Dramatic Expansion of Germinal Stem Cells by Ectopically Expressed Human Glial Cell Line-Derived Neurotrophic Factor in Mouse Sertoli Cells

Kentaro Yomogida, Yo Yagura, Yuko Tadokoro, and Yoshitake Nishimune

Department of Science for Laboratory Animal Experimentation, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan

ABSTRACT

Although the mammalian germinal stem cell (GSC) provides a good model to investigate the regulation of stem cells, the small number of these cells currently available hampers elucidation of the regulatory mechanism. Here, we show the dramatic amplification of GSCs in mouse testis following transfection of human glial cell line-derived neurotrophic factor cDNA into Sertoli cells using an efficient, in vivo electroporation technique. Transplantation analysis demonstrated not only GSC enrichment but also differentiation from stem cells into sperm. The GSC population, as estimated using a colony-formation assay, was approximately 20-fold greater than in cryptorchid testis, or approximately 500- to 1000-fold greater than in normal adult testis. This system should provide sufficient quantities of GSCs to accelerate our understanding of GSC properties, regulation mechanisms, and behavior control.

INTRODUCTION

The regulation of stem cell self-renewal and differentiation is a hot topic in developmental biology and medicine [1–4]. The mammalian germinal stem cell (GSC) system provides a good model for investigating the regulation of stem cells because of the availability of germ cell transplantation analyses and of many well-characterized mutant mice, morphological identification, and differentiation markers [3, 5–6]. However, the small size of the GSC population prevents us from elucidating mechanisms as in other stem cell systems [7–9]. Previously, the best source for GSCs was the testis of the cryptorchid mouse, which provides 25- to 50-fold more GSCs than normal testis [9].

Glia cell line-derived neurotrophic factor (GDNF) was recently identified as a candidate regulator of GSC proliferation and differentiation [10–12]. The GDNF is distantly related to the transforming growth factor β superfamily and promotes the proliferation and differentiation of several types of neurons in the nervous system [13–15]. In mammalian testis, the supporting Sertoli cells, which provide the microenvironment for GSCs, secrete GDNF, and the receptor complex composed of Ret receptor tyrosine kinase and a glycosylphosphatidylinositol-linked coreceptor, GFR-α1, is expressed on undifferentiated spermatogonia, including the cell population with GSC activity [6, 10]. If the level of GDNF expression is too low in heterogenous GDNF knockout mice, the number of undifferentiated spermatogonia is reduced [10]. In contrast, if the level is too high in transgenic mice in which constitutively expressed human (h) GDNF cDNA is introduced, active proliferation of undifferentiated spermatogonia-like cells occurs [10]. However, amplified, morphologically undifferentiated spermatogonia in the hGDNF transgenic mouse were not normal GSCs; rather, they were transplantable, spermatogonia-like cells that had no capacity for spermatogenesis and were fated to promote testicular tumors [10–12]. Here, we show GDNF-regulated proliferation of GSCs and demonstrate normal spermatogenesis from amplified GSCs.

We have improved an in vivo electroporation both as a new functional assay for gene products and as a promoter assay of Sertoli cell-specific genes in vivo that can be used without producing transgenic animals [16]. Under our conditions, transgenes specifically introduced into Sertoli cells are stably expressed for a long time. To determine the role of GDNF in mammalian spermatogenesis and to increase the GSC population, we applied the in vivo electroporation technique in the present study.

MATERIALS AND METHODS

Animals

C57BL/6 male mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan) at 12 days or 2 mo of age. Enhanced green fluorescence protein (EGFP) transgenic mice were raised in our animal facility [17]. To obtain recipient mice for germ cell transplantation, 2-mo-old C57BL/6 male mice were injected intraperitoneally with 40 mg/kg of busulfan. The busulfan-treated mice were used as recipients 4 wk after injection. All the animal experiments conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation (Research Institute for Microbial Diseases, Osaka University).

In Vivo Electroporation

For in vivo electroporation, we used three circular-form expression vectors: pCXN-EGFP [18], an hGDNF expression vector [10], and a vector without inserted cDNA as a control. For injection, all DNA was dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) at a concentration of 1 mg/ml with 0.04% trypan blue. The mice were anesthetized, and the testes were exposed under a dissecting microscope. A glass micropipette was inserted into the rete testis for injection into each testis. At least 80% of the seminiferous tubules were filled with DNA solution, as was easily verified with the colored dye, and were charged with an electric pulse generator (Electroporator CUY-21; Tokiwa-Science, Fukuoka, Japan) using a pair of tweezer-type electrodes (CUY650; Tokiwa-Science, Fukuoka, Japan).
FIG. 1. The expression and effects of transgenes. Expression vectors, EGFP, or hGDNF in circular form were transfected into the testes of 12-day-old mice and observed after 4 wk. The transgenes stably expressed the transcripts, as shown by Northern blot analysis (A), and the proteins, as shown by Western blot analysis (B), in the testes. A low-magnification fluorescence stereomicroscopy image (C) of transfected testes 4 wk after electroporation is also shown, as are cross-sections (D) of transfected testes under UV light (upper) followed by counterstaining with hematoxylin (lower). Transfected Sertoli cells were detected as fluorescence-positive cells. Clusters (arrows) and alignments (arrowheads) of undifferentiated spermatogonia and a few tubules with no fluorescent cells supporting normal spermatogenesis (asterisk) were identified in testes transfected with hGDNF cDNA. Bar = 1 mm (C) and 100 μm (D).

Northern Blot Analysis

Four weeks after electroporation, the testes were sampled together with untreated control testes. Total RNA was isolated from each testis with a Trizol reagent (Invitrogen, Carlsbad, CA), and aliquots (15 μg) were electrophoresed on a 1% agarose/formaldehyde gel. After transfer to a Zeta-Blotting Membrane (Bio-Rad Laboratories, Hercules, CA), 32P-labeled EGFP cDNA and hGDNF cDNA probes were hybridized with the membrane. This experiment was repeated three times with two mice each, and the results were reproducible.

Western Blot Analysis

Each testis was homogenized in lysis buffer (10 mM Tris-HCl [pH 7.4], 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA, and 5 mM PMSE), and 100 μg of the protein were loaded in each lane. After 15% SDS-polyacrylamide gel electrophoresis, Western blot analysis was done on Immobilon Transfer Membranes (Millipore, Billerica, MA). The GDNF was detected with chicken polyclonal antibody to hGDNF (Promega, Madison, WI), followed by horseradish peroxidase (HRP)-conjugated antibody (Promega) and NADH (Wako, Osaka, Japan). This analysis was performed three times with two mice each, and the results were reproducible.

Fluorescent Stereomicroscopic Observation under Ultraviolet Light

Transfected or transplanted testes were observed using a fluorescence stereomicroscope with ultraviolet (UV) light excitation and photographed with a Leica DC200 (Leica Microscopy System Ltd., Wetzlar, Germany) attached to the stereomicroscope.

Histological Analysis of EGFP-Transfected Cells

Four weeks after electroporation, the testes were fixed with 4% paraformaldehyde, embedded in glycol methacrylate, and cut into sections (thickness, 5 μm). Testes with EGFP-labeled cells were observed using fluorescence stereomicroscopy under UV light. After the EGFP fluorescence had been photographed, the same section was stained with hematoxylin and observed under a photomicroscope.

Immunohistochemistry

The hGDNF-transfected testes were fixed in Bouin solution (six testes from three mice) or 4% paraformaldehyde (eight testes from four mice) and embedded in paraffin or methyl methacrylate (MMA) resin. The sections (thickness, 5 μm) were removed from the paraffin or MMA resin and incubated with TRA98 rat monoclonal antibody (1:500) [19], anti-GFR-α antibody (1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-rat antibody (1:50; Santa Cruz Biotechnology), and anti-Santa Cruz Biotechnology) and anti-proliferating cell nuclear antigen (PCNA) antibody (1:400; Santa Cruz Biotechnology) for 8 or 48 h at 4°C. For ACK2 [20] staining, the four testes were covered with OTC compound (Tissue-Tec; Sakura, Tokyo, Japan), quickly frozen, and sectioned (thickness, 7 μm) in a cryostat. The frozen sections were incubated with ACK2 (1:50) for 24 h at 4°C. Signals were detected with HRP-conjugated anti-rat immunoglobulin G or an avidin/biotin-conjugated HRP complex system (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) with dianaminobenzidine or streptavidin, Alexa Fluor 568 conjugate (Molecular Probes, Eugene, OR).

Germ Cell Transplantation Assay

Donor cells for transplantation were prepared from four hGDNF-transfected testes (two mice) 4 wk after electroporation. After decapsulation, the seminiferous tubules were placed in Dulbecco modified Eagle medium (DMEM) buffered with 20 mM Hepes at pH 7.3 containing collagenase type I (1 mg/ml) and hyaluronidase (1 mg/ml) and then incubated for 15 min at 37°C with agitation at 5-min intervals. The seminiferous tubules were washed twice in PBS and then incubated in PBS containing 0.25% trypsin for 15 min at 37°C with manual agitation at 5-min intervals. After adding a half-volume of DMEM containing 10% fetal bovine serum, the cell suspension was filtered through a nylon mesh (pore size, 30 μm). The collected cell pellet was resuspended in injection medium (138 mM NaCl, 8.1 mM Na2HPO4, 2.7 mM KCl, 1.1 mM KH2PO4, 0.1 mM EDTA, 5.5 mM glucose, 5 mg/ml of bovine serum, 100 μg/ml of DNase I, and 0.4 mg/ml of trypan blue) at a concentration of 106 cells/ml. To transplant the germ cells, we injected them into recipient testes (n = 10; five mice) in the same way that we injected the DNA. Ten weeks after transplantation, we sampled the testes for observation by fluorescence stereomicroscopy and histological analysis of EGFP-labeled cells, as described above.

Colony-Formation Assay

Experimental cryptorchidism of EGFP transgenic mice was performed 2 mo after birth, as described previously [6]. Two months after the operation, the artificially induced cryptorchid testes were electroporated with hGDNF cDNA, as described above. After 4 wk, the testicular cells were prepared, and the total cell number recovered per testis was counted. For transplantation, each cell suspension was resuspended in injection medium at a concentration of 106 cells/ml, and 106 cells (10 μl) were injected into a recipient testis. Eight weeks after transplantation, the testes were sampled for observation by fluorescence stereomicroscopy. To count green fluorescent colonies, we decapsulated the testes and observed the unraveled seminiferous tubules in transfected testis under a fluorescence stereomicroscope. Quantitative data were statistically analyzed using an unpaired Student t-test in Excel software (Microsoft, Redmond, WA).
RESULTS

Direct Effects of Transfected hGDNF in Mouse Sertoli Cells on Spermatogenesis

To clarify the direct effects of GDNF on undifferentiated spermatogonia, we transfected hGDNF expression vector with an EGFP reporter gene into the Sertoli cells of 12-day-old C57BL/6 mouse testes using an in vivo electroporation technique [16]. Four weeks after electroporation, we detected abundant expression of transgenes in Northern (Fig. 1A) and Western (Fig. 1B) blot analyses. Efficient transfection of cDNA into Sertoli cells in the testis was easily detected by EGFP fluorescence under UV light (30 mice, 30 pairs of testes analyzed) (Fig. 1C). The EGFP fluorescence was also used to detect hGDNF cotransfection. In cross-sections of testes electroporated solely with the EGFP reporter gene as a control, fluorescence-positive Sertoli cells with normal spermatogenesis were observed (30 mice, 30 one-side testes analyzed) (Fig. 1D, left). In contrast, we identified many undifferentiated spermatogonial cell clusters in association with fluorescent Sertoli cells in all the testes transfected with GDNF cDNA (30 mice, 30 other-side testes analyzed). The cell clusters piled up in the lumen of seminiferous tubules, and some alignments of type A spermatogonia were observed along the tubule walls near the fluorescence-positive Sertoli cells (Fig. 1D, right). Spermatogenesis was interrupted in various parts of the tubules (Figs. 1D and 2A). Because a few seminiferous tubules with no fluorescent Sertoli cells performed normal spermatogenesis (Fig. 1D, right asterisk), electroporated hGDNF must be a causal factor in the disruption of germ cell differentiation, as suggested previously [10, 12].

To confirm the hGDNF-induced expansion of the undifferentiated spermatogonial population in seminiferous tubules, we verified the expression of GDNF receptors, GFR-α1 and Ret, on the cell surface of proliferated, undifferentiated spermatogonia [6]. All clustered cells and a string of spermatogonia along the tubule wall expressed both receptors (Fig. 2, B and C) along with the PCNA (Fig. 2D).
The proliferating cells were morphologically classified as type A spermatogonia (Fig. 2A). Indeed, all the cells in clusters were recognized by the germ cell-specific monoclonal antibody TRA98 [19] (Fig. 3A) and showed the surface c-kit receptor-negative stem cell phenotype [5, 6] (Fig. 3B). Although the microscopic appearance of testicular cross-sections was similar to that of testicular cross-sections of GDNF transgenic mice [10] in which spermatogonia-like cells were propagated autonomously [11], the clustered spermatogonia in the present study were quite different. They were thought to be normal spermatogonia stimulated to proliferate by paracrine secretion of transfected hGDNF into the Sertoli cells. To confirm this, EGFP-labeled spermatogonia were prepared from the testes of EGFP transgenic mice electroporated with hGDNF. These germ cells are easily transplanted into the seminiferous tubules of busulfan-treated mouse testes, and their fate can be tracked by fluorescence [17]. Ten weeks after transplantation, GSC proliferation was detected by the fluorescence of donor green germ cells in the seminiferous tubules of the testis (Fig. 3C), and complete spermatogenesis of green germ cells was observed (Fig. 3, D and E).

**Estimate of GSC Increase**

To estimate the increase in the GSC population, we performed semiquantitative colony-formation assays of proliferating spermatogonia derived from the cryptorchid testes of EGFP transgenic mice electroporated with hGDNF. Many more GSCs are found in the cryptorchid testis than in the normal adult mouse testis [9], because only undifferentiated spermatogonia can survive the heat shock stress (Fig. 4A) [6]. Four weeks after electroporation of hGDNF expression vector, we observed many green cell clusters in the tubules of electroporated cryptorchid testes (Fig. 4B). The recovery of testicular cells, consisting mainly of spermatogonia with a few somatic cells, was approximately 3-fold greater from hGDNF-electroporated cryptorchid testes than from controls (P < 0.01) (Table 1). Eight weeks after transplantation of these green germ cells, the recipient testes were observed under a fluorescence stereomicroscope (Fig. 4, C and D). We counted the green colonies as fluorescence-positive lesions in whole-mount, unraveled seminiferous tubules. Although the length of fluorescence-positive seminiferous tubules was similar in both green cell transplantation experiments, the numbers differed markedly. Stem cell activity in testicular cells transplanted into seminiferous tubules, as estimated by colony formation and spermatogenesis, was approximately 6.6-fold higher than in control cryptorchid testis (P < 0.001) (Fig. 4, C and D, and Table 1). These results indicate that GSCs increased approximately 20-fold following transfection of hGDNF into cryptorchid testis.

**DISCUSSION**

It has been demonstrated that hGDNF is a candidate GSC-propagating factor [6, 10]. Although spermatogonia-like cells proliferated in the hGDNF transgenic mouse, they had no ability to produce mature sperm and were fated to promote seminomatous tumors [11]. To obtain sufficient numbers of mouse GSCs, we introduced hGDNF expression vector into Sertoli cells specifically using an in vivo electroporation technique [16]. As expected, the ectopic expression of hGDNF expanded the normal GSC population in the mouse testis. Our semiquantitative colony-formation assay indicated that the GSC population in the cryptorchid testis was expanded approximately 20-fold with the transfection of hGDNF cDNA into Sertoli cells (Table 1). Because the GSC population in the cryptorchid testis is 25- to 50-fold higher than in normal testis [9], the expanded population was approximately 500- to 1000-fold that in the normal testis. This expansion method should provide sufficient numbers of GSCs for study.

Our observations clearly demonstrated that GSC proliferation prevented differentiation; the extraordinary stimulation of proliferation by transfected GDNF had a detrimental effect on GSC differentiation (Figs. 1D, 2A, and 3B). This effect would be an advantage for enriching the GSC population in testicular cells, not only from cryptorchid testes (Fig. 3) but also from transfected normal testes, without producing artificial cryptorchidism surgically (Figs. 1 and 3). The supporting Sertoli cells play an important role in regulating GSC proliferation and differentiation via the FSH/FSH-receptor/GDNF pathway [6]. Homeostatically controlled endogenous GDNF secreted by supporting Sertoli cells might be required to maintain GSC behavior in a microenvironment (stem cell niche).

In GSC studies, as in all stem cell biology, the lack of a suitable culture system prevents a more thorough investigation of GSC properties. The GSC enrichment method presented here could easily supply a sufficient population of GSCs in adult testis, with a short preparation period. Using a cell sorter system and germ cell transplantation

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**TABLE 1. Colony-formation assay of testicular cells prepared from green mice transplanted into recipient testes.**

<table>
<thead>
<tr>
<th>Source</th>
<th>No. tested&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of recovered testicular cells per testis (&lt;×10&lt;sup&gt;4&lt;/sup&gt;)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. of recipient testes observed</th>
<th>No. of colonies&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Calculated recovery of colony-forming cells per testis</th>
<th>Expansion of GSC population&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryptorchid</td>
<td>12</td>
<td>165 ± 62 (1.00)</td>
<td>12</td>
<td>2.83 ± 6.52 (1.0)</td>
<td>467</td>
<td>1.00</td>
</tr>
<tr>
<td>Cryptorchid electroporated with hGDNF&lt;sup&gt;e&lt;/sup&gt;</td>
<td>8</td>
<td>489 ± 279 (2.96)</td>
<td>8</td>
<td>18.75 ± 6.52 (6.6)</td>
<td>9169</td>
<td>19.63</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of donor testes prepared.

<sup>b</sup> Value = mean ± SD; ratio in parentheses.

<sup>c</sup> Total number of fluorescent colonies with spermatogenesis observed in whole-mount, seminiferous tubules isolated from a recipient testis transplanted with 10<sup>4</sup> cells (mean ± SD); stem cell activity normalized to cryptorchid control is in parentheses.

<sup>d</sup> Relative ratio to control cryptorchid testes.

<sup>e</sup> Testicular cells were prepared 4 wk after electroporation.
techniques, detailed characterization of testicular stem cell populations expanded using GDNF should be possible. Application of this method will also accelerate our understanding of the supporting mechanisms of somatic cells, like Sertoli cells, in maintaining the GSC microenvironment [8, 9, 21]. Easier handling of purified adult GSCs will facilitate the analysis of regulatory factors and the search for factors that maintain stem cell activity in adult tissue, which will help us to establish a culture system for mammalian GSCs. Furthermore, in combination with the efficient retroviral transduction of male GSCs [22, 23], the unlimited amplification of normal GSCs presented here may help us to develop new technologies to generate transgenic or gene knockout mice. It will also provide a useful model system for analyzing the mechanisms involved in infertility caused by genetic or environmental factors or drug toxicity.

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REFERENCES