2.5. Trouble shooting

One of the problems in culture initiation is overgrowth of somatic cells. Somatic cells grow faster than SSCs *in vitro* and overwhelm the culture. This can be alleviated, in part, by reducing the serum concentration and/or enhancing the GDNF concentration. However, SSCs, at present, cannot grow in the complete absence of serum. Although two different "serum-free" culture systems have been reported (Kanatsu-Shinohara *et al.*, 2005a; Kubota *et al.*, 2004), one of these cultures were maintained using serum to stop the trypsin reaction at each passage in one study (Kubota and Brinster, 2008). Because the same medium cannot support feeder-free GS cell culture without serum, we also cannot exclude the possibility that residual serum remaining with the feeder cells promote GS cell propagation (Kanatsu-Shinohara *et al.*, 2005a). Besides somatic cells, loss or reduced GDNF activity often causes problems. Because GDNF is essential for SSC self-renewal, low concentrations of GDNF are detrimental to GS cell culture.

When the culture reaches confluency, c-Kit expression, a marker of differentiating spermatogonia, is occasionally upregulated, suggesting that some levels of differentiation occur in culture. At the same time, GS cell growth is suppressed. In this situation, remove the medium and replace with fresh medium. In most cases, a decrease in the number of differentiating cells is observed, and the stem cells revive by GDNF action. If the GS cells look unhealthy, change the medium or add GDNF. GS cells are very tough, and in most cases will recover.

2.6. Optional: Feeder-free culture and GS cell suspension culture

GS cells can be maintained under a feeder-free condition or in suspension culture (Kanatsu-Shinohara *et al.*, 2005a, 2006b). For feeder-free culture, the culture plates should be coated with 20 μg/ml laminin (#354232; BD Biosciences, Franklin Lakes, NJ) for 1–2 h at room temperature (0.8 ml laminin solution per well of a 6-well culture plate). Remove the laminin solution and immediately plate the GS cells at 3×10^5 per well. The culture can be passed with trypsin. For the suspension culture, plate 2×10^5 GS cells directly onto a Petri dish. GS cells aggregate to make clumps, but continue to proliferate slowly. These cultures can be passed without trypsin, and GS cell clumps can be disaggregated using simple pipetting with P1000 tips. Although the proliferation rates are lower than MEF culture (doubling time, 2.7 days for MEF culture, 5.6 days for feeder-free culture, and 4.7 days for suspension culture), GS cells maintained feeder-free or in suspension continue to proliferate for more than 6 months without losing SSC activity.

2.7. Optional: Establishing GS cells from adult testes

GS cells can also be established from adult testes, but enrichment using gelatin-coated plates is not effective. Because the SSC frequency is low in adult testes (about 2–3 in 10^4 cells; Tegelenbosch and de Rooij, 1993; Meistrich and van Beek, 1993), purification using either magnetic beads or a fluorescence-activated cell sorter is necessary. Several SSC markers are useful for SSC enrichment. For example, a relatively high rate of enrichment has been achieved in our lab using magnetic beads sorted with α6- or β1-integrins and CD9 (Kanatsu-Shinohara *et al.*, 2004b; Shinohara *et al.*, 1999). Thy-1 is also expressed in SSCs, but seems to work better after Percoll separation (Ryu *et al.*, 2004). A higher enrichment efficiency (about 166-fold) can be achieved with cell sorting using a combination of several markers (Ryu *et al.*, 2004; Shinohara *et al.*, 2000), but enrichment by magnetic beads is sufficient for culture initiation in many cases.

Purified cells are transferred to gelatin- or laminin-coated plates and cultured in GS cell culture medium. In a manner similar to the initiation of GS cell culture from neonatal testes, germ cell colonies are formed on top of the flat somatic cell layer. However, because adult germ cells proliferate more slowly than neonatal or pup germ cells in the initiation of culture, more time is required to establish a GS cell culture from adult testes than from neonatal testes. Retrieving GS cell colonies is sometimes helpful using glass needles in cases of somatic cell overproliferation.

2.8. Optional: Derivation of mGS cells and its application in gene targeting

mGS cells were initially discovered in our attempt to produce knockout GS cells. Unusual colonies may rarely occur during the initiation of a GS cell culture; typical GS cell colonies show grape-like clumps, whereas pluripotent cell colonies appear like ES cells, or as an epiblast cell sheet (Fig. 2.1C), a derivative of the inner cell mass. Once these colonies develop in culture, they outgrow GS cells after several passages because they proliferate faster. Although they can grow in GS cell culture medium, changing the medium (DMEM + 15% FBS) and supplementing it with LIF is advisable to maintain their undifferentiated state. GDNF is no longer necessary after mGS cells are established, and established mGS cells can be maintained in the same manner as ES cells (Fig. 2.1D).

Although both GS and mGS cells are derived from testicular germ cells, they have different characteristics. GS cells are unipotent and committed to spermatogenesis, whereas mGS cells are pluripotent and can differentiate into various cell types. They behave exactly like ES cells, except they have a partial androgenetic imprinting pattern and characteristic centromeric DNA hypomethylation, reflecting their postnatal male germ cell origin (Kanatsu-Shinohara *et al.*, 2004a; Yamagata *et al.*, 2007). Because SSC activity is lost

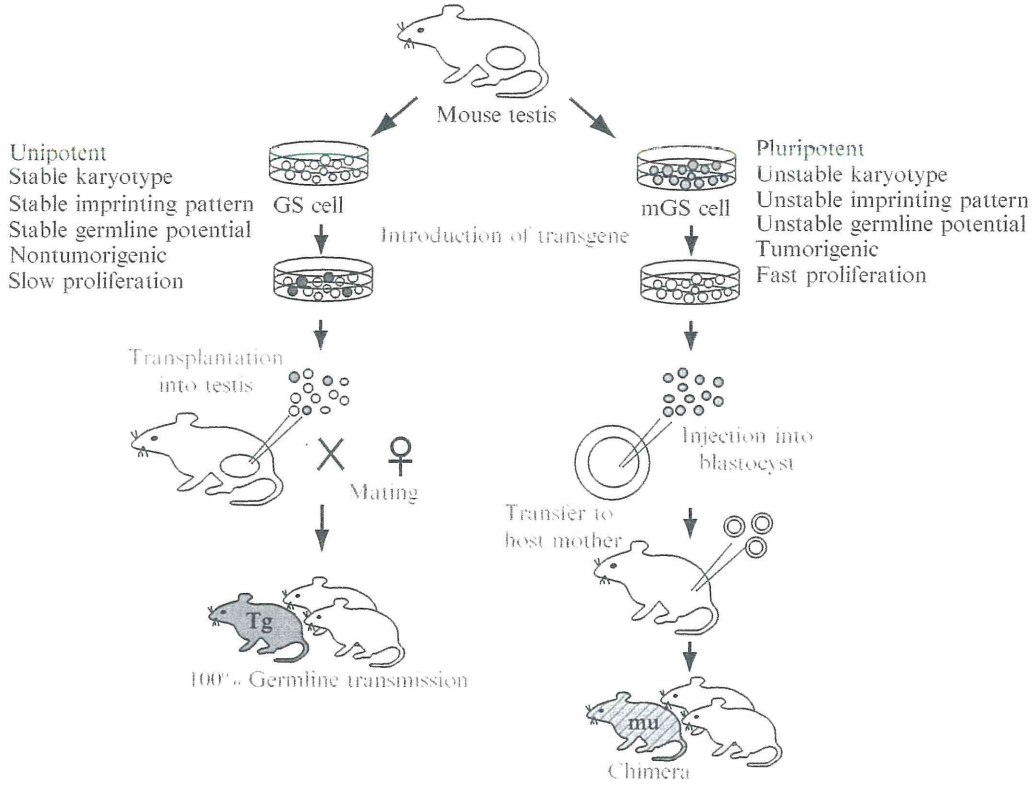

Figure 2.2 GS and mGS cell differences in their utility for animal transgenesis.

in mGS cells, they produce teratomas instead of sperm when transplanted into testes (Fig. 2.2). They produce germline chimeras following injection into blastocysts, in a manner similar to ES cells. The application of conventional methods used for ES cells allows for the production of knockout mice or double-knockout cells from mGS cells (Takehashi *et al.*, 2007b).

3. Gene Transduction and Genetic Selection of GS Cells

SSCs can be transduced with a retrovirus (Nagano *et al.*, 2000), lentivirus (Nagano *et al.*, 2002), adenovirus (Takehashi *et al.*, 2007a), or adeno-associated virus vector (Honoramooz *et al.*, 2008), and with plasmid vectors by various methods, including the calcium phosphate method, lipofection, and electroporation (Kanatsu-Shinohara *et al.*, 2005c). Knockout mice were produced with either a virus-based gene trap vector or with a gene-targeting plasmid vector. However, because viral origin promoters are repressed in germ cells and early embryonic cells, choosing promoters that are not suppressed in germline cells is necessary. According to our experience, most expression vectors used in ES cells will express foreign genes in SSCs.

Genetic selection with drugs is relatively difficult with GS cells. While ES cells grow robustly like transformed cells and can proliferate from single cells, GS cell proliferation is modest and is influenced by cell density. Therefore, one should avoid an extremely low cell density during culture. When only a few cells are recovered after drug selection, we add nontransfected "carrier" cells to enhance the growth of transfected cells (Kanatsu-Shinohara *et al.*, 2005c). These carrier cells are removed by repeated drug selection, which usually takes about 2 months.

Here, we describe the protocol for gene transduction of a gene targeting vector and the genetic selection of GS cells using a neomycin-resistant gene.

3.1. Step 1. Gene transduction to GS cells

GS cells can be transduced using the Cell Line T Nucleofector Kit (#VCA-1002; Lonza, Münster, Germany). Briefly, dissociate the GS cells with trypsin and suspend them in IMDM + 2% FBS. Remove as much of the MEF as possible because MEF abrogates GS cell survival. Cells ($4–8 \times 10^6$) should be transferred to a tube and centrifuged. Wash the cells twice with PBS, and suspend the pellet in 100 μl of Nucleofector solution (#VCA-1002; Lonza). Then add 5 μg of the gene targeting vector. The gene targeting vector is constructed following the conventional method used for ES cells, and we are able to isolate clones with a homologous recombination using both circular and linearized vectors. Plasmid DNA is suspended in sterile water or Tris–EDTA (TE). Transfer the cell suspension to a cuvette and treat with an electroporator (Nucleofector II device; Lonza) using program A-23. GS cells can also be efficiently transduced using an electroporator (Bio-Rad, Hercules, CA); the cells are suspended in PBS and treated with a single 500 μF pulse. After electroporation, culture the cells with conventional GS cell medium on MEF. The cells are passed in fresh MEF (1:1) the next day.

3.2. Step 2. Drug selection

Start the drug selection after the cells have recovered from the damage of electroporation (about 7–10 days). At 2–3 days after passage, add 40–120 μg/ml G418 (Geneticin, #83-5027; Invitrogen). If the culture reaches confluency, regardless of the G418, the cells should be passed (1:1). Puromycin (#P7255; Sigma; dissolve 1 mg/ml water) can also be used as a selection marker at 0.1–0.2 μg/ml. In contrast, if the drug efficiently reduces cell density, leave the culture plate for about 3 weeks and only change the medium, until transduced cell colonies have formed.

Treat the culture with 0.25% trypsin, and pass to fresh MEF at a 1:1 dilution; add nontransfected GS cells at a density of about 3×10^4 cells/cm^2. Restart the drug selection 2–3 days after the passage. Repeat the same procedure until a sufficient number of transfected GS cells is obtained at 100% purity. Overall, about 2–3 months is needed to obtain about 10^6 cells of an established clone.

3.3. Step 3. DNA isolation and detection of homologous recombination

Genomic DNA is isolated from cultured cells. More than about 20 μg DNA can be obtained from 2×10^6 GS cells. The clones inserted with a homologous recombination are screened with the polymerase chain reaction (PCR) or Southern blotting.

4. Spermatogonial Transplantation and Offspring Production

SSCs will migrate into the germline niche after microinjecting them into the seminiferous tubules and create germ cell colonies. This method is conceptually similar to hematopoietic stem cell transplantation (Till and McCulloch, 1961), in that donor stem cells are reintroduced into a microenvironment that lacks endogenous stem cells. This technique provided the first functional SSC assay. Furthermore, because the recipient animals eventually produce offspring from donor SSCs, this technique also allowed the possibility to manipulate SSCs to produce offspring.

4.1. Donor cell preparation

Having a donor marker during germ cell transplantation is advisable. Because single germ cell colonies originate from single SSCs (Kanatsu-Shinohara *et al.*, 2006c; Nagano *et al.*, 1999), a marker allows for the quantitative assessment of donor SSC colonization. Moreover, it also helps identify abnormal germ cell development.

Several transgenes, including LacZ or enhanced green fluorescent protein (EGFP), are used. Each has its advantage. For example, EGFP allows for offspring production using microinsemination or serial transplantation, whereas LacZ allows for better histological presentation of donor cell colonization.

Single cell suspensions are prepared using an enzymatic digestion as described above. The reaction can be stopped by adding DMEM + 10–20% FBS. Cells must be filtered though a 40-μm nylon mesh (#BD352340; BD Biosciences) before transplanting, so that cell aggregates do not clog the injection needle. Cells are usually suspended in DMEM + 10% FBS. The concentration of cells can be as high as 10^8 cells/ml.

Although fresh testicular cells can be transplanted relatively easily, freeze–thawed cells or cultured cells often clog the injection needle, which may occasionally be resolved by adding a small amount of DNase (about 0.7 mg/ml; #DN25; Sigma) to the cell suspension. Although the cells can be stored on ice for several hours, injecting the cells as soon as the cell suspension is made is better.

4.2. Recipient preparation

Donor SSCs must be histocompatible with the recipient animal. Although testes are immune-privileged organs, allogeneic SSCs can be rejected despite successful transplantation (Kanatsu-Shinohara *et al.*, 2003b). Although several cases of successful transplantation have been reported, using immunodeficient nude/scid mice is advisable when the donor cells are not histocompatible with the recipients. This allows for a xenogeneic transplantation and produces fertile spermatozoa (Clouthier *et al.*, 1996; Shinohara *et al.*, 2006).

Furthermore, elimination of endogenous SSCs is prerequisite. Transplanted SSCs are believed to compete for available niches with endogenous SSCs, and reducing the number of endogenous SSCs is thought to increase the transplantation efficiency (Shinohara *et al.*, 2002). This can be achieved by busulfan or radiation treatment. Additionally, one can use congenitally infertile mutants such as WBB6F1-W/W^{V} (designated as W) mice (Brinster and Zimmermann, 1994). While local radiation of testis specifically eliminates germ cells (Creemers *et al.*, 2002), busulfan treatment sometimes induces anemia caused by its systemic side effect on bone marrow. This can be a problem with nude mice that are relatively weak to busulfan-induced damage. However, most germ cell transplantation experiments depend on busulfan-treated recipients as described in the following protocol.

4.2.1. Step 1. Busulfan preparation

Busulfan (#B2635; Sigma) is first dissolved in dimethyl sulfoxide (DMSO) at 8 mg/ml. Because of its strong toxicity, one must wear gloves and a mask for protection. Once it is dissolved in DMSO, an equal amount of distilled water is added to the busulfan solution at 4 mg/ml. Upon mixing, the solution will generate heat. It is advisable to use the busulfan solution before it starts to form precipitates.

4.2.2. Step 2. Injection of the busulfan solution into recipient animals

Busulfan solution is injected intraperitoneally into animals. The amount of busulfan varies according to the animal background. However, we generally use doses ranging from 44 to 50 mg/kg. Inbred mice are more sensitive to busulfan than F_1 hybrids or animals in closed colonies. Although spermatogenesis can be suppressed at a lower dose, it may regenerate from endogenous SSCs after several weeks, which interferes with SSC colonization and the subsequent development of germ cell colonies. In addition, one must consider that the complete elimination of endogenous spermatogenesis is undesirable to successfully produce offspring in both mice and rats (Brinster and Avarbock, 1994; Ryu *et al.*, 2007).

4.3. Transplantation

A single donor cell suspension can be introduced into the seminiferous tubules via three routes: the seminiferous tubules, rete testis, or efferent duct (Ogawa *et al.*, 1997). In the original report (Brinster and Zimmermann, 1994), the cells were injected directly into the seminiferous tubules by exposing the tubules to air via tunica albuginea removal. However, this method is time-consuming and is the most difficult. The most popular method is efferent duct injection. The efferent duct provides passage for mature spermatozoa from the testis to the epididymis. After microinjecting the donor cell suspension, the seminiferous tubules are filled in a retrograde manner. Therefore, transplanted cells migrate in the opposite direction to normal sperm transport.

The injection can be performed manually with a 1-ml syringe equipped with a glass needle. This allows for easier guidance of the injection pipette and is recommended for the novice. Alternatively, automatic microinjection equipment used commonly for producing transgenic offspring (model 5242; Eppendorf, Hamburg, Germany) can be used. This has an advantage in that it provides a more accurate quantitative injection and better pressure control.

Here, we describe the method used to inject into the efferent duct.

4.3.1. Step1. Glass needle preparation

We regularly use glass needles (1 mm in diameter using a Sutter puller; model P-97 and #BF200-156-10; Sutter Instruments, Novato, CA) for transplantation. However, the needle edge does not need to be sharpened, as is often necessary for pronuclear DNA injection. The glass needle can be of any size or shape as long as cells can go pass through the needle tip into the seminiferous tubules.

4.3.2. Step 2. Efferent duct exposure

After anesthetizing the animal, make a straight midline incision (1.5–2 cm) in the center of the abdomen. Using blunt forceps, expose the testis by pulling out the fat attached to the epididymis. Dissect out the efferent duct using fine forceps. Finding the efferent duct is sometimes difficult in aged, fat animals. Excessive dissection will damage the duct and is not advisable.

4.3.3. Step 3. Insertion of the donor cell suspension into the glass needle

Insert the donor cell suspension from the rear end of the glass needle. Optionally, add trypan blue to the donor cell suspension to help visualize the fate of the transplanted cells. However, trypan blue may cause inflammation in some cases (Kanatsu-Shinohara *et al.*, 2008b), and including the dye in the suspension solution is not advisable for regular experiments.

The transplantation volume varies depending on the recipient type. In busulfan-treated testis, as much as 10 μl of donor cell suspension can be injected to fill 80–90% of the tubules. For a W testis, we regularly inject 4 μl because it is smaller than a busulfan-treated testis.

4.3.4. Step 4. Injection into the efferent duct

Insert the glass needle into the efferent duct (Fig. 2.3). Placing the epididymis on the medial side of the testis generally helps expose the duct. If the needle is injected too deeply along the efferent duct through the rete testis, cells will leak out into the interstitial tissue and will not colonize the seminiferous tubules.

If clogging occurs during the injection, pull out the injection needle, and after removing the clog by breaking the pipette tip, reinsert it into the efferent duct multiple times as long as the efferent duct maintains its original shape and rigidity. It is advisable not to fill 100% of the tubules because this can limit blood supply or induce inflammation in the recipient testis. High pressure in the testis can be relieved by making a small incision in the tunica using a 26–30-gauge needle. The accidental injection of air does not seem to cause a problem, but should be avoided.

Pull out the needle and move the testis back into the abdominal cavity. One need not place the testis back in the scrotum. Pull out another testis for transplantation. After the injection, return the testis to the body cavity. Muscle layers as well as skin must be sutured. Typically, transplantation into

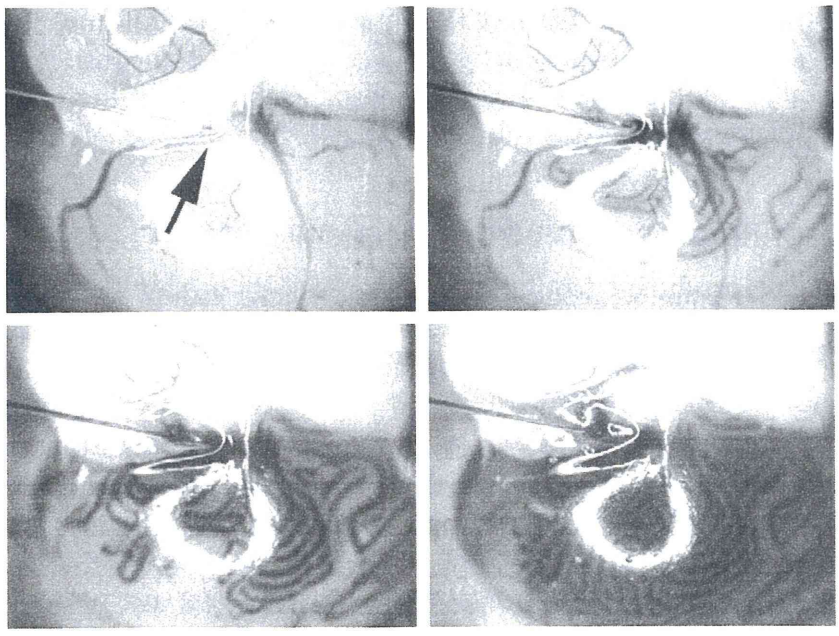

Figure 2.3 Spermatogonial transplantation. A glass needle is inserted into the efferent duct of a W mouse. The donor cell suspension is visualized by adding trypan blue solution, which gradually fills up the seminiferous tubules by increasing the injection pressure. Arrow indicates the glass needle tip.

one animal (with two testes) takes about 5–10 min for an experienced manipulator. Researchers are encouraged to refer to a detailed protocol if necessary (Ogawa *et al.*, 1997).

4.4. Optional: Measurement of SSC activity by analyzing the recipient testes

According to the donor cell marker type, colonization can be visualized by X-gal staining (LacZ) or under a UV light (EGFP). Within 2–3 weeks following transplantation, donor SSCs form a network or chains of spermatogonia on the basement membrane (Nagano *et al.*, 1999). By 1 month, the cells start to differentiate vertically toward the tubule center. By 3 months posttransplantation, donor-derived spermatozoa appear in the seminiferous tubules. Although the distribution of SSCs in the colony remains unknown, SSCs are believed to preferentially undergo self-renewal divisions at both ends of the colony and start differentiation in the center (Nagano *et al.*, 1999). During the course of colony development, the length of the colony gradually increases (1.73 mm/month), and colonies tend to merge after the long term. Colonies can be safely determined at least 6 weeks after transplantation.

An important issue here is the definition of germ cell colony because numerous donor cell clusters are present in the seminiferous tubules and one must set a criteria to define a "germ cell colony." We slightly modified the criterium of Nagano *et al.* (1999) and define a colony as when the donor cells occupy the entire seminiferous tubule and the colony is larger than 0.1 mm.

Note that not all transplanted SSCs make germ cell colonies: 5–10% of the SSCs are thought to have a repopulating ability (Nagano *et al.*, 1999; Ogawa *et al.*, 2003). In the case of GS cells, about 1–2% of the transplanted cells repopulate seminiferous tubules (Kanatsu-Shinohara *et al.*, 2005b).

4.5. Offspring production from recipient mice

Offspring are produced from donor-derived cells either by crossing the recipient mice with wild-type females via natural mating or microinsemination. Both busulfan-treated recipients and W mice will produce offspring from donor SSCs. Transplantation into pup testes results in a more rapid fertility recovery, possibly due to the absence of tight junctions between Sertoli cells in immature testes (Shinohara *et al.*, 2001). Theoretically, about 50% of the F_1 offspring from founders are heterozygous because the transgene is transmitted to half of the haploid germ cells after meiosis. Unlike ES and mGS cells, the F_1 offspring from the founder are not chimeras, and offspring are directly produced from the transplanted donor cells. However, it is necessary to confirm donor cell origin by PCR or Southern blotting because endogenous SSCs may regenerate to produce fertile sperm. Offspring were produced using primary testis cells by transplanting a few

hundred SSCs (Ogawa *et al.*, 2000; Shinohara *et al.*, 2001). Single GS cells can also produce offspring after drug selection and *in vitro* expansion (Kanatsu-Shinohara *et al.*, 2005c, 2006a).

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—Original Article—

Unstable Side Population Phenotype of Mouse Spermatogonial Stem Cells *In Vitro*

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Abstract. Stem cells of the side population (SP) phenotype are found in many self-renewing tissues and can be identified by their unique ability to effectively exclude the dye Hoechst 33342. We previously established a method for expanding spermatogonial stem cells (SSCs) *in vitro*, but the frequency of SSCs is only about 1 to 2%, limiting detailed SSC analyses. In this study, we sought to isolate SSCs from *in vitro* cultures by exploiting their ability to exclude Hoechst 33342. In contrast to the findings of previous *in vivo* studies, we found that SP cells developed in a stochastic manner *in vitro*. Moreover, SP cells in culture were not enriched in SSCs, but they were interconvertible with non-SP cells. Although SP cells were consistently found in testes after transplantation of cultured cells, they were not enriched in SSCs. These results show that SSCs have an unstable SP phenotype and provide evidence that SSCs change their phenotype characteristics in response to their microenvironment.

Key words: Developmental biology, Gametogenesis, Sertoli cell, Spermatogenesis, Testis

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Spermatogonial stem cells (SSCs) are spermatogonia that continuously undergo self-renewal [1, 2]. Hence, although they comprise a very small percentage of the cells in the testis, SSCs provide an unlimited source of spermatogenesis throughout the male lifetime. Despite their biological importance, however, SSCs are difficult to study because distinguishing them from committed progenitor cells is technically problematic. No SSC-specific markers are available, and SSCs cannot be identified by their morphology, only by their ability to self-renew themselves. However, the technique of germ cell transplantation is widely used to identify SSCs, based on their biological function [3]. In this technique, dissociated testis cells are transplanted into the seminiferous tubules of infertile recipient testes. Those transplanted cells that reinitiate spermatogenesis after transplantation are SSCs by definition.

Development of the germ cell transplantation technique has been accompanied by development of methods for enriching SSCs [4–6] and identification of several SSC surface antigens. Although stem cells have traditionally been purified on the basis of their surface antigens, they can also be purified using fluorescent dyes. In particular, stem cells in many self-renewing tissues, including bone marrow and various types of epithelium, can be found within a side population (SP) of cells defined by the ability to exclude Hoechst 33342 [7, 8], which is a function associated with members of the ATP-binding cassette (ABC) transporter family, such as MDR1 and ABCG2/Brcp1. Although several groups have examined whether Hoechst 33342 exclusion can be used for enrichment of SSCs, results have been inconsistent. In fact, SSCs were initially thought to be non-SP cells [6], but they are now considered to be SP cells [9–12].

In 2003, a system for long-term culture of SSCs was established [13]. Development of this system was based on the observation of glial cell line-derived neurotrophic factor (GDNF) transgenic/knockout (KO) mice [14]: overexpression of GDNF in transgenic mice induced the accumulation of undifferentiated spermatogonia and produced germ cell tumors, whereas the decreased expression of GDNF in heterozygous KO mice depleted the spermatogonia and caused male infertility. SSCs from testes at any postnatal stage were then found to proliferate in the presence of self-renewal factors, including GDNF and fibroblast growth factor 2 (FGF2). These cells, designated as germline stem (GS) cells, can expand by a factor of 10^{85}-fold after 2 years in culture [15]; they can also be genetically modified to produce transgenic and KO mice and are potentially pluripotent [16, 17]. This GS cell culture technique allows researchers to greatly expand SSCs for molecular or biochemical analysis.

Although all GS cells are spermatogonia, the frequency of SSCs in GS cell cultures is only approximately 1–2% [18], and obtaining a purified SSC population has not been possible. In the present study, we sought to identify SSCs in a GS cell population by exploiting the ability of SP cells to exclude Hoechst 33342 dye. We determined the SSC activity of the SP cells using germ cell transplantation. We also examined the SP cell phenotype and SSC activity of GS cells after transplantation into seminiferous tubules.

Materials and Methods

Cell culture

The GS cells used in this study were derived from the transgenic mouse line C57BL/6 Tg14(act-EGFP)OsbY01, which was backcrossed to a DBA/2 background [13]. These cells were maintained on dishes coated with laminin (20 μg/ml; BD Biosciences, Franklin Lakes, NJ, USA). For culturing after cell sorting, sorted cells were

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Table 1. Summary of transplantation experiments

Experiment	Donor cells	No. of experiments	No. of cells injected[a]	No. of testes injected	No. of testes colonized (%)	No. of colonies/ testis/10^5 cells[b]	P value by t-test
In vitro	SP	4	150 – 1300	20	8 (40)	560.3 ± 432.0	0.12
	Total EGFP	4	1000 – 2400	20	16 (80)	142.5 ± 22.6	
In vivo (WT)	SP	3	650 – 2000	18	0 (0)	0	0.40
	Total EGFP	3	32000 – 70000	16	3 (19)	0.8 ± 0.4	
In vivo (Ras)	SP	3	640 – 5900	17	2 (12)	10.1 ± 9.1	0.30
	Total EGFP	3	5900 – 30000	17	2 (12)	0.5 ± 0.3	

Values are means ± SEM. [a] Approximate number of cells was calculated by assuming 4 μl of cells was transplanted. [b] Number of individual colonies in each testis. Results were normalized to 10^5 cells injected/testis.

maintained on mitomycin C-treated mouse embryonic fibroblasts (MEFs). For combined staining experiments, we used GS cells from the transgenic mouse line B6-TgR(ROSA26)26Sor (The Jackson Laboratory, Bar Harbor, ME, USA), which was back-crossed to a DBA/2 background. The growth factors used were mouse epidermal growth factor (EGF; 10 ng/ml), human FGF2 (10 ng/ml), and rat GDNF (15 ng/ml; all from PeproTech, Rocky Hill, NJ, USA). GS cells expressing constitutively active *Akt* or *H-RasV12* under CAG promoter were previously described [19].

Magnetic cell sorting (MACS) and SP cell analysis by flow cytometry

Cultured cells were dissociated by treatment with 0.25% trypsin, and testis cells were dissociated by two-step enzymatic digestion with collagenase type IV and trypsin, as previously described [20]. For MACS, primary spermatogonia were collected from 7-day-old DBA/2 pup testes using a rat anti-EPCAM antibody (G8.8; BioLegend, San Diego, CA, USA) and Dynabeads sheep anti-rat IgG (Invitrogen, Carlsbad, CA, USA), as previously described [4]. For SP cell analysis, single-cell suspensions were suspended at 10^6 cells/ml in Dulbecco's Modified Eagle's Medium containing 2% fetal bovine serum (FBS) and 10 mM Hepes. SP cells were detected as those remaining unstained after a 90-min incubation with 4 μg/ml Hoechst 33342 (Sigma, St. Louis, MO, USA) at 37 C. For combined staining, 0.1 μg/ml rhodamine 123 (Rh 123; Invitrogen) was added during the last 20 min of the Hoechst 33342 incubation. For immunostaining of SP cells, Hoechst 33342-stained cells were incubated with allophycocyanin- or Alexa 647-conjugated antibodies against KIT (2B8), EPCAM (G8.8), ITGB1 (HMβ1-1), ITGA6 (GoH3), CD9 (MZ3) and FUT4 (MC480; BioLegend) after washing the cells with ice-cold phosphate-buffered saline containing 1% FBS. Propidium iodide (1 μg/ml; Sigma) was added to exclude dead cells before analyses. Cells were analyzed on a FACSAria 2 flow cytometer equipped with a 375-nm UV laser (7 mV; BD Biosciences). The Hoechst dye was excited at 375 nm, and its dual emission wavelengths were detected using 450/20 (Hoechst 33342 blue) and 660/20 (Hoechst 33342 red) filters.

Germ cell transplantation

Single-cell suspensions of germ cells were microinjected into the seminiferous tubules of WBB6F1-W/W^v (W) mice (Japan SLC, Shizuoka, Japan) through the efferent duct [20]. Approximately 75–85% of the tubules were filled in each recipient testis. The recipient mice were treated with anti-CD4 antibody (GK1.5; a gift from Dr T Honjo, Kyoto University) to induce tolerance to the allogeneic donor cells [21]. Immediately after transplantation, some of the recipients were made cryptorchid by suturing the testicular fat pad to the lateral abdominal wall, as previously described [22]. All animal experimentation protocols were approved by the Institutional Animal Care and Use Committee of Kyoto University.

Analysis of recipient testes

Donor cell-derived colonies in the recipient testes were detected by enhanced green fluorescence protein (EGFP) fluorescence under UV light. Germ cell clusters were defined as colonies when they were longer than 0.1 mm and occupied the entire circumference of the seminiferous tubule. For histological analyses, samples were fixed in formalin, embedded in paraffin and sectioned. All sections were counterstained with hematoxylin and eosin.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol (Invitrogen), and first-strand cDNA was produced using Superscript II (Invitrogen). The specific RT-PCR primers are listed in Table S1. PCR was performed using a single incubation at 95 C for 5 min, followed by 30 cycles of 94 C for 30 sec, 60 C for 30 sec and 72 C 1 min.

Statistical analysis

Results are presented as means ± SEM. Data were analyzed using the Student's *t*-test.

Results

Identification of SP cells in GS cell cultures

SP cells in the testis population have been shown to express *Abcg2*, which is thought to be responsible for the SP phenotype [10]. To determine what types of ABC transporters are expressed in GS cells, we carried out RT-PCR analysis. The analysis showed that several ABC transporters are expressed in the testis (Fig. 1A). GS cells were found to express *Mrp1, Mrp4, Mrp5* and *Abcg2*, but not *Mrp2, Mrp3 or Mrp6*. This pattern of gene expression is different from that observed in EPCAM-expressing spermatogonia (Fig. 1A), which exhibit the SP cell phenotype [11].

To determine whether GS cells exhibit the SP phenotype, GS cells expressing the gene for EGFP were cultured on laminin for 1–6 days (Fig. 1B), recovered by trypsin digestion, and stained with Hoechst 33342. Unlike the whole testis cell population, which yielded at least six distinct regions on a flow cytometry dot plot [9], GS cell cultures exhibited a simpler dot plot pattern, indicating a more uniform composition of the stained cells (Fig. 1C). However, SP cells were found in only 57.1% (24 out of 42) of the GS cell cultures, indicating that the SP population developed stochastically. At least three independent GS cell lines showed stochastic expression. Development of the SP population was not influenced by the length of time after passage, but was inhibited by the addition of verapamil (Fig. 1D), which inhibits dye efflux by ABC transporter activity [7]. In cultures with SP cells, the frequency of the SP population ranged from 0.3 to 22.3% (2.1 ± 0.9%; n=24), whereas non-SP cells were consistently found in all of these cultures.

Because *Akt* regulates the SP cell phenotype in hematopoietic and neuronal cells [23, 24], we examined the effect of *Akt* overexpression on SP cell development. However, GS cells stably expressing active *Akt* failed to develop SP cells in three independent experiments (Fig. 1E), suggesting that the mechanism regulating SP cell development in GS cell culture differs from that in hematopoietic or neuronal cells.

To characterize the SP cells, we stained them with several spermatogonia markers. Despite the significant difference in the Hoechst 33342 staining patterns of the SP vs. non-SP cells, both types of cells expressed the same surface markers, ITGA6, ITGB6, EPCAM and CD9 (Fig. 2A). They also exhibited strong expression of KIT, which is normally used as a marker for differentiating spermatogonia and spermatocytes. Neither SP nor non-SP cells expressed FUT4, a marker for primordial germ cells and embryonic stem cells. Efflux of Rh 123, which is often used to enrich stem cells in several tissues [25], was more pronounced in SP cells than in non-SP cells (Fig. 2B). Rh 123^{low} cells were not detected in the non-SP cell population.

Reversibility of the SP phenotype in GS cell culture

Because previous studies suggested that the SP population has higher stem cell activity, we expected that SP cells in GS cell cultures would be more undifferentiated than non-SP cells and that they would differentiate into non-SP cells. To test this hypothesis, we fractionated EGFP-expressing GS cells on laminin-coated dishes according to their Hoechst 33342 staining patterns (Fig. 3A). In these experiments, 2.5 to 5.0 × 10^3 SP cells and 1.8 to 2.9 × 10^5 non-SP cells were sorted per experiment. The separated SP and non-SP cells were then cultured on MEFs and examined for their phenotypes. The sorted cells were cultured on MEFs rather than on laminin because they survived better on MEFs, possibly because they were damaged during sorting [5].

The colonies that developed from the cultured SP and non-SP cells were morphologically indistinguishable and did not vary significantly in growth rate. To observe the SP phenotype of the developed colonies, the cells were stained again with Hoechst 33342 on days 29 and 34 after sorting. In two separate experiments, the cultured SP cells differentiated into non-SP cells, whereas the cultured non-SP cells produced both SP and non-SP cells. As they did on laminin, GS cells stochastically produced SP cells on MEFs and yielded a pattern of Hoechst 33342 similar to that of the original unsorted GS cell cultures. These results indicate that SP and non-SP cells are interconvertible *in vitro*.

SSC activity of SP cells in GS cell culture

To determine whether the SP cell population is enriched in SSCs, we used germ cell transplantation to compare the SSC activity of SP cells with that of total GS cells (Fig. 3B). These two populations of cells were microinjected into the seminiferous tubules of infertile W mice lacking endogenous spermatogenesis. In four separate experiments, a total of 20 recipient testes received transplantation of each cell type.

The recipients were sacrificed 2 months after transplantation. The isolated recipient testes were then analyzed for donor cell colonization levels by counting the number of EGFP-expressing germ cell colonies under UV light. Transplantation of the SP and total GS cell populations produced 560.3 ± 432.0 and 142.5 ± 22.6 (n=20) SSC-derived colonies per 10^5 donor cells, respectively. Although the frequency of SSC colony production was higher for the SP cells, the difference between the two cell types was not statistically significant (P=0.12; Table 1), indicating that SP cells are not enriched in SSCs.

Histological analyses of the recipient testes showed normal differentiation of the transplanted SP cells (Fig. 3C). No spermatogenesis was observed in the nontransplanted control W testes.

SP cell phenotype after transplantation in vivo

To examine the effect of the microenvironment on SP cell development, we transplanted EGFP-expressing GS cells into seminiferous tubules (Fig. 4A). In these GS cells, the EGFP gene is driven by the CAG promoter and is expressed at all stages from the spermatogonium to the round spermatid [21]. By gating EGFP-expressing cells, we excluded the possibility of somatic cell contamination of the germ cells.

Transplanted SSCs are known to form chains or networks of spermatogonia on the basement membrane 2–3 weeks after transplantation, when SSCs are thought to be preferentially undergoing self-renewal division [26]. However, at around 1 month, some of the cells in the colony start to differentiate vertically into the lumina. Normal spermatogenesis with spermatogenic cycles is restored as soon as 3 months after transplantation, which corresponds to approximately three cycles of spermatogenesis.

We transplanted approximately 4 × 10^6 EGFP-expressing GS cells grown on laminin into the seminiferous tubules of W mice. Some of the testes were surgically fixed at a higher position in the abdomen to induce cryptorchidism, which destroys differentiating germ cells [22]. Recipients were sacrificed at the early (7–14 days) or late (3–4 months) phase after transplantation, and the testes were dissociated into single cells using a collagenase type IV/trypsin, a two-step digestion technique that is commonly used to prepare single-cell suspensions for germ cell transplantation [20].

At the early phase after transplantation, approximately 1.8–2.0 × 10^6 cells were recovered per testis, and the proportion of EGFP-expressing cells was small (Fig. 4B). The overall Hoechst 33342

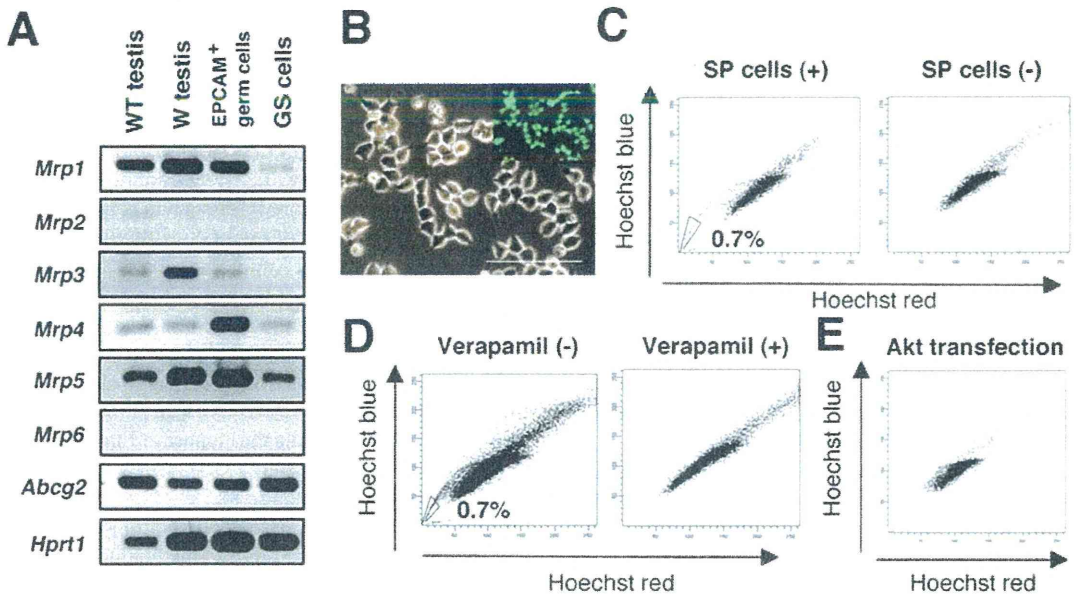

Fig. 1. SP cell phenotype of GS cells. A: RT-PCR analysis of transporter gene expression demonstrating that GS cells express *Mrp1*, *Mrp4*, *Mrp5* and *Abcg2*. B: EGFP-expressing GS cells on a laminin-coated dish exhibit strong green fluorescence under UV light (inset). C: Flow cytometric analysis of GS cells stained with Hoechst 33342. GS cells were imaged by using filters for Hoechst red and Hoechst blue emission. Whereas some of the GS cell cultures contained Hoechst 33342-excluding cells (SP cells: left), other did not (right). D: Inhibition of SP cell development by verapamil. After GS cells were dissociated, the recovered cells were stained with Hoechst 33342 in the absence (left) or presence (right) of verapamil. (E) Absence of SP cells in a population of GS cells stably expressing active *Akt*. Bar=50 μm (B).

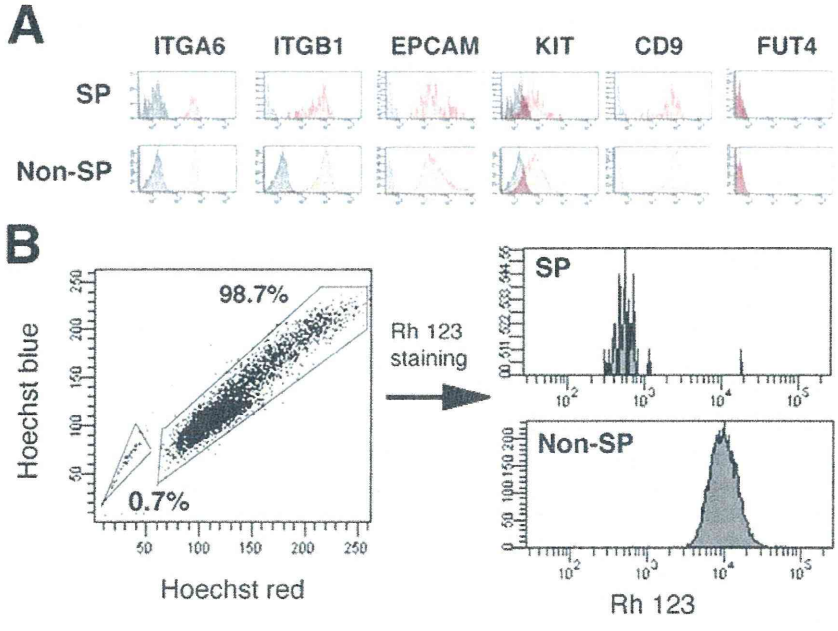

Fig. 2. Phenotypes of SP cells in GS cell culture. A: Expression of surface markers on SP and non-SP cells. The black-shaded area indicates control staining. No significant differences between SP and non-SP cells are evident. B: Rh 123 efflux characteristics of SP and non-SP cells. GS cells from ROSA26 mice were stained with Hoechst 33342 and Rh 123.

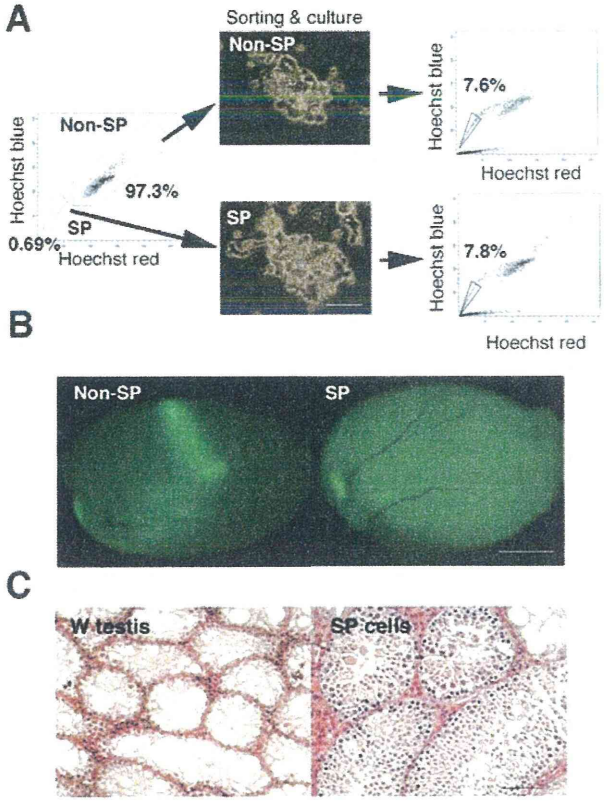

Fig. 3. Functional analyses of SP cells in GS cell culture. A: Reversibility of the SP cell phenotype. GS cells cultured on laminin-coated dishes were sorted into SP and non-SP cells according to their Hoechst 33342 staining patterns. The SP and non-SP cells were cultured for 29 and 34 days *in vitro* and stained again with Hoechst 33342. B: Macroscopic appearance of recipient W testes transplanted with SP (right) and total EGFP-expressing cells (left). Approximately 0.2–1.3 × 10^3 SP or 1.0–2.4 × 10^3 non-SP cells were transplanted into each testis. Green fluorescence indicates colonization by donor cells. C: Histological appearance of the recipient testes. W testes transplanted with SP cells produce colonies undergoing normal spermatogenesis (right). In contrast, non-transplanted W testes demonstrate no evidence of ongoing spermatogenesis (left). Bars=50 μm (A), 1 mm (B) and 100 μm (C).

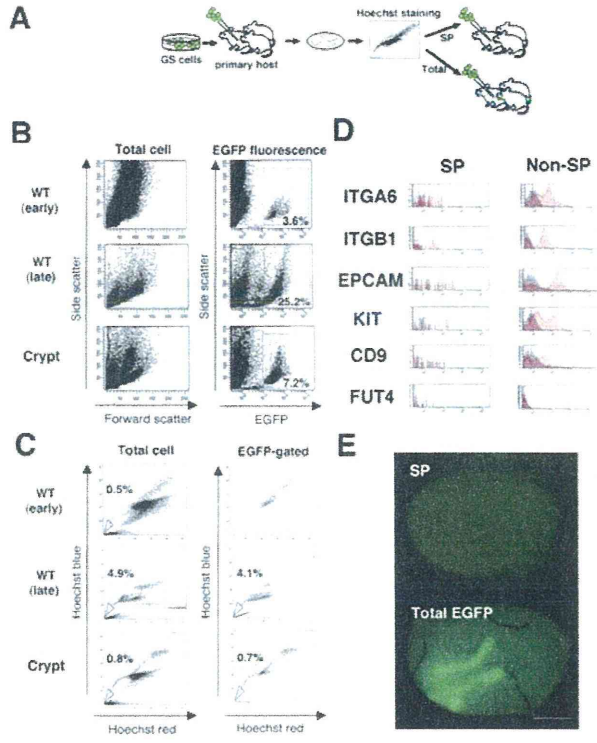

Fig. 4. SP cell analysis after transplantation of WT GS cells. A: A diagram showing the experimental strategy. EGFP-expressing GS cells were transplanted into the primary W recipient mice. Some of the recipients were made cryptorchid to eliminate differentiated germ cells. Recipient testes were dissociated at early (7–14 days) or late (3–4 months) phases after transplantation and stained with Hoechst 33342. After cells expressing EGFP were gated, SP and total EGFP-expressing cells in the EGFP-expressing cell population were sorted and transplanted into secondary W recipients. B: Comparison of light-scattering properties (left) and EGFP fluorescence (right) of dispersed testis cells from WT and cryptorchid recipients. Donor-derived cells were gated based on EGFP fluorescence and side scatter for SP cell identification. C: Comparison of Hoechst 33342 staining patterns of dispersed testis cells from WT and cryptorchid recipients. D: Expression of surface markers on SP and non-SP cells in WT W recipients. The black-shaded area indicates control staining. E: Macroscopic appearance of recipient testes that were transplanted with SP (top) and total EGFP-expressing cells (bottom). Approximately 0.7–2.0 × 10^3 SP or 3.2–7.0 × 10^4 non-SP cells were transplanted into each testis. Green fluorescence indicates colonization by donor cells. Bar=1 mm (E).

staining pattern of the recovered cells indicated no SP cells (Fig. 4B and C). At the later phase after transplantation, the recipient testes were significantly larger, and the two-step digestion technique described above yielded 2.8–5.9 × 10^6 cells per testis. The cryptorchid testes yielded only 1.0–1.5 × 10^6 cells per testis because germ cell differentiation was limited by the high temperature of the body cavity. In contrast to our findings *in vitro*, all nine experiments performed *in vivo* yielded SP cells. The pattern of Hoechst 33342 staining in normal W recipients appeared more complex in the later stage of transplantation than in the early stage (Fig. 4B and C), reflecting the colonization and differentiation of the transplanted GS cells over time. Although cryptorchid testes also yielded SP cells, the staining pattern of the dissociated testicular cells of the cryptorchid testes differed somewhat from that of the normal testes.

When only EGFP-expressing cells were gated for analyses, both wild-type (WT) and cryptorchid testes cells yielded staining patterns that were distinctly different from those of the total cells (Fig. 4B). Despite the elimination of some subpopulations by EGFP gating, the EGFP-expressing cell populations from both WT and

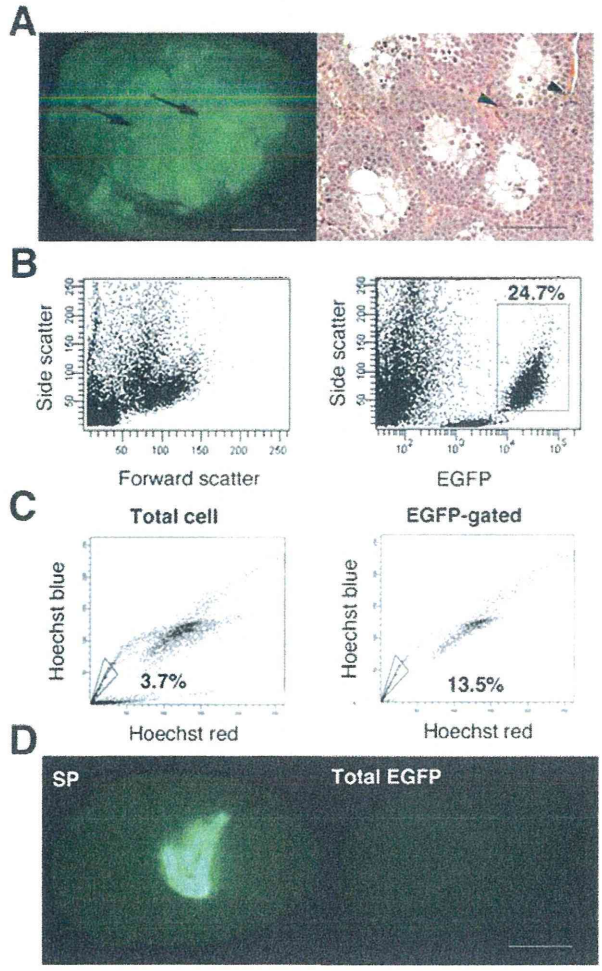

Fig. 5. SP cell analysis after transplantation of *H-RasV12*-transfected GS cells into W testes. A: Macroscopic (left) and histological (right) appearance of a recipient testis transplanted with *H-RasV12*-transfected GS cells. Arrows indicate abnormal germ cell clumps in the seminiferous tubules; arrowheads indicate invasion into interstitial tissue. Note the abnormal spermatogenesis in the seminiferous tubules. B: Light-scattering properties (left) and EGFP fluorescence (right) of dispersed testis cells from recipients that were transplanted with *H-RasV12*-transfected cells. Donor-derived cells were gated based on EGFP fluorescence and side scatter for SP cell identification. C: Hoechst 33342 staining patterns of dispersed testis cells from recipient testes that were transplanted with *H-RasV12*-transfected cells. Although SP cells were found in total testis cells (left) and in EGFP-expressing testis cells (right), the overall pattern of Hoechst 33342 staining was significantly different from that observed after transplantation of WT GS cells (see Fig. 4C). D: Macroscopic appearance of recipient testes transplanted with SP (left) and total EGFP-expressing cells (right). Approximately 0.6–5.9 $\times$ 10^3 SP or 0.6–3.0 $\times$ 10^4 non-SP cells were transplanted into each testis. Green fluorescence indicates colonization by donor cells. Bars=100 μm (A, right) and 1 mm (A, left; D).

cryptorchid testes consistently yielded SP cells (Fig. 4C). In WT recipients, the SP cells comprised 0.06 to 4.7% (1.9 ± 0.6%; n=7) of the EGFP-expressing cells, and they expressed typical SSC markers including ITGB1, ITGA6, and EPCAM. Compared with GS cells, however, CD9 is only weakly expressed in both SP and non-SP cells, suggesting that these populations have a reduced SSC concentration. Although they weakly expressed KIT, no FUT4 expression was detected (Fig. 4D).

To examine the SSC activity of SP cells found in the testis after GS cell transplantation, the SP cells were separated from the WT primary recipient testes 3 to 4 months after GS cell transplantation and implanted into secondary recipient testes (Fig. 4E). We gated EGFP-expressing cells and sorted 0.5–1.5 $\times$ 10^4 SP cells (0.8 ± 0.3 $\times$ 10^4 cells; n=3) and 2.4–5.3 $\times$ 10^5 total EGFP-expressing cells (3.4 ± 0.9 $\times$ 10^5 cells; n=3). All of the sorted cells were clearly positive for EGFP expression, and the total EGFP-expressing cell population was implanted into secondary recipient testes as a control. In three separate experiments, a total of 18 and 16 recipient testes were transplanted with SP cells or total EGFP-expressing cells, respectively. The secondary recipient testes yielded no colony after SP cell transplantation, whereas total EGFP cells yielded 0.8 ± 0.4 colonies per 10^5 donor cells (n=16; Table 1). The difference was not statistically significant (P=0.40).

SP cell phenotype in seminomatous tumors formed after transplantation

In our final set of experiments, we used GS cells overexpressing activated *H-Ras* (*H-RasV12*) to examine whether the SP phenotype could be used to enrich the population of cancer stem cells. *H-RasV12*-transfected GS cells proliferate even in the absence of exogenous cytokines, and produce seminomatous tumors after transplantation into W mice [19]. Three to four months after 4 $\times$ 10^6 of these cells were transplanted into W mice, the testes were recovered (Fig. 5A and B), and the EGFP-expressing cells were gated (Fig. 5B) and analyzed for their Hoechst 33342 staining pattern. *H-RasV12*-transfected GS cells exhibited SP cell development in each of four independent experiments. The Hoechst 33342 staining pattern appeared simpler than that observed after transplantation of WT GS cells (Fig. 5C), possibly reflecting abnormal spermatogenesis from the *H-RasV12*-transfected cells. SP cells comprised 0.7–13.5% (4.4 ± 3.0%; n=4) of the total EGFP-expressing cells.

The numbers of SP cells recovered by cell sorting was 0.4–4.4 $\times$ 10^4 cells (2.1 ± 1.2 $\times$ 10^4; n=3) per testis. On the other hand, 0.2–1.5 $\times$ 10^5 total EGFP-expressing cells (7.2 ± 3.9 $\times$ 10^4; n=3) per testis were sorted as a control for transplantation. The SP and total EGFP-expressing cells were collected and microinjected into W mice in three sets of experiments involving a total of 17 recipient testes for each cell type. Analyses of the recipient testes showed that transplantation of SP cells and total EGFP-expressing cells produced 10.1 ± 9.1 and 0.5 ± 0.3 colonies per 10^5 donor cells (n=17). However, the difference between the two cell types was not statistically significant (P=0.30)(Fig. 5D, Table 1).

Discussion

In this study, we examined the SP phenotype of a pure spermatogonia population *in vitro* using GS cell culture, and we transplanted GS cells cultures into testes so that we could follow SP phenotypic changes occurring in response to the seminiferous tubule microenvironment. Although the analysis of SP cells in the testis can be complicated by contaminating Leydig stem cells, which also exhibit an SP phenotype [27], we were able to exclude these cells from our analyses because we gated only EGFP-expressing germ cells developed from transplanted GS cells. Although some of our GS cell cultures contained SP cells and we consistently found SP cells *in vivo* after transplantation of WT or *H-RasV12*-expressing GS cells, SP cells from these sources were not enriched in SSCs. Thus, the SP phenotype does not appear to be a reliable criterion for defining SSCs both *in vitro* or *in vivo*.

Previous studies on the feasibility of SSC enrichment via the SP phenotype have yielded inconsistent results. Whereas three studies demonstrated significant SSC enrichment in both pup and adult testes [9, 10, 12], two other studies failed to show SSC enrichment in the SP population [6, 25]. EPCAM-expressing undifferentiated spermatogonia from adult testis were also reported to contain SP cells, but no transplantation was conducted to confirm SSC enrichment in that study [11]. In the present study, SP cells appeared stochastically *in vitro*, but consistently *in vivo* 3–4 months after transplantation, which was when the donor cells were undergoing steady spermatogenesis. Consistent SP cell development was also observed after transplantation of *H-RasV12*-expressing GS cells. These results confirm previous reports that SP cells are produced not only by Leydig cells but also by germ cells [6, 9–12, 25]. However, despite the consistent development of SP cells in seminiferous tubules, transplantation of SP cells did not enrich the SSCs or cancer stem cell population in the recipient testes.

At present, it is difficult to reconcile the contradictory results of this and some of the previous studies; however, it was pointed out that the use of cryptorchid testes as SSC donor in some studies may have influenced SSC biology in those studies [10]. Hoechst 33342 toxicity might also contribute to the inconsistency of the SP phenotype [25]. However, we did not note any differences between cryptorchid and WT testes with regard to SP phenotype, and we confirmed that cell sorting using Hoechst 33342 at the experimental concentration of 4 μg/ml had no apparent toxic effects, at least at the time of transplantation, by trypan blue staining. Although further studies are needed to explain the various experimental discrepancies, our results indicate that the SP phenotype is not practically useful for identifying SSCs in GS cell culture. Moreover, our failure to observe SSC enrichment after GS cell transplantation calls into question the biological significance of the SP phenotype. Although it is possible that GS cells are culture-adapted/modified germ cells, they reinitiate normal spermatogenesis after transplantation, and we observed consistent SP cell development, as other studies have reported. As previously suggested [28], caution is necessary when an SP separation method is used for SSC identification *in vivo*.

Our results also provide evidence for SP-phenotypic stochasticity of GS cells. The SP phenotype was not only unstable, but was also interconvertible with the non-SP phenotype. We previously demonstrated a similar phenotypic reversibility in SSCs [29]; in GS cell cultures, SSCs exhibited nontraditional phenotypes and stochastically expressed KIT, a marker of differentiating spermatogonia and early spermatocytes. The KIT^+ and KIT^- GS cells had comparable levels of SSC activity, and spermatogenesis occurred after transplantation of either population [29]. Although another group recently reported that rat SSCs differentiate in a stochastic manner [30], their findings were distinct from ours with regard to the reversibility of SSC fate. Whereas SSCs in the rat study were irreversibly committed to differentiation and died synchronously, the KIT phenotype in our previous study was interconvertible: KIT^+ cells turned into KIT^- cells without losing SSC activity [29]. In this context, the reversibility of the SP phenotype in the present study is reminiscent of the reversibility of KIT expression, confirming the fluctuation of the SSC phenotype *in vitro*.

SP phenotypic reversibility has also been reported in hematopoietic stem cells (HSCs). SP cells in bone marrow are remarkably enriched in HSCs [7, 8]. Although SP cells were initially thought to differentiate unidirectionally into non-SP cells, quantitative transplantation studies have revealed that both SP and non-SP cells exhibit similar levels of HSC activity [31]. Moreover, the non-SP and SP phenotypes are reversible *in vivo* after transplantation. Although SP cells developed only inconsistently in our GS cell cultures, they develop consistently in hematopoietic systems. Although the lack of HSC culture systems prevents direct comparison with the spermatogenic system, these studies showed that the SP phenotype does not specify all HSCs. In fact, the SP phenotype is not the only unstable characteristic of HSCs; HSCs also exhibit dynamic changes in the expression of CD34 [32], a marker for activated HSCs that is not expressed in mitotically quiescent HSCs. It is possible that SSCs might have a similarly flexible phenotype and do not necessarily show fixed marker expression.

The findings of the present study extend our previous study and provide additional evidence that SSCs exhibit different phenotypes in different biological microenvironments. We speculate that the stochastic changes observed in GS cells *in vitro* are attributable to excessive stimulation of SSC self-renewal. Indeed, SSCs *in vivo* are constantly exposed to differentiation triggers, while the differentiation of SSCs *in vitro* is probably inhibited by their continuous exposure to GDNF. Although a recent study suggested that SSCs differentiate stochastically *in vivo* during steady-state spermatogenesis [33], we do not know whether SSC commitment occurs in the same manner *in vitro*. Comparison of *in vivo* and *in vitro* phenotypes may provide clues to the mechanisms by which SSCs are triggered to self-renew or differentiate and will further enhance our understanding on SSC fate commitment and its regulation.

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