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REVIEW

Sperm Biology

Application of three-dimensional culture systems to study mammalian spermatogenesis, with an emphasis on the rhesus monkey (*Macaca mulatta*)

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***In vitro* culture of spermatogonial stem cells (SSCs) has generally been performed using two-dimensional (2D) culture systems; however, such cultures have not led to the development of complete spermatogenesis. It seems that 2D systems do not replicate optimal conditions of the seminiferous tubules (including those generated by the SSC niche) and necessary for spermatogenesis. Recently, one of our laboratories has been able to induce proliferation and differentiation of mouse testicular germ cells to meiotic and postmeiotic stages including generation of sperm in a 3D soft agar culture system (SACS) and a 3D methylcellulose culture system (MCS). It was suggested that SACS and MCS form a special 3D microenvironment that mimics germ cell niche formation in the seminiferous tubules, and thus permits mouse spermatogenesis *in vitro.* In this review, we (1) provide a brief overview of the differences in spermatogenesis in rodents and primates, (2) summarize data related to attempts to generate sperm *in vitro,* (3) report for the first time formation of colonies/clusters of cells and differentiation of meiotic (expression of CREM-1) and postmeiotic (expression of acrosin) germ cells from undifferentiated spermatogonia isolated from the testis of prepubertal rhesus monkeys and cultured in SACS and MCS, and (4) indicate research needed to optimize 3D systems for *in vitro* primate spermatogenesis and for possible future application to man.**

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INTRODUCTION

Spermatogenesis is an intricate process of male germ cell proliferation and differentiation that leads to the generation of sperm.[1] The process involves several types of undifferentiated and differentiating germ cells located in the seminiferous tubules within the testis. Spermatogonial stem cells (SSCs) are primitive diploid germ cells attached to the basement membrane of the seminiferous tubule and located in specific "niches" within these testicular structures. SSCs are the cells that initiate and maintain the process of spermatogenesis throughout adulthood.[2–9] Stem cells divide to generate two types of daughter cells: new stem cells and progenitor cells. It had generally been considered that progenitor cells were only capable of proliferating before they committed to the path of differentiation. However, recent studies of mice combining pulse-labeling to track spermatogonial lineage with live-imaging of the *in situ* testis suggest that the relationship between stem and progenitor cells maybe much more plastic than initially thought.[6]

In mammalian species, spermatogenesis relies on the appropriate expansion of undifferentiated and differentiating spermatogonia prior to the entry of germ cells into meiosis and subsequent spermiogenesis.[1] Spermatogonial proliferation and differentiation and the control of these processes have been studied primarily using rodent models. The extent to which the results of such studies may be directly translated to man, however, is uncertain. There are differences in spermatogenesis between rodents and primates.[10,11] In contrast to rodents, spermatogenesis in primates is not initiated until several years after birth, and the abbreviated first wave of spermatogenesis observed in the former species[12,13] would seem to have no adaptive value in long-lived primates. Spermatogonial differentiation in primates is highly dependent on pituitary gonadotropin secretion and following hypophysectomy in the rhesus monkey only Sertoli cells and undifferentiated spermatogonia are observed in the testis.[10] In the rat, on the other hand, meiotic cells are found in the absence of gonadotropin stimulation.[14] Each cycle of the seminiferous epithelium in rodents is initiated by transformation of undifferentiated spermatogonia into the first generation of differentiating spermatogonia, while in the rhesus monkey (and other highly evolved primates presumably) the cycle is initiated with a mitotic division.[11] In adult rodents, the testis appears to be functioning at its spermatogenic ceiling but in the rhesus monkey this is not the case, as reflected by the finding that unilateral orchidectomy in this macaque postpubertally results in an increase in testicular volume of the contralateral testis.[15] Spermatogenesis in the adult rhesus monkey may also be increased by selectively increasing stimulation with FSH, but interestingly not with LH.[16]

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The use of nonhuman primates in biomedical research poses special problems. These animals represent a limited and expensive resource, and their large size, long lifespan, genetic intractability and out-bred nature require the development of robust *in vitro* approaches in order to better understand the biology of spermatogonia in these species. In this regard, xenotransplantation of primate testicular cells and xenografts of primate testicular tissue to the testis or subcutaneous sites of recipient mice, respectively, have been reported.[4,7,17–23] Such studies have demonstrated that when baboon, rhesus monkey or human testicular germ cells were transplanted into the rete testis of mice they formed small colonies of spermatogonia in the seminiferous tubules of the recipient, but further differentiation was not observed.[4,23] Most importantly, however, autologous and allogeneic SSC transplantation into rhesus monkey testes regenerated spermatogenesis, with sperm derived from one allogeneic donor shown to initiate blastocyst development when injected into oocytes.[20] Several factors should be considered to improve the outcome of xenotransplantation, and these have been discussed in considerable detail by others previously.[24]

Autologous grafts of marmoset testicular tissue to orthotopic, but not ectopic, sites, led to complete spermatogenesis, suggesting that the site of transplantation rather than the endocrine milieu may be important for development of the transplanted testicular tissue.[22] More recently, testicular tissue from peripubertal rhesus monkeys, which had been collected and cryopreserved approximately 2 years before the animals were castrated, was autologously transplanted into the scrotum approximately 2–3 months postcastration. Five months after transplantation, sperm were observed in some seminiferous tubules of the orthotopic grafts.[21]

Until recently, three-dimensional (3D) culture systems have only been applied to the study of SSC activity in mice.[25–27] The finding that 3D cultures supported the proliferation of mouse testicular germ cells and their differentiation to meiotic and postmeiotic stages including generation of sperm like-cells, indicates that these *in vitro* systems offer great potential for investigating the mechanisms that control male germ cell differentiation. A major purpose of this article is to review previous work with 3D cultures of rodent cells and to describe our preliminary attempts to apply 3D culture systems to the study of primate spermatogonia. First, however, we briefly describe the process of spermatogenesis in rodents and primates.

Spermatogenesis

Spermatogonial cell types

Spermatogonia are classified into undifferentiated and differentiating subtypes. In mice, three types of undifferentiated spermatogonia are traditionally recognized (A single, As; A pair, Apr; A aligned; Aal) and these spermatogonia comprise <1% of the entire population of testicular cells in this species.[2] There is continuing debate about whether the stem cell pool is restricted to As, as classically proposed by Huckins (1971)[28] and Oakberg (1971)[29] or might be expanded to include Apr and some Aal.[6,8,30] During sequential divisions, the progenitor cells remain connected by intercellular bridges to form Apr and clones of Aal spermatogonia (syncytia of 2, 4, 8 and up to 16 cells).[28,29] The kinetics of progenitor spermatogonial divisions do not appear to be synchronized with the seminiferous epithelial cycle (see below). Aal spermatogonia transform as clones giving rise to the first generation of differentiating spermatogonia (type A1), which undergo a series of mitotic divisions yielding an additional 5 generations of differentiating spermatogonia (type A2, A3, A4, Intermediate and B). Intermediate and type B spermatogonia are morphologically distinct appearing as large interconnected cohorts of cells in tubules that may be visualized in the longitudinal plane in preparations known as "whole mounts".[2]

Schemata of spermatogonial proliferation and differentiation in primates are based largely on the classical work of Clermont and colleagues 40 to 50 years ago.[31] In macaques and men, two morphologically distinguishable types of undifferentiated spermatogonia have been traditionally proposed, the types A dark (Ad) and A pale (Ap). Both cell types are present on the basement membrane of the primate seminiferous tubule but differ mainly in the nuclear architecture and staining intensity with hematoxylin. Although both are commonly referred to as SSCs,[4,11,32–35] the precise relationship between these spermatogonial types is unknown and their "relative stemness" has not been established empirically. In the rhesus monkey, Ap spermatogonia undergo mitosis to generate the first of four generations of differentiating type B spermatogonia, known as B1, B2, B3 and B4.[10,31] In man, only 1 generation of type B spermatogonia has been reported.[36]

Seminiferous epithelium cycle

Spermatogenesis in mammals occurs in a synchronized, cyclic pattern where the cellular associations of differentiating germ cells are maintained in a progressive and repeated fashion.[1,2] Accordingly, it is possible to classify the seminiferous epithelium into numerous discrete "stages" based upon the cellular complement observed in a given segment of the seminiferous tubule. In the mouse, 12 discrete stages of the seminiferous epithelium were identified, 14 stages in rats, 12 stages in the Old World monkeys (olive baboon, and stump-tailed, rhesus and cynomolgus macaque) and 6 in chimpanzee and human.[4] It should be noted that in rodents, prosimians, and most Old World monkeys, cross-sections of the seminiferous tubules show only a single spermatogenic stage. However, in New World monkeys, great apes and man cross-sections of seminiferous tubule may show multiple stages.[37,38] However, the significance of this species variability in the organization of the seminiferous tubule to the cellular and molecular processes governing germ cell differentiation is unclear.

Little is known about the clonal distribution of Ad and Ap spermatogonia in the primate testis. It was initially reported that pairs and quartets of Ad and Ap spermatogonia are present in seminiferous tubules of the rhesus macaque.[33,34] Later, results from low-dose irradiated testis of the rhesus monkey showed that following recovery clones of various sizes of both Ad and Ap were observed in the seminiferous tubules similar to the clonal organization of spermatogonia in rodents.[39] Recently, Schlatt and his colleagues have described clonal arrangements of spermatogonia in testes from the adult rhesus monkey and marmoset.[40] However, the progression of spermatogonial clones has not been systematically studied in any species of primate.

Biomolecular markers for testicular germ cells

Relative extensive research with rodents examining specific molecular markers of the cell surface, cytoplasmic and nuclear proteins has led to the broad molecular characterization of spermatogonia phenotype at different stages of differentiation. These markers include α6-integrin, β1-integrin, cluster of differentiation 9 (CD9), cadherin 1 (CDH1), glial cell line-derived nerve factor family receptor alpha 1 (GFR-α1), G protein-coupled receptor 125 (GPR125), c-KIT, neurogenin3 (NGN3), promyelocytic leukemia zinc finger protein (PLZF), octamer-binding transcription factor 4 (OCT4), stimulated by retinoic acid gene 8 (*STRA8*) and thymocyte differentiation antigen 1 (THY-1) [see 3 for a review]. However, until very recently, little was known

about the markers of spermatogonial proliferation and differentiation in primates. Contemporary studies have shown that some of the rodent spermatogonial markers were also expressed in the human and monkey testes.[3,4,19,41,42] In the testis of the adult rhesus monkey, most Ad and ~50% of Ap were GFR-α1+, PLZF+, NGN3−, c-KIT−: a phenotype restricted to As spermatogonia in the mouse.[19] In addition, similar to longer-chain progenitor cells of the rodent (Aal) that express c-KIT, some Ap in the adult rhesus testis were GFR-α1+, PLZF+, NGN3+, c-KIT+. Thus, Hermann *et al.*[19] proposed that the SSC pool in the rhesus monkey may comprise all Ad and at least 50% of Ap spermatogonia, and that the SSC pool in primates is considerably larger than that in mouse testis. On the other hand, it seems that the progenitor pool is larger in rodents compared to macaques.[19] Recently, it was demonstrated that, as in mouse and macaque, GFR-α1, GPR125, THY-1, α6-Integrin, CD133, SSEA4, VASA, DAZL, TSPYL2 and PLZF are expressed in human testicular germ cells.[3,4,43] β1-Intergin, c-KIT, OCT-4 and testis-specific protein, Y-encoded (TSPY), however, were differentially expressed in rodent and human testis.[3] This may suggest differences between rodents and human in markers expressed by their respective SSCs and their progeny.

Recently, it was demonstrated for the common marmoset monkey that some of the molecular markers of spermatogonia discussed above were also expressed by cells derived from connective tissue of the testis that are known as testicular multipotent stromal cells.[44,45] Such findings underline the importance in studies of germ cell differentiation of selecting markers for specific germ cell types and using well-characterized antibodies for identification of the markers.

Dynamic niches for mammalian spermatogenesis

SSCs reside within niches that are generated primarily by the somatic Sertoli cells (but also interstitial and peritubular cells), which elaborate a microenvironment that is conducive to SSC activity. The niche is extremely sensitive to local and systemic physiological and/or pathological factors that may affect both the quality and location of the niche, and thus may change the normal progression of stem cell development.[7,46,47] It is considered that niches are governed locally by interactions between somatic cells and the germ cells. These interactions are likely to be achieved through secretion of several paracrine factors by the Sertoli cells and other somatic elements that target developing germ cells either directly and/or indirectly after cross-talk between the niche cells generates an integrated signal to the developing germ cells.[7,47–52] Some of the well-known factors produced by these cells and affecting SSC self-renewal and differentiation include glial cell line derived nerve factor (GDNF), fibroblast growth factor 2 (FGF2), and insulin-like growth factor-I (IGF-I) (expressed mainly by Sertoli cells), colony stimulating factor-1 (CSF-1) (mainly by Leydig cells), leukemia inhibitory factor (LIF) (mainly by peritubular cells), and vascular endothelial growth factor (VEGF) (mainly by vasculature interstitial tissue and Sertoli cells).[7] In addition, matrix components such as fibronectin, collagens, and laminins, and adhesion molecules expressed by interstitial and tubule cells also contribute to the SSC niche.[7,48]

In rodents, SSC and other undifferentiated spermatogonia (Ap/Aal) are located on the basement membrane in seminiferous tubules in close vicinity to the interstitial blood vessels, and as they undergo differentiation they migrate to different niches, which in turn can support further differentiation.[5,53–56] Whether this anatomical relationship between SSCs and the interstitial vasculature is observed in primates, where the SSC reside within the population of Ad and Ap, remains to be determined. Additional niches may be associated with successive generations of germ cells as they move toward the lumen of the seminiferous tubules. These putative niches are likely to be critical for the regulation of the entire spermatogenic process.[57] In this regard, the characteristics of such niches in terms of paracrine factors and matrix and adhesion components are likely to depend on whether the niche is regulating SSC fate or proliferation of differentiating spermatogonia.[7,48,58]

The process of spermatogenesis in both rodents and primates requires gonadotropin stimulation.[10] Leydig cells are regulated primarily by LH, while the major endocrine control of Sertoli cells is provided by FSH, in combination with testosterone derived from the Leydig cell in response to LH stimulation. Endocrine regulation of somatic testicular cells affects the type and levels of the paracrine factors produced by these cells and thus undoubtedly determine, in part, the niches they contribute too.

Development of spermatogenesis in vitro

Attempts to develop spermatogenesis *in vitro* were initiated almost a century ago, when investigators placed fragments of seminiferous tubules into organ culture.[59] Subsequently, culture conditions were modified by, for example, addition of gas-liquid interphases and addition of amino acids and hormones to the media.[60–64] Notwithstanding, these approaches led only to the development of early stage spermatids. Very recently, however, Sato *et al.*[65–67] reported complete spermatogenesis with the generation of fertile sperm from the culture of mouse seminiferous tubules.

Another approach to achieving spermatogenesis *in vitro* has been to employ 2D systems to culture isolated germ cells from seminiferous tubules of rodents. Those laboratories developing such cell culture approaches were faced by many obstacles that had to be overcome. Most significant was the need to identify SSCs, which are present in very low numbers in the testes of rodents. In this regard, the recognition of markers for SSCs such as GFR-α1, CD9, and THY-1, led to their enriched isolation and facilitated their use *in vitro*.[68] Although it has been estimated that there are approximately 35 000 As spermatogonia in the testis of adult mice, only about 3000 of these are considered to be SSCs (0.01% of total testis cells).[69,70] Additionally, the conditions that are critical for promoting proliferation and differentiation of SSCs *in vivo* remain elusive and are, therefore, unlikely to be fully replicated *in vitro*.[47] Nevertheless, isolated testicular germ cells have been cultured in the presence of a battery of growth factors (GDNF, LIF, SCF, GF, FGF, etc) and/or on various layers of feeder cells such as vero cells (a cell line derived from the kidney of the African green monkey), mouse embryo fibroblasts (MEF), Sertoli cells, and Leydig cells.[48,71–73] Although the latter conditions have led to germ cell proliferation, entry into meiosis and differentiation of postmeiotic cells, complete spermatogenesis was not achieved.[27,48,71–73]

Recently, Sadri-Ardekani *et al.*[74,75] reported proliferation of SSC isolated from normal men and prepubertal patients with cancer when cells were cultured in StemPro media, while Koruji *et al.*[76] using laminin-coated dishes found that SSCs from azoospermic patients proliferated in the presence of GDNF, bFGF, EGF and LIF. Both groups used a 2D culture system comprised of laminin-coated dishes. Using RNA and protein markers, Sadri-Ardekani *et al.*[74,75] demonstrated the ability of their system to support the proliferation of SSC for approximately 7 months. Xenotransplantation into recipient mice was used to demonstrate the functionality of human SSCs and calculate their number, which increased by 53-fold within 19 days and by nearly 20 000-fold within 64 days. The calculation of SSC number was based on the number of spermatogonial colonies that developed in the xenotransplanted mice after SSCs had migrated to the basal compartment

of the recipient testes, and the 5% efficiency at which human SSCs colonize the mouse testis.[74] Another study using a culture system that contained human Sertoli cells as a monolayer reported the proliferation of SSC obtained from azoospermic patients in the absence of exogenous growth factors.[77] On the other hand, Lim *et al.*[78] reported proliferation of SSCs isolated from obstructive and nonobstructive azoospermic patients and cultured on laminin-coated dishes (without Sertoli cells) in the presence of growth factors such as GDNF, LIF, EGF and FGF.

While spermatogonia enter meiosis on the basement membrane, completion of this critical step in spermatogenesis requires that the differentiating germ cells migrate through the blood-testicular barrier. This process and subsequent movement of the postmeiotic germ cell toward the lumen of the seminiferous tubule is governed by cell-cell interactions between Sertoli cells and maturing germ cells,[79,80] and signals in the extracellular matrix comprised of several growth/ differentiation factors in the specific microenvironment.[45] So far, these factors and signals have not been fully defined. It is reasonable, however, to suggest that developing germ cells are affected by different and specific niches during their migration to the lumen of the seminiferous tubule. These local and specific microenvironments are likely involved in the regulation of all stages of male germ cell development: since many are probably incomplete or missing under 2D *in vitro* culture conditions, alternative approaches have been sought with the aim of providing optimal conditions for SSC behavior *in vitro*.

In the intact testis, male germ cells are located inside a 3D structure formed by the seminiferous tubule. Therefore, it was reasoned that 3D culture of SSCs may provide an environment that more closely mimics the *in situ* conditions. Recently, one of our laboratories described two novel 3D culture systems: a soft agar culture system (SACS) and a methylcellulose culture system (MCS)[25,27,47] (**Figure 1** and **Table 1**). The SACS was composed of two layers: a solid lower layer (layer 1, 0.5% (w/v) agar), which contained RPMI (Roswell Park Memorial Institute medium 1640) and FCS (fetal calf serum), and a soft upper layer (layer 2, 0.37% (w/v) agar), which also contained RPMI. The MCS system was composed of only one layer. Cultures were maintained at either 35°C or 37°C. Seminiferous tubular cells (STC) from mouse testis were isolated using a two-step enzyme digestion and experiments were performed as follows: isolated STC were cultured in either the lower or upper layer of SACS containing in some cases hormones (FSH + hCG). In addition, GFR-α1-enriched or GFR-α1-depleted cells were also cultured without hormones (**Table 1**). FCS was added to SACS in the study by Abu Elhija *et al.*[25]

In contrast to conventional cell cultures where the dish is coated with gelatin, collagen, matrigel, or other support materials, the 3D matrices provided in SACS and MCS exist as a thick layer (several millimeters to several centimeters), in which the germ and supporting cells are embedded (**Figure 1**). It was suggested that these 3D systems provide niches that recapitulate to some extent the microenvironment and spatial arrangement of the *in vivo* conditions in the seminiferous tubule where germ cells are embedded in Sertoli cells.[1] 3D culture systems might also provide an improved structural environment for cell-cell interactions essential for clonal expansion and differentiation of germ cells. Additionally, these systems may allow the organization of germ cells into densely packed clusters that provide conditions which facilitate the delivery of oxygen, nutrients, and other factors and thus enable the maintenance of their survival and proliferation. Also, these clusters may provide and maintain germ cell–germ cell contacts necessary during differentiation.[47]

The foregoing approach has allowed one of our laboratories to achieve male germ cell development (proliferation and differentiation to sperm), although, to date, efficiency of the spermatogenic process in 3D

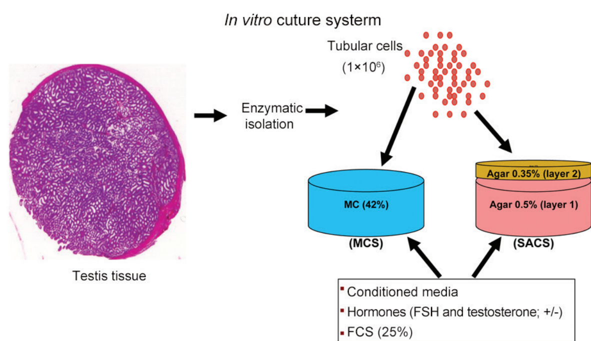

Figure 1: Schematic of *in vitro* methyl cellulose and soft agar culture systems (MCS and SACS, respectively). MCS was composed of 42% methylcellulose (MC), 25% fetal calf serum (FCS) and 38% RPMI (Roswell Park Memorial Institute medium 1640). SACS was composed of two layers: a solid lower layer (layer 1) (0.5% (w/v) agar) consisting of RPMI containing 25% FCS, and a soft upper layer (layer 2) (0.35% (w/v) agar). Both cultures were performed in 24-well plates. Testicular tissue from juvenile rhesus monkeys was enzymatically digested as described by Hermann *et al.*[19] and isolated seminiferous tubular cells were used for culture in MCS (10^6 cells per well per 500 µl) or in the upper phase of the SACS (layer 2) (10^6 cells per well per 200 µl) with or without hormones (FSH and testosterone) (in layer 1) in 5% CO_2 at 37°C.[25,26].

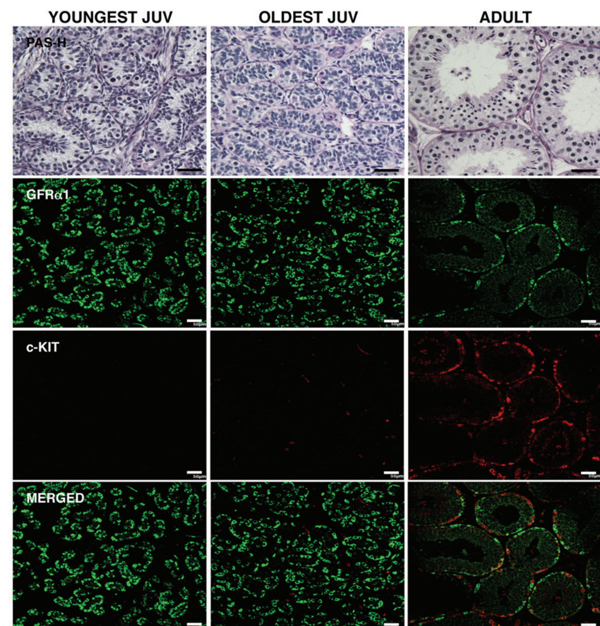

Figure 2: Prepubertal status of testis from the youngest (left-hand panels) and oldest (center panels) juvenile monkey used to isolate germ cells for 3D culture. A section from the testis of an adult monkey is shown for comparison (right-hand panels). PAS-hematoxylin staining (1st row, ×40) confirmed the presence of seminiferous cords containing only Sertoli cells and undifferentiated spermatogonia in testes from the juveniles. Confocal projections (×20; 1 µm optical sections) illustrating the distribution of GFR-α1 (2nd row) and c-KIT (3rd row) immunostaining in 5 µm sections of testis confirmed the absence of differentiating spermatogonia in the testis of the juveniles as revealed by the absence of c-KIT positive cells. c-KIT immunopositive spermatogonia were pronounced and numerous in the seminiferous tubules of the adult. Merged staining for GFR-α1 and c-KIT is presented in the bottom row of the figure. Scale bar: 50 µm.

systems has been low and identification of sperm has required fixation of the cells generated in culture. Therefore, it has not been possible to isolate sperm postculture in order to assess sperm quality using intracytoplasmic sperm injection.[25,27] To date, the work with these 3D systems should, therefore, be considered as providing proof of principle for the *in vitro* generation of sperm-like cells from mouse spermatogonial cells.

Pilot studies of juvenile rhesus monkey male germ cell differentiation using 3D culture system

This part of the review describes our initial attempts with SACS and MCS (**Figure 1** and **Table 1**) to examine proliferation and differentiation of testicular germ cells from the rhesus monkey, a representative highly evolved primate. We considered that the juvenile rhesus monkey would be a good model to explore the feasibility of using 3D culture systems to study primate spermatogenesis for the following reason. The juvenile phase of prepubertal development in this monkey, as in man, is characterized by a protracted hypogonadotropic and hypoandrogenic state[81] that provides an ideal baseline for examining the initiation of spermatogenesis. The only germ cells present in the testis of the juvenile rhesus monkey (typically 6 to 36 months of age) are type A undifferentiated spermatogonia that are proliferating in a relatively gonadotropin-independent manner.[82,83] Spermatogonial differentiation, however, may be readily induced by stimulation with LH or testosterone, either alone or in combination with FSH.[15,83,84] Qualitatively, the germ cell complement of the juvenile testis is similar to that of the neonatal and infant testis, but at the earlier postnatal stages of development, the testis is exposed to adult-like levels of LH/testosterone and FSH.[83] Spermatogonial differentiation, however, is not initiated at these early stages of postnatal development because androgen receptor signaling by the Sertoli cell has not "matured".[85]

MATERIALS AND METHODS

In the studies to be described we isolated STC from testes of juvenile (13–33 months age; $n = 6$) rhesus monkeys immediately after castration. STC were enzymatically isolated as described by Hermann *et al.*[4] STC of juvenile testes, which comprise mainly undifferentiated spermatogonia, Sertoli cells and peritubular cells, were cultured in SACS or MCS and incubated (37°C, 5% CO_2) for 4–8 weeks in the presence or absence of hormones (recombinant macaque FSH [5 ng ml^{-1}] and testosterone [T; 10^{-7} m mol l^{-1}]). With regard to the temperature of the culture, proliferation and differentiation of mouse SSCs in 3D systems at 35°C or 37°C appears to be similar,[25,27] so a general laboratory incubator set at 37°C was used. Prior to STC isolation, a fragment of a testis from each monkey was fixed in 4% paraformaldehyde, and later paraffin-embedded. Five micrometer sections were used for PAS-hematoxylin staining or dual fluorescence immunohistochemical staining for GFRα1 (a marker of undifferentiated spermatogonia) and c-KIT (a marker of differentiating spermatogonia) as described in a previous study from one of our laboratories.[86] Details of the antibodies used are provided in **Table 2**. As previously reported for the testis of juvenile monkeys,[82,83] PAS-hematoxylin staining revealed that the testes of all animals were comprised of seminiferous cords that contained

Table 1: Experimental conditions used for culture of isolated seminiferous tubular cells in SACS and MCS

3D system	*Animal used*	*Number of layers*	*Cells and 3D layer (1 or 2) used*	*Hormones (layer)*		*FCS*	*Culture temperature (°C)*	*Reference*
				FSH+hCG	*FSH+T*			
SACS	Mouse	2	Tubular cells (2)	No	No	Yes (1, 2)	37	24
SACS	Mouse	2	GFR-α1-enriched tubular cells (2)	No	No	No	35	26
SACS	Mouse	2	GFR-α1-enriched tubular cells (2) GFR-α1-depleted tubular cells (1)	No No	No No	No No	35	26
SACS	Mouse	1	Testicular cells (1)	No	No	No	35	26
SACS	Mouse	1	Testicular cells (1)	Yes (1)	No	No	35	26
MCS	Mouse	1	Testicular cells (1)	No	No	No	35	26
MCS	Mouse	1	Testicular cells (1)	Yes (1)	No	No	35	26
SACS	Monkey	2	Tubular cells (2)	No	No	Yes (1, 2)	37	Current paper
SACS	Monkey	2	Tubular cells (2)	No	Yes (1)	Yes (1, 2)	37	Current paper
MCS	Monkey	1	Tubular cells (1)	No	No	Yes	37	Current paper
MCS	Monkey	1	Tubular cells (1)	No	Yes (1)	Yes	37	Current paper

SACS: soft agar culture system; MCS: methylcellulose culture system; FSH: follicle stimulating hormone; FCS: fetal calf serum; hCG: human chorionic gonadotropin; T: testosterone; GFR-α1: glial cell line derived nerve factor family receptor alpha 1

Table 2: Antibodies used in the present study

First antibody	*Species*	*Company*	*Catalog number*	*Concentration (μg ml^{-1})*	*Second antibody*
GFR-α1	Mouse monoclonal anti-human	R and D Systems, Minneapolis, MN, USA	MAB7141	4 (cells); 2.5 (tissue)	Donkey anti-mouse Alexa Fluor 488 IgG (Invitrogen, Carlsbad, CA, USA)
VASA	Rabbit polyclonal anti-human	Abcam, Cambridge, MA, USA	ab13840	1	Cy3; donkey anti-rabbit antibodies; (Jackson ImmunoResearch, West Grove, PA, USA)
SALL4	Mouse monoclonal anti-human	Abcam, Cambridge, MA, USA	ab57577	0.5	Donkey anti-mouse Alexa Fluor 488 IgG (Invitrogen, Carlsbad, CA, USA)
c-KIT, CD117	Polyclonal rabbit anti-human (1:400)	DAKO, Carpinteria, CA, USA	Ref #A4502	30	Affinipure Cy3 donkey anti-rabbit IgG (Jackson Laboratory Inc.)
CREM-1	Rabbit polyclonal anti-mouse	Santa Cruz Biotechnologies, Inc., Santa Cruz, Calfornia, USA	sc-440	4	Cy3; donkey anti-rabbit antibodies; (Jackson ImmunoResearch, West Grove, PA, USA)
ACROSIN	Mouse monoclonal anti-human	Biosonda (Santiago, Chile)	AcrC5F10	1	Donkey anti-mouse Alexa Fluor 488 IgG (Invitrogen, Carlsbad, CA, USA)

GFR-α1: glial cell line derived nerve factor family receptor alpha 1

Sertoli cells and undifferentiated type A spermatogonia only. Moreover, all cords contained many spermatogonia positive for GFR-α1 but all were negative for c-KIT. Representative PAS-hematoxylin and dual immunofluorescence stained testis sections from the youngest and oldest juvenile monkey used in this study are compared to those from an adult as shown in **Figure 2**. These results confirm that the only germ cells present in the testis prior to 3D culture were type A undifferentiated spermatogonia.

Developed colonies in MCS were harvested, suspended in RPMI and centrifuged at 3000 RPM for 10 min. The pellet was then collected and washed with RPMI and recentrifuged. The new pellet was diluted in 0.5 ml RPMI. Part of the suspension (100 μl) was mounted on SuperFrost[R] Plus slides, dried at room temperature, fixed in cold methanol for 20 min. and then stored at 4°C until stained. Immunofluorescence staining using specific antibodies (**Table 2**) for various markers of germ cell differentiation was performed as described in a previous study by one of our laboratories.[25]

RESULTS

Types of colonies/clusters developed

Monkey testicular germ cells cultured for 4–8 weeks in SACS (**Figure 3a**) or MCS (**Figure 3b**) formed colonies/clusters of cells of variable sizes. Colonies containing more than 10 cells but <50 cells were considered as small (S), colonies containing more than 50 but <150 cells were considered as medium (M) and those containing more than 150 cells were considered as large (L). In some cases, germ cell colonies were identified in the upper layer of SACS while somatic cells (mainly Sertoli and peritubular cells) were present in the lower layer of the system. In other cases, however, germ and somatic cells were both found in the upper layer of SACS. Similarly, in MCS, germ cell colonies in methylcellulose were either separated from somatic cells that were adhered to the plastic below the methylcellulose or juxtaposed to the adherent somatic cells; the later organization indicating that physical contact between the germ cell colonies and somatic cells may occur.

Effect of hormones and duration of incubation on the number of developed colonies

Colonies/clusters of cells developed in MCS regardless of the presence or absence of hormones (FSH [5 ng ml^{-1}] and testosterone [10^{-7} mol l^{-1}]), and these were counted after 4 and 5 weeks of culture. As depicted in **Figure 4a**, in the absence of hormone, the number of S colonies increased with the duration of cultures ($P < 0.01$). However, the number of M colonies was unrelated to the duration of culture, and the number of L colonies appeared to be inversely related to the duration of culture ($P < 0.05$) (**Figure 4a**). In the presence of hormone, on the other hand, the number of colonies of all sizes was independent of the duration of culture (**Figure 4b**). The number of S and L colonies in the absence of hormones were significantly higher compared to those in the presence of hormones ($P < 0.001$) for 5 and 4 weeks, respectively (**Figure 4a** and **4b** respectively). To confirm proliferation of germ cells in these cultures, it will be necessary to perform additional experiments and assess mitotic indices, using either an endogenous marker of proliferation, such as Ki-67 or an exogenous S-phase label, such as BrdU.

Expression of premeiotic, meiotic and postmeiotic markers in MCS

We analyzed germ cell differentiation in MCS since it was technically easier to recover large numbers of cells and colonies in this system compared to SACS. Colonies of all sizes and cells were harvested after 30 days of culture, and the degree of differentiation of the recovered germ cells was examined using immunofluorescence. A battery of specific antibodies was used for this purpose (**Table 2**). Before culture, VASA, SALL4 and GFR-α1 positive cells (premeiotic cells) were present in isolated STC from juveniles (**Figure 5a**). The same premeiotic markers were also observed after 30 days of culture with or without hormones (**Figure 5b**). The percentage of recovered cells expressing these markers after 30 days of culture (without hormones) was increased compared to before culture: for VASA from 5.4% ± 3% to 19.0% ± 11% (3 cultures from 3 monkeys), for SALL4 from 0.5% to 13% ± 7% (3 cultures from 3 monkeys), and for GFR-α1 from 8.5% ± 3% before culture to 16% ± 7% after 30 days of culture (5 cultures from 5 monkeys). These results demonstrate that undifferentiated Type A spermatogonia survive in MCS and indicate, but do not prove that they may proliferate in this culture system. Cells positive for CREM-1 (meiotic cells) and acrosin (postmeiotic cells) were not present in isolated STC from juvenile monkey testes before culture (**Figure 5a**, middle panel). These cells, however, were present after culture with and without hormones (**Figure 5b**): the percentage of recovered cells positive for CREM-1 at 30 days of culture (without hormones) was 30% ± 9% (5 cultures from 5 monkeys), while that of acrosin positive cells with a rounded morphology recovered at this time was 27% ± 10% (3 cultures from 3 monkeys) (**Figure 5b**). In one experiment, CREM-1 positive cells (14% ± 8%) were recovered as early as day 14 of culture. As a positive control for CREM-1 and acrosin, we used tubular cells and semen from adult monkeys, respectively (**Figure 5a**, lower panel).

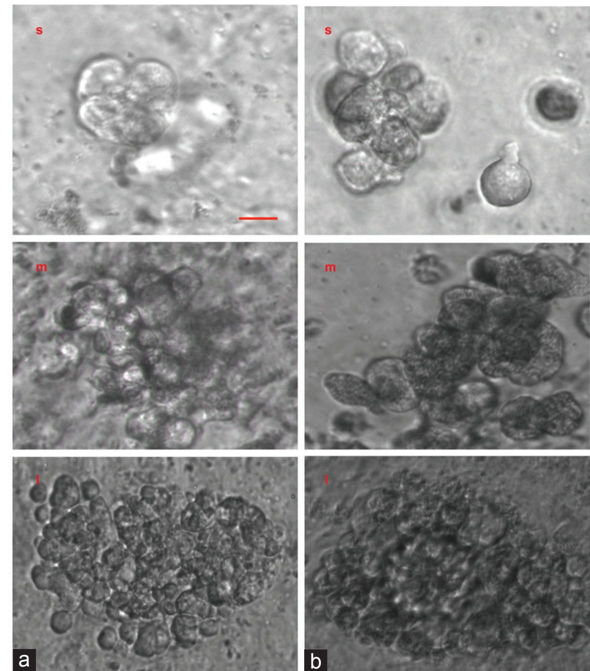

Figure 3: Juvenile rhesus monkey seminiferous tubular cell colony development in SACS (**a**) and MCS (**b**). Isolated seminiferous tubule cells were cultured as described in Figure 1. The sizes of the colonies developed in SACS (upper layer) and in MCS were evaluated after 4–8 weeks of culture. Colonies were designated as small (S) when they contained more than 10 cells but <50; medium (M) when they contained between 50 and 150 cells; and large (L) when they contained more than 150 cells. Scale bar (bottom right): 20 μm.

DISCUSSION

The present study using the MCS 3D culture system demonstrates for

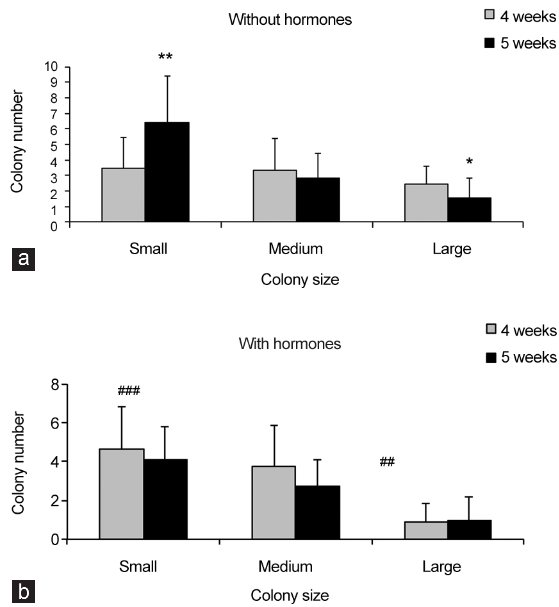

Figure 4: Effect of duration (4 or 5 weeks) of culture without (**a**) and with (**b**) hormones (FSH [5 ng ml^{-1}] and testosterone [10^{-7} mol l^{-1}]) on the number of germ cell colonies (small, medium and large) developed from juvenile rhesus monkey seminiferous tubule cells in MCS (mean ± SE). *compared between 4 and 5 weeks (*$P < 0.05$; **$P < 0.01$). #compared between with and without hormones (##$P < 0.01$; ###$P < 0.001$).

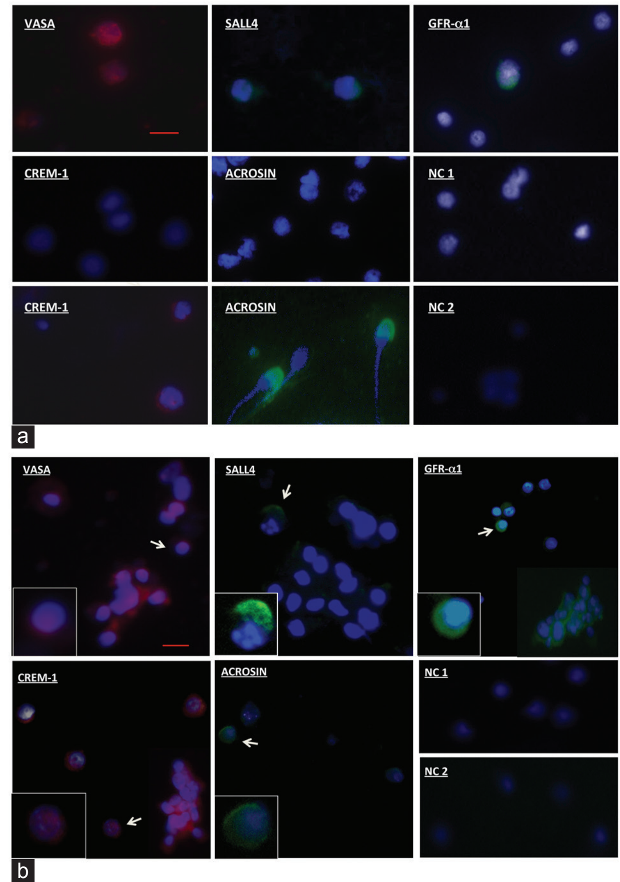

Figure 5: Immunofluorescence staining of juvenile rhesus monkey seminiferous tubular cells. (**a**) Isolated seminiferous tubular cells from juvenile monkeys were stained before culture in MCS by specific antibodies (**Table 2**) for markers of progressive germ cell differentiation, including VASA, SALL4, and GFR-α1 (premeiotic), CREM-1 (meiotic), and ACROSIN (postmeiotic) (upper and middle panels). As a positive control for CREM-1 and ACROSIN, semen and seminiferous tubular cells from adult monkeys were used (lower panel). Scale bar: 20 μm. (**b**) Colonies that developed within 30 days in MCS were collected, and the cells stained by specific antibodies for VASA, SALL4, GFR-α1, CREM-1, and ACROSIN. White arrows indicate a cell positive for respective marker and insets show the magnified cell. Scale bar: 20 μm. NC, negative control (primary antibody omitted or relevant IgG was added instead of the primary antibody) (NC1 represent mouse host antibodies, and NC2 represent rabbit host antibodies).

the first time that undifferentiated Type A spermatogonia harvested from the testes of the juvenile rhesus monkeys survive and commit to a pathway of differentiation under *in vitro* conditions, with both meiotic and postmeiotic cells being consistently observed after 30 days in culture. The kinetics of the germ cell development observed was not inconsistent with the *in vivo* situation where formation of pachytene spermatocytes from Type Ap spermatogonia takes a little over one cycle of the seminiferous epithelium (10.5 days) that characterizes this species of macaque.[31,87,88] Also of note, differentiation was induced in the absence of testosterone and FSH stimulation; however, the important question of whether the colonies were generated by a SSC or simply by differentiation of undifferentiated Type A spermatogonia devoid of "stemness" remains to be established.

Our inability to identify elongated spermatids in colonies of monkey germ cells in MCS could be related to inadequacies of the culture system that prevent the final stages of germ cell differentiation from occurring. Alternatively, as suggested in a previous study of mice using SACS[25] and discussed above, the inability to see spermatozoa in MCS may be related to either difficulties in microscopically identifying these cells in the thick agar layer or their very limited production. However, the case may be, these studies with rhesus monkey STC cells suggest that 3D systems mimic, at least in part, the *in vivo* conditions of the primate seminiferous tubule that promote the differentiation of Type A spermatogonia to spermatocytes. In addition, our findings also indicate that isolation of STC from the juvenile monkey testis and placing them into 3D culture removes the undifferentiated spermatogonia at this stage of development from a microenvironment that prevents their differentiation, or the survival of their differentiated progeny, in the *in situ* gonad. This being the case, it might be speculated that isolation of seminiferous tubular cells and their subsequent culture in 3D systems, results in reduction in signal transduction in pathways that have been implicated to suppress spermatogonial differentiation, such as that for GFR-1.[89] Future studies might be directed at testing this hypothesis by (1) examining the growth factor content of 3D culture systems in which germ cell differentiation occurs and (2) comparing differences in gene expression by Sertoli cells *in vivo* with that under 3D culture conditions. We also suggest that it may be informative to determine the extent to which germ cell differentiation in 3D culture system proceeds when purified germ cell preparations are cultured in the absence of somatic cells. Once a baseline is established "add back" experiments with specific populations of somatic cells, or respective conditioned media might then be performed. Further insight into factors that regulate male germ cell differentiation may be gained by exploring a phenomenon reported in the late 90s that injection of spermatocytes, or the nuclei of these meiotic cells, into activated oocytes was able to facilitate the completion of male meiosis.[90,91] One of

the current limitations of the present study was the low number of germ cells in the final 3D cultures, which prevented a more comprehensive analysis of these cultures. Future studies, as outlined above, are expected to resolve this problem.

In summary, the successful maintenance of SSCs in culture and the capacity to induce their self-renewal, proliferation and differentiation, under controlled conditions is likely to deepen our understanding of spermatogonial biology. This approach may also provide new insight into the biology of stem cells in nongonadal tissues. Additionally, 3D culture systems may lead to new therapeutic strategies for specific types of male infertility, including patients with azoospermic syndromes and cancer patients (prepubertal boys and adults), who cannot generate sperm to be cryopreserved but who have SSCs. In moving this field forward, we should always be cognizant of potential risks. Technologies to achieve pregnancy using intracytoplasmic injection of sperm harvested or generated from testes of subfertile/infertile men may pose a genetic risk to the offspring.[92,93] In addition, the generation of sperm *in vitro* may disrupt the normal epigenetic characteristics of male gametes that could lead to an increased risk of genomic imprinting disorders and abnormal embryogenesis.[92,93] Therefore, before such approaches are translated to the clinic a full understanding of epigenetic programming of male germ cells developed *in vitro* needs to be at hand.

In vitro culture systems may also be helpful in assessing the mechanism of action of contraceptive agents that induce infertility at the testicular level. Finally successful spermatogenesis *in vitro* could also be used for animal conservation and preservation of endangered species.

AUTHOR CONTRIBUTIONS

MH pioneered the use of SACS and MCS for spermatogenesis *in vitro*, made substantial contributions to the conception and design of the study and the experiments; carried out the MCS experiments and immunofluorescence staining; performed the statistical analysis; the interpretation of the data, and participated in drafting and critically revising the paper for key intellectual content. TMP made substantial contributions to the conception and participated in designing the study, interpreting the data and critically revising the paper for key intellectual content. SN performed the immuno- and histochemical staining of testicular tissue. All authors read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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