies aimed at *in vitro* spermatogenesis has resulted in a sufficient number of mature gametes. Numerous studies have revealed worthy pieces of information, building up a body of information on conditions that are required to maintain and mature male germ cells *in vitro*. In this review, we report on previously published and unpublished experiments addressing murine germ cell differentiation in three-dimensional (3D) *in vitro* culture systems. In a systematic set of experiments, we examined the influence of two different matrices (soft agar and methylcellulose) as well as the need for gonadotrophin support. For the first time, we demonstrate that pre-meiotic male germ cells [revealed by the absence of meiotic marker expression (e.g. Boule)] obtained from immature mice pass through meiosis *in vitro*. After several weeks of culture, we obtained morphologically normal spermatozoa embedded in the matrix substance. Complete maturation relied on support from somatic testicular cells and the presence of gonadotrophins but appeared independent from the matrix in a 3D culture environment. Further research efforts are required to reveal the applicability of this culture technique for human germ cells and the functionality of the spermatozoa for generating offspring.

Key words: *in vitro* spermatogenesis / gonadotrophins / soft agarculture system (SACS) / methylcellulose culture system (MCS)

Introduction

Male fertility preservation is considered an important topic in reproductive medicine/biology. New strategies for maintenance of livestock, for conservation of rare species and for fertility protection in men have been experimentally addressed in model systems (Sofikitis *et al.*, 2003; Ehmcke and Schlatt, 2008). In respect to clinical applications, a steadily increasing number of patients facing infertility due to oncological therapy have provoked a number of studies to explore the cellular mechanisms during male germ cell differentiation and to generate new strategies for preservation and expansion of male germ cells. Two main experimental strategies have been followed to achieve these goals: (i) testicular germ cell transplantation or testicular grafting into host animals (Wistuba and Schlatt, 2002; Dobrinski and Travis, 2007) or (ii) germ cell or testicular tissue culture (Staub, 2001; Sofikitis *et al.*, 2003, 2005; Georgiou *et al.*, 2007). *In vitro* germ cell culture systems and/or transplantation techniques could provide future options for genome preservation and fertility treatment in adult, adolescent and pre-pubertal cancer patients and infertile male patients.

Xenotransplantation provides developing germ cells with microenvironments similar to the situation in the donor. However, the transfer

of germ cells into a foreign species carries the risk of retroviral transmission or contamination of the germ line cells with contagious agents. These risks do not exist for autologous testicular grafting. On the other hand, autologous transfer increases the risk for malignant relapse as contaminating tumour cells from the testicular tissue could be reinfused into the lymphatic system. Thus far, several approaches have been designed to solve the problem of possible cancer cell contamination but none has revealed success rates acceptable for clinical use (Fujita *et al.*, 2005; Fujita *et al.*, 2006; Hou *et al.*, 2007; Hou *et al.*, 2009). Despite the risk of transferring cancer cells or viruses, techniques of transplantation or grafting of testicular tissues have already resulted in completion of spermatogenesis in several animal models *in vivo* (Honaramooz *et al.*, 2002; Schlatt *et al.*, 2003; Snedaker *et al.*, 2004; Honaramooz *et al.*, 2007; Luetjens *et al.*, 2008).

The *in vitro* generation of sperm from germ line stem cells which would require the entire process of spermatogenesis to occur in a culture dish remains a challenge. As yet, numerous *in vitro* studies have attempted differentiation of male germ cells into mature spermatozoa. Promising results have been published for *in vitro* maturation of round and elongating spermatids. In patients with round spermatid arrest, it has been possible to overcome the developmental blockade at the level of round spermatids and the cultured cells continue differentiation into mature spermatozoa (Cremades *et al.*, 1999). In subsequent studies, *in vitro* matured spermatids were shown to have low fertilization potential but, in the cases of successful fertilization, had normal blastocyst formation potential (Cremades *et al.*, 2001) Although *in vitro* differentiation of male germ cells from earlier developmental stages have been described (Aslam and Fishel, 1998; Tesarik *et al.*, 1998a, b; Tanaka *et al.*, 2003), cultured male germ cells undergoing a completed spermatogenic cycle starting from spermatogonial stem cells (SSCs) and differentiating into mature spermatozoa have not been demonstrated so far.

This review focuses on studies dealing with strategies to differentiate male germ cells *in vitro* by using single-cell suspensions of immature rodent testicular cells in three-dimensional (3D) culture systems. We and others have recently published a report showing that novel culture systems provide promising strategies for *in vitro* germ cell differentiation (Lee *et al.*, 2006; Stukenborg *et al.*, 2008). Improving such approaches might offer future perspectives to preserve male fertility.

History of experimental approaches towards *in vitro* spermatogenesis

Almost a century ago, the first studies on *in vitro* spermatogenesis aimed at achieving a better understanding of testicular germ cell proliferation and differentiation. Organ cultures of testicular tissue from a variety of species revealed *in vitro* maintenance of the spatial structure of the seminiferous epithelium and persistence of cell to cell communications of somatic and germ cells (Goldschmidt, 1915; Champy, 1920; Michailow, 1937). Although these cultures presented rather simple settings in comparison to today's standards of *in vitro* studies, these early experiments already resulted in progression of spermatogonia into meiosis. However, spermatogenesis arrested in meiosis and no completion of spermatogenesis was achieved under these culture conditions.

In the 1960s and 1970s, the group of Anna and Emil Steinberger performed a series of studies demonstrating crucial effects on the developmental and functional status of the cultured testicular tissue. These studies also revealed the *in vitro* effects of temperature and hormones on the survival of testicular tissue and on the extent of germ cell proliferation and differentiation. It was shown that an incubation temperature lower than body core temperature was beneficial for testicular germ cell differentiation and that the addition of gonadotrophins had no direct positive effect on germ cell differentiation (Steinberger, 1975). They also analysed whether the developmental potential of testicular tissue fragments improves when cultivated in the presence of isolated Sertoli cells (Steinberger *et al.*, 1964; Steinberger and Steinberger, 1966, 1970). The importance of a low testicular temperature for successful completion of spermatogenesis *in vivo* as well as *in vitro* was confirmed in subsequent studies (Nakamura *et al.*, 1978; Mieusset and Bujan, 1995). Consequently, testicular cells should be cultured close to scrotal temperature at 35°C.

A second factor important for successful maintenance and culture of the germ line is the developmental status of the testicular tissue, which is related to the donors' age. Several studies have demonstrated that pre-pubertal germ cells are twice as viable as more advanced germ cells (Creemers *et al.*, 2002). The proportion of undifferentiated spermatogonia is 100-fold higher in immature mouse testes (Day 10 post-parturition) compared with adult testes (de Rooij and Russell, 2000; McLean *et al.*, 2003; Aponte *et al.*, 2005). The high number of testicular stem cells and the better survival rates imply that immature testes provide better starting material for *in vitro* approaches on testicular germ cells compared with adult testes. Many studies have also explored the role of growth factors and hormones on spermatogonial expansion and the fate decision to undergo self-renewal and differentiation. For example, it has been shown already in 2003 that activin exerts a stimulatory effect on spermatogonial differentiation, whereas GDNF has the opposite effect (Nagano *et al.*, 2003). Since this topic presents a wide field by itself, it cannot be covered extensively in this review.

In summary, these studies have provided insight into the general requirements of testicular cells *in vitro*, namely the presence of somatic and germ cells, a lower temperature compared with other cell types and an origin from an immature donor. Logically, the next step should be the definition of optimal culture conditions to support the spermatogenic progress *in vitro*.

From conventional cultures to 3D culture systems

The majority of studies performed from the early 1980s onwards used conventional culture methods, e.g. coated or uncoated plastic culture dishes in which germ cells and/or somatic cells were cultured alone or with feeder cells. The aim of germ cell cultures in the clinical context is for *in vitro* matured male germ cells to be available for assisted reproductive techniques. The immature germ cells are obtained by biopsy of the testes of infertile men with arrested germ cell development. When post-meiotic germ cells at the level of round and elongating spermatids have been cultured, they have developed into mature gametes (Cremades *et al.*, 1999, 2001). These studies have shown that the later

developmental steps can occur *in vitro* under conventional culture conditions.

Conventional cultures, however, do not provide the spatial arrangements which testicular cells encounter in their natural environment (reviewed in Staub, 2001). This fact may have a negative impact on germ cell development *in vitro*. Especially meiotic cells are engulfed in Sertoli cells as large interconnected clones with no contact to the basement membrane or the adluminal compartment. Such a complex microenvironment cannot be reconstructed in conventional cultures. As previously published, conventional experimental settings, making use of various supporting cell types elucidated physiological and developmental characteristics of undifferentiated spermatogonia (de Rooij and Russell, 2000), have allowed establishment of SSC lines (Shinohara *et al*., 2000; Nagano *et al*., 2003; Kubota *et al*., 2004a, b; Kanatsu-Shinohara *et al*., 2005) and provided a tool to explore male germ cells at different spermatogenic stages (Tres and Kierszenbaum, 1983; Gerton and Millette, 1984; Hue *et al*., 1998; Tesarik *et al*., 1998a, b; Feng *et al*., 2002; Sousa *et al*., 2002; Sa *et al*., 2008). Studying spermatogenesis *in situ* has lead to the understanding that apparently the spatial arrangement of the testicular cells is of enormous importance for the regulation and completion of germ cell maturation (for review see Wistuba *et al*., 2007). From studies on spermatogonia as well as spermatocytes, it has been concluded that improved culture conditions should provide a microenvironment that resembles the 3D *in situ* organization of the seminiferous epithelium (Hofmann *et al*., 1992; Lee *et al*., 2006; Lee *et al*., 2007; Stukenborg *et al*., 2008).

In the past, 3D culture systems were established as clonogenic assays to explore the complex mechanism of multipotent haematopoietic cell proliferation and differentiation (Parent-Massin, 2001). By testing such culture systems on male germ cells, several groups have provided unequivocal evidence that male germ cells in a 3D culture system developed to the level of elongating spermatids (Hofmann *et al*., 1992; Lee *et al*., 2006; Lee *et al*., 2007; Stukenborg *et al*., 2008).

In contrast to conventional cell cultures where the coating of the dish is a thin layer of gelatine, collagen, matrigel or other matrix substances, the 3D matrix provided in the soft agar culture system (SACS) or the methylcellulose culture system (MCS) presents a thick layer (several millimetres to several centimetres) in which the cultured cells are embedded (Stukenborg *et al*., 2008; Fig. 1). These culture systems present microenvironments for the embedded germ cells, which might resemble the complex spatial conditions in the testis where germ cells are embedded in the seminiferous epithelium as large and highly synchronized cohorts. Culturing single-cell suspensions from immature testes in such 3D culture systems provides a tool to avoid the ischaemia which hampers the long-term organ culture of testicular tissue and allows for the organization of germ cells as densely packed clusters providing an opportunity to create and maintain the potentially important germ cell–germ cell contacts necessary during differentiation. In the studies discussed in this review, we addressed the supporting and limiting effects of the 3D matrix and the potential benefit of co-culture of somatic testicular cells together with differentiating germ cells. Since the primary cells providing support for developing male germ cells *in vivo* are Sertoli cells, we specifically focused on the role of Sertoli cells in co-culture with germ cells (Stukenborg *et al*., 2008). Germ cells expanded more prominently in the 3D matrix when Sertoli cells were present.

We hypothesize that under improved culture conditions in respect to physical as well as physiological conditions (low temperature, appropriate endocrine and paracrine milieu, 3D arrangements supporting cell–cell contacts), germ cells will not only survive but will enter and pass through meiosis and spermiohistogenesis *in vitro*.

An additional and important prerequisite for the initiation of the spermatogenic progress *in vitro* is a microenvironment resembling features of the spermatogonial niche provided to the testicular stem cells under natural conditions (Guan *et al*., 2006; Lee *et al*., 2007; Wistuba *et al*., 2007; Chu *et al*., 2008; Stukenborg *et al*., 2008). Only when the stem cell niche is reconstructed, can a constant and long-lasting replenishment of dying or differentiating germ cells be established and a continuous generation of *in vitro* generated gametes be achieved. It is therefore imperative to replicate *in vitro* the different niches and microenvironments provided by the complex spatial arrangement of the seminiferous epithelium which gives rise to three different epithelial compartments (basal, intraepithelial and adluminal).

Original data from studies on male germ cell differentiation using 3D culture systems

This review describes several additional experiments following four different experimental designs (ED1–4, Fig. 1, Table I) which extend our previously published data on the culture of mouse spermatogonia obtained from immature testes (Stukenborg *et al*., 2008). In ED1, fractions of spermatogonia obtained by positive enrichment using Gfrα-1 antibodies and magnetic-activated cell sorting (MACS, Kubota *et al*., 2004a; Buageaw *et al*., 2005; Oatley *et al*., 2007; Kokkinaki *et al*., 2009) were cultured in the absence of somatic cells (Fig. 1, Table I: ED1). The second experimental setup involved a co-culture of testicular somatic cells with or without direct contact to the MACS-derived spermatogonial cells (Fig. 1, Table I: ED2). Since our initial experiments provided evidence that co-cultures providing direct contact of germ and somatic cells lead to more extensive germ cell expansion compared with germ cells separated from somatic cells (Stukenborg *et al*., 2008), we decided to perform the subsequent experiments by adding all cells isolated from mouse immature seminiferous tubules to a single compartment. Therefore, in ED3 and ED4, no MACS enrichment was applied and a crude cell suspension containing somatic and germ cells was added to the gel phase (Fig. 1, Table I: ED3 and ED4). After two step digestion of the 7–9 days post-parturition mouse testes, this cell preparation consisted primarily of Sertoli cells and spermatogonia. The fraction of germ cells consisted of pre-meiotic germ cells at the level of differentiating A1–A4 spermatogonia and B-spermatogonia. The fourth ED was identical to the conditions of ED3 except that the culture medium was supplemented with gonadotrophins (5 IU/l hCG and 5 IU/l rhFSH).

Application of MACS requires the presence of specific cell-surface markers expressed exclusively by a distinct cell type. Unfortunately, no cell-surface marker is available that is exclusively expressed by SSCs (Sofikitis *et al*., 2005). Antibodies directed against Thy-1, CD-9 and Gfrα-1 have been described as useful markers for the enrichment of SSCs (Meng *et al*., 2000; Kanatsu-Shinohara *et al*., 2004; Kubota *et al*., 2004a; He *et al*., 2007). Flow cytometric and immunohistochemical analysis have revealed that Gfrα-1 is a reliable marker for
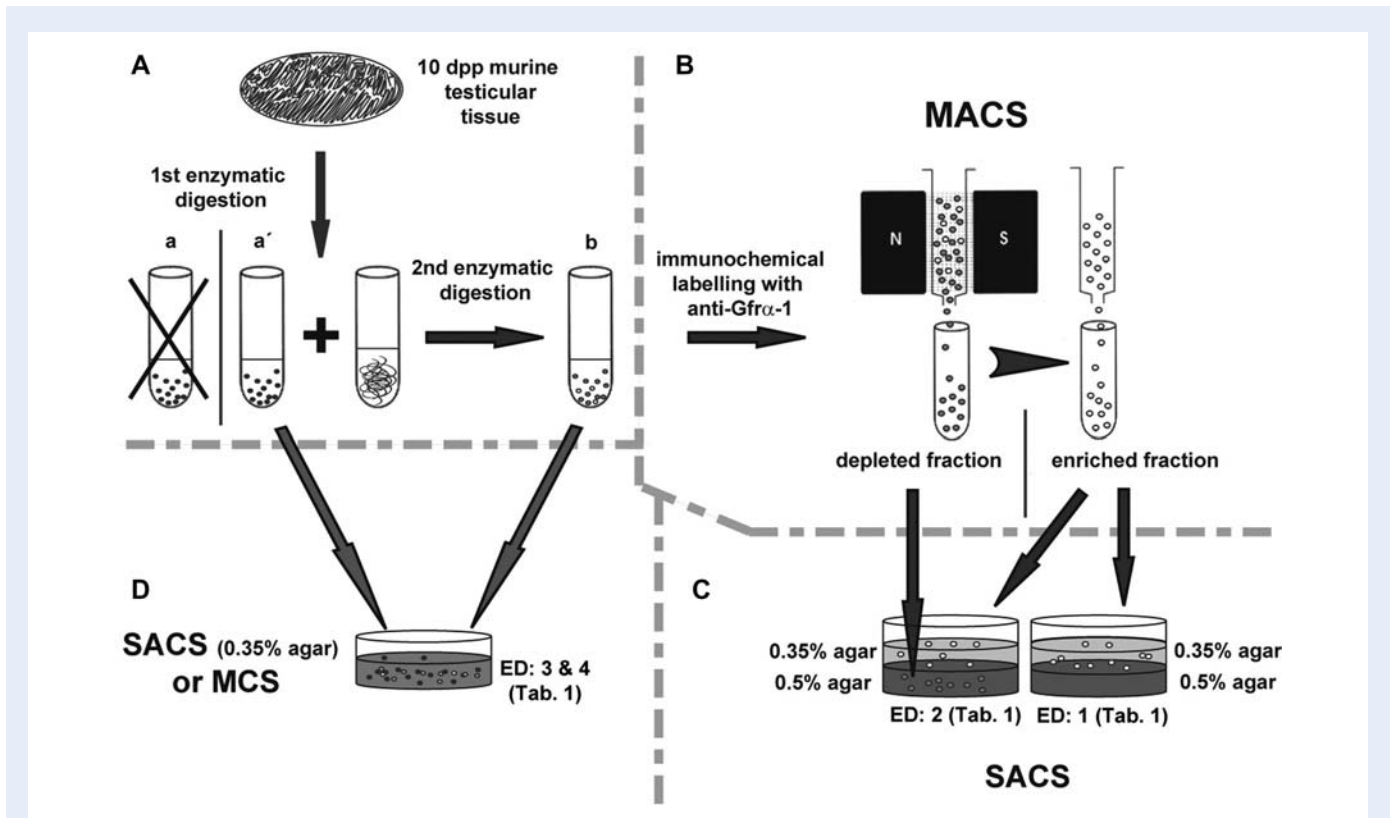
Figure 1 Experimental design of the soft agarculture system (SACS) and the methylcellulose culture system (MCS).
Single-cell suspensions of murine testicular tissues were prepared via a two-step digestion [first step: collagenase type IA (1 mg/ml) and DNase (0.5mg/ml); separation of supernatant from tubular fragments by unit gravity sedimentation generated fraction a (discarded) or á (used as interstitial cell fraction)]; tubular fragments were subjected to second digestion: collagenase type IA (1.0 mg/ml), DNase (0.5 mg/ml) and hyaluronidase (0.5 mg/ml) (fraction b)] (**A**). Undifferentiated spermatogonia were labelled with anti-Gfrα-1 antibodies and enriched via MACS (**B**). Four different experimental designs (ED1–4; for more details see Table I) were used to culture undifferentiated spermatogonia (enriched fraction) placed in the gel phase (0.35% agar) with or without somatic cell support from the depleted fraction placed in the solid agar phase (0.5% agar) over a time period up to 16 days (**C**). Interstitial (á) and intratubular (b) single-cell suspensions of murine testicular cells, obtained after enzymatic digestion (A), were combined in the SACS as well as the MCS to study male germ cell differentiation *in vitro* (**D**). dpp, days post-parturition.

Table I Experimental conditions of 3D culture approaches used for male germ cell culture in our studies: (i) SACS; (ii) MCS

Culture system	Experimental design (ED)	Numbers of layers	Cell fractions (layer)	hCG (5 IU/l)	rhFSH (5 IU/l)
SACS	1	2	Gfrα-1 enriched (MACS) (1)	No	No
			No cells (2)	No	No
SACS	2	2	Gfrα-1 enriched (MACS) (1)	No	No
			Gfrα-1 depleted (MACS) (1)	No	No
SACS	3a	1	Testicular cells (1)	No	No
MCS	3b	1	Testicular cells (1)	No	No
SACS	4a	1	Testicular cells (1)	Yes	Yes
MCS	4b	1	Testicular cells (1)	Yes	Yes

undifferentiated spermatogonia and is superior to Thy-1 and CD-9 (Stukenborg *et al.*, 2008; Kokkinaki *et al.*, 2009). We therefore decided to perform MACS using Gfrα-1 antibodies and to obtain an enriched fraction of SSCs by positive selection for our experiments.

The depleted fraction obtained during MACS is depleted of SSCs and contains all somatic cells present in the crude cell preparation. We used the depleted fraction as a source of testicular somatic cells for our experiments (Fig. 1).

The SACS is separated into two distinct zones: a solid lower phase of 0.5% agar and an upper gel phase of 0.35% agar (Huleihel *et al.*, 1993). This biphasic arrangement allowed us to add different cell fractions to each compartment (ED2). In all experiments, we added unsorted (ED3 and ED4) or MACS sorted (ED1 and ED2) germ cell fractions to the upper gel phase. In ED2, the depleted fraction was added to the lower solid compartment. This fraction contains primarily Sertoli cells. In all experiments, the cells in the upper gel phase were analysed for various end-points at different time points from 1 day up to several weeks.

During the first 24 h of culture, cell numbers decreased. Apoptosis was shown to be the major mechanism for the cell loss as we detected abundant TUNEL-positive cells irrespective of the experimental conditions (Stukenborg *et al.*, 2008). In ED1 and ED2, the addition of somatic cells to the solid lower phase resulted in more extensive colony formation and improved spermatogenic differentiation of the MACS-enriched germ cell fraction in the upper gel phase. The improved survival and differentiation of germ cells in the presence of somatic cells was observed from 24 h to 16 days of culture (Stukenborg *et al.*, 2008), which is consistent with findings obtained from conventional culture experiments (Dirami *et al.*, 1999; Izadyar *et al.*, 2003). Thus, the presence of somatic cells but not necessarily the direct contact appears to be mandatory for efficient proliferation of male germ cells *in vitro*.

We then tested methylcellulose as an alternative matrix for germ cell culture. We observed that the nature of the matrix material was not critical. MCS and agar both provided a 3D structure which supported clonal outgrowth for colony formation and differentiation of the germ cells. This finding is in agreement with the work of Lee *et al.* (2006, 2007). In their experiments, they utilized collagen as culture matrix and also found outgrowth and differentiation. However, the use of MCS provided us with an opportunity to resuspend the cultured cells from the 3D matrix and to perform a more detailed characterization of the cells using flow cytometry.

In additional experiments, we extended our culture approach to explore the effects of gonadotrophins on germ cell development in serum-free SACS and MCS. In contrast to the previous experiments (Stukenborg *et al.*, 2008), we now added single-cell suspensions to the gel phase, which consisted of all testicular cell types from the immature murine testis. Within 1 day of culture, cells arranged themselves to form dense aggregates in the matrices. One aspect investigated in this setting was the functionality of Leydig cells by hCG stimulation. As has been shown in previous studies, stimulation of isolated testicular cells with hCG stimulates testosterone production (Steinberger, 1975) and evokes an anti-apoptotic effect on male germ cells (Print and Loveland, 2000). In our studies, stimulation with 5.0 IU/l hCG resulted in high testosterone production which could be detected 6 h after initiation of culture. Testosterone levels were maintained at this level for 16 days *in vitro*. Titration revealed that a minimum of 1.0 IU/l hCG was needed to stimulate a testosterone response *in vitro* within 12 h of culture. These data reveal that our freshly isolated cells contain steroidogenically active Leydig cells which will provide testosterone to the 3D culture systems.

In order to detect the progression of germ cells from immature to mature stages, the protein expression of markers identifying specific stages of germ cell development was immunohistochemically examined at different time points of culture. In freshly isolated cell fractions,

no cells positive for Boule, a marker for spermatocytes, and Crem, a marker for round spermatids, were detected (Delmas *et al.*, 1993; Wistuba *et al.*, 2002; Xu *et al.*, 2003). Boule-positive pachytene spermatocytes and Crem-positive round spermatids were, however, localized in colonies of SACS after 3 weeks of culture. These cells were only found in ED3 and ED4 (Fig. 1, Table I: ED3 and ED4), indicating that development to post-meiotic stages only occurs when somatic and germ cells are mixed into the same culture compartment where they can interact closely. Figure 2B shows positive colonies in ED3 developing to post-meiotic germ cells in the absence of hormones. Complete maturation of germ cells into spermatozoa was observed only in ED4 after extended culture periods of >40 days. Here, the cultures were initiated with somatic and germ cells mixed into the gel phase and were supported with gonadotrophins. Morphological assessment as well as markers revealed development into late post-meiotic round and elongating spermatids which expressed Protamine-1, a marker for elongating spermatids (Brehm and Steger, 2005) (Fig. 2B). The micrographs in Fig. 2 reveal the *in vitro* generation of morphologically identifiable spermatozoa. This finding confirms our previously published results using mRNA expression profiles to determine progression of spermatogenesis in our cultures. We revealed a progression of spermatogenesis and the presence of somatic cells by detection of markers for spermatogonia (Oct3/4, Kit, CD-9, Gfrα-1, α-6-integrin and Dazl), spermatocytes (Prohibitin, Scp-3 and Srf-1) and spermatids (Ldh, Protamine-2 and Sp-10) as well as for Sertoli cells (ABP) and peritubular cells (α-smooth-muscle actin; Stukenborg *et al.*, 2008). We detected meiotic gene expression in cultures following ED3 but not in cells maintained under ED1 and ED2. We observed spermatozoa only in MCS and SACS cultures when they were supplemented with gonadotrophins (5 IU/l hCG and 5 IU/l rFSH), indicating a potentially crucial role of hormones for germ cell development.

The temporal progression of *in vitro* spermatogenesis closely resembled the natural sequence of events. Meiotic pachytene spermatocytes and early post-meiotic round spermatids were present at earliest after 3 weeks of culture (Fig. 2). Elongating spermatids and spermatozoa were not observed before 31 days of culture (Fig. 2C). When we consider that murine spermatogenesis *in vivo* takes 34 days and assume that the kinetics *in vitro* are similar to the *in vivo* situation, we can calculate that the spermatozoa obtained in our cultures derived from spermatogonia. Further studies are needed to provide proof of the fertilization potential of *in vitro*-derived spermatozoa.

To confirm the observations made by histology and immunohistochemistry, we analysed cells cultured in MCS by flow cytometry (Fig. 3). Germ cell differentiation can be assessed by flow cytometry through determination of the nuclear DNA content using propidium iodide staining. The cells are separated into diploid (2C), double diploid/tetraploid (4C) and haploid (1C) cell fractions (Chandolia *et al.*, 2006; Yeung *et al.*, 2007). In all cultures, the diploid cells presented the majority in number accounting for 65–70% of the total cell population. We observed a slight increase of haploid cells (1C) with and without gonadotrophins over the 16-day culture period. We assume that the increase of haploid cells in both conditions reflects the completion of differentiation of a few pre-meiotic germ cells present in the original cell population (Fig. 3A). The most informative and crucial parameter for assessing the continuous meiotic
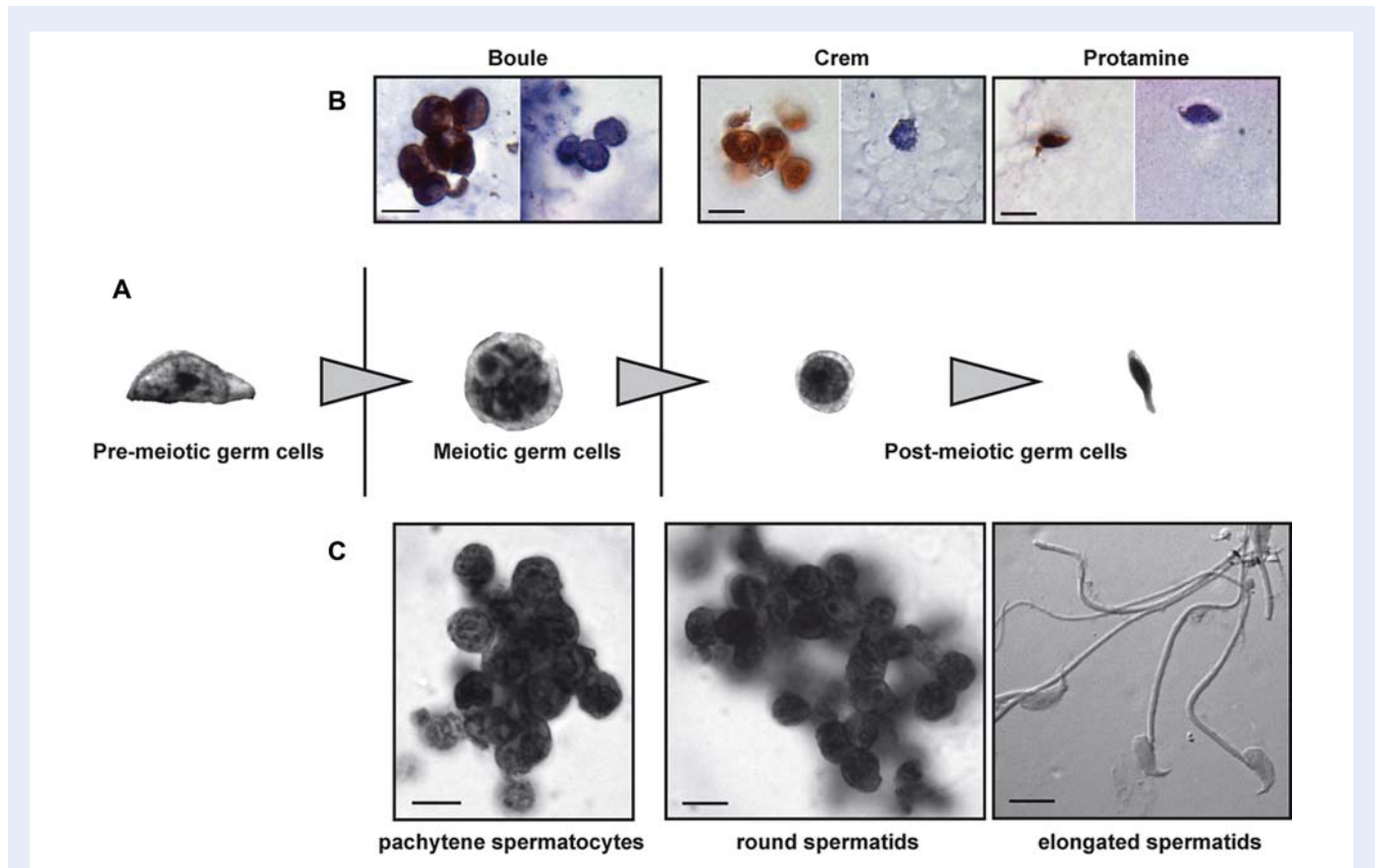
Figure 2 Immunohistochemical and morphological analysis of cells from the soft agarculture system (SACS).
Testicular germ cells were isolated from immature (Days 7−9) CD-1 mice; Schematic illustration of murine spermatogenesis divided into pre-meiotic, meiotic and post-meiotic stages (**A**). Immunohistochemical staining of cross-sections of germ cell colonies obtained from the SACS. The micrographs show the localization of Boule, Crem1 and Protamine-1 protein expression on the left side of each panel. The brown precipitate represents positive staining and haematoxylin (blue colour) was used as counterstain. Negative controls revealing specificity of the staining after omission of the primary antibodies are shown on the right site of each panel (**B**). Micrographs showing the morphological features of germ cell colonies isolated from SACS. The cytoplasmic and nuclear patterns reveal progression of germ cell cohorts *in vitro*. The cohorts of pachytene spermatocytes and round spermatids were observed in SACS after 21 days of culture and spermatozoa were observed after 31 days of culture (**C**). Scale bars: 10 μm.

entry of spermatogonia into differentiation, and therefore a measure of the functionality of the stem cell niche in our cultures, is reflected by the pattern of tetraploid (4C) cells (Fig. 3B), which primarily represent spermatocytes. Cultures supplemented with gonadotrophins resulted in a stable and later increasing number of 4C cells, whereas in the absence of hormones, the cultures showed a constant decline in the number of 4C cells. The flow cytometric evaluation of 4C and 1C cells is consistent with our molecular, morphological and immunohistochemical data analysis. Although we did not detect any meiotic or post-meiotic markers or morphologically identifiable meiotic cells in the testes of the immature mice, meiotic and post-meiotic markers as well as morphologically identifiable spermatocytes and spermatids were seen in our cultures. The continuous presence of tetraploid cells indicates that hormones provide the support needed for expansion of germ cell progenitors which replenish the pool of cells entering meiosis. In SACS and MCS cultures, gonadotrophin support was not required for completion of meiosis *in vitro* but for maintenance of meiotic germ cell colonies. Without continuous generation of newly differentiating germ cells, *in vitro* generation of sperm is impossible. We therefore conclude that the presence of somatic cells and their stimulation by gonadotrophins is crucial for *in vitro* generation of sperm by SACS or MCS culture systems.

In conclusion, we established a novel 3D culture approach allowing pre-meiotic mouse male germ cells to pass through meiosis *in vitro* and to mature into morphologically normal spermatozoa. The differentiating germ cells need to be supported by somatic testicular cells and gonadotrophins, irrespective of the nature of the matrix. One aim of *in vitro* maturation of male germ cells has been achieved in our studies: the completion of the entire spermatogenic sequence from spermatogonial stage to morphologically normal spermatozoa. The technique now has to be translated into human spermatogenesis and spermatozoa and has to be functionally proven in further studies.

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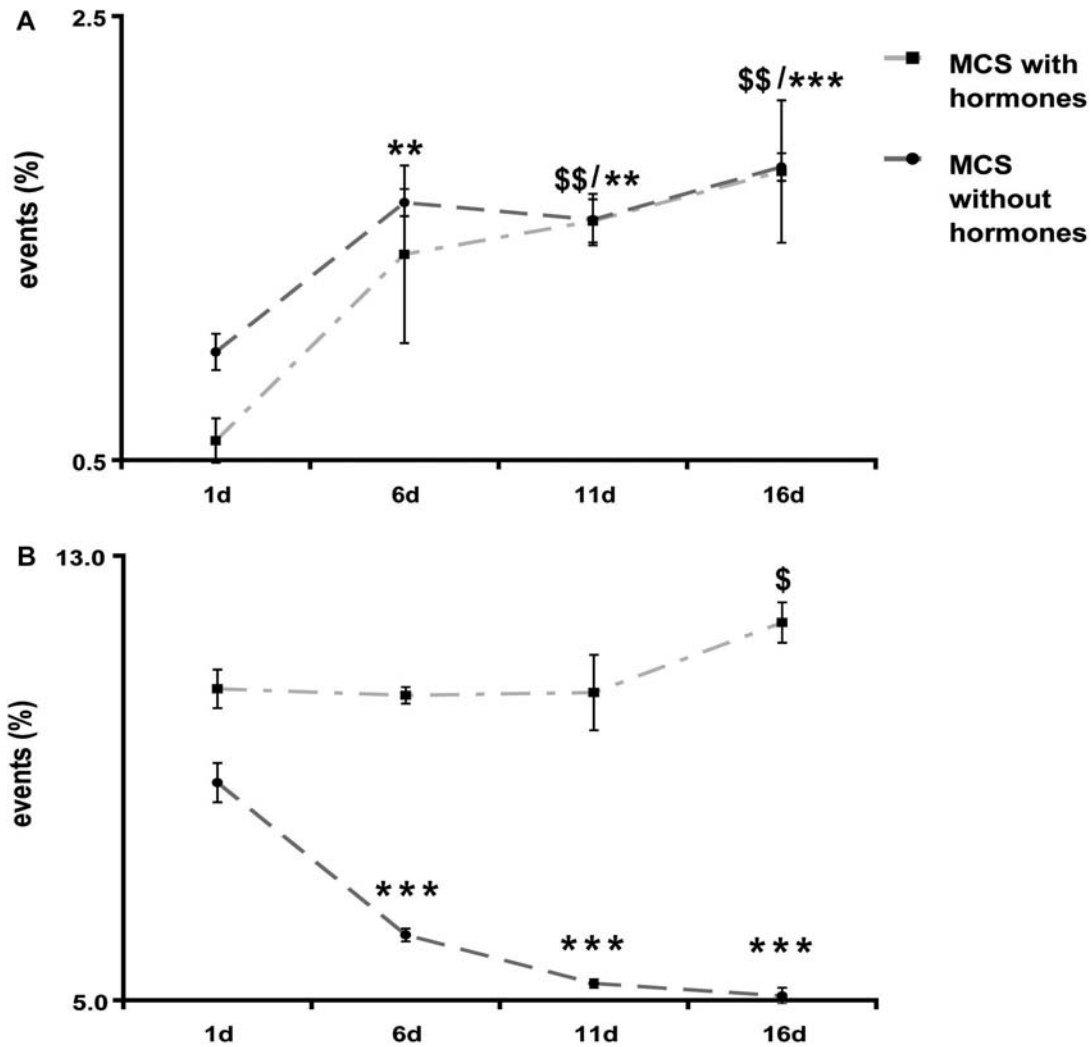
Figure 3 Flow cytometric analysis of haploid (1C) and tetraploid (4C) cells during the first 16 days of methylcellulose culture system (MCS). The number of 1C cells increased with and without supplementation of gonadotrophins (**A**). The number of 4C cells decreased without gonadotrophins, whereas addition of gonadotrophins resulted in a stable and, later on, growing population of 4C cells (**B**). Pooled results are shown as means ± SEM of three experiments. Statistical analysis was performed by one-way repeated measurements ANOVA and subsequent *post hoc* test (Tukey) using SigmaStat3 software (Statcon, Witzenhausen, Germany). Data points with different superscripts indicate statistically significant differences to Day 1. The symbol '$' indicates significant changes in the presence of hormones ($\$\$\$P < 0.001$; $\$\$P < 0.01$; $\$P < 0.05$). The symbol "*" indicates changes in the absence of hormones (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$).

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