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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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; 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 sections were counterstained with hematoxylin and eosin. 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.
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 on the basement membrane 2–3 weeks after transplantation [21]. By gating EGFP-expressing cells, we excluded the possibility of somatic cell contamination of the germ cells.

### 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 × 10⁵ SP cells and 1.8 to 2.9 × 10⁵ 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 colonies. 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 10⁵ 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 × 10⁶ 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 × 10⁶ 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.
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
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 ($0.8 \pm 0.3 \times 10^4$ cells; n=3) and $2.4–5.3 \times 10^5$ total EGFP-expressing cells ($3.4 \pm 0.9 \times 10^5$ 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 $0.8 \pm 0.4$ 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 ($2.1 \pm 1.2 \times 10^4$; n=3) per testis. On the other hand, $0.2–1.5 \times 10^5$ total EGFP-expressing cells ($7.2 \pm 3.9 \times 10^4$; 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 $10.1 \pm 9.1$ and $0.5 \pm 0.3$ 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 vitro 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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