Restoration of fertility in infertile mice by transplantation of cryopreserved male germline stem cells

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BACKGROUND: The development of a spermatogonial transplantation technique has provided new possibilities for the treatment of male infertility. Previous studies have shown that spermatogonial stem cells could reinitiate spermatogenesis after cryopreservation and reintroduction into the seminiferous tubules of infertile recipient males, and this raised the possibility of banking frozen stem cells for male infertility treatment. It remains unknown, however, whether germ cells from freeze–thawed stem cells are fertile, leaving the possibility that the procedure compromises the integrity of the stem cells. METHODS AND RESULTS: Dissociated mouse testis cells were cryopreserved and transplanted into infertile recipient testes. The freeze–thawed testis cell populations contained higher concentrations of stem cells than fresh testis cell populations. Offspring were obtained from freeze–thawed stem cells transplanted into infertile males, and fertility restoration was more efficient in immature (5–10 days old) than in mature (6–12 weeks old) recipients. However, offspring were also obtained from infertile adult recipients using in-vitro microinsemination. CONCLUSIONS: This first successful application of frozen stem cell technology in the production of offspring by spermatogonial transplantation suggests the superiority of immature recipients for clinical applications. Thus, the combination of cryopreservation and transplantation of stem cells is a promising approach to overcome male infertility.

Key words: cryopreservation/infertility/stem cell/testis/transplantation

Introduction

Sperm cryopreservation has proven to be a valuable procedure for the management of infertility, for the protection of fertility in oncology patients, and for couples undergoing IVF (Bunge and Sherman, 1953; Fossa et al., 1989; Royère et al., 1996; Lass et al., 2001). However, there is a lack of methods by which the germlines of patients with few sperm may be preserved. Examples include prepubertal patients or those with clinical conditions such as severe spermatogenic maturation arrest or who lack sperm. The infertility of these patients cannot be overcome by the use of traditional assisted-reproduction technology.

The development of a spermatogonial transplantation technique has provided a new treatment strategy for male infertility (Brinster and Zimmermann, 1994). Following the transplantation of dissociated testis cells into a seminiferous tubule microenvironment, the spermatogonial stem cells colonize and initiate spermatogenesis (Nagano et al., 1999). Since spermatogonial stem cells self-renew and differentiate into proliferating spermatogonia, they provide a limitless supply of mature spermatozoa. Thus, spermatogonial stem cell transplantation may be useful for the treatment of different types of male infertility; for example, it was used to treat a mouse model of Sertoli cell-only syndrome (Ogawa et al., 2000; Shinohara et al., 2001). The mice that underwent transplantation recovered normal fertility and produced progeny for the rest of their lives. In addition to congenital infertility, this technique should also benefit oncology patients who are undergoing stem cell-destroying irradiation or chemotherapy, by prior isolation of stem cells and autotransplantation (Aslam et al., 2000). Transplantation of stem cells should rescue fertility in a manner similar to that of bone marrow stem cell transplantation. Therefore, the establishment of methods to preserve stem cells and restore fertility has important clinical implications.

Brinster and colleagues first demonstrated that frozen stem cells retain the ability to carry out spermatogenesis and to produce spermatozoa (Avarbock et al., 1996). Surprisingly, in contrast to the difficulties associated with the freezing of sperm, the protocol used for freezing germline cells was very simple, and similar to those generally employed for somatic cells. Further studies indicated that the same procedure could be applied to the freezing of spermatogonial stem cells from several other species, such as rats, hamsters, cattle, primates and humans (Dobrinski et al., 1999; 2000; Ogawa et al., 1999;
Brook et al., 2001; Nagano et al., 2001; 2002; Izadyar et al., 2002; Schlatt et al., 2002a). Despite the potential shown by this technology in initial studies, no attempts to mate recipients of cryopreserved germ cells have been reported, and it remains unknown to date whether germ cells developed from frozen stem cells are fertile. It is possible that the freeze–thaw procedure significantly compromises stem cell survival or proliferation, leading to infertility after transplantation. In addition, the effects of cryopreservation on stem cell viability or proliferation have not been well characterized.

In the present study, the effect of freezing on stem cell colonization efficiency was evaluated, and the feasibility of restoring fertility via the transplantation of frozen stem cells examined. It was demonstrated that cryopreserved stem cells retain significant regenerative potential. Moreover, the successful birth of healthy offspring from frozen stem cells by natural mating supports the future application of frozen stem cells in the treatment of male infertility.

Materials and methods

Donor mice and cell collection

Donor cells used for the quantification of stem cells (first experiments) were collected from the testes of a transgenic mouse line B6-Tg(Rosa26)26Sort (designated ROSA) purchased from The Jackson Laboratory (Bar Harbor, ME, USA). This mouse expresses the E. coli LacZ transgene in all of the spermatogenic cells in the seminiferous tubules (Nagano et al., 1999). The transplanted donor cells can be detected by LacZ staining with the substrate 5-bromo-4-chloro-3-indolyl α-D-galactosidase (X-gal) (Nagano et al., 1999). Since the LacZ transgene is expressed at all stages of the donor spermatogenic cells, spermatogenesis from donor stem cells can be identified by LacZ staining after incubation with X-gal (Nagano et al., 1999).

Donor cells used for fertility experiments (second experiments) were isolated from the testes of a transgenic mouse line C57BL/6 Tg14(act-EGFP)OsbY01 (designated Green) provided by Dr M Okabe (Osaka University) (Okabe et al., 1997). The spermatogonia and spermatocytes of these mice express the gene for the enhanced green fluorescent protein (EGFP), the amount of which gradually decreases after meiosis. Only the male pups (6-day-old) or adults (6–8 weeks of age) that were positive for the transgenes were used for transplantation experiments. Cells were collected from the testes of 6-day-old Green mouse pups or from the cryptorchid testes of adult Green mice, 2–3 months after the operation. It has been shown that these testes are enriched for stem cells, due to the absence of differentiated germ cells (Shinohara et al., 2000; 2001); therefore, they should improve colonization efficiency and facilitate offspring production. Cryptorchid testes were produced as previously described (Shinohara et al., 2000).

Single-cell suspensions were prepared from the donor testis by two-step enzymatic digestion, as described previously (Ogawa et al., 1997). In brief, testis cells were digested with 1 mg/ml collagenase (type IV; Sigma, St Louis, Missouri, USA) for 15 min, followed by 0.25% trypsin/1 mmol/l EDTA (both from Invitrogen, Carlsbad, CA, USA) for 10 min.

Cryopreservation of testis cells

For cryopreservation, dissociated testis cells were suspended in a cell cryopreservation solution (Cellbanker; DIA-IATRON, Tokyo, Japan, www.mk-iatron.jp), which contains dimethyl sulphoxide and 10% fetal calf serum (FCS). Aliquots of ~1 ml cell suspension, containing 10^7 donor testis cells, were transferred to 1.5-ml polypropylene cryotubes (Sumitomo Bakelite, Tokyo, Japan), placed in a freezing container, and frozen at –80°C for 1 day, after which the cryotubes were plunged into liquid nitrogen. The cryotubes were thawed in a water-bath at 37°C, and 10 ml of Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FCS (DMEM/FCS) was added drop-wise. After washing by centrifugation, the cells were suspended in DMEM/FCS and kept on ice until transplantation into the testes. Cell viability was assessed by trypan blue staining. Only live germ cells were counted for transplantation.

Recipient mice

Donor cells were transplanted into the testes of C57BL/6 (B6) mice (6–12 weeks old) or WBB6F1-W/W^+ (W) mice (either 5–10 days old or 6–12 weeks old; see Table I for experimental design) (Silvers, 1979), which were purchased from the Shizuoka Laboratory Animal Center (Hamamatsu, Japan). The B6 males were treated with busulfan (44 mg/kg bodyweight) at 6 weeks of age to destroy the endogenous germ cells, and used at least 1 month after busulfan injection. Busulfan treatment allows donor cell colonization by destroying the endogenous spermatogonial stem cells (Bucci and Meistrich, 1987; Brinster and Zimmerman, 1994; Shinohara et al., 2002b). Thus, it mimics the disrupted spermatogenesis that occurs in oncology patients. Two different stages of the W recipients, namely immature (5–10 days old) and mature (6–12 weeks old) mice were also used. W mice lack endogenous spermatogenesis due to mutations in the c-kit tyrosine kinase gene (Geissler et al., 1988), which is normally expressed on germ cells. Both chemically castrated and congenitally infertile recipients have been shown to be capable of generating spermatogenesis from transplanted fresh stem cells (Brinster and Avarbock, 1994; Mahato et al., 2000; Ogawa et al., 2000; Shinohara et al., 2001).

Transplantation procedure

The experimental design for transplantation is summarized in Table I. In experiments using B6 recipients, approximately 10 µl of the donor cell suspension was introduced into the seminiferous tubules of the testis, whereas only 3 µl of the suspension was injected into the adult W testis, which is smaller. When immature W recipients (5–10 days old) were used as recipients, 2 µl of the donor cell suspension was injected. Cells were introduced into the efferent ducts of the recipient testes (Ogawa et al., 1997). In each testis, 75–85% of the tubules were filled with the cell suspension. The adult recipient mice were anaesthetized with a tribromoethanol injection (640 mg/kg), while the pup recipients were placed on ice to cause hypothermia-induced anaesthesia (Shinohara et al., 2001). In order to avoid long-term exposure to ice (which adversely affects the survival of recipients), the cells were injected only into the right testis in immature W recipients, whereas cells were injected into the both sides in mature recipients.

In the first experiments, frozen cells were suspended in DMEM/FCS at a concentration of 7.5×10^7 to 3.0×10^8 cells/ml, whereas the concentration of fresh cells was 10^7 cells/ml, because the recovery of frozen–thawed cells varied between experiments. In the second experiments, the frozen donor cells were suspended in DMEM/FCS at a concentration of either 10^7 cells/ml (B6 or mature W recipients) or 3×10^7 cells/ml (immature W recipients).

Microscopy

Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin wax and then cut into sections at 12-µm intervals. All sections were stained with haematoxylin and eosin. Four histological sections were taken from the testes of each mouse.
transferred to the oviducts of Day 1 pseudopregnant ICR females. Embryos that reached the 2-cell stage after 24 h in culture were (B6D2F1) oocytes, which were collected from superovulated females. Freeze–thawed spermatogonial stem cells (see text for details). Yanagimachi, 1995) of donor testis cells into C57BL/6
Microinsemination was performed by ICSI (Kimura and microinsemination, as described previously (Ogura fragments. The donor testis cell suspension was refrozen before germ cells collected mechanically by repeated pipetting of the tubule
Donor-derived EGFP-positive colonies were identified under a fluorescent microscope (MZ FLIII; Leica, Tokyo, Japan), and the germ cells collected mechanically by repeated pipetting of the tubule fragments. The donor testis cell suspension was refrozen before microinsemination, as described previously (Ogura et al., 1996). Microinsemination was performed by ICSI (Kimura and Yanagimachi, 1995) of donor testis cells into C57BL/6×DBA/2 F1 (B6D2F1) oocytes, which were collected from superovulated females. Embryos that reached the 2-cell stage after 24 h in culture were transferred to the oviducts of Day 1 pseudopregnant ICR females. All of the animal experimentation protocols were approved by the Institutional Animal Care and Use Committee of Kyoto University.

Results
Colonization efficiencies of fresh and cryopreserved spermatogonial stem cells
The effect of freezing on stem cell potential was evaluated in the first set of experiments. Donor cells were collected from the testes of adult ROSA mice and cryopreserved for 5 months in liquid nitrogen. After thawing, the mean (± SEM) viability index of the freeze–thawed testis cells, as indicated by trypan blue exclusion, was significantly reduced (67.4 ± 5.9%; n = 8) compared with that in fresh cells (93.6 ± 1.7%; n = 7; P < 0.01). The average recovery of the originally frozen cell population was 37.6 ± 5.1% (n = 8).

To compare the repopulation efficiency, both viable frozen and viable fresh cells were transplanted into the seminiferous tubules of congenitally infertile W recipients or busulfan-treated mouse recipients. Two experiments were performed, and the number of colonies was counted for each recipient testis, 2 months after transplantation. The analyses of W recipient testes revealed that the extent of colony generation from frozen stem cells was 11.7-times that from fresh stem cells (62.1 versus 5.3 colonies per 3 × 10^5 donor cells; P < 0.05) (Figure 1a and b; Table II). Likewise, the frozen cells showed higher stem cell activities in the busulfan-treated testis (45.9 versus 9.0 colonies per 1 × 10^5 donor cells); the number of colonies from frozen donor cells was 5.1-times higher than that from fresh donor cells (P < 0.01) (Table II).

### Table I. Experimental design

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Donor⁵</th>
<th>Recipient⁶</th>
<th>Type of cells injected</th>
<th>Donor cell concentration (cells × 10^5/ml)</th>
<th>Volume injected (µl)</th>
<th>No. of animals injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>First experiment</td>
<td>ROSA, adult, untreated</td>
<td>W, adult</td>
<td>Frozen</td>
<td>7.5–30</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>B6, adult busulfan</td>
<td>W, adult</td>
<td>Frozen</td>
<td>7.5–30</td>
<td>10</td>
<td>4</td>
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<tr>
<td></td>
<td>B6, adult busulfan</td>
<td>B6, adult busulfan</td>
<td>Fresh</td>
<td>100</td>
<td>3</td>
<td>6</td>
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<tr>
<td></td>
<td>B6, adult busulfan</td>
<td>B6, adult busulfan</td>
<td>Fresh</td>
<td>100</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Second experiment</td>
<td>GFP, adult cryptorchid</td>
<td>B6, adult busulfan</td>
<td>Frozen</td>
<td>100</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>B6, adult busulfan</td>
<td>W, adult</td>
<td>Frozen</td>
<td>100</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>B6, adult busulfan</td>
<td>B6, adult busulfan</td>
<td>Frozen</td>
<td>30</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>W, pup</td>
<td>W, pup</td>
<td>Frozen</td>
<td>30</td>
<td>2</td>
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<td></td>
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<td>GFP, pup</td>
<td>Frozen</td>
<td>30</td>
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</tr>
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</table>

First experiments were designed to evaluate effect of freezing on spermatogonial stem cells; second experiments were designed to derive offspring from freeze–thawed spermatogonial stem cells (see text for details).

ROS A adult: 6–8 weeks old; GFP adult: 14–20 weeks old; GFP pup: 6 days old.

W adult: 6–12 weeks old; B6 adult: 10–12 weeks old; W pup: 5–10 days old.

*Analysis of recipient testes*

In the first experiments, the recipient mouse testes were recovered 2 months after the injection of donor cells, and analysed by X-gal staining as described previously (Nagano et al., 1999). Individual blue-stained stretches of the seminiferous tubules (colonies) in the recipient testes represented spermatogenesis from transplanted stem cells, as the other testis cells could not regenerate spermatogenesis and the endogenous recipient spermatogenesis did not stain positive. Each blue colony is thought to arise from a single transplanted stem cell (Nagano et al., 1999). Therefore, the number of blue colonies represents the number of stem cells in the injected cell population.

In the second experiments, donor cell colonization was evaluated by histological sectioning, because recipients showed extensive donor cell colonization long after transplantation. Each slide was viewed at a magnification of ×400 for the analysis. To assess the level of spermatogenesis in the recipient testis, the numbers of tubule cross-sections with evidence of spermatogenesis (defined as the presence of multiple layers of germ cells in the entire circumference of the seminiferous tubule) or lacking evidence of spermatogenesis were recorded for three sections from each testis, and at least 500 seminiferous tubules were counted. Statistical analyses were performed using Student’s t-test.

*Microinsemination*

Donor-derived EGFP-positive colonies were identified under a fluorescent microscope (MZ FLIII; Leica, Tokyo, Japan), and the germ cells collected mechanically by repeated pipetting of the tubule fragments. The donor testis cell suspension was refrozen before microinsemination, as described previously (Ogura et al., 1996). Microinsemination was performed by ICSI (Kimura and Yanagimachi, 1995) of donor testis cells into C57BL/6×DBA/2 F1 (B6D2F1) oocytes, which were collected from superovulated females. Embryos that reached the 2-cell stage after 24 h in culture were transferred to the oviducts of Day 1 pseudopregnant ICR females.

Figure 1. Comparison of the stem cell activities of fresh and freeze–thawed ROSA testis cells after transplantation into infertile W recipient mice. (a) Macroscopic appearance of W recipient testes after transplantation of fresh (3 × 10^7 cells injected; left) or freeze–thawed (3 × 10^7 cells injected; right) ROSA testis cells. The blue coloration represents donor-derived spermatogenesis. Note the enhanced X-gal staining and increased level of donor cell-derived spermatogenesis from freeze–thawed donor testis cells, despite the lower concentration of injected cells. (b) Histological section of a recipient testis 2 months after transplantation with freeze–thawed stem cells. Note the normal appearance and organization of the germ cells, and the presence of spermatozoa (arrow). Haematoxylin and eosin staining. Scale bars: (a) = 1 mm; (b) = 25 µm.
Taken together, these results demonstrate that freeze–thawed testis cells had higher stem cell activities than fresh donor cells.

**Derivation of offspring from frozen stem cells**

In the second set of experiments, attempts were made to restore fertility to the two types of infertile recipients—the W and busulfan-treated B6 mice—using transplantation of frozen stem cells. The cells were frozen for 2 to 7 weeks, thawed, and microinjected into the seminiferous tubules of the infertile recipients. The viability indices of the pup and cryptorchid testis cells were 68.6 ± 7.3% (n = 3) and 71.0 ± 2.1% (n = 5) respectively. Interestingly, the average recovery was significantly higher than that seen in the first experiment for the mature adult testis; 72.8 ± 10.7% (n = 3) and 69.2 ± 5.5% (n = 5) of the original populations of pup and cryptorchid testis cells respectively were recovered after freeze–thawing. The recipients were housed with B6 wild-type females to determine whether spermatogonial transplantation restored fertility. At least two experiments were performed for each type of transplant.

The results of the fertility experiments are shown in Table III. Four of the eight (50%) W pup recipients sired offspring within 72–190 days of transplantation with the frozen donor cells. Three of the fertile males received adult cryptorchid donor cells, and offspring were also obtained from one recipient that received pup donor cells. The donor cell origin of the offspring was confirmed by green fluorescence under ultraviolet (UV) light. At the time of sacrifice, the mean weight of the fertile recipient testes was significantly higher (40.7 ± 7.3 mg; n = 4) than that of the infertile recipients (22.6 ± 2.2 mg; n = 4; P < 0.05) or the untransplanted controls (10.4 ± 0.8 mg; n = 7; P < 0.01) (Figure 2a). Histological analyses also revealed more extensive donor germ cell colonization of the fertile recipient testes (76.3 ± 6.6%; n = 4) than of infertile recipient testes (32.8 ± 3.0%; n = 4; P < 0.001) (Figure 2b and c). Spermatogenesis in the recipient testes arose exclusively
from donor stem cells, as the stem cells in the W recipient could not undergo spermatogenesis (Brinster and Zimmermann, 1994; Ogawa et al., 2000; Shinohara et al., 2001). However, the restoration of spermatogenesis occurred in all eight immature W recipient testes, and spermatozoa were observed in 87.5% (7/8) of epididymis sections, which suggests potential fertility. The four recipients that produced progeny remained fertile up to the time of analysis; that is, at least 228 days after transplantation (Figure 2d; Table IV), which indicates that the transplanted stem cells underwent continuous division and normal differentiation. Taken together, these results demonstrate that the transplantation of frozen stem cells from the testes of pups or cryptorchid adults restored normal fertility to congenitally infertile W recipients.

By contrast, the restoration of fertility was not efficient in adult recipients. Although one of nine adult W recipients achieved fertility through natural mating, the offspring was obtained 221 days after transplantation, and a smaller percentage of the epididymides contained spermatozoa as compared with the recipient pups (55.6 versus 87.5%). In addition, none of the busulfan-treated recipients became fertile after 7 months. Donor-derived spermatogenesis occurred in the testes of both types of recipients (Figure 3a), though the level of spermatogenesis was significantly lower than that in the pup recipients. A similar histological profile was found when one of the busulfan-treated recipient testes was examined 12 months after transplantation (Figure 3b and c).

In order to overcome the infertility of the busulfan-treated recipient mice, an in-vitro microinsemination technique was employed which is commonly used to derive offspring from infertile animals and humans (Palermo et al., 1992; Kimura and Yanagimachi, 1995). One of the busulfan-treated recipient mice was sacrificed 108 days after transplantation with frozen–thawed cells from pup testes, and live spermatogenic cells were recovered by repeatedly pipetting colonized tubule fragments, as identified by EGFP gene expression under UV light. The donor testis cell suspension containing differentiated germ cells was frozen and stored until microinsemination. After storage for 3 days, the mature spermatozoa or elongated spermatids from the frozen–thawed testis were injected into B6D2F1 oocytes. About 80% of oocytes developed into 2-cell forms within 24 h, irrespective of the male germ cells used. All 101 diploid zygotes constructed with 80 spermatozoa and 21 elongated spermatids were transferred into the oviducts; of these, 68 (67%) implanted into the uterus and a total of 31 pups was born (31% efficiency). Donor origin was confirmed by fluorescence under UV light. The offspring were proven to be fertile.

### Discussion

Spermatogonial stem cells represent an attractive cell population for the preservation of male germlines. These cells, due to their self-renewal activities, have an enormous capacity to regenerate from a small basal population, and to produce limitless numbers of mature spermatozoa (Meistrich and...
van Beek, 1993; de Rooij and Russell, 2000). In addition, unlike spermatozoa, frozen stem cells can undergo meiosis, thereby maintaining genetic diversity in the male germline. They are also easy to handle, as the same protocol and inexpensive equipment may be used to freeze spermatogonial stem cells from a variety of species (Ogawa et al., 1999; Dobrinski et al., 2000; Brook et al., 2001; Nagano et al., 2001; 2002; Izadyar et al., 2002; Schlatt et al., 2002a). Thus, spermatogonial stem cells have several unique advantages over frozen sperm. In the present experiments, a commercially available cryopreservation solution was used that is commonly used to freeze somatic cells. This was because it was also effective for freezing stem cells in testis pieces, despite low permeation into the tissue (Shinohara et al., 2002a). Furthermore, the use of a commercially available reagent simplified the freezing procedure. The present results indicated that spermatogonial stem cells are relatively resistant to freezing compared with other spermatogenic cells, and suggested that any freezing protocol commonly used for somatic cells would be applicable for stem cells. The present results also demonstrated the first successful application of a technique to produce offspring from cryopreserved stem cells, and indicated that the transplantation of freeze–thawed stem cells is a potentially useful approach to restoring fertility to infertile males.

The results of the first experiment showed that frozen testis cells contain significantly higher stem cell concentrations than fresh testis cells. This result was unexpected, because in a previous study the efficiency of frozen cell colonization was seen to be very low and variable (Avarbock et al., 1996). The high survival rate of stem cells contrasts with that of the more-differentiated spermatogenic cells, which undergo significant damage during the freeze–thaw procedure (Hafez, 1993; Ogura et al., 1996; Glenister and Thornton, 2000), and probably reflects a unique biological characteristic of stem cells. It is known that stem cells are highly resistant to a variety of agents that damage the testis; the stem cell is the last cell type to be destroyed after irradiation or chemical insult, and it can regenerate via self-renewing division to complete spermatogenesis (Meistrich and van Beek, 1993; de Rooij and Russell, 2000). Therefore, it is reasonable to speculate that a large proportion of the stem cells survived the cryopreservation procedure based on their higher viability, while the remainder of the testicular cells underwent significant damage and died. The net result was an increase in the relative concentration of stem cells in the freeze–thawed mixed cell population. This also explains the higher cell recovery rates of the freeze–thawed pup and cryptorchid testis cells, both of which lack differentiated germ cells. Thus, the superior resistance of stem cells to cytotoxic damage can explain their high survival rates, and confers an advantage on these cells in cryopreserved populations.

The ultimate goal of the frozen stem cell technology is to generate offspring from the preserved stem cells. Since spermatogonial transplantation is a potentially effective therapy for both congenital and acquired infertility in humans, an investigation was made as to whether the technique was useful for fertility restoration in the mouse infertility models of W and busulfan-treated mice. The testicular environments are probably abnormal in both models; busulfan treatment damages the Sertoli cells, and may reduce the efficiency of spermatogenesis (Nagano et al., 1999), whereas the Sertoli cells in W mice have never been exposed to germ cells and may be incapable of physiological responses to germ-cell signal (Ogawa et al., 2000). Thus, the seminiferous tubule microenvironments are probably different in the two models, which might affect the outcome of transplantation.

Interestingly, the immature W mice were able to sire offspring most efficiently by natural mating, although this result might reflect recipient age rather than recipient type. A previous study showed that the age of recipients was critical for the success of spermatogonial transplantation: immature W testes not only had a 10-fold higher stem cell colonization efficiency than mature W testes, but also allowed faster growth of stem cell colonies (Shinohara et al., 2001). Importantly, the higher success rate in immature recipients has a direct clinical implication for fertility protection in young cancer patients who do not have sufficient sperm for freezing purposes (Blatt, 1999). It is encouraging to find that only a few hundred stem cells (~1% of a donor testis) can restore the fertility of immature recipients as early as 3 months after transplantation. In contrast, the restoration of fertility to an adult generally takes at least 5 months and requires higher cell numbers, even when fresh donor cells are used (Ogawa et al., 2000; Shinohara et al., 2000). Taken together, these results indicate that frozen stem cells are fully functional after cryopreservation, and suggest the potential advantage of immature recipients for offspring production.

On the other hand, frozen stem cell transplantation was relatively inefficient in adult recipients. Although the donor testes used in this study were relatively enriched for stem cell activity, only one of the males became fertile, at 7 months after transplantation. The absence of sperm in the epididymides of many recipient mice suggests that donor-derived sperm production was insufficient. In addition, it is also possible that sperm in the spermatogenic colonies may not be normal. Indeed, missing layers of germ cells or abnormalities in the elongation phase of spermatogenesis were found in the spermatogenic colony after spermatogonial transplantation (Russell et al., 1996), and busulfan-treated adult recipients rarely become fertile after spermatogonial transplantation (Griswold et al., 2001; Brinster et al., 2003). Fertility in busulfan-treated recipients was achieved in cases when a lower dose of busulfan was used to maintain some level of endogenous spermatogenesis (Brinster and Avarbock, 1994; Kent Hamra et al., 2002; Zhang et al., 2003). Thus, low levels of donor-derived spermatogenesis and potential abnormalities in the sperm may account for the inefficiency of fertility restoration in the adult recipients.

In the present study, the infertility of the busulfan-treated adult recipients was successfully overcome by microinsemination (Palermo et al., 1992; Kimura and Yanagimachi, 1995), demonstrating that at least some spermatozoa in the busulfan-treated recipients are functionally normal. An important advantage of this technique is that it allows fertilization from a small number of spermatozoa that are generated in a tiny
segment of the seminiferous tubule. Therefore, it allows offspring production from infertile recipients that have a level of spermatogenesis that is insufficient for natural mating. Clearly, the restoration of natural fertility is one of the advantages of spermatogonial transplantation. However, as the results of the present study show, not all recipients become fertile after transplantation, and the success of the technique depends on extensive colonization by donor cells and long periods of stem cell proliferation, particularly in adult recipients. In contrast, microinsemination allows offspring production at 2–3 months, when mature sperm are first observed in spermatogenic colonies in mice (Nagano et al., 1999), thereby shortening the period for fertility restoration. Given that the technique is well established in humans (Palermo et al., 1992; Silber, 1995), microinsemination will be useful in overcoming the slow growth of stem cells in humans, and will complement spermatogonial transplantation in case of long-term infertility.

It is necessary to note here that the present success in mice may not be simply extrapolated to humans due to the different structure and biology of the human testis. In fact, the ability to restore fertility using spermatogonial transplantation has not been demonstrated for any species except mice and rats (Brinster and Avarbock, 1994; Kent Hamra et al., 2002; Zhang et al., 2003). Nonetheless, the present results highlight the promising aspects of using frozen stem cell technology in humans. As the results show, the technique will most likely benefit prepubertal patients in terms of fertility protection (Aslam et al., 2000), while adult patients may require microinsemination to obtain offspring. At present, there are other approaches to preserve male germ cells, such as testis piece freezing (Nugent et al., 1997; Hovatta, 2001; Honaramooz et al., 2002; Schlatt et al., 2002b; Shinohara et al., 2002a) or fresh tissue grafting (Honaramooz et al., 2002; Schlatt et al., 2002b; Shinohara et al., 2002a), and healthy isogenic (Shinohara et al., 2002a; Schlatt et al., 2003) or xenogenic offspring were born with these procedures (Shinohara et al., 2002a). However, spermatogonial transplantation is the only method that restores natural fertility to the host, and it will be important to identify circumstances when this technique may be applied in clinical situations.

The results from the present study showed clearly that it is essential to achieve extensive donor cell colonization in order to restore normal fertility, and that immature recipients have better success rates. Clearly, another critical factor to increase colonization is the number of stem cells in the donor cell population. The donor testes used in this study were relatively enriched for stem cell activity (Shinohara et al., 2000; 2001). However, the percentage of stem cells in the normal adult testis is significantly lower; stem cells comprise approximately 0.02% of the adult testis cell population (Meistrich and van Beek, 1993). As only a limited number of stem cells can be recovered from a small biopsy sample from a patient (Brook et al., 2001), the enrichment of stem cells alone does not necessarily ensure high stem cell recovery. From this viewpoint, one of the approaches to overcome this problem would be to develop methods to expand spermatogonial stem cells

in vitro. If it is possible to obtain large number of stem cells by in-vitro culture from a small biopsy, this will greatly enhance the level of donor cell colonization and offspring production. Studies are now in progress to culture stem cells to increase their number (Nagano et al., 1998; Has thorpe et al., 2000; Izadyar et al., 2003). Once established, the combination of in-vitro culture method with transplantation and freezing technologies of spermatogonial stem cells will be a powerful technique for fertility restoration.

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References


Transplantation of cryopreserved stem cells


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