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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.

Keywords: Spermatogenesis, Sertoli cell, Testis, Developmental biology, Gametogenesis

Introduction

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 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 backcrossed to a DBA/2 background. The growth factors used were mouse epidermal

96 growth factor (EGF; 10 ng/ml), human FGF2 (10 ng/ml), and rat GDNF (15 ng/ml; all from
97 PeproTech, Rocky Hill, NJ, USA). GS cells expressing constitutively active *Akt* or *H-RasV12* under
98 CAG promoter were previously described [19].
99
100 *Magnetic cell sorting (MACS) and SP cell analysis by flow cytometry*
101 Cultured cells were dissociated by treatment with 0.25% trypsin, and testis cells were
102 dissociated by two-step enzymatic digestion with collagenase type IV and trypsin, as previously
103 described [20]. For MACS, primary spermatogonia were collected from 7-day-old DBA/2 pup testes
104 using a rat anti-EPCAM antibody (G8.8; BioLegend, San Diego, CA, USA) and Dynabeads sheep
105 anti-rat IgG (Invitrogen, Carlsbad, CA, USA), as previously described [4]. For SP cell analysis,
106 single-cell suspensions were suspended at 10^6 cells/ml in Dulbecco's Modified Eagle's Medium
107 containing 2% fetal bovine serum (FBS) and 10 mM Hepes. SP cells were detected as those
108 remaining unstained after a 90-min incubation with 4 μg/ml Hoechst 33342 (Sigma, St. Louis, MO,
109 USA) at 37°C. For combined staining, 0.1 μg/ml rhodamine 123 (Rh 123; Invitrogen) was added
110 during the last 20 min of the Hoechst 33342 incubation. For immunostaining of SP cells, Hoechst
111 33342-stained cells were incubated with allophycocyanin- or Alexa 647-conjugated antibodies
112 against KIT (2B8), EPCAM (G8.8), ITGB1 (HMβ1-1), ITGA6 (GoH3), CD9 (MZ3) and FUT4
113 (MC480; BioLegend) after washing the cells with ice-cold phosphate-buffered saline containing 1%
114 FBS. Propidium iodide (1 μg/ml; Sigma) was added to exclude dead cells before analyses. Cells
115 were analyzed on a FACSAria 2 flow cytometer equipped with a 375-nm UV laser (7 mV; BD
116 Biosciences). The Hoechst dye was excited at 375 nm, and its dual emission wavelengths were
117 detected using 450/20 (Hoechst 33342 blue) and 660/20 (Hoechst 33342 red) filters.
118
119 *Germ cell transplantation*

120 Single-cell suspensions of germ cells were microinjected into the seminiferous tubules of
121 WBB6F1-W/W^{v} (W) mice (Japan SLC, Shizuoka, Japan) through the efferent duct [20].
122 Approximately 75-85% of the tubules were filled in each recipient testis. The recipient mice were
123 treated with anti-CD4 antibody (GK1.5; a gift from Dr. T. Honjo, Kyoto University) to induce
124 tolerance to the allogeneic donor cells [21]. Immediately after transplantation, some of the recipients
125 were made cryptorchid by suturing the testicular fat pad to the lateral abdominal wall, as previously
126 described [22]. All animal experimentation protocols were approved by the Institutional Animal
127 Care and Use Committee of Kyoto University.
128
129 *Analysis of recipient testes*
130 Donor cell-derived colonies in the recipient testes were detected by enhanced green
131 fluorescence protein (EGFP) fluorescence under UV light. Germ cell clusters were defined as
132 colonies when they were longer than 0.1 mm and occupied the entire circumference of the
133 seminiferous tubule. For histological analyses, samples were fixed in formalin, embedded in paraffin
134 and sectioned. All sections were counterstained with hematoxylin and eosin.
135
136 *Reverse transcriptase-polymerase chain reaction (RT-PCR)*
137 Total RNA was isolated using TRIzol (Invitrogen), and first-strand cDNA was produced
138 using Superscript II (Invitrogen). The specific RT-PCR primers are listed in Table S1. PCR was
139 performed using a single incubation at 95°C for 5 min, followed by 30 cycles of 94°C for 30 sec,
140 60°C for 30 sec and 72°C 1 min.
141
142 *Statistical analysis*
143 Results are presented as means ± SEM. Data were analyzed using the Student's *t*-test.

144

145 **Results**

146 *Identification of SP cells in GS cell cultures*

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

153 To determine whether GS cells exhibit the SP phenotype, GS cells expressing the gene
154 for EGFP were cultured on laminin for 1-6 days (Fig. 1B), recovered by trypsin digestion, and
155 stained with Hoechst 33342. Unlike the whole testis cell population, which yielded at least six
156 distinct regions on a flow cytometry dot plot [9], GS cell cultures exhibited a simpler dot plot
157 pattern, indicating a more uniform composition of the stained cells (Fig. 1C). However, SP cells
158 were found in only 57.1% (24 out of 42) of the GS cell cultures, indicating that the SP population
159 developed stochastically. At least three independent GS cell lines showed stochastic expression.
160 Development of the SP population was not influenced by the length of time after passage, but was
161 inhibited by the addition of verapamil (Fig. 1D), which inhibits dye efflux by ABC transporter
162 activity [7]. In cultures with SP cells, the frequency of the SP population ranged from 0.3 to 22.3%
163 ($2.1 \pm 0.9\%$; $n = 24$), whereas non-SP cells were consistently found in all of these cultures.

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

168 hematopoietic or neuronal cells.

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

177

178 *Reversibility of the SP phenotype in GS cell culture*

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

187 The colonies that developed from the cultured SP and non-SP cells were morphologically
188 indistinguishable and did not vary significantly in growth rate. To observe the SP phenotype of the
189 developed colonies, the cells were stained again with Hoechst 33342 on days 29 and 34 after sorting.
190 In two separate experiments, the cultured SP cells differentiated into non-SP cells, whereas the
191 cultured non-SP cells produced both SP and non-SP cells. As they did on laminin, GS cells

192 stochastically produced SP cells on MEFs and yielded a pattern of Hoechst 33342 similar to that of
193 the original unsorted GS cell cultures. These results indicate that SP and non-SP cells are
194 interconvertible in vitro.
195
196 *SSC activity of SP cells in GS cell culture*
197 To determine whether the SP cell population is enriched in SSCs, we used germ cell
198 transplantation to compare the SSC activity of SP cells with that of total GS cells (Fig. 3B). These
199 two populations of cells were microinjected into the seminiferous tubules of infertile W mice
200 lacking endogenous spermatogenesis. In four separate experiments, a total of 20 recipient testes
201 received transplantation of each cell type.
202 The recipients were sacrificed 2 months after transplantation. The isolated recipient testes
203 were then analyzed for donor cell colonization levels by counting the number of EGFP-expressing
204 germ cell colonies under UV light. Transplantation of the SP and total GS cell populations produced
205 560.3 ± 432.0 and 142.5 ± 22.6 (n = 20) SSC-derived colonies per 10^5 donor cells, respectively.
206 Although the frequency of SSC colony production was higher for the SP cells, the difference
207 between the two cell types was not statistically significant ($P = 0.12$; Table 1), indicating that SP
208 cells are not enriched in SSCs.
209 Histological analyses of the recipient testes showed normal differentiation of the
210 transplanted SP cells (Fig. 3C). No spermatogenesis was observed in the nontransplanted control W
211 testes.
212
213 *SP cell phenotype after transplantation in vivo*
214 To examine the effect of the microenvironment on SP cell development, we transplanted
215 EGFP-expressing GS cells into seminiferous tubules (Fig. 4A). In these GS cells, the EGFP gene is

216 driven by the CAG promoter and is expressed at all stages from the spermatogonium to the round
217 spermatid [21]. By gating EGFP-expressing cells, we excluded the possibility of somatic cell
218 contamination of the germ cells.

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

225 We transplanted approximately 4×10^6 EGFP-expressing GS cells grown on laminin into
226 the seminiferous tubules of W mice. Some of the testes were surgically fixed at a higher position in
227 the abdomen to induce cryptorchidism, which destroys differentiating germ cells [22]. Recipients
228 were sacrificed at the early (7-14 days) or late (3-4 months) phase after transplantation, and the
229 testes were dissociated into single cells using a collagenase type IV/trypsin, a two-step digestion
230 technique that is commonly used to prepare single-cell suspensions for germ cell transplantation
231 [20].

232 At the early phase after transplantation, approximately $1.8\text{-}2.0 \times 10^6$ cells were recovered
233 per testis, and the proportion of EGFP-expressing cells was small (Fig. 4B). The overall Hoechst
234 33342 staining pattern of the recovered cells indicated no SP cells (Fig. 4B and C). At the later
235 phase after transplantation, the recipient testes were significantly larger, and the two-step digestion
236 technique described above yielded $2.8\text{-}5.9 \times 10^6$ cells per testis. The cryptorchid testes yielded only
237 $1.0\text{-}1.5 \times 10^6$ cells per testis because germ cell differentiation was limited by the high temperature of
238 the body cavity. In contrast to our findings in vitro, all nine experiments performed in vivo yielded
239 SP cells. The pattern of Hoechst 33342 staining in normal W recipients appeared more complex in

240 the later stage of transplantation than in the early stage (Fig. 4B and C), reflecting the colonization
241 and differentiation of the transplanted GS cells over time. Although cryptorchid testes also yielded
242 SP cells, the staining pattern of the dissociated testicular cells of the cryptorchid testes differed
243 somewhat from that of the normal testes.

244 When only EGFP-expressing cells were gated for analyses, both wild-type (WT) and
245 cryptorchid testes cells yielded staining patterns that were distinctly different from those of the total
246 cells (Fig. 4B). Despite the elimination of some subpopulations by EGFP gating, the
247 EGFP-expressing cell populations from both WT and cryptorchid testes consistently yielded SP
248 cells (Fig. 4C). In WT recipients, the SP cells comprised 0.06 to 4.7% (1.9 ± 0.6%; n = 7) of the
249 EGFP-expressing cells, and they expressed typical SSC markers including ITGB1, ITGA6, and
250 EPCAM. Compared with GS cells, however, CD9 is only weakly expressed in both SP and non-SP
251 cells, suggesting that these populations have a reduced SSC concentration. Although they weakly
252 expressed KIT, no FUT4 expression was detected (Fig. 4D).

253 To examine the SSC activity of SP cells found in the testis after GS cell transplantation,
254 the SP cells were separated from the WT primary recipient testes 3 to 4 months after GS cell
255 transplantation and implanted into secondary recipient testes (Fig. 4E). We gated EGFP-expressing
256 cells and sorted 0.5-1.5 × 10^4 SP cells (0.8 ± 0.3 × 10^4 cells; n = 3) and 2.4-5.3 × 10^5 total
257 EGFP-expressing cells (3.4 ± 0.9 × 10^5 cells; n = 3). All of the sorted cells were clearly positive for
258 EGFP expression, and the total EGFP-expressing cell population was implanted into secondary
259 recipient testes as a control. In three separate experiments, a total of 18 and 16 recipient testes were
260 transplanted with SP cells or total EGFP-expressing cells, respectively. The secondary recipient
261 testes yielded no colony after SP cell transplantation, whereas total EGFP cells yielded 0.8 ± 0.4
262 colonies per 10^5 donor cells (n = 16; Table 1). The difference was not statistically significant (P =
263 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 4×10^6 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 × 10^4 cells (2.1 ± 1.2 × 10^4; n = 3) per testis. On the other hand, 0.2-1.5 × 10^5 total EGFP-expressing cells (7.2 ± 3.9 × 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 continuous 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

312 also contribute to the inconsistency of the SP phenotype [25]. However, we did not note any
313 differences between cryptorchid and WT testes with regard to SP phenotype, and we confirmed that
314 cell sorting using Hoechst 33342 at the experimental concentration of 4 μg/ml had no apparent toxic
315 effects, at least at the time of transplantation, by trypan blue staining. Although further studies are
316 needed to explain the various experimental discrepancies, our results indicate that the SP phenotype
317 is not practically useful for identifying SSCs in GS cell culture. Moreover, our failure to observe
318 SSC enrichment after GS cell transplantation calls into question the biological significance of the SP
319 phenotype. Although it is possible that GS cells are culture-adapted/modified germ cells, they
320 reinitiate normal spermatogenesis after transplantation, and we observed consistent SP cell
321 development, as other studies have reported. As previously suggested [28], caution is necessary
322 when an SP separation method is used for SSC identification in vivo.

323 Our results also provide evidence for SP-phenotypic stochasticity of GS cells. The SP
324 phenotype was not only unstable, but was also interconvertible with the non-SP phenotype. We
325 previously demonstrated a similar phenotypic reversibility in SSCs [29]; in GS cell cultures, SSCs
326 exhibited nontraditional phenotypes and stochastically expressed KIT, a marker of differentiating
327 spermatogonia and early spermatocytes. The KIT^+ and KIT^- GS cells had comparable levels of SSC
328 activity, and spermatogenesis occurred after transplantation of either population [29]. Although
329 another group recently reported that rat SSCs differentiate in a stochastic manner [30], their findings
330 were distinct from ours with regard to the reversibility of SSC fate. Whereas SSCs in the rat study
331 were irreversibly committed to differentiation and died synchronously, the KIT phenotype in our
332 previous study was interconvertible: KIT^+ cells turned into KIT^- cells without losing SSC activity
333 [29]. In this context, the reversibility of the SP phenotype in the present study is reminiscent of the
334 reversibility of KIT expression, confirming the fluctuation of the SSC phenotype in vitro.

335 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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