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Isolation of male germ-line stem cells; influence of GDNF

Marie-Claude Hofmann[a,*], **Laura Braydich-Stolle**[a], and **Martin Dym**[b]

[a]Department of Biology, The University of Dayton, 300 College Park, Dayton, OH 45469-2320, USA

[b]Department of Cell Biology, Georgetown University Medical Center, Washington, DC 20057, USA

Abstract

The identification and physical isolation of testis stem cells, a subset of type A spermatogonia, is critical to our understanding of their growth regulation during the first steps of spermatogenesis. These stem cells remain poorly characterized because of the paucity of specific molecular markers that permit us to distinguish them from other germ cells. Thus, the molecular mechanisms driving the first steps of spermatogenesis are still unknown. We show in the present study that GFRα-1, the receptor for GDNF (glial cell line-derived neurotrophic factor), is strongly expressed by a subset of type A spermatogonia in the basal part of the seminiferous epithelium. Using this characteristic, we devised a method to specifically isolate these GFRα-1-positive cells from immature mouse testes. The isolated cells express Ret, a tyrosine kinase transmembrane receptor that mediates the intracellular response to GDNF via GFRα-1. After stimulation with rGDNF, the isolated cells proliferated in culture and underwent the first steps of germ cell differentiation. Microarray analysis revealed that GDNF induces the differential expression of a total of 1124 genes. Among the genes upregulated by GDNF were many genes involved in early mammalian development, differentiation, and the cell cycle. This report describes the first isolation of a pure population of GFRα-1-positive cells in the testis and identifies signaling pathways that may play a crucial role in maintaining germ-line stem cell proliferation and/or renewal.

Keywords

Type A spermatogonia; Stem cell; GFRα-1; GDNF; FGF2; Affymetrix microarray

Introduction

In the mammalian testis, continuous production of mature sperm throughout life is maintained by a small population of germ-line stem cells. These stem cells represent a discrete sub-population of type A spermatogonia, and reside in the basal part of the germinal epithelium, in contact with the basement membrane. The true identity of the testis stem cells has not been fully elucidated, and at least two models have been proposed for describing their renewal and/or differentiation. In one model, the stem cells of the testis are called A_{single} (A_s) spermatogonia (Huckins, 1971; Oakberg, 1971). They can renew themselves or differentiate into A_{paired} (A_{pr}) spermatogonia that remain connected by an intercellular bridge. The A_{paired} spermatogonia further divide to form $A_{aligned}$ spermatogonia, which give rise to more differentiated germ cells such as A_1–A_4 spermatogonia, type B spermatogonia, and spermatocytes that will undergo meiosis. A_{single}, A_{paired} and $A_{aligned}$ are sometimes collectively referred as undifferentiated spermatogonia (De Rooij and Grootegoed, 1998; De Rooij et al., 1999; Meng et al., 2000). In the other model, type A_s and A_{pr} are called A_0 (Clermont and Bustos-Obregon, 1968). These cells are believed to remain quiescent, or at the

* Corresponding author. Fax: +1 937 229 2021. Marie-Claude.Hofmann@notes.udayton.edu (M.-C. Hofmann).

most divide very slowly, unless an excessive loss of germ cells stimulates their proliferation, for example, after injury or irradiation (Clermont and Hermo, 1975; Dym and Clermont, 1970). In this model, the A_1–A_4 spermatogonia retain their stem cell properties, and the A_4 spermatogonia are able either to produce a new A_1 spermatogonia or go forward through spermatogenesis.

Self-renewal and differentiation of the testis stem cells is dependent on their close association with Sertoli cells in the seminiferous epithelium (Fritz, 1994). These processes are mediated by growth factors produced by the Sertoli cells, which induce or inhibit self-renewal, differentiation and further development of all germ cells (Jegou, 1993; Skinner, 1991).

Spermatogonial stem cells are unipotent: they are at the origin of only one cell lineage that will ultimately produce spermatozoa. They are capable of self-renewal and can regenerate spermatogenesis when transplanted into the seminiferous tubules of an infertile male (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). Spermatogonial stem cells exhibit a distinct phenotype such as the high expression of α-6 and β-1 integrins (Shinohara et al., 1999) and the lack of expression of the c-kit receptor (Ohta et al., 2000). Recently, Meng et al. (2000, 2001) reported that transgenic mice over-expressing glial cell line-derived neurotrophic factor (GDNF) exhibit an increase in proliferation of clusters of undifferentiated type A spermatogonia, containing mainly–but not exclusively–A_{single} cells. These clusters can evolve into testicular tumors resembling human seminoma (Tadokoro et al., 2002). More recently, Dettin et al. (2003) and Von Schönfeldt et al. (2004) demonstrated that a subset of type A spermatogonia expresses the receptors for GDNF.

GDNF binds to its co-receptor/receptor complex, made up of GFRα-1 and Ret, respectively (Baloh et al., 2000; Jing et al., 1996; Trupp et al., 1995). When occupied by its ligand, GFRα-1 activates Ret, which mediates the intracellular response (Robertson and Mason, 1997; Treanor et al., 1996; Worby et al., 1996). In the testis, GDNF is produced by the somatic Sertoli cells (Golden et al., 1999; Meng et al., 2000; Trupp et al., 1995; Viglietto et al., 2000).

Studies on the biology of type A spermatogonia, and in particular of the A_{single} cells, the putative stem cells, have been severely hampered because their number is low (0.03% of the total number of germ cells) (Tegelenbosch and De Rooij, 1993) and no unique marker exist. The identification and physical isolation of male germ-line stem cells are critical to our understanding of their growth regulation during the first steps of spermatogenesis. The ability to culture and manipulate these cells in vitro would allow us to unravel the molecular mechanisms driving spermatogenesis, and to characterize the signaling pathways inducing stem cell differentiation versus self-renewal. This, in turn, could help us understand the origin of certain testicular neoplasias, and the causes of male infertility. To start answering these questions, we attempted to isolate pure populations of GFRα-1-positive spermatogonia using immuno-magnetic beads, and to characterize these cells. After stimulation of the GFRα-1-positive cells with rGDNF, microarray analysis revealed the differential expression of a total of 1124 genes. The genes identified were verified by semi-quantitative RT-PCR and confirm that GDNF is a major inducer of spermatogonial proliferation and differentiation. Since the molecular mechanisms underlying these processes are unknown, we sought to identify signaling pathways that may play a crucial role in regulating the fate of GFRα-1-positive cells in the postnatal testis.

Material and methods

Expression of GFRα-1 and Oct-4 in whole-mount seminiferous tubules

The testes of adult and 6-day-old mice were excised, the seminiferous tubules isolated and immediately fixed in Dent's solution (1:4 DMSO-methanol). Whole-mounts were processed for immunocytochemistry according to the method of Davis (Davis, 1993). For the GFRα-1 staining, the primary antibody was a goat anti-mouse GFRα-1 antibody, which recognizes the C-terminus of the receptor, and used at dilutions of 1:100 to 1:250 (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody was a donkey anti-goat IgG conjugated to biotin (Santa Cruz Biotechnology, Santa Cruz, CA), and used at a dilution of 1:1000. For the Oct-4 staining, we used a rabbit anti-Oct-4 antibody from Active Motif (Carlsbad, CA), recognizing the human and mouse protein, at dilutions from 1:100 to 1:250. The secondary antibody was a biotinylated goat anti-rabbit IgG used at a dilution of 1:1000 (Vector Laboratories, Burlingame, CA). Finally, the samples were incubated with streptavidin-peroxidase, and the enzyme revealed with the AEC kit from Zymed (Zymed, San Francisco, CA), which gives a red precipitate on the reaction sites.

Isolation of type A spermatogonia

Type A spermatogonia were isolated using the STAPUT method that utilizes gravity sedimentation on a 2–4% BSA gradient (Dym et al., 1995). Immediately after STAPUT isolation, the spermatogonia were counted and resuspended in 30 ml D-MEM/F12 culture medium, supplemented with 5% fetal calf serum (FCS), 1 mM sodium pyruvate, 2 mM glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, and 100 mM non-essential amino acids. All tissue culture reagents and supplies were purchased from Fisher Scientific, Pittsburgh, PA. The cell suspension was placed into a 15-cm diameter tissue culture dish and incubated for 2 h at 34°C in order to eliminate residual adherent Sertoli cells (differential plating) (Dirami et al., 1999, 2001). A total of 60 male pups (6 day-old) were used for each isolation experiment, yielding an average of 5×10^6 type A spermatogonia with a purity of 90%.

Isolation of GFRα-1-positive spermatogonia

Magnetic beads (Dynabeads) were prepared in advance according to the manufacturer's instructions (Dynal, Brown Deer, WI). Briefly, streptavidin-coated magnetic beads were incubated for 1 h at room temperature with a biotinylated donkey anti-goat secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), at a concentration of 2.5 μg for 4×10^7 beads. After incubation, the beads were washed and resuspended at a concentration of 4×10^7 beads in 100 μl phosphate buffered saline (PBS) containing 0.1% BSA.

After STAPUT isolation and differential plating, type A spermatogonia were incubated for another 4 h at 34°C in D-MEM/F12 medium to ensure a complete cell recovery. Cells were centrifuged and resuspended in 1 ml culture medium. They were incubated overnight at 4°C with a 1:200 dilution of a goat anti-mouse antibody recognizing the C-terminus of the GFRα-1 receptor (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were then washed 3 times with PBS and incubated for 1 h on a shaker at room temperature with the magnetic beads coated with the secondary antibody (ratio = 10 beads per target cell). The number of target cells was estimated at around of 5×10^4 cells for 60 pups (De Rooij and Russell, 2000). After incubation, the bead-coated cells (GFRα-1-positive cells) were separated from the GFRα-1-negative cells with a magnet (Dynal, Brown Deer, WI). Both cell populations were gently washed 3 times with PBS, immediately fixed with 10% formaldehyde in PBS, and spotted on microscope slides coated with Biobond (Electron Microscopy Sciences, Fort Washington, PA). Slides were dried on a slide warmer and processed for immunocytochemistry.

Immunocytochemistry of isolated spermatogonia

STAPUT-isolated cells (total type A spermatogonial population) and the subpopulations of GFRα-1-positive and -negative cells were incubated overnight at 4°C with a goat anti-mouse c-kit antibody (Santa Cruz Biotechnology Santa Cruz, CA) at dilutions of 1:50 to 1:200. After washing with PBS, the cells were incubated with a rabbit anti-goat secondary antibody conjugated to biotin (Vector Laboratories, Burlingame, CA). Finally, the cells were incubated with streptavidin-peroxidase, and the enzyme revealed with an aminoethyl-carbazole substrate kit giving a red precipitate on the reaction sites (Zymed Laboratories, San Francisco, CA). Alternatively, the cells were incubated with a goat anti-mouse Ret antibody at concentrations of 1:50 to 1:200 (R&D Systems, Minneapolis, MN). The secondary antibody was a rabbit anti-goat antibody conjugated to biotin (Vector Laboratories, Burlingame, CA). Finally, the cells were incubated with streptavidin-peroxidase, and the enzyme revealed with the Vector VIP substrate kit that gives a purple precipitate on the reaction sites (Vector Laboratories, Burlingame, CA).

Culture of GFRα-1-positive spermatogonia with GDNF and other growth factors

GFRα-1-positive cells were isolated from 6-day-old mice testes as described above. They were seeded in microtiter plates at a concentration of 1000 cells/well in 100 μl StemPro medium (Invitrogen). Alternatively, D-MEM/F12 (HyClone, Logan, UT) was used as culture medium. In all cultures, the medium was complemented with 10% NU synthetic serum to allow for a controlled environment (Fisher Scientific, Pittsburgh, PA). After optimization, rat rGDNF was added at a final concentration of 100 ng/ml (R&D Systems, Minneapolis, MN). GDNF was also used in combination with other growth factors at the following final concentrations: bFGF (10 ng/ml, BD Biosciences, Franklin Lakes, NJ), LIF (10 ng/ml, Chemicon International, Temecula, CA), EGF (20 ng/ml, BD Biosciences, Franklin Lakes, NJ) and TGF-β (100 ng/ml, BD Biosciences, Franklin Lakes, NJ). After 5 days of culture, clusters (cell aggregates containing 5 to 20 cells) and colonies (aggregates containing 20 to 50 cells) were counted.

Microarray analysis of GDNF-stimulated cells

GFRα-1-positive cells were isolated from 6-day-old mice testes as described above. Two groups of cells were formed. One group of cells was incubated for 10 h with 100 ng/ml recombinant GDNF (R&D Systems, Minneapolis, MN) in DMEM/F12 medium with 10% Nu serum (Fisher Scientific, Pittsburgh, PA), at 34°C and 5% CO_2 in an humidified incubator. The second group (control) was incubated for 10 h in culture medium only. The DMEM/F12 medium was supplemented with 50 U/ml penicillin-streptomycin and 2 mM L-glutamine (Atlanta Biologicals, Norcross, GA).

RNA was isolated from the cell samples using the Zymo Mini RNA isolation kit (Zymo Research, Orange, CA). Two runs of RNA amplification were performed using the Ambion MessageAmp aRNA kit (Ambion, Austin, TX), yielding about 10 μg antisense RNA (aRNA) per sample. Five μg of aRNA were converted to cDNA, and biotinylated cRNA produced according to the manufacturer's instructions (Affymetrix, Santa Clara, CA).

After cleaning up and quantification, the biotinylated cRNA was fragmented and hybridized to U74Avs.2 gene chips at 45°C for 16 h. The cRNA was automatically washed and stained on a fluidics station, and then scanned on a GeneArray scanner (all from Affymetrix, Santa Clara, CA). Labeling and scanning services, as well as normalized data for comparison expression analysis were provided by Dr. P. Manickam (Neurologics Inc., Bethesda, MD). Genes expression was analyzed using the GeneSpring software (Silicon Genetics, Redwood City, CA). Probes showing more than 2-fold difference between unstimulated and stimulated cells were selected and classified into functional groups according to their cellular role.

Semi-quantitative RT-PCR

GFRα-1-positive cells were isolated and cultured overnight with or without 100 ng/ml GDNF as described above. Primers for the chosen genes were designed using the PRIDE and Net Primer on-line programs and obtained from Integrated DNA Technologies (IDT), Coralville, IA. Total RNA was isolated using the Zymo Mini RNA isolation kit, which allows for the isolation of RNA from 10^3 to 10^5 cells/sample (Zymo Research, Orange, CA). Total RNA samples were treated with 1 unit/1 μg RNA of RQ1 RNase-free DNase (Promega, Madison, WI) to degrade any genomic DNA present. We then used the Superscript One-Step RT-PCR system and Platinum Taq polymerase (Invitrogen, Carlsbad, CA) with 0.1 μg total RNA of each sample. For quantitation, we used the QuantumRNA 18S internal standards (Ambion, Austin, TX) and the optimal ratio of 18S primers/competimers. As negative controls, PCR was performed on RT reaction products obtained without the use of reverse transcriptase.

Gel analysis was performed with the LAS 3000 imaging system (FUJIFILM Medical Systems, Stamford, CT). Quantitation of the PCR products was performed using the ImageGauge software (FUJIFILM Medical Systems, Stamford, CT).

Statistical methods

For semi-quantitative RT-PCR, the data are presented as mean ± standard deviation. Each data point represents the average of three separate experiments. The two-sample t test (assuming unequal variances) as implemented in MS Excel, was used to determine statistical significance of observed differences in the mean values ($P < 0.05$, one-tailed). For in vitro culture assays with growth factors, each data point represents the average of three separate experiments, and is presented as mean ± standard deviation. Statistical significance among the number of clusters obtained with different growth factor combinations was determined using ANOVA and subsequent pair-wise analysis by LSD test (least significant difference), as implemented in the SPSS statistical software (SPSS, Chicago, IL). $P < 0.05$ (one-tailed for LSD tests) indicated statistical significance.

Results and discussion

Despite the central importance of male germ-line stem cells in genetics and developmental biology, our ability to study them directly has been hampered by the lack of unique markers and an adequate method of isolation. This report demonstrates the successful isolation of a subset of type A spermatogonia that satisfy many of the criteria assigned to male germ-line stem cells. When observed in situ in adult seminiferous tubules, these cells express the GFRα-1 receptor and are dispersed in the basal part of the seminiferous epithelium (Fig. 1A). Many of these isolated cells exhibit a high nuclear/cytoplasmic ratio. They present a thin rim of cytoplasm highly positive for the GFRα-1 receptor, as shown in Fig. 1B (arrow). The cell shown in Fig. 1B is a high magnification of one of the spots visualized in Fig. 1A. Not all single cells exhibit this high level of GFRα-1 staining. Often, the staining is confined in only one part of the cytoplasm, which could be attributed to a capping artefact due to fixation. Because of their localization in the basal part of the epithelium, their morphology, and the fact that they are not connected to other cells by intercellular bridges, we classify these cells as A_{single} spermatogonia, the testis stem cells. GFRα-1-positive cells can also appear in pairs. Since it is not known whether spermatogonial stem cells undergo asymmetric or symmetric division, the doublets could be daughter cells after a self-renewing division or A_{paired} cells already committed to spermatogenic differentiation. Our results confirm the immunohistochemical data of Von Schönfeldt et al. (2004) obtained with tissue sections of adult testis. In comparison, we observed a greater percentage of cells expressing the GFRα-1 receptor in the 6-day-old seminiferous epithelium (Dettin et al., 2003). This is due to the fact that the germ cells generated at this age represent only the first steps of spermatogenesis (A_{single}, A_{paired}, and some

$A_{aligned}$ spermatogonia). Meng et al. (2000,2001) previously described transgenic mice over-expressing GDNF. These mice exhibited a transient increase in the number of undifferentiated type A spermatogonia organized in clusters within the seminiferous tubules. A majority of the cells were A_{single} spermatogonia, although a breakage of the bridges between A_{paired} and $A_{aligned}$ cells due to a different microenvironment cannot be excluded. Our whole-mount data thus confirm that in the normal adult mouse testis, a small subpopulation of type A spermatogonia (the A_{single} and possibly the A_{paired} cells) express GFRα-1, which is the receptor for GDNF.

We also attempted to visualize the expression of the nuclear protein Oct-4 in whole mounts of seminiferous tubules (Fig. 1C). Oct-4 is a transcription factor belonging to the POU family, which regulates the expression of target genes by binding to the octamer motif ATGCAAAT within their promoter or enhancer regions (Herr and Mccleary, 1995). In the mouse, embryonic expression of Oct-4 is high at the preimplantation stages, especially in the ICM where it seems to maintain cellular pluripotency (Buehr et al., 2003;Pesce and Schöler, 2001;Wang and Schultz, 1996). After implantation, Oct-4 expression progressively decreases. After gastrulation, it is exclusively expressed in the nuclei of primordial germ cells (Schöler et al., 1990). In the testis after birth and in the adult, Oct-4 is expressed in the germline stem cells and in other type A spermatogonia (Pesce et al., 1998). Our results confirm that the expression of Oct-4 is more widely distributed than the expression of GFRα-1, since it is also seen in $A_{aligned}$ spermatogonia (Fig. 1C).

We then attempted to specifically isolate the GFRα-1-positive cells. As a first step, we used the Staput method initially devised by Dym et al. (1995) to isolate type A spermatogonia from 6-day-old mice testes (Bellvé et al., 1977). This technique allows for the isolation of a population containing at least 90% type A spermatogonia. After Staput isolation, immunocytochemistry revealed that about 90% of the cells expressed the c-kit receptor, which confirms that most cells isolated are pre-meiotic germ cells (Besmer et al., 1993). The age of the mice was chosen in order to avoid the presence of A1–A4 spermatogonia or more differentiated germ cells in the seminiferous epithelium and to maximize the number of stem cells available in the germ cell pool. Indeed, no differentiated spermatogonia or spermatocytes were present after careful morphological examination. The remaining c-kit-negative cells were residual Sertoli cells and type A spermatogonia, confirming that a subset of type A spermatogonia are negative for this marker (Ohta et al., 2000; Schrans-Stassen et al., 1999). This separation was followed by immunomagnetic beads isolation of the GFRα-1-positive cells according to the protocol we recently developed and modified as described above (Van der Wee et al., 2001) (Fig. 2A).

About 5×10^4 GFRα-1-positive cells could be retrieved from the type A spermatogonial population. Their identity was confirmed by determining the expression of Ret, the transmembrane receptor mediating GFRα-1 activation by GDNF, and a marker for pre-meiotic germ cells (Viglietto et al., 2000; Widenfalk et al., 2000). Ret-positive cells represented 92.2 ± 3.4% of the total number of GFRα-1-positive cells in replicate experiments ($n = 3$) (Figs. 2A and B, panel C). Further, the GFRα-1-positive cells were stained for c-kit. Interestingly, about half of the population (43.2 ± 6.8%) was c-kit-negative (Fig. 2B, panel A), while the rest (51.6 ± 0.2%) was c-kit-positive (Fig. 2B, panel B). In summary, GFRα-1-positive spermatogonia exhibit 2 phenotypes: GFRα-1^+/RET^+/c-kit^- cells or GFRα-1^+/RET/$^+$c-kit^+ cells. This confirms the finding of others that a subpopulation of undifferentiated type A spermatogonia is c-kit-negative (Schrans-Stassen et al., 1999; Tadokoro et al., 2002; Yoshinaga et al., 1991). Schrans-Stassen et al. (1999) have suggested that these c-kit-negative cells are A_{single} spermatogonia since they were never found in groups, and were always isolated when observed in testis sections. Further, Ohta et al. (2000) recently reported that in the mammalian testis, SCF stimulation of the c-kit receptor was necessary for the maintenance of differentiated germ

cells. However, the stem cells did not respond to the action of SCF, suggesting that the testis stem cells are c-kit-negative.

There are still conflicting data about the presence or absence of the c-kit receptor at the surface of A_{paired} spermatogonia [(Schrans-Stassen et al., 1999), and D.G De Rooij, personal communication]. However, combining our data on the expression of GFRα-1 in situ (Fig. 1) with the c-kit expression of the same cells after immunomagnetic isolation, we conclude that the c-kit-positive cells must be the A_{paired} spermatogonia. Taken together, the data presented here demonstrate that it is possible to isolate specifically the male germ-line stem cells and their direct progeny using an immunomagnetic bead technique. While the number of cells obtained is low, the purity of the GFRα-1 population is almost 100%, with no contamination from somatic cells or more advanced germ cells such as $A_{aligned}$ spermatogonia.

We then attempted to culture the GFRα-1-positive spermatogonia with GDNF and/or other growth factors, but without a feeder layer of Sertoli cells or STO fibroblasts (Nagano et al., 1998, 2003). Recently, several laboratories established methods allowing for the enrichment of male germ-line stem cells or improving their culture conditions (Kanatsu-Shinohara et al., 2003a,b; Shinohara and Brinster, 2000; Shinohara et al., 2000). Expansion techniques have used feeder layers, or a combination of growth factors, or serial transplantation procedures (Hasthorpe, 2003; Kanatsu-Shinohara et al., 2003a,b). However, the expansion of pure stem cells in culture is also possible, although the proliferation of the cells remains limited (Hasthorpe, 2003). In our hands, the GFRα-1-positive cells could grow without feeder layers and with GDNF alone for a maximum period of 21 days. After 3 days in culture, the cells produced aggregates of 5–20 cells (clusters) (Fig. 3B). After 6 days in culture, the clusters grew in number and some increased in size to become colonies (aggregates containing more than 20 cells). Interestingly, some of the aggregates produced rows of cells connected by cytoplasmic bridges resembling $A_{aligned}$ spermatogonia (Fig. 3C). After 21 days in cultures, some colonies were still viable and stained positive for the c-kit receptor (Fig. 3D). Thus, GDNF is stimulating the proliferation of pure GFRα-1-positive spermatogonia in vitro in controlled conditions, and might also be involved in driving the first steps of the differentiation process.

To determine which genes are involved in the response of germ-line stem cells to GDNF, we performed microarray analysis using the Affimetrix technology and the U74Avs.2 gene chip. GFRα-1-positive cells isolated from the 6-day-old mouse testis were treated with 100 ng recombinant GDNF for 10 h. We then isolated their RNA for differential gene expression analysis in comparison to untreated cells. Since the number of cells recovered by the immunomagnetic bead method was small, we performed 2 rounds of mRNA amplification before processing for DNA microarray. We were able to amplify the mRNA more than a 1000-fold, resulting in enough material for microarray analysis. After hybridization, we observed that a total of 1124 genes are differentially expressed (Fig. 4A). Probes showing more than 2-fold upregulation between unstimulated and stimulated cells were selected for further analysis (n = 378). Out of these 378 upregulated genes, 163 (~43%) have a known function. We then distributed the latter into 13 functional classes (Table 1). The classes containing the higher number of upregulated genes are the cell proliferation and the cell differentiation gene classes (Tables 2 and 3). We also could create a class for genes involved in stem cell fate and development (Table 4). We next focused on twenty-two genes that were more likely to play a role in stem cell proliferation and differentiation (Table 5). Semi-quantitative RT-PCR confirmed the differential expression of about 70% of the genes studied so far. Fig. 4B shows a representative collection of the genes that we examined. It is important to note that in all cases where differential expression was confirmed and statistically significant, the agreement between microarray and semi-quantitative RT-PCR data was qualitative rather than quantitative. In many cases, the differential expression measured by semi-quantitative RT-PCR is less important than the differential expression measured by microarray analysis. This might

be due to the fact that in oligonucleotides microarrays, the probability of cross-hybridization of short sequences with homologous sequences is high for certain genes and could account for a substantial percentage of the signal (Ding and Cantor, 2004). Taken together, our results confirm that GDNF is a major inducer of spermatogonial proliferation. For example, cyclin B1 and cyclin D3 are upregulated, while cyclin inhibitors such as p19, p21, and p15 might be down-regulated by GDNF (Table 2 and Fig. 4B). However, the up-regulation of cyclin E could not be confirmed. In addition, N-myc (brain and testis form of Myc) and L-Myc (liver form) were also upregulated by GDNF (Table 2 and Fig. 4B). Myc is well known for its direct role in the DNA replication machinery or its modulation of the transcriptional control of genes involved in cellular replication (Menssen and Hermeking, 2002). For example, several members of the cyclin family are direct targets of c-Myc in hematopoietic stem cells, and play a role in self-renewal (Kamijo et al., 2002;Satoh et al., 2004). Cyclins and Myc are often activated through a STAT3 or a STAT5-dependent signaling pathway (Calo et al., 2003). However, regulation of spermatogonial proliferation by GDNF could also be dependent on a Ras and PI3 kinase pathways since their activation has been shown in neurons and kidney cells (Hayashi et al., 2000;Tang et al., 2002). Because germ-line stem cells proliferate as they differentiate into A_{paired} and $A_{aligned}$ spermatogonia, it is not yet clear whether cyclin D3, B1 and N-Myc are involved in A_{single} spermatogonia cell self-renewal as well. Only a functional assay for germ-line stem cells such as testis transplantation after gene manipulation will be able to answer this question in the future.

In Fig. 4B, we present the differential expression of other genes that might be important for spermatogonial stem cell proliferation and differentiation. For example, the gene coding for TIAR, a RNA recognition motif/ribonucleoprotein-type RNA-binding protein is up-regulated (Beck et al., 1998). TIAR is essential for the survival and development of primordial germ cells, and thus might also be essential for the development of testis stem cells after birth. Other genes involved in cell proliferation include genes usually expressed in brain development, which is not surprising considering the fact that GDNF is primarily a neurotrophic factor.

Our microarray analysis also revealed that the Notch pathway, a pathway important for stem cell self-renewal and lineage determination in many mammalian tissues, might play a role in germ-line stem cell development. Indeed Notch expression has been detected in the testis by several investigators (Dirami et al., 2001; Hayashi et al., 2001; Von Schönfeldt et al., 2004). Notch is a transmembrane receptor activated by juxtacrine signaling (Weinmaster, 1997). Notch homologues promote germ cell mitosis vs. meiosis in *Caenorhabditis elegans* (Crittenden et al., 1994) and maintain spermatogonia in an undifferentiated state in *Drosophila* (Xu et al., 1992). The role of Notch in the mammalian germ-line is less clear. However, Notch seems involved in spermatogonial differentiation in rats and humans, since suppression of this signaling pathway results in maturation arrest of the germ cells (Hayashi et al., 2001, 2004). In addition, activated forms of Notch, which act as transcription factors, can activate the c-myc promoter (Satoh et al., 2004). In our hands, GDNF does not induce the differential expression of *notch*. However, it induces the up-regulation of the gene *numb*, which is an antagonist of *notch*. It also induces the up-regulation of *seven-in-absentia*, which is involved in *numb* modulation. *Numb* has also been implicated in defining daughter cell asymmetry after stem cell division, in *Drosophila* and mouse neural stem cells (Fuerstenberg et al., 1998). Taken together, the regulation of the Notch signaling pathway might be important in influencing the decision of a germ-line stem cell to self-renew or differentiate.

We also found that GDNF was able to up-regulate fibroblast growth factor receptor-2 (FGFR2) (Fig. 4B), suggesting that GDNF might render germ-line stem cells responsive to bFGF, and that a cooperation of both growth factors is essential for their proliferation. We thus cultured GFRα-1-positive cells in the presence of GDNF and GDNF+ bFGF for 5 days in StemPro medium and assessed cluster growth. As presented in Fig. 5, GDNF at a concentration of 100

ng/ml significantly increases the number of clusters formed. Further, bFGF added at a concentration of 10 ng/ml in this culture environment amplifies the effects of GDNF. Addition of other growth factors did not further increase the number of clusters produced, indicating that the combination GDNF/FGF is the limiting factor. It has been postulated that FGFR2 is expressed by human spermatogonial stem cells and that it may play a role analogous to Ret in regulating their clonal expansion and fate (Goriely et al., 2003). GDNF and bFGF are part of a cocktail of factors used recently by Kanatsu-Shinohara et al. (2003a,b) to establish long-term cultures of gonocytes A more recent report indicates that bFGF has a negative effect on the colonization ability of spermatogonial stem cells at concentrations higher than 1 ng/ml (Kubota et al., 2004). However, in this case, germ cell populations enriched in stem cells were cultivated on feeder layers of STO fibroblasts. Thus, the differences in conditions of incubation with bFGF might account for the discrepancy. In the nervous system, bFGF requires the presence of GDNF for promoting the survival of hippocampal neurons (Lenhard et al., 2002). Thus, our findings indicate that a cooperation between GDNF and bFGF is likely to maintain spermatogonial stem cell self-renewal or proliferation/differentiation.

In conclusion, we have been able to isolate specifically a subset of type A spermatogonia, which is positive for the membrane receptor GFRα-1. This cell population likely represents the spermatogonial stem cells. Our results show clearly that GDNF is involved in spermatogonial proliferation and differentiation. However, since the GFRα-1-positive germ cell population might be heterogeneous in respect to its ability to self-renew or differentiate, in the future it will be essential to isolate specifically the A_{single} spermatogonia (GFRα-1-positive and c-kit-negative cells). Our study also identified signaling pathways that may play a crucial role in regulating stem cell fate in the postnatal testis, and stresses the importance of bFGF and its cooperation with GDNF to maintain spermatogonial proliferation in vitro at its optimal level. Interestingly, since this growth factor combination enhances the number of clusters, rather than their size, it indicates that bFGF might be important to maintain the integrity of the stem cell pool rather than to stimulate spermatogonial proliferation.

A number of important questions remain unanswered in the field of male germ-line stem cells such as: What “keeps a stem a stem”? What cocktail of growth factors will allow for stem cell renewal or stem cell differentiation? What genes and proteins are essential for the spermatogonial stem cells? What signal transduction mechanisms are active during stem cell renewal vs. stem cell differentiation? The successful isolation of a pure population of male germ-line stem cells (spermatogonial stem cells) described in this work will facilitate the resolution of these queries.

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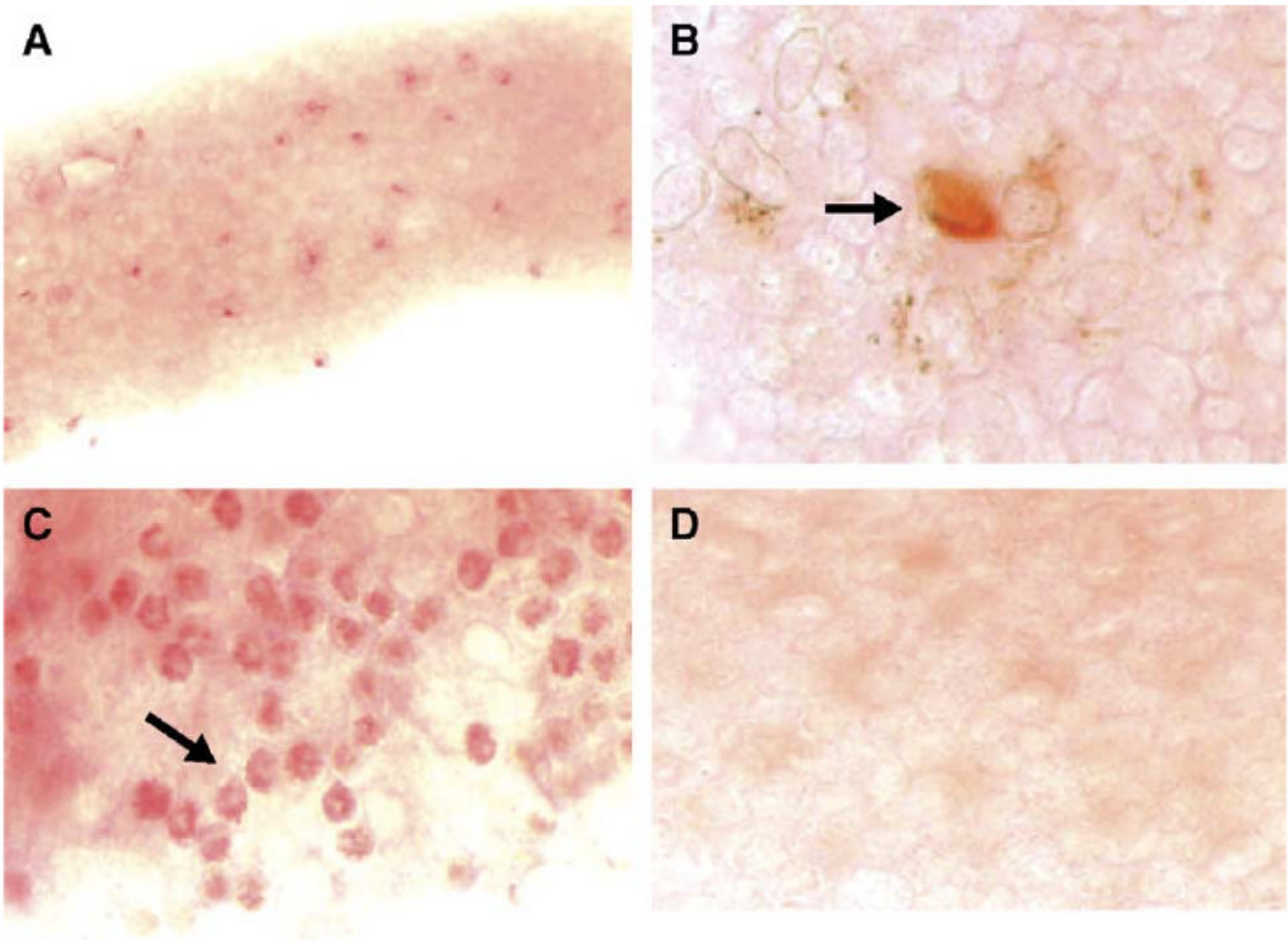

Fig. 1.

Expression of the GFRα-1 receptor in whole-mount adult mouse seminiferous tubules. Testes were decapsulated and seminiferous tubules fixed in Dent's fixative. The tubules were then rehydrated and stained for the GFRα-1 receptor. (A) In this overview, each one of the red, GFRα-1-positive dots represents a spermatogonial stem cell (A_{single} spermatogonium) or two daughter cells after division (×10). (B) Strong expression of the GFRα-1 receptor in a A_{single} spermatogonium (arrow). Magnification of 1A (×60). (C) Expression of the nuclear protein Oct-4 in A_{single}, A_{paired}, and $A_{aligned}$ spermatogonia (arrow) (×40). (D) Negative control without primary antibody (×60).

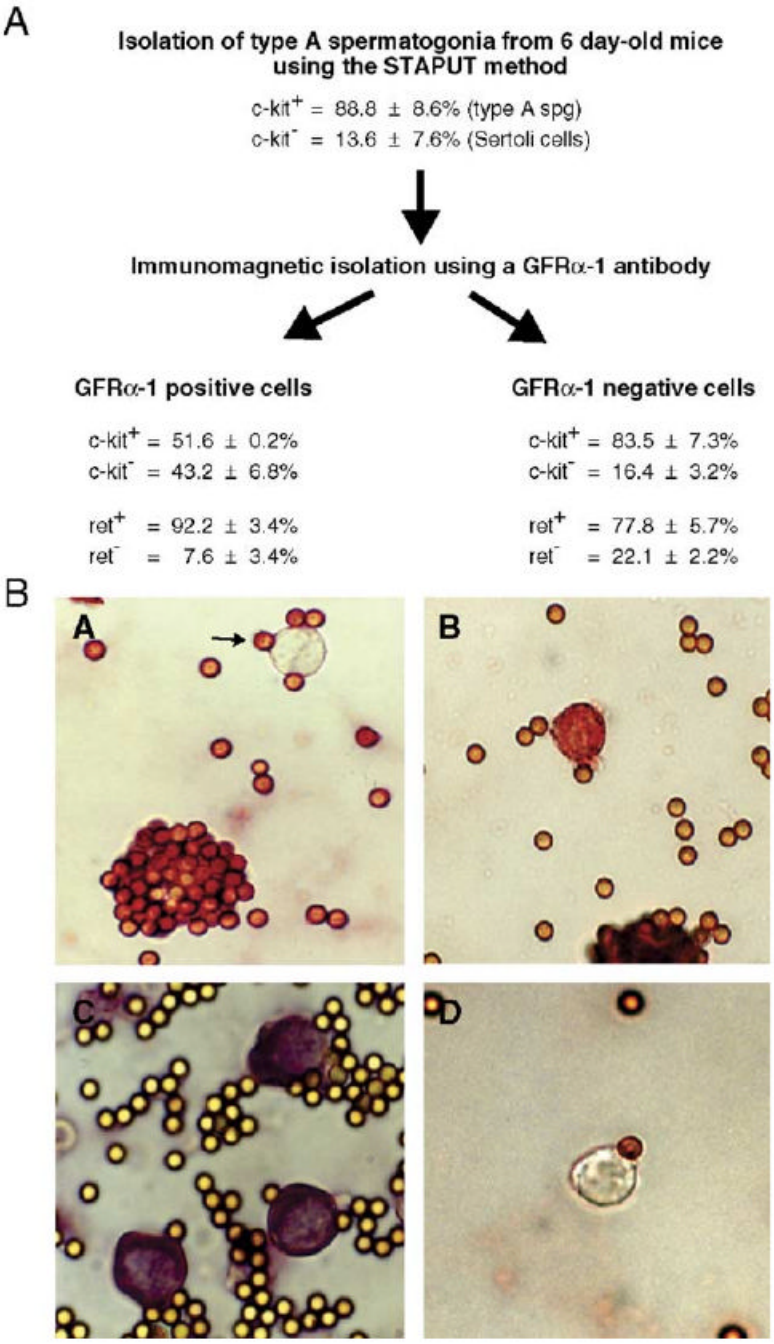

Fig. 2.

Specific isolation and characterization of GFRα-1-positive spermatogonia from 6-day-old mouse testes. (A) The Staput method allows the isolation of almost pure populations of type A spermatogonia. This first step eliminates most of the testicular somatic cells. The Staput isolation is followed by magnetic beads separation of the GFRα-1-positive cells. This subpopulation expresses the Ret receptor and contains c-kit-negative cells (stem cells, or A_{single} spermatogonia) and c-kit-positive cells (A_{paired} spermatogonia). (B) After isolation, the GFRα-1-positive spermatogonia were stained for surface receptors specifically expressed by germ cells in the early steps of spermatogenesis (Ret and c-kit receptors). (A) Spermatogonium positive for GFRα-1 (as seen by the beads still attached to the cell membrane) but negative for

c-kit (A_{single} spermatogonium) (×60). (B) Spermatogonium positive for GFRα-1 and the c-kit receptor (A_{paired} spermatogonium) (×60). (C) All GFRα-1 spermatogonia are positive for the Ret receptor (×60). (D) Negative control without primary antibody (×60).

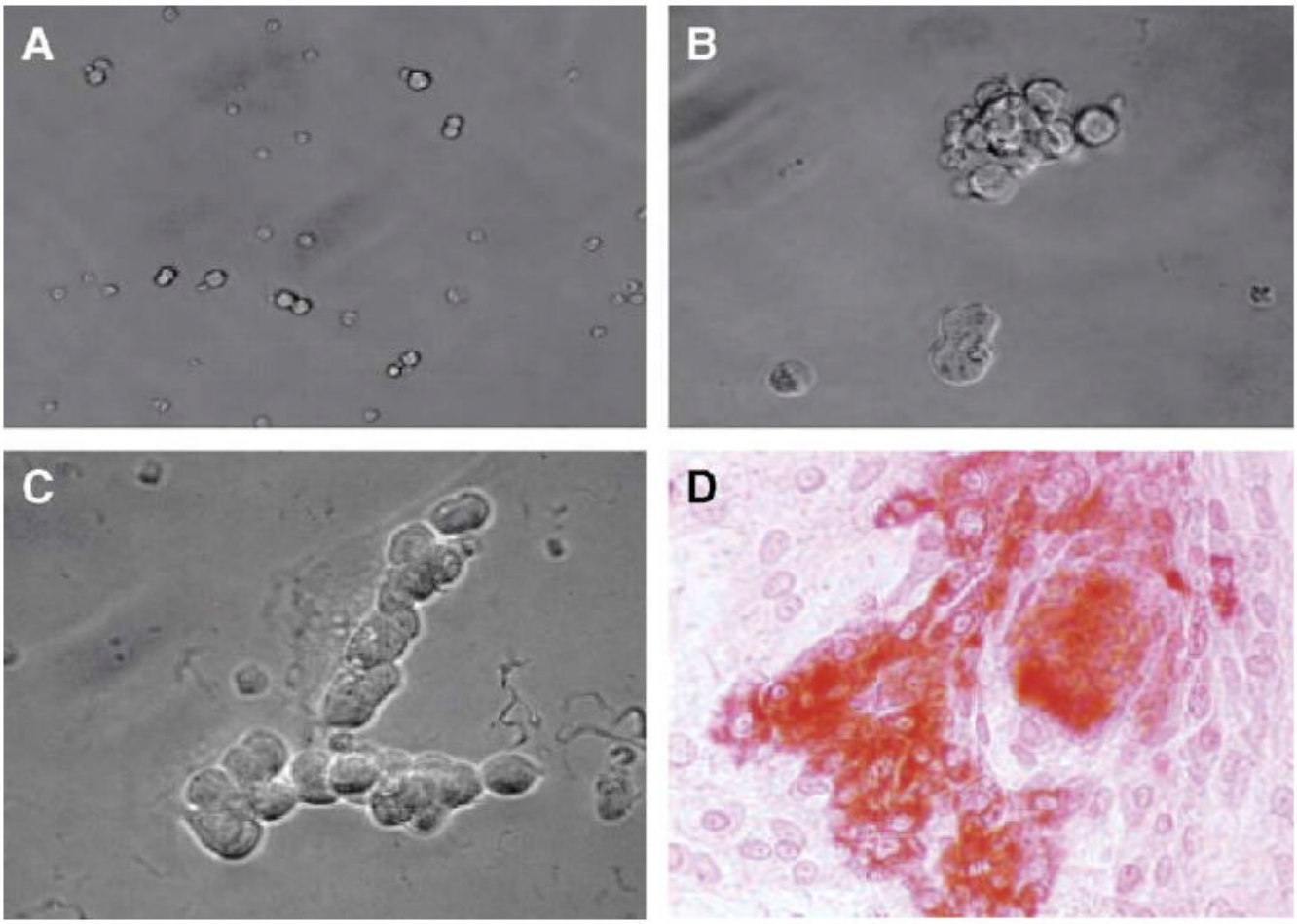

Fig. 3.

Proliferation and differentiation of spermatogonial stem cells in presence of GDNF in vitro. GFRα-1-positive spermatogonia were isolated with the Staput/magnetic bead method and cultured with GDNF (100 ng/ml) in controlled conditions for a period of 21 days. (A) 7-day-old culture without GDNF (negative control) (×20). (B) Growing cluster of spermatogonia representative of a 3-day-old culture (×40). (C) $A_{aligned}$ spermatogonia in a 6-day-old culture (×40). (D) A colony observed after 21 days of culture with GDNF and positive for the c-kit receptor (×20).

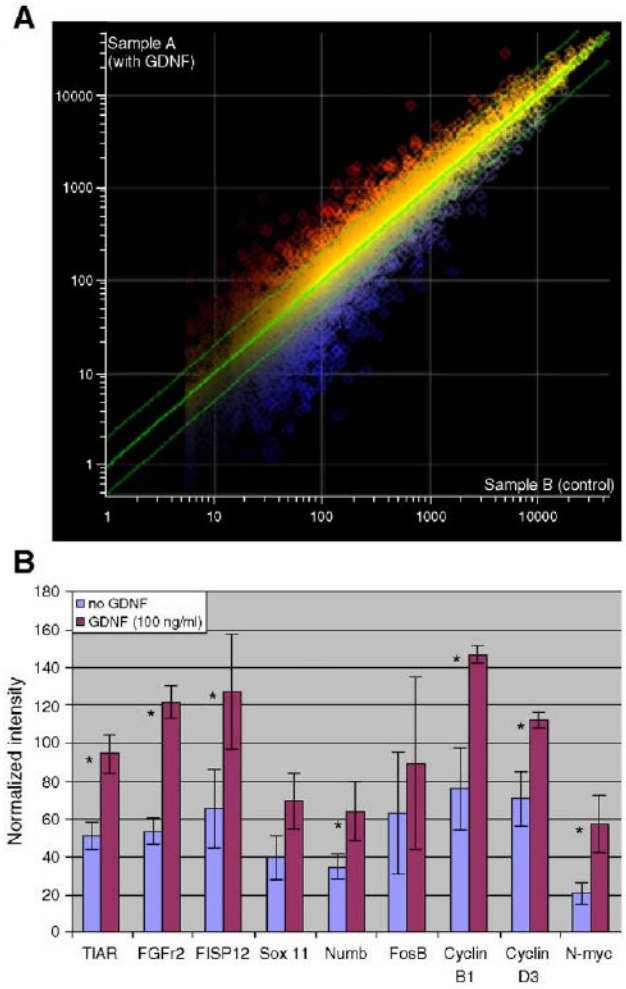

Fig. 4.

Microarray analysis of genes differentially regulated by GDNF. GFRα-1-positive spermatogonia were isolated with the Staput/magnetic bead method and cultured for 10 h with or without GDNF (100 ng/ml) in controlled conditions. RNA was isolated and processed for Affymetrix microarray with the U74Avs.2 gene chip. (A) Scatter plot showing the distribution of the 1124 genes that are differentially regulated by GDNF. Genes situated above the upper green line are up-regulated more than 2-fold. Genes situated below the lower green line are down-regulated more than 2-fold. (B) Semi-quantitative RT-PCR data confirming the up-regulation of certain genes of interest. The differential regulation of 70% of the genes investigated could be confirmed. A value of $P < 0.05$ (one-tailed) was assumed to indicate statistical significance (asterisks).

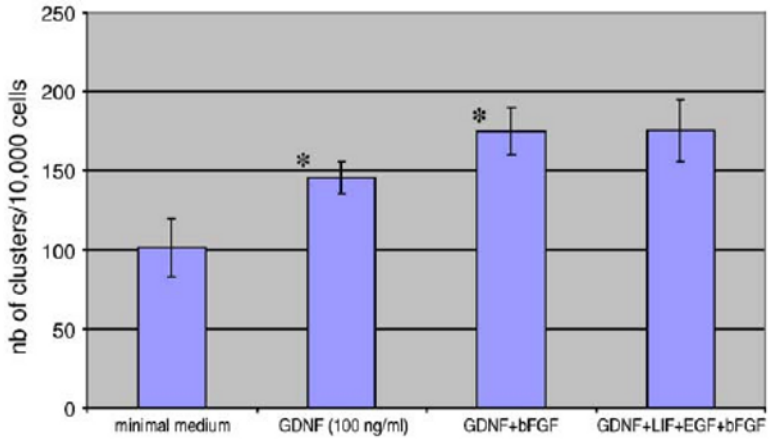

Fig. 5.

Growth of spermatogonial clusters in presence of GDNF and bFGF after 5 days of culture. GFRα-1-positive spermatogonia were isolated with the Staput/magnetic bead method and cultured for 5 days with or without GDNF (100 ng/ml) and bFGF (10 ng/ml) in controlled conditions. bFGF amplifies the effect of GDNF on cluster numbers, but not cluster size. Addition of other growth factors such as LIF and EGF did not further increase the number of clusters produced. The results obtained with GDNF and FGF are significantly different from the results obtained with GDNF alone or with minimal media. A value of $P < 0.05$ (one-tailed) was assumed to indicate statistical significance (asterisks).

Table 1

Classes of genes up-regulated by GDNF

Cell proliferation	19 genes	Cell/cell adhesion Cell/ECM adhesion	20 genes
Cell differentiation/development	24 genes	Cytoskeleton associated	8 genes
Stem cell fate	7 genes	Transcription/translation	16 genes
Germ cell-expressed	19 genes	DNA binding/repair	5 genes
Cell cycle	5 genes	RNA processing	9 genes
Signal transduction	7 genes	Metabolism/transport	36 genes
Apoptosis	6 genes		

We manually classified the 163 known genes that are up-regulated >2-fold with GDNF into 13 main classes corresponding to different cellular functions. Some genes can belong to two different classes.

Table 2

Cell proliferation/cell cycle group

Name	Fold expression	GenBank #
cyclin B1	14.10	X64713
FGF-inducible protein	8.03	U42383
ST2	7.05	Y07519
Ras-related protein	6.62	AF009246
IGF-binding protein	4.92	M32490
cyclin D3	4.60	M86183
N-myc (brain, testis)	4.60	M36277
L-myc (liver)	4.50	BC053059
egr-2	4.32	M24377
brain-derived neurotrophic factor	4.13	X55573
telomeric repeat-binding factor	3.81	AF003000
SRG3 (early embryogenesis)	3.69	U85614
c-fos B	3.58	X14897
amphiregulin (mitogen for neural stem cells)	3.55	L41352
cyclin E2	3.41	AF091432
FGFR-2	3.25	M86441
TIAR (PGC proliferation and survival)	3.17	U55861
Tsg118 (chromosome condensation in mitosis)	2.75	AF034580
FISP-12 (growth factor-induced)	2.73	M70642
egr-1	2.62	M28845
HRS (c-fos pathway, cytokine-induced)	2.54	AF020308
stromal cell-derived factor 1	2.35	L12029
proliferin	2.25	K03235
A-myb	2.25	X82327
p19ink*	−1.60	U19597
p15ink*	−2.00	AF059567
p21*	−2.60	U09507
p53*	−2.60	AF161020

Initial list of genes involved in driving cell proliferation and the cell cycle that are up-regulated 2-fold and more by GDNF. Cell cycle inhibitor genes (asterisks) have been added as well since they seem down-regulated, which provide for a more comprehensive picture.

Table 3

Cell differentiation/development group

Name	Fold expression	GenBank #
H19	9.07	X58196
socs-2	7.91	U88327
PTEN (tumor suppressor)	3.55	U92437
twist	3.52	M63649
latent TGF-beta-binding protein	3.46	AF022889
kruppel-like	3.16	U20344
sox-11	2.81	AF009414
TP2 homolog	2.81	X17069
NDPP-1	2.78	D10727
cd24 (differentiating neurons)	2.87	M58661
cystein-rich protein (LIM-associated)	2.62	D88793
BCNT	2.62	AB010828
cbfa-2 (core-binding factor 2)	2.55	D26532
LIM (ldb3)	2.46	AF053367
RBP (retinol-binding protein)	2.46	U63146
pontin52 (beta-catenin signaling)	2.35	AF100694
neuropilin (migration, axon guidance)	2.29	D50086
gadd45	2.28	U00937
myeloid differentiation protein	2.27	AJ001616
ZAP	2.16	AB033168
adam9	2.12	U41765
vbp-1 (tumor suppressor-binding protein)	2.12	U96760

Initial list of genes involved in driving cell differentiation that are up-regulated 2-fold and more by GDNF.

Table 4

Stem cell fate/development group

Name	Fold expression	GenBank #
numb (Notch antagonist, asymmetric cell division)	4.44	U70674
twist	3.52	M63649
amphiregulin (growth of neural stem cells)	3.45	L41352
TIAR (Primordial germ cell development)	3.17	U55861
seven-in-abstentia (Numb degradation, thus Notch activation)	2.60	Z19580/Z19581
praja-1 (stem cell marker)	2.28	U06944
mph2 (polycomb family)	2.03	AF060076

Initial list of genes that are known for determining stem cell fate that are up-regulated 2-fold or more by GDNF.

Table 5

Genes further investigated by semi-quantitative RT-PCR

Name	GenBank #	Status
numb	U70674	C
sox-11	AF009414	NC
twist	M63649	C
seven-in-abstentia	Z19580/Z19581	C
cbfa-2	D26532	NC
mph-2	AF060076	C
Fgfr2	M86441	C
TIAR	U55861	C
Ras-regulated protein	AF009246	C
socs-2	U88327	NC
srg-3	U85614	C
fisp-12	M70642	C
H19	X58196	C
egr-2	M28845	C
c-fosB	X14897	NC
A-myb	X82327	C
N-myc	M36277	C
gadd45	U00937	NC
cyclinD3	M86183	C
cyclinB1	X64713	C
cyclinE2	AF091432	NC
FGF-inducible protein	U42383	NC

From Tables 2–4, a total of 22 genes were selected for further analysis. C = confirmed. NC = not confirmed/not significant.