Germline niche transplantation restores fertility in infertile mice

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BACKGROUND: Stem cells interact closely with their microenvironment or niche, and abnormalities in niche compromise the self-renewing tissue. In testis, for example, Sertoli cells interact with germ cells, and defects in Sertoli cells compromises spermatogenesis, leading to male infertility. However, it has not been possible to restore spermatogenesis from endogenous stem cells in infertile testis with environmental defects

METHODS AND RESULTS: When healthy Sertoli cells from infertile white spotting (W) mouse were transplanted into the seminiferous tubules of infertile Steel (Sl) mouse testis that had defective Sertoli cells, spermatogenesis occurred from Sl stem cells in the recipient testis. On average, 1.1% of the recipient tubules showed spermatogenesis. Furthermore, in a microinsemination experiment with germ cells that developed in the testis, we obtained four normal offspring from 114 successfully injected oocytes. CONCLUSIONS: This study demonstrates that defects in male germline microenvironment can be corrected by Sertoli cell transplantation. Although further improvements are required to enhance the low efficiency of spermatogenesis, the ability to correct environmental defect by niche transplantation has important implications in developing new strategies for treating incurable disorders in self-renewing tissues.

Key words: infertility/niche/Sertoli cells/spermatogenesis/transplantation

Introduction
Spermatogenesis occurs within the seminiferous tubules, which are composed of germ cells, Sertoli cells, and peritubular cells. Although Sertoli cells comprise only 3% of the total testis cell population (Bellvé, 1993), they are the only somatic cells that interact directly with germ cells, thereby constituting the primary cellular component of germline niche (Spradling et al., 2001). Because they provide structural and nutritional support for spermatogenesis, the disruption of this crucial relationship results in male infertility. For example, the lack of expression of membrane-bound Steel factor on Sertoli cells in infertile Steel (Sl) mutant mice prevents the differentiation of spermatogonia that express c-kit receptor, resulting in azoospermia (Flanagan et al., 1991). In contrast, the germ cells in infertile white spotting (W) mice have mutations in the c-kit receptor, causing a similar condition (Geissler et al., 1988). However, when spermatogonial stem cells from Sl mice were transplanted into the testes of W mice that had normal Sertoli cells, complete spermatogenesis occurred and offspring were produced from the transplanted Sl stem cells (Ogawa et al., 2000). Thus, the interaction between Sertoli cells and germ cells is critical for spermatogenesis, and the restoration of physiological interactions between germ cells and Sertoli cells by germ cell transplantation allows the complete differentiation of spermatogonial stem cells. Interestingly, a similar relationship exists in other self-renewing tissues; defects in haematopoiesis and melanogenesis in Sl and W mutants can be rescued by the transplantation of cell populations from Sl mice into W recipient mice (McCulloch et al., 1965; Mayer and Green, 1968). However, the reciprocal transplantation of cells from W mice or wild-type mice into Sl recipients has failed to restore normal function (McCulloch et al., 1965; Mayer and Green, 1968), and it has not been possible to replace the defective microenvironment to rescue stem cells in situ.

Recently, we have shown the feasibility of transplanting Sertoli cells (Shinohara et al., 2003). Sertoli cells isolated from the donor testis could colonize the seminiferous tubules of the infertile recipient testis following microinjection. The colonizing activity was enhanced significantly when the recipient Sertoli cells were eliminated by cadmium treatment or when the donor Sertoli cells were prepared from immature testes in which the Sertoli cells were mitotically active. The dissociated donor cells reaggregated after transplantation to

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form a seminiferous tubule-like structure (‘minitubule’), and spermatogenesis was detected in the recipient testis. Although these studies demonstrated spermatogenesis in the tubule-like aggregates, it has not been possible to induce the recovery of spermatogenesis from endogenous stem cells. This was an important goal, since it is necessary to use endogenous germ cells in the host to rescue infertility in clinical situations. Our present results provide evidence that a similar process of reorganization occurred in vivo with heterologous testis cells in the seminiferous tubules of the recipient mice.

Materials and methods

Animals
Both WBB6F1-Sl/St^d^ and WBB6F1-W/W^v^ mice were purchased from Japan SLC (Shizuoka, Japan). Single cell suspensions were prepared from the testes of neonatal (0–2 day old) W and mature (4–6 week old) Sl mice using a two-step enzymatic digestion as previously described (Ogawa et al., 1997). In brief, the testis cells were digested with 1 mg/ml collagenase (type IV; Sigma, St Louis, MO, USA) for 15 min, followed by digestion with 0.25% trypsin/1 mmol/l EDTA (both from Invitrogen, Carlsbad, CA, USA) for 10 min. The cell suspensions were filtered through nylon mesh with a 30 μm pore size before transplantation.

For transplantation of the prepared testis cells, ~3 μl of the donor cell suspensions were injected into the seminiferous tubules of the testes of each of 4–6 week old Sl recipient mouse through the efferent duct (Ogawa et al., 1997). The cells were injected at a concentration of 8.3 × 10^7 cells/ml. In some experiments, 4 weeks after the transplantation of cells from W mice, the recipient Sl mice were given an additional transplantation of 3 × 10^5 testis cells from another Sl mouse. The injections filled 75–85% of the tubules in all recipient testes. The estimated concentration of Sertoli cells was ~3.7 × 10^7 cells/ml. In both cases, ~2.5 × 10^5 testis cells from W mice (approximately equivalent to one-half W testis) were microinjected into the testes of each Sl recipient.

A total of four experiments was performed. Eighteen Sl testes received only W testis cells, and six Sl testes received both W and additional Sl testis cells by injection into the seminiferous tubules. All of the animal experimentation protocols were approved by the Institutional Animal Care and Use Committee of Kyoto University.

Analysis
Two to 3 months after the transplantation, the mice were killed and the testes were fixed with 10% neutral-buffered formalin and processed for paraffin sectioning. Two histological sections were made from each recipient testis with an interval of 12 μm between sections. All sections were stained with haematoxylin and eosin. Each slide was viewed at a magnification of ×400 to determine the extent of spermatogenesis. The number of tubule cross-sections with or without spermatogenesis, defined as the presence of multiple layers of spermatogenic cells in the entire circumference of the seminiferous tubule, was recorded for one histological section from each testis.

Microinsemination
Two recipients that had been transplanted with testis cells from W mice were killed at 6 months after transplantation. The testes were mechanically dissociated, and live spermatogenic cells were recovered by repeated pipetting of the seminiferous tubules. Round spermatids, as identified by a small round nucleus with a uniquely shaped chromatin mass, were microinjected into oocytes derived from C57BL6×DBA/2 F1 mice. About 80% of oocytes survived the injection procedure irrespective of the day of experiment. Microinsemination was performed as previously described using round spermatids (Kimura and Yanagimachi, 1995). Embryos that reached the 2-cell stage after 24 h in culture were transferred to the oviducts of day 0.5 (the day following sterile mating) pseudopregnant (ICR) females. Live fetuses retrieved on day 19.5 were raised by lactating foster ICR mothers.

Figure 1. Overview of Sertoli cell transplantation. (A) Appearance of infertile white spotting (W) and infertile Steel (Sl) mouse mutants. W (left) and Sl (right) have defects in hematopoiesis, melanogenesis and gametogenesis attributable to defects in the c-kit and Sl genes respectively. Owing to defective melanogenesis, the W and Sl mutants have a white coat colour and are indistinguishable from each other on the basis of appearance, whereas the wild-type C57BL6 mouse (middle) has a black coat colour. (B) A schematic outline of Sertoli cell transplantation. The germ cells in W mice have a defect in the c-kit receptor and cannot transduce the signal from the Sl factor. On the other hand, the Sertoli cells in Sl mice do not express the membrane-bound Sl factor, and cannot induce normal germ cell differentiation. The transplantation of W Sertoli cells into the Sl testis triggers spermatogenesis from the endogenous Sl germ cells, resulting in the production of differentiated germ cells carrying defective Sl genes.
DNA analysis
The genomic DNA was isolated from tissue samples taken from the tail of each offspring using phenol/chloroform extraction, followed by ethanol precipitation. Ten micrograms of DNA were digested with EcoRI, and separated on 1.0% agarose gels. The DNA transfer and hybridization were performed as described previously (Kanatsu-Shinohara et al., 2002). An NciI-BglI fragment of the cDNA of the Sl gene (~430 bp, provided by Dr Y.Matsui) was used as a hybridization probe.

Results
Transplantation of W testis cells to Sl recipients
Owing to mutations in the Sl gene, spermatogenesis in Sl mice is impaired by dysfunction of the Sertoli cells (Flanagan et al., 1991) (Figure 1A, B). The testes of Sl mice are significantly reduced in size (Figure 2A), and no differentiating germ cells can be detected by histological analysis (Figure 2B). However, there are a small number of undifferentiated spermatogonia that can differentiate into mature spermatoozoa when they are exposed to normal Sertoli cells by germ cell transplantation (Ogawa et al., 2000) (Figure 2C). To rescue this spermatogenic defect in Sl mice, testis cells were collected from neonatal W mice, and a dissociated single-cell suspension was microinjected into the seminiferous tubules of 4–6 week old Sl mice. W mice have normal Sertoli cells, but their germ cells cannot differentiate beyond the spermatogonia stage owing to mutations in the c-kit gene (Ohta et al., 2003) (Figure 2D, E). Therefore, any spermatogenesis in the testes of Sl recipients must originate from the Sl spermatogonia, with support provided by the normal Sertoli cells transplanted from the W mice. At 20–30 days after transplantation, some of the recipients received an additional injection of Sl testis cells in order to increase the number of spermatogonial stem cells, because the total number of stem cells in the testes of Sl mice is only ~5% of that in the testes of wild-type mice (Shinohara et al., 2000). The recipient mice were killed at 2–3 months after transplantation, and their testes were analysed histologically to determine the level of spermatogenesis. This time period corresponds to two or three spermatogenic cycles in mice, which would allow sufficient time to recover spermatogenesis from the spermatogonial stem cells (Meistrich and van Beek, 1993; de Rooij and Russell, 2000). Although no evidence of spermatogenesis was found in Sl testis without transplantation (12 testes examined), the restoration of spermatogenesis was observed in 13 of 24 (54%) recipient testes. In testes with spermatogenesis, an average of 2.2% of seminiferous tubules contained germ cells (Figure 2F). The transplantation of additional Sl testis cells enhanced the recovery of spermatogenesis in the recipient Sl testis (Table I), suggesting that...
the number of spermatogonial stem cells in these mice is a limiting factor for restoration of fertility. Spermatogenesis in the Sl testis depended on the transplantation of testis cells from W mice, because no evidence of spermatogenesis was found after autologous transplantation of Sl testis cells. These results indicated that Sertoli cells from the W donor testes colonized the testes of the Sl recipients and provided membrane-bound wild-type Sl factor to the germ cells in the recipients, thus promoting differentiation.

The success of the restoration of spermatogenesis varied significantly among the samples; in some testes, up to eight tubule cross-sections (~5% of the tubules in the plane of the tissue section) contained various stages of spermatogenesis. These differentiated germ cells originate from spermatogonial stem cells, because other spermatogenic cells do not have the capacity for self-renewal and are lost by 35 days after transplantation (Meistrich and van Beek, 1993; de Rooij and Russell, 2000). The spermatogonia, spermatocytes and round spermatids appeared morphologically normal. We also found tubules containing elongated spermatids that were apparently normal in several sections, but their incidence was significantly lower than that of the round spermatids. Spermatogenesis was found in apparently normal seminiferous tubules, but other areas with disorganized spermatogenesis were also detected (Figure 2G, H). In these 'minitubules', the seminiferous tubules had enlarged lumens, and irregular basement membranes subdivided the seminiferous tubules into several minitubules. Although these tubules often contained spermatogonia and spermatocytes undergoing meiosis, the formation of minitubules was not necessarily required for spermatogenesis, because apparently normal spermatogenesis was found in areas without minitubule formation. In addition, we occasionally found other areas in which minitubules were present without evidence of spermatogenesis, suggesting that the formation of minitubules does not always accompany successful spermatogenesis.

**Table I. Spermatogenesis in infertile Steel (Sl) mice by infertile white spotting (W) mouse testis cell transplantation**

<table>
<thead>
<tr>
<th>Donor cells</th>
<th>No. of experiments</th>
<th>No. of injected testes</th>
<th>No. of testes with spermatogenesis (%)</th>
<th>No. of tubule cross-sections with spermatogenesis (%)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sl (control)</td>
<td>2</td>
<td>14</td>
<td>0 (0)</td>
<td>0 (0/1925)</td>
</tr>
<tr>
<td>Sl + W</td>
<td>1</td>
<td>6</td>
<td>5 (83.3)</td>
<td>2.5 ± 0.8 (24/979)*</td>
</tr>
<tr>
<td>W</td>
<td>3</td>
<td>18</td>
<td>8 (44.4)</td>
<td>0.6 ± 0.2 (16/2546)</td>
</tr>
<tr>
<td>Total (W donors)</td>
<td>4</td>
<td>24</td>
<td>13 (54.1)</td>
<td>1.1 ± 0.3 (40/3525)</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM. No evidence of spermatogenesis was found in untransplanted Sl testes (12 testes examined).

*aIn parentheses, total number of tubule cross-sections containing spermatogenesis in all recipient testes/total number of cross-sections examined in all recipient testes.

*bSignificantly more tubules showed spermatogenesis by additional transplantation of Sl testis cells versus W donor cells alone (P < 0.01 by t-test).

Discussion

In this study, we demonstrated that Sertoli cell transplantation rescues spermatogonial stem cells in the defective host microenvironment to allow spermatogenesis and the production of offspring from infertile animals. The result was unexpected, because previous transplantation studies in hematopoietic, melanogenic and spermatogenic systems have failed to demonstrate a beneficial effect in treating defective environment (McCulloch et al., 1965; Mayer and Green, 1968; Shinohara et al., 2003). While stem cell transplantation is a promising approach to the treatment of stem cell disorders (Weissman, 2000), difficulties in collecting and transplanting stromal cells limited the development of cell therapy for microenvironmental disorders (Dexter, 1982; Keating et al., 1982; Simmons et al., 1987). Our study now demonstrates that defects in the microenvironment of male germ cells can be corrected by stromal cell transplantation, which has important implications for the development of new techniques to treat disorders in other self-renewing tissues that are attributed to defective microenvironments.

Several methods have been used to manipulate the microenvironment of spermatogonial stem cells. We and others have shown that virus particles microinjected into the seminiferous...
The wild-type locus produced hybridization bands at 4.3 and 13 kb, 4.3 and 7 kb. From wild-type and Sl/Sl d mice and from the offspring shown in A, which suggest that the animals are either Sl/Sl or Sl+/+. Note white patches transplanted testis cells from W mice donors. (A) Offspring produced from Sl male mice that received spermatids harvested from testes of Sl transplant recipients. (B) The newborn offspring derived from the microinsemination. Note white patches on the belly, which suggest that the animals are either Sl/+ or Sl/Sl. (C) Southern blot analysis of EcoRI-digested genomic DNA from wild-type and Sl/Sld mice and from the offspring shown in A. The wild-type locus produced hybridization bands at 4.3 and 13 kb, the Sl locus produced no bands, and the Sld locus produced bands at 4.3 and 7 kb.

Figure 3. Offspring produced from Sl male mice that received transplanted testis cells from W mice donors. (A) The newborn offspring produced by microinsemination using round spermatids harvested from testes of Sl transplant recipients. (B) The mature offspring derived from the microinsemination. Note white patches on the belly, which suggest that the animals are either Sl/+ or Sl/Sl. (C) Southern blot analysis of EcoRI-digested genomic DNA from wild-type and Sl/Sld mice and from the offspring shown in A. The wild-type locus produced hybridization bands at 4.3 and 13 kb, the Sl locus produced no bands, and the Sld locus produced bands at 4.3 and 7 kb.

tubules can transduce Sertoli cells in situ (Ikawa et al., 2002; Kanatsu-Shinohara et al., 2002). The transduction of Sl Sertoli cells in Sl mice with the wild-type Sl gene induced spermatogenesis from Sl stem cells, and normal offspring were produced. Sertoli cells can also be transduced by microinjection of DNA and subsequent electroporation of entire testis (Yomogida et al., 2002). Although these approaches are useful in that they allow very efficient gene expression in Sertoli cells, there are at least two major drawbacks. First, the genetic rescue of defective Sertoli cell functions requires the identification of the responsible genes, which are currently not well characterized in humans. Second, the transduction of Sertoli cells is accompanied by the potential transduction of the germline cells. Recent studies have shown that virus vectors can be transmitted to offspring after the infection of stem cells (Nagano et al., 2001; Kent Hamra et al., 2002; Kanatsu-Shinohara et al., 2004). These animal studies strongly suggest the potential risk of germline transduction, which precludes the use of virus vectors in clinical situations.

Sertoli cell transplantation provides an alternative method to overcome these problems. The success of the Sertoli transplantation technique probably depends on the unique ability of testicular cells to conserve their histogenic capacities. It was originally shown >25 years ago that dissociated newborn testis cells could reorganize in vitro into histotypic structures under conditions of slow rotation (Ohno et al., 1978; Zenzes et al., 1978). The reconstituted tubule-like structure contained germ cells, Sertoli cells and myoid cells, among others. The morphogenic activity is suppressed at the onset of puberty, but it can be induced if germ cells are removed (Zenzes and Engel, 1981). In our previous study, we extended this observation by demonstrating that dissociated testis cells reorganize into tubule-like structures in vivo after transplantation into seminiferous tubules (Shinohara et al., 2003). The critical finding of this in vivo study, in contrast to the previous in vitro studies, was the induction of spermatogenesis in the tubule-like aggregates; whereas spermatogenesis could not be induced in the reaggregated tubules in vitro, spermatogenesis occurred efficiently in vivo. The normal Sertoli cells from the W mice mingled with the defective Sl Sertoli cells of the recipients to reform tubule structures and provide membrane-bound wild-type Sl factor to the germ cells of the Sl mice to permit spermatogenesis, indicating the considerable flexibility of the seminiferous tubule.

The most striking result of the present study was the production of offspring from the infertile Sl mice. The efficiency of offspring production was very low after microinsemination, which suggests that not all the germ cells may have undergone normal development. Another study also reported a lower development rate of embryos in microinsemination experiments using germ cells that developed after germ cell transplantation (Goossens et al., 2003). Nevertheless, our result clearly demonstrates that at least some of the germ cells produced after Sertoli cell transplantation were functionally normal. Thus, the transplantation technique not only provides a method for basic studies on spermatogenesis but may also provide a new method for the treatment of male infertility. Currently, very little is known about the genes responsible for human male infertility, and no effective treatments are available for infertile men with potential Sertoli cell defects. The testes of Sl mice have histological features similar to those observed in the clinical condition known as Sertoli cell-only syndrome, which is found in 5–15% of infertile men (Del Castillo et al., 1947). Although germ cell transplantation may be useful in some cases, the technique is inevitably accompanied by ethical problems and is only useful for infertile men with germ cell defects. Nevertheless,
given our results, it may be anticipated that healthy Sertoli cells from a donor testis could be transplanted into a heterologous recipient who has defective Sertoli cells to induce spermatogenesis from the normal stem cells of the patient. The present technique may also be used to achieve cross-species germ cell transplantation. When germ cells from phylogenetically distant donors, such as from primate or human, are transplanted into immunodeficient mouse recipients, their differentiation arrests at the stage of spermatogonial proliferation, possibly owing to the incompatibilities in the microenvironments (Nagano et al., 2001, 2002). The transplantation of Sertoli cells may enable differentiation of these donors in mouse surrogates, thereby providing a biological assay system to characterize human spermatogenesis.

While our results show the remarkable flexibility of the spermatogonial system, improving the efficiency of spermatogenesis is the next important step required to facilitate a wide range of studies using Sertoli cell transplantation. Although premeiotic germ cells continued to proliferate for several months and spermatogenesis occurred up to the round spermatid stage, it was less efficient after the elongated spermatid stage. One possible explanation is that the transplanted Sertoli cells failed to reconstruct the spatially coordinated, cyclic gene expression pattern of normal seminiferous tubules. Alternatively, the limited area of colonization established by the donor Sertoli cells could also influence germ cell differentiation. A previous transplantation study showed that extensive proliferation of the spermatogonia population occurred in small colonies in the recipient tubules, but that meiotic differentiation occurred exclusively in colonies longer than 1 mm (Nagano et al., 1999). However, in this study, Sertoli cell colonization occurs in a limited area without appropriate host conditioning. Endogenous Sertoli cells can be eliminated by treating the seminiferous tubules with cadmium; donor Sertoli cells can colonize extensive areas after such treatment (Shinohara et al., 2003). Cadmium treatment, however, also removes the endogenous germ cells and is thus not appropriate in the present case (Shinohara et al., 2003). It will be important to develop other methods to enhance Sertoli cell colonization without affecting the endogenous germ cell population. The resolution of these problems will not only improve the efficiency of Sertoli cell transplantation techniques but also increase our understanding of spermatogenesis.

Several new therapeutic approaches can be envisaged for human spermatogenic failure. A stem cell transplantation technique is available that allows offspring production from fresh or cryopreserved spermatogonial stem cells (Avarbeck et al., 1996; Ogawa et al., 2000; Kanatsu-Shinohara et al., 2003a). This technique will be particularly useful for restoring fertility to those who become infertile after malignancy therapy by chemicals or radiation. Although no method for fertility protection is currently available for prepubertal boys who do not have sperm, stem cell transplantation will provide a method to recover their fertility, since spermatogenesis occurs by transplantation of spermatogonial stem cells even from immature donors (Shinohara et al., 2001). Recent development of spermatogonial stem cell culture techniques enables in vitro expansion of stem cells from a small biopsy sample for autologous transplantation (Kanatsu-Shinohara et al., 2003b, 2004; Kubota et al., 2004; Ogawa et al., 2004). Future developments might even allow correction of defective genes at the spermatogonial stem cell level, and methods for genetic manipulations are being developed (Kanatsu-Shinohara et al., 2005). In contrast, the Sertoli cell transplantation technique will be used to correct spermatogenic failure due to defects in Sertoli cells. Unlike stem cell transplantation, defective Sertoli cells can be replaced with healthy Sertoli cells from heterologous donors with less ethical restriction. As the method to culture Sertoli cells develops, it will be possible to correct the defect in Sertoli cells to be used for autologous transplantation in vitro. Our successful production of offspring from infertile Sl mice demonstrates the usefulness of the Sertoli cell transplantation technique, and indicates a promising opportunity to develop a new strategy for the treatment of human male infertility.

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