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## **Irradiated Mouse Testes Efficiently Support Donor Spermatogenesis from Mice and Rats**

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**Abstract**

Testicular cell transplantation has been widely used to investigate the biology of spermatogonial stem cells, production of transgenic animals, and restoration of fertility in rodent models. One critical step in successful transplantation is the preparation of the recipient testes. Busulfan has been widely used, but irradiation has been often suggested as an alternative. There have only been limited reports of the use of irradiated animals as recipients for studying differentiation of transplanted cells and there has been no direct comparison of irradiation and busulfan as preparation methods. Mouse testes, given local 1.5 + 12-Gy fractioned irradiation, were compared to busulfan-treated testes as recipients using mouse-to-mouse and rat-to-mouse germ cell transplantation. The fractioned irradiation schedule resulted in depletion of endogenous spermatogenesis similar to that produced by busulfan doses of 50-55 mg/kg. When immature mouse or rat testicular germ cells were transplanted into the irradiated testes, donor cells derived from either rat or mouse spermatogonial stem cells were able to form colonies of differentiated spermatogenic cells 10-13 weeks after transplantation with similar efficiencies as in busulfan-treated testes. Locally irradiated testes could be considered as an alternative to busulfan for recipients of germ cell transplantation in animals that cannot endure the systemic toxicity of busulfan.

**Keywords:** irradiation, busulfan, testis, spermatogonial transplantation

## ***Introduction***

In the past ten years, study of the mechanisms and regulation of spermatogenesis has progressed, in part through the technique of testicular germ cell transplantation (Brinster, 2002). The technique provides an in vivo functional assay of spermatogonial stem cells and has been  
25 employed in distinguishing defects caused by intrinsic function of spermatogonial stem cells and the extrinsic environment; it is useful also in producing transgenic animals (Nagano et al, 2001a) and restoring fertility (Ogawa et al, 2000). So far, these achievements have been limited mainly to the mouse model, and to a limited extent the rat (Jiang and Short, 1995; Clouthier et al, 1996; Ogawa et al, 1999b). Application of male germ cell transplantation to other species would have  
30 application to clinical infertility, production of transgenic animals of economic importance, and preservation of endangered species. However, the repeated failure of mouse recipient testes to support donor spermatogenesis from nonrodents (Dobrinski et al, 1999; Dobrinski et al, 2000; Reis et al, 2000; Nagano et al, 2001b; Izadyar et al, 2002) has hampered these applications. Donor spermatogenesis was supported in goat-to-goat transplantation (Honaramooz et al, 2003),  
35 but the efficiency was low, probably because the testes of the prepubertal animals used as recipients were not depleted of endogenous germ cells.

A critical factor in male germ cell transplantation is the preparation of recipients (Ogawa et al, 1999b; Brinster et al, 2003). Maximal depletion of endogenous germ cells and emptying of stem cell niches for donor cells, with minimal damage to the local spermatogenic and systemic  
40 environment, is required. Donor stem cell engraftment and spermatogenesis were more successful in recipient testes treated to ablate endogenous stem cells than they were in untreated testes (Shinohara et al, 2002).

Ablation of endogenous stem cells can be done with genetic mutations or chemical or physical treatments. Mutant mice such as W/W<sup>v</sup> (Ohta et al, 2003)) provide a supportive  
45 environment for donor spermatogenesis (Ogawa et al, 2000) and have been widely used as recipients, but they must be immunocompatible with the donors (Kanatsu-Shinohara et al, 2005) and the infertility of these mice makes their production difficult (Brinster and Avarbock, 1994); no comparable genetic mutations are available in other species.

The sole effective chemical treatment used so far to prepare recipients is busulfan. Doses  
50 of busulfan of less than 40 mg/kg given to adult mice did not result in prolonged depletion of endogenous spermatogenesis in most tubules (Kanatsu-Shinohara et al, 2003b); higher doses often caused severe hematopoietic suppression requiring bone marrow transplantation, or ending in death (Ogawa et al, 1999a). Prenatal exposure to busulfan by treating the mother has also been done, but the dose had to be reduced to avoid pregnancy failure and endogenous spermatogenesis  
55 recovered in these mice (Brinster et al, 2003). In rats, busulfan is more toxic (Sternberg et al, 1958), and the therapeutic index is lower. When busulfan was given in a fractionated regimen to avoid severe systemic toxicity (Ogawa et al, 1999b; Zhang et al, 2003), endogenous spermatogenesis recovered in most of the tubules (Jiang, 1998); but when a high single dose was given to cause prolonged depletion of spermatogenesis in most tubules, bone marrow  
60 transplantation was required (Udagawa et al, 2001). Data on the effects of busulfan on spermatogonia in other species are limited (Stellflug et al, 1985; Anserini et al, 2002) and the doses used were close to lethal; in humans busulfan is given only with bone marrow transplantation. Hence, it is unlikely that busulfan would be widely used for recipient preparation in a variety of species.

65 Physical methods for preparing testes as recipients could avoid the systemic toxicity that usually is a problem with busulfan. Although testicular cooling depleted all germ cells from many tubules, no donor spermatogenesis was observed after transplantation into the cooled testes (Zhang et al, 2004). Localized radiation is effective at killing endogenous stem cells in mouse testes (Lu et al, 1980), however reports showing differentiation of transplanted spermatogonia in  
70 the depleted tubules are limited. In one study (Creemers et al, 2002) only two irradiated two mice were recipients in one testis each for transplantation with wild-type mouse spermatogenic cells and normal spermatogenesis was reported in 20-25% of tubules. In another study, transplanted mouse testicular cells colonized and showed differentiation in recipients irradiated with 3 Gy (Giuli et al, 2002), but this dose is too low to deplete a significant number of  
75 endogenous stem cells. The effectiveness of testicular irradiation for preparing recipients to efficiently support differentiation of transplanted spermatogonia has not been yet evaluated. We undertook this study to determine the ability of irradiated mouse testes to serve as recipients of transplantation of mouse and rat spermatogonia and to support the differentiation of the transplanted cells, and to compare the results with those from busulfan-treated recipients.

## 80 **Materials and Methods**

### *Animals*

Adult nude (Swiss *nu-nu*/Ncr) mice bred at the M. D. Anderson Cancer Center were used as recipients. Donors were transgenic mice (Tg (*ACTB-EGFP*)10sb/J) on a C57BL/6 background that express GFP under a chicken  $\beta$ -actin promoter (Okabe et al, 1997) (Jackson Laboratories, Bar Harbor, ME), or transgenic rats expressing GFP under the control of a CMV enhancer and ubiquitin-C promoter (Lois et al, 2002). The GFP-containing rats, originally on a Sprague-Dawley genetic background, were backcrossed to the inbred Lewis strain (Harlan Sprague Dawley, Indianapolis, IN) for 2 or 3 generations; immature offspring expressing GFP from these crosses were used as donors. All animals were caged in a controlled environment at the M. D. Anderson Cancer Center (12h light:12h dark) with unlimited access to food and water. All experiments were approved by the Institutional Animal Care and Use Committee of The University of Texas M. D. Anderson Cancer Center.

### *Preparation of Recipients*

The nude mice were irradiated without anesthesia. They were restrained in plastic chambers (Lucite boxes) localized on a metal shield with a 3-cm diameter hole, so that only the lower abdominal and scrotal areas were irradiated. Radiation was delivered at a dose rate of 5.6 Gy/min using a dual source  $^{137}\text{Cs}$   $\gamma$ -ray unit. To deplete endogenous stem cells by radiation, fractionated regimens are preferred over single-doses because the testes of many strains of mice and other species, in contrast to other tissues, are more sensitive to fractionated radiation (Withers et al, 1974; Meistrich et al, 1984). Preliminary data from our laboratory (G. Wilson and M. L. Meistrich, unpublished data) compared single doses, 2 equal daily fractions, and 4 equal daily fractions on spermatogonial depletion in C57BL/6 and Swiss/Ncr nude mice. Two doses of

8 Gy given 24 h apart were most effective in reducing tubular repopulation without other damage. Since even a small initial radiation dose induces the remaining spermatogonial stem cells to become radiosensitive, others have used an initial dose of 1.5 Gy, followed by a larger dose of 12-16 Gy 24 hours later (Creemers et al, 2002). In this study we used two fractions of irradiation (8+8, 1.5+12, or 1.5+14 Gy) given 24 h apart to mouse testes to determine an optimal dose to deplete endogenous germ cells without severely damaging the various somatic components of the testis.

Busulfan (Sigma, St Louis, MO) was first dissolved in dimethyl sulfoxide (DMSO) (Sigma), then an equal volume of sterile water was added to obtain a final busulfan concentration of 8 mg/ml. The aqueous dilution was maintained at a temperature slightly above 37°C until injection to prevent the busulfan from crystallizing and keep it in solution. Different doses were intraperitoneally injected to determine a dose of busulfan that would produce depletion of endogenous spermatogenesis similar to that of irradiation.

Mice were used for germ cell transplantation 3-5 weeks after either irradiation or injection of busulfan.

#### *Preparation of Donor Cells*

Immature mice (10-20 days old) or rats (10-11 days old) were used as donors. To preferentially harvest single cells from the tubules, the tunica was removed and the tissue was sequentially digested with enzymes at 35°C in a shaking water bath (Zhang et al, 2003). Two digestions were performed in DMEM/F12 medium (Gibco, Carlsbad, CA) containing DNase I (Cat #DN25, Sigma) at 100 µg/ml and 1% fetal bovine serum (FBS, HyClone, Logan, UT) with 0.05% to 0.1% collagenase IV (Cat #4188, Worthington Biochemical Corporation, Lakewood, NJ) for 20-30 min, and then with 0.05% to 0.1% of collagenase and 0.05% of hyaluronidase (Cat #2592,

Worthington) for 20 min. Tubules were finally digested with 0.1% trypsin (Cat #3704 Worthington) and 100 µg DNase I/ml in Dulbecco's PBS (Gibco) containing 1 mM EGTA (Cat # E-4378, Sigma), for 10-15 min. The final pellets of cells were resuspended in DMEM/F12 containing 10% of fetal bovine serum.

130 Trypan blue (Gibco) was added to a final concentration of 0.02%. After determining the cell concentration and viability, the cell suspension was kept on ice until transplantation. The cell viability ranged from 91% to 99% (average 96%) and the concentration ranged from  $3.6$  to  $5.8 \times 10^7$ /ml (average  $4.4 \times 10^7$ /ml).

### *Transplantation*

135 Mice were anesthetized with a mixture of ketamine (6.7 mg/ml) and xylazine (1.3 mg/ml) given at 0.15 ml/10 g body weight. After incision of the lower abdomen and exposure of testis, a glass needle (tip inner diameter 20-25 µm with a 25° angle) was inserted into the efferent duct, and donor cells were injected into rete testes using a FemotoJet® semi-automatic microinjector (Brinkmann Instruments Inc, Westbury, NY). An average of 8 µl (maximum 15 µl, minimum 1  
140 µl) of cell suspension, containing about  $40 \times 10^4$  cells, was injected into each recipient testis. Trypan blue was used as marker to monitor the success of the injection.

### *Macroscopic and Microscopic Assessment of Spermatogenesis*

Mice were killed at different times after irradiation or busulfan treatment, and body and testis weights were recorded. Testes were fixed in Bouin's or 4% paraformaldehyde solution at  
145 4°C and embedded in paraffin or plastic. Sections of 4-5 µm thickness were cut and stained with PAS-hematoxylin for counting germ cells in the seminiferous tubules. All tubules in a cross-section were counted (average, 145 tubules per cross section). The tubule differentiation index

(TDI) is the percentage of tubules showing differentiation, which is defined by the presence 3 or more germ cells in a tubule reaching the B spermatogonia stage or beyond.

150 Recipients were killed 10 weeks after mouse-to-mouse transplantation or 13 weeks after rat-to-mouse transplantation. For recipient testes in which colonies of donor cells were to be counted, the testis was placed in cold PBS containing 0.05% collagenase, the tunica albuginea was removed, and the testis gently teased apart at room temperature within 5-10 minutes, then removed and placed in cold PBS. After counting the number of GFP-positive colonies in the  
155 recipient testes under fluorescence, the testes were fixed in 4% paraformaldehyde solution overnight at 4°C and subjected to routine histologic processing. The other recipient testes were fixed directly in 4% paraformaldehyde solution after the tunica was removed in PBS.

#### *Immunohistochemical Assessment of Spermatogenesis*

After routine dewaxing of paraffin sections and rehydration, slides were boiled for 3 min  
160 in Antigen Retrieval Citra Plus solution (Cat #HK080-9K, BioGenex, San Ramon, CA) on a hot plate and allowed to cool for 1 hour. The sections were treated 5 min with 0.6% hydrogen peroxide (Sigma) to block endogenous peroxidase activity, and for 1 h with 5% bovine serum albumin (Sigma) or serum of the same species as the second antibody to block nonspecific background staining. Serial sections were stained with 2 different primary antibodies; either the  
165 rat anti-mouse monoclonal anti-GCNA1 antibody (1:100 dilution, a gift from Dr George Enders) or the rabbit polyclonal anti-GFP (1:5000 dilution; Cat #NB600-303, Novus Biologicals, Littleton, CO) was added onto tissues and incubated overnight at 4°C. ABC Elite kits, second antibodies, and 3,3'-diaminobenzidine (DAB) were all ordered from Vector Laboratories (Burlingame, CA) and used according to procedures recommended by the manufacturer.  
170 Sections were counterstained with hematoxylin. For immunofluorescence staining, goat anti-

rabbit IgG (Alexa Fluor<sup>®</sup> 488, Molecular Probes Inc, Eugene, OR) was used at a 1:500 dilution. Sections were counterstained with DAPI.

Germ cells were identified following anti-GCNA1 staining and the total TDI was calculated. The numbers of GFP-positive tubules was assessed in histologic sections after anti-  
175 GFP staining. The most advanced stage of GFP-positive differentiated cells was determined in each tubule by histologic criteria. All values were based on counting all tubules taken from 3 sections per testis, at least 25  $\mu\text{m}$  apart from each other.

### *Statistical Analysis*

Any significant differences between irradiated and busulfan-treated groups were  
180 determined using the SPSS statistical package and a Student's *t*-test, unless otherwise specified.

## Results

### *Optimal Doses of Irradiation or Busulfan to Deplete Endogenous Germ Cells*

First, different dose regimens of irradiation (two fractions of 8+8, 1.5+12 or 1.5+14 Gy, given 24 h apart) were given to 3 groups of mice. These mice were killed 5 weeks later for evaluation of the testes (Table 1). All groups showed lower testicular weights than did unirradiated controls ( $101 \pm 3$  mg). Almost all germ cells were depleted in the testes of 1.5+14 Gy-treated mice, and the TDI was only 0.5 %. However, in this group, 5.8% of tubules were calcified (showing loss of epithelial structure and dark-pinkish staining by PAS-hematoxylin) that was similar to our previous observation from other experiments on chemical-treated rats (Meistrich et al, 2003). The other two groups showed similar depletion of spermatogenesis with TDIs of about 6%. Since the percentage of calcified tubules appeared to be slightly lower in the 1.5+12 Gy group (1.7%) than in the 8+8 Gy-group (3.8%) and the 1.5+12 Gy regimen had been used by others to produce recipient mouse testes (Creemers et al, 2002), this dose was used in further studies. The fact that only 6% of tubules showed differentiating cells at 5 weeks after irradiation indicates that at the time of transplantation, endogenous cells were eliminated from most of the stem cell niches, which should enhance colonization by donor cells. The recovery of endogenous spermatogenesis in the 1.5+12 Gy group was examined at later times after irradiation; the TDI increased slightly to 10.6% at 8 weeks and to 52.8% at 18 weeks after irradiation, which corresponds to the time after irradiation when some of the transplanted groups would be killed.

Mice were given 4 different doses of busulfan and compared to another group of mice simultaneously irradiated with 1.5+12 Gy. We were surprised that all nude mice on this outbred genetic background survived the highest dose of 60 mg of busulfan/kg without needing bone

marrow transplants, appeared healthy throughout the experiment, and showed no long-term  
205 reduction in body weight (Table 2). The response to busulfan was much more variable than to  
irradiation. Each group had clear outliers with TDI values of 64% to 100%. Even when these  
were excluded, the coefficients of variation of the TDI values were higher in the busulfan groups  
than in the irradiation group. Clear dose-responses were seen in the reductions of TDI and testis  
weights with increasing busulfan dose. The lowest busulfan dose of 44 mg/kg reduced the TDI to  
210 only about 50% at 5 weeks after treatment, which indicated that stem cell killing was insufficient  
to largely eliminate endogenous spermatogenesis. TDI levels (about 5%) in the testes of most of  
the mice receiving 50-55 mg/kg doses of busulfan were similar to those of the 1.5+12 Gy-  
irradiated testes. None of the busulfan-treated testes had any calcified tubules, unlike the testes of  
8 of the 10 mice irradiated with 1.5+12 Gy, which showed calcified tubules at 5 weeks after  
215 irradiation ( $P < 0.001$ , Mann-Whitney test; Tables 1 and 2).

#### *Mouse Donor Cell Colonies in Irradiated or Busulfan-Treated Recipient Testes*

Since the testes of mice receiving 50-55 mg/kg doses of busulfan had similar TDI levels  
as the testes irradiated with 1.5+12 Gy, these two groups were used to produce recipient mice for  
quantitative comparison of the colonizing efficiency of mouse donor cells in testes treated with  
220 busulfan or irradiation. Body and testis weights of the 2 recipient groups were not significantly  
different (data not shown). Mouse donor cells were able to colonize tubules in both irradiated  
and busulfan-treated recipient testes (Figure 1a and b). The number of colonies counted by  
fluorescence microscopy on gently teased testicular tissue (Figure 1c and d) per  $10^4$  donor cells  
in the irradiated and busulfan-treated recipient testes did not differ significantly (Table 3). In  
225 histologic sections from both busulfan-treated and irradiated testes, it was apparent that some  
tubules contained differentiating spermatogenic cells derived from the donor and some showed

recovery of endogenous germ cells (Figure 2a and b). That these were differentiating germ cells was confirmed by the results of staining serial sections with anti-GFP and anti-GCNA1 (Figure 2c and d). Not all tubules containing donor cells showed complete spermatogenic differentiation. 230 Some had donor cells that appeared to remain in the spermatogonial stage or to differentiate only to the spermatocyte or round spermatid stage (Figure 2e). Some tubules showed, however, that donor stem cells were able to differentiate to the elongated spermatid stage (Figure 2c and f).

Counts of colonies by fluorescence microscopy showed similar numbers in the irradiated and busulfan-treated testes (Table 3). Histological analysis also showed that there were similar 235 numbers of cross-sections in which the germ cells were immunostained for GFP in irradiated and busulfan-treated recipient testes and that the percentages of these colonies in which differentiated cells were found were the same in both treatment groups. We conclude that busulfan-treated and irradiated recipient testes demonstrated no significant differences in donor cell colonization and in donor spermatogenic development.

#### 240 *Immature Rat-Derived Donor Cell Spermatogenesis in Recipient Mouse Testes*

To test whether the irradiated mouse testes could support donor rat spermatogenesis, testicular cells collected from immature rat testes expressing GFP were successfully transplanted into the tubules of irradiated and busulfan-treated mouse testes. Thirteen weeks later, fluorescence microscopy of testicular tissue showed that regions of tubules in both the busulfan- 245 treated (Figure 3a) and irradiated mice (Figure 3b) showed GFP fluorescence, which demonstrated that rat donor cells had colonized the recipient tubules. These were tubules repopulated with donor rat cells, as determined by anti-GFP antibody (green fluorescence), but both busulfan-treated and irradiated testes also contained tubules repopulated by host mouse cells (only DAPI staining) (Figure 3c and d). This was confirmed further when the germ cells were

250 identified by staining serial sections with anti-GFP and anti-GCNA1 (Figure 3e and f). Because  
the anti-GCNA1 antibody was originally developed against mouse germ cells (Enders and May,  
1994), it has species-specific sensitivity and is more reactive with mouse germ cells than rat  
germ cells. The GFP-positive tubules showed definite but weak staining for GCNA1,  
demonstrating that these were indeed germ cells. Pale GCNA1-staining of these germ cells  
255 further demonstrated that they developed from donor rat spermatogonial stem cells. However,  
anti-GCNA1 staining in tubules that were GFP-negative was much stronger, proving that they  
were derived from host mouse stem cells, although some tubules showed an admixture of donor-  
and host-derived spermatogenesis (Figure 3g, arrow). Some tubules with donor-derived  
spermatogenesis differentiated incompletely, even 13 weeks after transplantation (Figure 3g,  
260 arrowhead), but elongated spermatids with the definite nuclear shape of elongated rat spermatids  
could be found in some GFP-positive tubules (Figure 3h, arrows and inset).

Quantitative analysis of cross-sections revealed that about one-third of the tubules  
contained rat germ cells and most of the rat spermatogonial stem cells that colonized the  
irradiated recipient mice are capable of differentiation (Table 4). As these transplantation  
265 experiments were undertaken before the optimal busulfan dose was chosen and a 44-mg/kg dose  
was used, interpretation of any quantitative comparisons between the irradiated and busulfan-  
treated testes is limited. Nevertheless, it was noted that the colonization and differentiation  
efficiency of the rat stem cells was higher in the irradiated than in the busulfan-treated testes,  
though this may be due in part to the limited depletion of endogenous spermatogenesis by the  
270 busulfan dose.

## ***Discussion***

In this study, we showed that irradiated recipient mouse testes could support complete donor spermatogenesis. There were similar numbers of donor colonies and differentiation of cells within those colonies derived from transplanted mouse spermatogonial stem cells in irradiated and busulfan-treated recipients in which endogenous spermatogenesis had been depleted to a similar degree (Table 3). A similar percentage of tubules in irradiated mouse testes were colonized by stem spermatogonia from immature rats as from immature mice, when normalized to injected cell numbers, and most of these rat spermatogonial-derived colonies were able to produce differentiated germ cells (Table 4). Thus, irradiated mouse testes could support the colonization and differentiation of spermatogonial cells from donor mice or from immature rats as had been described for testes of busulfan-treated mice (Clouthier et al, 1996). The efficiency of irradiated mouse testes as recipients in supporting syngeneic or xenogeneic donor spermatogenesis is comparable to that of busulfan-treated mouse testes.

An issue with busulfan treatment, which has been the most common method of preparing the recipient testis since germ cell transplantation was developed (Brinster and Zimmerman, 1994), is the strain-dependence of its systemic and spermatogenic toxicity. Doses of 40-45 mg/kg were sufficient in C3H (Bucci and Meistrich, 1987) or C57BL/6 mice (Kanatsu-Shinohara et al, 2003b) to reduce the fraction of tubules showing recovery of spermatogenesis 5 weeks later to 1%. However, in ICR mice there was nearly complete recovery of spermatogenesis within 12 weeks after similar doses (Choi et al, 2004). In the Swiss/Ncr nude mice used here, 44 mg/kg resulted in endogenous recovery in about 50% of seminiferous tubules 5 weeks after treatment; 60 mg/kg was required to maintain depletion in 99% of the tubules in most mice. In some strains, busulfan doses of 40-45 mg/kg caused death of some mice (Bucci and Meistrich, 1987), so that

295 bone marrow transplantation was usually required for survival (Kanatsu-Shinohara et al, 2003a).  
In contrast, Swiss/Ncr nude mice could endure a single busulfan dose of 60 mg/kg without  
obvious toxicity. Nevertheless, the therapeutic index was not much improved, because higher  
doses were also required to deplete the seminiferous tubules of endogenous stem cells.

Our data with radiation confirmed that a small initial dose followed by a larger dose was  
300 as effective at depleting spermatogenesis as was a higher total dose given in equal fractions, with  
a lower tendency to produce calcified tubules (Table 1). However, the fractions of tubules  
depleted of spermatogenesis by 1.5 +12 Gy in our nude mice was lower than the values of 99%  
and 97% reported for Nc/CpbU mice or a different strain of nude mice (NMRI, Hsd/Cpb),  
respectively, at 12 weeks after irradiation and the value of 89% depletion for the NMRI nude  
305 mice at 21 weeks after irradiation (Creemers et al, 2002). The difference was due most likely to a  
background-strain specific response to fractionated radiation (Meistrich et al, 1984). Our  
preliminary results (G. Wilson and M. L. Meistrich, unpublished data) also showed that there  
was greater depletion of spermatogenesis in C57BL/6 than in the nude mice at equivalent  
radiation doses.

310 We noted that the recovery of endogenous spermatogenesis after irradiation was not  
significantly affected by transplantation. In testes transplanted with immature mouse testicular  
cells, 73% of tubules showed recovery, but about 9% (16% x 55%, Table 3) could be attributed  
to donor spermatogenesis, leaving about 64% of the tubules showing endogenous spermatogenic  
recovery. Similarly, in testes transplanted with immature rat testicular cells, 69% of the tubules  
315 showed recovery, but 27% (35% x 76%, Table 4) could be attributed to the donor, leaving about  
42% endogenous recovery. These values are similar to the 53% recovery observed without  
transplantation (Table 1).

The presence of calcification in a small percentage of tubules was the only negative effect of irradiation compared to busulfan. This was specific to the Swiss/Ncr nude mice; very little  
320 calcification was observed after irradiation of C57BL/6 mice with equivalent doses (G. Wilson and M. L. Meistrich, unpublished data). Although we were concerned that the calcification might affect the transplanted cells' ability to flow through the seminiferous tubules, the numbers of tubules or tubule cross-sections containing donor germ cells were still quite high. Calcified tubules have also been observed in rats treated with busulfan (Udagawa et al, 2001) and  
325 dibromochloropropane (Meistrich et al, 2003), but not in busulfan-treated mice nor in irradiated rats (Kangasniemi et al, 1996).

By showing that irradiation is as good as other commonly used methods for preparing mouse testes for spermatogonial transplantation, our data support its consideration for testing in species for which genetic mutants are not available or show a narrow therapeutic window for  
330 spermatogenic *vs.* systemic toxicity with chemical treatments. Indeed, the effects of radiation on the testes have been described in many species and dose-response data are available for such an approach. In the rat, although relatively low doses of irradiation caused spermatogenic arrest in certain strains (Kangasniemi et al, 1996), in other strains the surviving stem cells differentiated and repopulated the seminiferous tubules (Dym and Clermont, 1970; Delic et al, 1986), as was  
335 the case in the mouse. Even in a strain in which radiation caused spermatogenic arrest, hormonal treatment to suppress intratesticular testosterone was able to stimulate the differentiation of endogenous surviving stem cells (Meistrich and Kangasniemi, 1997), and the same should apply to transplanted cells (Ogawa et al, 1999b; Zhang et al, 2003). Doses for killing stem cells and the subsequent repopulation of tubules have been described in the dog (Lushbaugh and Casarett,  
340 1976), rabbit (Lyon and Cox, 1975), ram (van Vliet et al, 1988), boar (Erickson and Martin,

1984), bull (Erickson et al, 1972), rhesus (van Alphen et al, 1988; van Alphen et al, 1989), cynomolgus (Foppiani et al, 1999; Kamischke et al, 2003), and stump-tailed (Boekelheide et al, 2005) macaques, and human (Clifton and Bremner, 1983). For application to different species, the physical dosimetry is straightforward, and differences in pharmacokinetics, which are an issue with chemical agents like busulfan, do not have to be considered. Furthermore, local irradiation of testes avoids the systematic toxicity that often occurs in busulfan-treated animals.

Two studies have reported the use of irradiation to deplete cells from testes of other species for use as hosts for transplantation. In one case, monkey testes irradiated with 2 Gy were used as a host for autologous transplantation of germ cells (Schlatt et al, 2002). At this dose, there was significant recovery of endogenous spermatogenesis but only two out of the five monkeys showed significantly increased numbers of differentiated spermatogenic cells in the transplanted testis compared to the saline injected control. In a study of autologous transplantation into calf testes, the animals were hemicastrated and irradiated with 10-14 Gy and cells from the contralateral testis were injected into the irradiated one; five of six animals showed enhanced spermatogenesis (Izadyar et al, 2003). It should be noted that neither of these studies included a marker to unequivocally distinguish donor germ cells from the endogenous ones.

In summary, irradiation consistently produced well-functioning germ cell-depleted recipient mouse testes. The irradiated testes could support complete donor spermatogenesis derived from either donor mouse or rat spermatogonial stem cells, confirmed with the use of a GFP marker, with an efficiency similar to that of busulfan-treated recipient mouse testes. Our results suggest that irradiation is worthwhile pursuing as a possible effective method for preparing recipient testes in other species.

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Table 1. Response of mouse testes to different doses of radiation \*

Doses of irradiation	Time of killing after irradiation	Weight / testis (mg)	Tubule differentiation index (%)	Calcified tubules (%)
8 + 8 Gy	5 weeks	28 ± 1	6 ± 2	4 ± 2
1.5 + 14 Gy	5 weeks	25 ± 1	0.5 ± 0.2 †	6 ± 3
1.5 + 12 Gy	5 weeks	29 ± 2	6 ± 1	2 ± 1
1.5 + 12 Gy	8 weeks	26 ± 2	11 ± 2	4 ± 2
1.5 + 12 Gy	18 weeks	30 ± 2	53 ± 6 ‡	3 ± 2

\* Values given as mean ± SEM, n=5 in each group except the "1.5+12 Gy for 18 weeks" group (n=10).

510 † Significantly different from values after 8+8 Gy and 1.5+12 Gy at 5 weeks after irradiation,  $P < 0.02$ .

‡ Significantly different from values with the same doses at 5 and 8 weeks after irradiation,  $P < 0.001$ .

Table 2. Response of mouse testes to different busulfan doses: comparison with irradiation \*

Treatment	Dose	Body weight (g)	Weight / testis (mg)	Tubule differentiation index (%)	Calcified tubules (%)
Busulfan	44 mg/kg	32 ± 1	36 ± 4 † (32 ± 1) §	57 ± 15 ‡ (47 ± 14) §	0
Busulfan	50 mg/kg	31 ± 1	29 ± 2 † (27 ± 1) §	20 ± 14 ‡ (6 ± 3) §	0
Busulfan	55 mg/kg	31 ± 1	27 ± 2 † (26 ± 2) §	16 ± 12 ‡ (4 ± 4) §	0
Busulfan	60 mg/kg	30 ± 1	26 ± 4 † (22 ± 2) §	18 ± 18 ‡ (0.4 ± 0.4) §	0
Radiation	1.5+12 Gy	32 ± 1	24 ± 1	6 ± 2	7 ± 4

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\* All values given as mean ± SEM, n=5 except where noted. Mice killed 5 weeks after treatment except the 60-mg/kg group, which was killed at 8 weeks.

† Dose-response to busulfan: Spearman correlation coefficient: -0.75,  $P < 0.021$

‡ Dose-response to busulfan: Spearman correlation coefficient: -0.84,  $P < 0.008$

§ n=4 after exclusion of one mouse in each group that was an obvious outlier with TDI values ranging from 64% to 100%.

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Table 3. Development of colonies from mouse donor cells in recipient mouse testes at 10 weeks after transplantation

Treatment of recipients (No. of successfully injected testes)	Total tubule differentiation index (%)	No. of GFP colonies per testis	Viable cells injected per testis ( $\times 10^4$ ) *	No. of colonies per $10^4$ viable cells	% of tubule cross-sections that were GFP-positive	% of GFP-positive cross-sections with differentiated germ cells
Irradiation † (n=12) §	79 ± 5	10.4 ± 2.2	29 ± 4	0.37 ± 0.07	16 ± 2	55 ± 6
Busulfan ‡ (n=12) §	54 ± 10	9.6 ± 1.4	30 ± 4	0.43 ± 0.07	22 ± 3	52 ± 5

\* The number of viable donor cells was calculated from the injection volume, cell concentration, and cell viability.

† Two fractions of irradiation (1.5 + 12 Gy, 24 hours apart).

‡ 50-55 mg/kg doses of busulfan injection. Three mice (5 successfully injected testes) were treated with busulfan at a dose of 50 mg/kg, and 4 mice (7 testes) with a dose of 55 mg/kg.

§ Only 7 of 12 irradiated testes and 11 of 12 busulfan-treated testes were processed for GFP immunostaining.

Table 4. Development of immature rat donor germ cells in recipient mouse testes at 13 weeks after transplantation

Treatment of recipient * (No. of testes)	Weight per testis (mg)	Total tubule differentiation index (%)	Viable cells injected per testis ( $\times 10^4$ )	% of tubule cross-sections that were GFP-positive	% of GFP-positive tubule cross-sections with differentiated germ cells
Irradiation (n=14)	47 $\pm$ 3	69 $\pm$ 4	76 $\pm$ 7	35 $\pm$ 5	76 $\pm$ 4
Busulfan (n=8)	90 $\pm$ 16	93 $\pm$ 2	67 $\pm$ 10	16 $\pm$ 5	36 $\pm$ 10

\* Irradiation doses, 1.5 +12 Gy, 24 hours apart; busulfan dose 44 mg/kg.

## **Figure Legends**

Figure 1. Visualization of colonies of transplanted mouse germ cells by GFP fluorescence 10 weeks after transplantation. Testes were treated with busulfan (a, c) or irradiation (b, d). Fluorescence was visualized in testes with tunica removed (a, b), and colonies were counted in tubules after gently teasing the tissue apart (b, d).

Figure 2. Histologic and immunochemical examination of tubules in mouse testes transplanted with immature mouse germ cells. Tubules from busulfan-treated (a) or irradiated (b) transplanted testes stained with anti-GFP and Alexa 488 second antibody (green) and DAPI counterstaining (blue) showed the repopulation of some tubules with donor cells. Serial sections from irradiated transplanted testes stained with anti-GFP (c) or anti-GCNA1 (d). The DAB reaction product (brown staining), with hematoxylin counterstaining, showed that in some tubules (\*) complete spermatogenesis was derived from donor stem cells. Some tubules in irradiated testes (e) contained colonies of donor cells that appeared to remain in the spermatogonia stage (open arrowhead) or showed partial differentiation (arrowhead). At higher magnification (f), elongated spermatids derived from the donor mice were observed (arrows). (a, b) bar = 50  $\mu\text{m}$ ; (c, d, e) bar = 50  $\mu\text{m}$ ; (f) bar = 25  $\mu\text{m}$ .

Figure 3. Tubules from mice killed 13 weeks after transplantation with immature rat germ cells into busulfan-treated (a, c) or irradiated (b, d-h) mice. (a, b) GFP-fluorescent and nonfluorescent regions of tubules from the surface of intact testes with the tunica removed. Tubules from busulfan-treated (c) or irradiated (d) transplanted testes stained with anti-GFP and Alexa 488 second antibody (green) and DAPI counterstaining (blue) show the repopulation of some tubules with donor cells and other tubules with host cells. Serial sections from irradiated transplanted testes stained with anti-GFP (e) or anti-GCNA1 (f) and DAB reaction product (both brown

staining) with hematoxylin counterstaining. The same tubules (\*), in which spermatogenesis was derived from donor stem cells, as determined by GFP staining (e), were confirmed to be rat derived by weaker GCNA1 staining than to tubules with mouse germ cells (no asterisks). (g) Varying patterns of donor rat spermatogenesis in tubules from irradiated mice stained with anti-GFP show tubules with complete spermatogenesis (\*), tubules with incomplete spermatogenesis (arrowhead), and tubules containing both regions of rat spermatogenesis stained with anti-GFP and regions of endogenous mouse spermatogenesis (unstained region, arrow). (h) Anti-GFP stained tubule showing donor spermatogenesis with elongated spermatids with typical nuclear shapes for the rat (arrows and inset). (a, b) bar = 200  $\mu\text{m}$ ; (c, d) bar = 50  $\mu\text{m}$ ; (e, f, g) bar = 50  $\mu\text{m}$ ; (h) bar = 10  $\mu\text{m}$ .

Figure 1.

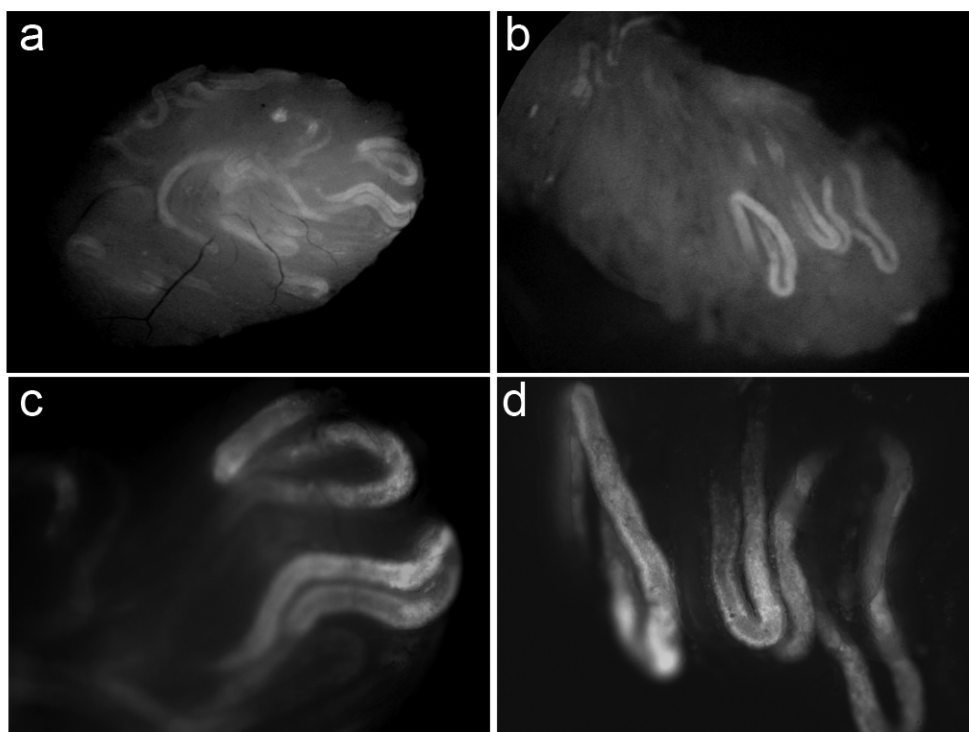


Figure 2.

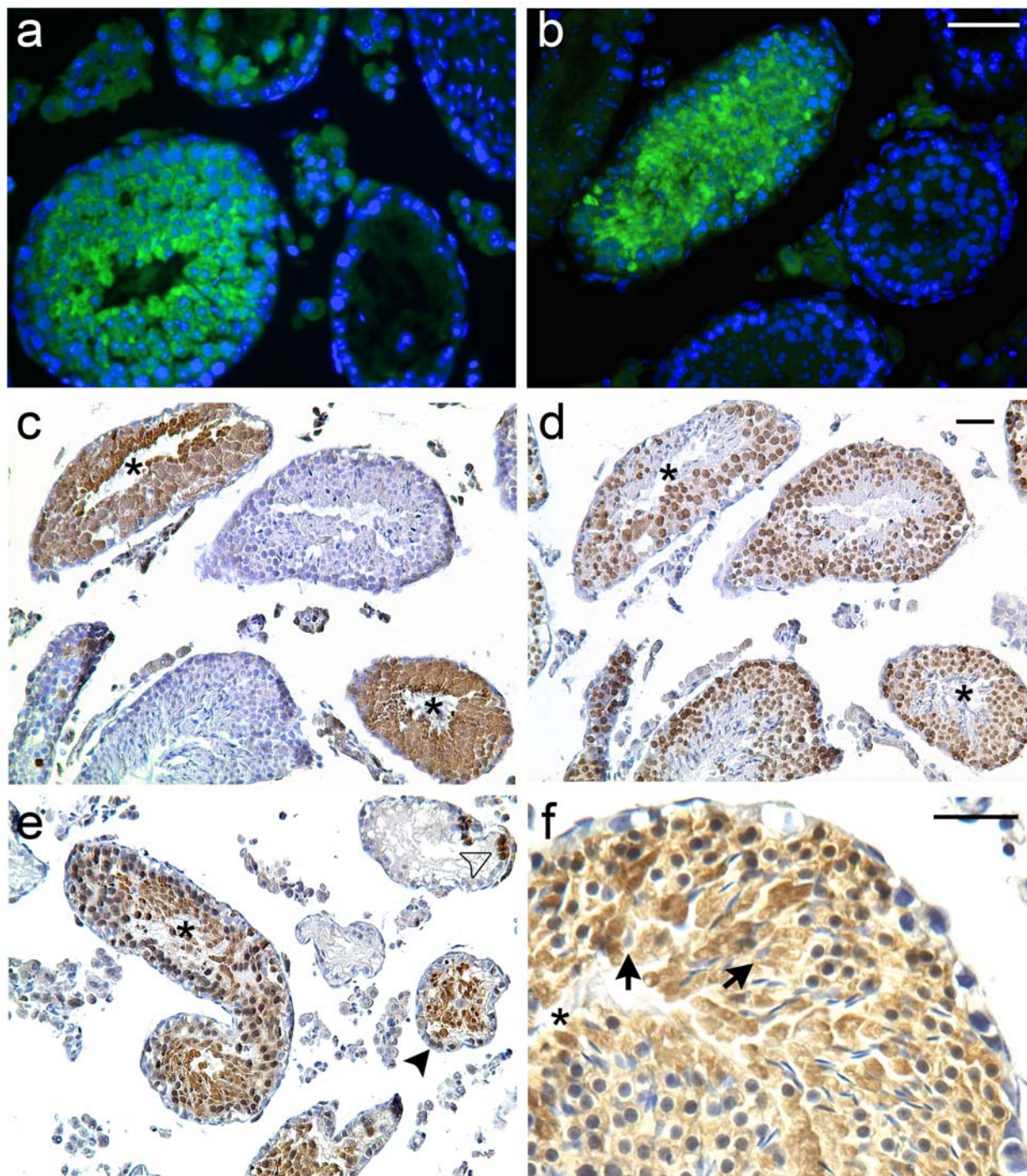


Figure 3.

