

Identification of Glial Cell Line-Derived Neurotrophic Factor-Regulated Genes Important for Spermatogonial Stem Cell Self-Renewal in the Rat¹

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ABSTRACT

Spermatogonial stem cells (SSCs) provide the foundation for spermatogenesis throughout the life of a male. Because SSCs of many species can colonize the mouse testis, and glial cell line-derived neurotrophic factor (GDNF) is responsible for stimulating SSC self-renewal in rodents, we reasoned that molecular mechanisms of SSC self-renewal are similar across species. GDNF-regulated genes have been identified in mouse SSCs; however, downstream targets of GDNF are unknown in other species. The objective of this work was to identify GDNF-regulated genes in rat SSCs and to define the biological significance of these genes for rat SSC self-renewal. We conducted microarray analysis on cultured rat germ cells enriched for SSCs in the presence and absence of GDNF. Many GDNF-regulated genes were identified, most notably, *Bcl6b* and *Etv5*, which are important for mouse SSC self-renewal. *Bcl6b* was the most highly regulated gene in both the rat and mouse. Additionally, we identified three novel GDNF-regulated genes in rat SSCs: *Bhlhe40*, *Hoxc4*, and *Tec*. Small interfering RNA treatment for *Bcl6b*, *Etv5*, *Bhlhe40*, *Hoxc4*, and *Tec* resulted in a decrease in SSC number, as determined by transplantation, without a change in total cell number within the culture. These data indicate that, like in the mouse SSC, *Bcl6b* and *Etv5* are important for rat SSC self-renewal, suggesting that these genes may be important for SSCs in all mammals. Furthermore, identification of three novel GDNF-regulated genes in the rat SSC extends our knowledge of SSC activity and broadens the foundation for understanding this process in higher species, including humans.

adult stem cells, germline, growth factors, microarray, rat, self-renewal

INTRODUCTION

The spermatogonial stem cell (SSC) is an adult tissue-specific stem cell that provides the foundation for spermatogenesis throughout the lifetime of a male. SSCs are members of a large group of cells termed spermatogonia, reside on the

basement membrane within a cognate niche in the seminiferous epithelium of the testis, and have the capability to self-renew or differentiate into daughter cells committed to become spermatozoa. The population of spermatogonia in the testis is comprised of three types of cells: 1) stem spermatogonia (A_s), the true SSC capable of self-renewal divisions; 2) proliferating spermatogonia (A_{pr} and A_{al}), that undergo mitotic division prior to entering the cycle of the seminiferous epithelium; and 3) differentiating spermatogonia ($A1-4$, B , intermediate), that have entered the cycle of the seminiferous epithelium [1]. Without the ability to undergo self-renewal, the SSC population is rapidly depleted, resulting in infertility. The process of SSC self-renewal is a multifaceted mechanism consisting of cell proliferation, prevention of apoptosis, and maintenance of the SSC phenotype. The regulation of the self-renewal vs. differentiation fate decision (which consists of some of the same mechanisms as self-renewal, such as cell proliferation) is presumed to be a highly organized process directed by factors within the stem cell niche [2]. Currently, very little is known about this important fate decision.

Direct study of SSCs within the stem cell niche has been hampered by the rarity of these cells within the testis and the lack of a definitive marker that can distinguish an SSC from a daughter proliferating spermatogonium committed to differentiation [3]. A further hindrance to study of SSCs has been the lack of methods of assay and maintenance. The development of a transplantation technique, in which labeled donor SSCs are transplanted into the testis of a sterile recipient, resulting in donor-derived spermatogenesis, has allowed for an in situ assay of SSCs [4–6]. This technique is the only definitive method to assay SSC presence and function in a given cell population, because only SSCs are capable of colonizing a recipient testis and maintaining long-term spermatogenesis. We recently developed defined, serum-free, in vitro culture systems that are ideal for studying mechanisms regulating self-renewal of mouse [7–8] and rat SSCs [9]. A drawback to the use of culture exclusively to study the biology of SSCs is that only approximately 1 in 10–15 germ cells in culture is thought to be a true SSC, with the remaining cells considered to be proliferating daughter cells [7]. Therefore, utilization of SSC culture followed by cell transplantation, to assay for the stem cell content of a given culture, is necessary to directly study SSC biology [2].

Several lines of evidence have implicated glial cell line-derived neurotrophic factor (GDNF) as the key regulator of SSC self-renewal. Using GDNF knockout and over-expression mice, Meng et al. [10] demonstrated that spermatogonial proliferation is regulated by GDNF. Additional work utilizing in vivo testis transfection of a GDNF expression vector followed by germ cell transplantation suggested that GDNF was important for SSC function [11]. Advances in the SSC culture system in our laboratory [7] provided definitive proof that mouse SSC self-renewal is dependent on GDNF. Subsequent work by us and others has demonstrated that

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GDNF is also essential for long-term self-renewal of rat and hamster SSCs [9, 12, 13]. Because of this conservation across rodent species, it is likely that GDNF is essential for SSC self-renewal in most, if not all, mammalian species. Furthermore, because of the ability of SSCs from a variety of species to colonize a mouse testis, it is also possible that the mechanism of GDNF-mediated SSC self-renewal is conserved among mammals [14–17].

Recently, we have begun to define the molecular regulatory process of mouse SSC self-renewal using microarray technology. Over the past decade, oligonucleotide microarray methodology has been used to characterize gene expression from whole testis tissue and individual testis cell populations [18–22]. These data sets provide a valuable tool for the discovery of differentially regulated genes across cell populations and treatments. Using microarray analysis of cultured mouse germ cells enriched for SSCs, we identified a subset of GDNF-regulated genes that are important for SSC self-renewal [23]. Furthermore, using kinase-specific inhibitors, we demonstrated that SRC family kinase (SFK) signaling mechanisms likely mediate expression of GDNF-regulated genes [24].

Although we know that GDNF supports the self-renewal of both mouse and rat SSCs, the degree of conservation of the regulation of this process between the two species is unknown. Because rat spermatogenesis occurs in mouse seminiferous tubules, and SSCs of many species are maintained and proliferate in the mouse seminiferous tubule, determining the identity of genes involved in SSC self-renewal in the rat is a critical step in extending our understanding of SSC self-renewal in higher species, including humans. Therefore, identification and subsequent analysis of GDNF-regulated genes was performed with cultured rat spermatogonia enriched for SSCs, which confirmed suspected similarities between mouse and rat SSC self-renewal, and expanded our knowledge of the regulators of this process in mammals.

MATERIALS AND METHODS

Materials and Animals

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO). Donor cells and tissues were isolated from Sprague Dawley wild-type, *Efl-EGFP*, or *Mtl-lacZ* transgenic rats. Immunodeficient NCr nude mice (CrTac:NCr-*Foxn1*tm) were purchased from Taconic Farms Inc. (Hudson, NY). All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Isolation and Culture of SSC-Enriched Testis Cell Fractions

The cell surface marker, EPCAM, is effective in isolating SSC-enriched testis cell populations from the rat [9]. Cultures of EPCAM⁺ clump-forming germ cells contain self-renewing SSCs capable of colonizing recipient testes after long-term culture. However, a majority of the cells within the culture are proliferating spermatogonia [9], and germ cell transplantation must be utilized to assay for the SSC. Cultures of rat EPCAM⁺ clump-forming germ cells were established from cells isolated from rat pups, as previously described [9] with few modifications, which are detailed in Supplemental *Materials and Methods* (supplemental data available online at www.biolreprod.org). Isolation of EPCAM⁺ cells was accomplished by magnetic-activated cell sorting and approximately 200 000 EPCAM⁺ cells were seeded per well of a 12-well culture dish containing 100 000 mitotically inactivated SIM mouse embryo-derived, thioguanine- and ouabain-resistant (STO) feeders. The EPCAM⁺ clump-forming germ cells were maintained in defined serum-free media (rSFM) with GDNF (20 ng/ml; R&D Systems, Minneapolis, MN), Gfra1 (150 ng/ml; R&D Systems), and FGF2 (1 ng/ml; BD Biosciences, San Jose, CA) [9].

Germ Cell Transplantation

The number of donor colonies of spermatogenesis in a recipient testis is a direct reflection of the SSC population in the originally injected cell population [25–27]. All germ cell transplantation experiments were conducted with long-

term (cultured at least 1 mo) cultures of EPCAM⁺ clump-forming germ cells established from 8- or 9-day-old Sprague Dawley rats carrying the *Escherichia coli LacZ* gene under the control of the metallothionein 1a promoter (*Mtl-LacZ*), similar to previous reports [9]. Cells (10 μ l; 1×10^6 /ml) were transplanted into each testis of adult busulphan-treated (50 mg/kg) nude mice. At 2–3 mo after transplantation, testes were removed and analyzed for donor-derived spermatogenesis by 5-bromo-4-chloro-3-indolyl β -d-galactosidase staining. Each experimental unit was transplanted into eight recipient testes, and each treatment was replicated two to three times. Colony number is the number of donor spermatogenic colonies in recipient testes per 10^5 cells originally cultured. Each colony represents the proliferation from a single SSC [25–27].

GDNF Withdrawal and RNA Isolation

Long-term cultures of wild-type Sprague Dawley EPCAM⁺ clump-forming germ cells were utilized for microarray analysis. Cells were isolated from cultures before and after an 18-h GDNF withdrawal, and at 2, 4, and 8 h after GDNF replacement, and frozen in Trizol. To isolate RNA for microarray, a combination of the Trizol method and RNeasy (Qiagen, Valencia, CA) columns was used, as previously described [23]. RNA was isolated using the Trizol method, as directed, for all other assays.

Microarray Processing and Analysis

After RNA isolation and cDNA production, (5 treatments, $n = 3$ /treatment), cDNA products were hybridized to Affymetrix Rat Genome 230 2.0 Arrays by the University of Pennsylvania School of Medicine Microarray Core Facility according to the Affymetrix GeneChip Expression manual (www.affymetrix.com). Microarray analysis was conducted at the Penn Bioinformatics core. The David Bioinformatics Database (<http://david.abcc.ncifcrf.gov>) was utilized for functional annotation clustering.

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was utilized to validate microarray, small interfering RNA (siRNA) knockdown, and in vivo expression of GDNF-regulated genes. After RNA isolation, samples were DNase (Ambion, Austin, TX)-treated to remove genomic DNA and reverse transcribed using oligo(dT) priming and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Primer sequences (Supplemental Table S1) were designed using Primer 3 software [28]. Expression levels of specific genes were assayed using SYBR green (Applied Biosystems, Foster City, CA) and an ABI 7300 sequence detection system (Applied Biosystems). Relative gene expression was determined by normalizing gene-of-interest expression levels to that of *Rps2* using the formula: Relative Expression = $1/2^{(Ct \text{ of the gene-of-interest}) - (Ct \text{ of } Rps2)}$.

Small Interfering RNA Transfection

To demonstrate that identified GDNF-regulated genes had biologic function, siRNA knockdown experiments were conducted on cultures of EPCAM⁺ clump-forming germ cells established from transgenic rats carrying either the *GFP* or *mt-LacZ* gene. Small interfering RNAs were purchased from Ambion (Silencer single nucleotide siRNAs; Austin, TX) or Dharmacon (siGENOME SMARTpool; cocktail-multi nucleotide siRNAs, Chicago, IL) (Supplemental Table S2). All siRNA sequences were validated using BLAST. Negative control siRNA was Ambion Silencer negative control scrambled nontargeting siRNA (product no. 4611). The transfection technique was performed as previously described [23], with few modifications, as detailed in the Supplemental *Materials and Methods*. Germ cells were transfected with gene-specific or negative control siRNA on Day 0 of the experimental timeline and subcultured to siRNA-free media 18 h later. The effect of siRNA knockdown on expression of specific genes was determined using qRT-PCR 18 h after transfection. The SSCs utilized for transplantation experiments were then allowed to self-renew for an additional 7 days. Because the siRNA transfection is transient in nature, cultures transfected with siRNA for genes important for SSC self-renewal would undergo a period of delay in SSC self-renewal, followed by a recovery in self-renewal ability. This delay would result in fewer SSCs per culture when compared with negative control-treated cultures after the 8-day culture period and subsequent transplantation analysis. By transfecting cells with a fluorescently labeled siRNA (Silencer FAM GAPDH siRNA; Ambion), transfection efficiency was determined to be $95 \pm 0.23\%$. Cultures established from rats carrying a *GFP* transgene were used to determine cell number and viability after transfection and cultures derived from rats carrying the *mt-LacZ* transgene were used to determine stem cell potential via the germ cell transplantation technique. Because the SSC concentration

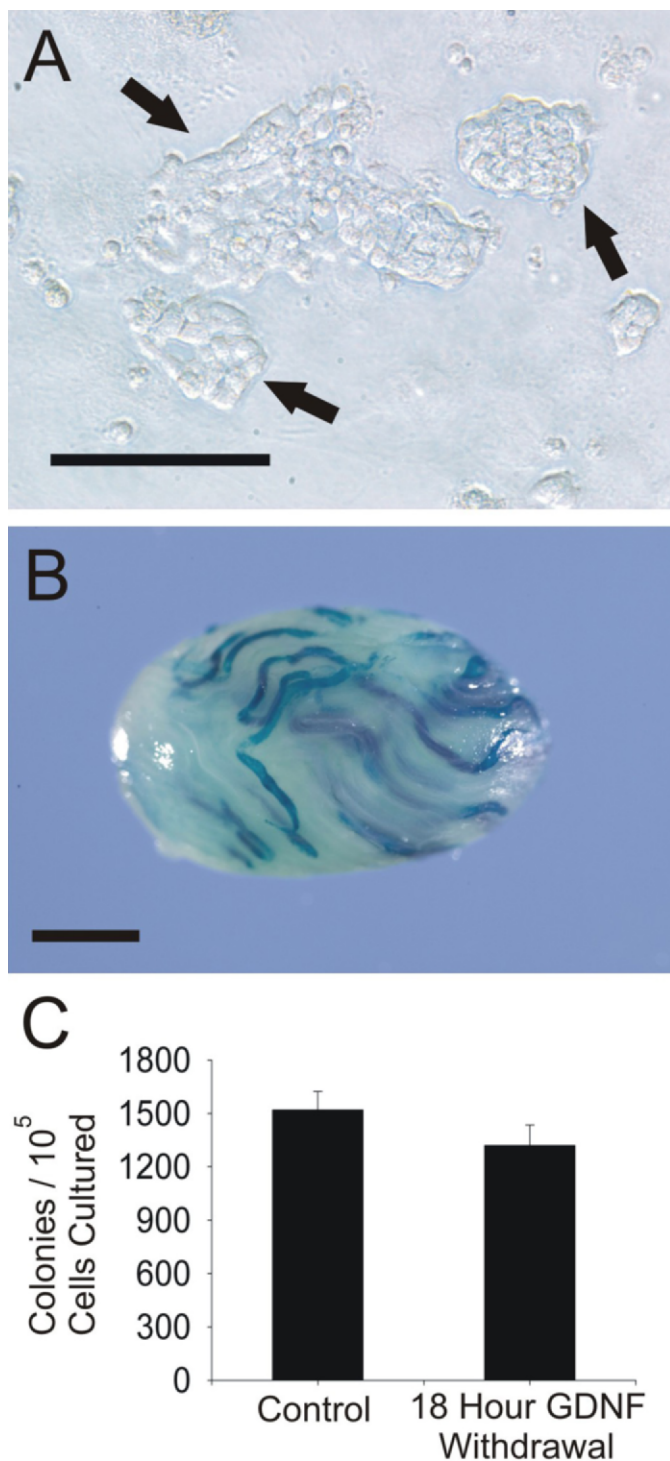


FIG. 1. Effect of GDNF withdrawal on SSC colonization. **A)** Cultures of rat EPCAM⁺ clump-forming germ cells contain both SSCs and proliferating daughter spermatogonia that form tightly connected clumps (arrows) that loosely adhere to the feeder layer. Bar = 100 μ m. **B)** True SSCs and their proliferating daughter spermatogonia are indistinguishable from each other unless assayed for using germ cell transplantation. After transplantation to the testes of busulfan-treated recipient nude mice, cultured rat SSCs form colonies of spermatogenesis that are easily visualized by using transgenic donor cells. In this case, the donor was transgenic for the *mt-LacZ* gene. Bar = 1.5 mm. **C)** Overnight withdrawal (18 h) of GDNF from cultured rat EPCAM⁺ clump-forming germ cells does not significantly influence the population of SSCs within the culture, as indicated by the lack of difference between colony numbers before and after GDNF withdrawal. Error bars represent the SEM.

varies between different primary cultures of EPCAM⁺ clump-forming germ cells, negative control siRNA (Ambion)-treated cultures were always run and analyzed in parallel to each gene-specific siRNA-treated culture, using cells from the same primary culture. By comparing the number of SSCs in negative control siRNA-treated cultures to gene-specific siRNA-treated cultures 8 days after treatment, we were able to directly evaluate the effect of specific gene knockdown on SSC activity.

Flow Cytometric Analysis of siRNA-Treated SSCs

In order to accurately determine the number of donor-derived cells in siRNA-treated cultures of EPCAM⁺ clump-forming germ cells, flow cytometry was used. Single-cell digests of cultured cells were assayed for GFP content using a FACSCalibur flow cytometer (BD Biosciences) on Days 0, 4, and 8 of siRNA treatment. Additionally, the percentage of apoptotic cells was determined on the same cells using a commercial nexin staining kit (Guava Technologies, Hayward, CA).

Immunofluorescence and Immunocytochemistry

Immunofluorescence was used to demonstrate the presence of the proteins for identified GDNF-regulated genes within EPCAM⁺ clump-forming germ cells. In order to confirm that proteins were in fact expressed in vivo, immunocytochemistry was performed on adult testis tissue. Descriptions of these techniques are provided in the Supplemental Materials and Methods.

Statistical Methods

All experiments were run in at least duplicate or triplicate using independently established cell cultures for each replicate. For determination of differences between colony numbers before and after GDNF withdrawal, ANOVA was performed using SPSS 15 (SPSS, Chicago, IL). When comparing data sets, univariate ANOVA was conducted and, if data sets contained multiple points, differences were determined using the least significant difference post hoc test. To determine colony number differences between negative control and siRNA treatments, data were first transformed using a square root transformation. No statistical differences were observed between Dharmacon and Ambion siRNAs; therefore, data from different siRNA companies were pooled. In Figures, error bars indicate the SEM.

Additional Methods

Detailed descriptions of EPCAM⁺ clump-forming germ cell isolation and culture, RNA isolation, microarray processing and analysis, flow cytometry, and immunoblotting of proteins are provided in the Supplemental Materials and Methods.

RESULTS

Identification of GDNF-Regulated Genes in Cultures of EPCAM⁺ Clump-Forming Germ Cells

To identify GDNF-regulated genes in cultures of EPCAM⁺ clump-forming germ cells, which contain the true SSC, GDNF withdrawal was conducted, followed by microarray analysis. In this culture system, SSCs grow within heterogeneous clumps of germ cells containing both the SSC and non-SSC proliferating spermatogonia (Fig. 1A). After transplantation to recipient mice, the true SSCs within the heterogeneous population of germ cells form colonies of complete spermatogenesis (Fig. 1B) [9]. In order for the microarray analysis to assess accurately gene expression in cultured SSCs, it was important to demonstrate that overnight GDNF withdrawal does not change the proportion of SSCs within the culture. Therefore, prior to microarray analysis, cultured *mt-LacZ* transgenic EPCAM⁺ clump-forming germ cells were transplanted into recipient mice before and after an 18-h GDNF withdrawal. No change in the number of colonies in recipient testes 3 mo after transplantation was observed, confirming that overnight short-term withdrawal of GDNF does not change the concentration of SSCs within the culture (Fig. 1C).

Upon confirmation of SSC maintenance following GDNF withdrawal, cultured EPCAM⁺ clump-forming germ cells were collected before and after an 18-h GDNF withdrawal, and at 2, 4, and 8 h after GDNF replacement. Microarray analysis indicated that approximately 21 000 genes are expressed in the cultured EPCAM⁺ clump-forming germ cells (Supplemental Table S3), 7900 of which were GDNF-regulated. However, expression of only 77 genes changed at least 2-fold after both GDNF withdrawal and replacement. The majority of these genes were positively regulated by GDNF, and expression recovered by 4 h after GDNF replacement (Supplemental Fig. S1). Functional clustering revealed that genes up-regulated by GDNF were classified into ion binding, cell cycle, and transcriptional regulation gene families (Supplemental Table S4).

EPCAM⁺ clump-forming germ cells expressed several genes previously reported to be expressed and/or GDNF-regulated in SSCs ([9, 23, 24]; Supplemental Table S5). Both *Bcl6b* (B-cell CLL/lymphoma 6, member B [zinc finger protein]) and *Etv5* (Ets variant 5), which are GDNF-regulated in the mouse SSC [23, 24], were similarly regulated in the rat EPCAM⁺ clump-forming germ cells. Additionally, some genes reported to be involved in SSC self-renewal in the mouse were not GDNF-regulated (*Lhx1* [23, 24], *Zbtb16* [previously known as *Plzf*] [29]), or not expressed in rat EPCAM⁺ clump-forming germ cells (*Nanos3* [30]). The SSC markers, *Epcam*, *Itga6*, *Thy1*, and *Cd9* [9, 31, 32], were expressed by EPCAM⁺ clump-forming germ cells, and, interestingly, *Itga6* and *Epcam* were also positively GDNF-regulated. The GDNF receptors, *Gfral* and *Ret*, were both expressed and negatively regulated by GDNF in rat EPCAM⁺ clump-forming germ cells. Genes that have been suggested to be involved in SSC differentiation were either down-regulated by GDNF (*Kit* [33]), or not expressed (*Neurog3* [34]). EPCAM⁺ clump-forming germ cells also expressed many genes that are important for stem cell function in hematopoietic, neural, and embryonic stem cell populations (Supplemental Table S6). Of particular interest were the expression patterns of the core embryonic stem (ES) cell pluripotency genes *Pou5f1* (previously known as *Oct4*), *Sox2*, and *Nanog*. Both *Nanog* and *Pou5f1* were expressed by the clump-forming germ cells; however, *Sox2* was not. Furthermore, neither *Nanog* nor *Pou5f1* were GDNF-regulated (Supplemental Fig. S2).

Conservation of GDNF-Regulated Genes Between the Mouse and Rat

Several genes (*Bcl6b*, *Egr2*, *Egr3*, *Etv5*, *Lhx1*, and *Tspan8*) were previously identified as GDNF-regulated in cultures of mouse germ cells enriched for SSCs [23]. *Bcl6b*, *Egr2*, *Egr3*, and *Etv5* were also positively GDNF-regulated in rat EPCAM⁺ clump-forming germ cells; however, *Lhx1* was not GDNF-regulated, and *Tspan8* was not expressed (Supplemental Table S7).

Because of the conservation of GDNF regulation of *Bcl6b* and *Etv5*, and the fact that *Bcl6b* was the most highly GDNF-regulated gene in both mouse [23] and rat analyses, we wanted to further characterize the expression of these genes in the rat and confirm their importance for SSC self-renewal in cultures of EPCAM⁺ clump-forming germ cells. The expression patterns for *Bcl6b* and *Etv5* before and after GDNF withdrawal in EPCAM⁺ clump-forming germ cells were validated using qRT-PCR (Table 1 and Supplemental Fig. S3), and the presence of the protein in the germ cell clumps was demonstrated using immunofluorescence (Fig. 2A). Additionally, *Bcl6b* and *Etv5* transcript expression was higher in cell

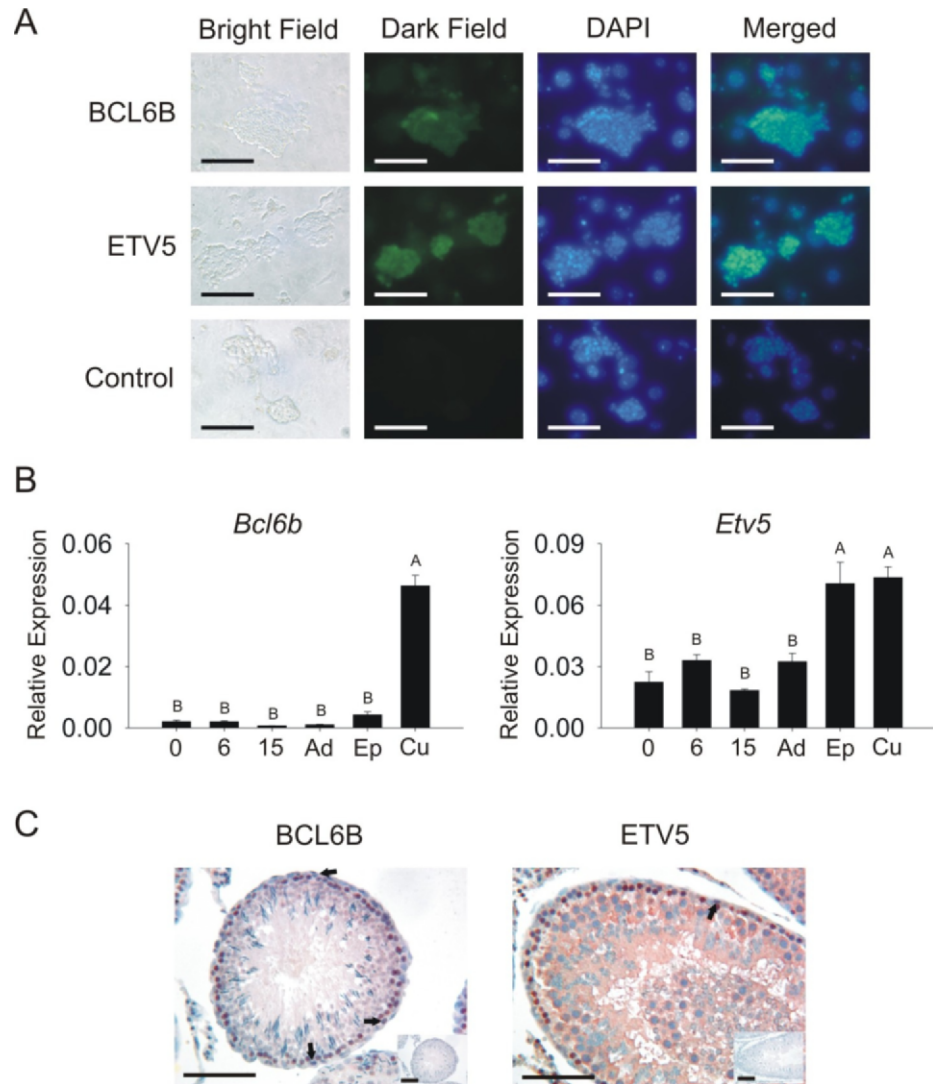
TABLE 1. Known GDNF-regulated genes expressed in mouse SSCs [23] identified as GDNF regulated in cultures of rat EPCAM⁺ clump-forming germ cells enriched for qRT-PCR.

Gene symbol	Gene name	Affymetrix probe set identification	GenBank no.	Raw signal ^a	-18 h ^b	2 h ^b	4 h ^b	8 h ^b
<i>Bcl6b</i> (also known as <i>Bazf</i>)	B-cell CLL/lymphoma 6, member B	1394375_x_at, 1386833_at, 1386832_a_at, 1381804_at	XM_001079767	1170.39	-9.56	2.32	4.03	3.83
<i>Etv5</i> (also known as <i>Erm</i>)	ets variant gene 5	1383447_at	NM_001107082.1	765.94	-1.46	-1.09	1.46	1.42

^a Average of the raw signals of gene expression on the microarray of three replicates of rat EPCAM⁺ clump-forming germ cells prior to GDNF withdrawal.

^b Fold change in gene expression after GDNF withdrawal and replacement: -18 h represents the change in expression after an 18-h GDNF withdrawal compared to before the withdrawal. 2 h, 4 h, and 8 h represent the change in gene expression level after GDNF was replaced for 2, 4, and 8 h compared to the 18-h withdrawal.

FIG. 2. Characterization of *Bcl6b* and *Etv5* transcript and protein expression in vivo and in vitro. **A**) BCL6B and ETV5 were expressed in cultures of EPCAM⁺ clump-forming germ cells. Column 1 = bright field; column 2 = dark field, gene-specific primary antibody (GFP); column 3 = 4',6'-diamidino-2-phenylindole (DAPI); column 4 = merged, overlay of columns 2 and 3. Control immunofluorescence images had rabbit IgG as primary antibody. Bars = 100 μ m. **B**) Quantitative RT-PCR of gene expression in testes from Day 0 (0), Day 6 (6), Day 15 (15), and adult (Ad) testes, fresh EPCAM⁺ cells (Ep), and cultured EPCAM⁺ cells (Cu) indicated that *Bcl6b* and *Etv5* transcripts were present in vivo and enriched in EPCAM⁺ clump-forming germ cells. Bars with different letters are significantly different. Error bars represent the SEM. **C**) BCL6B and ETV5 proteins appeared to stain at low levels in spermatogonia in adult testis tissue (arrows). Staining was more intense in other differentiated germ cells. Insets are representative images of negative control sections in which specific primary antibodies were replaced with IgG controls. Bars = 100 μ m.



populations enriched for SSCs, including fresh and cultured EPCAM⁺ cells, compared with whole testis (Fig. 2B). Spermatogonia along the basement membrane appeared to stain lightly for both BCL6B and ETV5 protein. Some differentiating stages of germ cells stained with greater intensity for these proteins (Fig. 2C).

To unequivocally demonstrate that *Bcl6b* and *Etv5* are required for self-renewal by the true SSC within the cultured clump-forming germ cells, siRNA-specific reduction in gene expression followed by germ cell transplantation was conducted. Cultures of EPCAM⁺ clump-forming germ cells were treated with siRNA for 18 h, cultured for 1 wk, and subsequently transplanted into recipient testes. Treated cultures were maintained for 1 wk prior to transplantation to evaluate potential effects of the treatment on self-renewal compared to negative control-treated cultures. Cultures transfected with siRNA for genes important for SSC self-renewal would undergo a period of delay in SSC self-renewal followed by a recovery in self-renewal ability, resulting in fewer SSCs per culture when compared with negative control-treated cultures after the 8-day culture period and subsequent transplantation analysis. The doubling time for rat SSCs in our culture system is 11 days; however, optimal growth is obtained if cells are subcultured every 8 days. Therefore, because 8 days represents nearly 73% of the self-renewal period, we felt that 8 days was

an adequate period of time to determine the effects of siRNA treatment.

If *Bcl6b* and *Etv5* are important for SSC self-renewal, siRNA treatment should result in fewer colonies of spermatogenesis upon transplantation compared to negative control-treated cultures. Small interfering RNA treatment reduced *Bcl6b* and *Etv5* gene expression by 37.2% and 42.9%, respectively, 18 h after transfection, and expression of the constitutively active gene, *Rps2*, did not change after siRNA treatment, indicating that no significant off-target effects were present (Fig. 3A and Supplemental Table S2). To evaluate changes in germ cell number due to siRNA treatment, cultures of GFP⁺ EPCAM⁺ clump-forming germ cells were analyzed by flow cytometric analysis on Days 4 and 8 after siRNA treatment. A dramatic reduction in cell numbers was observed in both negative control and gene-specific siRNA-treated cultures 4 days after treatment, presumably due to lipofectamine toxicity; however, *Bcl6b* and *Etv5* siRNA treatments did not significantly affect the number of GFP⁺ cells within the culture dish compared to controls at any time point examined (Fig. 3B). Additionally, no significant differences in the percentage of apoptotic cells were observed for *Bcl6b* or *Etv5* siRNA treatment compared to controls (Fig. 3C), indicating that neither *Bcl6b* nor *Etv5* function in apoptosis prevention within the SSC. By evaluating cells at 4 and 8 days,

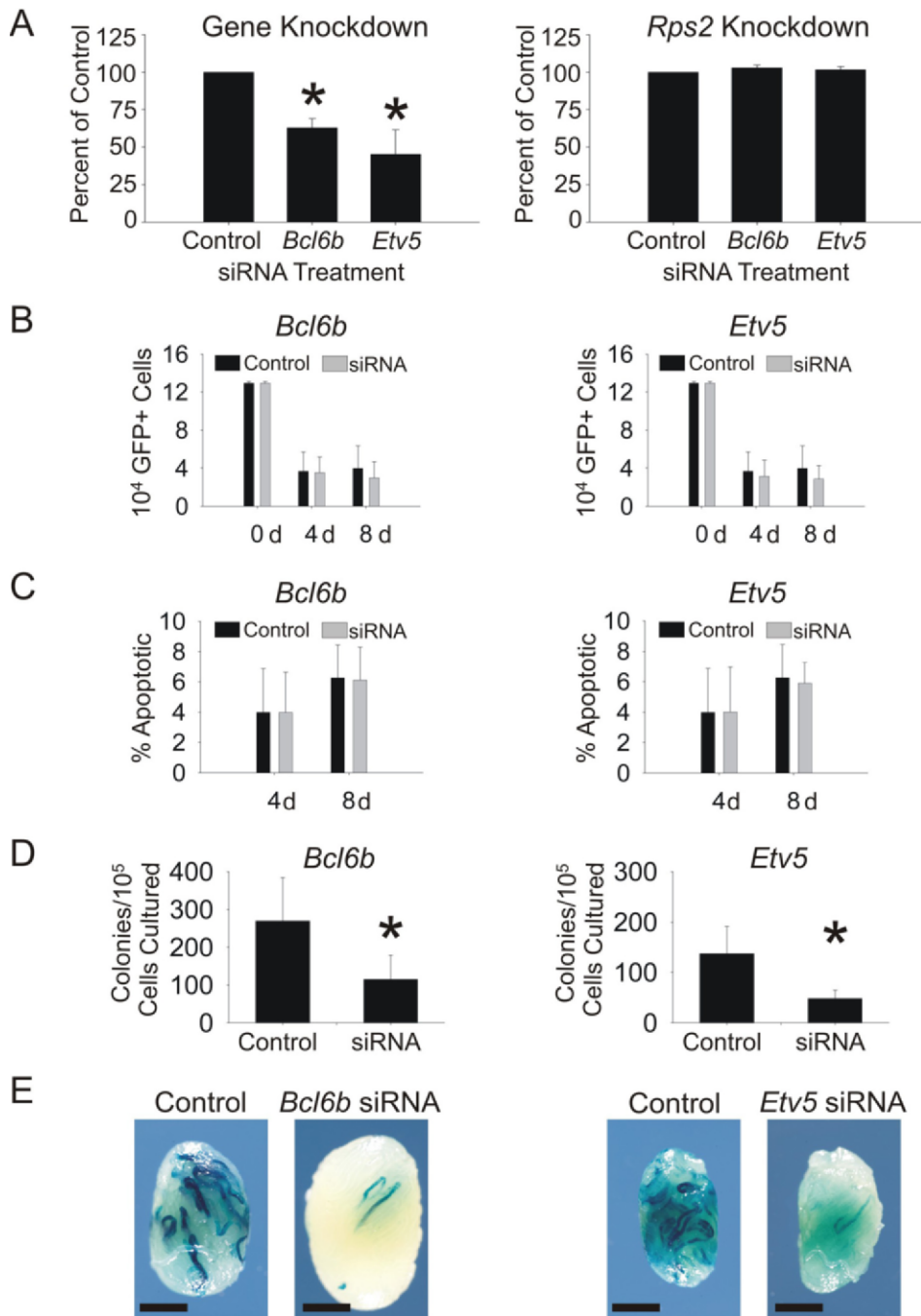


FIG. 3. Effect of *Bcl6b* and *Etv5* siRNA on rat SSC function. **A)** *Bcl6b* and *Etv5* siRNA significantly decreased expression of *Bcl6b* and *Etv5* in cultures of rat EPCAM⁺ clump-forming germ cells 18 h after transfection. Expression of *Rps2* was not changed by either *Bcl6b* or *Etv5* siRNA, indicating that no off-target effects were present. **B)** *Bcl6b* and *Etv5* siRNA did not significantly change the number of donor cells in cultures of Rat GFP⁺ EPCAM⁺ clump-forming germ cells at either 4 or 8 days after transfection. **C)** *Bcl6b* and *Etv5* siRNA did not significantly change the percentage of apoptotic cells (nexin positive) in cultures of Rat GFP⁺ EPCAM⁺ clump-forming germ cells at either 4 or 8 days after transfection. **D)** Both *Bcl6b* and *Etv5* siRNA significantly reduced the number of SSCs in cultures of EPCAM⁺ clump-forming germ cells 8 days after transfection as demonstrated by the SSC transplantation assay. **E)** Photomicrographs of representative testes transplanted with negative control or gene-specific siRNA demonstrate the data depicted in D. Bar = 2 mm. *Statistically significant differences; error bars represent the SEM.

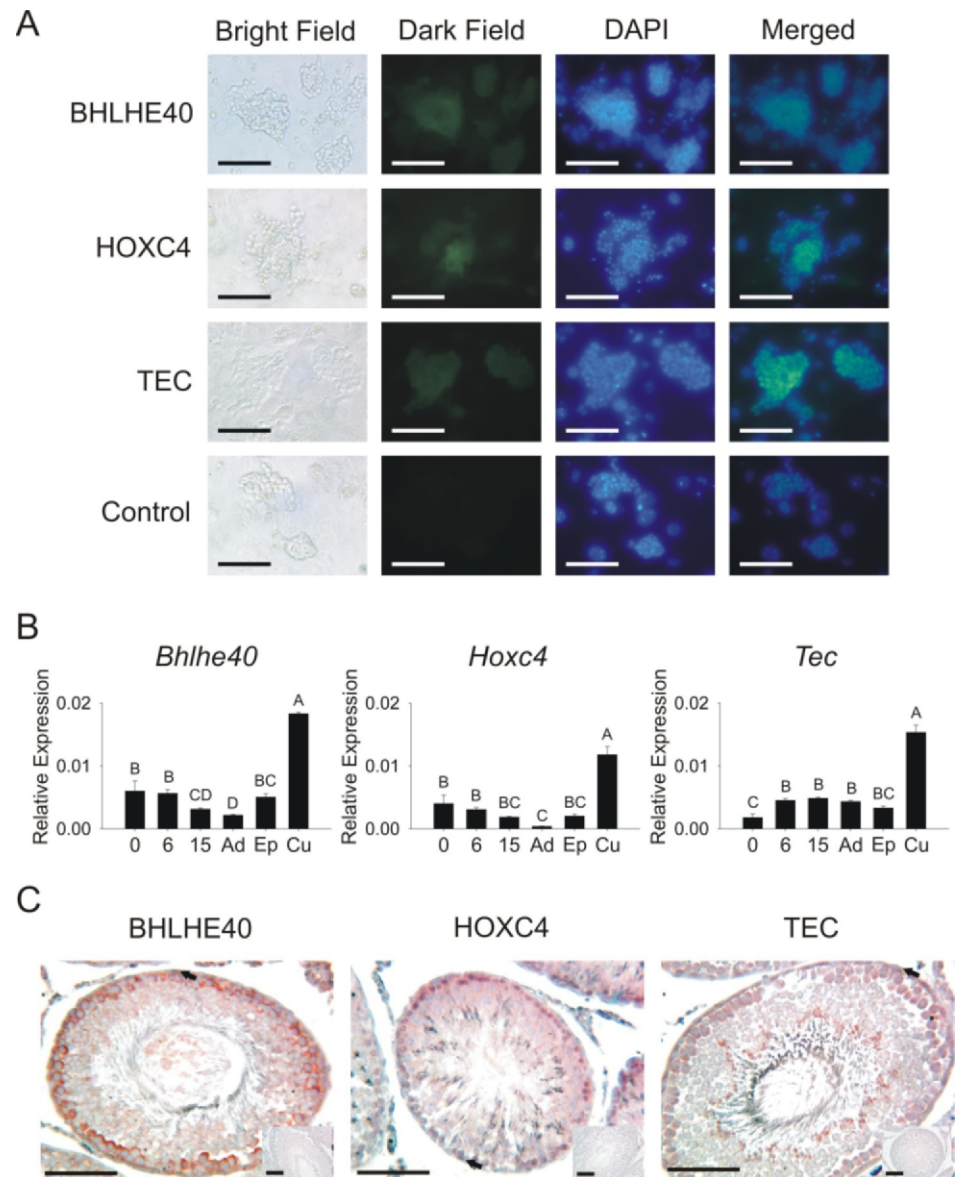
we were able to specifically look at the effects of gene-specific knockdown, rather than lipofectamine, on germ cell apoptosis. The number of spermatogenic colonies arising from transplanted *mt-LacZ*⁺ cultures treated with both *Bcl6b* and *Etv5* siRNA was significantly reduced compared with negative control siRNA-treated cultures (Fig. 3, D and E). Because of the slow-growing nature of the rat EPCAM⁺ clump-forming germ cell cultures, and technical transplant limitations, different primary cultures were used for each gene analysis. The concentration of stem cells is highly variable in cultures of EPCAM⁺ clump-forming germ cells; therefore, differences were observed in the numbers of colonies from negative control-treated cultures. However, because each gene-specific siRNA-treated culture was transplanted in parallel to a negative control-treated culture from the same primary culture, a direct comparison between colonies from specific negative control

siRNA and gene-specific siRNA-treated cultures can be made. These data indicate that *Bcl6b* and *Etv5* siRNA treatments result in a decrease in SSC number without a decrease in number of total cells or an increase in the percent apoptotic cells within the germ cell clumps compared to negative control treatments. Taken together, the data demonstrate that *Bcl6b* and *Etv5* function in SSC self-renewal in maintenance of SSC identity.

Identification of *Bhlhe40*, *Hoxc4*, and *Tec* as *GDNF*-Regulated and Important for Self-Renewal of Rat SSCs In Vitro

To expand our knowledge of the mechanism of *GDNF*-regulated SSC self-renewal, we identified, characterized, and biologically evaluated several genes previously unknown to be

FIG. 4. Characterization of *Bhlhe40*, *Hoxc4*, and *Tec* transcript and protein expression in vivo and in vitro. **A**) BHLHE40, HOXC4, and TEC proteins were expressed in cultures of EPCAM⁺ clump-forming germ cells. Column 1 = bright field; column 2 = dark field, gene-specific primary antibody (GFP); column 3 = DAPI; column 4 = merged, overlay of columns 2 and 3. Control immunofluorescence images had rabbit IgG as primary antibody. Bars = 100 μ m. **B**) Quantitative RT-PCR of gene expression in testes from Day 0 (0), Day 6 (6), Day 15 (15), and adult (Ad) testes, fresh EPCAM⁺ cells (Ep), and cultured EPCAM⁺ cells (Cu) indicated that *Bhlhe40*, *Hoxc4*, and *Tec* transcripts were present in vivo and enriched in EPCAM⁺ clump-forming germ cells. Bars with different letters are significantly different; error bars represent the SEM. **C**) BHLHE40, HOXC4, and TEC proteins appeared to stain at low levels in spermatogonia in adult testis tissue (arrows). Staining was more intense in other differentiated germ cells. Insets are representative images of negative control sections in which specific primary antibodies were replaced with IgG controls. Bars = 100 μ m.



involved in SSC self-renewal. Seven novel GDNF-regulated genes were validated by qRT-PCR (Table 2), and three (*Bhlhe40*, *Hoxc4*, and *Tec*) were selected for biological validation based on their known functions and similar micro-

array and qRT-PCR expression patterns (Supplemental Fig. S3). Immunofluorescence analysis confirmed that basic helix-loop-helix family, member e40 (BHLHE40), homeobox C4 (HOXC4), and tec protein tyrosine kinase (TEC) proteins were

TABLE 2. Novel GDNF-regulated genes identified as GDNF regulated in cultures of rat EPCAM⁺ clump forming germ cells enriched for SSCs that were validated by qRT-PCR.

Gene symbol ^a	Gene name	Affymetrix probe set identification	GenBank no.	Raw signal ^b	-18 h ^c	2 h ^c	4 h ^c	8 h ^c
<i>Bhlhe40</i>	Basic helix-loop-helix family, member e40	1369415_at, 1379483_at	NM_053328	95.52	-3.07	2.70	4.37	3.22
<i>Cd97</i>	CD 97 molecule	1372468_at	NM_001012164	638.15	-2.48	1.05	2.55	2.33
<i>Egr3</i>	Early growth response 3	1392791_at	NM_017086	3503.83	-7.21	9.96	9.97	4.50
<i>Gbp2</i>	Guanylate binding protein 2	1368332_at	NM_133624	89.05	-2.29	2.37	4.31	3.29
<i>Hoxc4</i>	Homeobox C4	1385927_at	XM_001068679	1703.88	-3.71	2.04	2.38	1.91
<i>Ldlr</i>	Low density lipoprotein receptor	1388218_at	NM_175762	92.33	-2.06	2.06	2.49	1.84
<i>Tcfap2c</i>	Transcription factor AP-2, gamma	1383937_at	NM_201420	25.34	-2.06	1.34	3.52	2.57
<i>Tec</i>	tec protein tyrosine kinase	1370916_at	XM_001069141	615.16	-2.27	1.13	2.24	2.26

^a *Bhlhe40*, *Hoxc4*, and *Tec* were chosen for further biological analysis.

^b Average of the raw signals of gene expression on the microarray of three replicates of rat EPCAM⁺ clump forming germ cells prior to GDNF withdrawal.

^c Fold change in gene expression after GDNF withdrawal and replacement: -18 h represents the change in expression after an 18-h GDNF withdrawal compared to before the withdrawal. 2 h, 4 h, and 8 h represent the change in gene expression level after GDNF was replaced for 2, 4, and 8 h compared to the 18-h withdrawal.

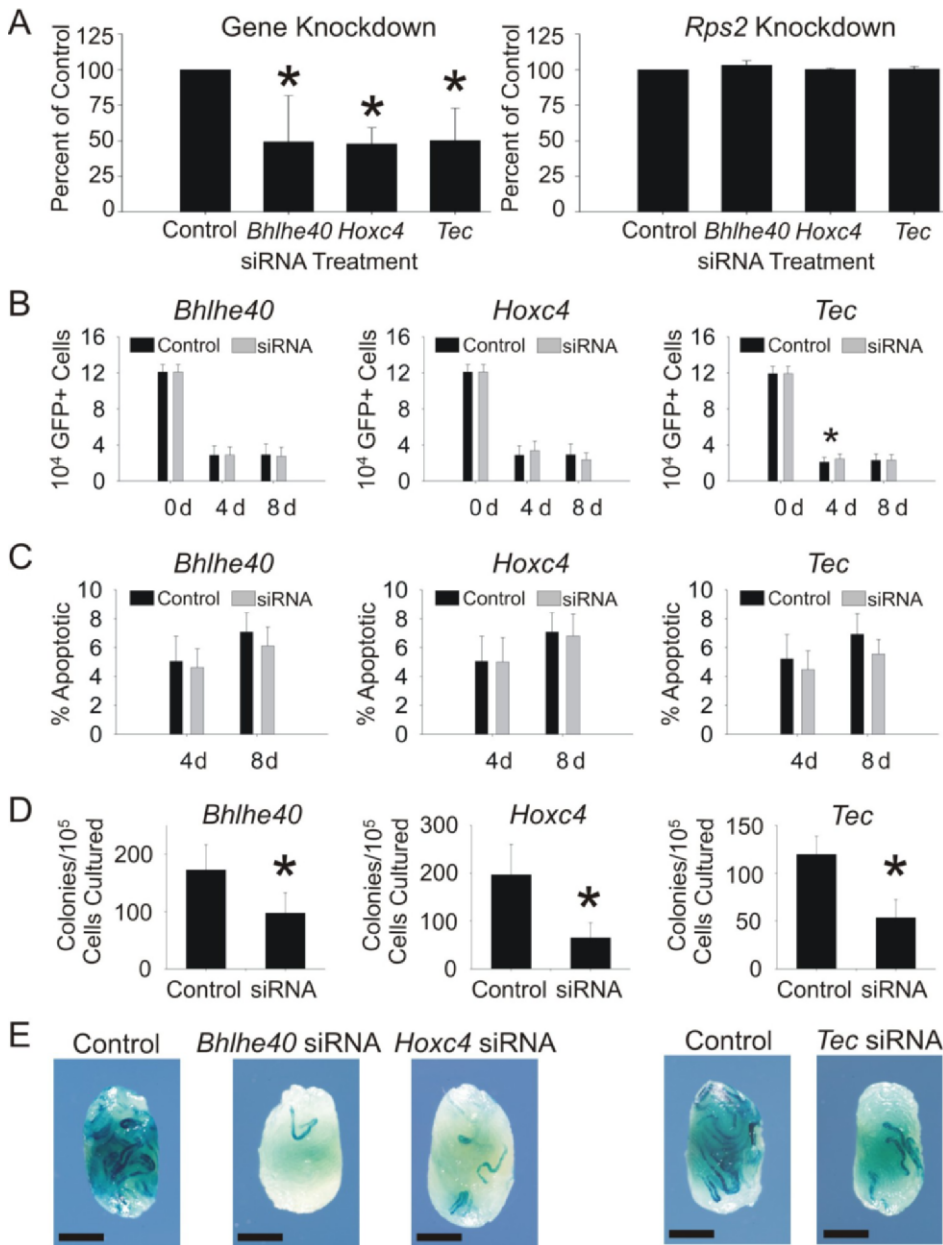


FIG. 5. Effect of *Bhlhe40*, *Hoxc4*, and *Tec* siRNA on rat SSC function. **A**) *Bhlhe40*, *Hoxc4*, and *Tec* siRNA significantly decreased expression of *Bhlhe40*, *Hoxc4*, and *Tec* in cultures of rat EPCAM⁺ clump-forming germ cells 18 h after transfection. Expression of *Rps2* was not changed by *Bhlhe40*, *Hoxc4*, or *Tec* siRNA, indicating that no off-target effects were present. **B**) *Bhlhe40* and *Hoxc4* siRNA did not significantly change the number of donor cells in cultures of Rat GFP⁺ EPCAM⁺ clump-forming germ cells at either 4 or 8 days after transfection. Cultures treated with *Tec* siRNA had more GFP⁺ cells than negative control-treated cultures at 4 days post-siRNA; however, this difference was not present after 8 days. **C**) *Bhlhe40*, *Hoxc4*, and *Tec* siRNA did not significantly change the percentage of apoptotic cells (Nexin Positive) in cultures of Rat GFP⁺ EPCAM⁺ clump-forming germ cells at either 4 or 8 days after transfection. **D**) *Bhlhe40*, *Hoxc4*, and *Tec* siRNA significantly reduced the number of SSCs in cultures of EPCAM⁺ clump-forming germ cells 8 days after transfection as demonstrated by the SSC transplantation assay. **E**) Photomicrographs of representative testes transplanted with negative control or gene-specific siRNA demonstrate the data depicted in **D**. *Bhlhe40*, *Etv5* (Fig. 3), and *Hoxc4* siRNA transplants were conducted at the same time with the same negative control; therefore, the same negative control image was used for all three treatments. Bar = 2 mm. *Statistically significant differences; error bars represent the SEM.

expressed by EPCAM⁺ clump-forming germ cells (Fig. 4A). In vivo transcript expression of *Bhlhe40* and *Hoxc4* was higher in younger testes and cultured germ cells compared with older testes (Fig. 4B). *Tec* transcript expression was highest in cell populations enriched for SSCs; however, little difference in expression was observed between Day 0 and adults (Fig. 4B). Spermatogonia along the basement membrane appeared to stain lightly for HOXC4, TEC, and BHLHE40 protein. Some more advanced stages of germ cells stained with greater intensity (Fig. 4C).

In order to validate the biological significance of *Bhlhe40*, *Hoxc4*, and *Tec*, siRNA treatment followed by germ cell transplantation was conducted, as described above. *Bhlhe40*, *Hoxc4*, and *Tec* siRNA treatments significantly reduced transcript expression in cultures of EPCAM⁺ clump-forming germ cells without reducing *Rps2* expression 18 h after transfection (Fig. 5A). Treatment of EPCAM⁺ clump-forming germ cells with *Bhlhe40* and *Hoxc4* siRNA did not affect the number of total cells within the culture well compared to

controls (Fig. 5B). Interestingly, treatment with *Tec* siRNA resulted in a significant increase in cell numbers 4 days after treatment compared with control (Fig. 5B); however, there was no difference at 8 days. Furthermore, no significant differences in the percentage of apoptotic cells were observed for *Bhlhe40*, *Hoxc4*, or *Tec* siRNA treatment compared to controls (Fig. 5C). Following transplantation, the number of spermatogenic colonies arising from gene-specific siRNA-treated cultures was reduced compared with negative control siRNA-treated cultures for all three genes (Fig. 5, D and E). These data indicate that *Bhlhe40*, *Hoxc4*, and *Tec* are indeed important for rat SSC self-renewal.

DISCUSSION

The rat SSC undergoes complete spermatogenesis within the mouse testis [6], whereas SSCs of more advanced species colonize the mouse testis, but undergo a variable degree of proliferation and little differentiation [14–17]. Additionally,

GDNF has been shown to regulate the proliferation of both rat and mouse SSCs in vitro [7, 9]. Therefore, we reasoned that oligonucleotide microarray analysis of SSCs from selected species, beginning with the mouse and rat, would aid in the overall understanding of mammalian SSC self-renewal. Using this technique, we have begun to elucidate the molecular mechanisms of GDNF-mediated SSC self-renewal in the mouse [23, 24]. The objectives of this work were to identify GDNF-regulated genes in cultures of rat EPCAM⁺ clump-forming germ cells enriched for SSCs and to define the biological significance of a subset of these genes for rat SSC self-renewal.

To evaluate the effect of GDNF on gene expression in SSCs, it was essential to demonstrate that short-term removal of GDNF did not change the proportion of SSCs within the culture. The 18-h GDNF removal did not significantly affect the number of SSCs within the culture, indicating that this time point would be appropriate for microarray profiling of genes directly regulated by GDNF. In contrast to the rat, removal of GDNF from cultures of mouse SSCs resulted in a significant increase in stem cell number within the culture, perhaps due to hypersensitivity of GDNF following withdrawal [23]. The lack of a response in the rat may be due to a slower SSC division rate in cultures of rat germ cells (~11 days [9]) compared with cultures of mouse germ cells (~6 days [7]).

Microarray analysis of GDNF-regulated genes in cultures of rat EPCAM⁺ clump-forming germ cells revealed several thousand GDNF-regulated genes. However, only 77 genes changed 2-fold or greater after both GDNF withdrawal and replacement. Many genes that have previously been implicated as germ cell markers (*Epcam*, *Itga6*, *Thyl*, and *Cd9*; [9, 31, 32]), or important for SSC self-renewal (*Bcl6b*, *Etv5*, and *Zbtb16*; [23, 29]), were also expressed in the rat EPCAM⁺ clump-forming germ cells; however, some genes that have been implicated in SSC function, such as *Nanos3* [30] and *Neurog3* [34], were not expressed. Hamra et al. [21] performed a microarray on laminin-binding (SSC-enriched) vs. laminin-nonbinding rat testis cells, and several genes expressed in their SSC-enriched cell population (*Bcl6b*, *Itga6*, *Egr3*, and *Gfral*) were also expressed by the EPCAM⁺ clump-forming germ cells in our study. In the work by Hamra et al. [21], functional transplantation analyses were used to confirm that the laminin-binding germ cells were indeed enriched for SSCs in order to identify germ cell genes linked to SSC function, including receptor subunits for GDNF and FGF2; however, subsequent functional transplantation analyses were not performed to determine which of the identified transcripts were required for SSC self-renewal. It is quite possible that the non-stem germ cells within our culture system also respond to GDNF withdrawal; thus, it is imperative that the importance of every gene of interest be independently validated using functional transplantation experiments.

Several genes that were identified as GDNF regulated in the mouse SSC were also GDNF regulated in cultures of rat EPCAM⁺ clump-forming germ cells—most notably, *Bcl6b* and *Etv5*. In fact, *Bcl6b* was the most highly regulated gene in both species [23]. Characterization of *Bcl6b* and *Etv5* in vitro confirmed that both transcripts and proteins for these genes are indeed expressed in spermatogonia in cultures of EPCAM⁺ clump-forming germ cells enriched for SSCs. Furthermore, siRNA treatment of EPCAM⁺ clump-forming germ cells, followed by germ cell transplantation, confirmed the importance of both *Bcl6b* and *Etv5* for SSC self-renewal in rats. Additionally, siRNA treatment had no influence on total cell number or apoptosis within the culture. Because siRNA treatments are transient in nature, it is unlikely that the

treatment affected the ability of the SSC to home to the SSC niche. Taken together, the data indicate that these genes most likely function in maintaining the rat SSC phenotype, rather than in preventing apoptosis or stimulation of cell proliferation. In the cultured mouse SSC, *Bcl6b* and *Etv5* siRNA resulted in delayed apoptosis and a decrease in cell numbers in vitro, in addition to a decrease in the total number of SSCs [23, 24]. These discrepancies are most likely due to the intrinsic differences between the culture systems. The proliferation rate of the SSC is much higher in the mouse culture (~6 days) than in the rat culture (~11 days); therefore, SSC inhibition would result in a more dramatic decrease in daughter cells within the mouse culture. Nevertheless, a direct role of these genes in the replication of the proliferating spermatogonia within the mouse, but not the rat, cannot be ruled out. To our knowledge, this is the first evidence that the intracellular mechanisms of SSC self-renewal are conserved between rats and mice, indicating that these mechanisms may be conserved in other mammals.

In an effort to increase our knowledge of GDNF-regulated genes in mammals, we identified several novel GDNF-regulated genes in cultures of rat EPCAM⁺ clump-forming germ cells. Three genes that were identified as GDNF regulated in the rat (*Bhlhe40*, *Hoxc4*, and *Tec*) were further examined for developmental expression, localization, and biological relevance. Proteins for all genes were demonstrated to be expressed in both cultured germ cells and germ cells within the testis. Protein from the identified genes (including *Bcl6b* and *Etv5*) appeared to be stained at low levels in spermatogonia of adult testes, but at higher levels by more differentiated germ cells. Because of the lack of a definitive marker for the SSC, it is impossible to demonstrate unequivocally, using histological techniques, that these proteins are indeed expressed by the SSC. However, it is possible to demonstrate functional significance of the genes, and thus their presence in SSCs, using siRNA treatments followed by quantitative transplantation experiments. Using siRNA knockdown of gene expression in cultured EPCAM⁺ clump-forming germ cells, followed by germ cell transplantation, we demonstrated that each gene evaluated was indeed important for SSC self-renewal. Because the siRNA transfection was transient in nature, cultures transfected with siRNA for genes important for SSC self-renewal would undergo a period of delay in SSC self-renewal, followed by a recovery in self-renewal ability. This delay resulted in fewer SSCs per culture when compared with negative control-treated cultures after the 8-day culture period and subsequent transplantation analysis. Differences in colony numbers are not due to differences in ability to colonize, but are due directly to a different number of SSCs within the siRNA-treated culture compared with the negative control. *Bhlhe40*, *Hoxc4*, and *Tec* were not identified as GDNF regulated in our previous mouse experiments [23]; however, array hybridizations for these genes were not adequate to determine significant differences. Thus, it is possible that *Bhlhe40*, *Hoxc4*, and *Tec* are also GDNF regulated in the mouse SSC, and further experimentation will clarify their roles. It is of note that *Bcl6b*, *Etv5*, *Bhlhe40*, *Hoxc4*, and *Tec* siRNA treatments all resulted in a decrease in SSC number within the culture dish without altering total cell number or the percent apoptotic cells. Because of this result, and the fact that all of these genes were highly regulated by GDNF, it is likely that they all function within the SSC to maintain the phenotype of the SSC, thus perpetuating the self-renewal fate decision rather than differentiation.

SSC self-renewal is a multifaceted process consisting of mechanisms such as cell proliferation, prevention of apoptosis,

and maintenance of SSC phenotype. It is possible that changes in expression of evaluated genes may result in changes in expression of cell surface markers necessary for translocation of the SSC to the SSC niche after transplantation. Because the only current method to assay for the SSC is germ cell transplantation, we must assume that any change in colonization due to treatment (compared to the control treatment) is a direct downstream effect of altering one of the mechanisms that make up the process of SSC self-renewal. Based on the fact that *Bcl6b*, *Etv5*, *Bhlhe40*, *Hoxc4*, and *Tec* siRNA treatments did not alter cell proliferation or apoptosis, it is thus likely that these molecules are important for maintenance of the SSC phenotype. Furthermore, because the germ cells within the culture are either SSCs or their differentiating daughter cells, a change in the number of SSCs without a change in the number of differentiating daughters indicates that the ratio of SSC to differentiating germ cell in the culture has changed, and thus a shift in the ratio of self-renewal to differentiation. However, due to limitations in the rat SSC culture system and transplantation, more detailed experiments must be conducted to elucidate the exact role of these genes in the process of SSC self-renewal. Additionally, even though Lipofectamine reduced cell numbers, the effect was the same for negative control and gene specific siRNA treated cultures, therefore we are confident that the results from the siRNA experiments do in fact reflect an importance of these genes for efficient SSC self-renewal, presumably by maintaining the SSC phenotype.

Biological roles for *Bcl6b* and *Etv5* have been explored using mouse knockout models [23, 35, 36]. Unfortunately, generation of knockout rats is challenging, and specific functional roles for these genes in rat SSC self-renewal cannot be confirmed in vivo. *Bcl6b* is a POZ family transcription factor, and experiments with mouse SSCs indicate that BCL6B is important for maintenance of the undifferentiated state, apoptosis, or proliferation of SSCs [23]. The fact that *Bcl6b* siRNA results in a decrease in proliferation of rat SSCs without directly affecting the proliferating daughter cells in the rat SSC culture indicates that, in the rat, *Bcl6b* is most likely important for maintenance of the undifferentiated state or influences proliferation of SSCs, but not for more differentiated germ cells. *Etv5* is a transcription factor that has been clearly demonstrated to be necessary for mouse SSC self-renewal [23, 24, 36]. Additionally, a role of *Etv5* in SSC niche function has also been proposed [35]. *Bhlhe40* is a basic helix-loop-helix transcription factor that, in the mouse, is expressed in activated CD34⁺ T cells. Similar to *Bcl6b*, which has a role in T-cell proliferation [37], loss of *Bhlhe40* in the mouse results in spontaneous activation of B and T cells, and it has been proposed that *Bhlhe40* is important for the maintenance of inactivated immune cells [38]. In the testis, *Bhlhe40* may have a similar function in maintaining the undifferentiated state of SSCs. *Hoxc4* is a transcription factor that is important for embryonic patterning of the thoracic vertebra and proliferation of cells in the esophageal musculature [39]. The mechanistic role of *Hoxc4* in SSCs is unclear, but results of the current study indicate that it is important for SSC self-renewal in the rat. *Tec* is a cytoplasmic protein tyrosine kinase that has been implicated in hematopoiesis, primarily in the development of the peripheral B-cell pool [40]. SFKs are important for GDNF signal transduction in the mouse SSC [24], and the structure of TEC is similar to that of SFKs [40]. It is possible that TEC kinase functions in the rat SSC similar to SFKs in the mouse SSC by mediating the GDNF signal. Further exploration of the function of these genes in SSCs will lead to a better understanding of their roles in GDNF-mediated SSC self-renewal.

To better understand the mechanism of pluripotency, we examined the levels of *Sox2*, *Nanog*, and *Pou5f1* in the cultures of rat EPCAM⁺ germ cells. Both *Nanog* and *Pou5f1* were expressed by the cultured cells; however, *Sox2* was not. This result is in contrast to cultures of Thy1⁺ mouse germ cells [23] in which *Sox2* and *Pou5f1* were expressed and *Nanog* was not. In ES cells, *Nanog* expression and its apparent role in maintaining self-renewal and pluripotency is regulated by the continued action of *Pou5f1* and *Sox2*. Lack of *Sox2* expression by cultures of rat EPCAM⁺ germ cells, combined with the observation that GDNF does not regulate *Pou5f1*, indicates that self-renewal of ES cells and rat SSCs are regulated in different manners.

Previously, we identified three factors (*Bcl6b*, *Etv5*, and *Lhx1*) that are GDNF regulated and important for SSC self-renewal through SFK signaling mechanisms in the mouse [23, 24]. The present work demonstrates that the function of *Bcl6b* and *Etv5* are likely similar between the mouse and the rat. In fact, *Bcl6b* was the most highly GDNF-regulated gene in both the rat and mouse SSC. These data indicate that GDNF-regulated SSC self-renewal is at least partially conserved between these two species. Furthermore, we have identified and biologically validated three more factors (*Bhlhe40*, *Hoxc4*, and *Tec*) that are GDNF regulated and important for SSC self-renewal in the rat, which further extends our knowledge of the mechanism of SSC self-renewal in mammals (Supplemental Fig. S4). The dramatic regulation of *Bcl6b* in both the mouse and rat, as well as the GDNF regulation of *Etv5*, suggests other exciting areas for continued investigation of SSC regulation. Using these two genes as an entry point should allow for discoveries about the downstream regulation of SSC self-renewal in the mouse and rat. This knowledge is extremely valuable for the continued development of SSC applications in more advancing species, including livestock and humans.

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