SCF對於精原幹細胞的增生及分化具有調節作用

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Regulation of proliferation and differentiation in spermatogonial stem cells: the role of c-kit and its ligand SCF

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SUMMARY

To study self-renewal and differentiation of spermatogonial stem cells, we have transplanted undifferentiated testicular germ cells of the GFP transgenic mice into seminiferous tubules of mutant mice with male sterility, such as those dysfunctioned at *Steel* (*Sl*) locus encoding the c-kit ligand or *Dominant white spotting* (*W*) locus encoding the receptor c-kit. In the seminiferous tubules of *Sl/Sl^d* or *Sl^{17H}/Sl^{17H}* mice, transplanted donor germ cells proliferated and formed colonies of undifferentiated c-kit (−) spermatogonia, but were unable to differentiate further. However, these undifferentiated but proliferating spermatogonia, retransplanted into *Sl* (+) seminiferous tubules of *W* mutant, resumed differentiation, indicating that the transplanted donor germ cells contained spermatogonial stem cells and that stimulation of c-kit receptor by its ligand was necessary for maintenance of differentiated type A spermatogonia but not for proliferation of undifferentiated type A spermatogonia. Furthermore, we have demonstrated that their transplantation efficiency in the seminiferous tubules of *Sl^{17H}/Sl^{17H}* mice depended upon the stem cell niche on the basement membrane of the recipient seminiferous tubules and was increased by elimination of the endogenous spermatogonia of mutant mice from the niche by treating them with busulfan.

Key words: c-kit, Stem cell factor, SCF, Spermatogenesis, Mouse, *Steel*,

INTRODUCTION

During mammalian spermatogenesis, spermatogonia proliferate and some undergo meiosis to give rise to haploid spermatids, which are then remodeled into spermatozoa. Spermatogonia have traditionally been subdivided into type A or B by the rare or abundant existence of heterochromatin in their nuclei, respectively. In rats and mice, intermediate type spermatogonia can also be defined. In the undifferentiated type A spermatogonial compartment, A_{single} (A_s), A_{paired} (A_{pr}) and $A_{aligned}$ (A_{al}) spermatogonia can be distinguished according to their topographical arrangement on the basement membrane of the seminiferous tubules (Oakberg, 1971; de Rooij, 1973). Although the A_s spermatogonia are considered to be the spermatogonial stem cells, regulation of their proliferation and differentiation is poorly understood.

Recently, a new technique has been developed for germ cell transplantation. Donor mouse germ cells were injected into seminiferous tubules of a recipient mouse testis (Brinster and Zimmermann, 1994); the transplanted cells underwent spermatogenesis and, therefore, the recipient mouse could transmit the donor haplotype to progeny (Brinster and Avarbock, 1994). In a previous paper, we established a technique for transplanting "green germ cells" of GFP transgenic mice, and demonstrated the proliferation and differentiation of colonized spermatogonial stem cells (Ohta et al., 2000). This spermatogenesis of transplanted spermatogonia in the recipient testes has permitted the study of stem cell repopulation in seminiferous tubules. However, stem cell proliferation and differentiation occur simultaneously and, therefore, it is difficult to separately analyze these two processes. A method for analysing cell proliferation without differentiation has been long waited.

A double gene dose of mutant alleles at either the *dominant-white spotting* (*W*) locus encoding the c-kit receptor or the *Steel* (*Sl*) locus encoding the stem cell factor (SCF) or c-kit ligand (KL) has been known to show pleiotropic effects on sterility, hypoplastic anaemia, and depletion of mast cells and melanocytes (see Besmer et al., 1993, for review). In spite of the similarity in the phenotypic expression between *W* and *Sl* mutant mice, the underlying mechanisms are quite different. Depletion of differentiated germ cells in *W/W^v* mice has been attributed to a defect in their precursor cells, whereas the *Sl/Sl^d* mutation causes a defect in the tissue microenvironment. In both cases, germ cells are few, if any, due to an impairment of the proliferation and migration of the primordial germ cells (Bennett, 1956; Mintz and Russell, 1957). In the testis, the SCF is produced by the Sertoli cells, whereas the c-kit receptor is

expressed on the germ cells from the differentiating type A spermatogonia through to pachytene spermatocytes but not expressed on the undifferentiated type A spermatogonia (Motro et al., 1991; Rossi et al., 1991; Tajima et al., 1991; Vincent et al., 1998). In the case of the Sl^{17H}/Sl^{17H} mutant which causes a splicing defect in the cytoplasmic tail of the SCF protein but with a normal extracellular domain, the mutant testis produces quite a number of undifferentiated spermatogonia but, after an initial wave of spermatogenesis, fails to continue spermatogonial differentiation (Brannan et al., 1992). In our previous paper, spermatogenesis was arrested at the stage of the undifferentiated type A spermatogonia, specifically A_{al} spermatogonia in the Sl^{17H}/Sl^{17H} mouse testis (de Rooij et al., 1999). Thus, these results implied that the SCF may be prerequisite for the continuous differentiation of testicular germ cells. Therefore, the use of *Sl* mutants as recipients for spermatogonial transplantation may be able to successfully separate their proliferation from differentiation.

In the present study, germ cell transplantation is performed using the "green" germ cells as the donor, obtained from GFP transgenic mouse, and mutant mice such as W/W^v, Sl/Sl^d and Sl^{17H}/Sl^{17H} as the recipients. Analyzing those transplanted germ cells, we demonstrate that SCF is a prerequisite for maintenance of c-kit-positive differentiated germ cells but not for proliferation of undifferentiated type A spermatogonia. Furthermore, the existence of the stem cell niche in the seminiferous tubules is strongly suggested.

MATERIALS AND METHODS

Mice

Male Sl/Sl^d and W/W^v mice were purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan, at 2 months of age. Sl^{17H}/Sl^{17H} mice were raised in our animal facilities and all mice older than 2.5 months of age were used as recipients or donors. To destroy the spermatogenic cells, 40 mg/kg busulfan was intraperitoneally injected once into the Sl^{17H}/Sl^{17H} mice. The busulfan-treated males were used as recipients 4 weeks after injection. Double transgenic mice (C57BL/6TgN(acro/act-EGFP)OsbN01) carrying both acrosin/eGFP (Acr3-EGFP) (Nakanishi et al., 1999) and pCXN-eGFP (Okabe et al., 1997) transgenes were used as donors of testicular germ cells at 7 days; testicular cells were easily distinguished at all stages due to the presence of eGFP (Ohta et al., 2000).

Transplantation of green germ cells

Donor cells for transplantation were prepared using a two-step digestion procedure to obtain a testicular cell suspension (Ohta et al., 2000). Briefly, after tunica albuginea was removed from a testis, the seminiferous tubules were placed in Dulbecco's modified Eagle's medium (DMEM) buffered with 20 mM Hepes at pH 7.3 containing collagenase type I (1 mg/ml) and hyaluronidase (1 mg/ml) and were then incubated for 15 minutes at 37°C, with manual agitation at 5-minute intervals. The seminiferous tubules were washed twice in calcium-free phosphate-buffered saline (PBS) and then incubated in PBS containing 0.25% trypsin for 15 minutes at 37°C, with manual agitation at 5-minute intervals. After adding a half volume of DMEM containing 10% fetal bovine serum, the cell suspension was filtered through a nylon mesh, 30 μm in pore size, to remove large clumps of cells. The filtrate was centrifuged at 600 ***g*** at 16°C for 5 minutes, and the cell pellet was then resuspended in injection medium (138 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.1 mM KH_2PO_4, 0.1 mM EDTA, 5.5 mM glucose, 5 mg/ml bovine serum albumin, 100 μg/ml DNaseI, and 0.4 mg/ml Trypan blue; Brinster and Avarbock, 1994) at a concentration of 10^8 cells/ml. The transplantation was performed according to the method of Ogawa et al. (1997) via the efferent ductules. Approximately 70-90% of seminiferous tubules were filled with the donor cell suspension as monitored by trypan blue.

Immunohistochemistry

To determine differentiation steps of spermatogonia, serial sections were stained with germ-cell-specific monoclonal antibody (TRA98: Tanaka et al., 1997) or anti-c-kit monoclonal antibody (ACK2: Nishikawa et al., 1991). For this, freshly dissected testes were covered with OTC (Tissue-Tec, Sakura, Tokyo, Japan), quickly frozen and sectioned at 10 μm in a cryostat. The sections were reacted with TRA98 at room temperature or with purified 10 μg ACK2 monoclonal antibody in 1 ml PBS at 4°C overnight for the c-kit receptor. Antibody was detected by the avidin-biotin-peroxidase complex method with diaminobenzidine using the Vectastain elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's recommendation.

Histological analysis and evaluation of colonized stem cells

Recipient *Sl* mutant mice were killed by cervical dislocation at 6 and 10 weeks after transplantation. The testes were fixed with 4% paraformaldehyde for 12 hours and were embedded in glycol methacrylate (Technovit 8100; Heraeus Kulzer GmbH). Histological sections of the whole testes were prepared at 5 μm in thickness. Three sections chosen randomly from the middle of the longitudinal axis of the testes, each separated more than 200 μm, were subjected to fluorescent microscopy, followed by counting colonized seminiferous tubules. After green fluorescence was photographed, the sections were stained with Hematoxylin and observed under a photomicroscope for detailed analysis.

In situ observation of transplanted testis with a fluorescent stereomicroscope under UV light

For in vivo observation and follow-up of the transplanted cells in the seminiferous tubules, mice were anesthetized and the testes were taken out by mid-abdominal incision, exposed to the excitation light of UV and photographed with a Leica DC 200 (Leica Microscopy System Ltd) or a PXL KAF 1400-G2 digital camera (Photometrics Co. Ltd) set to a fluorescent stereomicroscope. After the testes were returned to the scrotum, the abdominal wall was carefully sutured. Hereafter, photos of the same testis were taken in the same way at appropriate time intervals. In the case of the detailed observation of colonized seminiferous tubules, the transplanted testis was decapsulated and the seminiferous tubules were isolated in PBS. Fluorescence-positive tubules were collected on glass slides, mounted with 50% glycerol in PBS and observed under a fluorescent microscope.

RESULTS

Characterization of spermatogonia in donor transgenic and recipient *Sl* mutant mouse testes

TRA98 monoclonal antibody, exclusively recognizing all of the germ cell nuclei (Tanaka et al., 1997), and ACK2 monoclonal antibody, recognizing the extracellular domain of the murine c-kit receptor (Nishikawa et al., 1991), were used. In the testis of donor GFP transgenic mouse at 7 days of age, all germ and somatic cells were in green due to expression of eGFP (Fig. 1A,B) and approximately half of the germ cells were c-kit-positive spermatogonia (Fig. 1C,D; the numbers of germ cells and c-kit-positive cells per 100 tubules were 1207 and 652, respectively). In the testis, c-kit receptor was

expressed from the differentiated A_1 spermatogonia to the pachytene spermatocytes and Leydig cells, but not on the undifferentiated type A spermatogonia (Yoshinaga et al., 1991; Vincent et al., 1998). However, in the case of Sl/Sl^d mutant mice, all of the spermatogonia in tubular cross sections were undifferentiated type A spermatogonia, negative for c-kit receptor. Furthermore, approximately 85% of the tubular cross sections showed no germ cells but only Sertoli cells (Fig. 1E,F; Table 1).

SCF is a prerequisite for maintenance of differentiated type A spermatogonia but not for proliferation of undifferentiated type A spermatogonia

To elucidate the role of SCF in spermatogenesis, the green germ cells were transplanted into seminiferous tubules of Sl/Sl^d mutant testes. 6 weeks after transplantation, all of the donor green germ cells were still spermatogonia in the Sl/Sl^d mutant and did not differentiate further (Fig. 2A,B). Immunohistochemical analysis showed that these undifferentiated type A germ cells were also c-kit receptor negative (data not shown). c-kit receptor is expressed on the germ cells from the differentiating type A spermatogonia through to pachytene spermatocytes but not expressed on the undifferentiated type A spermatogonia. Therefore, these results could indicate that the SCF is prerequisite for maintenance of c-kit-positive differentiated germ cells. Consistent with this view, only undifferentiated type A spermatogonia were maintained in the testis of *Sl* mutant mice carrying the mutated ligand SCF (Table 1). Chronological observation under a fluorescent stereomicroscope showed that the fluorescent seminiferous tubules elongated laterally from 6 to 10 weeks after transplantation without any increase in the fluorescence intensity (Fig. 2C-E), indicating that no differentiation but only proliferation of spermatogonia took place in the seminiferous tubules of *Sl* mutants. In contrast, in the wild-type mouse, differentiation of germ cells was observed through to haploid spermatids (Fig. 2F; Ohta et al., 2000). Furthermore, histological analysis of testicular cross sections showed no differentiated germ cells in the *Sl* mutant from 6 to 10 weeks after transplantation even though the number of colonized tubules in a cross section was increased (Fig. 5). Taken together, these results indicated that SCF was a prerequisite for maintenance of c-kit-positive differentiated germ cells but not for proliferation of undifferentiated type A spermatogonia.

Table 1. Characterization of testicular cross section of *Sl* mutants

Genotype	Tubules containing spermatogonia (%)[a]	Tubules containing c-kit-positive spermatogonia (%)[b]
Sl/Sl^d	14.5±7.4%	0%
Sl^{17H}/Sl^{17H}	36.8±10.3%	0%
busulfan-treated Sl^{17H}/Sl^{17H}	0%	NT

Serial cross sections from 6 testes of 2.5-month-old Sl/Sl^d, Sl^{17H}/Sl^{17H} and busulfan-treated Sl^{17H}/Sl^{17H} mice were stained with TRA98 and ACK2. More than 800 tubular cross sections of each mouse were counted. A percentage of the number of tubules containing TRA98-positive[a] or ACK2-positive[b] cells against a total number of tubular cross sections was examined. NT, not tested. Each value indicates a mean value ± s.d. of six samples.

Identification of the spermatogonial stem cells

Furthermore, to test the differentiation capability of the transplanted spermatogonial stem cells proliferating in the seminiferous tubules of *Sl* mutant mice, these green germ cells were re-transplanted into seminiferous tubules of a W/W^v mouse. From 6 to 10 weeks after re-transplantation, the green germ cells not only proliferated and colonized the seminiferous tubules of W/W^v mouse (Fig. 3A,B) but also underwent complete spermatogenesis (Fig. 3C,D). These results indicate that the undifferentiated type A spermatogonia that had colonized *Sl* mutant testes were spermatogonial stem cells able both to proliferate and differentiate.

Stem cell niche on the basement membrane of the seminiferous tubules

Stem cells are thought to occupy a special environmental location, a so-called niche, generated by neighboring cells to enable the stem cells to undergo the self-renewal (Potten and Loeffler, 1990; Whetton and Graham, 1999). When donor green germ cells were transplanted into the seminiferous tubules of Sl/Sl^d mouse, they settled predominantly in the seminiferous tubules with no endogenous spermatogonia (Fig. 4; Table 2). This observation suggested that the presence of endogenous spermatogonia inhibited anchorage of the exogenous transplanted spermatogonia. This view was further reinforced with the observation that the colonization efficiency of green donor germ cells was about two times higher in Sl/Sl^d than in Sl^{17H}/Sl^{17H} mutant (Fig. 5) associated with the non-existence of endogenous undifferentiated germ cells (Table 1). Next, we tried to confirm the effect of endogenous germ cells on transplantation efficiency, we have eliminated these cells with busulfan. In the Sl^{17H}/Sl^{17H} mouse testis, approximately 37% of tubular cross sections had undifferentiated type A spermatogonia but no spermatogonia survived in the busulfan-treated Sl^{17H}/Sl^{17H} mutant mice (Table 1). As was expected, colonizing efficiency of the donor GFP-labeled germ cells in the seminiferous tubules was much higher in the busulfan-treated than in the non-treated Sl^{17H}/Sl^{17H} mutants (Fig. 5). In addition, the colonizing efficiency in the busulfan-treated Sl^{17H}/Sl^{17H} mouse was the same as in the Sl/Sl^d mouse (Fig.

Table 2. Colonization pattern of transplanted spermatogonial stem cells in the seminiferous tubules of Sl/Sl^d mouse

Exogenous	Endogenous	Exogenous + endogenous	Empty	Total‡
23±3.8	20.5±7.8	0.8±0.9	83.3±12.4	127.5±15.3
(18.4)	(15.8)	(0.6)	(65.2)	(100)
97%*		3%*		

Eight testes of Sl/Sl^d mouse at 14 weeks after transplantation were examined. All of the tubular cross sections in one selected section of each testis were counted (1020 in total). All cross sections of the seminiferous tubules were differentialy counted and the average number of cross sections ± s.d. (%) in each category were demonstrated; 'exogenous' having only transplanted green spermatogonia, 'endogenous' having only endogenous spermatogonia, 'exogenous+endogenous' having both green and non-green endogenous spermatogonia, and 'empty ' having no spermatogonia in the seminiferous tubules.

*,% of tubules in total tubules transplanted by exogenous green germ cells.

‡, average number of total tubular cross sections per one testicular cross section.

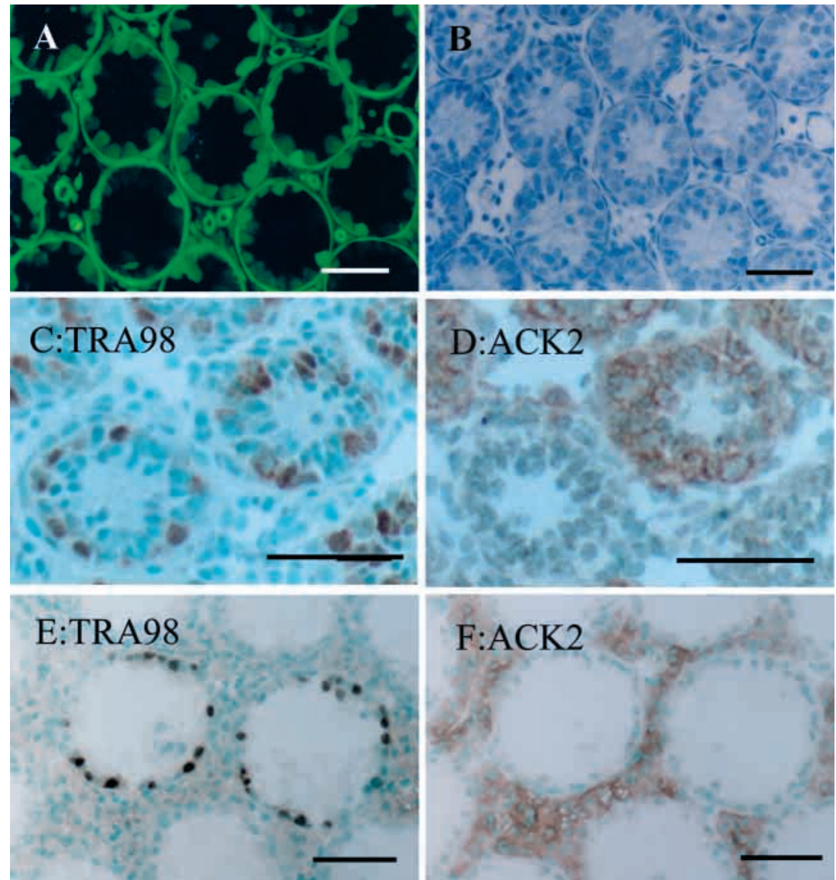

Fig. 1. Characterization of donor transgenic and recipient *Sl* mutant mouse testes. A fluorescent microscopic picture of a testicular cross section of a 7-day-old donor green mouse (A) and photomicroscopic picture of the same section stained with Hematoxylin (B). (C) Immunohistochemical staining of a 7-day-old donor testis with germ-cell-specific monoclonal antibody (TRA98). (D) Immunohistochemical staining of a 7-day-old donor testis with anti-c-kit monoclonal antibody (ACK2). The section D was an immediate subsequent section of the section C. Immunohistochemical staining of a recipient adult *Sl/Sl*^{d} mouse testis with TRA98 (E) and ACK2 (F). Scale bars, (A-D) 50 µm; (E,F) 100 µm.

5). Thus, the presence of the endogenous spermatogonia inhibited anchorage of the exogenous stem cells. These results indicate that the capacity of stem cell niche is limited so that, if part of the niche has already been occupied by the endogenous germ cells, colonization of exogenous transplanted germ cells is retarded. However, if the endogenous germ cells occupying the niche are eliminated by busulfan, the colonization efficiency is increased dramatically.

DISCUSSION

Deletions or mutations of a gene encoding the tyrosine kinase receptor, c-kit, or its ligand, stem cell factor (SCF), resulted in sterility in mice due to the loss of germ cells (Besmer et al., 1993). Studies of these mutants have clearly established the importance of c-kit/SCF interactions for normal development of primordial germ cells (PGCs) and spermatogonia. Detailed histological analyses of *W* and *Sl* embryos demonstrated

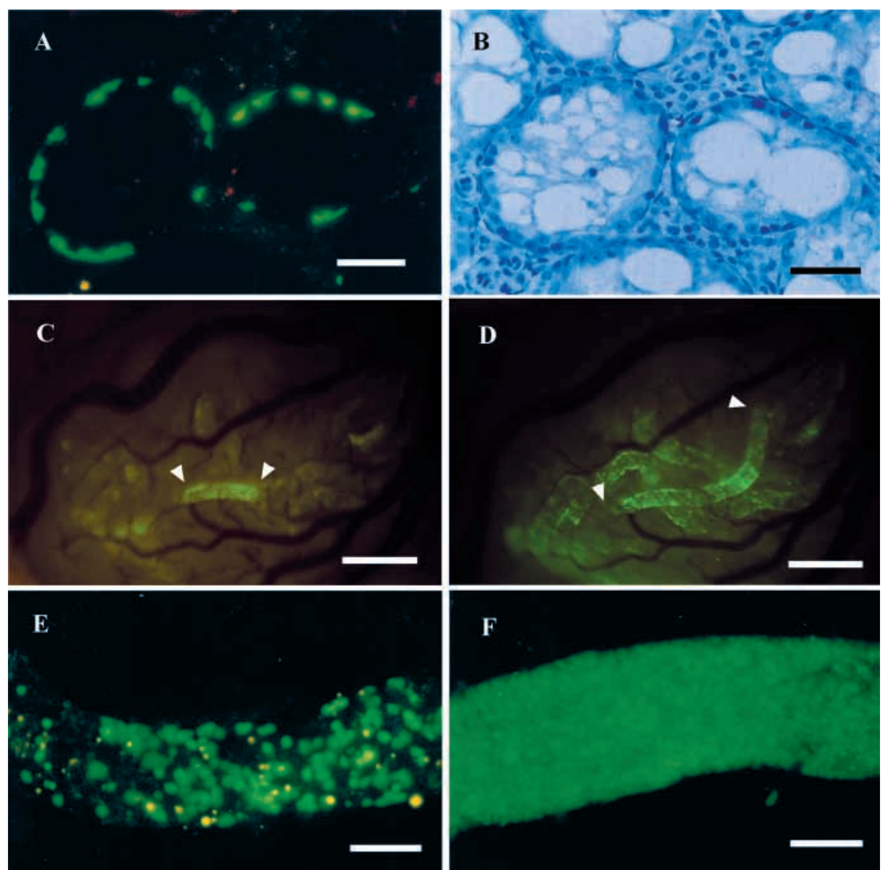

Fig. 2. Transplantation of green germ cells into the seminiferous tubules of *Sl* mutant mouse. 6 weeks after transplantation, donor green cells observed were only spermatogonia in the seminiferous tubules of *Sl/Sl*^{d} mouse. A fluorescent microscopic picture (A) and photomicroscopic picture of the same section stained with Hematoxylin (B) are shown. (C,D) Chronological observation of a whole testis at the same position under a fluorescent stereomicroscope after testicular green cell transplantation. 6 weeks after transplantation (C), donor green germ cells colonized the recipient seminiferous tubules. 10 weeks after transplantation (D), fluorescent seminiferous tubules were elongated. Arrowheads show both ends of a colonized fluorescent seminiferous tubule. An isolated seminiferous tubule of the *Sl/Sl*^{d} mouse (E) and wild-type mouse (F) at 10 weeks after transplantation. The yellow bright spots in E were, probably, degenerated cells. Scale bars, (A,B) 50 µm; (C,D) 0.5 mm; (E,F) 100 µm.

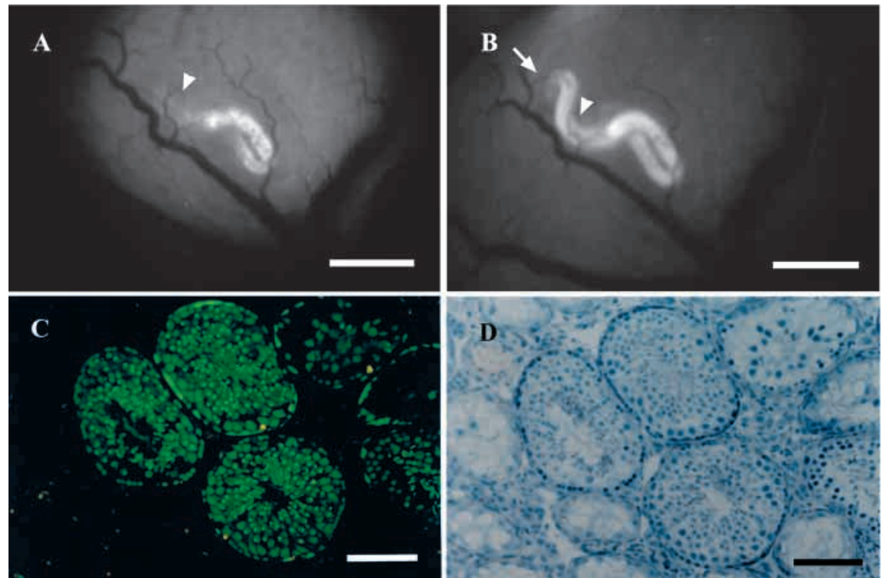

Fig. 3. Identification of the spermatogonial stem cells. Transplanted GFP-labeled undifferentiated type A spermatogonia that had proliferated in the seminiferous tubules of *Sl/Sl^d* mouse were re-transplanted in the seminiferous tubules of *W/W^v* mouse. Chronological observation under a fluorescent stereomicroscope after re-transplantation was carried out. 6 weeks after transplantation, donor green germ cells colonized the recipient seminiferous tubules of *W/W^v* mouse (A). An arrowhead shows an end of a fluorescent seminiferous tubule. At 10 weeks after transplantation (B), the fluorescent seminiferous tubule was more elongated (arrow) than that at 6 weeks after transplantation (arrowhead). 10 weeks after re-transplantation, donor green germ cells were able to complete spermatogenesis, as shown in a fluorescent microscopic picture (C) and a photomicroscopic picture of the same section stained with Hematoxylin (D). Scale bars, (A,B) 0.5 mm; (C,D) 100 μm.

that the c-kit/SCF system played a role in migration and/or proliferation of the primordial germ cells (PGCs) (Bennett, 1956; Mintz and Russell, 1957; McCoshen and McCallion, 1975). Consistent with this notion, in situ hybridization studies showed that c-kit was expressed in the PGCs before and after migration into genital ridges during embryogenesis (Orr-Urtreger et al., 1990). However, in contrast to the process of germ cell development during gestation, the role of c-kit/SCF system in spermatogenesis is not so well understood: phenotypic analysis of the mutant mice was unable to correlate the expression of c-kit and its ligand with their functional role in postnatal gonads, since virtually no germ cells were present in the gonads of *W* or *Sl* mice. Various approaches using *W* and *Sl* mice, including experimental cryptochidism (Nishimune et al., 1980), in vitro organ culture (Nishimune et al., 1984), aggregation chimera (Kuroda et al., 1988; Nakayama et al., 1988) or transplantation of seminiferous tubules (Kuroda et al., 1989), have been used to resolve this issue, but produced no direct clear answer. In our present report, we have succeeded in direct demonstrating the existence of functional spermatogonial stem cells in the population of undifferentiated type A spermatogonia, and further shown that SCF is necessary for differentiation of germ cells but not for spermatogonial stem cell proliferation. Consistent with our transplantation experiments, antibody-blocking studies of Yoshinaga et al. (1991) also supported the role of c-kit/SCF in postnatal male germ cell development. Although both the soluble and membrane-associated forms of SCF were blocked in their experiment, survival and/or proliferation of differentiated type A spermatogonia was only affected, whereas undifferentiated type A spermatogonia was not. In addition, in vitro culture studies by Tajima et al. (1994) indicated that c-kit-positive type A spermatogonia in the testes of 5-day-old mice required SCF for their proliferation, whereas c-kit-negative undifferentiated type A spermatogonia in the testes of 2-day-old mice did not require SCF for their self-renewal and differentiation.

Recently, it has been indicated that the signaling by SCF inhibits p53-induced apoptosis of erythroleukemia cell lines (Abrahamson et al., 1995) and suppresses p53-dependent radiation-induced apoptosis of bone marrow cells (Lotem and Sachs, 1993). Further, deficiency of p53 rescued the male infertility of *W^v/W^v* mice (Jordan et al., 1999). These results suggest that some male infertility could be due to apoptosis involving p53, and this p53 function could be suppressed by SCF. Thus, it is likely that SCF suppresses apoptosis of c-kit-positive cells, thus keeping these cells alive. Consistent with

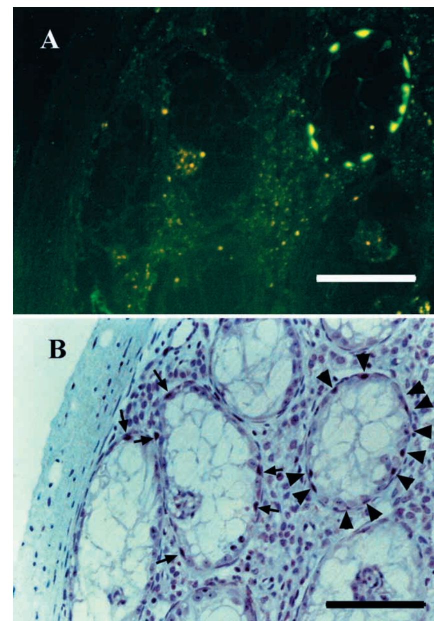

Fig. 4. Settlement of donor green spermatogonia in the mutant seminiferous tubules. 10 weeks after transplantation, a recipient *Sl/Sl^d* mutant was examined. Transplanted donor spermatogonia were settled predominantly in the seminiferous tubules without endogenous spermatogonia. A fluorescent microscopic picture (A) and a photomicroscopic picture of the same section stained with Hematoxylin (B) are shown. Arrows and arrowheads show endogenous and exogenous spermatogonia, respectively. Scale bars, 100 μm.

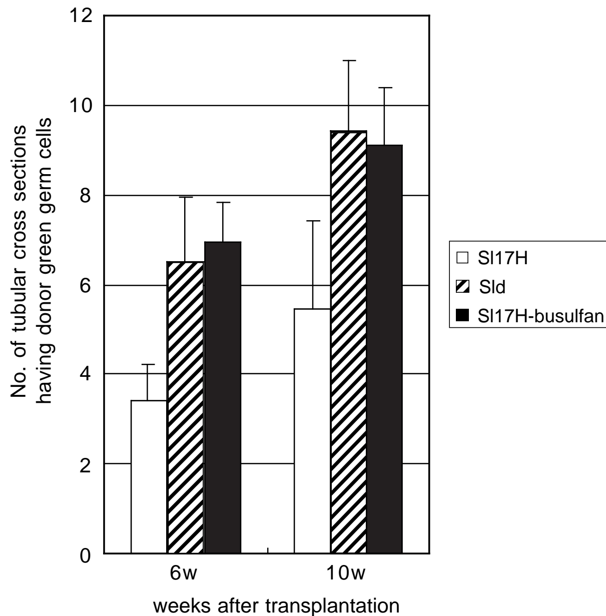

Fig. 5. Settling efficiency of donor green germ cells in the recipient seminiferous tubules. To evaluate transplantation efficiency, the number of fluorescent-positive seminiferous tubules per testicular cross section at a middle of a testis was determined. From a middle portion of one testis, three representative sections, more than 200 μm apart from each other, were chosen to determine the number of fluorescence-positive tubules. In *Sl* mutant mice, the number of total tubules in the middle of the longitudinal axis of the testes was 156.8±18.6 (n=5). 6 and 10 weeks after transplantation, 6 and 11 testes, respectively, were examined in each mutant. Opened, dashed and closed columns indicate Sl^{17H}/Sl^{17H}, Sl/Sl^{d} and busulfan-treated Sl^{17H}/Sl^{17H} mutants, respectively. Each bar indicates a mean value ± s.d. of the data.

this, in our present study, c-kit-positive differentiated germ cells were not observed in the endogenous germ cells of the testes of Sl/Sl^{d}, Sl^{17H}/Sl^{17H} or after transplantation of exogenous green germ cells. These results suggest that c-kit receptor-positive differentiated germ cells could not be maintained in the testis without SCF stimulation.

Undifferentiated type A spermatogonia can be subdivided into A_{single} (A_s), A_{paired} (A_{pr}) or $A_{aligned}$ (A_{al}) spermatogonia by their topographical arrangement on the basement membrane (Oakberg, 1971; de Rooij, 1973) and all of these exist in the Sl^{17H}/Sl^{17H} mutant testis (de Rooij et al., 1999). Spermatogonia in the Sl^{17H}/Sl^{17H} mutant were also able to undergo spermatogenesis, provided that they were transplanted into the seminiferous tubules of W/W^{v} mutant mouse (data not shown). However, at present, it is not yet clear whether all of the A_s, A_{pr} and A_{al} spermatogonia are stem cells. Recently, β1- and α6-integrins were identified as marker molecules of spermatogonial stem cells (Shinohara et al., 1999). Both integrins were expressed from the early male PGCs to the surface of the adult spermatogonia. In addition, flow cytometric analysis of the integrin-enriched cell populations indicated that there were very few c-kit-positive cells in the integrin-selected cell fraction (Shinohara et al., 1999). These data are consistent with our results that undifferentiated type A spermatogonia, including spermatogonial stem cells, did not express c-kit receptor. Furthermore, α3-, α5-, αV-, and β1-integrin subunits were identified on PGCs during or after migration (Anderson et al., 1999). The use of these molecules as markers may facilitate further fractionation and subsequent analysis of the enriched stem cell population to study characteristics of spermatogonial stem cells.

Survival and proliferation of hematopoietic stem cells (HSCs) in vivo are dependent on close association with the bone marrow stroma, which generates many regulatory factors and maintains adhesive interactions with the HSC compartment (Whetton and Graham, 1999). The interactions between the HSC and the stromal environment (the stem cell niche) resemble those of the spermatogonial stem cells and the Sertoli cells in the testis. In addition, β1-integrin is required for hematopoietic precursor cell migration to the fetal liver during development (Hirsch et al., 1996), colonization in hematopoietic organs after transplantation (Williams et al., 1991) and mobilization of these cells from bone marrow (Craddock et al., 1997). Thus, the molecular basis of the stem cell system between hematopoiesis and spermatogenesis appears to be very similar. In our previous paper, we have demonstrated that the spermatogonial stem cells proliferated along the basement membrane before differentiation (Ohta et al., 2000). Furthermore, in the present paper, we have demonstrated that the stem cell niche existed on the basement membrane of the seminiferous tubules. Taking these results together, it is possible that the spermatogonial stem cells may proliferate along the stem cell niche on the basement membrane by attaching to extracellular matrix molecules such as the integrin family proteins on the basement membrane. However, it is possible that this microenvironment or niche is formed by some supplement of the Sertoli cells, i.e., Sertoli cell-spermatogonial stem cell interaction or growth factors produced by Sertoli cells might contribute to the stem cell niche. In contrast, our observation on the transplanted green spermatogonial stem cells showed that all of them were directly attached on the basement membrane. Thus, the stem cell niche is thought to be formed on the basement membrane surround by the Sertoli cells.

In the present study, using the germ cell transplantation technique, we have determined the existence of a spermatogonial stem cell proliferation system that is independent of the differentiation process. Furthermore, we have demonstrated that the spermatogonial stem cells occupy the stem cell niche present on the basement membrane of the seminiferous tubules, and that the capacity of the niche to induce proliferation of the spermatogonia is limited. We have also showed that SCF stimulation is required for maintenance of differentiated germ cells but not for spermatogonial stem cell proliferation.

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