

Enrichment of Spermatogonial Stem Cells using Side Population in Teleost

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Enrichment of spermatogonial stem cells using side population in teleost¹**Running title: Enrichment of fish spermatogonial stem cells**

Summary sentence: The method for spermatogonial stem cell enrichment from type A spermatogonia using fluorescent intensity after Hoechst 33342 staining was established in rainbow trout.

Keywords: fish, flow cytometry, side population, Hoechst 33342 dye, spermatogonial stem cells, germ cell transplantation

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ABSTRACT

Spermatogenesis originates from a small population of spermatogonial stem cells; this population can maintain continuous sperm production throughout the life of fish via self-renewal and differentiation.

Despite their biological importance, spermatogonial stem cells are not thoroughly characterized

5 because they are difficult to distinguish from their progeny cells that become committed to differentiation. We previously established a novel technique for germ cell transplantation to identify spermatogonial stem cells based on their colonizing activity and their ability to initiate donor-derived gametogenesis in the rainbow trout (*Oncorhynchus mykiss*). Although spermatogonial stem cells can be retrospectively identified after transplantation, there is currently no technique to prospectively enrich
10 for or purify spermatogonial stem cells. Here, we describe a method for spermatogonial stem-cell enrichment using side-population. With optimized Hoechst 33342 staining conditions, we successfully identified side-population cells among type A spermatogonia. Side-population cells were transcriptomically and morphologically distinct from non-side-population cells. To functionally determine whether the transplantable spermatogonial stem cells were enriched in the side-population
15 fraction, we compared the colonization activity of side-population cells with that of non-side-population cells. Colonization efficiency was significantly higher with side-population cells

than with non-side-population cells or with total type A spermatogonia. In addition, side-population cells could produce billions of sperm in recipient. These results indicated that transplantable spermatogonial stem cells were enriched in the side-population fraction. This method will provide biological information that may advance our understanding of spermatogonial stem cells in teleosts. Additionally, this technique will increase the efficiency of germ-cell transplantation used in surrogate broodstock technology.

INTRODUCTION

Spermatogonial stem cells (SSCs) are responsible for maintaining spermatogenesis [1, 2].

25 SSCs can undergo self-renewal and produce differentiated germ cells that are committed to becoming
mature spermatozoa. However, despite their biological importance, SSCs are difficult to study because
distinguishing them from cells committed to differentiation is technically challenging. Currently, only a
few SSC markers are available in a limited number of animal species. In mice, germ-cell transplantation
can be used to retrospectively identify SSCs based on their biological function [3, 4]. Testicular germ
30 cells transplanted into the seminiferous tubules of infertile recipient mice can reinitiate spermatogenesis
and produce large numbers of functional sperm over a prolonged period, indicating that the transplanted
cells include a population of cells that behave as SSCs. This technique has allowed important
breakthroughs to be made in mammalian SSC research [5].

Reproductive strategies differ greatly among fish taxa. Some species have a defined breeding
35 season, and others are highly reproductively active throughout the year [6]. Most salmonid species
produce gametes only once during their life cycle (semelparity) [7-9]; however, some salmonids,
including trout and char, undergo repeated spawning (iteroparity) [10-12]. Moreover, some fish species
can perform sex reversal during their life cycle (hermaphroditic) [13]. Although SSCs might play

pivotal roles in the abovementioned reproductive strategies, they have not yet been well studied in fish.

40 Improving our knowledge of fish SSCs could help to reveal the mechanisms underlying fish reproductive diversity.

To identify SSCs in fish, we previously established a novel method of transplanting germ cells based on their biological function in the rainbow trout (*Oncorhynchus mykiss*) [14]. Not all but only a portion of the testicular germ cells transplanted into the peritoneal cavity of recipient embryos
45 migrated toward and then colonized the embryonic gonad; subsequently, these colonizing cells produced large numbers of functional sperm for several years. These results have provided functional evidence that the transplanted testicular germ cells included SSCs. Furthermore, no significant difference was observed between the percentage of recipient embryos with colonizing donor germ cells and the percentage of mature recipient fish that produced donor-derived sperm [14], demonstrating that
50 the colonizing germ cells behaved as SSCs in the recipient gonads.

In addition, a previous study revealed that among testicular germ cells, type A spermatogonia (A-SG) have colonizing activity, but type B spermatogonia, spermatocytes, and spermatids do not [15]. More importantly, only 0.046% of transplanted A-SG colonized the recipient gonad [14]; in contrast, about 10% of transplanted primordial germ cells, all of which are likely to have the colonization activity,

55 colonized the recipient gonad [16]. Therefore, A-SG constitute a heterogeneous population that includes cells with colonizing activity and those without. In other words, it appears that only some A-SG possess stem cell activity. Although colonizing SSCs can be retrospectively identified after transplantation and subsequent analyses, a technique to prospectively enrich for or purify teleost SSCs has not been developed.

60 Stem cells in many self-renewing tissues, including mouse bone marrow, can be found among side-population (SP) cells that are characterized by the ability to exclude Hoechst 33342 dye (H33342) [17]. Members of the ATP-binding cassette (ABC) transporter family are responsible for this exclusion activity. However, the utility of this method to enrich for rodent SSCs remains controversial [18-22]. Here, in order to prospectively enrich for teleost SSCs that can be used for transplantation, we analyzed
65 whether SSCs were enriched among SP cells.

MATERIALS AND METHODS

Ethics

All experiments described herein were carried out in accordance with the Guide for the Care and Use of Laboratory Animals from Tokyo University of Marine Science and Technology.

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Preparation of suspensions of testicular cells

Testes were collected from 10- to 15-mo-old *pvasa-GFP* transgenic rainbow trout; at this stage, germ cells are A-SG with strong GFP intensity [15, 23]. Not all but only a portion of the A-SG isolated at this stage can colonize and undergo self-renewal in recipient gonads following

75 transplantation [14]. Approximately 4 million testicular cells could be obtained from a single fish.

Testes from 15 to 30 fish were pooled and dissociated as described in Okutsu et al. [14]. Dissociated testicular cells were washed three times with L-15 medium (pH 7.8) (Life Technologies, Carlsbad, CA)

supplemented with 10% fetal bovine serum (FBS; Life Technologies), 25 mM HEPES (Sigma, St.

Louis, MO), and antibiotics (50 μ g/ml of ampicillin, 50 U/ml of penicillin, and 50 μ g/ml of

80 streptomycin; all antibiotics were from Wako, Osaka, Japan) to eliminate enzyme activity; cells were

collected after each wash via centrifugation.

Flow-cytometric analysis and cell sorting

Dissociated testicular cells were diluted to 1×10^6 cells/ml in L-15 medium (pH 7.8) (Life Technologies) supplemented with 10% FBS (Life Technologies), 25 mM HEPES (Sigma), and antibiotics; 1-ml samples were then dispensed into 2-ml plastic tubes, and stained with H33342 (Dojindo, Kumamoto, Japan). We initially tested combinations of the following conditions: H33342 at concentrations of 5, 10, 15, 20, 25, 50, or 100 $\mu\text{g/ml}$; staining times of 1.5, 2, 3, 4, 6, 8, 10, 12, or 15 h; and temperatures of 10, 14, 16, or 18°C. For subsequent experiments, we used an optimized staining condition, which was 5 $\mu\text{g/ml}$ H33342 for 10 h at 16°C. To inhibit the ABC transporters, verapamil (Sigma) was added to the H33342 staining solution at a final concentration of 30 $\mu\text{g/ml}$. Before each analysis, propidium iodide (PI; Dojindo) was added to each sample at a final concentration of 1 $\mu\text{g/ml}$ to eliminate dead cells from the sorting gates.

A MoFlo XDP (Beckman Coulter, Miami, FL) equipped with a 355-nm Xcyte laser and a 488-nm sapphire laser was used for flow cytometry and cell sorting. A 529-nm band-pass filter was used to detect GFP. Hoechst blue and Hoechst red were detected using 457- and 670-nm band-pass filters, respectively. Green fluorescence was measured using a logarithmic amplifier; Hoechst blue and

red were measured using linear amplifiers. Cells were sorted through a 100- μ m flow cell under 30-psi sheath-fluid pressure. Cell sorting was carried out at a rate of 2,000–5,000 counts/s using the Purify

100 Mode.

Short-term culture of A-SG

SP cells and non-SP cells were cultured essentially as described in Shikina et al. [24], but with several modifications. Cells were seeded at a concentration of 1.3×10^4 cells/well in 96 well plate and

105 cultured at 10°C in L-15 medium (pH 7.8) (Life Technologies) supplemented with 10% FBS (Life

Technologies), 25 mM HEPES (Sigma), and antibiotics. After 3 days of culture, the number of viable A-SG that expressed GFP was determined using the Guava PCA-96 flow cytometry system (Millipore,

Billerica, MA).

110 *Microarray Experimental Design*

Microarray analysis was performed using a custom-made 60-mer oligo microarray [25] (GPL17533; Agilent Technologies, Santa Clara, CA). Gene expression analysis is greatly affected by genetic heterogeneity among samples. However, no inbred strains of rainbow trout have been

established, and intrinsic genetic heterogeneity of the fish used in independent experiments was
 115 unavoidable. Therefore, to estimate and remove biases of parental fish groups from expression values
 (see Microarray Data and Statistical Analysis section), SP and non-SP cells were collected from three
 independent cell-sorting events that each involved three different groups of parental fish. Preparation of
 labeled cRNA and hybridization were performed as described in Hayashi et al. [25]. A GenePix 4000B
 scanner (Molecular Devices, Sunnyvale, CA) was used to scan each array. Each array was scanned
 120 twice at different sensitivity levels to improve the accuracy of signal measurements. Feature Extraction
 9.5 (Agilent Technologies) was used to extract signal data, and the data were deposited in the Gene
 Expression Omnibus (GEO) under accession no. GSE49565.

Processing and statistical analysis of microarray data

125 The gProcessedSignal values calculated from scanned images at different sensitivities were
 combined to obtain the raw expression value for each spot [26]. The raw values were then normalized
 across samples using quantile normalization. For each probe, the following linear model was fitted to
 the normalized expression value:

$$S_{ijkl} = C_i + G_j + C_i : G_j + N_l + \varepsilon_{ijkl}.$$

130 where S is the \log_2 signal intensity of the normalized expression value, C , G , and N are the cell type, group, and scan effects, respectively, ε is the residual, and $C:G$ is an interaction term. The estimated values for cell types were used for further statistical analyses. An empirical Bayes method (limma package in Bioconductor, <http://www.bopconductor.org>) was used for variance shrinkage. The P -values were corrected for multiple tests using Storey's false discovery rate to obtain q-values [27]. The

135 abovementioned procedures were performed using R, limma package in Bioconductor (<http://www.bopconductor.org>), and custom R scripts.

Morphological analysis of sorted cells

Hematoxylin-eosin (HE) staining was performed with smeared samples of sorted cells to

140 analyze the morphology of SP and non-SP cells. Approximately 1×10^5 sorted cells were fixed in Bouin's solution, and smears were prepared on glass slides coated with Matsunami adhesive silane (MAS) (Matsunami Glass Co. Ltd., Osaka, Japan). Samples were dried overnight at room temperature; slides were then rinsed with phosphate-buffered saline (PBS) before HE staining. Stained samples were analyzed as described by Loir to determine cell size and the number of dense bodies (nucleoli and

145 heterochromatin masses) per cell [28]. Cell size was measured using ImageJ software (National Institutes of Health, Bethesda, MD).

Germ-cell transplantation assay

Cells isolated from *vasa*-GFP-positive A-SG based on H33342 fluorescence were
150 transplanted into the peritoneal cavity of non-transgenic rainbow trout hatchlings as described by Okutsu et al. [14]. Isolated cells were suspended in Eagle's MEM (Nissui, Tokyo, Japan) supplemented with 20 mM HEPES (Sigma) and 5% FBS (Life Technologies). For each injection, approximately 150 cells were injected into the peritoneal cavity of one recipient non-transgenic hatchling in the present study. At 20 or 100 days post-transplantation (dpt), recipient embryos were dissected, and the
155 percentage of recipient fish that had transplanted A-SG colonizing their genital ridge among all injected recipient was determined by observing the recipient gonads under a fluorescence microscope (model BX-51-34FL; Olympus).

Progeny Tests

160 We used triploid, sexually undifferentiated recipients to determine whether transplanted,

donor SP cells could give rise to functional sperm. Triploid recipients were produced as described in Okutsu et al. [29]. To determine the sex of recipients that had matured to the hatchling stage, total genomic DNA was extracted from the fin of recipients and subjected to PCR analyses with *sdY*-specific primers, 5'-ATGGCTGACAGAGAGGCCAGAATCC-3' and

165 5'-CTTAAAACCACTCCACCCTCCAT-3'. *sdY* is a male-specific genomic sequence on the Y chromosome [30]. After recipients had matured at 2 years old, milt produced by each male recipient was obtained by applying abdominal pressure. To determine the production of spermatozoa derived from donor SP cells, total genomic DNA was extracted from the milt of recipients and subjected to PCR analyses with *Gfp*-specific primers, 5'-GACGTAAACGGCCACAAGT-3' and

170 5'-TCCAGCAGGACCATGTGAT-3'. *β -actin* was used as an internal control for PCR; the *β -actin* primers were 5'-AGCCCAAACCCAGCTTCTCA-3' and 5'-AGAGGTCACACTCGGGTTCATT-3'.

The number of donor-derived spermatozoa was calculated as described in Okutsu et al. [14]. To obtain offspring originating from sperm derived from donor SP cells, sperm obtained from triploid recipients were used for *in vitro* fertilization of eggs obtained from non-transgenic wild-type rainbow trout. As

175 donor SP cells had been obtained from donor fish that were hemizygous for *pvasa-GFP* (*Gfp*⁻) and heterozygous for a mutation that causes a dominant orange color[31] (OR/wild-type (WT)), F1 larvae

would be expected to exhibit a 50% ratio of the dominant donor phenotypes (OR/WT; *Gfp*^{-/-}) following Mendelian inheritance.

180 *Statistical Analysis*

In this study, multiple statistical methods were used. The statistical methods used for analysis of microarray data are described in "*Processing and statistical analysis of microarray data*" section. Dunnett's test was used for multiple comparison of the average percentage of PI-positive cells by comparisons with control in the experiment used to optimize Hoechst staining. The student's t-test was used for comparisons between verapamil-treated and untreated A-SG populations with regard to the percentage of SP cells. The student's t-test was also used for comparisons between SP and non-SP cells with regard to cell survival rates. Wilcoxon rank sum test, which is a non-parametric test for comparing two samples, was used for comparison between SP and non-SP cells with regard to cell size. The chi-square test was used for comparison between SP and non-SP cells with regard to the percentage of cells with only a single dense body and for comparison the rate of recipient fish with transplanted cells. Fisher's exact test was used for comparison the distribution of the number of recipients with each number of incorporated donor cells. Statistical significance levels were set to P -value < 0.05 and

q-value < 0.05.

RESULTS

195 *Identification of SP cells in A-SG*

In this study, to enrich for SSCs in rainbow trout, we focused on SP. To investigate whether a portion of A-SG in rainbow trout exhibit a SP phenotype, we optimized H33342 concentration and staining temperature and time to achieve maximum differential staining between SP and non-SP cells. Cells derived from mouse bone marrow usually take up stain when exposed to 5 $\mu\text{g/ml}$ H33342 for 1.5 h at 37°C [17]. However, rainbow trout are reared in cold fresh water (10°C), and rainbow trout germ cells are damaged at temperatures over 20°C [32]. In order to avoid damaging the rainbow trout germ cells, we modified the mouse protocol by changing only staining temperature, and used 5 $\mu\text{g/ml}$ H33342 for 1.5 h at 18°C. However, this condition was not enough to saturate H33342 staining of A-SG nuclei. The coefficient of variation (CV) value of G0/G1 cells was more than 20, and this staining condition did not split A-SG into SP cells and non-SP cells (Fig. 1 A-C). To determine the appropriate staining conditions that would saturating H33342 staining and minimize negative effects on cell viability, we tested combinations of the following conditions: H33342 concentrations of 5, 10, 15, 20, 25, 50, or 100 $\mu\text{g/ml}$; staining times of 1.5, 2, 3, 4, 6, 8, 10, 12, or 15 h; and temperatures of 10, 14, 16, or 18°C. Initially, we assessed the effect of H33342 concentration on testicular cell viability. For 1.5 h

210 (the shortest staining time) at 10°C (the lowest temperature), forward scatter (FS) and side light scatter (SS), which are indicators of the condition of cell membrane, were analyzed. At H33342 concentrations of 50 or 100 $\mu\text{g/ml}$, FS was reduced and SS was increased (Fig. S1 A-H). These results indicated that testicular cells were damaged with these H33342 concentrations. In addition, with a H33342 concentration of 20 or 25 $\mu\text{g/ml}$, the rate of PI-positive cells was significantly increased (Fig. S1I).

215 Therefore, we used only H33342 concentrations of 5, 10, or 15 $\mu\text{g/ml}$ to optimize staining time and temperature. We found that the following eight sets of staining conditions each resulted in saturated H33342 staining of A-SG nuclei and very minimal negative effects on testicular cell viability: 5 $\mu\text{g/ml}$ H33342 for 10 h 16°C, 5 $\mu\text{g/ml}$ H33342 for 10 h 18°C, 10 $\mu\text{g/ml}$ H33342 for 8 h 14°C, 10 $\mu\text{g/ml}$ H33342 for 3 h 16°C, 10 $\mu\text{g/ml}$ H33342 for 3 h 18°C, 15 $\mu\text{g/ml}$ H33342 for 6 h 14°C, 15 $\mu\text{g/ml}$ H33342 for 3 h 16°C or 15 $\mu\text{g/ml}$ H33342 for 2 h 18°C (Table S1). For each set of conditions, the CV value of G0/G1 was less than 7 (Table S1). When selecting the optimal set of staining conditions for subsequent experiments, we recognized that higher H33342 concentrations had negative effects on cell viability; therefore, we chose from among the two sets of conditions with the lowest H33342 concentration, 5 $\mu\text{g/ml}$ H33342 for 10 h 16°C and 5 $\mu\text{g/ml}$ H33342 for 10 h 18°C, and selected set with the lowest

225 temperature. When A-SG were stained with 5 $\mu\text{g/ml}$ H33342 for 10 h 16°C, the SP cells in A-SG could

be identified by their low fluorescence (Fig. 1 D and E). The percentage of SP cells was $5.65 \pm 0.61\%$ of all A-SG. To investigate whether ABC transporters caused the low fluorescence of SP cells, A-SG were stained with H33342 in the presence of verapamil, which is an inhibitor of ABC transporters. The verapamil treatment resulted in a significant decrease in the percentage of SP cells ($1.23 \pm 0.18\%$; P -value < 0.05 ; Student's t-test) (Fig. 1F), indicating that the low H33342 fluorescence of SP cells resulted from ABC transporter activity.

To prepare suspensions of testicular cells, testes were dissociated with trypsin for 2 h. Before H33342 staining, this treatment may have affected cell viability, and only healthy cells may have exhibited an SP phenotype. In other words, A-SG might be subdivided into healthy and unhealthy cells that correspond to SP cells and non-SP cells, respectively. To compare the health of SP cells with that of non-SP cells, we analyzed the survival rate after culturing each cell population *in vitro* for 3 days. After 3 days of culture, the cell size and GFP intensity were similar to those of the cells just after collection (data not shown). No significant difference was observed between the survival rate of SP cells and that of non-SP cells (Fig. 2A), suggesting that the difference between SP cells and non-SP cells was not caused by differences in cell viability.

To investigate whether SP cells were transcriptomically distinct from non-SP cells,

microarray analysis was used to compare genome-wide gene expression in SP cells with that in non-SP cells. The statistical analysis indicated that for 16,148 probes (28.9% of the all the printed probes) the level of expression differed significantly between the two cell types (q -value < 0.05 ; see Materials and Methods) (Fig. 2B) (Table S2). Among these 16,148 probes, 8,350 probes showed higher expression in SP cells than in non-SP cells (maximum value of \log_2 (SP / non-SP) was 3.76, minimum value was 0.23); additionally 7,798 probes showed lower expression (minimum value of \log_2 (SP / non-SP) was -5.66, maximum value was -0.22) in SP cells than in non-SP cells (Fig. 2B). This result indicated that the mRNA expression profile of SP cells differed significantly from that of non-SP cells.

250

Morphological characterization of SP cells

To investigate whether SP cells constituted an SSCs-enriched fraction, we characterized and compared between the morphology of SP and non-SP cells. Cell morphology was assessed, as described by Loir [28], with regard to cell size and the number of dense bodies per nuclei. The percentage of cells containing only a single dense body among all cells in a population was higher for the SP population (31.4%; $N = 102$) than for the non-SP population (15.4%; $N = 104$) (P -value < 0.05 ; Chi-square test) (Fig. 3 A–C). In addition, SP cells were significantly larger than non-SP cells (Fig. 3 A, B, and D).

Transplantation assay of SP cells in A-SG

260 To investigate whether SP cells had higher colonizing activity in recipient gonads, approximately 150 SP cells or non-SP cells isolated from *pvasa-GFP*-positive A-SG were transplanted into the peritoneal cavity of each recipient embryo. This number of transplanted cells was fewer than that in our previous study [14] to avoid saturating the colonization rate. At 20 dpt, GFP-labeled SP cells colonized the recipient gonads (Fig. 4 A and D). Notably, the colonization efficiency of SP cells was

265 significantly higher than that of non-SP cells or of unsorted A-SG (Table 1). Additionally, the number of cells that incorporated into the gonads of each recipient embryo was significantly greater for SP cells than for non-SP cells (Fig. 4G). For non-SP cells, the maximum number of incorporated cells per gonads of each recipient embryo was two; in contrast, the maximum number of incorporated SP cells was 10. Among all recipients transplanted with SP cells, 24.7% (23/93) recipients carried greater or

270 equal to two donor-derived germ cells in the gonads. Only 14.1% (12/85) or 2.9% (2/70) of the recipients that were transplanted with total A-SG or non-SP cells, respectively, carried greater or equal to two donor-derived germ cells in the gonads. Although it is difficult to completely exclude the possibility that some incorporated cells had proliferated, it is most likely that the number of cells in each

recipient reflected the number of incorporated cells.

275 To further investigate whether colonizing SP cells were able to proliferate and to form colonies of descendants, we examined recipient gonads at 100 dpt. A colony of transgenic cells formed from descendants of transplanted SP cells (Fig. 4 B and E). Importantly, transplanted SP cells from A-SG were also able to colonize the ovaries of female recipients and differentiated into oocytes (Fig. 5 C and F). The colony-formation efficiency of SP cells was significantly higher than that of non-SP cells or of unsorted A-SG in both sexes (Table 2). In addition, no significant difference was observed 280 between the colonization rate of SP cells at 20 dpt and at 100 dpt for all recipients, regardless of sex (P -value > 0.05; Chi-square test). These results indicated that SP cells that had colonized recipient gonads were able to proliferate and reinitiate gametogenesis.

 In order to determine whether colonizing SP cells differentiated into functional sperm in 285 recipient gonads, we used mature male recipients to perform progeny tests. We transplanted diploid SP cells from *pvasa-GFP* hemizygous (*Gfp*^{-/-}) fish that were also heterozygous (OR/WT) for a mutation that causes a dominant orange-colored phenotype [31] into non-transgenic wild-type triploid (-/-/-; WT/WT/WT) recipients that produce aneuploid sperm. Among the 16 recipient fish, 9 fish were male (Fig. 5A), and they each produced milt. To determine whether the sperm carried the *pvasa-GFP*

290 transgene, we initially performed PCR analysis. *Gfp* was evident in milt samples from only three of the
nine males (33.3%) (Fig. 5B). The average number of haploid sperm derived from donor SP cells was
 5.42×10^8 (range, 1.04 - 13.48×10^8). Each of the three *Gfp*-positive milt samples was used to inseminate
eggs obtained from a non-transgenic wild-type (-/-; WT/WT) rainbow trout. After the FI embryos
hatched, the ratio of *pvasa*-GFP-positive to *pvasa*-GFP-negative and the ratio of orange-colored trout to
295 wild-type trout were analyzed. Both ratios were nearly 1:1 (Fig. 5 C-H, Table 3). These results indicated
that 33.3% (3/9) of the male recipients produced donor-derived sperm that descended from colonizing
SP cells. Furthermore, this percentage was not significantly different from the percentage of recipients
colonized at 20 dpt (P -value > 0.05 ; Chi-square test) or of male recipients colonized at 100 dpt (P -value
 > 0.05 ; Chi-square test). In contrast, no male recipients transplanted with non-SP cells ($N = 6$) or with
300 unsorted A-SG ($N = 6$) produced donor-derived sperm.

DISCUSSION

We demonstrated that SP cells have higher colonizing activity than non-SP cells or unsorted A-SG and that they can initiate gametogenesis in gonads of recipient fish. In addition, colonizing SP cells were able to produce billions of functional sperm in individual recipient males. Once fish spermatogonia are committed to differentiation, they lose their self-renewal activity and possess only a limited ability to proliferate [33]. In rainbow trout, differentiated spermatogonia are reported to undergo mitosis no more than seven times, followed by two consecutive cycles of meiosis [28]; thus, one founder spermatogonium can produce up to 512 spermatozoa. In this study, the maximum number of incorporated SP cells per recipient was only 10. To produce billions of sperm, individual incorporated SP cells must have undergone mitosis more than 20 times, which was far more than predicted proliferation rate. Previously, we have also shown that colonizing germ cells produced functional gametes for at least three consecutive spawning seasons [14, 34]. The data indicated that colonizing SP cells subsequently 1) behaved as SSCs in recipient gonads; 2) had very high, possibly unlimited, capacity for self-renewal; and 3) could differentiate into spermatozoa. Therefore, we concluded that SSCs possessing high colonizing activity were enriched in the SP fraction. The SP analysis established in this study succeeded in prospectively enriching SSCs prior to using transplantation in fish.

In the transplantation assay, not only SP cells but also a portion of non-SP cells were incorporated into the recipient gonads and formed colonies. These data indicate that SSCs are also contained in non-SP cells. However, the colonization efficiency of SP cells was significantly higher than that of non-SP cells or of unsorted A-SG. Furthermore, the male recipients transplanted with SP cells produced donor-derived sperm; however, those with non-SP cells or with unsorted A-SG did not. Therefore, we concluded that SP cells have a greater percentage of SSCs possessing high colonizing activity than non-SP cells and unsorted A-SG.

Since SP cells are characterized by the ability of the ABC transporter to exclude H33342, it was possible that SP phenotype reflected only their healthiness. Consequently, SP cells may show the higher colonizing activity in transplantation assay. However, we detected no difference between the healthiness of SP cells and that of non-SP cells in an *in vitro* cell culture assay, suggesting that SP cells were an intrinsically different cell population from non-SP cells.

Although fish SSCs are expected to play pivotal roles in fish reproductive systems, very few studies have been conducted on these cells. The identification and isolation of SSCs are initial steps that are critical to further studies on teleost SSCs. Antibodies against the cell-surface antigens specific to SSCs are widely used to enrich for mouse SSCs [18, 35-39]. However, no such marker is currently

available for fish SSCs, and antibodies against mammalian antigens do not cross-react with rainbow trout homologs. Additionally, the vast genetic diversity among teleost species may hamper successful application of an antibody developed for one particular species to a wide variety of species; production of a versatile antibody that recognizes the SSCs of various fish species would be challenging. By contrast, SP analyses based on simple H33342 staining under the appropriate conditions may allow for SSCs to be enriched from total A-SG populations collected from different species. Although A-SG isolated from *pvasa-GFP* transgenic rainbow trout were used as the starting material in this study, we previously established a method for enrichment of A-SG that did not require transgenes. A-SG have light-scatter properties that distinguish them from other types of testicular cells [40]. Based on these light-scattering characteristics, A-SG can be isolated with a flow cytometer [40]. Combining A-SG purification by flow cytometry and SSC enrichment by SP isolation may become a powerful and applicable approach to enriching for SSCs from many fish species, even without transgenesis.

Morphological analysis revealed that the cells that possessed fewer dense bodies and were larger in size were enriched among SP cells. Loir [28] previously reported that A-SG were segregated into two types: A1-SG and A2-SG. A1-SG are larger in size and have fewer dense bodies visible within nuclei, and A2-SG are smaller in size and have more dense bodies. Loir speculated that A2-SG were

more differentiated than A1-SG, although there was no functional evidence supporting this hypothesis.

350 SP cells, which were enriched with transplantable SSCs relative to non-SP cells, were also enriched for cells that exhibited the characteristics of A1-SG; therefore, our results were consistent with Loir's hypothesis [28], and some or all A1-SG might behave as SSCs.

Despite previous efforts, whether mouse SSCs can be enriched via isolation of SP cells remains unclear [18-22]. The staining conditions used differed slightly among these mouse studies. A comparison of these experiments revealed that milder staining conditions: 5 $\mu\text{g/ml}$ H33342 at 32°C or 355 2.5 $\mu\text{g/ml}$ H33342 at 37°C, appeared to correlate with successful enrichment for SSCs in SP cells [19-21], whereas severer conditions: 4 $\mu\text{g/ml}$ H33342 at 35°C or at 37°C, did not [18, 22]. In each of these mouse experiments enrichment for SSCs was evaluated by functional assays, such as transplantation. Therefore, we speculated that some of the mouse SP cells were partially damaged with 360 severer staining conditions and that the damaged cells lost the ability to reinitiate spermatogenesis in recipient testis. Nevertheless, we suspect that each group was enriched with SSCs. Actually, our efforts to optimize the staining conditions revealed that each variable—staining temperature, H33342 concentration, and staining time—had a large effect on SP cell detection and A-SG viability. Within a narrow range of staining conditions, A-SG could be reproducibly separated into SP and non-SP cells

365 with little effect on viability. Therefore, to identify SP cells, it is essential to determine the appropriate set of staining conditions that allow SP cells to be separated from non-SP cells, but that do not affect cell viability.

Based on both on successful transplantation and on morphological characteristics, A-SG have previously been regarded as a heterogeneous population of cells [14, 28]. However, only indirect evidence for this supposition has been obtained previously because the techniques used in the previous studies could not separate A-SG into subpopulations. Here, we successfully used the SP phenotype to identify and harvest two distinct subpopulations from an A-SG population; these subpopulations differed from each other transcriptomically, morphologically, and functionally. Further characterization of these subpopulations will improve our understanding of fish SSCs and teleost SSC differentiation. The SSC-enrichment technique established in this study is important to the field of stem cell biology, and it is also a powerful tool for biotechnology. Previously, we established a germ cell transplantation technique that can produce gametes from commercially valuable and/or endangered species via surrogate parents in salmonids [29, 34]. This technology was recently applied to commercially important marine teleosts [41-44]. Furthermore, successful transplantation of A-SG into testes of adult recipients was also performed with pejerrey, tilapia, and zebrafish [45-47]. Combining our method for enrichment of SSCs

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that have higher colonizing activity and the techniques for germ cell transplantation could allow for the efficient production of gametes of valuable and/or endangered species.

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FIGURE LEGENDS

Fig. 1. Detection of SP cells in A-SG. (A) A-SG with GFP fluorescence were gated; x- and y-axis indicate GFP intensity and cell count, respectively. (B-F) Testicular cells derived from *pvasa-GFP* transgenic rainbow trout were stained with 5 $\mu\text{g/ml}$ H33342 for 1.5 h at 18°C (B and C) or with 5 $\mu\text{g/ml}$ H33342 for 10 h at 16°C (D-F). Verapamil was added to inhibit ABC transporters (F). GFP-positive A-SG, which were gated in (A) were analyzed at two emission wavelengths (x-axis, Hoechst red; and y-axis, Hoechst blue) (C, E, F). The CV values of G0/G1 were calculated (B, D); x- and y-axis indicate cell count and the intensity of Hoechst blue, respectively (B, D). The areas shown for SP and non-SP (E) indicate the gates used for flow-cytometric cell sorting.

Fig. 2. Comparison between SP cells and non-SP cells with regard to healthiness and transcriptome.

(A) Survival rate of SP or non-SP cells was relative value of culture cell number after 3 days with the initial cell number. * P -value > 0.05. Significance was calculated using the student's t-test. (B)

Distribution of \log_2 ratios of expression levels between SP and non-SP cells in microarray analysis.

X-axis indicates the \log_2 ratios of expression levels between SP and non-SP cells, which were calculated to remove the biases of parental fish groups from expression values. In all, there were 55,928 probes

(blue, red, and orange). The numbers of probes that showed significantly higher (red) or lower (orange) expression in SP cells than in non-SP cells were 8,350 or 7,798, respectively (q -value < 0.05 ; see Materials and Methods).

Fig. 3. Morphological characteristics of SP cells. Smear HE-stained SP cells (A) or non-SP cells (B). Scale bar, 10 μ m. (C) Distribution of the number of dense bodies per SP cell and non-SP cell. Red, yellow, green, and blue colors indicate 1, 2, 3, or 4+ dense bodies, respectively. (D) Size distribution of SP cells (red) and non-SP cells (blue). The number of SP cells and non-SP cells observed were 102 and 104, respectively. Significance was calculated using the Wilcoxon rank sum test. Significance was calculated by comparisons between SP and non-SP cells with regard to cell size. P -value < 0.05 .

Fig. 4. SP cells in recipient gonads after transplantation. GFP-labeled SP cells were incorporated into the recipient gonad at 20 dpt (A and D). Arrowheads indicate transplanted SP cells with GFP fluorescence. Broken lines indicate recipient gonad. Incorporated SP cells had proliferated in recipient testis by 100 dpt (B and E). SP cells isolated from A-SG proliferated and differentiated into oocytes in a recipient ovary at 100 dpt (C and F). Bright-field images (A–C) and corresponding GFP-fluorescent images (D–F) are

shown. Scale bars, 20 μm (A and D) and 100 μm (B, C, E, and F). (G) Number of cells incorporated into the gonads of a recipient embryo at 20 dpt. Each circle indicates the data point associated with an individual recipient. Y-axis indicates the number of GFP-labeled cells that were incorporated into the gonads of an individual recipient embryo. The numbers of recipients carrying greater or equal to one donor-derived germ cells were listed in Table 1. * P -value < 0.05; Significances were calculated using Fisher's exact test.

Fig. 5. Donor-derived sperm and F1 offspring obtained from progeny tests. (A) Sex of each recipient was analyzed by PCR with *sdY*-specific primers. This gene is a male-specific genomic sequence on the Y chromosome [30]. (B) PCR analysis of milt obtained from *sdY*-positive males with *Gfp*-specific primers. Each milt sample obtained from a *pvasa-GFP* transgenic rainbow trout or a non-transgenic rainbow trout was used as positive control (P.C.) or a negative control (N.C.), respectively. (C-H) Newly hatched F1 offspring derived from WT (C) and the triploid recipient with transplanted SP cells (D). Approximately 50% F1 offspring derived from the recipients exhibited orange-colored phenotype (arrow in D). Gonads of F1 offspring derived from WT (E and G) and triploid recipients with transplanted SP cells (F and H). Germ cells were labeled by GFP in the gonads of approximately 50%

of F1 offspring derived from the recipients (F and H). Broken lines indicate the gonad of the F1 offspring shown in C (E and G) and in D (F and H). Bright-field images (E and F) and corresponding GFP-fluorescent images (G and H) are shown. Scale bars, 20 μm .

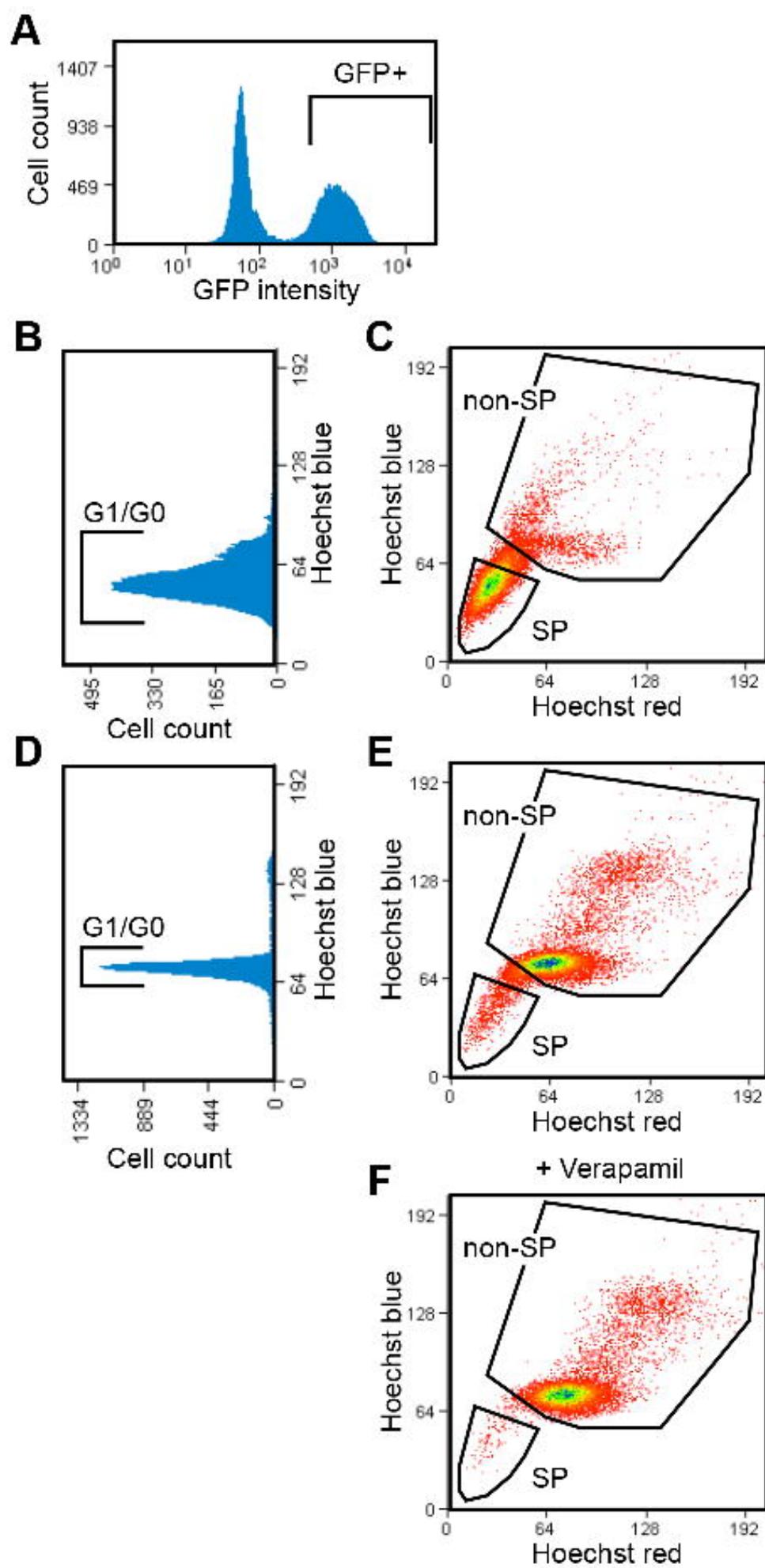


Figure 1

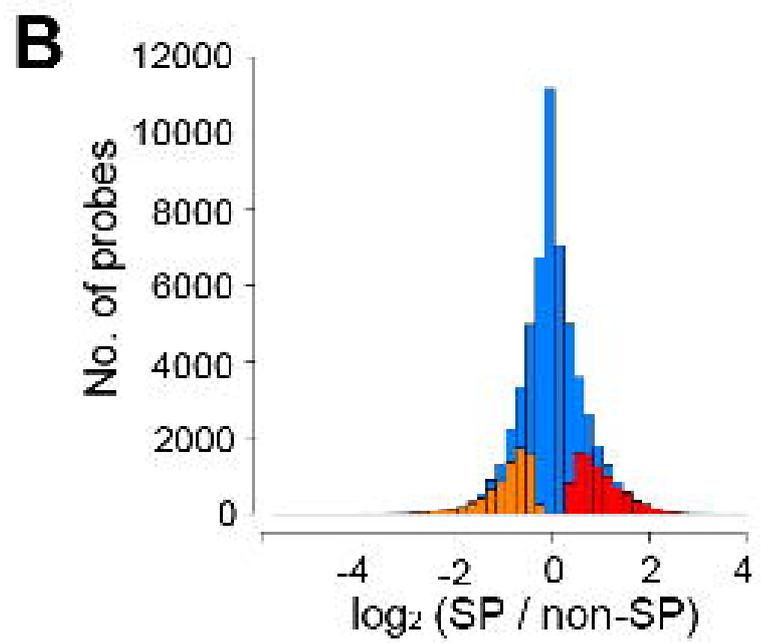
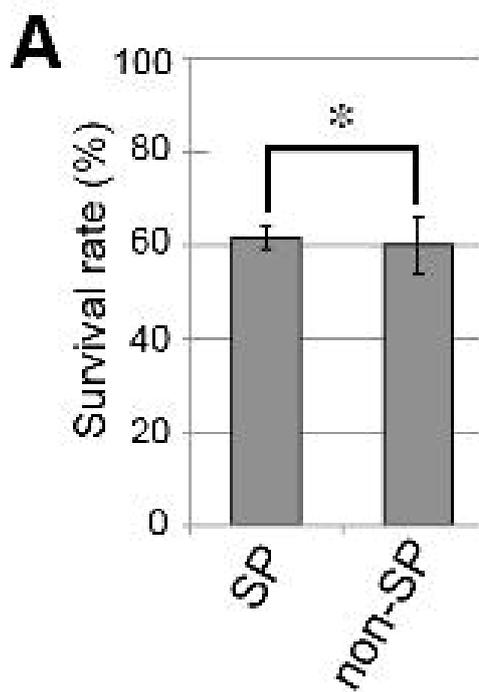


Figure 2

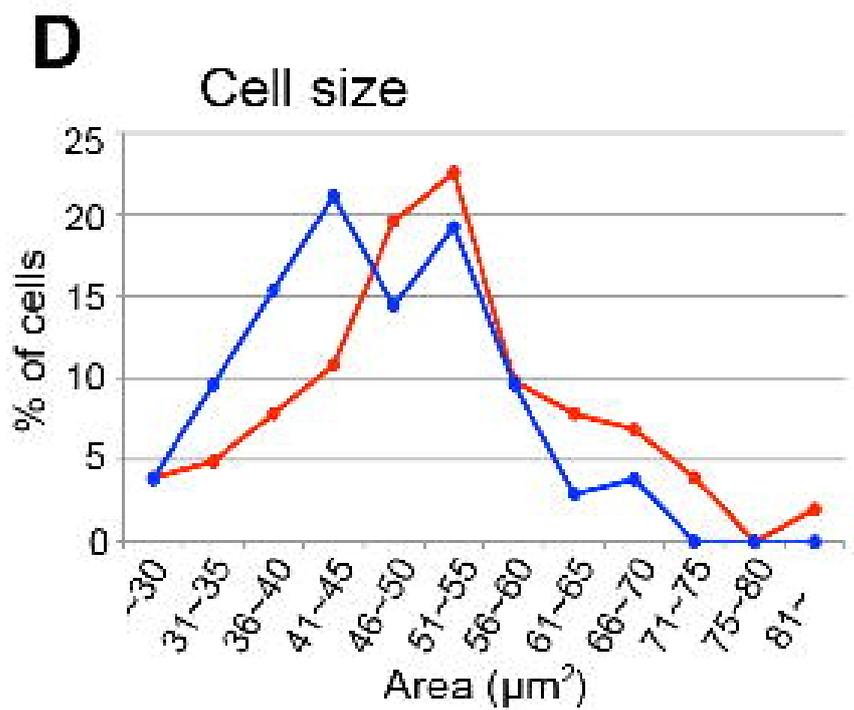
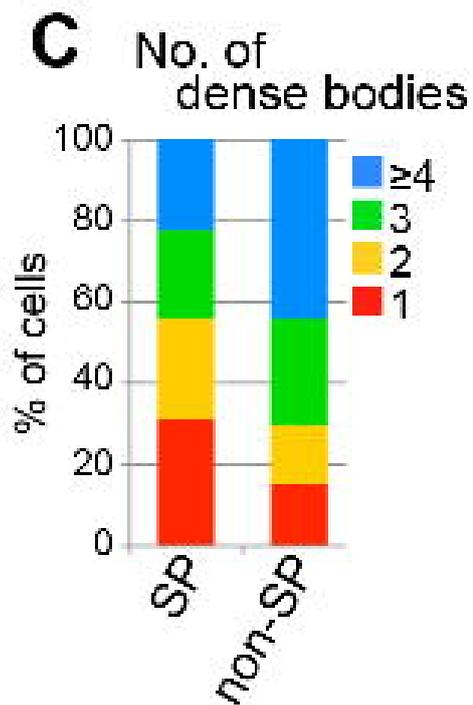
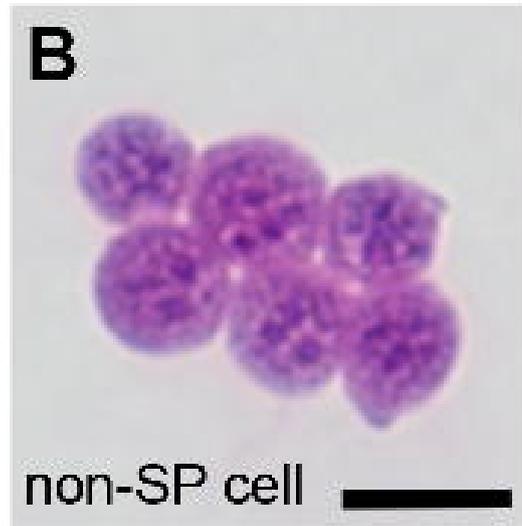
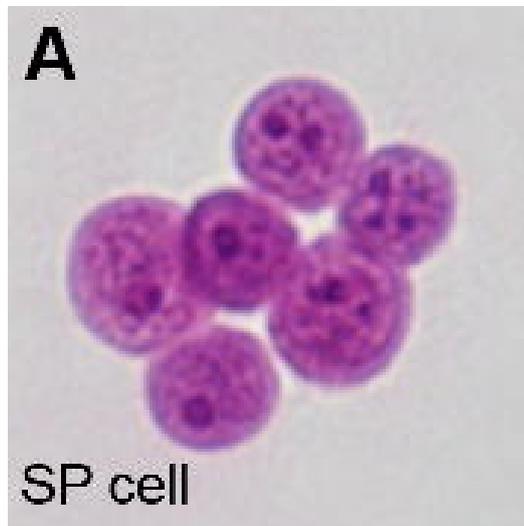


Figure 3

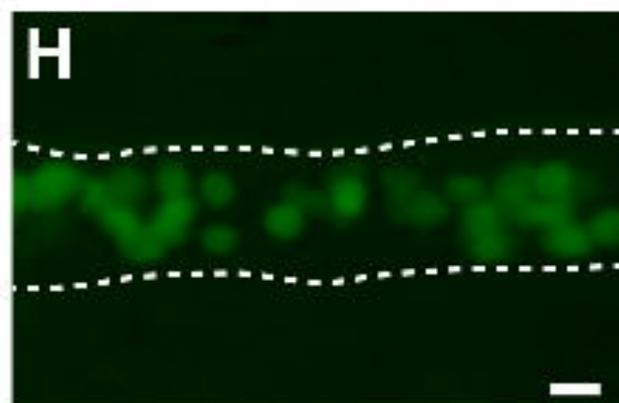
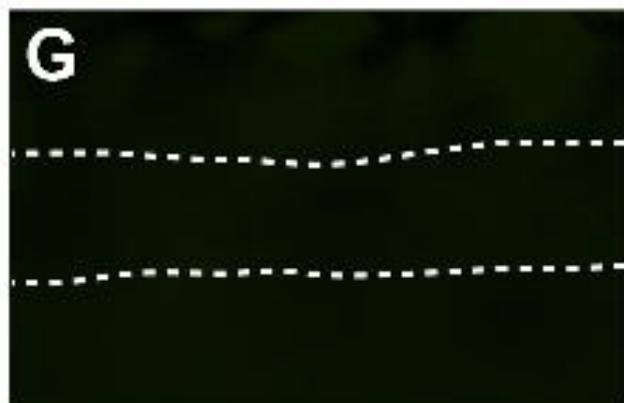
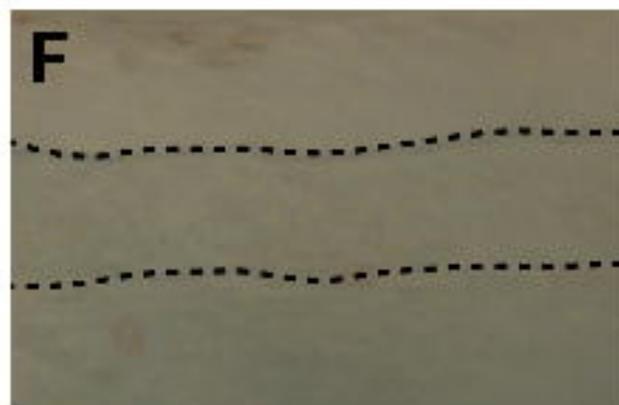
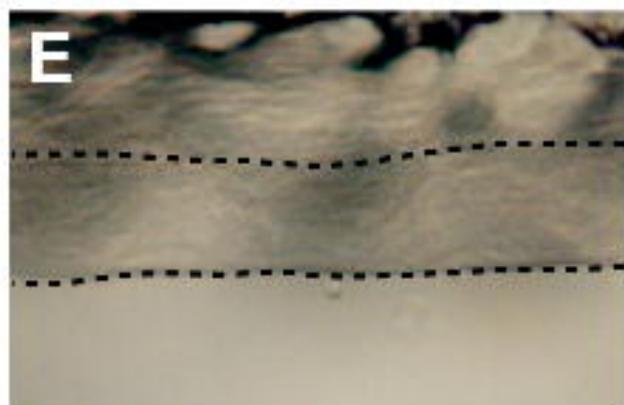
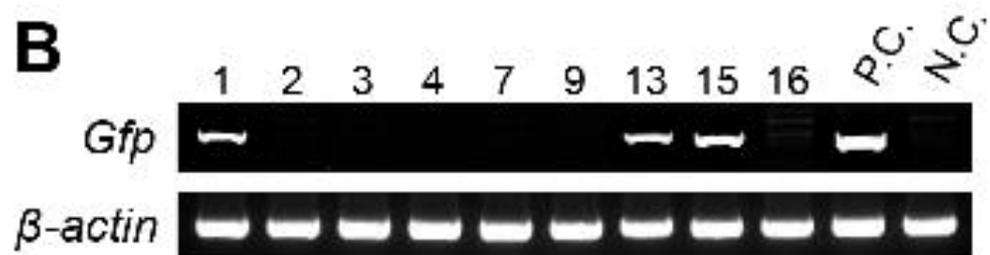
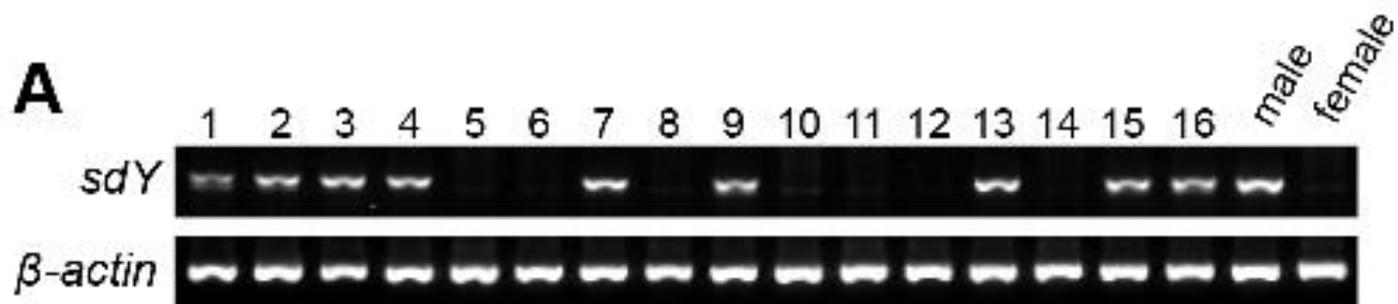


Figure 5

Table 1. Rates at which transplanted cells colonized recipient gonads at 20 dpt

Fraction	No. of fish	
	Total	With transplanted cells in gonads (%)
unsorted A-SG	85	18 (19.6±5.5)
SP	93	43 (46.2±5.7)
non-SP	70	5 (7.6±3.5)

* $P < 0.05$; Significance was calculated by using Chi-square test. Data are shown as mean \pm SEM

Table 2. Rates at which transplanted cells formed colonies at 100 dpt

Fraction	No. of male fish		No. of female fish	
	Total	With transplanted cells in testes (%)	Total	With transplanted cells in ovaries (%)
unsorted A-SG	19	1 (5.6±5.6)	13	2 (16.3±3.8)
SP	17	8 (48.6±26.4)	17	9 (52.8±2.8)
non-SP	14	0 (0)	14	2 (10.0±10.0)

* P < 0.05; Significance was calculated by using Chi-square test. Data are shown as mean ± SEM

Table 3. Percentage of donor-derived phenotypes among F1 offspring

	Total	GFP+ (%)	Orange (%)
Control	44	0 (0)	0 (0)
1	113	49 (43.4)	53 (46.9)
13	111	70 (63.1)	55 (49.5)
15	90	57 (63.3)	40 (44.4)

Sperm obtained from WT (control), and triploid recipients transplanted SP cells (1, 13, 15) were fertilized with eggs obtained from non-transgenic wild type rainbow trout.

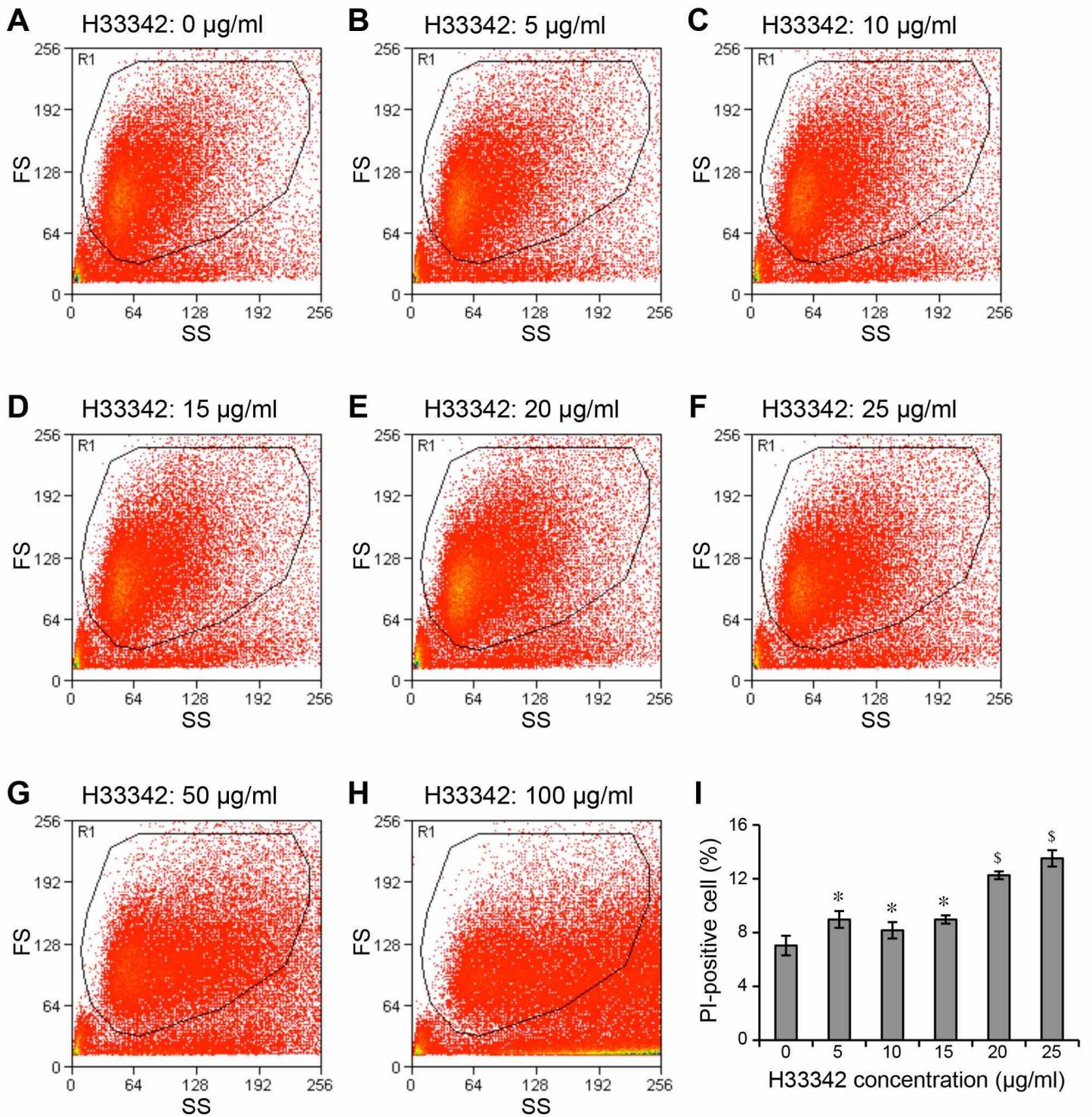


Figure S1

SUPPLEMENTAL FIGURE LEGENDS

Fig. S1. The effect of H33342 concentration on the viability of testicular cells for 1.5h staining at 10°C.

(A-H) The FS (y-axis) and SS (x-axis) of total testicular cells were analyzed under after treatment with H33342 at a concentration of 0 (A), 5 (B), 10 (C), 15 (D), 20 (E), 25 (F), 50 (G), or 100 (H) µg/ml. All cells were dissociated into single-cell suspension for staining and sorting. Almost all testicular cells without H33342 staining, were sorted by the R1 gate (A). (I) Among samples containing all testicular cells, the rate of PI-positive cells was analyzed after staining with H33342 at a concentration of 0, 5, 10, 15, 20, or 25 µg/ml. Significance was calculated using Dunnett's test by comparisons with control. * P -value > 0.05, \$ P -value < 0.05.

Table S1. CV values of G1/G0 and testicular cell viability for each set of H33342 staining conditions

The table cells highlighted with blue or pink indicate the staining conditions that resulted in saturated nuclear staining of A-SG with H33342 and very minimal effects on testicular cell viability.

The table cells highlighted with pink indicate the staining conditions that were used for subsequent experiments.

CV values of G1/G0 in GFP-positive cells (%)

		1.5h	2h	3h	4h	6h	8h	10h	12h	15h
10°C	5µg/ml	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	28.00	27.48
	10µg/ml	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	17.01	16.40
	15µg/ml	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	12.68	11.18
14°C	5µg/ml	N.D.	N.D.	N.D.	N.D.	20.01	12.09	11.19	9.50	7.40
	10µg/ml	N.D.	N.D.	16.25	11.85	8.19	6.97	6.38	6.48	6.55
	15µg/ml	N.D.	N.D.	14.19	8.69	6.35	6.12	6.19	6.26	6.49
16°C	5µg/ml	N.D.	19.78	13.89	10.13	9.24	8.37	6.03	6.47	6.57
	10µg/ml	15.35	10.26	6.77	6.42	6.13	5.97	6.12	6.47	6.83
	15µg/ml	10.90	7.07	6.14	5.68	6.21	6.45	6.73	6.73	7.17
18°C	5µg/ml	22.52	17.89	12.22	8.51	8.51	7.29	6.63	6.57	6.42
	10µg/ml	12.60	9.93	6.66	5.75	6.06	6.18	6.33	N.D.	N.D.
	15µg/ml	8.75	6.49	5.76	5.45	6.06	6.32	6.54	N.D.	N.D.

PI-positive rate in total testicular cells

		1.5h	2h	3h	4h	6h	8h	10h	12h	15h
10°C	5µg/ml	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	6.33	6.78
	10µg/ml	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	7.01	7.13
	15µg/ml	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	8.06	9.78
14°C	5µg/ml	N.D.	N.D.	N.D.	N.D.	7.42	6.00	6.75	7.32	8.65
	10µg/ml	N.D.	N.D.	5.51	5.27	6.01	6.45	7.75	9.75	11.08
	15µg/ml	N.D.	N.D.	7.29	6.70	6.35	9.51	11.16	17.57	21.74
16°C	5µg/ml	N.D.	5.86	5.97	5.76	6.73	7.67	7.02	10.31	11.52
	10µg/ml	7.18	7.26	7.13	6.17	7.46	7.71	10.54	14.69	19.55
	15µg/ml	6.65	6.47	7.85	7.62	10.20	16.44	24.27	34.15	44.79
18°C	5µg/ml	5.17	6.94	7.13	6.46	6.97	7.21	8.36	11.02	11.31
	10µg/ml	7.11	7.21	7.65	6.41	7.89	8.89	11.47	N.D.	N.D.
	15µg/ml	7.41	8.68	7.78	8.15	13.92	16.71	24.33	N.D.	N.D.

YGL02.845.C1	6.748893076	-2.935574641	-0.209871071	-0.773468903	0.959668812	0.394240968	0.937369506	1.62E-08	1.02E-05
YGL02_GNYAISO04I0D64	7.629185825	-2.942775513	-1.195590622	-3.410002067	-0.043551162	0.742151735	1.955228304	0.000392004	0.005515054
YGL02.855.C5	5.332164897	-2.942998391	-0.774492816	-0.621894044	0.907540517	1.416311796	1.658495424	4.49E-05	0.001215004
YGL02.3918.C1	7.701618791	-2.949462029	-0.847323938	-1.051277007	-0.015131513	1.051184003	2.188669102	0.000310471	0.004693389
YGL02_GNYAISO04I1VELI	9.588673397	-2.966989823	-1.718831275	-2.069234871	-0.01897129	1.300073749	2.290076331	1.48E-05	0.000561883
YGL02_GNYAISO04I1HLJ1	7.146727718	-2.968116477	-2.048369768	-2.552665279	0.730887777	0.62922192	2.521490649	4.08E-05	0.00113786
YGL02.5257.C1	6.227084831	-2.970987484	-0.160529555	-0.776957583	0.079382287	0.571649157	0.386396403	6.83E-07	7.91E-05
YGL02.11170.C1	7.315822673	-2.972370162	-0.123729304	-0.548660487	-0.158440207	0.774412204	1.638348361	6.18E-06	0.000317642
YGL02.1913.C1	6.609493023	-2.984690683	-1.891259713	-2.424277077	0.019373292	1.087843911	2.848635503	2.69E-05	0.000851493
YGL02_GNYAISO04H4DXN	5.92766216	-3.005582522	0.249682267	-0.522772998	1.679365922	-0.116406413	0.678124708	1.40E-08	9.43E-06
YGL02.153.C1	9.729904361	-3.012362283	-1.715062921	-1.922926991	0.267767186	0.999526169	1.993735643	3.82E-07	5.45E-05
YGL02_GNYAISO04IFZSO	5.901477136	-3.025839582	-3.034867116	-2.960634359	-0.066363104	2.995204432	3.474431696	1.13E-06	0.000109666
YGL02.15593.C1	6.225976196	-3.038898687	0.207496339	-2.172240079	-0.014743823	0.196945285	2.32504188	4.72E-09	6.11E-06
YGL02_GNYAISO04H7MFD	6.812828103	-3.054698911	-0.234488864	-0.658381612	0.48215985	0.047246112	0.634214078	6.24E-08	2.06E-05
YGL02_GNYAISO04H99GK	5.913709519	-3.060849771	-0.533538649	-2.730324211	0.356698266	0.964475463	2.540005052	1.13E-05	0.000469526
YGL02_GNYAISO04I3VPD	5.913549155	-3.06343232	-1.558130569	-1.64419802	0.648072571	1.644434835	2.079571717	0.000163118	0.002971065
YGL02.15481.C1	7.719418297	-3.090810072	0.00945855	-1.473687883	-0.071320312	0.513834525	0.906142882	4.67E-05	0.001243801
YGL02_GNYAISO04J5EXY	8.050829934	-3.092789057	-0.399841184	-1.652947501	0.230761825	-0.534523393	0.85170201	8.48E-10	3.26E-06
YGL02_GNYAISO04HZ4CI	5.886454569	-3.093641806	-1.665452022	-2.982275773	0.041117122	1.694454998	3.419410528	4.67E-07	6.17E-05
YGL02_GNYAISO04I156F	7.110049119	-3.098397708	-2.998201035	-2.706143249	-0.682499779	2.303490968	2.291123618	3.31E-07	4.98E-05
YGL02_GNYAISO04I5G4D	7.576377802	-3.146231033	-1.279796593	-3.586757565	-0.305626211	1.396211992	3.135715352	5.19E-05	0.001335572
YGL02.3369.C2	9.085641076	-3.157926386	-0.509094869	-1.871777327	0.017538353	-0.276734309	1.225459473	5.15E-10	3.18E-06
YGL02_GNYAISO04I2W3F	7.072048599	-3.164656674	-1.561859547	-2.263680059	0.323796945	1.320784098	2.953340183	4.86E-05	0.001280204
YGL02.843.C1	7.656265955	-3.168894847	-1.736532359	-1.404710684	-0.179312223	0.672352012	2.332367981	3.22E-05	0.000967639
YGL02_GNYAISO04JQVZE	6.609045786	-3.174201166	-2.716723953	-2.940826419	-0.39077156	2.296939159	2.549137568	1.42E-05	0.000548151
YGL02_GNYAISO04I3U8	6.273474088	-3.18236511	-0.382722431	-1.472717932	0.137807484	0.032510582	3.583188044	0.006261362	0.039826743
YGL02_GNYAISO04H1GDI	7.89560547	-3.191202812	-1.37160382	-1.106707466	-0.046249244	1.376302078	2.600964475	0.000107792	0.0022352
YGL02.6796.C1	7.799458832	-3.220298461	-1.67597416	-1.601754675	0.969776378	0.948046297	2.622470633	3.17E-05	0.000957602
YGL02.4231.C1	9.612062393	-3.228193571	-0.34281799	-1.758553609	-0.088252431	-0.194553847	1.211753616	4.38E-12	2.84E-07
YGL02_GNYAISO04H5XKH	6.346370412	-3.261960552	-0.375749448	-0.710519755	-0.076236761	0.802147525	1.857128077	6.45E-09	7.01E-06
YGL02_GNYAISO04M0G0	6.639477318	-3.290940176	-2.885870618	-2.686205215	-0.35102589	2.598548558	2.476707208	3.81E-06	0.000233174
YGL02_GNYAISO04H878E	7.218904899	-3.32025439	-4.287912436	-2.870879223	-0.13324774	3.254446551	2.14615459	1.88E-07	3.54E-05
YGL02_GNYAISO04J11TP	6.123794469	-3.349666625	-2.535180703	-2.993820419	0.275287336	2.590550416	3.741862487	1.04E-07	2.59E-05
YGL02_GNYAISO04H7LFM	7.597025225	-3.376457724	-0.43839907	-2.276853403	-0.201711921	0.248041407	2.834131561	3.07E-08	1.43E-05
YGL02_GNYAISO04I1VWSH	6.057462667	-3.377144351	-1.518745284	-1.104840767	0.497583289	1.388881894	0.974488427	6.60E-08	2.08E-05
YGL02.2667.C2	7.354146414	-3.433108425	-1.890027197	-2.339241707	-0.613438118	1.597216904	2.099825081	3.89E-05	0.001101353
YGL02_GNYAISO04L6ZQ	6.242611187	-3.467671145	-1.344079503	-0.563185861	0.666104508	1.697434724	2.002934551	3.82E-06	0.00023354
YGL02_GNYAISO04WYWNW	7.762073201	-3.614752392	-0.221880619	-1.784065006	0.117362304	2.329132755	2.263308565	2.85E-06	0.000193871
YGL02_GNYAISO044F7N	6.554329213	-3.685705472	-2.296447027	-2.631041517	0.199324837	2.217902155	3.036944162	1.01E-07	2.57E-05
YGL02_GNYAISO04H09TG	7.517918966	-3.769813455	-2.974312162	-3.135824335	-0.063244148	2.250918171	3.481608538	5.63E-09	6.70E-06
YGL02.7597.C1	7.259150508	-3.813893642	-2.059647094	-2.767169677	-0.312615032	2.210779376	3.012027647	4.57E-08	1.73E-05
YGL02_GNYAISO04I72JM	6.961733969	-3.902928953	-1.966544987	-3.019447408	0.22644437	2.180658328	3.231003707	3.24E-05	0.000971553
YGL02_GNYAISO04JEWE4	7.002914904	-4.065247896	-2.279227213	-3.071944479	-0.038934217	2.598285448	2.937362375	1.08E-08	8.24E-06
YGL02.5735.C1	9.182839691	-4.796705864	-2.109084645	-2.423495689	-0.111780426	1.527568254	2.844474537	2.39E-11	1.03E-06
YGL02_GNYAISO04IQCIR	8.067547395	-5.089536185	-2.39398774	-2.11863498	-0.007304989	2.227605884	2.082697291	2.36E-14	3.05E-09
YGL02_GNYAISO04H174S	11.06278543	-5.413248982	-2.352748899	-2.666301106	-0.29704552	2.126954413	3.450386423	5.32E-09	6.48E-06
YGL02_GNYAISO04JFK2N	8.320927868	-5.66116341	-2.075258176	-2.078117865	0.266765743	3.141209405	3.434981737	5.96E-07	7.22E-05