

# Responses to Glial Cell Line-Derived Neurotrophic Factor Change in Mice as Spermatogonial Stem Cells Form Progenitor Spermatogonia which Replicate and Give Rise to More Differentiated Progeny<sup>1</sup>

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## ABSTRACT

Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis. These cells are classically defined as a subset of morphologically defined A single ( $A_s$ ) spermatogonia, which can produce more SSCs or they can give rise to nonstem  $A_s$  cells that, upon replication, generate A paired ( $A_{pr}$ ) and then A aligned ( $A_{al}$ ) spermatogonia. These latter two cell types, along with the nonstem  $A_s$  cells, function as transit-amplifying progenitor cells. It is known that glial cell line-derived neurotrophic factor (GDNF) is essential for maintaining all of these cells, but it is unknown if or how the responses of these cells change as they progress down the pathway to differentiated type A1 spermatogonia. We address this issue by using a chemical-genetic approach to inhibit GDNF signaling in vivo and an in vitro approach to increase GDNF stimulation. We show that inhibition for 2 days suppresses replication of  $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia to an equal extent, whereas stimulation by GDNF preferentially increases replication of  $A_s$  and  $A_{pr}$  spermatogonia. We also test if inhibiting GDNF signaling causes  $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia to express Kit, an essential step in their differentiation into type A1 spermatogonia. Inhibition for 3 or 7 days produces a progressive increase in the percentages of  $A_s$ ,  $A_{pr}$ , and  $A_{al}$  undergoing differentiation, with the largest increase observed in  $A_{al}$  spermatogonia. Finally, we demonstrate that numbers of SSCs decrease more slowly than numbers of progenitor spermatogonia when GDNF signaling is inhibited. Taken together, these data suggest that there are significant changes in the responses to GDNF as SSCs give rise to progenitor spermatogonia, which replicate and gradually differentiate into type A1 spermatogonia.

*GDNF, progenitor spermatogonia, Ret, spermatogonial stem cells*

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## INTRODUCTION

The proper regulation of spermatogonial stem cells (SSCs) is required for maintaining male fertility. A substantial body of data indicates that the Sertoli cell product, glial cell line-derived neurotrophic factor (GDNF), plays an essential role in this regulation. GDNF is required for maintaining and expanding SSCs in vitro, for establishing the proper number of SSCs in vivo during puberty, and for maintaining these cells in the normal adult testis [1–4]. However, as of yet, we know little about how GDNF regulates the numbers, self-renewing replication, and differentiation of SSCs or their progeny in the normal adult testis. This information is essential to understanding how fertility is preserved in most men, but lost in others.

The classical model describing the self-renewing replication and differentiation of SSCs posits that they represent a subset of cells that are morphologically characterized as A single ( $A_s$ ) spermatogonia, solitary cells that are sparsely distributed between the somatic Sertoli cells and the basement membrane of the seminiferous tubule. These  $A_s$  spermatogonia are also characterized by their expression of genes that are required for maintaining a highly undifferentiated state. When  $A_s$  spermatogonia replicate, they either form more  $A_s$  cells and thereby self-renew, or they give rise to A paired ( $A_{pr}$ ) spermatogonia that are joined by a cytoplasmic bridge. Subsequent mitotic divisions generate chains of A aligned ( $A_{al}$ ) spermatogonia up to 32 cells in length [5]. Identification of the  $A_{pr}$  and  $A_{al}$  spermatogonia is facilitated by their expression of many of the same genes that characterize  $A_s$  cells. In the classical model, the  $A_s$  spermatogonia that undergo self-renewing replication are functionally defined as SSCs, while the  $A_s$  spermatogonia that divide and form  $A_{pr}$  spermatogonia, as well as the  $A_{pr}$  and  $A_{al}$  spermatogonia, function as transit-amplifying progenitor spermatogonia. However, the progression of  $A_s$  to  $A_{al}$  spermatogonia is not irreversible, as recent evidence shows that new  $A_s$  cells can be formed by severing the cytoplasmic bridges between individual  $A_{pr}$  and  $A_{al}$  spermatogonia [6], but the formation of type A1 spermatogonia is an irreversible step in spermatogonial development. Almost all type A1 spermatogonia are formed by the differentiation of  $A_{al}$  spermatogonia, though a small percentage is formed directly from  $A_s$  and  $A_{pr}$  spermatogonia [7].

One of the genes, the expression of which identifies spermatogonia as being highly undifferentiated, is  $GFR\alpha 1$ , the ligand-binding subunit of the GDNF receptor.  $GFR\alpha 1$  is expressed by most  $A_s$  and  $A_{pr}$  spermatogonia, as well as by many  $A_{al}$  spermatogonia in chains of four cells (in a normal, mature testis, few  $A_{al}$  spermatogonia in chains longer than four

cells express GFR $\alpha$ 1 [4]). The numbers of GFR $\alpha$ 1<sup>+</sup> A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia decrease rapidly when GDNF signaling is inhibited, indicating that all of these cells express a functional GDNF receptor and, in some way, are targets of GDNF [4]. However, it is not known whether in vivo GDNF regulates the proliferation, differentiation, and/or survival of all three of these cell types. Neither is it known whether the responses to GDNF remain the same or change as the SSCs give rise to progenitors, which replicate and gradually differentiate into type A1 spermatogonia. The experiments described in this paper address these important issues.

Our experiments examined the in vivo effects of blocking GDNF signaling on the replication and differentiation of GFR $\alpha$ 1<sup>+</sup> A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia. We compared the time course of the effect of inhibited GDNF signaling on numbers of these cells with the relative numbers of cells functionally defined as SSCs or as progenitor spermatogonia. We blocked GDNF signaling in vivo by use of a chemical-genetic approach that allows for the reversible inhibition of the activity of Ret, the tyrosine kinase subunit of the GDNF receptor [4]. While Ret interacts with GFR $\alpha$ 1 as well as GFR $\alpha$ 2, GFR $\alpha$ 3, and GFR $\alpha$ 4, knockouts of these latter three GFR $\alpha$  family members have no effect on male fertility [8–10]. Furthermore, the inhibition of Ret activity in the adult has no significant effect on the histology of any organ except the testis [4]. Therefore, chemical inhibition of Ret activity in the testis is equivalent to inhibition of GDNF signaling. To complement the effects of inhibition of GDNF signaling on replication of A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia, we also examined the effects of increased GDNF concentration achieved by culturing seminiferous tubules in the presence of excess GDNF.

In this paper, we report that altering GDNF signaling to morphologically defined A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia affects both their replication and differentiation. However, stimulation by GDNF has less of an effect on replication of A<sub>al</sub> spermatogonia than the other two cell types, while inhibition of GDNF signaling has the greatest effect on the differentiation of A<sub>al</sub> spermatogonia. Finally, our data support the conclusion that, when GDNF signaling is inhibited, numbers of functionally defined SSCs decrease more slowly than numbers of progenitor spermatogonia.

## MATERIALS AND METHODS

### *Animals and Their Treatment*

The chemical-genetic approach we used to block GDNF signaling in vivo employs mice that carry a point mutation (V805A) in the ATP-binding site of Ret, the tyrosine kinase subunit of the GDNF receptor. This mutation has no effect on normal Ret function, but it does allow this kinase to reversibly bind with high affinity to a bulky ATP competitive inhibitor, INA-PP1 [4].

Homozygous Ret (V805) mice (C57BL/6J background) were obtained from our animal colony. To inhibit GDNF signaling, male Ret (V805) mice, 90–120 days of age, were given daily subscapular injections of 43.7 mg/kg of the ATP competitive inhibitor, INA-PP1-HCl, which was synthesized and prepared for injection, as previously described [4] (hereafter, INA-PP1-HCl is referred to as INA-PP1). While this dose is lower than that used previously (63.5mg/kg) [4], dose-response analysis showed that the effects of these two doses were similar (Supplemental Fig. S1; Supplemental Data are available online at [www.biolreprod.org](http://www.biolreprod.org)). To study the effect of inhibiting GDNF signaling on cell replication, mice were injected i.p. with 20 mg/kg of the thymidine analog, 5-ethynyl-2'-deoxyuridine (EdU; Invitrogen, Carlsbad, CA) 24 h prior to tissue collection. C57BL/6J mice (100–120 days old) were purchased from Jackson Laboratories (Bar Harbor, MA) and used as the source of seminiferous tubules for culture. The Johns Hopkins University Institutional Animal Care and Use Committee approved the use of mice for all experiments.

### *Immunocytochemistry, Microscopy, and Image Analysis*

A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia were identified by their expression of GFR $\alpha$ 1, the ligand-binding domain of the receptor for GDNF, a growth factor that is required for the development and maintenance of SSCs in vivo [2–4]. We used previously described, immunocytochemical methods to identify GFR $\alpha$ 1<sup>+</sup> cells in intact seminiferous tubules that had been fixed in 4% paraformaldehyde in PBS [4]. In one experiment, we also used previously described methods to detect cells that express a second marker of undifferentiated spermatogonia, ZBTB16 [4]. EdU incorporation was detected using Alexafluor-555-Azide and Click-Chemistry (Invitrogen). A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia that had reached the final steps in their differentiation into type A1 spermatogonia were identified by their coexpression of GFR $\alpha$ 1<sup>+</sup> and the cell surface receptor, Kit, which is required for undifferentiated spermatogonia to form type A1 spermatogonia [5, 11]. For these studies, tubules were fixed in 10% methanol and 10% paraformaldehyde in PBS (1 h at 4°C), washed, and incubated for 1 h with 1% BSA in PBS and then overnight at 4°C with 1:50 rat anti-mouse Kit (ACK2; Millipore Corp, Billerica, MA) plus 1:100 anti-GFR $\alpha$ 1 (R&D Systems, Minneapolis, MN). The following day, tubules were washed with 1% BSA and 0.1% Triton X-100 in PBS (PBS-BT), and then incubated for 2 h at room temperature in 1:200 dilutions of AlexaFluor 555 donkey anti-goat IgG and AlexaFluor 488 donkey anti-rat IgG (Invitrogen). Tubules were mounted in VectaShield (Vector Labs, Burlingame, CA) after washing in PBS-BT. Negative controls (replacement of primary antibodies with nonimmune IgG) were run for all experiments.

Whole mounts of tubules isolated from animals treated in vivo with INA-PP1 or vehicle were imaged using a Zeiss LSM 710 Confocal Microscope equipped with argon and helium-neon lasers. Depending on the experiment, optical sections of 1.8 or 2.2  $\mu$ m were captured. Laser strengths were determined empirically at the beginning of the analysis of each experiment and then were used for analysis of all samples. Cultured seminiferous tubules and tubules analyzed for ZBTB16<sup>+</sup> cells were imaged as previously described [4]. GFR $\alpha$ 1<sup>+</sup> spermatogonia were classified as A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia, depending on whether they were not connected to another cell or were connected to one or multiple GFR $\alpha$ 1<sup>+</sup> cells, respectively.

iVision software (Biovision Technologies, Exton, PA) was used to determine the numbers of GFR $\alpha$ 1<sup>+</sup> cells/mm<sup>2</sup> of tubule surface and to measure the diameter of seminiferous tubules. We also used this software to measure the fraction of GFR $\alpha$ 1<sup>+</sup> cells that were either EdU<sup>+</sup> or Kit<sup>+</sup>. Relative levels of GFR $\alpha$ 1 expressed per cell were estimated by measuring fluorescence intensity. All measurements of fluorescence intensity were corrected for the background fluorescence of each image.

### *Morphological Analyses to Evaluate Loss of SSCs and Progenitor Spermatogonia and to Determine the Total Numbers of GFR $\alpha$ 1<sup>+</sup> A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> Spermatogonia in an Adult Mouse*

We used a previously described protocol to estimate the relative numbers of SSCs that remained when Ret (V805A) mice were treated for 3, 5, 7, or 9 days with INA-PP1 [4]. At 45 days after the first injection, testes were collected and analyzed histologically. The effect of treatment on relative numbers of SSCs was defined by the fraction of all tubules in testis cross sections that lacked all spermatogonia, spermatocytes, and spermatids (up to step 14; a detailed description of this protocol and a review of data that support its use for estimating loss of SSCs are provided in Supplemental *Materials and Methods*).

We used the same histological sections described above to determine if 5, 7, or 9 days of inhibited GDNF signaling caused a loss of progenitor spermatogonia prior to loss of SSCs. Loss of progenitor spermatogonia, but not SSCs, would result 45 days after the first injection in a transient decrease in numbers of spermatogenic cells, but not in maturation depletion. Therefore, we determined the numbers of pachytene spermatocytes and step-1 to step-8 spermatids per Sertoli cell. To exclude the effects of loss of SSCs on this analysis, we excluded from our analysis all tubules that lacked all germ cells except step-15 and step-16 spermatids (a detailed description of this protocol is provided in Supplemental *Materials and Methods*).

One of the goals of our experiments was to compare the total numbers of GFR $\alpha$ 1<sup>+</sup> A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia in control testes and in testes after 7 days of inhibited GDNF signaling. This goal was met by measuring the numbers of these cells per square millimeter of tubule surface, as described above, and multiplying this cell density by the total surface area of seminiferous tubules in each testis. To determine surface area, we used iVision software to measure the volume densities of seminiferous tubules in 1- $\mu$ m, plastic-embedded toluidine blue-stained sections of testes of Ret (V805A) mice. We also measured the testis weights and diameters of tubules from control and treated mice. From these data, we calculated total surface area of the

seminiferous tubules in control and treated testes. Multiplication of total surface area by numbers of  $GFR\alpha1^+$   $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia per square millimeter of tubule surface gave total numbers of these cells per testis. Details of the stereological methods are described in the Supplemental *Materials and Methods*.

### Seminiferous Tubule Cultures

To measure the effect of increased GDNF signaling on replication of  $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia, seminiferous tubules were cultured for 1–3 days in the presence or absence of 250 pg/ml of recombinant human GDNF (R&D systems). An equal volume of PBS was added to control cultures. Mature seminiferous tubules were isolated by microdissection and then encapsulated in 8  $\mu$ l of 1% alginate acid (Sigma-Aldrich, St. Louis, MO) by emersion in 500  $\mu$ l 1.5%  $CaCl_2$ , 0.9% PBS, for 4 min. Tubules were encased in alginate, because preliminary experiments showed that, when GDNF was added to tubules that were not encapsulated, almost all  $GFR\alpha1^+$  spermatogonia migrated into the culture medium, an event that was prevented by encapsulation. Subsequent to encapsulation, the tubules were washed for 4 min in 500  $\mu$ l of Dulbecco modified Eagle medium/F-12 (Invitrogen) and cultured on inserts (PICM03050; Millipore) in previously described, hormone-supplemented medium [12]. GDNF was added to one-half of the cultures. At 24 h prior to collection of tubules, EdU was added at a final concentration of 2  $\mu$ M.

### Statistical Analysis

Statistical analyses were performed using StatView (SAS Institute, Cary, NC). Data obtained from histological analyses of testes were analyzed by ANOVA. All other data were analyzed by nested ANOVA. All post hoc comparisons were performed using Fisher PLSD (protected least significant difference) test. Statistically significant differences were defined as  $P < 0.05$ .

## RESULTS

### The Effects of Altered GDNF Signaling on Replication of $GFR\alpha1^+$ $A_s$ , $A_{pr}$ , and $A_{al}$ Spermatogonia

We first examined whether the effect of GDNF on cell replication changed as  $GFR\alpha1^+$   $A_s$  gave rise to  $A_{pr}$  and then to  $A_{al}$  spermatogonia. To address this issue in vivo, Ret (V805A) mice were treated for 2 or 3 days with 1NA-PP1 or vehicle. At 24 h prior to tissue collection, the mice were injected with the thymidine analog, EdU. Figure 1A shows a representative 1.8- $\mu$ m optical section through the base of a tubule of a control mouse.  $GFR\alpha1$  (green) is concentrated in the plasma membranes of  $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia; EdU (red) is present in some of the nuclei, marking these cells as having completed most or all replicative DNA synthesis during the last 24 h of the experiment. Note that four of the  $A_{al}$  spermatogonia (see cells marked by asterisks) express a lower level of  $GFR\alpha1$ , raising the possibility that, on average,  $A_{al}$  spermatogonia express a reduced level of the ligand-binding domain of the GDNF receptor. This possibility was examined in detail by quantifying fluorescence intensities of all  $GFR\alpha1^+$   $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia on tubules of control and treated animals. Results show that  $A_{al}$  spermatogonia express significantly less  $GFR\alpha1$  than  $A_s$  cells (Supplemental Fig. S2). The amount of  $GFR\alpha1$  expressed by  $A_{pr}$  spermatogonia is intermediate to the amounts expressed by the other two cell types.

To quantify the effect of inhibiting GDNF signaling on replication of  $GFR\alpha1^+$   $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia, we determined the fraction of  $GFR\alpha1^+$   $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia that had incorporated EdU. Data (Fig. 1B) show that the progression of  $A_s$  to  $A_{al}$  spermatogonia is associated with a significant increase in the fraction of cells that are replicating. Furthermore, inhibition of GDNF signaling for 2 or 3 days reduced replication of  $GFR\alpha1^+$   $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia; on Day 3, replication of these cells was reduced to 19%, 15%, and 25% of controls, respectively. However, consistent with the fact that these cells have long cell cycle times, there was no

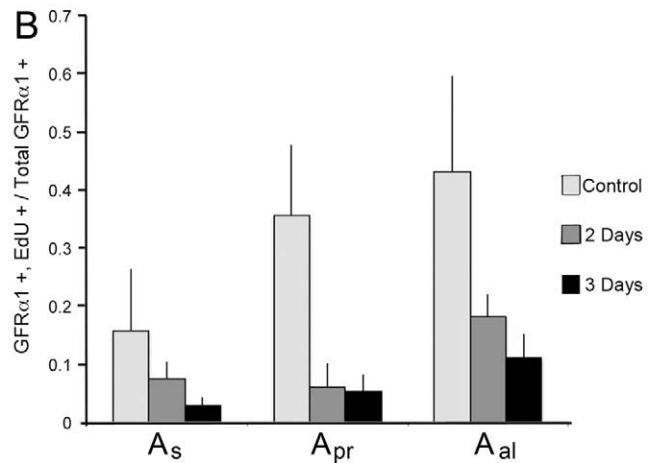
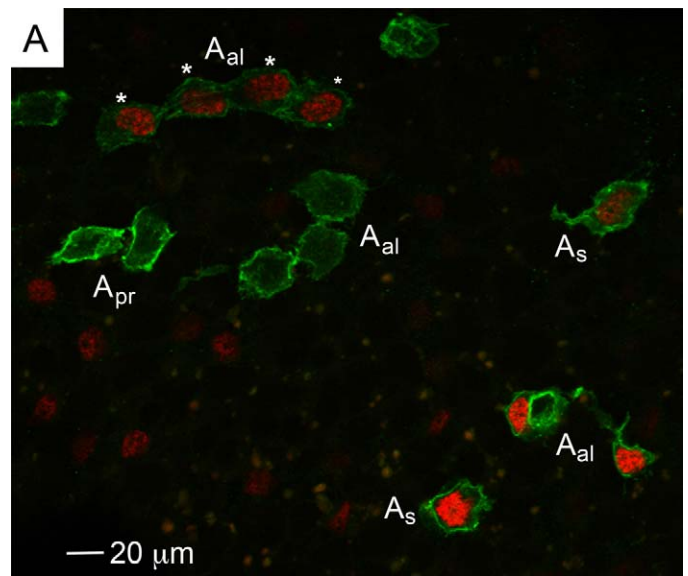


FIG. 1. The in vivo effect of inhibition of GDNF signaling on replication of  $GFR\alpha1^+$   $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia. **A**) Identification of replicating,  $GFR\alpha1^+$  spermatogonia on whole mounts of seminiferous tubules of control mice.  $GFR\alpha1$  was detected by immunocytochemistry (green), and replicating cells were detected by their incorporation of the thymidine analog, EdU (red).  $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia are labeled on this figure. The four cells marked by asterisks are replicating  $A_{al}$  spermatogonia that express low levels of  $GFR\alpha1$ . Optical sections obtained by confocal microscopy are 1.8- $\mu$ m thick. **B**) In vivo effect of inhibition of GDNF signaling for 2 or 3 days on the replication of  $GFR\alpha1^+$   $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia. Data (mean + SEM;  $n = 3$ /group) are presented as the fraction of  $GFR\alpha1^+$   $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia that incorporated EdU during the last 24 h of the experiment. ANOVA (cell type nested within treatment) shows that there was a significant effect of inhibiting GDNF signaling on replication of  $GFR\alpha1^+$   $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia. Post hoc comparisons demonstrated that, within a 24-h period, a lower fraction of  $A_s$  spermatogonia replicated than  $A_{al}$  spermatogonia.

significant effect of inhibition of GDNF signaling for 3 days on cell numbers [13] (Supplemental Fig. S3).

While inhibition of GDNF signaling had similar effects on the replication of  $GFR\alpha1^+$   $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia, this experiment left open the possibility that there were differences between the three cell types in their response to increased GDNF stimulation. This issue is important, because the expression of GDNF by murine Sertoli cells increases as their adjacent spermatogenic cells progress from stage VI to stage XII of the cycle of the

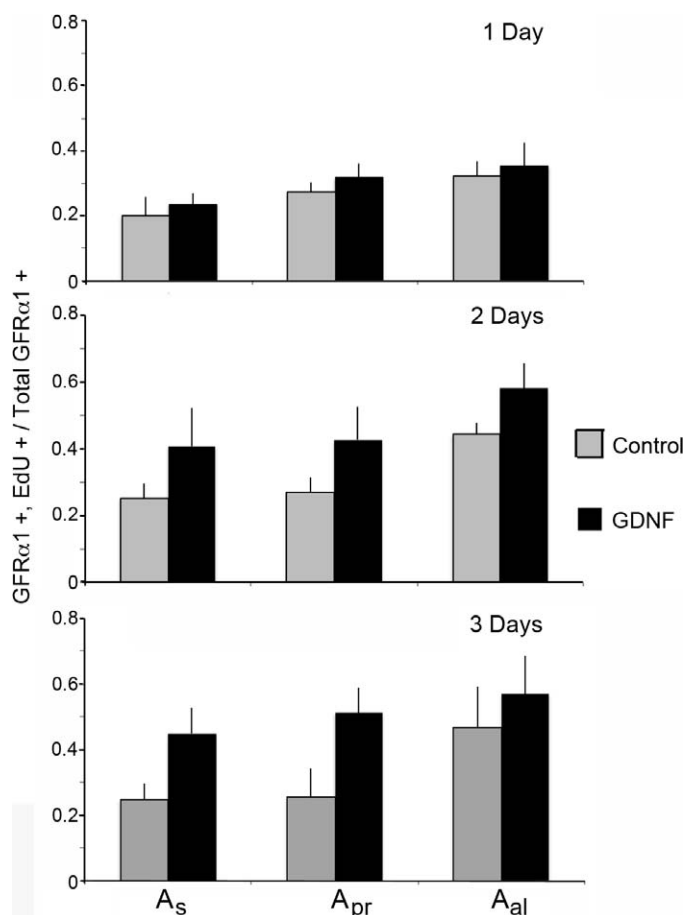


FIG. 2. Replication of GFR $\alpha$ 1<sup>+</sup> A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia on seminiferous tubules cultured in medium containing 250 pg/ml GDNF or vehicle (PBS; control) for 1, 2, or 3 days. EdU was present in the medium for the last 24 h of culture. Data (mean + SEM; n = 5/group) are expressed as the fraction of GFR $\alpha$ 1<sup>+</sup> A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia that incorporated EdU. ANOVA (cell type nested within days nested within treatment) shows that there were significant effects of treatment, of cell type, and of days on replication of the GFR $\alpha$ 1<sup>+</sup> spermatogonia. Furthermore, there was a significant interaction between the effects of days and treatment.

seminiferous epithelium, and because replication of A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia primarily occurs at stages of highest GDNF expression [7, 14, 15]. Therefore, to determine if increased GDNF concentration would stimulate replication of GFR $\alpha$ 1<sup>+</sup> A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia, and to test if there were differences between the three cell types in their response to increased GDNF concentration, we cultured tubules in the presence or absence of 250 pg/ml (21.6 pM) of recombinant GDNF for 1, 2, or 3 days and added EdU during the last 24 h of the experiment. Undifferentiated spermatogonia in control tubules were exposed to the GDNF secreted by their Sertoli cells. The published K<sub>d</sub> of GDNF for its receptor predicts that the amount of recombinant GDNF added to the cultures was sufficient to saturate approximately one-half of available receptors [16, 17]. Thus, adding this amount of GDNF to cultured tubules should mimic an effect on A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia of a physiologically relevant, stage-specific rise in GDNF production by Sertoli cells.

The results shown in Figure 2 reveal no significant effect of incubating tubules in GDNF for one day. However, incubation with GDNF for 2 or 3 days increases the

replication of GFR $\alpha$ 1<sup>+</sup> A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia by approximately 60%, 60%, and 31%, respectively. The reduced replication of A<sub>al</sub> spermatogonia in response to added GDNF was evident in a statistical interaction between treatment and cell type. We therefore conclude that an increase in GDNF concentration in a seminiferous tubule stimulates the replication of all three cell types, but that the A<sub>al</sub> spermatogonia are less responsive to this stimulus than A<sub>s</sub> and A<sub>pr</sub> cells

#### *The Effects of Inhibited GDNF Signaling on Differentiation of GFR $\alpha$ 1<sup>+</sup> A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> Spermatogonia*

An obligatory step in the differentiation of A<sub>s</sub>, A<sub>pr</sub>, or A<sub>al</sub> spermatogonia into type A1 spermatogonia is the expression of Kit, a cell surface receptor that mediates the responses of these cells to the Sertoli cell product, Kit ligand [5]. Therefore, we evaluated the effect of inhibiting GDNF signaling for 3 or 7 days on differentiation of GFR $\alpha$ 1<sup>+</sup> A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia by determining the fraction of these cells that also expressed Kit.

Figure 3 shows representative 2.2- $\mu$ m optical sections of the surface of seminiferous tubules of a control mouse, the spermatogonia of which express GFR $\alpha$ 1 alone, Kit alone, or both cell surface receptors. To facilitate interpretation, we examined separate images of the green (Kit; Fig. 3, A and E), red (GFR $\alpha$ 1<sup>+</sup>; Fig. 3, C and F) and combined channels (Fig. 3, B and D). Figure 3 (A–C) shows two A<sub>pr</sub> spermatogonia that do not express Kit (see black-on-white arrowheads) and a chain of five GFR $\alpha$ 1<sup>+</sup> A<sub>al</sub> spermatogonia. Three of the cells in this chain also express Kit (white arrowheads), while the other two cells express reduced levels of GFR $\alpha$ 1, but do not express Kit (white arrows). Figure 3 (D–F) shows a single GFR $\alpha$ 1<sup>+</sup>, Kit<sup>+</sup> spermatogonium (arrow) in the middle of a chain of cells, all the rest of which are GFR $\alpha$ 1<sup>-</sup> and Kit<sup>+</sup>. In our analyses, GFR $\alpha$ 1<sup>+</sup>, Kit<sup>+</sup> cells, such as the one in the middle of this chain, were defined as A<sub>al</sub> spermatogonia.

Figure 4A quantitatively describes the progressive effects of inhibiting GDNF signaling for 3 or 7 days on the differentiation of GFR $\alpha$ 1<sup>+</sup> A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia. In vehicle-treated mice, the fraction of GFR $\alpha$ 1<sup>+</sup> A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia that also expressed Kit was approximately 0.008, 0.02, and 0.19, respectively. After 3 days of inhibited GDNF signaling, these fractions increased to approximately 0.08, 0.2, and 0.5, and, after 7 days, the fractions were increased to 0.4, 0.56, and 0.95, respectively. Nested ANOVA of these data proved that there were significant effects of inhibition of GDNF signaling and of cell type (A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia) on differentiation, and that the effect of inhibition of GDNF signaling differed between cell types. The fact that, after 7 days of inhibited GDNF signaling, 95% of A<sub>al</sub> GFR $\alpha$ 1<sup>+</sup> spermatogonia were also Kit<sup>+</sup> predicted that, after 7 days of inhibition, GFR $\alpha$ 1<sup>+</sup> A<sub>al</sub> spermatogonia would exhibit the greatest absolute decrease in cell numbers. Consistent with this prediction, while treatment of Ret (V805A) mice for 7 days with 1NA-PP1 significantly reduced numbers of GFR $\alpha$ 1<sup>+</sup> A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia, the greatest absolute decrease occurred with A<sub>al</sub> spermatogonia, which were reduced from 61 cells/mm<sup>2</sup> of tubule surface area (vehicle-treated) to 16 cells/mm<sup>2</sup> (1NA-PP1-treated) (Fig. 4B). This same trend was evident qualitatively when we used expression of another marker, ZBB16, to identify A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia (Supplemental Fig. S4).

Nested ANOVA analysis verified that the inhibition of GDNF signaling reduced the numbers of all three GFR $\alpha$ 1<sup>+</sup> cell types, and that the effect of inhibiting GDNF signaling differed

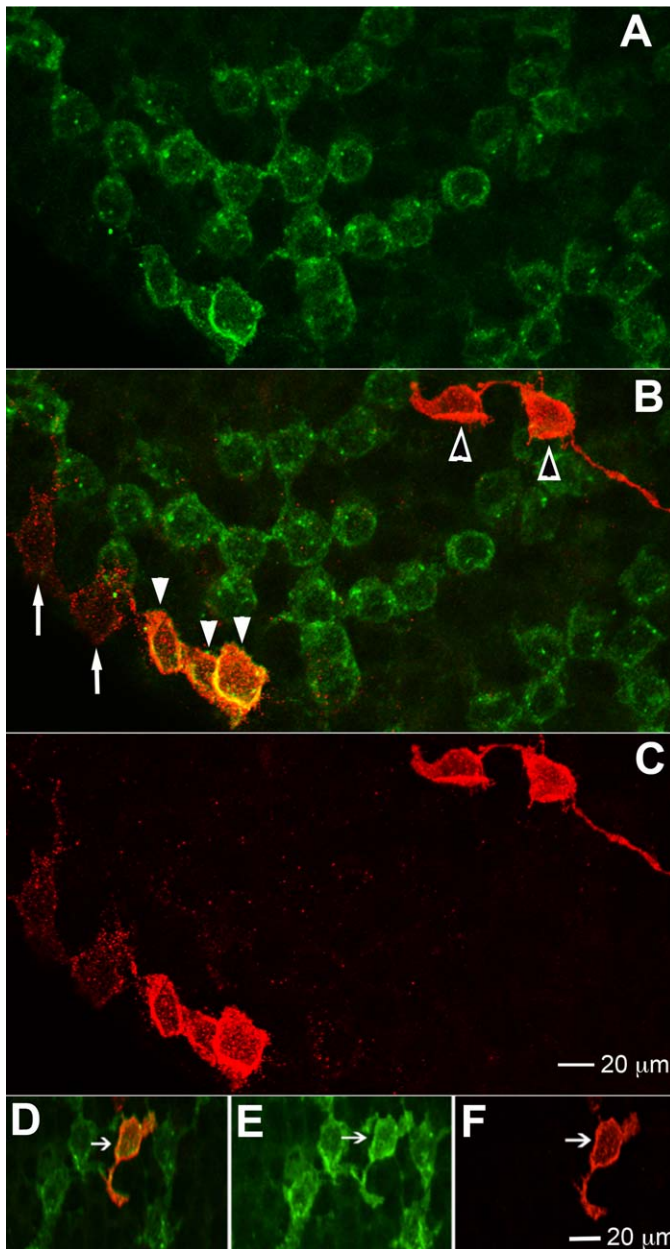


FIG. 3. Identification by immunocytochemistry and confocal microscopy of spermatogonia that express both GFR $\alpha$ 1 and Kit. The cells shown here are present on whole mounts of seminiferous tubules of control mice. The channels from the confocal microscope are presented separately and together. Optical sections are 2.2- $\mu$ m thick. **A** and **E** Kit alone (green). **C** and **F** GFR $\alpha$ 1 alone (red). **B** and **D** GFR $\alpha$ 1 and Kit together. In **B** and **D**, costained cells are orange. **B**) Black-on-white arrowheads point to a pair of GFR $\alpha$ 1<sup>+</sup>/Kit<sup>-</sup> cells. White arrowheads point to three GFR $\alpha$ 1<sup>+</sup>/Kit<sup>+</sup> cells in a chain of five cells. White arrows point to two cells in the same chain that express low levels of GFR $\alpha$ 1, but do not express Kit. **D**) A GFR $\alpha$ 1<sup>+</sup>/Kit<sup>+</sup> spermatogonium in the middle of a chain of GFR $\alpha$ 1<sup>-</sup>/Kit<sup>+</sup> cells. When samples were incubated with nonimmune IgGs rather than primary antibodies, and images were captured under identical conditions as those shown here, the images were black or showed only light punctate background staining.

between cell types. Subsequent experiments showed that this differential effect of inhibited GDNF signaling on A<sub>al</sub> spermatogonia continued when GDNF was inhibited for 9 days, as the few remaining GFR $\alpha$ 1<sup>+</sup> cells were predominantly A<sub>s</sub> and A<sub>pr</sub> spermatogonia (Supplemental Fig. S5). This particular effect on A<sub>al</sub> spermatogonia was consistent with

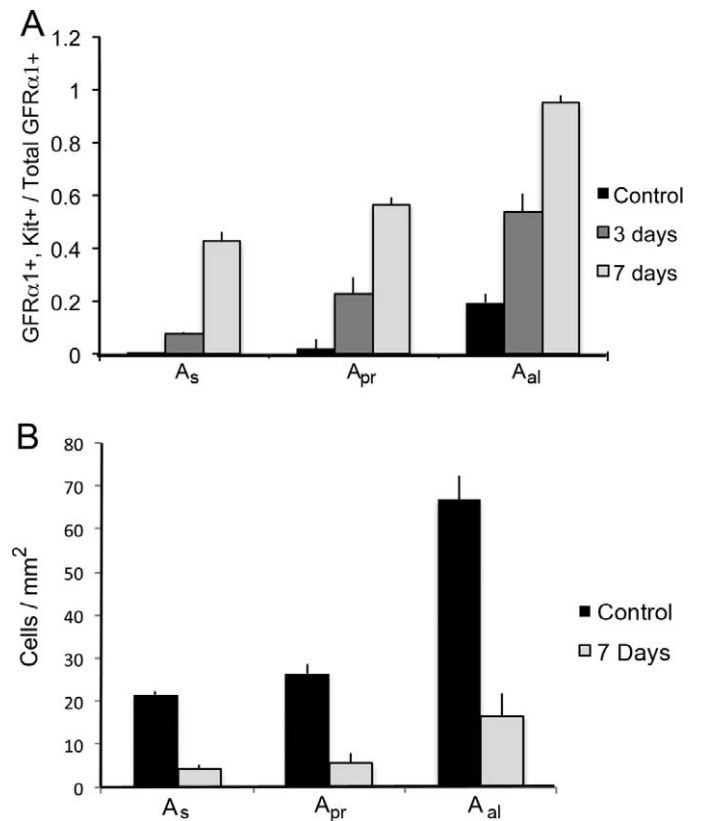


FIG. 4. Effect of inhibition of GDNF signaling for 3 or 7 days on differentiation of GFR $\alpha$ 1<sup>+</sup> A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia and on the numbers of these cells. **A**) Effects of 3 or 7 days of inhibited GDNF signaling on differentiation. Data (mean + SEM) are expressed as the fraction of GFR $\alpha$ 1<sup>+</sup> A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia that express Kit, and thus, are differentiating. After 7 days of inhibition, 43%, 57%, and 95% of the A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia, respectively, were differentiating. ANOVA (cell type nested within treatment) reveals significant effects of treatment and of cell type on differentiation. Furthermore, there was a significant interaction between the effects of cell type and treatment. **B**) Numbers of GFR $\alpha$ 1<sup>+</sup> A<sub>s</sub>, A<sub>pr</sub>, and A<sub>al</sub> spermatogonia per square millimeter of tubule surface in mice treated with vehicle or with 1NA-PP1 for 7 days. Data are expressed as mean + SEM (n = 3 mice per group). ANOVA (cell type nested within treatment) shows significant effects of treatment and of cell type, and a significant interaction between the effects of cell type and treatment. Post hoc comparisons showed that a significantly higher fraction of GFR $\alpha$ 1<sup>+</sup> A<sub>pr</sub> spermatogonia expressed Kit than of GFR $\alpha$ 1<sup>+</sup> A<sub>s</sub> spermatogonia, and that a significantly higher fraction of GFR $\alpha$ 1<sup>+</sup> A<sub>al</sub> spermatogonia expressed Kit than GFR $\alpha$ 1<sup>+</sup> A<sub>pr</sub> spermatogonia.

our observation that these cells were missing when GDNF signaling was inhibited for 11 days [4].

We also tested the hypothesis that inhibition of GDNF signaling increased apoptosis by A<sub>s</sub>, A<sub>pr</sub>, or A<sub>al</sub> spermatogonia. However, in both control and treated mice, less than 3% of these cells expressed the marker of apoptosis, activated caspase 3, and inhibition of GDNF signaling did not significantly increase this percentage. As this percentage was very low, and as we did not detect a significant increase in apoptosis with treatment, we conclude that apoptosis is not a significant regulator of numbers of GFR $\alpha$ 1<sup>+</sup> spermatogonia, and that this process is not increased when GDNF signaling is inhibited (see Supplemental Materials and Methods and Fig. S6).

TABLE 1. Numbers ( $\pm$ SEM) per testis of  $GFR\alpha1^+$   $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia in control Ret (V805A) mice and in mice that experienced 7 days of inhibited GDNF signaling.

Type of spermatogonia	Control mice	Mice with inhibited GDNF signaling
$A_s$	24 427 $\pm$ 2764	5035 $\pm$ 1256
$A_{pr}$	31 226 $\pm$ 6133	6379 $\pm$ 2888
$A_{al}$	75 443 $\pm$ 3386	18 886 $\pm$ 5972
Total	131 137 $\pm$ 12 284	30 301 $\pm$ 10 117

*Estimates of the Absolute Numbers of  $GFR\alpha1^+$  Cells in the Testes of Control Mice and Mice Treated for 7 Days with 1NA-PP1*

Recent reports indicate that there are between 3000 and 6000 functional SSCs in a mouse testis [18], and it is generally assumed that these cells represent but a small fraction of the total pool of the cells that are morphologically defined as  $GFR\alpha1^+$   $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia. To date, however, this assumption has not been tested. To do so, we used stereology to determine the total surface area of the seminiferous tubules in each testis and multiplied these areas by numbers of  $GFR\alpha1^+$   $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia per square millimeter of tubule surface (see Supplemental Table S1 for stereological data). This analysis revealed that there were, on average, 131 137  $GFR\alpha1^+$  spermatogonia in each testis of a control mouse; 24427 of these were  $A_s$  cells (Table 1). Inhibition of GDNF signaling for 7 days resulted in a 77% reduction in total numbers of  $GFR\alpha1^+$  spermatogonia. On average, testes of treated mice contained 5035  $GFR\alpha1^+$   $A_s$  spermatogonia.

*An Analysis of the Effects of Varying Periods of Inhibited GDNF Signaling on Relative Numbers of SSCs and Progenitor Spermatogonia*

The fact that inhibition of GDNF signaling for 7 days resulted in a 77% reduction in numbers of  $GFR\alpha1^+$  spermatogonia raised the question of whether a significant number of SSCs were lost or whether these cells were more resistant to loss of GDNF signaling than progenitor spermatogonia. Furthermore, when stem cells or progenitor cells were lost, what was the time course of this loss? To answer these

questions, Ret (V805A) mice were injected with vehicle or with 1NA-PP1 for 3, 5, 7, or 9 days and testes collected 45 days after the first injection. To estimate the percentage SSCs that had had been lost by the end of treatment with 1NA-PP1, we determined the percentage of cross-sections of seminiferous tubule that, on day 45 of the experiment, were devoid of all spermatogonia, spermatocytes, and step-1 to step-14 spermatids. Results (Fig. 5A) show that, in mice treated for 7 days with 1NA-PP1, there was no increase in this percentage. In fact, 87% of the tubules contained at least three generations of spermatogenic cells, including step-1 to step-12 spermatids (Supplemental Table S2). Thus, the process of spermatogenesis was not completely interrupted in the 7-day treatment group. In contrast, when animals that were treated for 9 days and testes examined on Day 45 of the experiment, 50% of the seminiferous tubules were depleted of all spermatogenic cells, with the possible exception of step-15 or step-16 spermatids. Taken together, these data suggest that inhibition of GDNF signaling for 9 days, but not for 7 days, led to a significant and substantial reduction in numbers of SSCs. This also suggests that the  $GFR\alpha1^+$   $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia that were lost during 7 days of inhibited GDNF signaling had been functioning as progenitor spermatogonia. If they were, there would be a subsequent reduction in numbers, but not a complete depletion of spermatogenic cells. To test this prediction, we enumerated round spermatids and pachytene spermatids per Sertoli cell in randomly selected tubules. We excluded from this analysis tubules that were devoid of spermatogonia, spermatocytes and step-1 to step-14 spermatids in order to avoid including the effect of loss of SSCs in these results (as noted above, only samples from animals treated for 9 days with 1NA-PP1 had a significant number of such tubules). Results (Fig. 5B) show that, when GDNF signaling was inhibited for 7 days and samples collected on Day 45 of the experiment, the numbers of round spermatids and pachytene spermatocytes per Sertoli cell were significantly reduced by 59% and 50%, respectively.

## DISCUSSION

The results of these studies show that numbers of  $GFR\alpha1^+$  or  $ZBTB16^+$   $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia were reduced when GDNF signaling was inhibited for 7 days. This loss resulted, 38 days later, in reduced numbers of pachytene spermatocytes and round spermatids, but not in maturation depletion of seminif-

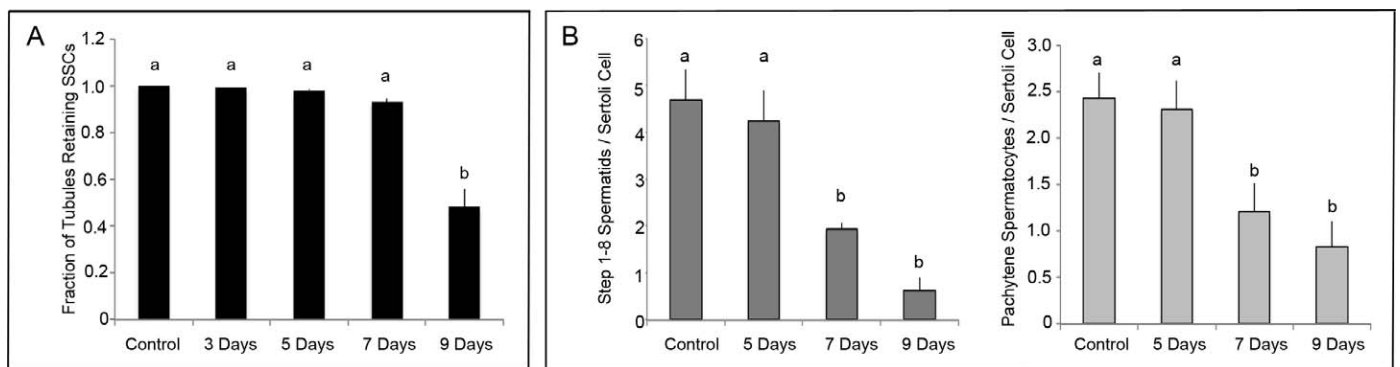


FIG. 5. An analysis of the effects of varying periods of inhibited GDNF signaling on relative numbers of SSCs and progenitor spermatogonia. **A)** Estimate of the fraction of SSCs that remained immediately after inhibition of GDNF signaling for 3, 5, 7, or 9 days. Loss of SSCs was estimated 45 days after the first injection. Data (mean  $\pm$  SEM;  $n = 3$ ) are presented as the fraction of seminiferous tubule cross-sections that lacked spermatogonia, spermatocytes, and step-1 to step-14 spermatids. Means with different superscripts differ statistically. **B)** Evidence for the loss of spermatogonial progenitors that occurred as a consequence of inhibition of GDNF signaling for 5, 7, and 9 days. Data (mean  $\pm$  SEM;  $n = 3$ /group) are expressed as the numbers of step-1 to step-8 spermatids per Sertoli cell and the numbers of pachytene spermatocytes per Sertoli cell. Loss of these cells was established 45 days after the first injection. Means with different superscripts differ statistically.

erous tubules. However, such depletion occurred when GDNF signaling was inhibited for 9 days. Taken together, these data indicate that numbers of SSCs were reduced when GDNF signaling was inhibited for 9 days, but not when it was inhibited for 7 days. This suggests that, during 9 days of inhibited GDNF signaling, loss of progenitor spermatogonia began before loss of SSCs. Thus, we interpret these data as indicating that SSCs are more resistant to loss of GDNF signaling than progenitor spermatogonia.

While inhibition of GDNF signaling for 7 days led, at the end of treatment, to a 74% decrease in numbers of  $GFR\alpha 1^+$  spermatogonia, 38 days later, numbers of pachytene spermatocytes and round spermatids per Sertoli cell were reduced only by 53% and 61%, respectively. Why was the apparent loss of  $GFR\alpha 1^+$  spermatogonia greater than the subsequent decrease in numbers of pachytene spermatocytes and round spermatids? One possible explanation is that, despite the high sensitivity of confocal microscopy, we did not detect some cells that expressed very low levels of  $GFR\alpha 1$ , causing us to overestimate the loss of  $GFR\alpha 1^+$  cells. A second possibility is that the numbers of spermatocytes and spermatids reflected both the effects of the loss of the  $GFR\alpha 1^+$  spermatogonia that functioned as progenitor cells as well as the partial rebuilding of their numbers when GDNF signaling was re-established. This suggestion is based on the following considerations. Totals of 17 and 25.5 days are required for type A1 spermatogonia to give rise to the pachytene spermatocytes and round spermatids, respectively, in a stage VII seminiferous tubule [19]. As we collected samples 38 days after the last day of treatment with 1NA-PP1, the pachytene spermatocytes and round spermatids that we counted were the descendants the type A1 spermatogonia that were formed approximately 21 and 12.5 days after GDNF signaling was re-established. That re-establishment would be expected to cause sequential expansion of the numbers of progenitor spermatogonia, type A1 spermatogonia, and then the more mature spermatogenic cells. This expansion would cause the decrease in numbers of pachytene spermatocytes and round spermatids to be less than the decrease in the numbers of progenitor spermatogonia that were present in the testes immediately after 7 days of treatment. Nonetheless, our data support the conclusion that inhibition of GDNF signaling for 7 days diminishes the numbers of functional progenitor spermatogonia.

The reasons that inhibition of GDNF signaling causes loss of progenitor spermatogonia and SSCs are evident in our analysis of the replication and differentiation of morphologically defined  $GFR\alpha 1^+$   $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia. In vivo, inhibition of GDNF signaling suppresses the replication of these cells to a similar extent. However, inhibition of GDNF signaling has the greatest effect on the fraction of  $A_{al}$  spermatogonia that are differentiating and on their absolute numbers. As cells that are morphologically defined as  $A_{al}$  spermatogonia are considered functionally to be progenitor cells, these results partially explain why progenitor spermatogonia respond more rapidly to loss of GDNF signaling than SSCs. However, eventually, the inhibition of GDNF signaling leads to a loss of SSCs, and we propose that this loss occurs because the SSCs cease to replicate and then differentiate into type A1 spermatogonia.

Our conclusion that in vivo SSCs are more resistant than progenitor spermatogonia to loss of GDNF signaling is consistent with in vitro studies that show that, when GDNF is withdrawn from cultures of spermatogonia enriched in SSCs, the total number of cells in the cultures decreased more rapidly than the numbers of functional SSCs, as assayed by their ability to restore spermatogenesis when transplanted into a germ cell-

deficient testis [20]. However, those authors also reported that in vitro GDNF caused the spermatogonia to aggregate into clusters, and that this aggregation suppressed the numbers of functional stem cells. Additionally, those authors reported that the clusters disaggregated when GDNF was removed from the culture medium. Thus, the apparent retention of SSCs in the absence of GDNF stimulation could have been due either to the enhanced resistance of SSCs to loss of GDNF signaling or to the disaggregation of the clusters allowing a greater fraction of the remaining cells to express the characteristics of SSCs. Thus, those results, while tantalizing, did not resolve the issue of whether SSCs have an enhanced resistance to loss of GDNF signaling. However, the confounding factor of the formation and disaggregation of cell clusters did not occur in our in vivo experiments, which provide direct proof that SSCs are more resistant to loss of GDNF signaling than their progeny, the progenitor spermatogonia.

Our conclusion that in vivo SSCs are more resistant to loss of GDNF signaling than spermatogonial progenitors raises the issue of the biological bases for the stem cell resistance. First, it is possible that, during the period that GDNF signaling is experimentally suppressed, the loss of spermatogonial progenitors causes Sertoli or other cells to increase their expression of growth factors, such as CXCL12, bFGF, and CSF1, all of which promote self-renewing replication of SSCs cells in vitro [20–24]. Second, it is possible that the stem and progenitor cells are equally responsive to these other growth factors, but that the concentrations of these growth factors are highest in the stem cell niche. This suggestion is consistent with the proposition that these niches face the interstitium and, thus, Leydig cells and blood vessels, both of which are potential sources of important growth factors [25]. An alternative possibility is that, because stem cells are the least differentiated cells in the spermatogenic lineage, they must progress through more steps, and thus require more time than progenitors to differentiate into type A1 spermatogonia. This suggestion is consistent with our observation that, in control mice, the percentage of  $GFR\alpha 1^+$   $A_s$  spermatogonia that are  $Kit^+$  is markedly lower than the percentage in  $GFR\alpha 1^+$   $A_{pr}$  and  $A_{al}$  spermatogonia. Steps that potentially need to be taken for differentiation to occur include the silencing of genes that promote “stemness,” by increased expression of the DNA methyltransferases, *Dnmt3a2* and *Dnmt3b*, and/or by the establishment of transcriptionally repressive histone modifications, such as H3K9me2 [5].

Our results provide the first estimate of the total number of  $GFR\alpha 1^+$  spermatogonia in the mouse testis. We recognize that  $GFR\alpha 1^+$  is not often expressed by  $A_{al}$  spermatogonia in chains longer than four cells, and thus our estimates do not account for all  $A_{al}$  cells. However, as GDNF is required for the in vivo maintenance of  $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia and, as in the testis,  $GFR\alpha 1$  is the functional ligand-binding domain of the GDNF receptor, the cells that maintain their undifferentiated state must express  $GFR\alpha 1$ . This paper provides the first determination of the total numbers of  $GFR\alpha 1^+$   $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia in the testis of an adult mouse with a C57Bl/6J genetic background. The stereological data, when combined with recent estimates of numbers of SSCs in a mouse testis [18], lead to the conclusion that, on average, one SSC is present every 260- to 520- $\mu$ m length of tubule. Furthermore, our data indicate that there are 24 427 and 5035  $GFR\alpha 1^+$   $A_s$  spermatogonia, respectively, in testes of control mice and mice that were treated for 7 days with 1NA-PP1. As our results suggest that it was primarily progenitor spermatogonia that were lost when GDNF signaling was inhibited for 7 days, and as it is estimated that

there are between 3000 and 6000 SSCs in the testis of a mouse [18], we suggest that, after 7 days of inhibited GDNF signaling, between 84% and 100% of the remaining  $GFR\alpha1^+$   $A_s$  spermatogonia functioned as stem cells. Furthermore, we suggest that, in a normal mouse testis that has not experienced a period of inhibited GDNF signaling, less than 25% of the  $GFR\alpha1^+$   $A_s$  cells function as SSCs.

We acknowledge that we have discussed our data from the vantage point of the classical model that stipulates that SSCs are a subset of  $A_s$  spermatogonia, and that numbers of these stem cells are primarily regulated by self-renewing replication. The classical model posits that nonstem  $A_s$  spermatogonia and  $A_{pr}$  and  $A_{al}$  spermatogonia are progenitor spermatogonia that represent early, sequential steps in spermatogonial differentiation. This model is supported by the fact that numbers of functional SSCs, as assayed by their ability to re-establish spermatogenesis when transplanted into a germ cell-deficient testis, are substantially lower than the total number of  $GFR\alpha1^+$  spermatogonia reported herein [18, 26]. Furthermore, the conclusion that  $A_s$ ,  $A_{pr}$ , and  $A_{al}$  spermatogonia differ from one another is supported by the following facts: only  $A_s$  spermatogonia express the transcriptional repressor, ID4, which is required for stem cell maintenance [27];  $A_s$  spermatogonia replicate more slowly than  $A_{al}$  spermatogonia; and the fraction of cells that express Kit increases significantly as  $A_s$  spermatogonia become  $A_{pr}$  spermatogonia and then  $A_{al}$  spermatogonia (Figs. 3 and 4).

However, we also acknowledge that live cell imaging has led to a different model for the formation and function of  $A_s$ ,  $A_{pr}$ , or  $A_{al}$  spermatogonia. This model posits that all  $GFR\alpha1^+$  spermatogonia function as SSCs, and that their numbers are controlled by stochastic processes that determine both the frequency of cell replication and the differentiation of these cells into  $Ngn3^+$  and then  $Kit^+$  cells. Additionally, this model proposes that numbers of  $A_s$  spermatogonia are primarily controlled by fragmentation of chains of  $A_{al}$  spermatogonia and not by self-renewing replication [6, 28, 29]. As noted above, other data obtained by stereological analysis and from functional stem cell assays argue strongly against the new hypothesis that all  $GFR\alpha1^+$  cells are stem cells. Nonetheless, this different model does provide an alternative explanation for why loss of GDNF signaling leads to a more rapid loss of progenitor spermatogonia than SSCs. It is possible that, when GDNF signaling is inhibited, SSCs and progenitor spermatogonia may be lost to cell differentiation at similar rates. However, at the same time, there may be an increased rate of formation of SSCs due to fragmentation of chains of  $GFR\alpha1^+$   $A_{al}$  spermatogonia and subsequent dedifferentiation of the liberated  $A_s$  cells into SSCs. Thereby, the stem cell pool would initially be buffered from losses due to cell differentiation. It should be noted, however, that this event would occur in the absence of GDNF signaling, which our data proves promotes loss, not formation, of SSCs. Thus, this alternative explanation requires that growth factors or cytokines besides GDNF regulate fragmentation of chains of  $A_{al}$  spermatogonia and the dedifferentiation of these liberated cells into SSCs. Nonetheless, when viewed from the perspective of cell populations, this alternative explanation still leads to the conclusion that loss of GDNF signaling is accompanied by a more rapid decrease in numbers of progenitor spermatogonia than in numbers of SSCs.

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