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A novel subpopulation lacking Oct4 expression in the testicular side population

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Abstract. We characterized murine spermatogonial stem cells (SSCs) using a multi-parameter selection strategy, combining Oct4 expression determined by monitoring green fluorescent protein (GFP) expression, and the testicular side population (SP) showing weak fluorescence on Hoechst 33342 dye staining, as markers of stem cell purification. Testicular cells were collected from Oct4/GFP transgenic mice and analyzed using a fluorescence-activated cell sorter (FACS). SP was detected in testicular cell suspensions at an average rate of 0.10%. Multicolor analysis indicated that 96% of SP cells were negative for Oct4. The cells did not express SSC marker genes, but expressed Bcrp1. While the main population was 93% positive for pyronin Y staining, this was limited to 51% in SP. We found a novel subpopulation with reduced RNA content lacking Oct4 expression in testicular SP. These results suggest that the cells isolated by FACS represent a novel population of SSCs in the G0 quiescent state.

Introduction

Spermatogonial stem cells (SSCs) form the foundation of spermatogenesis and are the only cells that undergo self-renewal throughout postnatal life in mammals and transmit genetic information to subsequent generations. Their characteristics are unique among stem cells, and these cells are essential for the propagation of a species. Previous studies suggested that SSCs are included in undifferentiated type-A spermatogonia (1), which are categorized as type-A single, type-A paired, and type-A aligned according to their morphological characteristics (2). However, the biochemical characteristics of SSCs remain largely unknown, and no useful cell-surface markers have yet been found to identify these cells. In addition, the very small number (0.03% of all germ cells) of SSCs in the testis makes it difficult to identify and isolate these cells for further manipulation (3).

In 1994, Brinster *et al* (4,5) reported that stem cells obtained from the testes of donor male mice repopulated those of genetically or chemically sterile mice. This transplantation technique confirmed that the extracted cells from the testes of donor males contained SSCs capable of repopulating the testes of sterile recipients to reproduce fertile offspring. These landmark publications provided the means to develop an *in vivo* functional assay to identify SSCs.

Previous attempts at *in vitro* or *in vivo* genetic modification of SSCs have met with little success partly because of the lack of availability of methods for identification of these cells. There have been several reports on the *in vitro* transduction of SSCs using lenti- or retroviruses followed by transplantation (6-9), but these methods have low transduction rates. Attempts to modify SSCs *in vivo* by direct injection of viral vectors (adeno-, retro-, lenti-, or adeno-associated viruses) into the seminiferous tubules of sterile male mice (10) were hampered by the inaccessibility of SSCs due to tight Sertoli cell junctions or the blood-testis barrier; the results indicated low rates of transduction in mouse pups (11) and no transduction of SSCs in adult mice (10). Transient transduction of testis cells has been achieved by other approaches, such as electroporation of the testes after injection of foreign DNA (12,13), indicating transduction only of somatic cells, such as Sertoli or Leydig cells, and differentiated germ cells, such as spermatozoa, spermatids, or spermatocytes. The difficulties in transducing SSCs *in vivo* are due largely to their location in seminiferous tubules. This inaccessibility of SSCs increases the importance of *in vitro* assays and makes the development of method for isolation of SSCs essential for further manipulation.

A method for isolation and enrichment of SSCs has been reported, but this method is only beginning to be established. On the other hand, octamer-binding protein 4 (Oct3/4 or Oct4) is a well-defined marker of multipotent undifferentiated cells and is expressed only by type-A spermatogonia in the adult testis (14,15). Ohbo *et al* (16) reported that Oct4-positive spermatogonia have repopulating activity. The testicular side population (SP) is a group of cells that are refractory to Hoechst 33342 staining and thus exhibit a low level of fluorescence, which is concentrated near the x-axis in the fluorescence-activated cell sorter (FACS) Hoechst blue vs.

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Hoechst red profile (17). SP has been shown to include a high concentration of hematopoietic stem cells (HSCs) among cells extracted from bone marrow. In a previous study using knockout mice, Zhou *et al* showed that the ATP-binding cassette (ABC) transporter, Bcrp1, is responsible for active Hoechst efflux in SP cells (18,19). It has been suggested that SP cells have characteristics common to stem cells in a variety of organs and species (20). Falciatori *et al* (21) and Lassalle *et al* (22) reported successful enrichment of SSCs using SP as a marker of stem cells, while Kubota *et al* (23) and Lo *et al* (24) reported the converse.

Oct4 is an appropriate marker for isolation of type-A spermatogonia, including SSC. In the present study, we used Oct4/GFP transgenic mice expressing green fluorescent protein (GFP) under the control of the Oct4 promoter (25). We isolated and characterized SSCs from murine testicular cells using a multi-parameter selection strategy, combining *in vivo* expression of Oct4 determined by monitoring GFP expression and the testicular SP showing weak fluorescence on staining with Hoechst 33342 dye as markers for stem cell purification. A novel cell population was found that showed a low level of Hoechst fluorescence, reduced RNA content, and was negative for Oct4.

Materials and methods

Animals. Oct4/GFP mice were a kind gift from Y. Matsui (Osaka Medical Center of Maternal and Child Health) and H.R. Scholer (University of Pennsylvania) (25). Testis cells were collected from (ICR x Oct4/GFP) F1 hybrids at 3, 7, and 10 days old or at the adult stage. ICR females were purchased from Charles River Japan (Yokohama, Japan). All animals were maintained at the Laboratory Animal Resource Center, University of Tsukuba, at a controlled temperature of 23±1˚C, humidity of 55±5%, and under a 14-h light:10-h dark cycle. Mice had free access to commercial chow (NMF; Oriental Yeast Company, Ltd., Tokyo, Japan) and autoclaved water. All of the study protocols were approved by the University Animal Experimental Committee of the University of Tsukuba.

Cell preparation. Cells were prepared from the testes of mice by the procedure of Ogawa *et al* (26) with minor modifications. Adult or juvenile mice were euthanized and the testes were removed immediately. In the case of pups, 8-20 testes were collected for each experimental analysis, whereas a maximum of 6 testes were collected from adults. The tunica albuginea was removed and the testes were cut into smaller pieces for efficient digestion. Testes were then incubated in Hank's-balanced salt solution without calcium or magnesium (HBSS; Gibco, Carlsbad, CA) containing 1 mg/ml collagenase type IV (Sigma, St. Louis, MO) at 32˚C for 15 min, with agitation at intervals of 5 min. After incubation, the testes were washed once in HBSS, and then further incubated in HBSS containing 0.25% trypsin and 1 mM ethyleneglycol-bis-(ß-aminoethylether)-N, N, N', N'-tetraacetic acid (EDTA) at 32˚C for 10 min, with agitation at intervals of 5 min. The activity of trypsin was then terminated by adding a 10-20% volume of fetal calf serum (FCS). Large pieces of undigested tubules were removed and the cell suspension was filtered through nylon mesh (pore size, 60 μm) to remove cell clumps. DNaseI (200-500 μg; Sigma) was then added to facilitate dispersion.

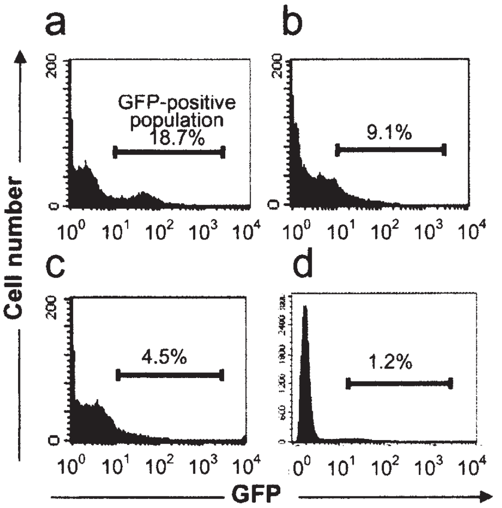

Figure 1. Flow cytometric analysis of GFP expression in total testicular cells of Oct4/EGFP transgenic mice. Testicular cells were collected and analyzed from 3-day-old (a), 7-day-old (b), 10-day-old (c), and adult (d) Oct4/EGFP transgenic mice. The percentages of cells positive for GFP expression are indicated.

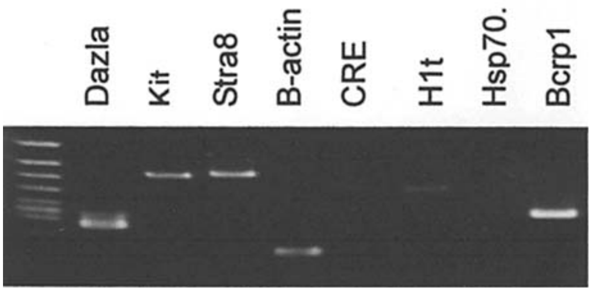

Figure 2. RT-PCR analysis of GFP-positive cells for SSC marker genes and spermatocyte and/or spermatid marker genes. RNA was extracted from sorted GFP-positive testicular cells from Oct4/EGFP transgenic mice. RT-PCR was performed for Dazla (260 bp, 30 cycles), Kit (650 bp, 35 cycles), Stra8 (637 bp, 35 cycles), ß-actin (142 bp, 30 cycles), CREM (254 bp, 30 cycles), H1t (509 bp, 30 cycles), Hsp70-2 (324 bp, 30 cycles), and Bcrp1 (326 bp, 35 cycles) genes.

Hoechst 33342 staining of testicular cells. Cell concentration was determined by counting cells stained with trypan blue using a hemocytometer. Cell suspensions were then centrifuged at 1,000 g for 5 min at 4˚C, and resuspended to give a final concentration of 1×10^6 cells/ml in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (Gibco) and 2% FCS (DMEM+). Cells were then stained with Hoechst 33342 (Sigma) at 5 μg/ml for 1 h at 32˚C. After incubation for 1 h, the cell suspensions were centrifuged at 1,000 g for 5 min at 4˚C, and re-suspended in DMEM+ to prohibit leakage of Hoechst 33342 dye. The final cell suspension was again filtered through 60 μm nylon mesh to remove any remaining cell clumps.

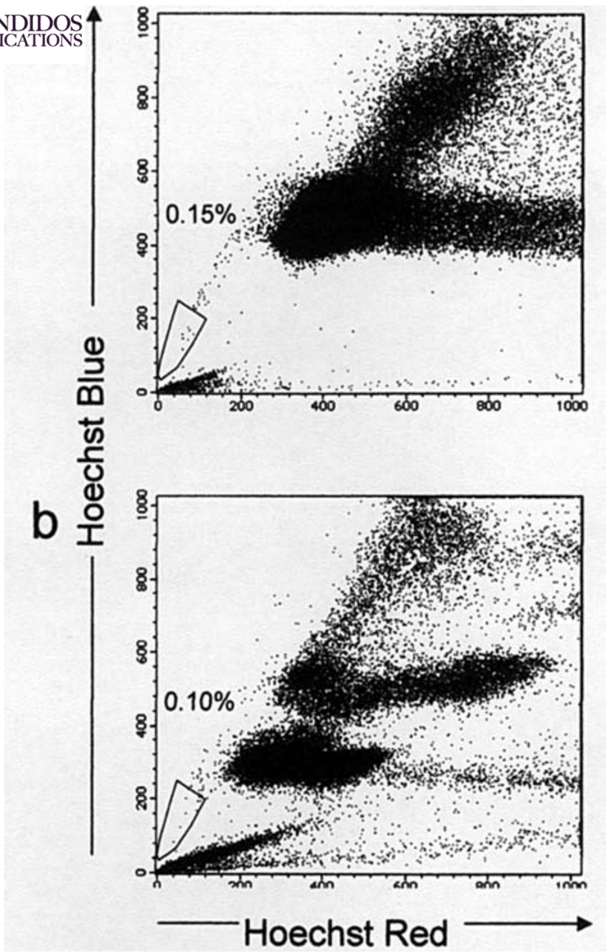

Figure 3. Flow cytometric analysis of Hoechst 33342 fluorescence in the testicular cells of Oct4/EGFP transgenic mice. Testicular cells were collected and analyzed by FACS Vantage SE, from 3-day-old (a) and adult (b) Oct4/EGFP transgenic mice. The vertical and horizontal axes show blue (Ho) and red (Ho/PI) fluorescence, respectively. The region of SP and its percentage are indicated.

Hoechst efflux inhibition. Inhibition of Hoechst efflux was performed by pre-incubating testis cells for 30 min at 32˚C in DMEM+ supplemented with verapamil (Sigma) at 25 or 75 μg/ml. The cell suspensions were then incubated with Hoechst 33342 in the presence of verapamil at 32˚C for 1 h.

Pyronin Y staining of testicular cells. After incubation of testis cells with Hoechst 33342 (5 μg/ml) for 45 min at 32˚C, pyronin Y (Sigma) was added at 1 μg/ml and cells were further incubated for an additional 45 min at 32˚C in the presence of Hoechst 33342.

Flow cytometry and cell sorting. Before analysis, propidium iodide (PI; Sigma) was added at a concentration of 1 μg/ml to exclude dead cells. Cell analyses and sorting were performed either on a dual-laser FACS LSR (Becton Dickinson, Franklin Lakes, NJ) equipped with a 488 nm argon laser and a 360 nm UV argon laser (for detection of GFP), or a triple-laser FACS Vantage SE (Becton Dickinson) equipped with a 488 nm argon laser, a 633 nm Hene laser, and a 360 nm UV argon laser. GFP fluorescence was determined using a 560 nm short pass filter and a 530/30 band pass filter. PI fluorescence was determined using a 610 nm short pass filter and a 610/20 band pass filter. Hoechst blue and red fluorescence emissions were determined using a combination of 640 nm long pass and 424/44 band pass, and 675/20 band pass filters. Pyronin Y fluorescence was determined using a 610 nm short pass filter and a 585/42 band pass filter.

RNA extraction and RT-PCR. For RNA extraction, cells were sorted with a FACS Vantage SE. Cells were centrifuged at 3,000 g for 5 min at 4˚C, and total RNA was purified using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. First-strand cDNA was synthesized using 10 mM dNTPs and Oligo (dT) Primer (Invitrogen, Carlsbad, CA). After 5 min of denaturation at 65˚C, the reaction mixtures were put on ice for 5 min. Then, 5X first-strand buffer and DDT (Invitrogen) were added, the reaction mixtures were heated to 50˚C, SuperScriptIII Reverse Transcriptase (Invitrogen) was then added, and the mixtures were incubated at 50˚C for 1 h. Finally, the reaction mixtures were heated at 70˚C for denaturation, and cDNAs were prepared by PCR with primers for Dazla, Kit, Stra8, ß-actin, CREM, H1t, Hsp70-2, and Bcrp1 (all primers were from Hokkaido System Science, Sapporo, Japan). PCR was performed according to standard procedures.

Results

The ratio of GFP-expressing cells decreases with age in male Oct4/EGFP mice. Homozygous male Oct4/EGFP transgenic mice were crossed with female ICR mice to produce F1 hybrids. Male offspring were sacrificed at 3, 7, or 10 days old, or at the adult stage and the percentages of testicular cells expressing GFP were determined at each stage. As the testes matured, the ratio of cells positive for GFP expression decreased from 18.7% at 3 days postnatally to 1.2% in adults (Fig. 1). In addition, the GFP-positive and GFP-negative cell populations were clearly separated in the testes of 3-day-old males. However, in the testes of 7- and 10-day-old or adult males, the populations of GFP-positive and -negative cells were not clearly divided, and some cells showed weak expression of GFP.

GFP-positive cells express marker genes for SSCs. Dazla, Kit, and Stra8 are expressed in pre-meiotic germ cells with reconstituting capacity for spermatogenesis, and thus are marker genes for SSCs (27-29). H1t, a variant of testis-specific histone H1, is transcribed only in pachytene spermatocytes (30). Cyclic AMP-responsive element modulator (CREM) and heat-shock protein 70-2 (Hsp70-2), are expressed in post-meiotic cells from spermatocytes I to spermatids (31,32). To determine whether the GFP-positive testicular cells in Oct4/GFP mice included SSCs, the expression of SSC marker genes in GFP-positive cells was examined by RT-PCR. The GFP-positive cells from the testes of adult Oct4/GFP transgenic mice were sorted by flow cytometry, and total RNA was extracted from approximately 30,000 cells from the GFP-positive population. The GFP-positive cells were positive for mRNAs encoding the SSC marker genes, Dazla, Kit, and Stra8 (Fig. 2), while those of spermatocyte and/or spermatid marker genes, CREM, H1t, and Hsp70-2, were not detected

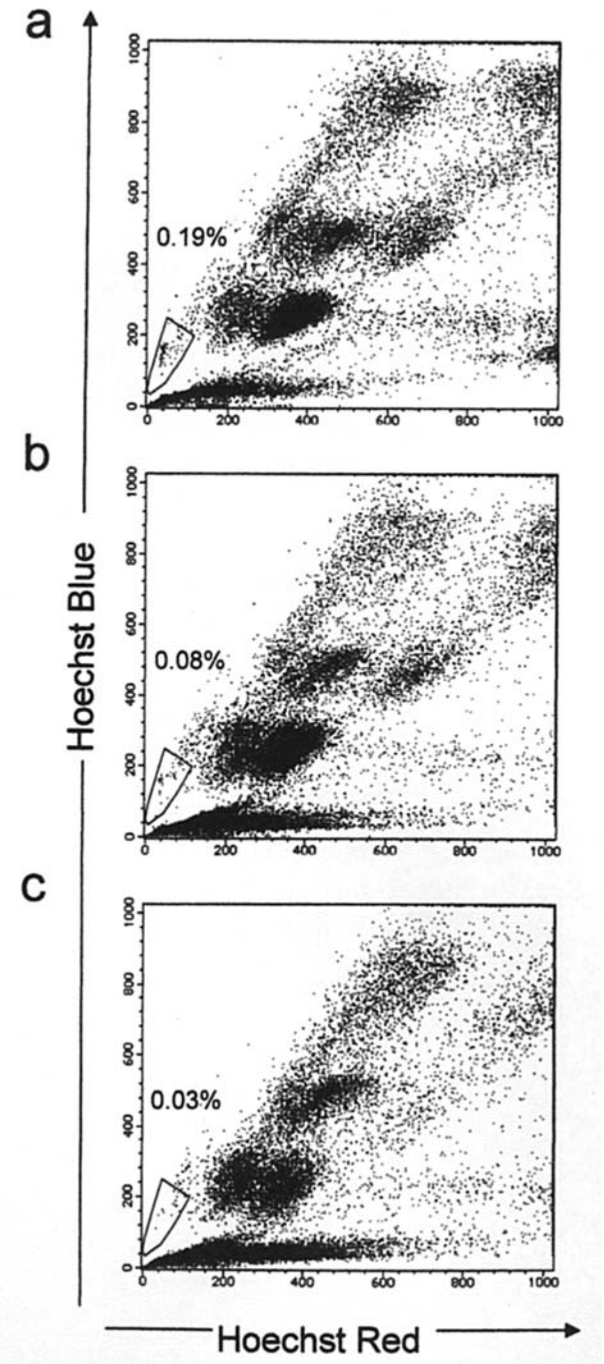

Figure 4. Flow cytometric analysis of the inhibition of Hoechst 33342 efflux by verapamil. Total testicular cells were collected from Oct4/EGFP transgenic mice and stained with Hoechst 33342 without inhibitor (a), or with 25 μg/ml (b) or 75 μg/ml of verapamil (c). The region of SP and its percentage are indicated.

or were present at very low levels. These results indicated that the majority of GFP-positive cells were pre-meiotic cells, including SSCs. We also observed strong Bcrp1 gene expression in GFP-positive cells. Bcrp1, a member of the ABC transporter family, has been reported to be responsible for Hoechst efflux from HSCs (33), and its expression suggests that SP cells are included in the GFP-positive population.

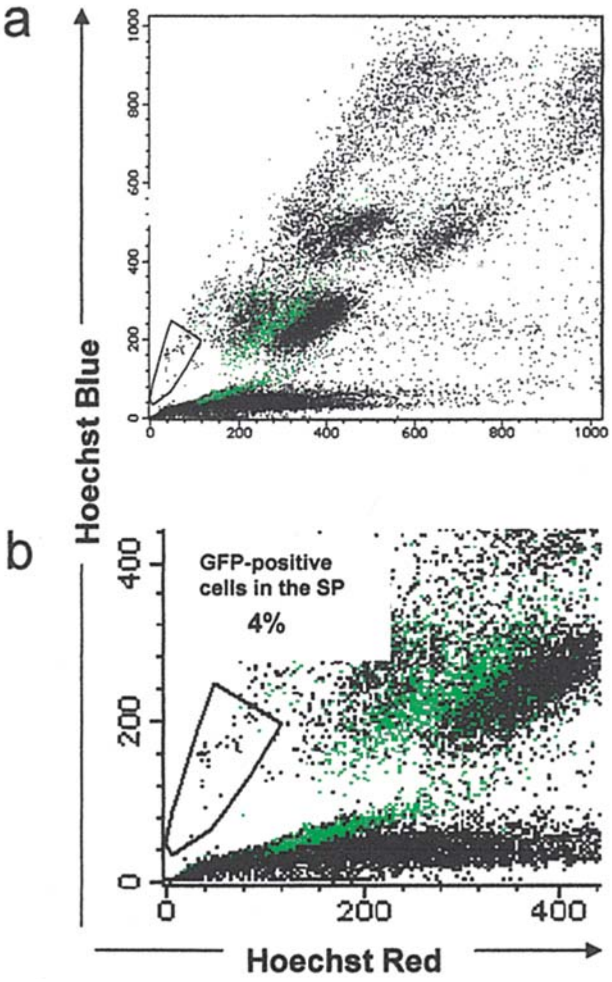

Figure 5. Flow cytometric analysis of GFP expression in SP cells. Total testicular cells were collected from Oct4/EGFP transgenic mice, stained with Hoechst 33342, and analyzed by FACS Vantage SE. (a) Total testicular cells stained with Hoechst 33342. SP cells were gated. Green and black dots indicate GFP-positive and GFP-negative cells, respectively. (b) Enlargement of the SP region from plot (a). The average percentage of GFP-positive cells in SP is shown.

SP is detected in testicular cells. SP may be a useful marker to obtain SSCs in a variety of animals and at increased concentration. To examine the presence of SP cells in mouse testes, cells were extracted from the testes of both adult and juvenile Oct4/GFP transgenic mice, stained with Hoechst 33342, and analyzed by FACS Vantage SE. The Hoechst blue versus red profile, displayed with blue on the vertical axis and red on the horizontal axis, indicated the presence of SP cells as well as the main cell population in both adult and juvenile mice (Fig. 3). In 3-day-old pup testes, diploid cells were detected in the main population and SP was clearly detected at an average frequency of 0.15% (Fig. 3a). In the adult testes, the main population included haploid, diploid, and tetraploid cells, and SP was detected at an average frequency of 0.10% (Fig. 3b). Although we found several subpopulations in the Hoechst profile of whole adult testicular cells, SP was clearly separated from the main subpopulations. Thus, SP cells can be isolated from other testicular cells irrespective of age.

SP is successfully inhibited by verapamil. To confirm the characteristics of the SP cells obtained, adult testicular cells were treated with a low (25 μg/ml) or a high (75 μg/ml)

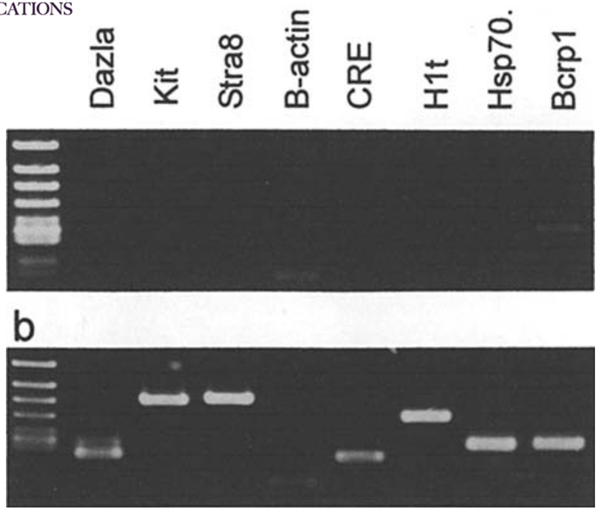

Figure 6. RT-PCR analysis of SP cells for SSC marker genes and spermatocyte and/or spermatid marker genes. RNA was extracted from SP cells (a) sorted by FACS Vantage SE and unsorted total testicular cells (b) from the testes of adult Oct4/EGFP mice and RT-PCR was performed for Dazla (260 bp, 35 cycles), Kit (650 bp, 40 cycles), Stra8 (637 bp, 40 cycles), ß-actin (142 bp, 35 cycles), CREM (254 bp, 35 cycles), H1t (509 bp, 35 cycles), Hsp70-2 (324 bp, 35 cycles), and Bcrp1 (326 bp, 40 cycles) genes. Samples of 30,000 cells were used for RNA extraction.

concentration of verapamil, which has an inhibitory effect on ABC transporters (33). The results showed a reduction in SP cells of 58% at the low concentration of verapamil (from 0.19 to 0.08%; Fig. 4a and b), and a reduction of 84% at the high concentration (from 0.19 to 0.03%; Fig. 4c). Thus, verapamil induced SP cell loss in a dose-dependent manner, confirming that testicular SP has the characteristics of stem cells.

SP consists of GFP-negative and GFP-positive cells. Multicolor analysis was performed on FACS Vantage SE to examine GFP expression in SP cells. Approximately 500,000 cells from the testes of adult male Oct4/GFP transgenic mice were analyzed after Hoechst dye staining (Fig. 5). The results indicated that 4% of SP cells expressed GFP, while the remaining 96% did not (Fig. 5; black and green spot indicate GFP-negative and GFP-positive cells, respectively). GFP-expressing cells were located far from the tip of SP, close to the haploid and diploid populations, and no signals for GFP were detected at the tip of the SP (Fig. 5b).

SP cells do not express marker genes for SSCs. To determine the gene expression profiles of SP cells, we examined differentiation marker genes for spermatogenesis in both SP cells and the whole testicular cell population. Approximately 30,000 SP cells from the testes of adult Oct4/GFP mice were sorted by flow cytometry, and RT-PCR assay showed that the mRNAs of the pre-meiotic marker genes, Dazla, Kit, and Stra8, and the spermatocyte and/or spermatid marker genes, CREM, H1t, and Hsp70-2, were expressed in the whole testicular cell population (Fig. 6b) but not in SP cells (Fig. 6a). Despite the lack of expression of differentiation marker genes, SP cells showed expression of Bcrp1, which is responsible for Hoechst efflux in stem cells (Fig. 6a).

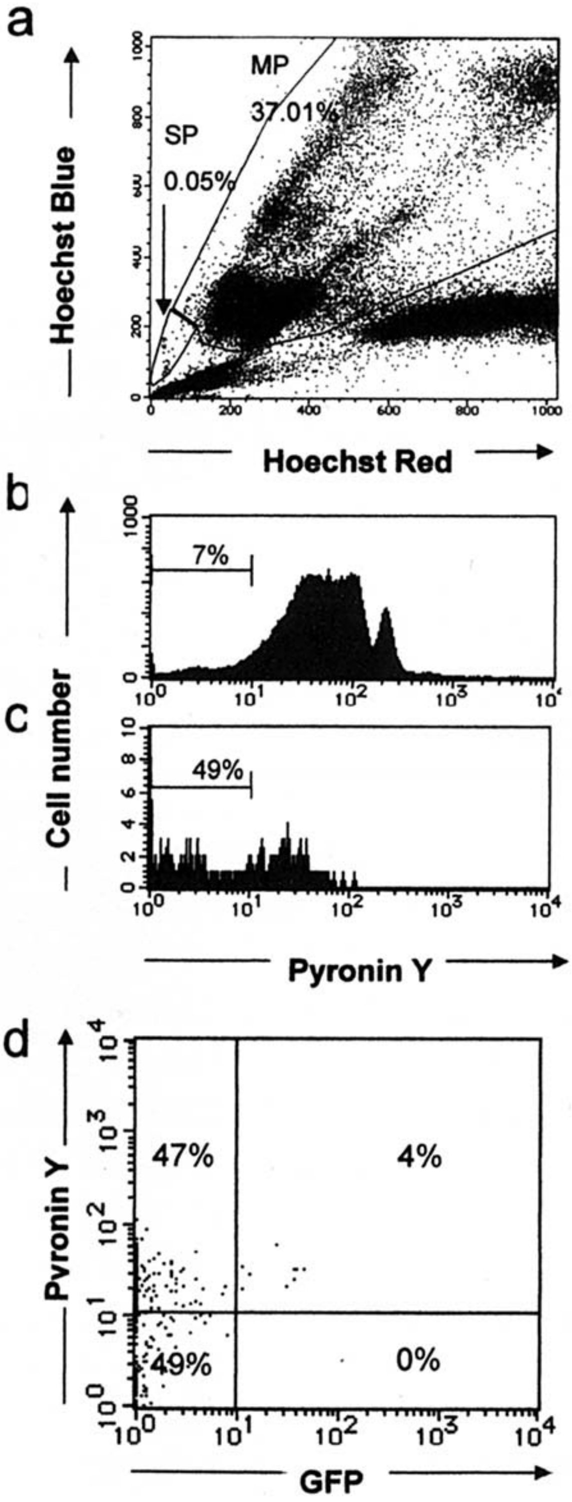

Figure 7. Flow cytometric analysis of the incorporation of pyronin Y in SP and main population cells. Total testicular cells were collected from adult Oct4/EGFP mice and stained with Hoechst 33342 and pyronin Y for analysis by FACS Vantage SE. SP and main population cells were gated, and their percentages are shown (a). Dead cells stained with PI and cell debris were excluded. The histograms show pyronin staining intensity of cells from the main population (b) and SP (c). The pyronin Y and GFP emission patterns of SP cells are shown in (d). The percentages of each quadrant are indicated.

SP cells are negative for pyronin Y staining. To investigate the cycling status of SP cells, whole testicular cells of Oct4/GFP adult mice were stained with Hoechst 33342 and pyronin Y prior to flow cytometric analysis. Pyronin Y is known to stain RNA and is therefore useful to determine the cycling status of cells (34). First, the Hoechst fluorescence intensity was analyzed in pyronin Y- and Hoechst 33342-stained testicular cells by flow cytometry. The main population and SP were gated to analyze their RNA content (Fig. 7a). Multicolor analysis revealed that the SP was 49% negative for pyronin Y staining (Fig. 7c), while the main population was positive for pyronin Y staining with only 7% of the cells showing no staining (Fig. 7b). Surprisingly, all SP cells expressing GFP were positive for pyronin Y staining, accounting for 4% of the whole SP (Fig. 7d). GFP-negative SP cells (51%) were also negative for pyronin Y staining (Fig. 7d). Furthermore, pyronin Y-positive cells in the SP region showed lower intensity of pyronin Y staining than those of the main population (Fig. 7b and c). These observations indicated that the majority of SP cells had a reduced RNA content, suggesting that these cells from the adult testes are largely quiescent.

Discussion

Stem cells show differential efflux of the fluorescent DNA-binding dye Hoechst 33342 as compared with non-stem cells. On the other hand, Oct4 is apparently critical for maintenance of the pluripotential phenotype. In the present study, we used multiple-parameter selection to isolate and characterize murine SSCs, combining the expression of Oct4 determined by GFP and SP phenotype. The results demonstrated that SP from murine testicular cells, detectable by FACS, consists of a large number of Oct4-negative cells and a small number of Oct4-positive cells (Fig. 5). The Oct4-negative SP cells showed reduced RNA contents as compared to the main population or Oct4-positive SP cells (Fig. 7). In addition, SP did not express SSC marker genes, but were positive for Bcrp1 gene expression (Fig. 6).

Oct4 is a transcription factor involved in the regulation of pluripotency during embryonic development (15) and is detected in both pluripotent cells and other early germ cells (14,15). In the adult murine testes, undifferentiated spermatogonial cells expressing Oct4 are distributed on the basement membrane of the seminiferous tubules (14). The timing of the stages of spermatogenesis in mice has been characterized in detail morphologically (35). The testes of laboratory mice at 0 days old contain only immature somatic cells and undifferentiated spermatogonia type A1. Spermatogonia type A2 to A4 and type B begin to appear at 3 days old. At 10 days old, spermatocytes begin to appear, and at 8 weeks of age, the testes have matured completely and all types of germ cells are observed. In the present study, we used transgenic mice carrying the GFP gene fused with an 18-kb genomic fragment encompassing Oct4 (Oct4/GFP) to select SSCs expressing Oct4. Yoshizumi *et al* reported GFP expression in both male and female germ cells at all developmental stages in Oct4/GFP mice except in male germ cells in the testes after differentiating into type A spermatogonia (25). In the present study, the ratio of GFP-expressing cells decreased with age, from 18.7% at 3 days old to 1.2% in the adult testes (Fig. 1). Furthermore, the GFP-positive fraction was clearly separated from the GFP-negative fraction in 3-day-old mice, but not in 7- or 10-day-old or adult mice. These results suggest that the majority of GFP-positive cells detected in the present study were spermatogonia and that isolation of undifferentiated spermatogonia is affected by the development of testicular cells.

RT-PCR (Fig. 2) analyses showed that GFP-positive cells express marker genes for SSCs, indicating that these cells included SSCs. Therefore, gating for the expression of the GFP/Oct4 transgene is an appropriate approach for enrichment of SSCs. However, the method of isolation of SSCs using GFP as an SSC marker is limited in two ways. First, all except the Oct4/GFP transgenic mice lack the transgene. Thus, a marker for SSC that can be used in a wider variety of species is required. Second, the percentage of cells expressing Oct4/GFP (1% in adults) among the total testicular cell population is much higher than the estimated percentage of SSCs (<0.1%) (3). Ohbo *et al* (16) attempted to isolate SSCs using the same Oct4/GFP transgene as used in the present study. Their testicular GFP-positive population consisted of Kit-positive and Kit-negative cells. Furthermore, they reported that the GFP-positive and Kit-negative population was more efficient for repopulation than the GFP-positive and Kit-positive population in the testicular cells of Oct4/GFP transgenic mice (16). Therefore, the GFP-positive population detected in the present study may have consisted of SSCs and committed spermatogonial cells, and the frequency of SSCs is thus speculated to be low. On FACS sorting, the Oct4 selection strategy is thus insufficient to purify SSCs from the murine testis.

To minimize the above limitations, the SP phenotype as a stem cell marker is available for isolation of SSCs. Goodell *et al* developed a method of isolating murine HSCs by dual-wavelength FACS analysis with the fluorescent DNA binding dye, Hoechst 33342 (17). The SP isolated from bone marrow cells was shown to consist of cells with lymphoid and myeloid hematopoietic reconstituting activity *in vivo*. Recently, Falciatori *et al* (21) and Lassalle *et al* (22) independently examined the hypothesis that SSCs have the same Hoechst dye efflux ability as HSCs. They demonstrated that testicular SP cells were able to home, proliferate, and differentiate into sperm cells when transplanted into the seminiferous tubules of genetically and chemically sterile mice.

As the testicular SP satisfies the definitive conditions for SSCs, we expected to observe Oct4/GFP transgene expression in the SP of testicular cells. Surprisingly, the ratio of GFP-positive cells was limited to 4% of the testicular SP of Oct4/GFP transgenic mice, with the remaining 96% negative for the Oct4/GFP transgene (Fig. 5). Furthermore, SP did not show expression of SSC markers or spermatocyte and/or spermatid markers. However, expression of the Bcrp1 gene, a member of the ABC transporter family (Fig. 6), was observed in the SP confirming the capacity of these cells for efficient Hoechst dye efflux. We hypothesized that the low/no expression of SSC marker genes, including Oct4, is a result of the low level of RNA expression due to the quiescent state of the testicular SP.

Stem cells are thought to reside in regulatory microenvironments 'niches' generated by stable stromal neighbors. Two groups reported that osteoblastic cells are a regulatory component of the hematopoietic stem cell niche *in vivo* (36,37). Recently, Arai *et al* demonstrated that HSCs expressing the yrosine kinase, Tie2 (comprising a side population were quiescent (38). Ang-1, the ligand for Tie2, enhanced the ability of HSCs to become quiescent and also induced their adhesion to bone, protecting them from the stress that suppresses hematopoiesis. The quiescent state has been suggested to be indispensable for the maintenance of HSCs. Although previous studies indicated that the quiescent state is important to maintain and protect SSCs from stress, it is possible that the SSCs share this characteristic with HSCs as this is a common characteristic shared with other stem cells, such as mesenchymal stem cells (39). Our results clearly demonstrate that the testicular SP, especially those cells negative for Oct4/GFP, has a decreased RNA content in comparison with the main population or SP positive for Oct4/GFP (Fig. 7). These results support our hypothesis that the low/undetectable expression of SSC marker genes is the result of the low RNA expression level in the quiescent SSCs of the testicular SP.

Matsuzaki *et al* (40) demonstrated that SP cells, especially those at the tip, are undifferentiated HSCs with the highest capacities for proliferation and multi-lineage differentiation capable of reconstituting the hematopoietic system. They proposed that the strongest dye efflux ability was a marker for the most primitive HSCs that have a strong ability to home to appropriate sites *in vivo*. In contrast with HSCs, there have been few reports addressing the primitive mechanism of spermatogenesis of SSCs in mice. As shown in multicolor analysis of testicular cells of Oct4/GFP mice stained with Hoechst, there were hardly any GFP-positive cells at the tip of the SP, which showed the strongest dye efflux ability (Fig. 5). In addition, the 'tip' SP showed the lowest RNA content as compared to SP cells located far from the tip (data not shown). These results suggest that the 'tip' SP of testicular cells is comprised of the most primitive SSCs that have the strong ability to home, proliferate, and differentiate into sperm.

Our results show that the mouse testis includes populations that exhibit different characteristics of SSCs. Our hypotheses are as follows: First, the testicular SP consists of two subpopulations of SSCs in the quiescent state (Oct4-negative cells) or with proliferation capacity (Oct4-positive cells). Second, Oct4 expression is necessary to suppress the commitment to the most primitive SSCs, but not in the quiescent state. Third, the most primitive SSCs are those at the tip of the SP, and these cells are quiescent SSCs with the highest capacity for reconstituting spermatogenesis *in vivo*. To confirm these hypotheses, SP cells negative and positive for Oct4 should be transplanted into mouse testes and examined for their ability to reconstitute spermatogenesis.

In conclusion, we isolated and characterized testicular cells by FACS using a multi-parameter selection strategy, combining the *in vivo* expression of Oct4 determined by monitoring GFP expression and *in vitro* SP. Our results indicate that SP can be isolated from whole testicular cells of Oct4/GFP mice irrespective of age, and this SP is comprised of a novel subpopulation negative for Oct4/GFP. This Oct4-negative subpopulation is potentially useful for production of genetically modified mammals, and will be a useful marker in the further isolation and characterization of murine SSCs.

Acknowledgements

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