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Spermatogenesis following male germ-cell transplantation

(spermatogonia/stem cells/testes/transgenic mice)

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ABSTRACT **In the adult male, a population of diploid stem-cell spermatogonia continuously undergoes self-renewal and produces progeny cells, which initiate the complex process of cellular differentiation that results in mature spermatozoa. We report here that stem cells isolated from testes of donor male mice will repopulate sterile testes when injected into seminiferous tubules. Donor cell spermatogenesis in recipient testes showed normal morphological characteristics and produced mature spermatozoa. This methodology, besides opening new avenues of basic research into spermatogenesis and stem-cell self-renewal, may prove useful as a tool for biomedical science and biotechnology.**

The only source of potentially totipotent diploid cells in the adult mammalian body resides within the basal layer of the seminiferous tubules of the testis. These cells are known as spermatogonia in the prepubertal and adult period and can be divided into three primary types: stem cell spermatogonia, proliferative spermatogonia, and differentiating spermatogonia (1). The first two groups are frequently designated as undifferentiated spermatogonia. It is the stem cell spermatogonia that are most resistant to a variety of agents which damage the testis, and often these cells survive when other germ cell types are destroyed. The less frequent division of this stem cell population is believed to be one reason for their ability to survive deleterious influences (1, 2). While it is difficult to be certain which of the spermatogonia are capable of self-renewal, a number of studies indicate that those cells classified on morphological grounds as stem cell spermatogonia and proliferative spermatogonia are capable of renewing themselves (1–4). Beginning before puberty and continuing in the adult animal, the stem cell spermatogonia undergo continuous replication, thereby maintaining their number, in a process known as stem cell renewal (3, 4). In addition, a fraction of the proliferative spermatogonia population undergoes a complicated differentiation process resulting in the production of mature spermatozoa. The entire process of self-renewal and differentiation into spermatozoa constitutes spermatogenesis (1–4). An accurate estimate of the number of stem cell spermatogonia is difficult because the morphological characteristics of the individual cell types are not well defined, and the changes that spermatogonia undergo as they differentiate are gradual (1, 2).

Through the process of spermatogenesis the spermatogonia become spermatozoa, and following fertilization their genes, complemented by female homologs, participate in embryogenesis and thus ultimately contribute to the differentiation of every cell in the body. In this sense these cells can be considered totipotent. In contrast, although female oocytes present in the adult contribute to the genetic makeup of progeny, they are not self-renewing; there is a finite number present at birth, and this pool decreases during the lifetime of the female. Stem cell spermatogonia are thus essentially the only self-renewing cell type in the adult capable of providing a genetic contribution to the next generation.

Because of the unique characteristics and potential of stem cell spermatogonia, the ability to recover these cells, manipulate them *in vitro*, and transfer them to another testis would provide a valuable technique to study the process of spermatogenesis. Furthermore, modifications of these cells prior to transfer could influence the development of eggs fertilized by spermatozoa arising from the altered stem cells. We describe here the requisite first step in this approach, a method to transplant testis cells from one male to another, and we demonstrate that spermatogenesis occurs from donor cells in the recipient male.

MATERIALS AND METHODS

Cell Preparation. Donor cells were prepared from the testes of mice by the procedure of Bellve *et al.* (5) with minor modifications. Male mice for these experiments were obtained between postnatal days 4 and 12, because immediately following birth the testes are small and the seminiferous tubules contain primarily gonocytes, whereas adult mice contain many advanced stages of spermatogenesis not capable of acting as stem cells (see Fig. 1 and Fig. 2 *A* and *D*). Experimental mice were euthanized, and the testes were rapidly removed and placed in calcium-free phosphate-buffered saline (GIBCO catalogue no. 21300-025). Between 10 and 20 testes were collected for each experiment. The tunica was removed from each testis or reflected away from the tubules, and the testes were placed in Dulbecco's modified Eagle's medium buffered with Hepes (20 mM) and containing collagenase type 1 (1 mg/ml; Sigma catalogue no. C-0130). Incubation was for 15 min at 37°C, with manual agitation at 5-min intervals. The incubation volume was ≈10 times the testes volume. After incubation the testes were removed, washed twice in calcium-free phosphate-buffered saline, and then incubated in 0.25% trypsin with 1 mM EDTA in the same buffer for 5–10 min at 37°C, with manual agitation at 5-min intervals. The action of trypsin was terminated by adding sufficient fetal bovine serum to increase the incubation volume by 50%. Large pieces of undigested tunica or tubules were removed with forceps. The cell suspension was centrifuged at 600 × *g* for 5 min at 16°C, the supernatant was removed, and the cells were resuspended in 200 μl of sperm cell medium in a microcentrifuge tube on ice. Sperm cell medium contained 138 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.1 mM KH_2PO_4, 0.1 mM EDTA, 5.5 mM glucose, bovine serum albumin (5 mg/ml), DNase I (100 μg/ml), and trypan blue (0.4 mg/ml) at pH 7.4. The cells were stored concentrated on ice until the time of injection, then 20–50 μl of the concentrated suspension was added to 500 μl of sperm

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Abbreviations: *W/W*, homozygous and compound heterozygous W mice; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

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cell medium. Cell concentration in this diluted cell suspension ranged from 10^6 to 10^7 cells per ml. Trypan blue was used to follow the progress of seminiferous tubule filling and to monitor the number of dead cells in the suspension, which was generally <5%.

Donor and Recipient Mice. Two types of mice were used as germ cell donors, and two types served as corresponding recipients (see Table 1). In protocol 1, the donor mice were all of the C57BL/6 histocompatibility genotype, C57BL/6 (black), C57BL/6 (albino), or C57BL/6 (tan belly), and they were introduced into mutant W mice, which are immunologically compatible with C57BL/6 mice. The mutant *W* mice were maintained on the C57BL/6 genetic background or the closely related WB/ReJ background (The Jackson Laboratory). In these experiments recipient males carried the *W*, W^v, and W^{44} mutant alleles in the homozygous or compound heterozygous condition, and spermatogenesis does not occur in the testes of these mice (reviewed in ref. 6; R.L.B., unpublished data). Very few germ cells of the most primitive stages can be found in the testes of W/W^v mice (7, 8). In protocol 2, the donor cells were obtained from mice carrying a transgene that directed the expression of the *Escherichia coli* β-galactosidase gene (*lacZ*) to round spermatids and later stages of spermatogenesis, which allowed these cells to be stained blue in animals heterozygous for the transgene. These transgenic lines were maintained on a C57BL/6 × SJL genetic background and were designated ZFlacZ. Donor cells from ZFlacZ mice were microinjected into the testes of F_1 males (C57BL/6 × SJL) that had been injected at 4–6 weeks of age with busulfan (40 mg/kg, intraperitoneally), which destroys spermatogenic stem cells (9, 10). The busulfan-treated males were used as recipients beginning 4 weeks after injection. In mice treated with busulfan at 40 mg/kg, spermatogenesis is rarely seen. In addition, endogenous spermatogenesis that may occur in these animals can be distinguished from ZFlacZ donor cell spermatogenesis because endogenous sperm cells will not stain for the presence of β-galactosidase. The testes and seminiferous tubules of homozygous and compound heterozygous *W* mice (hereafter referred to as *W/W*) and of mice treated with busulfan are significantly reduced in size because of the absence of spermatogenesis (adult testes are 10–15% of normal size; seminiferous tubules are about 50% normal diameter; Figs. 1 and 2).

Cell Transplantation Procedure. To transfer male germ cells from donor testes into a recipient testis, we developed a technique to microinject cells directly into individual seminiferous tubules. The recipient mouse was anesthetized, and the testis was exteriorized through a midline abdominal incision, immobilized, and oriented to align a group of tubules with the injection pipette (Fig. 3). The pipette size and construction were similar to that originally devised for injection of stem cells into blastocysts (11). The pipette was filled with germ cell solution by using a thin needle, and the pipette was fastened in an Eppendorf pipette holder attached by tubing to a pressure injector (Eppendorf model 5242) (Fig. 4). Under a dissecting microscope, the tunica was reflected off the testis to expose the seminiferous tubules. A section of tubule was entered with the tip of the pipette, and pressure in the injection tubing was raised until the cell suspension flowed into the tubule; the flow was monitored by observing the color change. When flow of the solution into the tubule

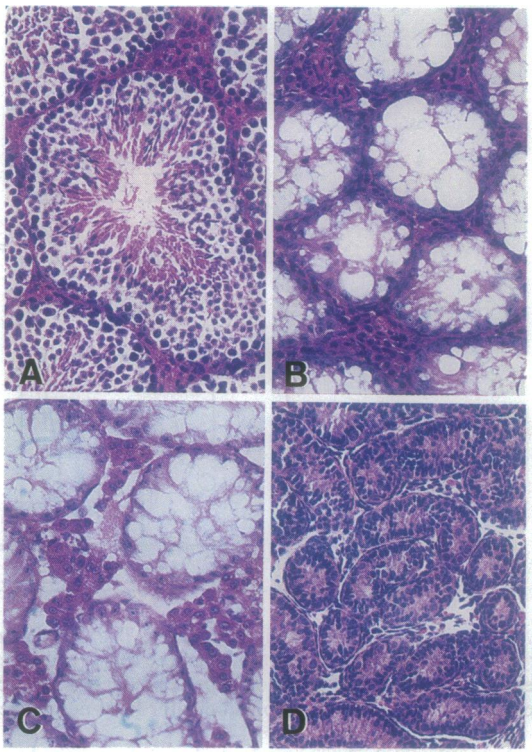

FIG. 2. Microscopic appearance of control and recipient testes. (*A*) Control C57BL/6 adult, age 12 weeks. Note active spermatogenesis in seminiferous tubules. (*B*) Mutant W/W^v [(C57BL/6 × WB/ReJ)F_1], age 10 weeks. Note absence of spermatogenesis. (*C*) Busulfan-treated C57BL/6 males, age 12 weeks, 6 weeks after treatment. Note absence of spermatogenesis. (*D*) Neonatal (C57BL/6 × SJL)F_1, age 2 days. Gonocytes, the prenatal form of testis stem cell, are the only germ cell stage. (Hematoxylin and eosin; ×140.)

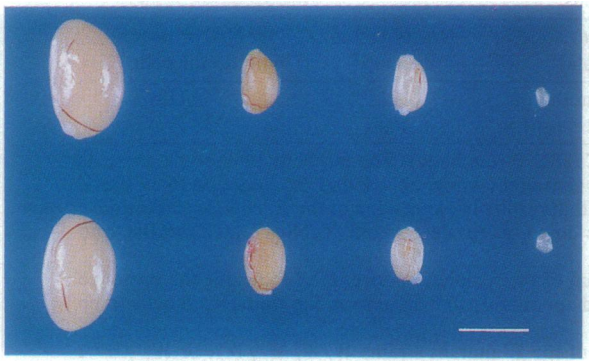

FIG. 1. Control testes and testes lacking spermatogenesis. (*Left*) Adult (C57BL/6 × SJL)F_1 hybrid, age 10 weeks. (*Left Center*) Adult W/W^v mutant, age 10 weeks. (*Right Center*) Adult (C57BL/6 × SJL)F_1, busulfan treated at 4 weeks, age 24 weeks. (*Right*) Neonatal (C57BL/6 × SJL)F_1, age 2 days. (Bar = 0.5 cm.)

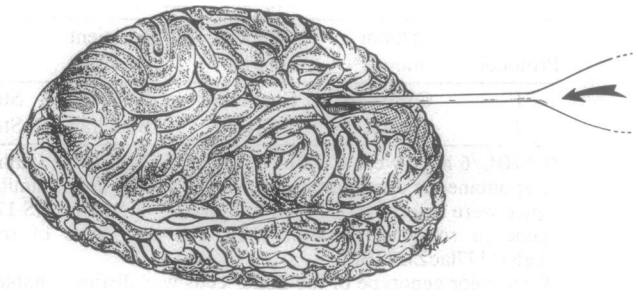

FIG. 3. Injection of recipient testis with donor cells. A straight length of seminiferous tubule was penetrated with a microinjection pipette, which was drawn from a 10-cm length of siliconized borosilicate glass with 1-mm outside diameter and 0.75-mm inside diameter (11). Each length of glass was drawn to make two pipettes, and the thin ends were chipped to produce a sharp 40-μm tip. The cell suspension was introduced into the pipette with a long 28-gauge needle.

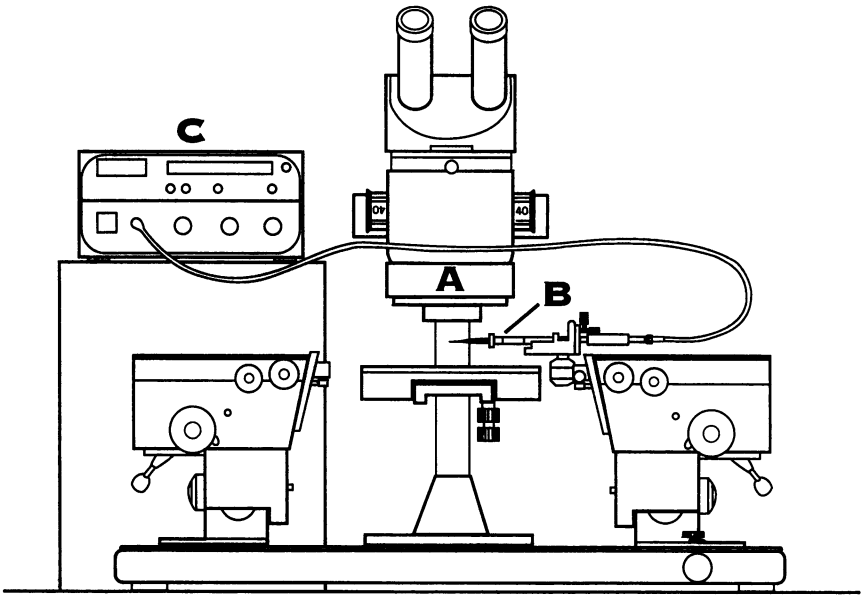

FIG. 4. Apparatus for microinjecting testes. The anesthetized mouse with testis exteriorized was placed on a platform and illuminated with a fiber optic ring light attached to the objective lens of a dissecting microscope (A). Magnification used was approximately ×120. The injection pipette was secured by an Eppendorf pipette holder (B) connected to an Eppendorf pressure injector (C). The movement of the injection pipette was controlled with a Leitz micromanipulator from either the right or the left.

stopped, another tubule was selected and the procedure was repeated. Between 25% and 90% of the surface tubules were routinely filled with the cell solution. Good filling of the tubules resulted in the appearance of dye-colored solution in the epididymis.

Analysis of Recipient Mice. Because the process of spermatogenesis from stem cell to mature spermatozoa takes 35 days, recipient males were maintained between 48 and 230 days following injection of donor germ cells before sacrifice and analysis. Testes from mice that had received C57BL/6 donor cells were fixed in neutral buffered 10% formalin, and 5-μm histological sections were cut and stained with hematoxylin and eosin. Two to four cross sections of each testis were examined for the presence of spermatogenesis in the tubules. Testes from mice that had received ZFlacZ donor cells were fixed and processed as described (13). In brief, the testes were fixed for 1–2 hr in 4% paraformaldehyde, washed, and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal). Each testis was examined at 120-fold magnification for the presence of tubules that stained blue. In some instances, 5-μm sections were cut and stained with neutral fast red. The number of tubule cross sections that stained blue was generally greater than the number of tubules visibly blue on the surface, reflecting the tortuous nature of the tubule path within the testis.

RESULTS AND DISCUSSION

Injections of donor cells isolated from C57BL/6 males were made into 22 *W/W* testes, and in 4 of these spermatogenesis was observed in the seminiferous tubules (Table 1). The number of *W/W* recipients available was a limiting factor in this protocol, because generation of *W/W* mutant males was difficult (only one in eight progeny carries two mutant alleles at the locus and is male). However, the testis of this genotype was a desirable recipient because spermatogenesis was completely absent (Fig. 2*B*) and the mechanism by which sterility was achieved did not rely on chemical treatment of the animal. In the testes that were colonized, the characteristic arrangement of the successive stages of sperm cell differentiation was normal (1, 2, 14), and mature spermatozoa were seen in the lumen of the seminiferous tubule (Fig. 5*A*). Testes

Table 1. Colonization of recipient testes by transplanted donor cells

Protocol	Donor mouse*	Genetic marker†	Recipient mouse‡	Characteristic of male§	No. of testes injected¶	No. of testes with donor cells (%)‖
1	C57BL/6	Coat color	*W/W*	Sterile by mutation	22	4 (18)
2	ZFlacZ	*lacZ*	Busulfan	Sterile by treatment	104	38 (37)

*C57BL/6 mice were C57BL/6 (black), C57BL/6-c^{2J} (albino), or C57BL/6 (tan belly). The tan belly phenotype arose as a spontaneous dominant mutant in our colony and is similar to the commonly known black and tan mutation (6). ZFlacZ mice were heterozygous for a transgene (−3.5-kb cEMS 177/*lacZ*) that results in expression of the *E. coli* β-galactosidase gene in round spermatids (12). The two lines of transgenic mice were designated TgN(c177lacZ)226Bri and TgN(c177lacZ)227Bri.

†Coat color genotype of the donor cells was distinguishable from that of the recipient mouse. *lacZ* indicates the presence of the β-galactosidase transgene in donor cells, which allows round spermatids that arise from the transferred cells to be stained blue.

‡*W/W* males were homozygous or compound heterozygous males of the *W* mutant strains *W/W*, W^v/W^v, or W^{44}/W^{44}. Busulfan-treated males were (C57BL/6 × SJL)F_1 hybrids.

§*W/W* males do not show spermatogenesis in their testes. Busulfan males were treated with busulfan (40 mg/kg) at 4 weeks, which destroys spermatogenic stem cells in the testes and abolishes spermatogenesis (9, 10).

¶Testes of *W/W* mice were injected with donor cells when males were 4 weeks of age or older. Testes of busulfan males were injected with donor cells 4 weeks or more after treatment with busulfan.

‖Testes of recipient males were examined 48–230 days after injection of donor cells.

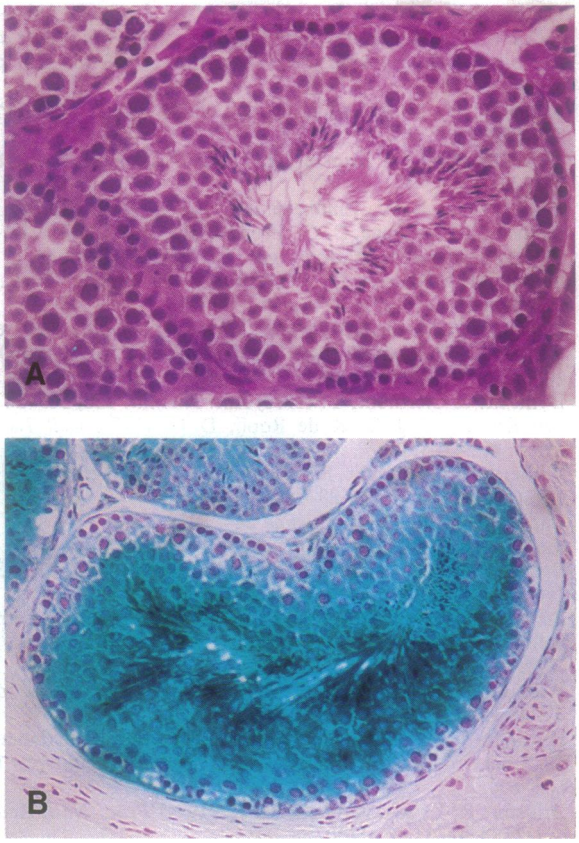

FIG. 5. (*A*) Spermatogenesis in the testes of a *W/W* mouse following injection of donor cells isolated from the testes of C57BL/6 males. The testes of control *W/W* mice do not show spermatogenesis (Fig. 2*B*). Note normal architecture of seminiferous tubule with correct association of various spermatogenic stages and mature spermatozoa. (Hematoxylin and eosin, ×300.) (*B*) Spermatogenesis in the testes of a busulfan-treated C57BL/6 × SJL male following injection of ZFlacZ donor cells. The testes of mice treated with busulfan (40 mg/kg) do not show spermatogenesis (Fig. 2*C*). Furthermore, the blue color of the spermatogenic stages positively identifies them as arising from the donor cells. Note normal spermatogenic process and mature spermatozoa. (X-Gal followed by neutral fast red; ×210.)

of *W/W* mice lack any mature stages of spermatogenesis (Fig. 2*B*).

A greater number of testes of busulfan-treated mice were injected, because this class of recipients could be generated at will. Colonization of recipient testes was found in 38 of the 104 testes that received donor cells. In tubules colonized by transferred cells, the organization of the spermatogenic stages within the seminiferous tubules was normal and mature spermatozoa were produced (Fig. 5*B*). Mice treated with busulfan (40 mg/kg) normally do not contain mature stages of spermatogenesis (Fig. 2*C*). Because the donor cells in this protocol carry a transgene that produces β-galactosidase in round spermatids, the mature spermatogenic stages at the center of the seminiferous tubule stained blue following treatment with X-Gal (Fig. 5*B*). When staining was intense, the blue color diffused to immature stages as well. Endogenous stem cells not destroyed by busulfan (e.g., because of chemical leakage at the time of injection) would not stain. Thus, for protocol 2, the busulfan recipients were more readily available than *W/W*, they carried no mutant alleles related to spermatogenesis, and donor cells could be unequivocally identified within seminiferous tubules.

The percentage of successful transfers for protocol 2 was twice that found for protocol 1. Both the donor cells and recipient testes were from inbred mice in protocol 1, whereas the cells and testes were of hybrid origin in protocol 2. The significance of this difference is difficult to establish, because of the small number of animals available in protocol 1. Considering both protocols together, one-third of the testes injected showed colonization by donor cells. In those testes in which donor stem cells proliferated, 1–20 tubules demonstrated some degree of spermatogenesis. The number of tubule cross sections visible in a testis ranged from 150 to 300 depending on the plane of section. Therefore, up to 10% of the tubules were colonized in the best cross sections.

These studies demonstrate that stem cells can be harvested from donor testes, maintained *in vitro* for several hours, transferred to a sterile recipient testis, and subsequently establish normal spermatogenesis with the production of mature spermatozoa [see ref. 25 (the following paper in this issue)]. A remarkable aspect of the colonization that was observed was the faithful reconstruction of the complex cellular associations found in normal spermatogenesis (1, 2, 14). This was achieved despite the dissociation and manipulation of the cell suspension that occurred during the process of isolation and transfer. The most reasonable explanation is that individual spermatogonia with stem cell potential establish themselves on the basal membrane of the recipient seminiferous tubule and begin to replicate, thereby providing for both renewal of the stem cell population and production of a subset of progeny that differentiate into spermatozoa (3, 4, 15, 16).

The technique described above should allow a detailed examination of the ability of various testicular cell populations to reestablish spermatogenesis. Methods are available for separation of seminiferous tubule cell populations by sedimentation velocity (5), and an antibody specific for mouse germ cell nuclear antigen has recently been described (8). Combining these two procedures with microscopic evaluation of isolated pools of cells should allow subgroups of spermatogonia to be identified and isolated. Reintroduction into recipient testes of small numbers of these cells could then be undertaken by either protocol 1 or 2. The number of cells needed to identify a population as having stem cell capability should be small, since a single spermatogonial stem cell is theoretically capable of forming 4096 spermatids, and clones of as many as 650 spermatids have been identified (1). Thus, the clonal expansion of individual stem cells should be easily identifiable (1, 15, 16).

While studies to improve the efficiency of testis repopulation by donor cells should be pursued, an obvious next step is the development of a culture system for stem cell spermatogonia. Under appropriate physiological cues, these stem cells redirect their activity in favor of self-renewal over the initiation of spermatogenesis; this occurs in response to an insult which depopulates the testis, and the biological aim is repopulation (15–17). Simulating those cues should favor the proliferation of stem cell spermatogonia in culture.

It is useful to compare spermatogonial culture and transfer with the current system, which utilizes embryonic stem cells to introduce germline changes. If spermatogonia can be cultured and manipulated—e.g., via targeted homologous recombination of DNA sequences—and individual modified clones of cells can be selected in a manner similar to embryonic stem cells (18, 19), then these cells could be used to create mice with germline modifications. However, while embryonic stem cells are returned to a blastocyst to colonize the embryo, cultured spermatogonia would be returned to the testis. Use of preimplantation embryos, for embryonic stem cell work as well as direct DNA microinjection into fertilized eggs (20, 21), can be prohibitively expensive and difficult, particularly with larger animals (22–24). Testicular cell transfers might ultimately provide an alternative approach, and stem cell spermatogonia could thus become a valuable biological tool.

The technique of spermatogonial transfer clearly allows normal spermatogenesis following transfer of testis stem cells and provides the first step in the development of methods to utilize this remarkable population of cells. The range of possible experimental uses of this technique certainly extends beyond those mentioned above and is likely to include a number of areas in biotechnology and transplantation biology.

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