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Immortalization of Mouse Germ Line Stem Cells

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Abstract

In the mammalian testis, the germ line stem cells are a small subpopulation of type A spermatogonia that proliferate and ultimately differentiate into sperm under the control of both endocrine and paracrine factors. To study the early phases of spermatogenesis at the molecular level, an in vitro system must be devised whereby germ line stem cells can be either cultured for a prolonged period of time or expanded as cell lines. In the study reported here, we chose to immortalize type A spermatogonia using the Simian virus large T-antigen gene (*LTag*) under the control of an ecdysone-inducible promoter. While the cells escaped the hormonal control after a finite number of generations and expressed the *LTag* constitutively, their growth remained slow and the cells exhibited morphological features typical of spermatogonia at the light microscopic level. Moreover, the cells expressed detectable levels of protein markers specific for germ cells such as *Dazl*, and specific for germ line stem cells such as Oct-4, a transcription factor, and *GFR α -1*, the receptor for glial cell line-derived neurotrophic factor (GDNF). Further analysis confirmed the spermatogonial phenotype and also revealed the expression of markers expressed in stem cells such as *Piwi12* and *Prme11*. Since the cells respond to GDNF by a marked increase in their rate of proliferation, this cell line represents a good in vitro model for studying aspects of mouse germ line stem cell biology.

Keywords

Type A spermatogonia; SV40; Large T antigen; Testis Germ line stem cell; *GFR α -1*; Glial cell line-derived neurotrophic factor (GDNF)

Introduction

Stem cells are unique cell populations that are able to undergo both self-renewal and differentiation and are found in the embryo, as well as in the adult animal. In the early mammalian embryo, pluripotent embryonic stem cells are derived from the blastocyst stage and have the ability to form any fully differentiated cell of the body. As the embryo develops, stem cells become restricted in their ability to form different lineages (multipotent stem cells). Multipotent stem cells are also found in a wide variety of adult tissues such as bone marrow and brain. However, in the adult animal, the ability of certain stem cells to differentiate can be restricted to only one cell lineage (unipotent stem cells). Examples of mammalian unipotent stem cells include the stem cells residing in the gut epithelium, the skin, and the seminiferous epithelium of the testis.

A widely accepted model for spermatogonial development has been originally proposed by Huckins and Oakberg [1–3]. In this scheme, the A_{single} (A_s) spermatogonia are the putative stem cells. They can self-renew or differentiate into A_{paired} (A_{pr}) spermatogonia that remain connected by an intercellular bridge. The A_{pr} spermatogonia further divide to form chains of 4, 8, or 16 A_{aligned} (A_{al}) spermatogonia. Further, the A_{al} cells will differentiate into type A_1 spermatogonia. The A_1 spermatogonia resume division to form A_2 to A_4 spermatogonia. Next, A_4 cells divide to form intermediate (In) spermatogonia, and In spermatogonia divide to produce type B spermatogonia. Finally, type B spermatogonia divide to form primary spermatocytes that will enter meiosis. Spermatogonial stem cells and their progeny are contained in the basal part of the germinal epithelium, in contact with the basement membrane, and they are in close association with the somatic Sertoli cells [4]. Regulatory mechanisms mediated by growth factors produced by the Sertoli cells induce or inhibit the proliferation, differentiation, and further development of the germ cells [5, 6].

Spermatogonial stem cells can generate spermatogenesis when transplanted into the seminiferous tubules of an infertile male [7, 8]. Currently, there is a limited understanding of the molecular mechanisms that control the development of these cells into mature sperm. Spermatogonial stem cells exhibit a distinct phenotype such as the high expression of β -1 and α -6 integrins [9–11] and specific light-scattering properties [12]. While the stem cell identity of the A_s spermatogonia has not yet been rigorously demonstrated, their morphology and location in the seminiferous epithelium make them good candidates for being stem cells [3]. A_s spermatogonia express $GFR\alpha$ -1, the receptor for glial cell line–derived neurotrophic factor (GDNF) [13, 14]. As the A_s differentiate into A_{pr} and A_{al} spermatogonia, they start expressing the surface receptor c-kit, which binds to stem cell growth factor (SCF) produced by Sertoli cells [15–17].

The ability to isolate, culture, and manipulate the germ line stem cell in vitro would allow us to unravel the molecular mechanisms that drive the first steps of spermatogenesis and to characterize the signaling pathways that induce spermatogonial differentiation versus self-renewal. In turn, this could help us understand the origin of certain testicular neoplasias and the causes of male infertility. To look at these issues, an in vitro system in which these cells could be maintained in long-term cultures would be ideal. In the study reported here, we attempted to establish a mouse spermatogonial stem cell line using the large T antigen under the control of an inducible promoter. The resulting immortalized cells express detectable levels of protein and RNA specific for A_s and A_{pr} spermatogonia such as the $GFR\alpha$ -1 membrane receptor. Further analysis revealed the expression of additional markers accounting for a stem cell phenotype such as the genes *oct-4*, *piwi12*, and *prame11* that have a role in stem cell maintenance and renewal in the germ line and other tissues.

Materials and Methods

Construction of pIND-LTAg

The plasmid pIND-LTAg was constructed from the pIND vector (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>), which contains the *neo^r* gene and an ecdysone-inducible promoter upstream of the multiple cloning site [18]. Ponasterone A, an analog of the *Drosophila* hormone ecdysone (Invitrogen), served as the inducer. pIND-LTAg was derived from the plasmid pSV3-neo (American Type Culture Collection [ATCC] no. 37150) and pIND by excising the 3.3 kb LTAg sequence out of pSV3-neo and ligating it into the *Bam*HI site of pIND. To check if the pIND-LTAg was functional, the plasmid was cotransfected into MDA-231 breast carcinoma cells with the plasmid pVgRXR (Invitrogen) that codes for the ecdysone receptor and the *zeocin^r* gene.

Cell Isolation, Transfection, and Subcloning

Animal investigations were conducted according to the NCR (National Research Council, National Academy Press) Guide for Care and Use of Laboratory Animals. Type A spermatogonia were isolated from 6-day-old Balb/c mice testes using the STAPUT method that utilizes gravity sedimentation on a 2%–4% bovine serum albumin (BSA) gradient [19]. Briefly, the testes from 60 pups were decapsulated and minced. Leydig cells and peritubular myoid cells were eliminated by a two-step enzymatic digestion using collagenase (1 mg/ml), hyaluronidase (1.5 mg/ml), and trypsin (1 mg/ml). The remaining cell suspension, containing mainly germ cells and Sertoli cells, was subjected to gravity sedimentation for 2.5 hours on a 2%–4% BSA gradient to separate the germ cells from the Sertoli cells. Cells were collected using a fraction collector. After STAPUT separation, the fractions containing only type A spermatogonia were pooled and plated for 2 hours on fetal calf serum (FCS)–coated plates to eliminate possible remaining Sertoli cells by adherence. Six-day-old mice were chosen since at this age only A_s, A_{pr}, and some A_{al} spermatogonia are found in the seminiferous epithelium. This isolation method allows us to isolate populations of type A spermatogonia with a purity exceeding 95%. Cotransfection with the pIND-LTAG plasmid and the pVgRXXR plasmid was performed with Lipofectin (Invitrogen), and the transfected cells were selected with the antibiotics zeocin (100 µg/ml) and G418 (100 µg/ml).

Cell Lines and Tissues

Several cell lines were tested in this study: the putative germ cell line C18-4, the Sertoli cell line 15P1 [20], the Sertoli cell line SF7 [21], and the NIH 3T3 fibroblast cell line (ATCC no. CRL-1658). The cell lines were grown in Dulbecco's modified Eagle's medium containing 1 mM sodium pyruvate, 50 U/ml penicillin-streptomycin, 100 mM nonessential amino acids, and 2 mM L-glutamine (Atlanta Biologicals, Norcross, GA, <http://www.atlantabio.com>) with 5% FCS (Atlanta Biologicals). The Sertoli cell lines and NIH 3T3 cells were used as negative controls. In addition, freshly isolated Sertoli cells, whole testis, and brain and kidney extracts were tested as positive and negative controls.

Immunocytochemistry

Cells were grown on FCS-coated coverslips until 80% confluency, then fixed with ice-cold methanol for 5 minutes. Reactions were performed according to standard protocols using the immunoperoxidase techniques. The antibodies used were a goat anti-mouse GFR α -1 and a goat anti-mouse c-kit from Santa Cruz Biotechnology (Santa Cruz, CA, <http://www.scbt.com>). We also used a rat anti-c-kit antibody (ACK45) from Pharmingen (San Diego, <http://www.bdbiosciences.com/pharmingen>), a goat anti-mouse Ret antibody from R&D Systems (Minneapolis, <http://www.rndsystems.com>), a goat anti-Oct-4 antibody from Santa Cruz Biotechnology, a rabbit anti-Oct-4 from Active Motif (Carlsbad, CA, <http://www.activemotif.com>), and two rabbit polyclonal anti-Dazl antisera [22].

Reverse Transcriptase Polymerase Chain Reaction

Total RNA was collected from the C18-4 cell line, 6-day-old mouse testis, the SF7 Sertoli cell line, and freshly isolated mouse Sertoli cells using TriReagent according to the manufacturer's protocol (Molecular Research Center, Cincinnati, <http://www.mrcgene.com>). Total RNA samples were treated with 1 unit per 1 µg RNA of RQ1 RNase-free DNase (Promega Corp., Madison, WI, <http://promega.com>) to degrade any genomic DNA present. cDNA was synthesized from 5 µg of total RNA using SuperScript II reverse transcriptase (RT) and oligo(dT) primers to selectively reverse transcribe mRNA (Invitrogen). Two µl of the cDNA obtained from each sample was amplified for 35 cycles (denaturation at 94°C for 30 seconds, annealing at specific temperatures for 45 seconds, and elongation at 72°C for 45 seconds) using primers and conditions already described by others: *c-kit* [23]; *LDH-C4*:

GenBank no. AF190799; α -actin [24]; and 3β HSD [25]. As a positive control and to check for the presence of contaminating genomic DNA, polymerase chain reaction (PCR) primers for *cyclophilin*, a housekeeping gene, were also used (GenBank no. 10090). As negative controls, PCR was performed on RT reaction products obtained without the use of RT.

The sequences of the primer pairs used for PCR amplification of each cDNA were devised by Wang et al. [26]. As a positive control, *Gapd*, a housekeeping gene, was used. As negative controls, and for each sample, PCR was performed on RT reaction products obtained without the use of RT. Each gene was tested in triplicate, using three different RNA preparations per cell line and tissue.

Immunoprecipitation and Western Blotting

Cells were cultivated in cell culture media completed as described above. The media were removed, and the cells were washed three times with ice-cold phosphate-buffered saline. Cells were disrupted with ice-cold immunoprecipitation buffer containing 150 mM NaCl, 15% v/v of mammalian protease inhibitors (Sigma-Aldrich, St. Louis, <http://www.sigma-aldrich.com>), and phenylmethyl sulfonyl fluoride at a final concentration of 1.0 μ g/ml (Sigma-Aldrich). Proteins were recovered according to standard procedures, and their concentration was measured with the Bio-Rad DC assay (Bio-Rad Laboratories, Hercules, CA, <http://www.bio-rad.com>).

The GFR α -1 protein was immunoprecipitated using 10 μ l of primary antibody (goat anti-mouse, Santa Cruz Biotechnology) and protein A agarose using standard methods. As positive controls, we used extracts of testis, brain, and kidney. As negative controls, we used protein extracts from the Sertoli cell line 15P1 and NIH3T3 fibroblasts. The concentrated proteins were run on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to a nitrocellulose membrane. The membrane was then probed with a 1:500 dilution of biotinylated rabbit anti-mouse GFR α -1 antibody (Pharmingen), followed by incubation with a 1:500 dilution of streptavidin-peroxidase (Vector Laboratories, Burlingame, CA, <http://www.vectorlabs.com>). Peroxidase activity was revealed with a solution containing 0.5 μ g/ml 4-chloronaphthol, 0.015% hydrogen peroxide, and 16.67% methanol in Tris-buffered saline at pH 7.5.

The Dazl and GDNF proteins were immunoprecipitated and probed using the same method. The antisera Dazl no. 149 and Dazl no. 150, kindly provided by Dr. Reijo-Pera [22], were used for the immunoprecipitation of Dazl and for the probing of the membrane after transfer. Similarly, a rabbit anti-mouse GDNF antibody (Santa Cruz Biotechnology) was used for the immunoprecipitation and probing of GDNF.

The Oct-4 protein was immunoprecipitated with a goat anti-Oct-4 antibody (Santa Cruz Biotechnology) and revealed on the nitrocellulose membrane with a rabbit anti-Oct-4 antibody (Active Motif).

Growth Curves

The C18-4 cells were seeded at 10,000 cells per well in 24-well plates with complete cell culture media and 10% FCS. Experimental wells also contained 100 ng/ml recombinant rat GDNF or 100 ng/ml SCF (R&D Systems). Each day for the next 6 days, three of the experimental wells and three of the control wells were trypsinized and the cells were counted using trypan blue exclusion. The cell numbers are presented as mean $\pm$ standard deviation.

Transient Transfections

Transient transfections of the line C18-4 with the expression vector pTracer containing the green fluorescent protein (GFP; Invitrogen) were performed using the calcium phosphate method [27]. At 3–5 days after transfection, the cells were observed in situ using an inverted phase contrast/fluorescence microscope, and expression of GFP was visualized using an excitation wavelength of 490 nm.

Results

Cell immortalization

In our immortalization strategy, the ecdysone receptor from *Drosophila* was expressed from a vector called pVgRxR [18]. The ecdysone-responsive promoter, which drives the expression of the large T-antigen gene, is on a second vector called pIND-LTA_g. Both vectors were stably cotransfected into freshly isolated type A spermatogonia that were induced to express the LTA_g when treated with Ponasterone A, an analog of ecdysone. While the cells escaped the hormonal control after a finite number of generations and expressed the LTA_g constitutively, their growth remained slow, with a doubling time of about 3 days. After several rounds of subcloning by limiting dilution, a clone was obtained that we called C18-4. The cells appeared round to polygonal, with a round nucleus, and a large nucleus-to-cytoplasm ratio in phase contrast microscopy (Fig. 1).

Immunocytochemistry

The purified C18-4 cells were stained by immunocytochemistry for the presence of the germ cell-specific Dazl protein using antisera nos. 149 and 150 [22]. Figure 2 shows that Dazl is expressed in the nucleus when it is stained with antiserum no. 149 (Fig. 2A), and it is expressed in the nucleus and the cytoplasm when it is stained with anti-serum no. 150 (Fig. 2C). The cells were also positive for GFR α -1, the coreceptor of Ret, and a marker for germ line stem cells (Fig. 3A). Further, they stained positive for the Ret transmembrane receptor, a marker for spermatogonia and spermatocytes (Fig. 3C). The cell nuclei, in particular the nucleoli, stained positive for Oct-4, an early marker for the germ line (Fig. 3E). The expression of the c-kit receptor could not be revealed using immunocytochemistry, but it was detected by RT-PCR in early passages only (Fig. 4).

Immunoprecipitation

The expression of GFR α -1 and Dazl in the C18-4 cell line was confirmed by immunoprecipitation (Fig. 5). To increase the specificity of detection, GFR α -1 was immunoprecipitated using a polyclonal antibody made in goat, then revealed after blotting using a polyclonal antibody made in rabbit. In Figure 5A, lane E, we show that the C18-4 cell line produces a protein exhibiting a molecular weight of 42 kDa that corresponds to the molecular weight of GFR α -1. As positive controls, we used protein extracts from developing brain, kidney, and testis (lanes B–D, respectively). As negative controls, we used NIH 3T3 cells and the Sertoli cell line 15P1 (lanes F and G) [20].

The Dazl protein was immunoprecipitated and revealed after blotting using antiserum no. 149 [22]. As seen in Figure 5B, a strong band with a molecular weight of approximately 33 kDa was revealed in the testis extract, corresponding to the molecular weight of Dazl [22]. The same band was observed using C18-4 extracts (Fig. 5B). Similarly, the Oct-4 protein was revealed after immunoprecipitation (Fig. 5C).

Finally, immunoprecipitation failed to detect the expression of GDNF in the purified cells, indicating that the C18-4 cells are not contaminated by Sertoli cells.

Reverse Transcriptase Polymerase Chain Reaction

Neither β HSD, a marker for Leydig cells, nor α -actin, a marker for peritubular myoid cells, could be found by RT-PCR, further indicating that the cells are of germ cell origin. The absence of LDHC4 expression indicated that the cells are not spermatocytes.

To further confirm the spermatogonial origin of the C18-4 cell line, we investigated by RT-PCR the expression of 36 spermatogonial-specific genes, which were previously identified by Wang and colleagues [26] in the 6-day-old mouse testis. There was a RT-PCR product for the housekeeping gene *Gapd* in both cell lines and in the neonatal testis samples, indicating that the RNA isolated contained intact transcripts (data not shown). RT-PCR further revealed that all 36 spermatogonial-specific genes tested were expressed in the 6-day-old testis, as expected. None of these genes were expressed in the SF7 Sertoli cell line, confirming the specificity of the transcripts. The expression of 10 spermatogonial-specific genes was detected in the C18-4 cell line. The results of these 10 are reported in Table 1.

One of the genes expressed by the C18-4 cells was the *Ott* gene (Fig. 6A), which is transcribed in vivo in the germ cells of testis and ovary [28]. The primers used in this study can detect the presence of two bands by RT-PCR, corresponding to two isoforms, with sizes of 551 and 503 bp, respectively [28]. As shown in Figure 6A, two bands of the correct size are expressed by the C18-4 cells (lane B). However, only one band (551 bp) was detectable in the neonatal testis sample (lane D). Sertoli cells do not express the *Ott* gene (lane F). Figure 6B shows that the C18-4 cell line also expresses *Sycp1*, a gene coding for a protein of the synaptonemal complex, which is already expressed in spermatogonia [29]. The band obtained is of 311 bp, as expected (lane B). The same band is visible for the 6-day-old testis sample (lane D), but not in Sertoli cells (lane F). Figure 6C represents the expression of the gene *Fth117* that codes for a testis-specific ferritin-binding protein [26]. *Fth117* is expressed in the C18-4 cells, giving the expected band of 292 bp (lane B) by RT-PCR. *Fth117* expression is found in the 6-day-old testis but is not found in Sertoli cells (lanes D and F, respectively). In addition to spermatogonial-specific genes with a known function, the C18-4 cell line expressed five novel genes that belong to the *Tex* (testis-expressed) gene family (Fig. 6D) [26]. None of those genes were expressed by the Sertoli cell line.

Interestingly, the C18-4 spermatogonial cell line also shows the expression of the *piwi12* (*piwi-like 2*) gene (Fig. 6E). *Piwi* belongs to a family of genes that is involved in stem cell maintenance and renewal in *Drosophila* [30]. Two mouse homologues of *piwi* have recently been cloned: *miwi* (mouse *piwi*) and *mili* (*miwi-like*) [31–33]. The primers used in this study are derived from the *Drosophila* sequence and give a band of 241 bp. As expected, *Piwi12* was also detected in the neonatal testis but not in Sertoli cells. Additionally, the C18-4 cells express a *PRAME*-like gene (Fig. 6F). *PRAME* is a protein expressed in the testis, as well as in several carcinomas, in acute myeloid leukemias, and in CD34⁺ hematopoietic stem cells [34–37].

Influence of GDNF and SCF on the Behavior of the C18-4 Cells

To determine if the C18-4 cell line responds to growth factors, we cultivated the cells in the presence or absence of 100 ng/ml GDNF in the culture medium. When GDNF is present, the rate of proliferation of the cells increases significantly ($p < .005$) (Fig. 7A). No influence on cell morphology or expression of the c-kit receptor was observed. Interestingly, the growth effect of GDNF is detected only when the culture medium also contains 10% FCS. No change in the rate of proliferation can be detected when FCS is replaced by a defined serum (Nu serum), which contains a minimal amount of cytokines. Further, addition of SCF to the culture medium did not stimulate the proliferation of the cells (Fig. 7B). SCF did not induce the cells to differentiate since RT-PCR analysis did not reveal the expression of LDHC4, a

germ cell-specific isozyme that is detected at the earliest in preleptotene spermatocytes [38, 39].

Discussion

One of the major problems encountered when working with type A spermatogonia and germ line stem cells is the limited number of cells available. As a consequence, little is known about the biology of these cells and the cellular and molecular pathways underlying the first steps of spermatogenesis. We have now established a cell line from the 6-day-old mouse testis, which shows morphological features of type A spermatogonia at the light microscopic level and expresses germ line stem cell-specific genes. To ascertain that this cell line is of germ cell origin, we investigated the expression of the proteins Dazl and Ret by immunocytochemistry and immunoprecipitation.

In the mouse, *Dazl* is an autosomally located gene that has a predominant role in spermatogenesis and oogenesis [40–42]. Previous studies have revealed that, in the mouse testis, the protein is predominantly found in the nucleus and the cytoplasm of fetal gonocytes. In the adult animal, the protein is mainly present in the nucleus of spermatogonia [41]. Ret is a transmembrane receptor activated through the binding of its coreceptor, GFR α -1, to GDNF [43]. Ret is expressed by all premeiotic germ cells in the testis, including pachytene spermatocytes [44]. We show here that the established cell line expresses both Dazl and Ret proteins, indicating that the cells are of germ cell origin. Further, the pattern of expression of Dazl (in the nucleus only with antiserum 149, and in both the nucleus and the cytoplasm with antiserum 150) indicates that the cells derive from early spermatogonia. In the cytoplasm, Dazl formed dot-like structures as previously described by Ruggiu et al. [42, 45], which could be due in part to oligomerization of the protein with itself. Since the cells do not express markers specific for spermatocytes such as LDHC4, we conclude that the cell line is truly of spermatogonial origin. The germ cell origin is further confirmed by the fact that the Oct-4 nuclear protein is expressed and that no marker specific for somatic cells was found, by either immunoprecipitation or RT-PCR. The expression of Oct-4 is weak in the nucleoplasm but strong in nucleoli and other granular structures in the nucleus, and this pattern is consistent with the findings of Parfenov et al. [46] in active oocytes.

To determine if the C18-4 line is derived from a germ line stem cell, we probed the cells for GFR α -1, a surface receptor that is specifically expressed by A_s and A_{pr} spermatogonia in the testis [14]. GFR α -1 is the receptor for GDNF, which is produced by Sertoli cells [13, 47, 48]. GDNF is a pleiotropic factor that influences proliferation, survival, and differentiation in a number of target tissues during development [49, 50]. GDNF interacts with the GFR α -1 receptor, which, in turn, mediates stimulation of the Ret receptor tyrosine kinase [43]. The C18-4 cell line was positive for GFR α -1 using immunocytochemistry and immunoprecipitation, indicating that they are A_s or A_{pr} spermatogonia. The cells responded to GDNF by significantly increasing their rate of proliferation. This behavior is similar to the behavior of A_s spermatogonia of transgenic mice overexpressing GDNF [47]. We also probed the cells for their expression of c-kit, the receptor for SCF. In germ cells, c-kit seems to be expressed by all spermatogonia except for A_s, which are the putative stem cells [23, 51]. Thus, c-kit is considered to be a marker of more differentiated spermatogonia. While c-kit could not easily be detected by immunocytochemistry, the transcript was revealed by RT-PCR in early passages, then disappeared. Addition of SCF to the culture medium did not increase proliferation or stimulate differentiation. Further, GDNF could not increase the expression of the c-kit receptor. Therefore, a more comprehensive approach such as microarray analysis might be useful to assess the potential of the C18-4 cells to differentiate in the presence of either GDNF or SCF, or both. In addition, transplantation of the C18-4 cells into an infertile testis might provide the optimal environment for their differentiation.

In this study, we also examined the expression profile of 36 spermatogonial-specific genes that have been previously identified by Wang and colleagues [26]. Ten of these genes were expressed in the C18-4 germ cells. The loss of expression of many of those genes may be the consequence of immortalization, or there may be a lack of specific external stimuli, such as growth factors produced by Sertoli cells, that are important for the expression of these particular genes. However, the overall pattern of gene expression indicates that the C18-4 cell line exhibits general properties of type A spermatogonia. Interestingly, the C18-4 cells express a gene belonging to the *piwi* gene family (*piwi12*, or *piwi-like 2*). This family defines a novel class of genes that are conserved during evolution and seem to be required for stem cell renewal, gametogenesis, and RNA interference. In *Drosophila*, the germ line stem cells of *piwi* mutants (male and female) differentiate without producing self-renewing divisions. This situation eventually leads to gonads lacking germ cells [30, 52]. Homologues of *piwi* have been identified in *Caenorhabditis elegans* (*rde-1*) [53], in human (*hiwi*) [54], and in the mouse (*miwi* and *mili*) [31–33]. Moreover, the C18-4 cells express another gene that is specific for undifferentiated progenitors such as *PRAME*-like. The PRAME protein belongs to the cancer/testis antigens group, a category of tumor antigens normally expressed in male germ cells of the testis but not in somatic cells. However, the gene is overexpressed in a number of malignancies, including acute myeloid leukemias, and is associated with tumor progression [55]. PRAME is also expressed in normal, CD34⁺ bone marrow cells [36] and is associated with stem cell proliferation. Thus, the expression of *piwi-like* and *PRAME-like* genes indicates that the C18-4 cell line has a stem cell component.

We recently reported the establishment of another mouse spermatogonial cell line, which was immortalized using the *mTert* gene [56]. This cell line, named S4, does not express much of the GFR α -1 receptor, but it does express c-kit and responds to SCF by differentiating in vitro. Therefore, we believe that the C18-4 cell line is derived from A_s spermatogonia, while the S4 line is derived from A_{pr} or A_{al} spermatogonia. The C18-4 cell line will be useful to dissect the molecular mechanisms underlying germline stem cell proliferation, while the S4 cell line will be useful to understand differentiation and the molecular mechanisms that lead to meiosis. In addition to our cell lines, a cell line derived from rat spermatogonia with stem cell characteristics has also recently been produced [57]. Thus, testicular stem cell lines from two major model organisms are now available for further developmental studies.

In summary, based on the data presented here, our new germ cell line has characteristics of germ line stem cells. The lack of specific markers available to distinguish the A_s from the A_{pr} spermatogonia impairs our ability to characterize these cells more precisely. However, to date, this is the only germ cell line expressing the GFR α -1 and Ret receptors. Further, this cell line responds to GDNF by proliferating at a rate that is significantly higher than the control without growth factor. Interestingly, no response can be detected when the medium contains GDNF and a defined serum such as NU serum instead of FCS. Since NU serum contains no or very few cytokines, this finding confirms the observation that GDNF often acts in synergy with other growth factors that are likely present in FCS [58, 59]. Although the cells are unable to differentiate to meiotic spermatocytes thus far in vitro, probably due to their expression of the large T antigen, we believe that they will be helpful for promoter studies of genes specifically expressed in the early phases of spermatogenesis. They also provide a good model to understand how *Dazl* regulates its target mRNAs, to unravel signaling pathways triggered by GDNF, and, possibly, to study the function of novel genes identified through expression arrays of primary spermatogonial stem cells. As shown in Figure 8, the C18-4 cells are transfectable since they can express, at least transiently, the GFP protein from an expression vector.

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Figure 1. Morphology of the C18-4 cells in phase contrast microscopy. The cells are round to polygonal, with a round nucleus, and a large nucleus-to-cytoplasm ratio.

Figure 2.

C18-4 cells, immunostained for the Dazl RNA-binding protein. **(A):** Staining for Dazl, a general marker for germ cells, revealed by immunocytochemistry using antiserum no. 149 and horseradish peroxidase. **(B):** Negative control without primary antibody. **(C):** Staining for Dazl, a general marker for germ cells, revealed by immunocytochemistry using antiserum no. 150 and horseradish peroxidase. **(D):** Negative control without primary antibody.

Figure 3.

C18-4 cells, immunostained for GFR α -1, the Ret transmembrane receptor, and the Oct-4 transcription factor. **(A):** Staining for GFR α -1, revealed by immunocytochemistry and horseradish peroxidase ($\times 100$). **(B):** Negative control without primary antibody ($\times 100$). **(C):** Staining for Ret, revealed by immunocytochemistry and horseradish peroxidase ($\times 100$). **(D):** Negative control without primary antibody ($\times 100$). **(E):** Staining for Oct-4, revealed by immunocytochemistry and horseradish peroxidase ($\times 40$). **(F):** Negative control without primary antibody ($\times 40$).

Figure 4. *c-kit* gene expression in the C18-4 cells visualized by reverse transcription polymerase chain reaction. Lane A: size markers; lane B: adult testis; lane D: C18-4 cell line; lane F: freshly isolated Sertoli cell. Lanes C, E, and G: negative controls without reverse transcriptase.

Figure 5.

Immunoprecipitation of GFR α -1, Dazl, and Oct-4 using C18-4 protein extracts. **(A):** Immunoprecipitation of GFR α -1. Lane A: molecular weight markers; lane B: adult kidney; lane C: developing brain (6-day-old); lane D: adult testis; lane E: C18-4 cell line; lane F: 15P1 Sertoli cell line; lane G: NIH 3T3 cell line. **(B):** Immunoprecipitation of Dazl. Lane A: molecular weight markers; lane B: adult testis; lane C: C18-4 cell line. **(C):** Immunoprecipitation of Oct-4. Lane A: C18-4 cell line; lane B: adult testis; lane C: molecular weight markers.

Figure 6.

Expression of spermatogonia-specific genes in the C18-4 cell line, visualized by RT-PCR. RNA samples were isolated from the C18-4 cells, from 6-day-old testes as positive control, and from the SF7 Sertoli cell line as negative control. After reverse transcription, the cDNA samples were amplified using the primers described by Wang et al. [26]. **(A):** RT-PCR for *Ott* (PCR product = 551 and 503 bp). **(B):** RT-PCR for *Sycp1* (PCR product = 311 bp). **(C):** RT-PCR for *Fth117* (PCR product = 292 bp). **(D):** RT-PCR for *Tex13* (PCR product = 220 bp), *Tex14* (PCR product = 635 bp), *Tex15* (PCR product = 411 bp), *Tex16* (PCR product = 660 bp), and *Tex19* (PCR product = 184 bp). **(E):** RT-PCR for *Piwil1* (PCR product = 241 bp). **(F):** RT-PCR for *Prame11* (PCR product = 448 bp). Lanes A: size markers (100 bp ladder); B: C18-4 cell line; C: negative control for C18-4; D: 6-day-old testis; E: negative control for 6-day-old testis; F: SF7 Sertoli cells; G: negative control for SF7 Sertoli cells. Abbreviation: RT-PCR, reverse transcriptase polymerase chain reaction.

Figure 7.

Rates of proliferation of the C18-4 cells with GDNF or SCF. **(A):** In the presence of GDNF, the rate of proliferation of the C18-4 cells increases significantly in comparison with the control cultures. **(B):** No changes could be observed when the cells are cultured with SCF. Abbreviations: GDNF, glial cell line–derived neurotrophic factor; SCF, stem cell factor.

Figure 8.
Transient expression of green fluorescent protein in the C18-4 cells.

Spermatogonial genes expressed in the C18-4 cell line, arranged in alphabetical order; 6-day-old testes were used as positive control, and the Sertoli cell line SF7 as negative control

Table 1

No.	Gene	SF7 (negative control)	Testis (positive control)	C18-4	Protein function
	<i>Gapd</i>	+	+	+	Glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene)
1	<i>Fth117</i>	-	+	+	Testis-specific ferritin (iron metabolism)
2	<i>Oit</i>	-	+	+	Putative meiotic gene, ovary and testis
3	<i>Piwi12</i>	-	+	+	Stem cell maintenance and renewal
4	<i>Prame11</i>	-	+	+	Expressed in testis and melanoma cells
5	<i>Sycp1</i>	-	+	+	Synaptonemal complex protein, transverse filaments
6	<i>Tex13</i>	-	+	+	Novel 186-residues protein
7	<i>Tex14</i>	-	+	+	Novel protein containing two protein kinase domains
8	<i>Tex15</i>	-	+	+	Novel 2,785-residues protein
9	<i>Tex16</i>	-	+	+	Novel 1,139-residues protein, rich in serine
10	<i>Tex19</i>	-	+	+	Novel 351-residues protein with a coiled-coil region

+ gene expressed; -, gene not expressed.