Identifying genes important for spermatogonial stem cell self-renewal and survival

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Contributed by Ralph L. Brinster, April 25, 2006

Spermatogonial stem cells (SSCs) are the foundation for spermatogenesis and, thus, preservation of a species. Because of stem cell rarity, studying their self-renewal is greatly facilitated by in vitro culture of enriched biologically active cell populations. A recently developed culture method identified glial cell line-derived neurotrophic factor (GDNF) as the essential growth factor that supports in vitro self-renewal of SSCs and results in an increase in their number. This system is a good model to study mechanisms of stem cell self-renewal because of the well defined culture conditions, enriched cell population, and available transplantation assay. By withdrawing and replacing GDNF in culture medium, we identified regulated expression of many genes by using microarray analysis. The expression levels of six of these genes were dramatically decreased by GDNF withdrawal and increased by GDNF replacement. To demonstrate the biological significance of the identified GDNF-regulated genes, we examined the importance of the most responsive of the six, bcl6b, a transcriptional repressor. By using siRNA to reduce transcript levels, Bcl6b was shown to be crucial for SSC maintenance in vitro. Moreover, evaluation of Bcl6b-null male testes revealed degeneration and/or absence of active spermatogenesis in 24 ± 7% of seminiferous tubules. These data suggest that Bcl6b is an important molecule in SSC self-renewal and validate the biological relevance of the GDNF-regulated genes identified through microarray analysis. In addition, comparison of data generated in this study to other stem cell types suggests that self-renewal in SSCs is regulated by distinctly different molecular mechanisms.

Results

Overnight (18-hr) Withdrawal of GDNF Does Not Adversely Affect the SSC Potential of Cultured Thy1 + Germ Cells. To determine the effect of growth factor changes on SSC potential in vitro, Thy1+ germ cells from donor ROSA male mice, which express a LacZ marker gene in all germ cells, were cultured. In this system, germ cells grow as clumps of proliferating cells with tightly adhering membranes (Fig. 1A). SSC presence in these clump-forming germ cell populations was confirmed by transplantation to recipient testes (2, 3), after which, donor stem cells generated colonies of spermatogenesis (Fig. 1B).

To evaluate retention of SSC potential after growth factor withdrawal, clump-forming cells were deprived of GDNF and GDNF-family receptor α1 (GFRα1) for 18 hr (overnight), followed by transplantation into recipient testes. Loss of SSC potential in the cultured cells because of this withdrawal would be detected as a decrease in the ability to form colonies of spermatogenesis in the busulfan-treated recipient testes. A reduction in SSC potential after GDNF/GFRα1 withdrawal was not detected (Fig. 1C), indicating that gene-activity changes in experimental cell populations would likely reflect immediate GDNF effects and not permanent alteration in stem cell potential.

Conflict of interest statement: No conflicts declared.

Abbreviations: ESC, embryonic stem cell; GDNF, glial cell line-derived neurotrophic factor; GFRα1, GDNF-family receptor α1; qRT-PCR, quantitative real-time PCR; siRNA, short interfering RNA; SSC, spermatogonial stem cell.

Data deposition: Microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE4799).

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tial (e.g., cell death or irreversible differentiation). Surprisingly, a significant increase in colonization occurred, suggesting that the cultured cells were more receptive to transplantation after a brief period of GDNF withdrawal.

Microarray Profiling Reveals GDNF/GFRα1-Regulated Genes in Self-Renewing SSCs in Vitro. To identify genes involved in SSC self-renewal, established cultures of clump-forming germ cells (~2 mo old) from inbred C57 mice (non-ROSA) were used to avoid possible influence of transgene expression. We first removed GDNF and GFRα1 from cultures of actively dividing germ cell clumps and, subsequently, replaced the factors. At the beginning of and during the process, pure populations of germ cells were separated from STO feeders by gentle pipetting, which yielded a homogenous cell population in which 96 ± 1%g (see Fig. 4, which is published as supporting information on the PNAS web site) of the cells displayed the typical SSC surface antigenic phenotype of αv-integrin−/−/dim αv-integrin+/Thy1+/+ (4, 12). It has been demonstrated that the SSC surface phenotype and ability to generate donor-derived colonies of spermatogenesis in recipient testes is maintained at a constant level for at least 6 mo in serially cultured SSCs (4). These cell populations were subsequently used for oligonucleotide microarray analysis to study changes in gene transcript levels. SSC presence in the cell populations was confirmed by transplantation into W recipient male testes (Fig. 1D), which are naturally sterile; therefore, any spermatogenesis must arise from donor SSCs.

When GDNF and GFRα1 were omitted from the medium for 18 hr (overnight), significant changes in the expression level of 278 transcripts were identified. The majority (199) of transcripts was down-regulated ≥2-fold, whereas a smaller number (79) of transcripts was up-regulated ≥2-fold. Several genes related to differentiation, for example, neurogenin 3 (ngn3) (13, 14), were up-regulated by GDNF/GFRα1 removal and, therefore, may be early/initial signals for SSC differentiation. In contrast, several genes associated with stem cell self-renewal and/or maintenance of pluripotency were down-regulated, for example, sox2, a transcription factor shown to be essential for embryonic stem cell maintenance of pluripotency (15). This latter group of genes may include regulators of GDNF-mediated self-renewal in SSCs.

After overnight withdrawal of GDNF/GFRα1, the growth factors were replaced in the culture medium and germ cells subsequently collected at 2, 4, and 8 hr to identify early, mid, and late regulated genes, respectively. Expression data at each time point were compared with the 0 hr (overnight withdrawal) treatment, and genes significantly up- or down-regulated ≥2-fold were identified. Venn diagrams were used to evaluate genes regulated uniquely at specific time points or commonly among multiple time points (see Fig. 5, which is published as supporting information on the PNAS web site). The majority of up-regulated genes were specifically changed at 4 hr of GDNF/GFRα1 treatment; whereas, for the majority of down-regulated genes, the changes occurred at 8 hr. Six transcripts were first significantly down-regulated by GDNF/GFRα1 withdrawal and then significantly up-regulated at the three time points by growth factor replacement (Table 1). Based on the regulated response of these genes in the absence and presence of

<table>
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<th>Gene*</th>
<th>GenBank no.</th>
<th>Raw signal in germ cell clumps</th>
<th>18-hr withdrawal</th>
<th>2-hr replacement</th>
<th>4-hr replacement</th>
<th>8-hr replacement</th>
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<td>−6.23</td>
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<td>Etv5</td>
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<td>−5.92</td>
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<td>2.26</td>
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* Bcl6b (B cell CLL/lymphoma 6, member B), Egr2 (Early growth response 2), Egr3 (Early growth response 3), Etv5 (Etv variant 5, ER_M), Lhx1 (LIM homeobox 1), Tspan8 (Testraspanin 8). Bcl6b, Egr2, Egr3, and Tspan8 expression was validated by qRT-PCR.

1 Average raw signal (relative value of mRNA abundance) from microarray experiments in three samples of in vitro clump-forming germ cells not subjected to GDNF/GFRα1 withdrawal (mean ± SEM).

2 Fold change of GENESPRING-normalized values (comparative value of mRNA levels) for cultured SSCs before and after GDNF/GFRα1 withdrawal.

3 Fold change of GENESPRING-normalized values (comparative value of mRNA levels) for cultured SSCs after replacement of GDNF/GFRα1 after withdrawal.
GDNF/GFRα1, we hypothesized that the six factors are important for SSC self-renewal.

Entire data sets can be accessed through the National Center for Biotechnology Information at the Gene Expression Omnibus (GEO) web site (www.ncbi.nlm.nih.gov/geo) accession no (GSE4799). Data sets of GDNF-regulated genes are available in Data Sets 1–3, which are published as supporting information on the PNAS web site.

Reduction of bcl6b Transcript Levels Affects SSC Clump Maintenance in Vitro. To demonstrate the biological significance of the identified GDNF-regulated genes, the transcriptional repressor bcl6b was examined for effects on SSC maintenance in vitro. Of the six highly regulated genes, bcl6b was down-regulated the greatest after overnight withdrawal of GDNF/GFRα1. We verified the regulated response of bcl6b by GDNF/GFRα1 stimulation using quantitative real-time (qRT)PCR. The expression pattern agreed closely with the microarray data (Fig. 2A), in which overnight withdrawal resulted in a 6.8-fold decrease, followed by a 2.1-, 3.6-, and 4.4-fold increase at 2, 4, and 8 h, respectively, of GDNF/GFRα1 treatment.

To study the importance of Bcl6b on SSC maintenance, we used a specific short interfering (si)RNA oligonucleotide duplex to suppress mRNA levels in cultures of clump-forming germ cells from ROSA donors. BLAST analysis of the sense siRNA oligonucleotide revealed no homology to any known mammalian gene other than bcl6b; thus, nonspecific reductions of other genes likely did not contribute to observed biological effects. Transfection efficiency in clump-forming germ cells was 67.9 ± 1.8% (mean ± SEM; n = 3), determined by flow cytometry after transfection of a cy3-labeled scrambled oligonucleotide. Transfection with bcl6b siRNA resulted in a decrease of 54 ± 0.1% (n = 3) in bcl6b mRNA levels compared with cells transfected with scrambled negative control siRNA as measured by qRT-PCR after 48 h. After transfection with bcl6b siRNA, there was a reduction in both size and number of germ cell clumps after 7 days compared with cells transfected with negative control siRNA (Fig. 2B). As a result, viable germ cell number progressively declined in bcl6b siRNA-treated cultures and was significantly reduced at 5 (P = 0.02) and 7 (P = 0.01) days compared with negative control siRNA-treated cultures. After 7 days, <17% (0.33 of 2.0) of cells remained in bcl6b siRNA-treated cultures (Fig. 2C). In addition, the percentage of apoptotic germ cells significantly (P = 0.002) increased and was almost doubled (22.0 vs. 12.9) in bcl6b siRNA-treated cultures compared with control siRNA cultures after 5 days (Fig. 2D). The doubling time in vitro for mouse SSCs is 6 days (4); therefore, the 7-day culture period encompassed a complete self-renewal period. These data demonstrate that inhibition of bcl6b has a significant impact on germ cell maintenance in vitro and suggest that Bcl6b is an important factor for SSC self-renewal and survival.

To confirm that siRNA-induced reduction of bcl6b mRNA results in decreased SSC numbers in culture, as suggested by the decrease in germ cell clump size and total germ cell number, we used the SSC transplantation assay. Established cultures of germ cells from ROSA donors were transfected with either bcl6b or negative control siRNA molecules and maintained in vitro for 7 days, followed by transplantation into infertile recipient testes. Treatment with bcl6b siRNA resulted in an 88% (2.0 vs. 17.2 average colony number) reduction of colonies within recipient testes compared with controls based on the number of cells originally seeded in culture (Fig. 2E). In addition, the concentration of SSCs in the population of transplanted germ cells (colonies generated per 10^5 cells) was also reduced in bcl6b siRNA-treated cultures (23.5 ± 1.9, mean ± SEM; n = 2 experiments, 15 total testes) compared with control siRNA cultures (49.5 ± 13.4; n = 2 experiments, 15 total testes) 7 days after transplantation. Thus, in the bcl6b siRNA-treated cultures, the PNAS web site.

Fig. 2. Evaluation of Bcl6b function in SSC self-renewal in vitro. (A) Regulation of bcl6b transcript expression by GDNF/GFRα1 identified by using oligonucleotide microarray analysis (filled triangles) was confirmed with qRT-PCR (open triangles). Error bars are mean ± SEM. The levels of regulated expression detected by the two techniques were nearly identical. After overnight withdrawal of GDNF/GFRα1 (0 hr), a sharp decline in expression occurred compared with cells not subjected to withdrawal (−18 hr). Replacement of GDNF/GFRα1 after overnight withdrawal resulted in significant up-regulation of gene expression at 2, 4, and 8 hr. (B) Treatment of germ cell clumps (arrows) with bcl6b siRNA dramatically reduced the size and number that formed during 7 days of culture compared with cells transfected with a control siRNA. (Scale bars, 100 μm.) (C) Confirming visual observations, total viable cell number progressively declined in bcl6b siRNA cultures compared with control siRNA cultures and was significantly reduced after 5 (P = 0.02) and 7 (P = 0.01) days in bcl6b siRNA-transfected cells compared with controls. Day 5, 0.72 ± 0.18 cells per 10^5 cells cultured, mean ± SEM; n = 3 (bcl6b siRNA) vs. 1.51 ± 0.14 cells per 10^5 cells cultured; n = 3 (control siRNA); day 7, 0.33 ± 0.04 cells per 10^5 cells cultured; n = 3 (bcl6b siRNA) vs. 2.0 ± 0.25 cells per 10^5 cells cultured; n = 3 (control siRNA). (D) The percentage of apoptotic cells after reduction of bcl6b transcript levels with siRNA was not different compared with controls after 3 days [16.3 ± 0.01%; mean ± SEM; n = 3 (bcl6b siRNA) vs. 13.5 ± 0.01%; n = 3 (control siRNA)] but progressively increased, becoming significant (P = 0.001) on day 5 of culture [22.0 ± 0.01; mean ± SEM; n = 3 (bcl6b siRNA) vs. 12.9 ± 0.01%; n = 3 (control siRNA)]. (E) The number of biologically active SSCs significantly (P = 0.006) declined during 7 days in culture after expression with bcl6b siRNA (2 ± 0.1 colonies per 10^5 cells cultured; n = 2 experiments, 15 total testes) compared with controls (17.2 ± 0.2 colonies per 10^5 cells cultured; n = 2 experiments, 15 total testes). Asterisks denote significant differences.
types. Similar abnormalities were not observed in wild-type males revealed abnormal seminiferous tubules, some with degenerating seminiferous epithelium with only spermatozoa, others contained degenerating seminiferous epithelium with only spermatozoa, and others devoid of all germ cells, containing only Sertoli cells. All male testes contained 24% of SSCs in transplanted germ cells is reduced 50% (23.5 of 49.5) after 7 days. The reduction of bcl6b mRNA levels has a dramatic effect on germ cell maintenance in vitro, and the effect is greater on SSCs than on nonstem germ cells.

Loss of Bcl6b Function Results in Impaired Spermatogenesis in Vivo. Recently, targeted disruption of Bcl6b expression was reported in mice (16). These Bcl6b-null mice are capable of siring offspring, but mating of null mice produced lower numbers of pups per litter (4.3 ± 0.5 pups per litter, n = 3) compared with the yield of pups from mating of wild-type C57 mice (5.9 ± 0.7 pups per litter, n = 3). To examine specifically the in vivo effect of Bcl6b deletion in males, adult (4-mo-old) wild-type (C57BL/6) and bcl6b-null male mice contained 24% (mean ± SEM; n = 3 males) abnormal seminiferous tubules per testis histological cross section. Wild-type control males (n = 3) contained no abnormal tubules. [Scale bars, 50 μm (B) and 25 μm (C).]

~12% (2.0 of 17.2) of the SSCs remain, and the concentration of SSCs in transplanted germ cells is reduced ~50% (23.5 of 49.5) after 7 days. The reduction of bcl6b mRNA levels has a dramatic effect on germ cell maintenance in vitro, and the effect is greater on SSCs than on nonstem germ cells.

Gene Expression Comparisons of Cultured SSCs with Other Stem Cell Types and Undifferentiated Spermatogonia Reveal Distinct Differences. Studies with embryonic stem cells (ESCs), hematopoietic stem cells, and neuronal stem cells have identified several genes that are essential for stem cell proliferation and maintenance of pluripotency (see Table 2, which is published as supporting information on the PNAS web site). It has been suggested that regulation of molecular mechanisms may be conserved among different types of stem cells. Our microarray data revealed that many factors identified in other stem cells were also expressed by SSCs, but only a few of these were influenced by GDNF/GFRα1. Two of the three core transcription factors of ESC pluripotency (17), oct4 and sox2, were expressed by the cultured SSCs; however, expression of the third critical molecule, nanog, could not be detected on the microarray chips (confirmed by RT-PCR; see Fig. 6, which is published as supporting information on the PNAS web site). The expression of sox2 was significantly reduced 5.95-fold after overnight withdrawal of GDNF/GFRα1, but oct4 was unaffected by GDNF/GFRα1 withdrawal or replacement.

Several molecules have been identified as being expressed by undifferentiated spermatogonia in the mouse testis, and some have been shown to be essential for their survival. Expression of these molecules by SSCs has not been conclusively determined in previous studies because of limitations of the methods used. Using the generated microarray data, we examined whether several of these molecules are expressed in SSC-enriched cultures (see Table 3, which is published as supporting information on the PNAS web site). Both ret protooncogene (ret) and gfra1 genes, the receptor duplex for GDNF, were expressed by the cultured cells. Although ret expression was unaffected by GDNF/GFRα1 withdrawal and replacement, endogenous expression of gfra1 was significantly influenced. The transcript for c-kit receptor, which is essential for differentiating spermatogonia, was questionably expressed by the cultured SSCs receiving a present call for one probe set on the microarray but an absent call for another c-kit-specific probe set. Corresponding c-kit protein expression could not be seen by immunofluorescent staining (data not shown) of the germ cell clumps. The transcript for several factors; dazl, plef, and taf4b, the absence of which have been shown to result in failure of spermatogenesis, possibly because of breakdown in spermatogonial function, were expressed by the cultured cells; however, none were influenced by GDNF/GFRα1 withdrawal or replacement. Interestingly, stra8, a putative marker for SSCs (18), was represented on the microarrays, but expression was not detected in any of the SSC-enriched culture samples, and this observation was further confirmed by RT-PCR (Fig. 6).

Discussion

Microarray profiling was used to investigate GDNF-regulated gene expression in established cultures of self-renewing mouse SSCs, providing a foundation for identifying molecular pathways controlling SSC self-renewal. To study GDNF regulation, it was necessary first to demonstrate that growth factor removal for 18 hr from culture media did not adversely affect stem cell potential of the cultured cell populations. Using a SSC transplantation assay, we were able to demonstrate that this was, indeed, the case. Therefore, gene activity changes in the experimental cell population likely reflect immediate GDNF effects and not permanent alteration in stem cell potential (e.g., cell death or irreversible differentiation). The significant increase observed in colony number after GDNF withdrawal could result from an increased sensitivity of the cells to GDNF at the time of transplantation compared with control cells that were constantly exposed to GDNF. Testes of busulfan-treated recipient males express GDNF at higher (≥5-fold) levels than wild-type mice (19), which may have led to improved initial colonizing efficiency by cells subjected to withdrawal compared with control cells that were continuously exposed to GDNF before transplant. Receptor hypersensitivity is also observed in breast cancer cell lines when deprived of estrogen for a period (20).

From the data generated in our microarray experiments, we were able to identify GDNF regulation of many genes, including...
the transcriptional repressor bcl6b, in germ cell clumps. To demonstrate the functional importance of the identified factors on SSC survival and self-renewal, Bcl6b was chosen for further analysis. Using siRNA-mediated reduction of bcl6b transcript levels in self-renewing SSCs, we demonstrated a dramatic effect on SSC maintenance in vitro. In addition, evaluation of Bcl6b-null mouse testes revealed an impairment of spermatogenesis in which many tubules contained degenerating stages of spermatogenesis or Sertoli cell-only phenotypes. These two phenotypes, particularly the Sertoli cell-only phenotype, occur in other conditions of stem cell loss, such as GDNF-null and Plzf-null male mice (21, 22). Therefore, although Bcl6b is not the only critical molecule for SSC function, it is clearly an important component in maintaining normal SSC biology and spermatogenesis. Bcl6b is also expressed by a subpopulation of memory T lymphocytes, in which it mediates an enhanced proliferative secondary response to viral infection (16). Thus, Bcl6b may have a role in regulating the function of other stem-cell and undifferentiated-cell populations.

Because GDNF has been established as the primary regulator of SSC self-renewal (4), dramatic alteration in bcl6b transcript levels resulting from withdrawal and replacement of the growth factor identifies an important molecule involved in the self-renewal mechanism of SSCs. The mechanism by which Bcl6b affects SSC biology remains unclear and may include influencing proliferation, maintenance of an undifferentiated state, regulation of apoptosis, or a combination of all three. Both Bcl6b and Plzf are members of the BTB/POZ domain transcriptional repressors, and ablation of Plzf expression in mice has been shown to result in male infertility (22, 23). Thus, two members of this family have now been shown to influence male fertility, and evidence suggests that the effect involves SSC self-renewal. Because SSCs from many species are able to proliferate in the mouse testis (7–11) and GDNF has been shown to support both mouse and rat SSC self-renewal in vitro (4–6, 24), there is a high probability that Bcl6b is a conserved regulator of SSC self-renewal among mammalian species.

A more dramatic effect on SSC function from impairing Bcl6b function was observed in vitro than in vivo (in the Bcl6b-null mice). This effect may be due to emphasis of the culture system on SSC self-renewal, whereas this event is rare in the process of spermatogenesis in vivo. These observations may also reflect a greater susceptibility of SSCs than differentiating spermatogonia to impaired Bcl6b function. Molecules having important function in many types of spermatogonia might have a more dramatic effect on male fertility, compared with SSC-specific factors, because the SSC is much less abundant than its differentiating daughters. Moreover, in vivo, other available factors may act as signals to partially replace or compensate for the lack of Bcl6b function.

Gene expression in a SSC-enriched cell population from the rat testis was evaluated by Hamra et al. (25), and Bcl6b was implicated as a possible factor important for SSC function. However, a functional assessment of the role of Bcl6b or relation to GDNF was not investigated. In addition, the effect of overnight GDNF exposure on gene expression in a mouse pup germ cell suspension was investigated by using microarray profiling, but no alteration of bcl6b expression was reported (26), possibly because SSCs were present at a low concentration. The majority (~52%) of the cell population in that study was c-kit-positive when evaluated by immunohistochemistry, and previous research has demonstrated that SSCs are c-kit-negative (27). Similarly, regulation of numb, a Notch signaling antagonist, by GDNF in germ cells was reported. Because Notch signaling influences cell differentiation, it was suggested that GDNF regulates numb expression in SSCs to maintain self-renewal. In contrast, in our SSC populations, no effect of GDNF on numb expression was observed. Although numb is expressed by the SSCs, neither withdrawal nor replacement of GDNF had any effect on its expression level. The absence of GDNF-regulated bcl6b expression or the up-regulation of numb expression in the mixture of c-kit-positive and -negative cells may reflect a dominance of the c-kit-positive cells in the microarray profile (26).

Distinct differences were identified in the expression pattern of genes in SSCs and expression of genes essential for self-renewal and maintenance of pluripotency in other stem cells. Sox2 interaction with Oct4 has been shown to be essential for maintenance of pluripotency in ESCs (17), and both molecules are regarded as vital. Although the transcript for oct4 was expressed by SSCs, its levels were unaffected by either GDNF/GFRα1 withdrawal or replacement, suggesting that Oct4 may not be an essential factor in SSC self-renewal in vitro. In ESCs, interaction between Sox2 and Oct4 controls the transcription of nanog (17), another factor essential for pluripotency (28). In contrast, nanog transcript expression was absent in self-renewing SSCs in our study and in a previous report (29). These data suggest that the Sox2/Oct4/Nanog interaction essential for ESC self-renewal and maintenance of pluripotency is not a conserved mechanism of self-renewal in SSCs. Sox2’s effect on SSC self-renewal must be through another mechanism. Thus, our studies provide evidence that self-renewal in SSCs is regulated by transcription factors that are different from those identified as important for ESCs. Similarly, other adult stem cells, which also might be considered postnatal stem cells, may each use largely unique molecular mechanisms to control self-renewal, perhaps related to subsequent signals used for differentiation of the lineage.

Studying the expression of specific molecules by SSCs in vivo is difficult because of their rarity in the testis. However, examination of the spermatogonia population has implicated the expression of several molecules by both undifferentiated spermatogonia and SSCs. The transcripts for many of these molecules were expressed by SSC-enriched germ cell cultures in this study. Interestingly, gfra1 expression was up-regulated by GDNF, suggesting a feedback pathway for SSC response to GDNF. Previous research has demonstrated that, in vivo, SSCs are c-kit-negative (27). In this study, c-kit expression was questioned from the microarray analysis, and protein expression was not observed by using immunofluorescent staining, confirming a previous study showing that c-kit expression by cultured SSCs is very weak (4). The transcription factor Stra8 has been implicated as a marker for SSCs and has recently been used to isolate putative SSCs from the adult mouse testis (30). The expression of stra8 mRNA could not be detected in any SSC-enriched germ cell culture analyzed in this study by either microarray or RT-PCR. Thus, expression of Stra8 by SSCs or the role it may play in their biological function remains debatable.

The results of our studies provide a rich source of gene expression data for an extremely important adult stem cell population, one responsible for fertility and species continuity. The well-defined culture conditions, the purity of the cell population analyzed, and the transplantation assay to confirm function provide a powerful system to analyze gene expression and mechanisms that regulate stem cell self-renewal. The specific regulation of the transcription factor bcl6b by GDNF, which has been shown to be the primary growth factor necessary for self-renewal of mouse and rat SSCs, identifies an important molecule in self-renewal and provides entrance for unraveling the pathway that controls the ability of SSCs to sustain male fertility.

Methods
Spermatogonial Stem Cell Cultures and Transplantation Analyses. Cultures of self-renewing SSCs were established from day-6 mouse pup donors as described in ref. 4. Donor pup strains used in this study were inbred C57BL/6 mice (C57; The Jackson Laboratory) and hybrid F1, progeny of B6.129S7-Gtrosa26 (designated ROSA;
The Jackson Laboratory. Recipients were busulfan-treated (60 mg/kg of body weight) 129 SvCP (129 × C57 and naturally sterile W^257/W^257 males (W) (2–4). Methods for establishing SSC cultures and transplantation analyses are available in Supporting Materials and Methods, which is published as supporting information on the PNAS web site.

**Microarray Analysis of GDNF-Regulated Genes.** Total RNA was isolated from germ cell clumps deprived of growth factors for 18 hr (n = 3) and after GDNF/GFRα1 replacement for 2, 4, and 8 hr (n = 3 samples per time point). Samples were also collected from clump-forming germ cells not subjected to the 18-hr withdrawal of GDNF/GFRα1 (n = 3). After stimulation, clump cells were removed from STO feeders by gentle pipetting (5), followed by isolation of total RNA. Details of the RNA isolation procedure are available in Supporting Materials and Methods.

Gene expression in SSC samples was measured by using Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA). Each sample was hybridized to an individual microarray chip. Target preparation, hybridization, and scanning were conducted according to the Affymetrix GeneChip Expression technical reference. Target preparation, hybridization, and scanning were conducted according to the Affymetrix GeneChip Expression technical reference. Supportive Materials and Methods.

**Additional Methods.** Detailed descriptions of methods for qRT-PCR analysis of bcl6b expression, functional analysis of Bel6b by siRNA, and evaluation of Bel6b knockout mice are available in Supporting Materials and Methods.

We thank Drs. R. Behringer, E. Sandgren, H. Kubota, and J. A. Schmidt for manuscript review and helpful comments; C. Freeman and R. Naroznowski for assistance with animal maintenance and experimentation; J. Hayden for photography; and P. Bell for histological preparations [produced in the University of Pennsylvania Institute for Human Gene Therapy Morphology Core, funded by National Institutes of Health (NIH) Grant 5-P30-DK-77474-07]. This work was supported by NIH Grants HD044445 (National Institute of Child Health and Human Development) and AG024992 (National Institute on Aging) and by the Robert J. Kleberg, Jr. and Helen C. Kleberg Foundation.