GDNF Family Receptor alpha1 Phenotype of Spermatogonial Stem Cells in Immature Mouse Testes[1]

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ABSTRACT

Spermatogonial stem cells (SSCs) are essential for spermatogenesis, and these adult tissue stem cells balance self-renewal and differentiation to meet the biological demand of the testis. The developmental dynamics of SSCs are controlled, in part, by factors in the stem cell niche, which is located on the basement membrane of seminiferous tubules situated among Sertoli cells. Sertoli cells produce glial cell line-derived neurotrophic factor (GDNF), and disruption of GDNF expression results in spermatogenic defects and infertility. The GDNF signals through a receptor complex that includes GDNF family receptor α1 (GFRA1), which is thought to be expressed by SSCs. However, expression of GFRA1 on SSCs has not been confirmed by in vivo functional assay, which is the only method that allows definitive identification of SSCs. Therefore, we fractionated mouse pup testis cells based on GFRA1 expression using magnetic activated cell sorting. The sorted and depleted fractions of GFRA1 were characterized for germ cell markers by immunocytochemistry and for stem cell activity by germ cell transplantation. The GFRA1-positive cell fraction coeluted with other markers of SSCs, including ITGA6 and CD9, and was significantly depleted of KIT-positive cells. The transplantation results confirmed that a subpopulation of SSCs expresses GFRA1, but also that the stem cell pool is heterogeneous with respect to the level of GFRA1 expression. Interestingly, POU5F1-positive cells were enriched nearly 15-fold in the GFRA1-selected fraction, possibly suggesting heterogeneity of developmental potential within the stem cell pool.

gamete biology, spermatogenesis, testis

INTRODUCTION

Spermatogenesis is maintained through continuous activity of spermatogonial stem cells (SSCs). The SSCs reside in stem cell niches located on the basement membrane of seminiferous tubules and among the basal portions of Sertoli cells [1, 2]. The crucial role of testicular stem cells is to maintain their own pool by self-replication and to provide germ cell progenitors that undergo mitotic cell divisions and, ultimately, differentiate into spermatozoa [1, 3–6].

Although SSC function is crucial for the maintenance of spermatogenesis, relatively little is known about their biology [1, 6, 7], mainly because studies of testicular stem cells in rodents are hampered by the difficult discrimination between stem cells and differentiating spermatogonia. Only subtle morphological differences exist between SSCs (A_s), undifferentiated spermatogonia (A_{pr} and A_{al}), and differentiating A1 through A4 spermatogonia [8], and thus far, no specific markers have been described to distinguish unequivocally SSCs from the differentiating progeny [1, 5, 9, 10]. Furthermore, the low number of stem cells in the testes is an obstacle to the study of testicular stem cells, because only two or three stem cells exist in 10^4 testis cells in adult mice [11, 12].

Research on embryonic and adult stem cells has provided valuable information about shared characteristics between different types of stem cells. Several surface proteins, including ITGA6 (also known as α6-integrin) and ITGB1 (also known as β1-integrin) and CD9, are commonly found on stem cells, and these markers may facilitate interactions between stem cells and their cognate niches, which typically are located on tissue basement membranes [13–15]. Using antibodies against these surface proteins, in combination with fluorescence activated cell sorting or magnetic activated cell sorting (MACS), has allowed systematic characterization of stem cell populations and enrichment of freshly isolated testicular stem cells. Unequivocal determination of stem cell enrichment or depletion was tested by germ cell transplantation, which is an elegant tool for

estimating the relative and absolute numbers of testicular stem cells in a cell suspension [7, 10, 16, 17].

Recently, glial cell line-derived neurotrophic factor (GDNF) was proposed to be an important growth factor for communication between spermatogonia and Sertoli cells. The GDNF promotes the proliferation of undifferentiated spermatogonia both in vitro and in vivo [2, 6, 9, 18]. Originally, GDNF was identified as a growth factor promoting neuronal cell survival and mediating kidney development [9, 18]. It signals via a surface receptor complex composed of a glycosyl-phosphatidyl inositol-linked receptor of the GDNF family α1–4 (GFRA1–4) and a ret tyrosine kinase receptor [2, 6, 18, 19]. In the testis, GDNF is secreted by Sertoli cells [2, 9, 19], and the GFRA1 receptor is expressed in undifferentiated spermatogonia [9, 18]. Indeed, recent reports suggest that GFRA1 is expressed by SSCs [18–21], but this remains to be confirmed by in vivo functional analysis. In the present study, we evaluate the GFRA1 phenotype of SSCs in mouse pup testes and examine whether this marker provides stem cell enrichment by MACS fractionation and germ cell transplantation. We define the subtypes of spermatogonia in the isolated cell fractions, using additional markers and immunofluorescent microscopy (stem cells, ITGA6 and CD9; differentiating cells, KIT). In addition, we use POU5F1 (also known as Oct4) antibodies on fixed-cell preparations to detect a potential enrichment of germ stem cells, because some studies have demonstrated expression of this pluripotency marker by undifferentiated spermatogonia in tissue sections [9, 19, 22].

MATERIALS AND METHODS

Animals

Ten-day-old CD1/ICR (Charles River Laboratories, Wilmington, MA) and ROSA26 (stock no. 002073; Jackson Laboratories, Bar Harbor, ME) mouse pups were used for preparation of testis cell suspensions. Mice (129 × C57BL/6) used as transplantation recipients were from Jackson Laboratories. Most procedures were performed under a protocol granted by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh School of Medicine (no. 0402568). The germ cell transplantation procedures were approved by the IACUC of Magee-Womens Research Institute in accordance with the Guide for Care and Use of Laboratory Animals of the National Academy of Sciences (assurance no. A3654-01).

Preparation of Single Cell Suspensions

The pups were killed by decapitation after inhalation of carbon dioxide gas from a compressed gas cylinder. The testes were dissected and placed into digestion medium consisting of a 1:1 mixture of Dulbecco modified Eagle medium (DMEM; catalog no. 10-013-CV; Mediatech (800) Cellgro, Mediatech Inc., Herndon, VA) and Ham F12 (catalog no. 10-080-CV; Mediatech (800) Cellgro).

The testes were decapsulated, and the tissue was minced using small scissors. To achieve a single cell suspension, we performed a two-step enzymatic digestion. In brief, the testicular tissue was transferred into digestion medium containing collagenase type 1A (1 mg/ml; catalog no. C2674; Sigma-Aldrich Co., St. Louis, MO) and DNase1 (1.3 mg/ml; catalog no. 104132; Roche Diagnostics GmbH, Indianapolis, IN). This digestion step was performed at 37°C for approximately 30 min and was terminated when no large tissue fragments were observed and all seminiferous tubules had been digested into small, tubular fragments. After the first digestion, interstitial cells were separated by sedimentation at unit gravity for 5 min, at which time the supernatant was discarded and the remaining tissue resuspended in DMEM/Ham F12. The second digestion was performed for 45 min in medium containing collagenase type 1A (1 mg/ml), DNase1 (7.3 mg/ml; Roche Diagnostic GmbH), and hyaluronidase type 1-S (1–3 mg/ml; catalog no. H-3506; Sigma-Aldrich). The single cell suspension obtained after the second digestion step was centrifuged (200 × g, 10 min) and resuspended in digestion medium plus 10% fetal calf

serum for 5 min. Total cell number was established using a hemocytometer (Hausser Scientific, Horsham, PA). Cell viability was determined by trypan blue exclusion (catalog no. 25-900-CI; Mediatech (800) Cellgro). The cells were finally centrifuged (200 × g, 10 min) and resuspended in digestion medium.

Spermatogonial Stem Cell Isolation and Enrichment

An aliquot of the single cell suspension was removed and maintained on ice throughout the remainder of the procedure. This fraction of cells was designated the presorted fraction.

All remaining cells were used for a positive-selection strategy using MACS. The procedure was performed in accordance with the protocols of the supplier of the magnet and magnetic columns (Miltenyi Biotec, Auburn, CA). In principle, indirect magnetic labeling using primary antibodies against GFRA1 and secondary antibodies carrying magnetic particles was performed before positive-selection columns (MS) were used to obtain depleted and sorted fractions of the cells. In brief, the cell suspension was incubated with a rabbit polyclonal antiserum directed against GFRA1 (catalog no. SC-10716; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500 in MACS buffer (50 mM PBS and 150 mM NaCl, [overall pH 7.4], supplemented with 2 mM EDTA and 0.5% BSA) for 10 min at 6–10°C. The cells were then washed by adding MACS buffer and centrifuged (200 × g, 10 min), followed by incubation with the secondary antibody of goat anti-rabbit immunoglobulin (Ig) G conjugated to microbeads (1:5; Miltenyi Biotec) for 15 min at 6–10°C. Control samples underwent the same procedure except for omission of the primary antibody in the first incubation step. Cells were finally washed and resuspended in ice-cold, degassed MACS buffer before the sorting procedure.

The MS magnetic columns (Miltenyi Biotec) were placed in the magnet and flushed three times with 500 μl of ice-cold, degassed MACS buffer. Then, the cell suspension was added. Cells positive for GFRA1, which carry magnetic beads, remain in the matrix of the MACS column as long as it is maintained in the magnetic field. Unlabeled cells pass through the column into the collection tube (depleted fraction). To increase the purity of the sorted cell fraction, the magnetic column was rinsed three times with 500 μl of degassed MACS buffer while in the magnetic field. The retained cells were finally eluted by removing the column from the magnetic field, adding 500 μl of degassed MACS buffer, and flushing the columns under additional pressure from a plunger. This fraction of cells was designated the sorted fraction.

Immunocytochemistry

Immunocytochemistry with GFRA1, CD9, ITGA6, and KIT antibodies was performed to determine the number of positive cells in the three fractions (presorted, depleted, and sorted) obtained after MACS. Cells were aliquoted into four Eppendorf tubes. Each tube of presorted and depleted fractions contained at least 10^7 cells per 100 μl, whereas those of sorted fraction contained $0.7–2 × 10^6$ cells per 100 μl. The cells in each fraction were incubated with primary antibodies: 1) rabbit polyclonal anti-GFRA1 (1:100; Santa Cruz Biotechnology), 2) mouse monoclonal anti-CD9 (1:100; catalog no. 9310-01; Southern Biotechnology Associates, Inc., Birmingham, AL), 3) mouse monoclonal anti-CD49f UNLB (ITGA6; 1:100; Southern Biotechnology Associates, Inc.), and 4) rat polyclonal anti-KIT (1:100; Southern Biotechnology Associates). Cells were incubated with primary antibodies for 10 min at 6–10°C, washed with PBS, then incubated with secondary antibodies conjugated with biotin for the same time period and under the same conditions. The secondary antibodies are goat anti-rabbit IgG biotin conjugated (1:100; catalog no. B8895; Sigma, St. Louis, MO), goat anti-mouse IgG biotin conjugated (1:100), and goat anti-rat IgG biotin conjugated (1:100). After washing in PBS and centrifugation (200 × g, 10 min), the cells were incubated with Streptavidin Alexaflour 488 (1:100; Molecular Probes, Eugene, OR) for 10 min at 6–10°C. After immunocytochemistry, cells were fixed in 3–5% formaldehyde solution for 15 min at room temperature, then were washed by PBS and spun down at 200 × g for 3 h. The pellets were resuspended in Vectashield mounting medium with 4′,6′-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) before being transferred onto glass slides and sealed under coverslips. The slides were analyzed for positive cells under a fluorescent microscope (Nikon Eclipse E800).

POU5F1 Staining by Immunocytochemistry

Mouse spermatogenic cells were adhered to 0.1% poly-L-lysine-coated coverslips in humidifying chamber at 37°C for 15 min and then fixed in 100% cold methanol (−20°C) for 20 min. Following permeabilization with

TABLE 1. Number of testes from day 10 mice used for each independent experiment and number of cells in the presorted and enriched fractions obtained in each experiment.

Experiment	No. of testes used for experiment	Total no. of cells after 2nd digestion ($\times 10^6$)	No. of cells isolated per testis ($\times 10^6$)	No. of cells in GFRA1-enriched fraction ($\times 10^5$)	Relative no. of cells present in enriched cell fraction (%)
1	72	97.5	1.35	8.7	0.88
2	32	81.0	2.53	7.3	0.89
3	41	47.8	1.16	7.0	2.42
4	27	33.0	1.18	7.0	2.11
5	24	30.5	1.27	9.5	3.10
Total	196	290			
Mean ± SEM			1.5 ± 0.3	7.9 ± 0.5	1.9 ± 0.5

1% Triton X-100 in PBS for 40 min, the cells were blocked with 5% goat serum and 0.5% BSA in PBS for 40 min at room temperature. The cells were then stained using primary antibody, mouse monoclonal anti-human POU5F1 (1:50; Santa Cruz Biotechnology) overnight at 4°C, washed three times with PBS for 5 min each wash, and then incubated with secondary antibody, goat anti-mouse Alexa-488 or Alexa-594 (1:100; Molecular Probes) for 1 h at 37°C. The samples were washed with PBS three times for 5 min each time to remove the excess secondary antibody. To visualize DNA, 5 μM of TOTO-3 (Molecular Probes) in PBS were added to all samples, which were then incubated at room temperature for 20 min, washed three times for 10 min with PBS to remove the unbound molecules, and mounted with Vectashield mounting medium with DAPI and sealed with nail polish. As a positive control, we used human embryonic stem cell line H7. For the negative control, the primary antibody was omitted. Both positive and negative staining followed the above staining protocol. The images were captured using a Leica TCS-NT confocal microscope.

Cell Counting

Evaluation of cell counts was performed in a blinded fashion. Slides were evaluated first under low-power magnification (25×) to locate the area with positive cells. Then, higher-power magnification (40×) was used for cell counting. Screening of cells started randomly at the top-right corner of the counting area. The slide was then moved manually toward the left-hand side until outside of the counting area. Next, the slide was moved downward, and the counting continued from the left to the right end of the counting area. The sideward and downward progression of fields occurred without overlapping areas of view. The total number of cells (DAPI-positive nuclei) and the number of immunopositive and immunonegative cells were estimated in each field. The total number of cells counted on each slide was at least 700–1000 for the presorted and depleted fractions. Fewer cells were seen on slides made from the enriched cell fraction, but at least 300 cells were evaluated for each of these slides.

Germ Cell Transplantation

Donor cells for spermatogonial stem cell transplantation were from 10-day-old ROSA26 mice, which corresponds in age to that of the CD1/ICR mice used for the cell isolation and enrichment studies. This line of transgenic animals expresses the *Escherichia coli lacZ* transgene in many cell types, including all stages of spermatogenesis [23]. The *lacZ* transgene encodes the β-galactosidase enzyme that converts the colorless substrate 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) to a blue precipitate, allowing unequivocal identification of donor germ cells in recipient seminiferous tubules that do not express *lacZ*. Single cell suspensions from ROSA26 mouse pup (10 days postpartum) donors were produced by enzymatic digestion, as described above. The total cell population was then fractionated into GFRA1 sorted and depleted fractions for transplantation into recipient mouse testes to determine the relative concentrations of SSCs. Cell fractions were suspended (1–5 × 10^7 cells/ml) for transplantation in minimum essential medium α containing 10% fetal bovine serum.

Recipient mice (129 × C57; Jackson Laboratories) were treated with busulfan (50 mg/kg; Sigma, St. Louis, MO) at 5–6 wk of age to remove endogenous germ cells and create depleted stem cell niches for donor germ cell engraftment. Approximately 6 wk after busulfan treatment, the donor testis cell suspension (8 μl) was introduced into recipient seminiferous tubules by efferent duct injection as described previously [24].

Testes of recipient mice were collected 2 mo after transplantation and stained with X-gal to visualize blue donor-derived colonies of spermatogenesis. Transgenic donor SSCs are defined by their capacity to produce and maintain blue colonies of spermatogenesis, where each colony is clonally derived from a single spermatogonial stem cell [25]. Differentiated germ cells do not produce colonies of spermatogenesis, and endogenous
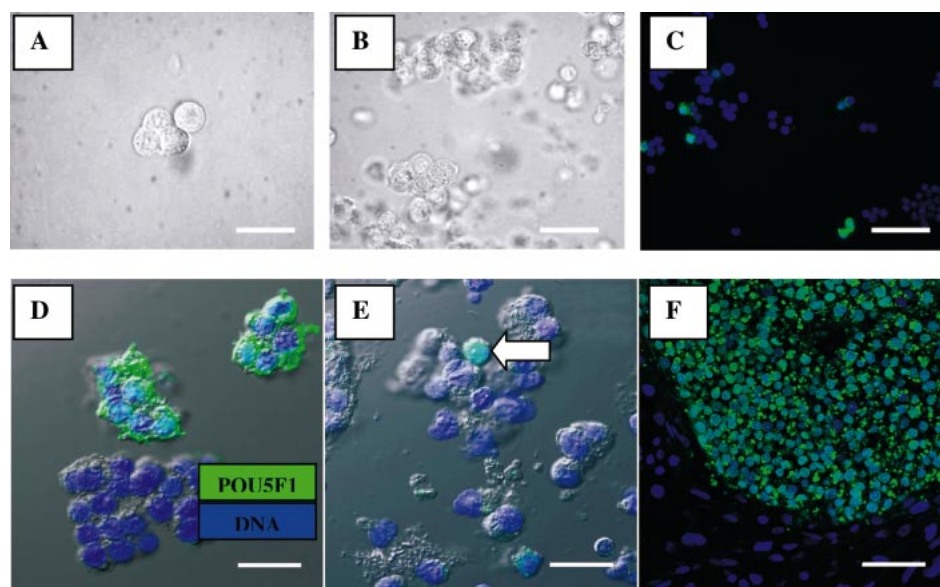

FIG. 1. Phase-contrast micrographs of fresh cell preparations after MACS sorting. **A**) Sorted fraction. **B**) Depleted fraction. **C**) Fluorescent micrograph of sorted cell fraction stained for GFRA1. Positive staining is seen as green fluorescence; nuclei are counterstained with DAPI (blue fluorescence). **D–F**) The pluripotency of POU5F1 expression was tested on spermatogonial cells of GFRA1 sorted and depleted fractions. Smears of methanol-fixed cells were stained with POU5F1 antibody and visualized by green fluorescence. Expression of POU5F1 was photographed using confocal microscopy (Leica TCS-NT). The POU5F1-positive cells in sorted fraction (**D**), POU5F1-positive cells in depleted fraction (**E**; arrow), and a confocal picture of POU5F1-positive control staining (**F**) of differentiating (negative) and nondifferentiating (positive) human embryonic stem cells (line H7) are shown. The green color indicates embryonic stem cells expressing POU5F1. Bar = 20 μm (**A**), 30 μm (**B**), 90 μm (**C**), 25 μm (**D** and **E**), and 50 μm (**F**).
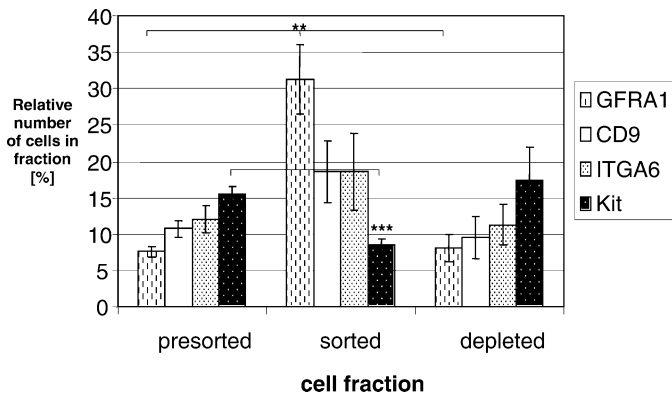
FIG. 2. Bar chart of the relative numbers of immunopositive cells stained by four antibodies (GFRA1, CD9, ITGA6, and KIT) in three cell fractions. **$P < 0.01$, ***$P < 0.001$.
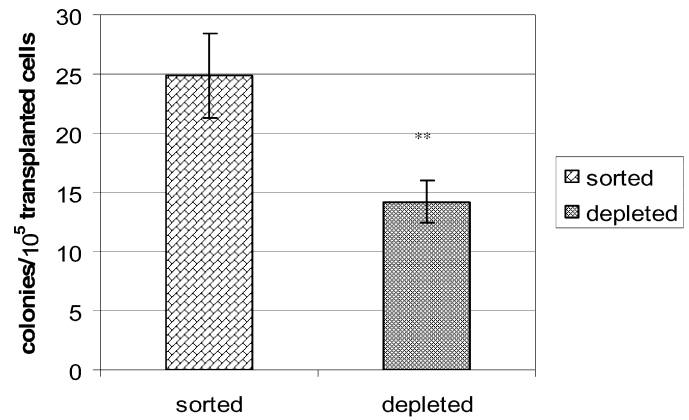

FIG. 3. Bar graph showing the outcome of germ cell transplantation experiments. Compared to the depleted fraction, a significantly higher number of germ cell colonies was obtained after transplantation of GFRA1-enriched cells. Data represent the number of blue colonies (mean $\pm$ SEM) per 10^5 transplanted cells. **$P < 0.01$.

germ cells do not express the *lacZ* transgene. Donor-derived spermatogenic colonies in recipient testes were counted using a dissecting microscope. Because the number of cells recovered in each fraction varied, colony number was normalized to 10^5 cells injected per testis.

Statistics

Data in the present study are shown as the mean $\pm$ SEM. Student *t*-test was used to analyze the difference between positive cells of each antibody in three fractions, the difference between all antibodies in the same fraction, the difference of POU5F1 staining in sorted and depleted fractions, and the difference of colonization between GFRA1-enriched and -negative cell fractions. A *P* value of less than 0.05 was accepted as a statistically significant difference.

RESULTS

Fractionation of Cells Using MACS

We report here the results of five independent experiments (Table 1). In total, 196 testes from Day-10 mice were used for the experiments. An average of 1.5 million cells was obtained from each testis after the second digestion step. Use of MACS with an antibody directed against GFRA1 and an indirect labeling approach with a secondary antibody conjugated to magnetic microbeads resulted in a small fraction of positively selected cells comprising 0.88–3.1% (mean, 1.9%) of the original cell preparation (Fig. 1A). The remaining cells were eluted into the depleted fraction (Fig. 1B and Table 1).

Immunocytochemical Characterization of the Isolated Cell Fractions

Subpopulations of intact live cells in presorted, sorted, and depleted fractions were characterized by immunocytochemistry. Four different antibodies, directed against GFRA1, CD9, ITGA6, and KIT, were visualized by immunofluoresence. These epitopes have been described previously as positive (ITGA6 and CD9) or negative (KIT)

TABLE 2. Relative number of POU5F1 immunopositive cells in isolated cell fractions.

Experiment	GFRA1-enriched fraction (%)	Depleted fraction (%)
1	9.15	0.87
2	10.17	0.24
3	12.74	1.04
Mean $\pm$ SEM	10.69 $\pm$ 1.30	0.72 $\pm$ 0.30

markers of SSCs [7, 10, 17, 19, 26]. In addition, KIT is known as a marker of differentiating spermatogonia [4, 8, 27, 28]. The determination of labeling indices for these markers enabled us to characterize the phenotype of cells in the presorted, GFRA1-sorted, and depleted fractions. A representative example of the immunocytochemical staining pattern is depicted in Figure 1C.

In the presorted fraction, 7.65% $\pm$ 0.73% of cells were GFRA1 positive. The sorted fraction contained 31.26% $\pm$ 4.77% GFRA1-positive cells, indicating a highly significant ($P < 0.01$), fourfold enrichment from the presorted fraction (Fig. 2). No significant depletion of GFRA1-positive cells was observed in the depleted fraction (8.12% $\pm$ 1.92%). The total number of cells in the enriched fraction was approximately 800 000 cells, representing less than 2% of the cells in the presorted fraction (Table 1). Because no depletion of GFRA1-positive cells was observed, these numbers indicate that the efficiency of the sorting procedure was low. As a consequence, the depleted fraction is nearly identical to the presorted fraction, because it contains 98% of the total presorted cell populations and is not significantly depleted of GFRA1-positive cells.

Staining for the additional spermatogonial stem cell markers CD9 and ITGA6 revealed a coenrichment in the GFRA1-enriched fraction. The percentage of CD9-positive cells increased from 10.69% $\pm$ 1.12% in presorted fraction to 18.6% $\pm$ 4.2% in sorted fraction. Similarly, ITGA6-positive cells increased from 11.98% $\pm$ 1.82% to 18.56% $\pm$ 5.27% (Fig. 2). Although these changes were consistent for each experiment, they did not reach statistical significance. However, a statistically significant depletion of KIT-positive cells was observed between the presorted fraction (15.55% $\pm$ 1.03%) and the GFRA1-sorted fraction (8.53% $\pm$ 0.76%, 1.82-fold depletion, $P < 0.001$) (Fig. 2). As expected from the above observations, the depleted fraction contained very similar numbers of CD9-, ITGA6-, and KIT-positive cells compared to the presorted fraction (Fig. 2).

Detection of POU5F1-Positive Cells in Isolated Cell Fractions

The presence of POU5F1 was determined on smears of methanol-fixed cells obtained from GFRA1-sorted and -depleted cell fractions. Representative examples of the immunocytochemical staining results are shown in Figure 1,

D–F. The specificity of the staining was confirmed by positive-control staining of POU5F1 on embryonic stem cells. The stained cells were analyzed by confocal and immunofluorescent microscopy and revealed a highly significant ($P < 0.001$), 15-fold enrichment of POU5F1-positive cells in the sorted fraction compared to the depleted fraction (Table 2).

Validation of Spermatogonial Stem Cell Enrichment by Germ Cell Transplantation

Two independent germ cell transplantation experiments were performed to quantify the number of SSCs in GFRA1-sorted and -depleted cell fractions. These experiments revealed a significantly ($P < 0.01$) greater concentration of stem cells in the enriched cell fraction (24.88 colonies per 10^5 cells transplanted) compared to the depleted fraction (14.21 colonies per 10^5 cells transplanted) (Fig. 3).

DISCUSSION

Several recent studies have demonstrated that GDNF is an important factor for promoting spermatogonial self-renewal and that its receptor GFRA1 is expressed on undifferentiated spermatogonia, possibly including SSCs [18–21]. We encountered approximately 8% GFRA1-positive cells in the presorted cell suspension of Day-10 mouse pup testis cells. In accordance with the background information, we designed experiments to characterize the GFRA1-positive cells for germ cell and pluripotency markers as well as for stem cell activity. Testes at this age are developmentally enriched for stem cells [29]. The seminiferous cords at this stage are still growing, creating more stem cell niches that will be occupied by proliferating SSCs.

The MACS has been used effectively for cell isolation in many tissues and organs, such as bone marrow, muscle, and liver [30–32]. The MACS is specifically useful for stem cell isolation, because stem cell numbers typically are very low. The efficiency of sorting intact cells depends on the availability of specific surface markers on the membranes of stem cells. In addition, MACS has been described as a successful tool for enrichment of SSCs [19, 33], and using this method, it was shown that SSCs share some surface protein antigens that also are present on other stem cells [10, 17].

To our surprise, the sorted fraction contained only 2% of the cells in the original cell suspension. This number is considerably lower compared to that of cells expressing GFRA1 in the presorted fraction, as determined by immunocytochemistry (8%) (Figs. 1 and 2). This result may indicate low antibody-epitope recognition or low-level expression of the GFRA1 epitope in positive cells. However, fractionation of cell populations based on variances in expression level can provide a powerful enrichment strategy [7, 34]. We therefore characterized the presorted, sorted, and depleted fractions by immunocytochemistry with various stem cell markers and for stem cell activity by germ cell transplantation. The GFRA1-positive cells were enriched up to fourfold compared to the presorted fraction. An enrichment of undifferentiated germ cells was supported by coelution with other spermatogonial markers, which were enriched at a lower rate (CD9, 1.74-fold; and ITGA6, 1.55-fold) that did not reach statistical significance. Relative to the depleted fraction, the GFRA1-positive fraction also exhibited a nearly 50% depletion of KIT, a marker of differentiating spermatogonia, providing additional confirmation that this fraction was enriched for undifferentiated

spermatogonia. Finally, the outcome of the germ cell transplantation experiments indicated a modest but significant enrichment of stem cells in the GFRA1-positive fraction compared to the depleted fraction. In accordance to the low amount of total cells in the sorted fraction as well as the low degree of enrichment of stem cells in that same fraction, we detected many cells in the depleted fraction that were positive for stem cell markers. We calculated from our cell numbers and the labeling indices that only 1 of 20 GFRA1-positive cells would be directed into the sorted fraction and that 19 of 20 would be found in the depleted fraction. This relative number was calculated from the absolute number of GFRA1-positive cells in the presorted fraction (8% of approximately 60 million [i.e., 4.8 million]) and the absolute number of GFRA1-positive cells in the sorted fraction (30% of approximately 0.8 million [i.e., 240 000]). This indicates that the depleted fraction is widely identical to the presorted fraction, because it contains approximately 98% of all isolated cells and approximately 95% of the GFRA1-positive cells that were present in the presorted total cell suspension. Taken together, our results indicate that GFRA1-targeted selection of cells by MACS leads to an enrichment of stem cells. However, stringency as well as efficiency of the sorting strategy appear to be low.

Primordial germ cells are the primitive germ cells in the early embryo. When these cells are placed in culture and exposed to the appropriate factors, they share many characteristics with embryonic stem cells. Both show pluripotent properties, including expression of alkaline phosphatase and the POU homeodomain transcriptions factor, POU5F1 [21, 35]. In addition, primitive germ cells share embryonic stem cell pluripotency characteristics in culture; they form embryoid bodies and differentiate into several cell lineages [36, 37]. The SSCs are the male-specific descendants from primitive germ cells and are found only in the postnatal testis [4]. In the present study, we demonstrate that POU5F1 immunoreactivity is dramatically enriched (14.85-fold) in a small population of mouse pup testis cells recovered in the GFRA1 sorted fraction. These observations raise the intriguing possibility both that SSCs in the postnatal mouse testis are a heterogeneous cell population and that those cells exhibiting high GFRA1 expression and POU5F1 expression have a unique developmental potential. For example, Kanatsu-Shinohara et al. [38] recently demonstrated that a small subfraction of stem cells in the postnatal mouse testis can give rise to embryonic stem-like cells in culture.

In summary, we have confirmed GFRA1 expression in SSCs and shown that GFRA1 antibodies can be used to obtain enriched fractions of SSCs by MACS. Our results indicate the existence of a distinct subpopulation of SSCs in the mouse pup testis that expresses a higher level of GFRA1 and is positive for the pluripotency marker POU5F1. It will be highly interesting in future studies to confirm the existence of subpopulations of SSCs that maintain different degrees of developmental potential.

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