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Germline transmission of donor haplotype following spermatogonial transplantation

(testis/stem cells/spermatogenesis/transgenic mice/fertility)

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ABSTRACT **Spermatogenesis is a complex, highly organized, very efficient process that is based upon the capacity of stem cell spermatogonia simultaneously to undergo self-renewal and to provide progeny that differentiate into mature spermatozoa. We report here that testis-derived cells transplanted into the testis of an infertile mouse will colonize seminiferous tubules and initiate spermatogenesis in >70% of recipients. Testis-derived cells from newborn mice were less effective in colonizing recipient testes than cells from 5- to 15- or 21- to 28-day-old mice. Increasing the number of Sertoli cells in the donor cell population did not increase the efficiency of colonization. Unmodified embryonic stem cells were not able to substitute for testis-derived cells in colonizing testes but instead formed tumors in syngeneic as well as nonsyngeneic hosts. Finally, with recipients that maintained endogenous spermatogenesis, testis cell transplantation yielded mice in which up to 80% of progeny were sired by donor-derived spermatozoa. The technique of spermatogonial cell transplantation should provide a means to generate germline modifications in a variety of species following development of spermatogonial culture techniques and should have additional applications in biology, medicine, and agriculture.**

Mature spermatozoa represent the product of a long developmental process that begins with differentiation of male primordial germ cells thought to arise from embryonic ectoderm (epiblast) in the 7-day-old mouse embryo (1, 2). During migration to the genital ridge, the number of primordial germ cells increases from about 100 on fetal day 7 to ≈25,000 on fetal day 13.5 (3, 4). Soon after arriving in the genital ridge, male germ cells stop dividing and remain quiescent within the seminiferous tubules of the fetal testis until several days after birth. During this period they are referred to as gonocytes (5, 6). Following birth the gonocytes divide and differentiate into spermatogonia, which then begin the complicated process of spermatogenesis. The spermatogonia, which form the basal layers within the seminiferous tubule, have been classified into three principal types: stem cell spermatogonia, proliferative spermatogonia, and differentiating spermatogonia (5, 7). The first two types appear to undergo self-renewal, and the proliferative spermatogonia simultaneously provide a population of differentiating spermatogonia, which are irreversibly committed to undertake the steps resulting in production of mature spermatozoa. During this process, spermatogonia undergo extensive replication, and most of the increase in cell number that characterizes spermatogenesis occurs at this time (7). Ultimately the spermatogonia form primary spermatocytes, which initiate the meiotic process and produce round spermatids. Round spermatids, in turn, undergo spermiogenesis, yielding the morphologically and functionally distinctive mature spermatozoa. Throughout this process, differentiating cells are supported by Sertoli cells and form highly ordered, characteristic cellular associations within the seminiferous tubule (6–8). In the mouse, the time from spermatogonial differentiation to production of mature spermatozoa is 35 days, with roughly one-third of the time in spermatogonial mitosis, one-third in meiosis, and one-third in spermiogenesis (5–7).

Considering the high efficiency of sperm production (on the order of 10^7 spermatozoa produced daily in the rat per gram of testicular tissue; ref. 9) and the fact that this process is active from puberty to old age, spermatogenesis is truly a remarkable phenomenon. This process is based on the stem cell spermatogonia. These cells undergo self-renewal throughout life, and because of their ability to transmit genes from one generation to the next they represent the only replicating potentially totipotent population of cells in the adult body and may in this sense be thought of as immortal (1). To study the function of these cells during spermatogenesis and to establish a system to exploit their unique ability to transmit genes to successive generations (10), we have developed a technique to transfer cells from donor testes into an infertile recipient testis. We report here that spermatogonia appear more efficient than gonocytes in repopulating a recipient testis and that progeny result from transplanted donor spermatogonia.

MATERIALS AND METHODS

Cell Preparation. Male germ cells were isolated from mice of three different ages: between embryonic day 18 (E18) and postnatal day 2 (P2), between P5 and P15; and between P21 and P28. The basic procedures used have been described (11). Several modifications were introduced to increase the number and improve the handling of isolated cells. In brief, 12–40 testes were collected (the larger numbers from the youngest animals), the tunica was removed, and the exposed tubules were subjected to collagenase (1 mg/ml) treatment followed by trypsin (0.25%) digestion. The released cells were centrifuged at 600 × *g* at 16°C for 5 min. After centrifugation, the supernatant was removed and the cells were suspended in injection medium. The composition of the medium was based on a formula originally developed for mouse eggs (12) and subsequently modified for use with primordial germ cells (13). These cell types have a requirement or preference for pyruvate and lactate as energy substrates, and male germ cells may share this characteristic because of their origin from primordial germ cells. The composition of the injection medium was 132 mM NaCl/7.8 mM Na_2HPO_4/2.6 mM KCl/1.1 mM KH_2PO_4/0.1 mM EDTA/0.25 mM pyruvate/3 mM lactate/1 mM glutamine/5.5 mM glucose/0.5% bovine serum albumin with DNase I

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Abbreviations: E*n*, embryonic day *n*; P*n*, postnatal day *n*; *W*/*W*, homozygous and heterozygous *W* mice; X-Gal, 5-bromo-4-chloro 3-indolyl β-D-galactoside.

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(200 μg/ml) at pH 7.4. The cells were resuspended in 1.6 ml of the injection medium, and aliquots (400 μl) were transferred to microcentrifuge tubes on ice. Cell concentration values for different-age testes were determined on samples of cells collected in an identical manner on days when injections were not performed. There was variation in cell concentration from day to day resulting from the number of testes available and the age of the donor males. An attempt was made to maximize the number of cells available in order to inject a concentrated solution, since the number of stem cells was unknown but thought to be small. One tube containing 400 μl was used to inject the testes of a single mouse. Before the cell suspension was introduced into the injection pipette, 50 μl of filtered (4%) trypan blue was added to the tube and the suspension was agitated. The dye allowed visualization of solution flow in the seminiferous tubule and was used to determine the number of dead cells in the injection suspension, which was generally <5%. It was not possible to measure the volume of cell suspension that entered the tubules, but the percent of surface tubules that filled with solution was recorded for each recipient testis.

Sertoli cells were isolated and maintained as described (14). They were added to the germ cells just before injection into the tubules. Embryonic stem cells (AB-1) were a gift from Alan Bradley and were maintained as described (15, 16).

Transplantation Procedure. Two separate protocols were used to transfer donor cells into recipient mice. In protocol 1, donor cells were isolated from the testes of C57BL/6 mice that were homozygous for a dominant mutant that resulted in tan belly hair. These cells were transferred into testes of compound heterozygous or homozygous mutant *W* mice (designated *W/W*), which do not show spermatogenesis (reviewed in ref. 17; R.L.B., unpublished data). In protocol 2, donor cells were isolated from mice (designated ZFlacZ) that were heterozygous for an *Escherichia coli* β-galactosidase transgene (*lacZ*), which allowed round spermatids to be stained blue following incubation with substrate (18). Donor cells from these mice were injected into testes of (C57BL/6 × SJL)F_1 males that had been treated with busulfan (40 mg/kg, intraperitoneally) at 4–6 weeks of age. This treatment destroys spermatogenic stem cells (19).

Cell Injection. To minimize the number of injection sites and to increase the efficiency of injecting concentrated cell suspensions, a glass pipette with a 1-mm outside diameter and a 40-μm tip was secured in a micropipette holder [WPI Instruments (Waltham, MA) catalogue no. MPH6S]. The tip of a 1-ml plastic syringe was then inserted into the other end of the micropipette holder, and the syringe was cut at the 0.4-ml mark. The cell solution containing the dye was deposited in the syringe barrel, which was then screwed onto the metal end of an Eppendorf capillary holder attached by tubing to a pressure injector (Eppendorf model 5242). To expose the tubules, only one to three small incisions (1–3 mm) were made in the tunica per testis. With these modifications, an average of 80% of the surface tubules could be filled with concentrated cell suspension (Table 1).

Analysis of Recipient Testes. To allow donor cells to undergo at least one cycle of spermatogenesis, recipient males were maintained a minimum of 50 days following injection before sacrifice. In protocol 1, the testes were fixed and 5-μm microscopic sections cut and stained with hemotoxylin and eosin. The number of tubule cross sections showing any stages of spermatogenesis was recorded. In protocol 2, the testes were incubated with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal), and the number of tubules that stained blue recorded, up to a maximum of 12; beyond this, it was difficult to identify individual tubules because of their tortuous course, and these testes were recorded as 12+. For some mice in protocol 2, microscopic sections were cut and stained with neutral fast red. The number of tubule cross sections that stained blue generally was greater than the number of tubules visibly blue on gross examination. Progeny of recipient males (protocol 2 only) were analyzed either by incubating testes with X-Gal or by assaying for the presence of the transgene (18).

RESULTS

Donor cells were collected from mouse testes at three representative developmental stages. The first, between E18

Table 1. Spermatogonial stem cell transplantation into recipient testes

Donor cell age, days	Concentration of donor cells,* no. × 10^{-6}	Donor cell strain†	No. of testes injected	Percent surface area covered‡	Time to analysis,§ days	No. of testes with donor cells¶ (%)	No. of tubules with donor cells‖
E18–P2	35 ± 16 [10–51]	C57BL/6	46	78 ± 20 [30–100]	81 ± 20 [54–130]	6 (13)	3.5 ± 2.3 [1–7]
		ZFlacZ	40	73 ± 21 [40–100]	81 ± 14 [60–110]	18 (45)	3.2 ± 2.5 [1–9]
P5–15	71 ± 18 [44–108]	C57BL/6	47	80 ± 19 [30–100]	117 ± 64 [70–300]	41 (87)	14.5 ± 16.2 [1–70]
		ZFlacZ	49	80 ± 15 [50–100]	87 ± 37 [60–197]	49 (100)	8.1 ± 4.0 [1–12+]
P21–28	83 ± 32 [45–123]	C57BL/6	11	86 ± 7 [75–95]	99 ± 43 [70–180]	7 (64)	16.7 ± 6.4 [9–26]
		ZFlacZ	52	83 ± 15 [40–100]	80 ± 25 [60–155]	52 (100)	5.1 ± 3.4 [1–12+]

Values are mean ± SD; range is in brackets.

*No. of determinations of cell concentration was six for E18–P2, nine for P5–15, and six for P21–28.

†C57BL/6 testis cells were transferred to the seminiferous tubules of homozygous or compound heterozygous *W/W* mice that lack spermatogenesis (protocol 1). ZFlacZ testis cells from mice of C57BL/6 × SJL genotype were transferred to the seminiferous tubules of (C57BL/6 × SJL)F_1 hybrid mice in which endogenous spermatogenesis had been destroyed by busulfan treatment (protocol 2). ZFlacZ mice were heterozygous for a transgene (−3.5-kb cEMS177/lacZ), and the transgenic lines were designated TgN(c177lacZ)226Bri and TgN(c177lacZ)227Bri (18).

‡Percent of the surface seminiferous tubules in the recipient testis filled by the injected cell suspension.

§No. of days from injection of donor cells to analysis of the recipient testis for presence of spermatogenesis. This represents the time available for donor cell proliferation.

¶Effects of donor cell age and donor cell strain (C57BL/6 vs. ZFlacZ) were both significant by analysis of variance ($0.05 > P > 0.01$).

‖No. of tubules with spermatogenesis in those recipient testes that had evidence of donor cell colonization. In C57BL/6 donor cell recipients, the tubule cross sections with evidence of spermatogenesis were counted. In ZFlacZ donor cell recipients, the individual tubules stained blue were counted up to a maximum of 12. Tubules in testes with >12 stained could not be accurately counted. Therefore, in this column the values for C57BL/6 cells are not directly comparable to those for ZFlacZ cells. The difference between the C57BL/6 values for the E18–P2 age group and the other two groups was significant ($P < 0.001$). The difference between the ZFlacZ values for the E18–P2 age group and the P5–15 age group was significant ($P < 0.001$), and the difference between the E18–P2 age group and the P21–28 age group was also significant ($0.02 > P > 0.01$) (analyses by *t* test). The ZFlacZ statistical comparisons underestimate the true difference, because in testes with 12+ tubules staining, the exact number of tubules with spermatogenesis was too great to count accurately.

and P2, provided primarily gonocytes and mitotically active Sertoli cells. The second, between P5 and P15, provided germ cells and Sertoli cells in transition between the perinatal and adult stages. The third, between P21 and P28, provided quiescent Sertoli cells and a germ cell distribution approaching that of mature testes. The average time from cell transplantation to analysis was ≈90 days, with a range of 54–300 days (Table 1). This represented 2–10 spermatogenic cycles. In total, 245 testes were injected with donor cells, of which 173 (71%) showed evidence that transplanted cells had survived, colonized recipient seminiferous tubules, and initiated spermatogenesis (Table 1). Cells isolated from perinatal testes (E18 to P2) colonized 24 of 86 injected testes, with an average of 3 to 4 tubules (out of 150–300 visible in a cross section) showing evidence of donor cell spermatogenesis. Donor cells isolated from P5 to P15 and P21 to P28 displayed a greater ability to colonize recipient testes: 90 of 96 and 59 of 63 testes, respectively, showed evidence of donor cell spermatogenesis. Donor cell age and strain (protocol 1 vs. protocol 2) both had a significant effect on the number of testes colonized (Table 1). The number of tubules supporting spermatogenesis for P5–15 and P21–28 cells was also significantly greater than for the perinatal cells. Although the concentration of donor cells injected was lower for suspensions from perinatal animals, reflecting the small size of donor testes, the percent of surface tubules filled with cell suspension (≈80%) and the percent live cells, as indicated by trypan blue, were similar for all three donor-stage cell preparations.

After colonization of recipient seminiferous tubules, donor cells generally established a morphological pattern representative of normal spermatogenesis. For protocol 1, all stages of sperm cell differentiation could be identified in microscopic cross sections of the seminiferous tubules. None of these stages was seen in control testes not injected with cells, because there is no endogenous spermatogenesis in *W* mice or in tubules of injected testes not populated with donor cells. Likewise, ZFlacZ donor cells (protocol 2) established normal spermatogenesis in busulfan-treated recipients with typical sperm cell associations and morphology of the tubules, and mature spermatozoa were produced (Fig. 1*A*). The testes of mice injected with busulfan but not receiving donor cells do not generally have tubules with spermatogenesis (ref. 19; R.L.B., unpublished data). In mice receiving a lower dose of busulfan (e.g., when there was leakage at the time of injection), some tubules could be repopulated by surviving endogenous stem cells. However, these tubules would be distinct, since round spermatids and subsequent stages derived from endogenous stem cells do not stain blue with X-Gal. The success of the transferred donor cells in colonizing the recipient testis varied, and some testes from ZFlacZ mice demonstrated up to 100 cross sections containing various stages of spermatogenesis. In testes with the most extensive colonization, by either C57BL/6 or ZFlacZ donor cells, mature spermatozoa were transported to the epididymis (Fig. 1*B*). No sperm were seen in epididymal tubules when recipient testes were not colonized by donor cells.

As anticipated from the observation of spermatozoa in the epididymides of some experimental mice, spermatozoa could also be identified in the ejaculate of these recipient males. Initially, the number of spermatoza was insufficient to result in progeny (by normal mating). An approach to facilitate production of offspring from donor-derived spermatozoa would be to provide carrier spermatozoa. As a consequence of occasional busulfan leakage at the time of injection or variability of individual animal susceptibility, a few experimental recipients may acquire fertility following tubule repopulation by endogenous stem cells. If donor cells have also become established, a fraction of tubules with spermatogenesis will stain blue (Fig. 2), and the ejaculate will contain

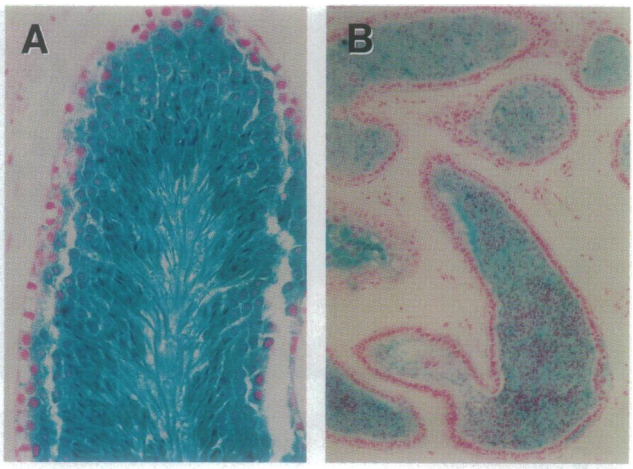

FIG. 1. Spermatogenesis in the testis of a recipient male following transfer of ZFlacZ donor cells. (*A*) Testis from (C57BL/6 × SJL)F_1 busulfan-treated male 6 months after donor cell transfer. Note active spermatogenesis and mature spermatozoa. Round spermatids and more mature stages stain blue. When stain is intense, immature stages also appear blue. (*B*) Tubules from epididymis of the mouse with testis shown in *A*. Many mature spermatozoa appear as red dots in the lumen of the tubules. Background blue color in the lumen is the result of β-galactosidase activity arising from the cytoplasm of immature sperm cells. Mature spermatozoa do not stain for β-galactosidase because they lack sufficient cytoplasm. (X-Gal followed by neutral fast red; *A*, ×125; B, ×55.)

spermatozoa derived from endogenous and transplanted stem cells. Half the male progeny that develop from eggs fertilized with spermatozoa of ZFlacZ donor stem cell origin should have testes that stain blue with X-Gal, because transferred ZFlacZ cells are heterozygous for the transgene. Testes from progeny of a recipient in which spermatogenesis occurred from endogenous and transplanted stem cells are shown in Fig. 3; however, only 1 of 122 male offspring carried the transgene. To specifically generate recipients that would reestablish endogenous spermatogenesis, protocol 2 was used; 10 males were treated with busulfan at 30 mg/kg, and their testes were injected with donor cells from P5–15 or P21–28 mice. Testes of 7 recipients were examined 65–290 days after injection. All were enlarged relative to animals treated with busulfan at 40 mg/kg, indicating a significant level of spermatogenesis. The testes contained 8.2 ± 4.0 (mean ± SD) blue-staining tubules. Of the 3 remaining males, 1 that received P5–15 cells is still infertile, 1 that received P21–28 cells has sired 6 of 39 transgenic progeny, and the third, which received P5–15 cells, has sired 6 of 15 transgenic progeny. Offspring were born beginning ≈8 months after donor cell injection. With this approach, up to 80% of the progeny of the male with the most successful colonization are from donor-derived spermatozoa, half of which carry the transgene.

Although injected cell suspensions contained donor Sertoli cells, several experiments were performed in which Sertoli cells (10^5–10^7) isolated as described were added to the donor cell suspension to determine whether this would enhance repopulation. With protocol 1 and donor cells from P5–15 mice, 10 of 12 recipient testes demonstrated spermatogenesis, with 13.0 ± 12.7 (mean ± SD) tubule cross sections colonized per testis. With protocol 2 and the same age donor cells, 24 of 25 testes had stained tubules with 9.0 ± 3.7 tubules stained per testis. The average time of analysis of the recipients after donor cell injection was 112 days. In general, the degree of colonization with and without additional Sertoli cells was similar (compare with Table 1).

Embryonic stem cells are closely related or identical to embryonal carcinoma cells, which arise from male primordial

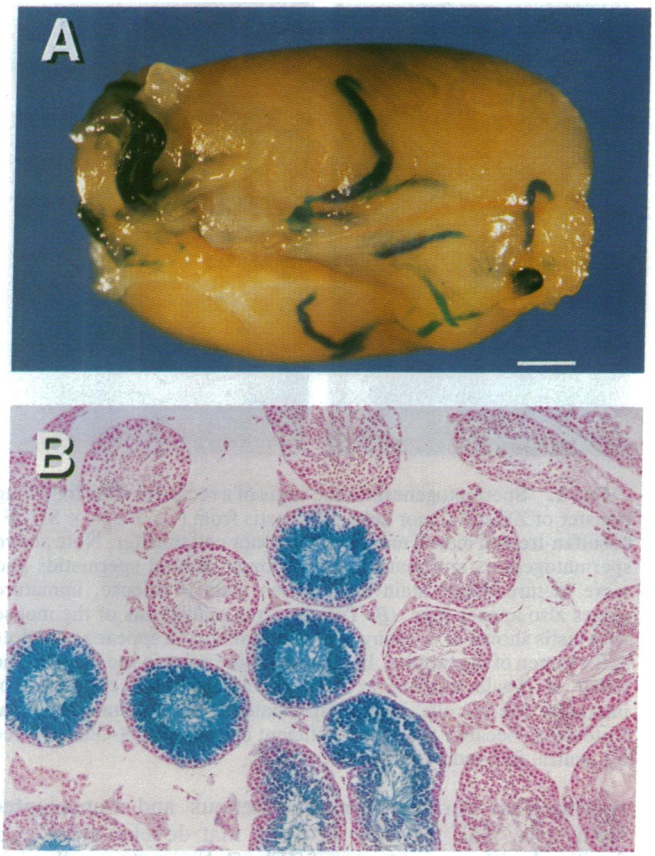

FIG. 2. Testis of busulfan-treated recipient male injected with ZFlacZ donor cells 14 months previously. (*A*) Gross appearance. The testis is ≈80% of normal size, because an inadequate busulfan effect permitted the reinitiation of spermatogenesis from endogenous stem cells (unstained tubules). Blue tubules indicate spermatogenesis from ZFlacZ donor spermatogonial stem cells. The testis has been partially transected longitudinally to allow for penetration of fixative and stain (X-Gal). (Bar = 1 mm.) (*B*) Microscopic appearance. Blue tubule cross sections represent spermatogenesis from donor stem cells. Red tubule cross sections reflect spermatogenesis from endogenous stem cells. The ratio of blue to red tubules across several complete testis cross sections in this area was ≈1:12. Other areas of the testis have fewer blue tubules. (X-Gal followed by neutral fast red; ×30.)

germ cells (1). Both types of stem cells, as well as primordial germ cells, will colonize blastocysts and differentiate into all cell types of the body, including male germ cells (1, 15, 20). Therefore, the ability of embryonic stem cells to become spermatogonia was examined by injecting embryonic stem cells (AB-1; ref. 15) into the seminiferous tubules of *W/W* and *W/W*^{54} mice. The *W*^{54} allele is carried on the 129/SvCP genetic background, from which AB-1 cells arise, and therefore the *W/W*^{54} cross would be immunologically tolerant to AB-1 cells. The *W/W* mice are of C57BL/6 genotype and would not be tolerant to AB-1 cells. Cell suspensions (15–60 × 10^6 cells per ml) were injected (0.4 ml per mouse) into the tubules of 10 *W/W*^{54} recipients to determine whether any spermatogenesis would develop. All of the mice died or had large testicular and/or abdominal tumors within 30 days of injection. In similar experiments, 9 *W/W* mice were injected with AB-1 cells. One of these animals died, and the remainder were examined between 24 and 130 days after injection. All but one of these animals developed tumors of the testes and/or in the abdominal cavity. Regardless of whether the testes were examined soon after injection (30 days) or at a later time (130 days) there was no sign of spermatogenesis, even in tubules not affected by tumorigenesis. Subcutaneous

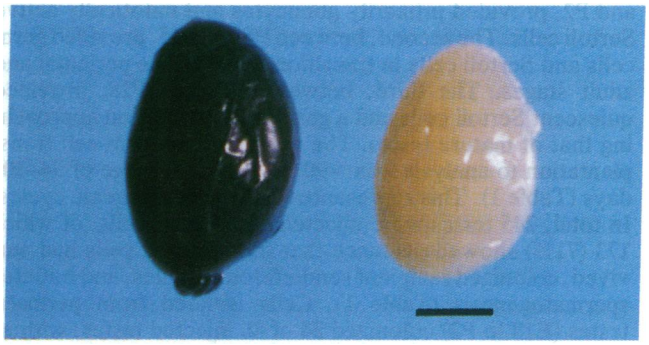

FIG. 3. Testes from progeny of the male described in Fig. 2. The testis on the left stained blue with X-Gal, indicating the presence of the transgene in all cells. The testis on the right is from another offspring and is not blue because it does not contain the transgene. The seminiferous tubules of the parent male displayed a mosaic pattern of staining resulting from donor stem cell and endogenous stem cell areas of spermatogenesis (see Fig. 2). Progeny was ≈10 weeks old, born 11 months after donor cell transfer. (Bar = 0.25 cm.)

injection of AB-1 cells (2 × 10^6 cells) produced tumors in 8 *W/W*^{54} mice in 30 days but not in 7 *W/W* mice in 60–90 days, indicating that the *W/W* mice were resistant to tumor formation from these cells by this route of administration.

DISCUSSION

The studies described above demonstrate that stem cells can be harvested from donor testes, maintained *in vitro*, transferred to a recipient testis, establish normal spermatogenesis, and produce functional spermatozoa that fertilize eggs and result in offspring. The production of young from the transplanted spermatogonial stem cells and the high percentage of recipient spermatozoa arising from the transplanted cells indicate that colonization of the seminiferous tubules can be very effective. Future efforts should increase the percentage of recipient males with high-level colonization and thereby increase the usefulness of spermatogonial transplantation.

In these studies, donor cell populations were mixed, containing Sertoli cells and various stages of differentiating sperm cells that varied with the age of the donor testes (5, 6, 11). Among this diverse group of cell types, only cells with stem cell potential could initiate spermatogenesis. Spermatocytes, meiotic stages, and spermatids are not capable of self-renewal. Thus, even if donor cells representing later stages of spermatogonia established an interaction with recipient Sertoli cells and continued to differentiate, they would become fully mature and be shed into the lumen by 35 days after transfer (6, 7). Spermatogenic elements visible in recipient testes after this time would necessarily have arisen from donor cells with stem cell potential.

The age of the testes from which donor cells were isolated had an influence on the efficiency of colonization of recipient testes. At birth the germ cells are gonocytes, but during the first week of life these cells differentiate into spermatogonia, which divide and begin to differentiate (5, 6). Thereafter, the spermatogonial cell population expands and differentiating stages of spermatogenesis appear successively. In prepubertal mice this first wave of spermatogenesis is synchronized, with spermatocytes appearing at about day 10, spermatids at about day 20, and mature spermatozoa at about day 35 (6, 11). Sertoli cells also undergo changes during this period. They continue to divide until between P10 and P14, after which they become mitotically quiescent (8). Thus, by recovering donor cells at birth, P5–15, and P21–28, three different mixed cell populations were tested for stem cell potential. Surprisingly, the results suggest that gonocytes, despite their primitive stage of development, were less efficient as donor cells

than older populations of cells containing spermatogonia. However, it has not been determined whether the stem cell populations in the three developmental stages tested have a differential sensitivity to the harvesting protocol employed. Although the number of dead cells as indicated by trypan blue staining was similar for the three cell preparations, differential killing of small populations of stem cells may not have been detectable. Also, stem cells are likely to make up a different fraction of the total cell population at each stage (5–7). These factors may also account for the lower efficiency of C57BL/6 donor cells in colonizing recipients. However, an additional element in this effect could be an inferior ability of the tubules in *W* recipients to support spermatogenesis.

Spermatogenesis was morphologically normal in many tubules examined (7), indicating effective colonization of seminiferous tubules. The donor cell population contained both germ cells and Sertoli cells; therefore, it could not be determined whether the interacting Sertoli–spermatogonial units that established a spermatogenic colony and repopulated the tubule were composed of donor germ cells interacting with endogenous Sertoli cells, with transferred Sertoli cells, or both. However, when additional Sertoli cells were coinjected with germ cells they did not have a dramatic effect on colonization efficiency. This suggests that Sertoli cells were not a limiting factor and may indicate that endogenous Sertoli cells were adequate to support recolonization.

Because of the close relationship of embryonic stem cells, embryonal carcinoma cells, and primordial germ cells, including their ability to differentiate into many tissue types (1, 15, 20), we examined the effect of the seminiferous tubule environment on their differentiation pathway. Under these experimental conditions, and in contrast to the blastocyst, the tubule environment was not able to control the growth and differentiation pathway of AB-1 cells. *In vitro* manipulation of embryonic stem cells may be required before this cell type can be directed to differentiate appropriately within the tubule microenvironment (10).

The most striking result of these experiments was production of offspring from donor cell-derived spermatozoa. The ability to achieve an effective level of seminiferous tubule colonization and to produce efficiently donor cell-derived progeny will facilitate a wide range of experimental approaches using spermatogonial transplantation. The opportunity now exists to develop a culture system that allows expansion of spermatogonial stem cells and selection of specifically modified clones of individual cells; spermatogonial culture could not be developed without a technique to test the biological potential of cell populations isolated. A variety of agents (e.g., growth factors) can also be examined to ascertain their ability to modify the function of donor cells and recipient tubules. An important extension of spermatogonial transplantation will be repopulation of testes of immunotolerant mice with donor cells from other species. In a properly prepared recipient, xenogeneic donor cell-derived spermatozoa may be matured along with endogenous spermatozoa in the epididymis, and the egg of the foreign species would provide the specificity of spermatozoal selection during *in vitro* or *in vivo* fertilization. The chicken will be an important species with which to examine the feasibility of this approach, since even testicular spermatozoa placed in the chicken oviduct are capable of fertilizing the egg (21).

Production of offspring from spermatogonia transplanted to a recipient testis establishes a method to study and utilize these remarkable stem cells. Since spermatogonia constitute the only self-renewing population of cells in the adult body capable of contributing to succeeding generations, the ability to manipulate these cells *in vitro* before transfer to a recipient will represent a challenge and an opportunity to scientists interested in spermatogenesis, infertility, embryo development, and germline modifications.

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