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Culture of mouse spermatogonial stem cells

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Abstract

Spermatogenesis occurs within the seminiferous tubules of mammals by a complex process that is highly organized, extremely efficient and very productive. At the foundation of this process is the spermatogonial stem cell that is capable of both self-renewal and production of progeny cells, which undergo differentiation over a period of weeks to months in order to generate mature spermatozoa. It had been thought that germ cells survive only a brief period in culture, generally less than a few weeks. However, an accurate assessment of the presence of spermatogonial stem cells in any cell population has only recently become possible with development of the spermatogonial transplantation technique. Using this technique, we have demonstrated that mouse spermatogonial stem cells can be maintained in culture for approximately 4 months and will generate spermatogenesis following transplantation to the seminiferous tubules of an appropriate recipient. Extensive areas of cultured donor cell-derived spermatogenesis are generated in the host, and production of mature spermatozoa occurs. Cultivation of the testis cells on STO feeders is beneficial to stem cell survival. These results provide the first step in establishing a system that will permit spermatogonial stem cells to be cultivated and their number increased in vitro to allow for genetic modification before transplantation to a recipient testis.

Keywords

Spermatogonia; testis; stem cell; culture; mouse; transplantation

Introduction

Within the testes of mammals, two organ systems exist which interact in a complex manner (Ewing et al., 1980; de Kretser & Kerr, 1994). One system produces spermatozoa within the seminiferous tubules of the testes. The second system produces hormones, primarily androgens, and the cells of this system lie between the tubules. Androgens have wide ranging physiological effects and are important for spermatogenesis. Production of spermatozoa is a complex process that begins with division and differentiation of the spermatogonial stem cell on the basement membrane within the seminiferous tubule of the testis (Russell et al., 1990). The stem cell has the unique ability both to self-renew and to produce progeny that undergo differentiation to spermatozoa (Morrison et al., 1997).

Currently, there are no morphological, biochemical or antigenic characteristics to identify the stem cell of spermatogenesis. Therefore, the stem cell is defined only by function.

Spermatogonial stem cells represent a small percentage of cells in the testes of any animal. In the mouse there are about 10^8 cells in the testis and approximately 2×10^4 are thought to be stem cells (Meistrich & van Beek, 1993; Tegelenbosch & de Rooij, 1993). Thus, most germ cells in the seminiferous tubules are mitotic spermatogonia, spermatocytes undergoing meiosis, and highly differentiated terminal stages such as spermatids (Huckins, 1971; Clermont, 1972). From a single stem cell in the rat, theoretically 1024 primary spermatocytes can be formed, but considerably fewer are produced because of cell loss (Russell et al., 1990). Nonetheless, an enormous proliferation of spermatogonia occurs, followed by meiosis of spermatocytes and a complex differentiation of spermatids to form spermatozoa. This entire process of spermatogenesis is supported by somatic Sertoli cells and occurs over a period of weeks to months, depending on the species, in a highly integrated manner (Bardin et al., 1993; Russell & Griswold, 1993). Because of the central importance of this process to survival of a species, repeated attempts have been made to reproduce spermatogenesis, in whole or in part, in vitro.

The cells of most interest are the spermatogonial stem cells, which are capable of regenerating the entire process of spermatogenesis and normally undergo replication throughout adult life. While a number of studies have been performed on testis cells in culture, it has been impossible to determine whether the critical spermatogonial stem cell remains viable once it is removed from the normal environment of the seminiferous tubule (Kierszenbaum, 1994). Several experiments have identified physical associations and even cellular junctional attachments between germ cells and Sertoli cells under culture conditions (Smith et al., 1992). Other studies have demonstrated maturation or brief differentiation sequences in vitro (Hofmann et al., 1992, 1994; Rassoulzadegan et al., 1993). Often cells remaining in culture retain some morphological characteristics of spermatogonia, such as large nuclear to cytoplasmic ratio, and it seems likely these primitive cells can be maintained in culture for several days or perhaps a few weeks (Kierszenbaum, 1994). Since stem cell is a functional definition, these cells cannot be currently identified by any physical or biochemical criteria. However, development of the spermatogonial transplantation technique has provided a functional assay by which the stem cell potential of any cell population can be assessed (Brinster & Avarbock, 1994; Brinster & Zimmermann, 1994). Therefore, it is now feasible to determine whether the spermatogonial stem cell can survive outside the seminiferous tubule and maintain full capability to regenerate spermatogenesis. Here we describe initial studies to answer this question and to begin to define culture characteristics for these cells.

Materials and methods

Mice and cell collection

To provide testis cells for culture, two strains of donor mice were used. One strain was the transgenic mouse line B6, 129-TgR(ROSA26)26Sor, designated ROSA26, from The Jackson Laboratory (Bar Harbor, Maine, USA) that was developed using gene trapping techniques and contains the *E. coli* LacZ (lacZ) structural gene. In this mouse many cell

types produce (β -galactosidase (β gal) and can be stained blue with 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-gal) (Zambrowicz et al., 1997). In adult testes all stages of spermatogenesis can be stained blue, and in neonatal testes it is clear that the stem cell stains intensely blue (Nagano & Brinster, 1998). Therefore, in culture studies the testis cells of these mice can be distinguished from feeder cells by incubation with X-gal and the characteristics of the testis cells examined at various time points. A second strain of mice used was the transgenic mouse line B6, SJL-TgN(c177lacZ)227Bri from The Jackson Laboratory that contains a zinc finger (ZF) promoter coupled to the *E. coli* LacZ (lacZ) structural gene (ZFlacZ). This fusion gene is expressed at high levels in round spermatids and, therefore, these and subsequent stages of spermatogenesis can be readily stained blue (Brinster & Avarbock, 1994; Brinster & Zimmermann, 1994). Other cells in the testis do not stain; thus spermatogenesis derived from these cells can be unequivocally distinguished following transplantation to recipient animals. Both ROSA26 and ZFlacZ testis cells can be identified following transplantation to a recipient, but ZFlacZ donor cells provide an unequivocal indication that the transplanted cells have produced late stages of spermatogenesis. Therefore, ZFlacZ testis cells were used for culture when the cells were to be transplanted.

The appropriate histocompatible recipient mouse for ZFlacZ donor cells is a C57BL/6 $\times$ SJL F₁ hybrid. Recipient mice are treated with busulfan to destroy endogenous spermatogenesis (Bucci & Meistrich, 1987). This allows transplanted spermatogonia to reach the basement membrane of the seminiferous tubule (Brinster & Avarbock, 1994).

Donor cells were collected from transgenic testes by a modified two-step enzymatic isolation procedure (Bellvé et al., 1977; Bellvé, 1993); details of the cell isolation and enrichment procedure have been previously described (Brinster & Avarbock, 1994; Ogawa et al., 1997). The enriched and dissociated cells were concentrated by centrifugation and then resuspended in medium at the desired concentration for culture.

Culture conditions

Following collection of testis cells, they were allocated to culture flasks (25 cm²), tissue culture plates (60 mm diameter, 27 cm²) or wells (2 cm²) of a 24-well plate (Falcon Plastics, New Jersey). The number of testis cells seeded was approximately 25×10^6 cells/25 cm² flask or plate for cells from adult animals 4 weeks or older, and 5×10^6 cells/25 cm² for cells from neonatal animals (5–15 days old) in 5 ml of medium. For cultures in wells, 10^5 to 10^6 cells were placed in each well in 1 ml of medium. In some cultures, mitomycin-treated STO feeders were used (Robertson, 1987). The feeders were seeded on the culture surface at a concentration of 5×10^5 cells/cm² before (1–5 days) testis cells were added. Culture medium was Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) with 6 mM glutamine, 30 (μ g/ml) penicillin and 50 (μ g/ml) streptomycin in one series of experiments. In another series of experiments, pyruvate (0.5 mM) and lactate (6 mM) were added to the culture medium because of their beneficial effect in culturing female germ cells (Brinster, 1972). Medium was changed two or three times each week, and the cells were maintained at 32°C in an atmosphere of 5% carbon dioxide in air.

In some experiments, cultured cells were stained for β gal activity to distinguish testis cells from feeders. In order to stain the cells, medium was removed, the cell layer washed once with phosphate-buffered saline (PBS), fixed with 0.5% glutaraldehyde for 5 min, washed twice with PBS, and then incubated with X-gal until blue color developed (Soriano et al., 1991).

Transplantation and analysis

The cultured cells were maintained for various periods and then transplanted to recipient testes. To prepare the cultured cells, they were harvested by trypsin (0.25%) digestion, washed in DMEM/FBS medium, pelleted by centrifugation, and resuspended in medium for injection into recipient testes (Brinster & Avarbock, 1994; Nagano & Brinster, 1998). The concentration of cells in the injection medium ranged from 2×10^6 to 51×10^6 cells/ml (Table 1), and approximately 10 μ l of cell suspension was introduced into the seminiferous tubules of a recipient testis (Ogawa et al., 1997). The technique for injection of cell suspensions into the seminiferous tubules has been previously described (Brinster & Avarbock, 1994; Ogawa et al., 1997). Following cell transplantation, the recipient mice were maintained for 100–162 days, and the testes were then analyzed by X-gal staining (Brinster & Avarbock, 1994; Ogawa et al., 1997).

Experimental design

In the experiments reported here, three variables were addressed. First, the effect of STO feeder cells on growth and appearance of ROSA26 testis cells in culture was examined. In addition, ZFlacZ testis cells were used to determine the influence of STO feeders on the ability of cultured cells to produce late stages of spermatogenesis in recipient testes. Second, the effect of pyruvate and lactate in culture medium on the ability of cultured stem cells to survive and produce spermatogenesis was assessed. Third, the age of donor mice from which testis cells were collected for culture was examined for an effect on recipient testis colonization.

Results

Culture of cells

For examination of the characteristics of testis cells in culture, 24-well tissue culture plates were employed in order to provide multiple samples of the same preparation to examine at several times after initiation of culture. In these studies, cells were harvested from ROSA26 mice approximately 6 weeks after birth because all stages of germ cells from this transgenic line can be stained by X-gal to provide identification during culture. The cells were dispersed to individual wells at several concentrations in 1 ml of DMEM/FBS medium containing pyruvate and lactate. The surface area of each well is approximately 2 cm², and cells at a wide range of concentration successfully attach and proliferate on the surface. However, it was found that 10^6 cells/ml (well) provided the most consistent seeding of the surface and subsequent establishment of a cell layer. This was particularly true when there were no feeder cells to facilitate attachment of germ cells.

To determine the effect of STO feeders on the characteristics of testis cells in culture, 10^6 testis cells/ml were placed on feeders in individual wells and examined periodically. There was a distinct difference in the appearance of the cultures in wells with and without STO feeders 2 days after the cells were seeded in the wells, (Fig. 1A and B). In the absence of STO feeders, the cell layer was thin and small areas of the plastic surface appeared not to be covered by cells; round putative germ cells were predominantly in clusters and adherent to flatter cells spreading on the plastic surface (Fig. 1A). When STO feeders were present, a larger number of round cells adhered to the feeders and other cells spreading on the feeders (Fig. 1B). This assessment of cells in culture was confirmed by staining with X-gal (Fig. 1C and D). The round putative germ cells stained well and were mostly dark blue. This dark staining is characteristic of the germ cell population in the testes of ROSA26 mice (Nagano & Brinster, 1998). Flat cells adhering to the plastic were light blue but could be more readily seen following X-gal staining, and the association of the round cells with these spreading adherent cells was apparent. The number of testis cells present in wells containing STO feeders was greater than in wells without feeders. In particular, the number of round blue cells appeared to be greater (Fig. 1D).

During subsequent weeks, the testis cells proliferate and spread on the bottom of the well, and become confluent. In general, the testis cells form a layer of flat cells on which rest several other types of cells, including round cells. After 3 or 4 weeks, the cultures remain in a relatively steady state. Some cells die and are removed when medium is replaced, but cell division appears to maintain the total population and general appearance of the cell layer. Rarely, the testis cells will lift from the plastic; in which case, they can be digested with trypsin and replated.

The appearance of the cell layer with phase-contrast microscopy after 1 month's cultivation was rather similar whether or not STO feeders were used (Fig. 2A and B). In general, the presence of a feeder layer seemed to increase the number of testis cells in the well and the number of round cells. However, staining of cell layers with X-gal after 1 month in culture clearly demonstrated the presence of a greater number of round cells in wells in which the testis cells were placed on STO feeders (Fig. 2C and D).

Transplantation of cultured testis cells

For these experiments, testis cells were obtained from mice that express the ZFlacZ transgene. Following transplantation of cells from this transgenic mouse to a recipient, only round spermatids and later differentiation stages express the transgene and stain blue following incubation with X-gal (Fig. 3A and B). This provides a clear demonstration that donor-cell-derived spermatogenesis has proceeded to late stages of differentiation in recipient testes. In order to obtain sufficient cells for transplantation following in vitro cultivation, tissue culture flasks (25 cm^2) were employed. Adult cells were plated at approximately 10^6 cells/cm^2 and neonatal cells at approximately $2 \times 10^5\text{ cells/cm}^2$. In both situations, the cell layer became confluent within 1 week.

In the first series of experiments, the culture medium was DMEM/FBS without pyruvate or lactate, and cells were plated directly on the plastic surface or on STO feeders. Following incubation periods of 10–25 days, the cells were harvested and transplanted to the

seminiferous tubules of C57BL/6 $\times$ SJL recipient male testes. The results are shown in Table 1 (experiments 1 to 5). The concentration of cells injected in each experiment is indicated, and approximately 10 μ l of cell suspension can be introduced into the seminiferous tubules of a testis (Ogawa et al., 1997). Thus, in these experiments between 8×10^4 cells (8×10^6 cells/ml $\times 10^{-2}$ ml; experiment 1) and 51×10^4 cells (experiment 4) were introduced into the tubules of a single testis.

The injected testes of these experimental recipient mice were analyzed by incubation with X-gal 100–135 days following transplantation of the cultured cells. Of the 23 recipient testes in this series of experiments, three were colonized by cells cultured 19 or 25 days on STO feeders (experiments 1 and 5). Each of the testes had more than 12 tubules colonized, and the blue tubules intertwined making it difficult to discriminate and count individual tubules. Histological examination of these testes demonstrated numerous seminiferous tubule cross-sections that stained blue indicating colonization by cultured donor cells (Fig. 4A and B). Since ZFlacZ cells were transplanted, only spermatid stages actually stain. However, the degree of staining in any tubule depends on incubation conditions, particularly penetration of the substrate X-gal. When staining is intense, the blue leaches into the more immature cell types in the tubule, as seen in some tubules in Fig. 4. The fixation procedure for β gal staining does not preserve good morphological characteristics, but it is clear that normal spermatogenesis has been achieved from cultured testis cells (Fig. 4B).

In the second series of experiments, the culture medium was DMEM/FBS containing pyruvate and lactate, and STO feeders were used for some cultures. The cells were maintained for periods of 62–132 days and passaged one to three times before harvest and transplantation to recipient testes. The results are shown in Table 1 (experiments 6 and 7). The concentration of cells injected was slightly lower in this series, and from 2×10^4 to 7×10^4 cells were introduced into the tubules of a single testis. The injected recipient testes were analyzed for colonization 108–162 days following transplantation of cultured cells. Areas of donor-cell-derived spermatogenesis were seen in three of 14 recipient testes that received transplanted cells, and the number of tubules colonized ranged from four to more than 12. A typical pattern of recipient testis colonization is shown in Fig. 3C and D. In many cases, particularly after careful dissection, it is clear that each stretch of blue tubule is separated by non-stained areas. At the end of the blue stretch of tubule, staining is less intense and gradually fades into the non-stained tubule. This reflects the spread of colonization by donor cells at the ends of the blue area where spermatids appear first in small numbers and, therefore, produce faint staining. Histological examination of testes from the second series of experiments provided data similar to the first series (Fig. 4C and D). Staining of donor-cell-derived spermatogenesis was found in a number of tubule cross-sections (Fig. 4C), and the intensity of color varied among the tubules (Fig. 4A and C). The morphological appearance of the spermatogenesis is often normal (Fig. 4B), but in some cases abnormalities are observed (Fig. 4D). There was no significant difference in the appearance or degree of recipient testis colonization by donor cells from the two series of experiments using different culture media. Likewise, the age of the mice from which the donor testis cells were collected for culture did not have a significant effect on recipient testis colonization (Table 2). However, the use of a STO feeder layer for testis cell culture

was an essential factor in maintaining stem cells and had a significant effect ($p = 0.001$) on recipient testis colonization (Table 2).

Discussion

The results clearly demonstrate that spermatogonial stem cells can be maintained in vitro for long periods. This is a remarkable finding considering the long-held view that germ cells do not remain in the culture environment for more than a few weeks at most (Kierszenbaum, 1994). The absence of a functional test of stem cell activity prevented an accurate assessment of cells previously maintained in vitro, and the morphological characteristics of testis cells in culture provided no signs of stem cell activity; there are no obvious colonies or centers of differentiation present (see e.g., Fig. 1 and 2). Thus, in the absence of distinctive characteristics suggestive of stem cell proliferation in culture and the inability to subject the cells to an appropriate assay, it is not surprising that investigators believed germ cells had disappeared in long-term culture. However, it is well known that the stem cell of spermatogenesis is remarkably resistant in vivo to many detrimental environmental factors and is the last of the germ cells to be destroyed by radiation, carcinogenic agents, heat, and other injuries to the testis (Ewing et al., 1980; de Kretser & Kerr, 1994). Consequently, perhaps it is not unexpected that these cells may be similarly able to survive under various in vitro conditions. In fact, earlier experiments had already established that these stem cells withstand the potentially harsh conditions present during transplantation from a fertile to an infertile testis and can also be cryopreserved for long periods (Brinster & Avarbock, 1994; Avarbock et al., 1996). Thus, this latest finding that stem cells can survive for months in culture provides further evidence of their durability and suggests that additional manipulations of these cells are possible.

A surprising and an important result from these experiments is that the stem cell survived under relatively simple culture conditions. No growth factors, hormones, or other unusual components were added to the culture media. However, this observation is tempered by two factors. First, fetal bovine serum was a significant (10%) component of the media, and many supportive factors for cell maintenance and division are present in serum. Thus, these ingredients, present at the concentration in serum, may be important to the stem cells. Second, and perhaps most important, all the successful cultures that resulted in spermatogenesis following transplantation were maintained on STO feeder cells (Table 2). These results provide strong evidence that the feeder layer is critical to spermatogonial stem cell maintenance. STO feeder cells are known to be beneficial for cultures of embryonic stem cells and primordial germ cells (Matsui et al., 1992; Resnick et al., 1992). This positive effect may result from growth factors and cytokines secreted by the feeders or from cellular associations between the stem cell and the feeder cells (Robertson, 1987; Smith et al., 1992). For example, the bound form of c-kit ligand (steel factor) has been demonstrated to be essential for the differentiation of male germ cells during spermatogenesis (Williams et al., 1992); however, the importance of this factor for spermatogonial stem cells is unclear (Yoshinaga et al., 1991). Studies employing various types of feeder cell lines producing soluble or bound c-kit ligand will help to address this question. Furthermore, the use of other cell lines (e.g., Sertoli feeders), may delineate the requirements for stem cell survival and replication in vitro.

A remarkable aspect of the results is the efficiency with which success was obtained. In four of the seven experiments performed, cultured cells produced spermatogenesis following transplantation, and seminiferous tubule colonization was obtained whether fetal or adult testis cells were cultured (Tables 1 and 2). When STO feeders were used, six of 13 cultures produced spermatogenesis; a result which clearly demonstrates a reproducible system capable of analyzing important elements necessary to improve culture conditions of stem cells. In several of the testes, colonization from cultured cells was extensive, and more than 12 individual areas in tubules demonstrated donor-cell-derived spermatogenesis. Since a blue area in a tubule represents colonization by at least one stem cell, a minimum of 12 stem cells must have been present in the injected cultured cells. If colonization efficiency is 10–40% (Ogawa et al., 1997), the number of stem cells in the injected population could have been 30–120. The suspension of cultured cells that produced the result shown in Figure 3 contained 6×10^6 cells/ml or 6×10^4 cells in the 10- μ l volume injected into the seminiferous tubules. Since only about two stem cells are believed to be present in 10^4 testis cells (Meistrich & van Beek, 1993; Tegelenbosch & de Rooij, 1993), 30–120 stem cells in the injected cultured population is greater than found in the testis. Therefore, the number of stem cells injected into the seminiferous tubules of a testis in experiment six may have been 2.5–10 times greater than that found in the donor testis, and similar numbers of stem cells may have been injected in experiments 1 and 5.

While these calculations might suggest replication and expansion of stem cell number during culture, caution is necessary. First, the degree of seminiferous tubule colonization following donor cell transplantation shows considerable variability even under the same experimental protocol. Second, the high number of stem cells from cultured cells could reflect more efficient stem cell attachment and survival rather than replication, since many more testis cells were placed in culture than were harvested. While our studies do not directly demonstrate stem cell replication in culture, the possibility is not ruled out. A conceivable scenario is that the stem cell slowly divides in culture and some of the replicated cells differentiate and are eventually lost, a situation that resembles spermatogenesis in vivo (de Rooij et al., 1989; Russell et al., 1990). The number of stem cells remaining at any time in culture then reflects not only their survival but also a balance between mitotic activity of the stem cell and differentiation into progeny; clearly a complex situation. However, the ability to maintain the spermatogonial stem cell of neonatal as well as adult testes in vitro for long periods of time, in a relatively defined system and in significant numbers, sets the stage for critical studies to define the criteria that will increase the efficiency of the process. An improved culture system would allow the production of large numbers of stem cells in vitro and the eventual modification of genes in these cells. Such an advance will be enormously important in biology, medicine and agriculture.

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Fig. 1.

Appearance of ROSA26 testis cells after 2 days in culture. **A** and **C** were cultured without STO feeders; **B** and **D** were cultured with STO feeders; **A** and **B** are phase-contrast microscopy of living cells; **C** and **D** are X-gal-stained cells. Each culture well initially received 10^6 testis cells. Note increased number of round cells present in wells with STO feeders. Many of these round cells are likely to represent various differentiated stages of germ cells. Scale bar = 60 μ m.

Fig. 2.

Appearance of ROSA26 testis cells after 1 month in culture. **A** and **C** were cultured without STO feeders; **B** and **D** were cultured with STO feeders; **A** and **B** are phase-contrast microscopy of living cells; **C** and **D** are X-gal stained cells. Each culture well initially received 10^6 testis cells. Note increased number of round cells still present in wells with STO feeders after 1 month. Germ cells are generally believed to maintain a round appearance in culture. All round cells are not likely to be germ cells. Scale bar = 60 μm .

Fig. 3.

Colonization of busulfan-treated recipient mouse seminiferous tubules by cultured testis cells from ZFlacZ donor. **A** Control C57BL/6 $\times$ SJLF₁ testis in which germ cells do not stain with X-gal for the presence of *E. coli* β -galactosidase; **B** Transgenic ZFlacZ adult donor testis showing intense blue staining from β -galactosidase activity in spermatid stages of germ cell population. **C** Busulfan-treated C57BL/6 $\times$ SJLF₁ recipient testis into which ZFlacZ testis cells cultured for 111 days were transplanted (Table 1, experiment 6, left testis). Cultured donor cells were originally collected from 10-day-old mice. Recipient was analyzed by X-gal staining approximately 3.5 months after cultured cell transplantation. Blue stretches of tubules indicate individual areas of colonization by cultured spermatogonial stem cells. Tubules without spermatogenesis or tubules that have reestablished recipient stem cell-derived spermatogenesis do not stain. **D** Tunica albuginea has been removed to reveal the extent of colonization, and to demonstrate that at the ends of the blue stretches of tubule the blue color diminishes indicating continued spread of stem cells and colonization. **A – D**, X-gal stain. Scale bar = 1 mm.

Fig. 4.

Donor-cell-derived spermatogenesis following transplantation of cultured testis cells to recipient mice. ZFlacZ testis cells were cultured and transplanted into the seminiferous tubules of busulfan-treated recipient mice. **A** Testis cells were collected from 7-week-old mice and cultured for 19 days before transplantation (Table 1, experiment 1). Recipient was analyzed by X-gal staining 110 days later. Only donor stem-cell-derived germ cells will stain blue. Cross-sections in this area of the testis demonstrate spermatogenesis from cultured donor cells. **B** Seminiferous tubule from the same testis as (**A**) demonstrating the normal appearance of spermatogenesis and many mature spermatozoa. **C** Testis cells were collected from 10-day-old mice and cultured for 111 days before transplantation (Table 1, experiment 6). Cross-sections in this area of the testis demonstrate cultured donor stem-cell-derived spermatogenesis. **D** Seminiferous tubule from the same testis as (**C**) showing an area of donor cell-derived spermatogenesis with atypical cellular arrangement (see Russell et al., 1996 for ultrastructural details of transplanted germ cell spermatogenesis). The intensity of X-gal staining of donor-cell-derived spermatogenesis varies among tubules because of differences in X-gal substrate penetration. Morphology of spermatogenesis is not optimal because fixation for X-gal staining does not preserve testis structure well. **A–D**, X-gal followed by neutral fast red stain. Scale bars: **A** and **C** = 100 µm; **B** and **D** = 20 µm.

Spermatogenesis from cultured spermatogonial stem cells following transplantation to recipient seminiferous tubules

Table 1

Experiment	Age of testes	Days cells in culture (passages)	Presence of STO feeders [†]	Concentration of cells/ml injected ($\times 10^6$)	Number of testes injected	Number of testes colonized [‡]	Number of tubules with donor Spermatogenesis [§]	
							Right	Left
1	7 weeks	19 (0)	+	8	2	2	12+	12+
2	6 weeks	10 (0)	—	28	6	0	—	—
3	6 weeks	22 (0)	—	35	6	0	—	—
4	7 weeks	19 (0)	—	51	8	0	—	—
5	8 days [*]	25 (0)	+	13	1	1	12+	—
6	10 days	111 (1)	+	6	2	2	4	12+
		132 (2)	+	5	2	0	—	—
7	11 days	62 (1)	+	7	2	1	5	0
		62 (1)	—	5	2	0	—	—
		76 (2)	+	4	2	0	—	—
		99 (3)	+	2	2	0	—	—
		99 (3)	—	6	2	0	—	—

* Accuracy ± 3 .

[†] + indicates testis cells were cultured on a feeder layer of mitomycin-treated STO cells. When cells were injected into the seminiferous tubules, the mixed population of cells in culture was digested and used for transplantation.

[‡] Number of testes with areas of cultured cell-derived spermatogenesis.

[§] Number of tubules in recipient testis with cultured donor cell-derived spermatogenesis. Individual tubules that stained blue were counted up to a maximum of 12. The number of tubules in testes with greater than 12 stained could not be accurately counted because of intermingling of tubules, and these testes were designated 12+.

Table 2

Effect of STO feeders and age of donor testes on culture of spermatogonial stem cells

Age of donor testes	Number of colonized testes/Number of testes injected		
	+STO	–STO	Total
Adult	2/2	0/20	2/22
Neonate	4/11	0/4	4/15
Total	6/13	0/24	6/37

Data summarized from Table 1.

Adult, 6–7 weeks old; neonate, 8–11 days old. Male mice become fertile at 4–6 weeks of age. + STO, testis cells cultured on STO feeders; – STO, testis cells cultured without STO feeders. Effect of donor cell age was not significant. Effect of STO feeders was significant ($p = 0.001$; χ^2 , Fisher exact test).