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Irradiated Mouse Testes Efficiently Support Spermatogenesis Derived From Donor Germ Cells of 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 transplant recipients for studying differentiation of transplanted cells, and there has been no direct comparison of irradiation and busulfan as preparation methods. Mouse testes treated with local fractionated irradiation (1.5 + 12 Gy) were compared with busulfan-treated testes as recipients using mouse-to-mouse and rat-to-mouse germ cell transplantation. The fractionated 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 treatment for animal recipients of germ cell transplants that cannot endure the systemic toxicity of busulfan.

Key words: Irradiation, busulfan, testis, spermatogonial transplantation.

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Introduction

During the past 10 years, study of the mechanisms and regulation of spermatogenesis has progressed, in part, because of the technique of testicular germ cell transplantation (Brinster, 2002). The technique provides an in vivo functional assay of spermatogonial stem cells and has been used in distinguishing defects caused by intrinsic function of spermatogonial stem cells and the extrinsic environment; it is also useful 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 model (Jiang and Short, 1995; Clouthier et al, 1996; Ogawa et al, 1999b). Performance of male germ cell transplantation in other species would be useful in the treatment of 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 has hampered these applications (Dobrinski et al, 1999; Dobrinski et al, 2000; Reis et al, 2000; Nagano et al, 2001b; Izadyar et al, 2002). Donor spermatogenesis was supported in goat-to-goat transplantation (Honaramooz et al, 2003), 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 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 by introducing genetic mutations or with chemical or physical treatments. Mutant mice, such as the W/W^{v} line (Ohta et al, 2003), provide a supportive environment for donor spermatogenesis (Ogawa et al, 2000) and have been widely used as recipients, but they must be

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immunocompatible with the donors (Kanatsu-Shinohara et al, 2005), and the infertility of these mice makes their production difficult (Brinster and Avarbock, 1994). No other species with comparable genetic mutations are available.

The sole effective chemical treatment used so far to prepare male germ cell transplant recipients is busulfan. Busulfan doses 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 resulted 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 thus resulted in recovery of endogenous spermatogenesis in the offspring (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 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. Busulfan is given to humans only in conjunction with bone marrow transplantation. Thus, it is unlikely that busulfan will be widely used in a variety of species for recipient preparation.

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 the depleted tubules are limited. In one study (Creemers et al, 2002), only 2 irradiated mice were recipients (1 testis of each mouse) 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 (Giuili et al, 2002), but this dose was too low to deplete a significant number of endogenous stem cells. The effectiveness of testicular irradiation for preparing recipients to efficiently support differentiation of transplanted spermatogonia has not yet been evaluated. We undertook this study to determine the ability of irradiated mouse testes to serve as recipients of mouse and rat spermatogonial transplants and support the differentiation of the transplanted cells and to compare these results with results for busulfan-treated recipients.

Materials and Methods

Animals

Adult nude (Swiss *nu-nu*/Ncr) mice bred at the University of Texas M. D. Anderson Cancer Center (Houston, Tex) were used as recipients. Donors were transgenic mice (Tg (*ACTB-EGFP*)1Osb/J) on a C57BL/6 background that express green fluorescent protein (GFP) under the control of a chicken β-actin promoter (Okabe et al, 1997) (Jackson Laboratories; Bar Harbor, Me) or transgenic rats expressing GFP under the control of a cytomegalovirus enhancer and a 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, Ind) 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 (12 h of light and 12 h of darkness) with unlimited access to food and water. All experiments were approved by the Institutional Animal Care and Use Committee of the M. D. Anderson Cancer Center.

Preparation of Recipients

The nude mice were irradiated without anesthesia. They were restrained in plastic chambers (Lucite boxes) placed 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}Cs γ-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 compared the affects of a single dose, 2 equal daily fractions, and 4 equal daily fractions on spermatogonial depletion in C57BL/6 and Swiss/Ncr nude mice (G. Wilson and M. L. Meistrich, unpublished data). Two doses of 8 Gy given 24 hours apart were most effective in reducing tubular repopulation without other damage. Because even a small initial radiation dose induces the remaining spermatogonial stem cells to become radiosensitive, other investigators 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 2 fractions of irradiation (8 + 8 Gy, 1.5 + 12 Gy, or 1.5 + 14 Gy) administered 24 hours 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 (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 greater than 37°C until injection to prevent the busulfan from crystallizing and to 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, Calif) containing DNase I (Cat #DN25; Sigma) at 100 μg/mL and 1% fetal bovine serum (HyClone, Logan, Utah) with 0.05%–0.1% collagenase IV (Cat #4188; Worthington Biochemical Corp, Lakewood, NJ) for 20–30 minutes and then with 0.05%–0.1% of collagenase and 0.05% of hyaluronidase (Cat #2592; Worthington) for 20 minutes. Tubules were finally digested with 0.1% trypsin (Cat #3704; Worthington) and 100 μg of DNase I/mL in Dulbecco's phosphate buffered saline (PBS, Gibco) containing 1 mM EGTA (Cat #E-4378; Sigma) for 10–15 minutes. The final pellets of cells were resuspended in DMEM/F12 containing 10% fetal bovine serum.

Trypan blue (Gibco) was added to a final concentration of 0.02%. After the cell concentration and viability was determined, the cell suspension was kept on ice until transplantation. The cell viability ranged from 91% to 99% (mean viability, 96%), and the cell concentration ranged from 3.6×10^7 to 5.8×10^7 cells/mL (mean cell concentration, 4.4×10^7 cells/mL).

Transplantation

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 semiautomatic microinjector (Brinkmann Instruments Inc, Westbury, NY). A mean of 8 μL (range, 1–15 μL) of cell suspension containing approximately 40×10^4 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 solution or 4% paraformaldehyde solution at 4°C and embedded in paraffin or plastic. Sections with a thickness of 4–5 μm were cut and stained with periodic acid-Schiff–hematoxylin for counting germ cells in the seminiferous tubules. All tubules in a cross-section were counted (mean number, 145 tubules per cross-section). The tubule differentiation index (TDI) is the percentage of tubules showing differentiation, which is defined as the presence 3 or more germ cells in a tubule reaching the B spermatogonia stage or beyond.

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 was gently teased apart at room temperature within 5–10 minutes and removed and placed in cold PBS. After the number of GFP-positive colonies in the recipient testes were counted 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 minutes in Antigen Retrieval Citra Plus solution (Cat #HK080-9K; BioGenex, San Ramon, Calif) on a hot plate and allowed to cool for 1 hour. The sections were treated for 5 minutes with 0.6% hydrogen peroxide (Sigma) to block endogenous peroxidase activity and for 1 hour 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 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, Colo) was added to tissues and incubated overnight at 4°C. ABC Elite kits, second antibodies, and 3,3′-diaminobenzidine were all ordered from Vector Laboratories (Burlingame, Calif) and used according to procedures recommended by the manufacturer. Sections were counterstained with hematoxylin. For immunofluorescence staining, goat anti-rabbit IgG (Alexa Fluor 488; Molecular Probes Inc, Eugene, Ore) was used at a 1:500 dilution. Sections were counterstained with DAPI.

Germ cells were identified after anti-GCNA1 staining, and the total TDI was calculated. The number of GFP-positive tubules was assessed in histologic sections after anti-GFP staining. The most advanced stage of GFP-positive differentiated cells was determined in each tubule by histologic criteria. All values were obtaining by counting all tubules taken from 3 sections at least 25 μm from each other per testis.

Statistical Analysis

Testing for statistically significant differences ($P < .05$) between the irradiated group and the busulfan-treated group was performed using the SPSS statistical package (version 11.5; SPSS Inc, Chicago, Ill) and a Student's *t* test, unless otherwise specified.

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

Irradiation Dose	No. of Testes	Interval Between Irradiation and Death (wks)	Testis Weight (mg)	Tubule Differentiation Index (%)	Calcified Tubules (%)
8 + 8 Gy	5	5	28 ± 1	6 ± 2	4 ± 2
1.5 + 14 Gy	5	5	25 ± 1	0.5 ± 0.2†	6 ± 3
1.5 + 12 Gy	5	5	29 ± 2	6 ± 1	2 ± 1
1.5 + 12 Gy	5	8	26 ± 2	11 ± 2	4 ± 2
1.5 + 12 Gy	10	18	30 ± 2	53 ± 6‡	3 ± 2

* Data are mean ± SEM.

† Significantly different from values after 8 + 8 Gy and 1.5 + 12 Gy 5 weeks after irradiation ($P < .02$).

‡ Significantly different from values with the same doses at 5 and 8 weeks after irradiation ($P < .001$).

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

Treatment Group	Dose	Body Weight (g)	Testis Weight (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

* Data are mean ± SEM, unless otherwise indicated. Data are for 5 testes in each treatment group, unless otherwise indicated. Most mice were killed 5 weeks after treatment. Mice in the 60-mg/kg group were killed 8 weeks after treatment.

† Dose response to busulfan: Spearman correlation coefficient: −0.75, $P < .021$.

‡ Dose response to busulfan: Spearman correlation coefficient: −0.84, $P < .008$.

§ Data for 1 mouse in each group were excluded because they were obvious outliers, with tubule differentiation index values ranging from 64%–100%.

Results

Optimal Doses of Irradiation or Busulfan to Deplete Endogenous Germ Cells

First, different dose regimens of irradiation (2 fractions of 8 + 8 Gy, 1.5 + 12 Gy, or 1.5 + 14 Gy) were given 24 hours apart to 3 groups of mice. These mice were killed 5 weeks later for evaluation of the testes (Table 1). All treatment groups had a lower mean testicular weight (±SEM) than did unirradiated controls (101 ± 3 mg). Almost all germ cells were depleted in the testes of mice treated with 1.5 + 14 Gy, 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 periodic acid-Schiff–hematoxylin), which was similar to our observation in previous experiments involving chemical-treated rats (Meistrich et al, 2003). The other 2 groups showed similar depletion of spermatogenesis, with TDIs of approximately 6%. Because the percentage of calcified tubules appeared to be slightly lower in the group treated with 1.5 + 12 Gy (1.7%) than in the group treated with 8 + 8 Gy (3.8%), and because 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 group treated with 1.5 + 12 Gy 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 with another group of mice simultaneously irradiated with 1.5 + 12 Gy. We were surprised that all nude mice with this outbred genetic background survived the highest dose of 60 mg of busulfan per kg of body weight without needing bone marrow transplants, appeared healthy throughout the experiment, and showed no long-term reduction in body weight (Table 2). The response to busulfan was much more variable than the response to irradiation. Each group had clear outlier data, with TDI values of 64%–100%. Even when these data 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 only

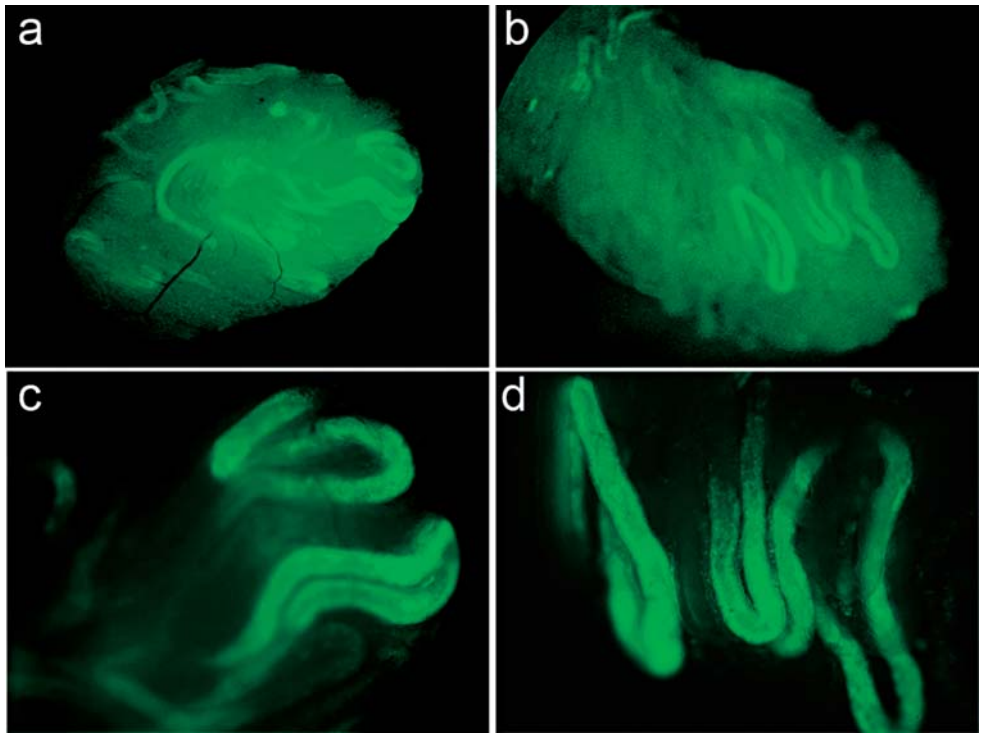

Figure 1. Visualization of colonies of transplanted mouse germ cells by GFP fluorescence 10 weeks after transplantation. Testes were treated with busulfan **(a, c)** or irradiation **(b, d)**. Fluorescence was visualized in testes with tunica removed **(a, b)**, and colonies were counted in tubules after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

approximately 50% at 5 weeks after treatment, which indicated that stem cell killing was insufficient to largely eliminate endogenous spermatogenesis. TDI levels (approximately 5%) in the testes of most of the mice receiving busulfan doses of 50–55 mg/kg were similar to those in the testes irradiated with 1.5 + 12 Gy. 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 5 weeks after irradiation ($P < .001$, by the Mann-Whitney U test) (Tables 1 and 2).

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

Because the testes of mice receiving 50–55 mg/kg doses of busulfan had similar TDI levels as the testes of mice irradiated with 1.5 + 12 Gy, these 2 groups were used to produce recipient mice for quantitative comparison of the colonizing efficiency of mouse donor cells in testes treated with busulfan or irradiation. Body weights 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 sections of histologic specimens 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. 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). Histologic analysis also showed that there were similar 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

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

Treatment Group*	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	Tubule Cross-Sections That Were GFP-Positive (%)*	GFP-Positive Tubule Cross-Sections With Differentiated Germ Cells (%)*
Irradiation‡	12	79 ± 5	10.4 ± 2.2	29 ± 4	0.37 ± 0.07	16 ± 2	55 ± 6
Busulfan§	12	54 ± 10	9.6 ± 1.4	30 ± 4	0.43 ± 0.07	22 ± 3	52 ± 5

* Only 7 of 12 irradiated testes and 11 of 12 busulfan-treated testes were processed for GFP immunostaining.
† Calculated on the basis of the injection volume, cell concentration, and cell viability.
‡ Treatment consisted of 2 fractions of irradiation (1.5 + 12 Gy, 24 h apart).
§ Treatment consisted of 50–55 mg/kg injections of busulfan. Three mice (5 successfully injected testes) were treated with busulfan at a dose of 50 mg/kg, and 4 mice (7 testes) were treated with a dose of 55 mg/kg.

and irradiated recipient testes demonstrated no significant differences in donor cell colonization and donor spermatogenic development.

Immature Rat Testes–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 revealed that regions of tubules in both the busulfan-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 was present), but both busulfan-treated and irradiated testes also contained tubules repopulated by host mouse cells (only DAPI staining was present) (Figure 3c and d). This was confirmed further when the germ cells were 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 with 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 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-derived and host-derived spermatogenesis (Figure 3g, arrow). Some tubules with donor-derived spermatogenesis differentiated incompletely, even 13 weeks after transplantation (Figure 3g, 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 approximately one-third of the tubules contained rat germ cells and that most of the rat spermatogonial stem cells that colonized the irradiated recipient mice were capable of differentiation (Table 4). Because these transplantation experiments were undertaken with a 44-mg/kg dose of busulfan, before the optimal busulfan dose was chosen, 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 testes than in the busulfan-treated testes, although this may be due, in part, to the limited depletion of endogenous spermatogenesis by the 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 similar 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 spermatogonia–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 has 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.

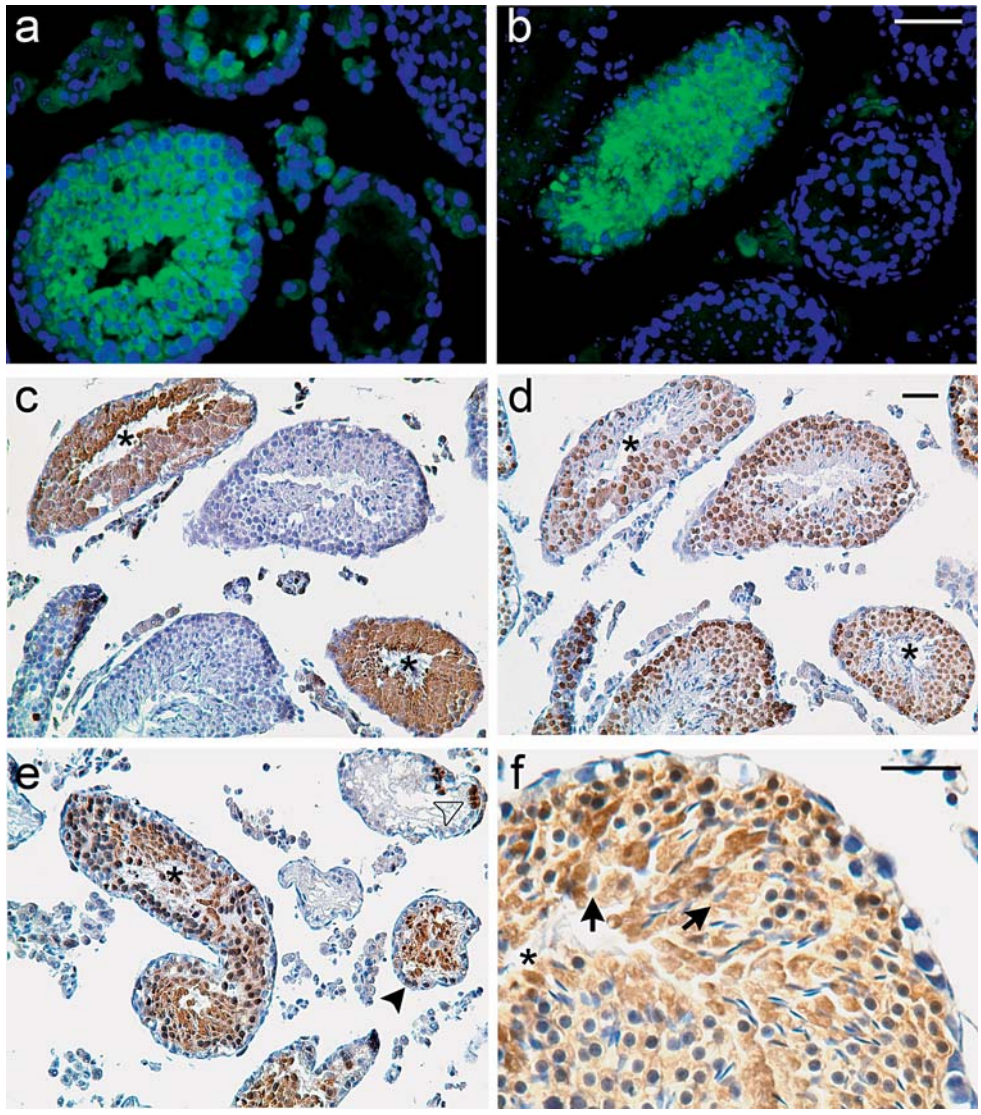

Figure 2. Histologic and immunochemical examination of tubules in mouse testes transplanted with immature mouse germ cells. Tubules from busulfan-treated **(a)** or irradiated **(b)** transplanted testes stained with anti-GFP and a second antibody (Alexa 488) (green) and DAPI counterstaining (blue) showed the repopulation of some tubules with donor cells. Serial sections from irradiated transplanted testes stained with anti-GFP **(c)** or anti-GCNA1 **(d)**. The 3,3′-diaminobenzidine 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 (shaded arrowhead). At higher magnification **(f)**, elongated spermatids derived from the donor mice were observed (arrows). **(a–e)** Scale bar = 50 μm; **(f)** scale bar = 25 μm.

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 mice (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 in our study, a 44 mg/kg dose resulted in endogenous recovery in approximately 50% of seminiferous tubules 5 weeks after treatment; a 60 mg/kg dose was required to maintain depletion in 99% of the tubules in most mice. In some strains, busulfan doses of 40–45 mg/kg caused the death of some mice (Bucci and Meistrich, 1987), and bone marrow transplantation was usually required for survival of other mice (Kanatsu-Shinohara et al, 2003a). In contrast, Swiss/Ncr nude mice could endure a single busulfan dose of 60 mg/kg

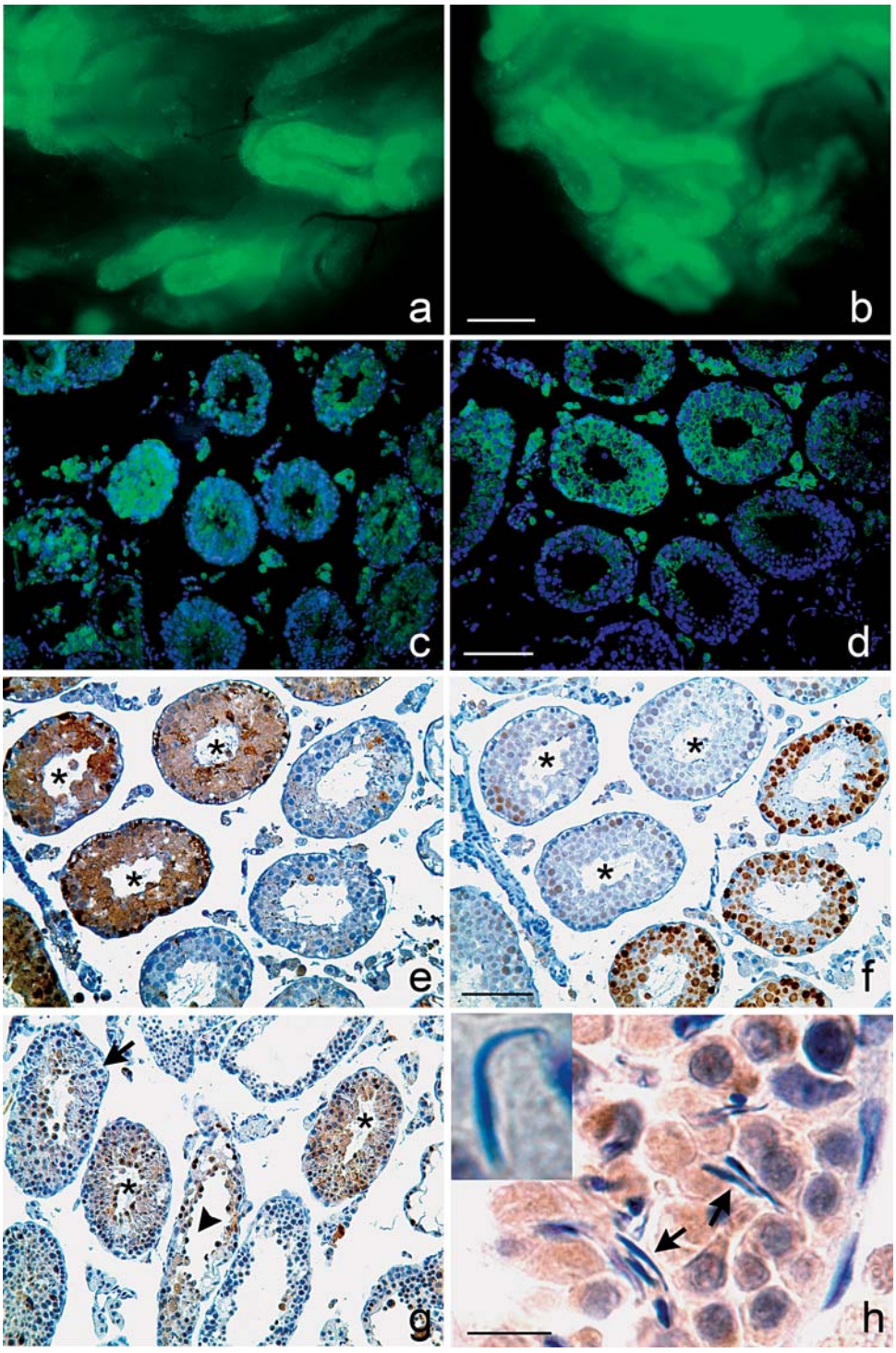

Figure 3. Tubules from mice killed 13 weeks after transplantation of immature rat germ cells into busulfan-treated **(a, c)** or irradiated **(b, d–h)** mice. **(a, b)** GFP-fluorescent and -nonfluorescent regions of tubules from the surface of intact testes with the tunica removed. Tubules from busulfan-treated **(c)** or irradiated **(d)** transplanted testes stained with anti-GFP and a second antibody (Alexa 488) (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 3,3′-diaminobenzidine reaction product (brown staining for both) 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 were tubules with mouse germ cells (no asterisk). **(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

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

Treatment Group	No. of Testes	Weight per Testis (mg)	Total Tubule Differentiation Index (%)	Viable Cells Injected per Testis ($\times 10^4$)	Tubule Cross-Sections That Were GFP-Positive (%)	GFP-Positive Tubule Cross-Sections With Differentiated Germ Cells (%)
Irradiation*	14	47 ± 3	69 ± 4	76 ± 7	35 ± 5	76 ± 4
Busulfan†	8	90 ± 16	93 ± 2	67 ± 10	16 ± 5	36 ± 10

* Treatment consisted of 1.5 + 12 Gy administered 24 hours apart.
† Treatment consisted of a 44-mg/kg dose.

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 from the irradiated mice confirmed that a small initial dose followed by a larger dose was as effective at depleting spermatogenesis as 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 less than the 99% and 97% depletion reported for Nc/CpbU mice and for a different strain of nude mice (NMRI, Hsd/Cpb), respectively, 12 weeks after irradiation and less than the 89% depletion for the NMRI nude mice 21 weeks after irradiation (Creemers et al, 2002).The difference was most likely due to a background strain–specific response to fractioned radiation (Meistrich et al, 1984). Our preliminary results also showed that there was greater depletion of spermatogenesis in C57BL/6 than in the nude mice at equivalent radiation doses (G. Wilson and M. L. Meistrich, unpublished data).

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 approximately 9% (ie, the percentage of GFP-positive tubule cross-sections [16%] multiplied by the percentage of GFP-positive tubule cross-sections with differentiated germ cells [55%]; Table 3) could be attributed to donor spermatogenesis, leaving approximately 64% of the tubules showing endogenous spermatogenic recovery. Similarly, in testes transplanted with immature rat testicular cells, 69% of the tubules showed recovery, but 26% (ie, the percentage of GFP-positive tubule cross-sections [35%] multiplied by the percentage of GFP-positive tubule cross-sections with differentiated germ cells [76%]; Table 4) could be attributed to the donor, leaving an endogenous recovery of aproximately 42%. 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. This was specific to the Swiss/Ncr nude mice; very little 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 calcification might affect the ability of the transplanted cells 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 with dibromochloropropane (Meistrich et al, 2003) but not in busulfan-treated mice or 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 the consideration of irradiation for testing in species for which genetic mutants are not available or that have a narrow therapeutic window for 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), the surviving stem cells differentiated and repopulated the seminiferous tubules in other strains (Dym and Clermont, 1970; Delic et al, 1986), as was 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,

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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)** Scale bar = 200 μm; **(c–g)** scale bar = 50 μm; **(h)** scale bar = 10 μm.

2003). Doses for killing stem cells and the subsequent repopulation of tubules have been described in the dog (Lushbaugh and Casarett, 1976), rabbit (Lyon and Cox, 1975), ram (van Vliet et al, 1988), boar (Erickson and Martin, 1984), bull (Erickson et al, 1972), rhesus macaque (van Alphen et al, 1988; van Alphen et al, 1989), cynomolgus macaque (Foppiani et al, 1999; Kamischke et al, 2003), stump-tailed macaque (Boekelheide et al, 2005), and humans (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 such as 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 2 of the 5 monkeys showed significantly increased numbers of differentiated spermatogenic cells in the transplanted testis, compared with the saline-injected control monkey. 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 testis; 5 of 6 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 germ cells.

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 (as 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 to investigate as a possible effective method for preparing recipient testes in other species.

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