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Gdnf Upregulates c-Fos Transcription via the Ras/Erk1/2 Pathway to Promote Mouse Spermatogonial Stem Cell Proliferation

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Key Words. GDNF • Spermatogonial stem cells • Ret • Ras/ERK pathway • CREB/ATF-1 family • c-fos

ABSTRACT

Glial cell line-derived neurotrophic factor (GDNF) plays a crucial role in regulating the proliferation of spermatogonial stem cells (SSC). The signaling pathways mediating the function of GDNF in SSC remain unclear. This study was designed to determine whether GDNF signals via the Ras/ERK1/2 pathway in the C18-4 cells, a mouse SSC line. The identity of this cell line was confirmed by the expression of various markers for germ cells, proliferating spermatogonia, and SSC, including GCNA1, Vasa, Dazl, PCNA, Oct-4, GFRα1, Ret, and Plzf. Western blot analysis revealed that GDNF activated Ret tyrosine phosphorylation. All 3 isoforms of Shc were phosphorylated upon GDNF stimulation, and GDNF induced the binding of the phosphorylated Ret to Shc and Grb2 as indicated by immunoprecipitation and Western blotting. The active Ras was induced by GDNF, which further activated ERK1/2 phosphorylation. GDNF stimulated the phosphorylation of CREB-1, ATF-1, and CREM-1, and c-fos transcription. Notably, the increase in ERK1/2 phosphorylation, c-fos transcription, bromodeoxyuridine incorporation, and metaphase counts induced by GDNF, was completely blocked by pretreatment with PD98059, a specific inhibitor for MEK1, the upstream regulator of ERK1/2. GDNF stimulation eventually upregulated cyclin A and CDK2 expression. Together, these data suggest that GDNF induces CREB/ATF-1 family member phosphorylation and c-fos transcription via the Ras/ERK1/2 pathway to promote the proliferation of SSC. Unveiling GDNF signaling cascades in SSC has important implications in providing attractive targets for male contraception as well as for the regulation of stem cell renewal vs. differentiation. STEM CELLS *2008;26:266–278*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Spermatogenesis is a cellular process by which spermatogonial stem cells (SSC) divide and differentiate into spermatozoa. SSC are unique since they are the only stem cells in the body that undergo self-renewal throughout life and transmit genetic information to offspring [1–3]. A better understanding of the molecular mechanisms controlling self-renewal, differentiation, or apoptosis of SSC, is essential for the regulation of spermatogenesis as well as for the potential use of the SSC to produce embryonic-like stem cells. Glial cell line-derived neurotrophic factor (GDNF) is the 1st molecule known to regulate the cell fate decision of SSC [4, 5]. In vivo data from transgenic mice indicate that GDNF mediates the renewal and differentiation of undifferentiated spermatogonia in a dose-dependent manner [4]. While GDNF-deficient mice show partial depletion of SSC, the mice over expressing GDNF display an accumulation of undifferentiated spermatogonia [4]. GDNF can also promote the proliferation of undifferentiated spermatogonia in vivo [6] and stimulates DNA synthesis in Ret-expressing spermatogonia [7]. In vitro, it has been demonstrated by our group and others that GDNF facilitates the expansion of SSC over a long culture period [8, 9]. However, the signal transduction pathways that lead to the GDNF-induced DNA synthesis and proliferation of SSC remain largely an enigma.

GDNF signals through a multicomponent receptor complex comprised of the Ret receptor tyrosine kinase and a member of the GFRα family of glycosylphosphatidylinositol (GPI)-anchored receptors, which are required for GDNF binding to Ret [10, 11]. In nervous tissue, there are multiple-pathways described for GDNF signaling. GDNF can trigger intracellular signaling through a Ret-independent pathway via GPI-linked protein GFRα1, which leads to activation of Src family tyrosine kinase and mediates various downstream responses to promote cell survival [12, 13]. We have recently shown that GDNF uses Src tyrosine kinase and phosphatidylinositol 3-kinase activation to upregulate *N-myc* expression in SSC [14]. In neuronal cell lines that coexpress Ret and GFRα1, GDNF signals through the Ret-dependent pathway to induce intracellular signal cascades [10, 13, 15]. Within the seminiferous tubules, GDNF is secreted by Sertoli cells [4, 16]. We and others have shown that the SSC but not differentiating germ cells express its co-receptors, GFRα1 and Ret [9, 17, 18]. It has been suggested that GDNF mediates Ret signaling via GFRα1 to regulate the cell fate of undifferentiated spermatogonia [17]. Nevertheless, the down-

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stream events triggered by GDNF/GFRα1/Ret need to be elucidated. To uncover signaling cascades in SSCs has implications for manipulating signaling pathways to spur transdifferentiation and regeneration. Further, these studies uncover new molecular targets for pharmacological inhibitors that might be useful for male contraception.

The small guanosine triphosphatase protein Ras is known as a key mediator for proliferation and differentiation [19–21], and the extracellular signal-regulated kinases (ERK), an important member of the mitogen-activated protein kinases, is involved in modulating a variety of cellular functions, including cell proliferation, differentiation, and cell cycle progression [22, 23]. It has been suggested that ERK is essential for the proliferation of c-kit expressing type A_1-A_4 spermatogonia stimulated with stem cell factor (SCF) [22]. In the current study, we sought to ascertain whether GDNF signals through the Ras/ERK1/2 pathway in the C18-4 cells, a murine spermatogonial stem cell line. We found that GDNF activated the tyrosine phosphorylation of Ret and Shc, and that GDNF induced the binding of the phosphorylated Ret to Shc and Grb2. GDNF stimulation subsequently activated the Ras/ERK1/2 pathway, which led to the phosphorylation of transcription factors cAMP responsive element-binding protein one (CREB-1), the activating transcription factor-1 (ATF-1), and cAMP response element modulation protein one (CREM-1), as well as the transcription of the immediate early gene c-fos. The active Ras and ERK1/2 phosphorylation induced by GDNF were further confirmed in freshly isolated type A spermatogonia from 6-day-old mice. The inhibitor PD98059 for the MEK1/ERK1/2 pathway completely blocked the upregulation in ERK1/2 phosphorylation, c-fos transcription, bromodeoxyuridine incorporation, and metaphase counts induced by GDNF. Furthermore, GDNF increased the expression of cell cycle regulators, including cyclin A and cyclin-dependent kinase 2 (CDK2) but not cyclin D1, and PD98059 blocked the upregulation of cyclin A and CDK2 expression. Our data demonstrate that GDNF/GFRα1/Ret signaling activates the Ras/ERK1/2 pathway to promote DNA synthesis and cell proliferation.

MATERIALS AND METHODS

The C18-4 Cells

The C18-4 cell line was established by transfecting mouse spermatogonial stem cells with a plasmid allowing the expression of the SV40 large T antigen under the control of a ponasterone A-driven promoter [24].

Animals

BALB/c mothers with 6-day-old male pups were obtained from the Charles River Laboratories, Inc. (Wilmington, MA, USA). All animal care procedures were carried out pursuant to the National Research Council's Guide for the Care and Use of Laboratory Animals, and the experimental protocols were approved by the Animal Care and Use Committee of Georgetown University.

Immunofluorescence

The immunofluorescence procedures used on the C18-4 cells, SF7 cells (a Sertoli cell line) [25], and adult mouse germ cells were described in our previous publication [26]. The antibodies included: GCNA1 (1:200 dilution) from Dr. George C. Enders; Vasa (catalog no. sc-48705, 2 μg/ml; Santa Cruz Biotechnology Inc., Santa Cruz, CA); Dazl (catalog no. sc-6156, 5 μg/ml; Novus Biologicals, Littleton, CO); PCNA (catalog no. NA03, 1 μg/ml; EMD Biosciences, Inc., San Diego); Oct-4 (catalog no. sc-8629, 2 μg/ml; Santa Cruz Biotechnology Inc.); GFRα1 (catalog no. sc-6156, 2 μg/ml; Santa Cruz Biotechnology Inc.); Ret (catalog no. AF482, 2 μg/ml; R&D Systems, Minneapolis); Plzf (catalog no. OP128L, 1 μg/ml; Calbiochem, San Diego); c-kit (catalog no. sc-168, 2 μg/ml; Santa Cruz Biotechnology Inc.); mouse IgG2a (catalog no. 401,123, 1 μg/ml; EMD Biosciences, Inc.); normal rat IgM (catalog no. sc-3885, 1:100 dilution; Santa Cruz Biotechnology Inc.); normal goat IgG (catalog no. sc-2028, 2 μg/ml; Santa Cruz Biotechnology Inc.); normal mouse IgG (catalog no. sc-2025, 2 μg/ml; Santa Cruz Biotechnology Inc.). All the secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. DAPI was used to stain the cell nuclei, and the cells were observed for epifluorescence using an Olympus Fluoview 500 Laser Scanning Microscope (Olympus, Melville, NY). Immunofluorescence was also carried out to determine the expression change and subcellular localization of cyclin A in the C18-4 cells with 100 ng/ml of recombinant rat GDNF (R&D Systems) treatment for 6 hours and 24 hours and in the GDNF-untreated cells using anti-cyclin A (Santa Cruz Biotechnology Inc.).

RNA Extraction and RT-PCR

Total RNA was extracted from the GDNF-treated or -untreated C18-4 cells using Trizol reagent (Invitrogen, Carlsbad, CA). RT-PCR was performed according to the protocol we described previously [26]. The forward and reverse primers of the chosen genes, including Vasa, Dazl, PCNA, Oct-4, GFRα1, Ret, Plzf, c-kit, Gapdh, and c-fos were designed and listed in Supplementary Data - Table 1. PCR products were separated by electrophoresis on 1.2% agarose gels. The gels were exposed to a Transilluminator (Fisher Scientific, Pittsburg), and pictures were taken with a Photo-Documentation Camera (Fisher Scientific). The data were scanned with an Epson Perfection 3,200 PHOTO, and densitometric analyses were processed with Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA).

Isolation of Type A Spermatogonia from 6-Day-Old Mice and Germ Cells from 60-Day-Old Mice

Type A spermatogonia and Sertoli cells were isolated from the testes of 6-day-old mice using mechanical dissociation and enzymatic digestion containing collagenase, trypsin, and hyaluronidase [27, 28]. Type A spermatogonia were further separated from the Sertoli cells by differential plating and the purity of type A spermatogonia was around 95% as evaluated by immunocytochemical staining using an antibody to GCNA1. Adult germ cells were isolated from the testes of 60-day-old mice using a 2-step enzymatic digestion containing collagenase, trypsin, and hyaluronidase and differential plating [27, 28].

Immunoprecipitation and Western Blotting

The C18-4 cells were cultured in the DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 unit/ml penicillin, and 100 mg/ml streptomycin, in 75 cm^2 cell culture flasks until cell confluence was up to 80%. The cells were starved in serum-free DMEM/F12 for 16 hours prior to the incubation with 100 ng/ml GDNF at 34°C for 5 minutes and 15 minutes and lysed with 1 ml of RIPA lysis buffer (Santa Cruz Biotechnology Inc.). After 30 minutes lysis on ice, cell lysates were cleared by centrifugation at 12,000 g, and the concentration of protein was measured by the Bio-Rad Bradford assay (BioRad Laboratories, Hercules, CA).

Fifty micrograms of cell lysate from each sample were used for SDS-PAGE, and Western blots were performed according to the protocol we described previously [26]. The chosen antibodies included phospho-Ret (Tyr 1,062) (Santa Cruz Biotechnology Inc.), phospho-Smad2 (Cell Signaling Technology, Inc., Danvers, MA), and phospho-CREB-1 (Ser-133) (Santa Cruz Biotechnology Inc.). After extensive washes in PBS, the blots were detected using the Western Blotting Luminol Reagent (Santa Cruz Biotechnology Inc.) and exposed to autoradiography films. The membranes were stripped using the Restore Western Blot Stripping Buffer (Pierce Biotechnology, Inc., Rockford, IL) and reprobed for the detection of Ret (Santa Cruz Biotechnology Inc.), Smad2/3 (Cell Signaling

Technology, Inc., Danvers, MA), or β-actin (IMGENEX Corp, San Diego). The films were scanned with an Epson Perfection 3,200 PHOTO, and densitometric analyses were processed with Adobe Photoshop 7.0.

To check whether the C18-4 cells still express the SV40 large T antigen, Western blots were carried out with the C18-4 cells, SF7 cells, and NIH 3T3 cells using anti-SV40 T antigen (Calbiochem, San Diego,) pursuant to the procedure mentioned above.

The C18-4 cells were pretreated with or without PD98059 (BIOSOURCE, Camarillo, CA) for 30 minutes and then treated with or without 100 ng/ml of GDNF for the indicated time periods. Western blot analysis was carried out using antibodies to phospho-ERK1/2 (Thr 202/Tyr 204), cyclin A, cyclin D1, and CDK2, respectively. All the antibodies were purchased from Santa Cruz Biotechnology Inc. The blots were re-probed for detecting ERK2 or β-actin using antibodies to ERK2 (Santa Cruz Biotechnology Inc.) or β-actin.

Type A spermatogonia were cultured in 100 mm Petri dishes with DMEM/F12 containing 10% FBS and 2 mM L-glutamine for 3 hours to allow restoring and attaching to the dish. The cells were starved in serum-free DMEM/F12 for 8 hours, and then 100 ng/ml GDNF was added to the medium for 5 minutes and 15 minutes. Western blotting was performed using antibody to phospho-ERK1/2 (Thr 202/Tyr 204). The blots were re-probed for detecting ERK2 using an antibody to ERK2.

For immunoprecipitations with anti-Shc, 5 μl of anti-Shc (Upstate Biotechnology Inc., Lake Placid, NY) was added to 500 μl of cell lysates from each sample overnight at 4°C. The immunocomplex was captured by adding 50 μl of the prepared Protein A agarose beads slurry (Upstate Biotechnology Inc.) for 2 hours at 4°C. The beads were washed 5 times with RIPA lysis buffer and boiled for 5 minutes in protein sample buffer. Half the immunoprecipitates was fractionated by SDS-PAGE, and Western blots were performed using anti-phospho-Shc (Tyr 317) (Upstate Biotechnology Inc.) or anti-phospho-Ret (Tyr 1,062). The blots were reprobed for the detection of Shc using an antibody to Shc.

Immunoprecipitations with anti-Grb2 (Upstate Biotechnology Inc.) were carried out using 2 μg of anti-Grb2 that was added to 300 μl of cell lysates from each sample overnight at 4°C. The immunocomplex was captured by adding 50 μl of the prepared Protein G MicroBeads (Miltenyi Biotec Inc., Auburn, CA) for 30 minutes on ice and proceeded to magnetic separation using μMACS Separator (Miltenyi Biotec Inc.). Half the immunoprecipitates was fractionated by SDS-PAGE, and Western blots were performed using anti-phospho-Ret (Tyr 1,062). The blots were reprobed for the detection of Grb2 using an antibody to Grb2.

The Ras Activation Assay

The C18-4 cells and type A spermatogonia were treated with 100 ng/ml of GDNF for 5 minutes and 15 minutes or without GDNF treatment, and then lysed with 1 ml of Lysis/Binding/Washing Buffer (Assay Designs, Inc., Ann Arbor, MI). The cell lysates from each sample were incubated with GST-Raf1-RBD and an Immobilized Glutathione Disc for 1 hour at 4°C using the StressXpress Ras Activation Kit (Assay Designs, Inc.). Half the eluted samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with a specific antibody to Ras (Assay Designs, Inc.).

Bromodeoxyuridine (BrdU) Incorporation Assay

The C18-4 cells were grown on coverslips in 6-well culture plates with DMEM/F12 containing 10% FBS for 6 hours to allow the cells to attach to coverslips. The cells were starved in serum-free DMEM/F12 for 16 hours prior to pretreatment with or without 100 μM of PD98059 for 30 minutes, and then 100 ng/ml GDNF and 30 μg/ml of BrdU (Sigma, Saint Louis) or 30 μg/ml of BrdU alone was added to the medium. After 16 hours of culture, immunocytochemical staining was performed using a monoclonal anti-BrdU (Sigma, Saint Louis) pursuant to the DBA kit (Zymed Laboratories Inc., South San Francisco). The percentage of BrdU-positive cells was counted out of 500 cells, and the data were presented from 3 experiments.

Metaphase Counting

The C18-4 cells were cultured in 100 mm Petri dishes with DMEM/F12 containing 10% FBS for 6 hours. The cells were starved in serum-free DMEM/F12 for 16 hours prior to pretreatment with or without 100 μM of PD98059 for 30 minutes, and then 100 ng/ml GDNF were added to the medium. Colcemid was added to the culture medium for 1 hour prior to cell harvest. After 24 hours of culture, the cells were processed using standard metaphase counting procedures. The percentage of cells in metaphase was counted out of 500 cells. The data were presented from 3 experiments.

Statistical Analysis

All the values were presented as mean ± SEM, and statistically significant differences ($p < .05$) among the GDNF treatment group, the control, and the cells pretreatment with PD98059, were determined using the analysis of variance (ANOVA) and Tukey post-tests.

RESULTS

The C18-4 Cells Express GCNA1, Vasa, Dazl, PCNA, Oct-4, GFRα1, Ret, Plzf, and SV40 Large T Antigen

We first confirmed the identity of the C18-4 cells using various markers for germ cells, proliferating spermatogonia, as well as SSC. RT-PCR analyses showed that the mRNA of Vasa, Dazl, PCNA, Oct-4, GFRα1, Ret, Plzf, and c-kit was present in the C18-4 cells (Fig. 1A). Immunocytochemical staining revealed that the C18-4 cells expressed GCNA1 (Fig. 1B), Vasa (Fig. 1D), Dazl (Fig. 1F), PCNA (Fig. 1H), GFRα1 (Fig. 1J), Ret (Fig. 1L), Oct-4 (Fig. 1N), and Plzf (Fig. 1P), reflecting that the C18-4 cells are phenotypically SSC. In contrast, c-kit protein was not detected in this cell line (Fig. 1R). Furthermore, Western blots showed that the C18-4 cells expressed the SV40 large T antigen (Fig. 1Y).

GDNF Activates the Tyrosine Phosphorylation of Ret and Shc and Induces the Binding of the Phosphorylated Ret to Shc and Grb2 in the C18-4 Cells

We then examined whether GDNF can activate the tyrosine phosphorylation of Ret and Shc in the C18-4 cells. Western blots clearly showed that the phosphorylation of Ret (170 kDa) at tyrosine 1,062 was activated upon GDNF stimulation for 5 minutes and 15 minutes (Fig. 2A). Furthermore, immunoprecipitation with anti-Shc and Western blotting using anti-phospho-Shc revealed that all the 3 isoforms (46, 52, and 67 kDa) of tyrosine phosphorylation of Shc were activated by GDNF treatment for 5 minutes and 15 minutes (Fig. 2C). Meanwhile, immunoprecipitation with anti-Shc and Western blotting using anti-phospho-Ret showed that the phospho-Ret attached to Shc was increased by GDNF (Fig. 2C). Similarly, immunoprecipitation with anti-Grb2 and Western blotting using anti-phospho-Ret revealed that the phospho-Ret attached to Grb2 was also increased by GDNF (Fig. 2F, upper panel). Densitometric analyses showed significant differences for each of the experiments upon the addition of GDNF at the indicated time intervals (Fig. 2B, 2D, 2E, 2G). To confirm the specific activation of these proteins by GDNF in the C18-4 cells, we checked whether Smad2 phosphorylation was activated by GDNF. As shown in Figure 2H, no activa-

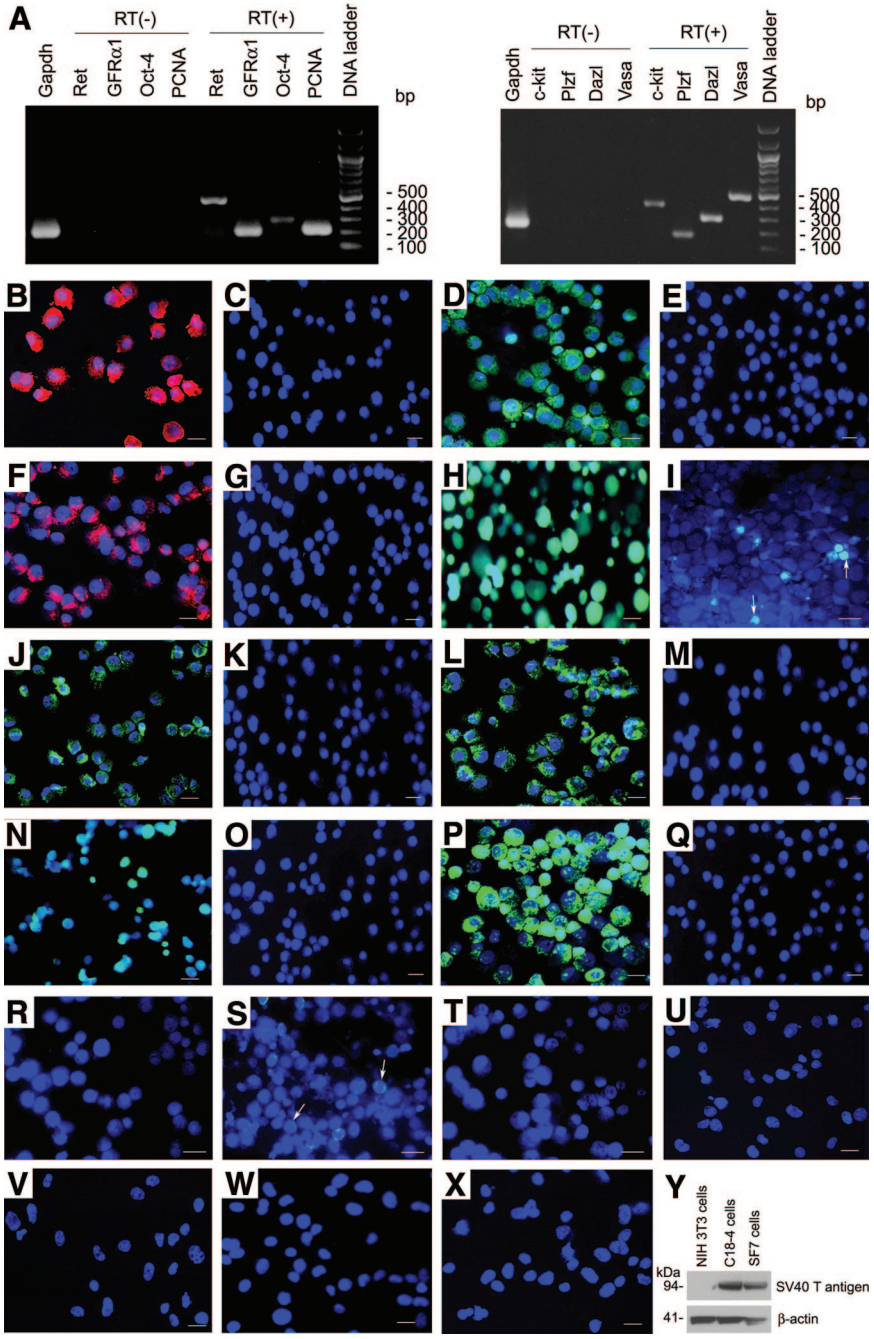

Figure 1. Identification and characterization of the C18-4 cells. **(A):** RT-PCR analysis shows the mRNA of Ret, GFRα1, Oct-4, PCNA, c-kit, Plzf, Dazl, and Vasa in the C18-4 cells. All the RNA samples were treated with DNase to remove the potential contamination of genomic DNA before reverse transcriptase (RT), and the RNA samples without RT but with PCR were used as a negative control. Gapdh was used as a loading control of total RNA. **(B–T):** Immunocytochemical staining shows the protein expression of GCNA1 (red fluorescence, nuclei) **(B)**, Vasa (green fluorescence, cytoplasm) **(D)**, Dazl (red fluorescence, cytoplasm) **(F)**, PCNA (green fluorescence, nuclei) **(H)**, GFRα1 (green fluorescence, surface cytoplasm and plasma membrane) **(J)**, Ret (green fluorescence, cytoplasm) **(L)**, Oct-4 (green fluorescence, nuclei) **(N)**, and Plzf (green fluorescence, nuclei) **(P)** in the C18-4 cells, while c-kit **(R)** was not detected in these cells. No staining of GCNA1 **(C)**, Vasa **(E)**, Dazl **(G)**, GFRα1 **(K)**, Ret **(M)**, Oct-4 **(O)**, and Plzf **(Q)** was observed in the SF7 cells, confirming the specific expression of these proteins in C18-4 cells. Some of the mouse adult germ cells were positive for PCNA (green fluorescence, nuclei) **(I)** and c-kit (green fluorescence, cytoplasm) **(S)**, and the positive cells for PCNA and c-kit were indicated by arrows. Replacement of the primary antibody with PBS results in no staining in the C18-4 cells **(T)**. No staining of normal rat IgM **(U)** (as a negative control for GCNA1), mouse IgG2a **(V)** (as a negative control for PCNA and Plzf), normal goat IgG **(W)** (as a negative control for Vasa, Dazl, GFRα1 and Oct-4), and normal mouse IgG **(X)** (as a negative control for Ret), was observed in the C18-4 cells. Staining with DAPI (blue fluorescence) was used to identify cell nuclei. Scale bars in **(B–X)** = 10 μm. **(Y):** Western blots show the expression of SV40 large T antigen in the C18-4 cells. NIH 3T3 cells were used as a negative control, and SF7 cells served as a positive control. The same membrane was re-probed with anti-β-actin and showed β-actin expression in all samples.

tion of Smad2 phosphorylation was observed in the C18-4 cells treated with GDNF for 5 and 15 minutes.

GDNF Activates the Ras/ERK1/2 Pathway in the C18-4 Cells and in Freshly Isolated Type A Spermatogonia

Next, we tested whether GDNF could activate the Ras/ERK1/2 pathway in the C18-4 cells and type A spermatogonia. The Ras activation assay uses a GST-fusion protein containing the Ras-binding domain (RBD) of Raf1 to affinity precipitate active Ras (GTP-Ras) from cell lysates. The GST-Raf1-RBD fusion protein is incubated with cell lysate and an Immobilized Glutathione Disc. The pulled-down active or GTP-Ras is detected by Western blots using a specific Ras antibody. Immunoprecipitation with GST-Raf1-RBD and immunoblotting using Ras antibody showed that the active Ras (GTP-Ras, 21 kDa) was induced by GDNF as early as 5 minutes and then decreased at 15 minutes (Fig. 3A), indicating that GDNF induces a rapid and transient Ras activation. Furthermore, the active Ras induced by GDNF was confirmed in the freshly isolated type A spermatogonia from 6-day-old mice (Fig. 3E, 3F). Western blots using anti-phospho-ERK1/2 showed that the phosphorylation of ERK1/2 (44 kDa and 42 kDa) was increased by GDNF stimulation for 5 minutes and 15 minutes (Fig. 3C). To verify this result, we pretreated the C18-4 cells with PD98059, a specific inhibitor for MEK1, the upstream regulator of ERK1/2. As expected, the phosphorylation of ERK1/2 by GDNF was completely blocked by pretreatment with inhibitor PD98059 (Fig. 3C, 3D). In addition, the phosphorylation of ERK1/2 activated by GDNF was also confirmed in the freshly isolated type A spermatogonia from 6-day old mice

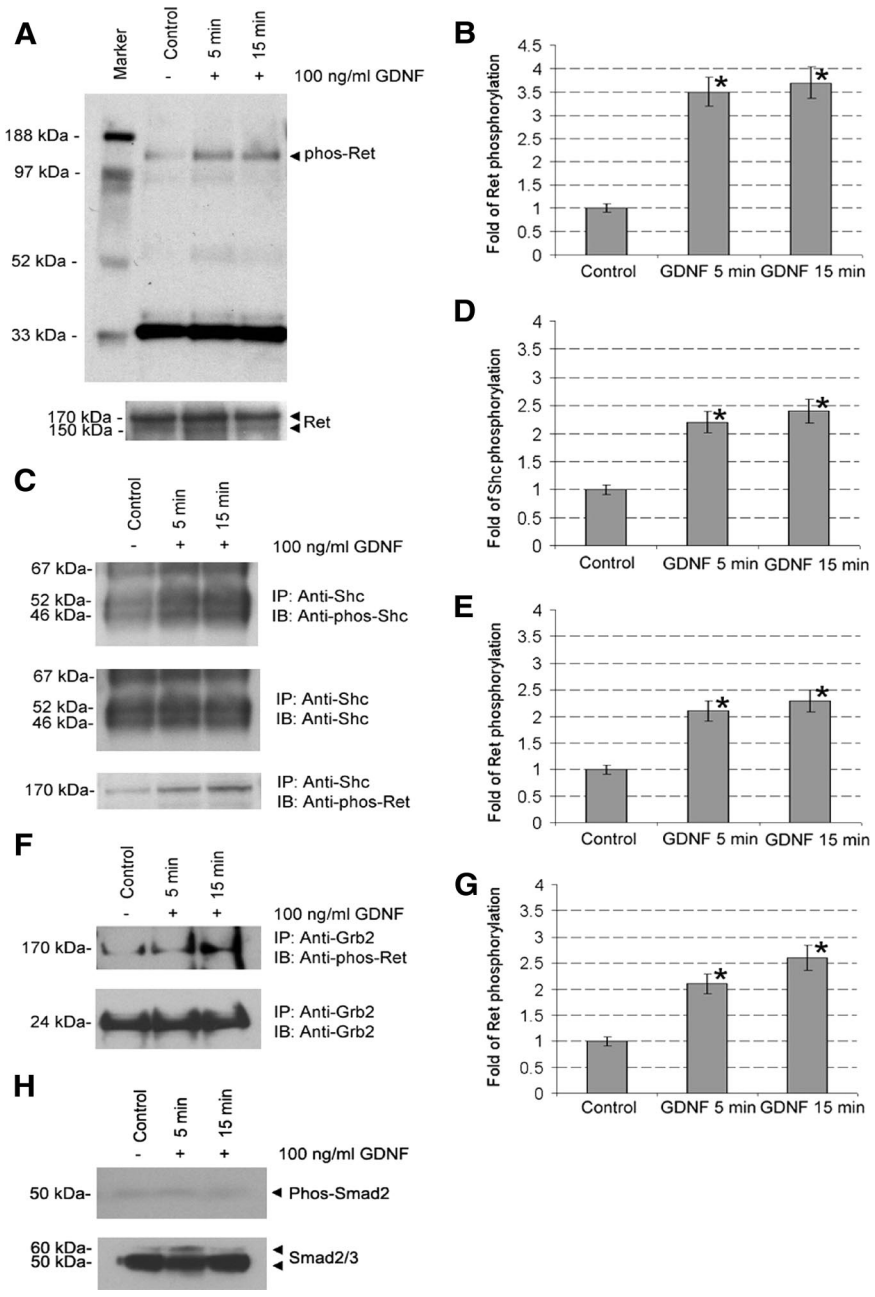

Figure 2. The tyrosine phosphorylation of Ret and Shc is activated by GDNF stimulation in the C18-4 cells and both Shc and Grb2 are co-immunoprecipitated with the phosphorylated Ret in the C18-4 cells after GDNF stimulation. **(A):** The C18-4 cells were serum-starved for 16 hours and then treated with 100 ng/ml GDNF for 5 and 15 minutes or without GDNF treatment. Fifty micrograms of total protein from the GDNF treated or untreated cells were resolved by 4–12% SDS-PAGE and blotted with anti-phospho-Ret (upper panel). The same membrane was re-probed with anti-Ret and showed Ret protein expression in all lanes (lower panel). The molecular mass standards are shown on the left, and the results are representative of 3 independent experiments. **(B):** The GDNF-induced phosphorylation of Ret relative to control (1.0) after normalization to the signal obtained with Ret. Statistically significant differences ($p < .05$) between the GDNF-treated and -untreated group were indicated by asterisks. **(C):** Five hundred micrograms of total protein from the GDNF treated or untreated cells were immunoprecipitated with anti-Shc, and half the immunoprecipitates was blotted with anti-phosphor-Shc (upper panel). The same membrane was reprobed with anti-Shc and showed Shc expression in all lanes (middle panel). The same membranes were reprobed with anti-phospho-Ret and they displayed the tyrosine phosphorylation of Ret that were pulled down together with Shc during the immunoprecipitation (lower panel). IP: immunoprecipitation; IB: immunoblotting. The molecular weights are shown on the left, and the results are representative of 3 independent experiments. **(D–E):** The GDNF-induced phosphorylation of Shc (52 kDa) and phospho-Ret relative to control (1.0) after normalization to the signal obtained with Shc (52 kDa). Statistically significant differences ($p < .05$) between the GDNF-treated and-untreated group were indicated by asterisks. **(F):** Three hundred micrograms of total protein from the GDNF treated or untreated cells were immunoprecipitated with anti-Grb2, and half the immunoprecipitates was blotted with anti-phosphor-Ret, showing the tyrosine phosphorylation of Ret that was pulled down together with Grb2 during the immunoprecipitation in the C18-4 cells (upper panel). The same membrane was re-probed with anti-Grb2 and showed Grb2 expression in all lanes (lower panel). IP: immunoprecipitation; IB: immunoblotting. The molecular weights are shown on the left, and the results are representative of 3 independent experiments. **(G):** The GDNF-induced phosphorylation of Ret relative to control (1.0) after normalization to the signal obtained with Grb2. Statistically significant differences ($p < .05$) between the GDNF-treated and -untreated groups were indicated by asterisks. **(H):** Western blots show no activation of Smad2 phosphorylation by GDNF in C18-4 cells. The same membrane was reprobed with anti-Smad2/3 and showed Smad2/3 expression in all lanes.

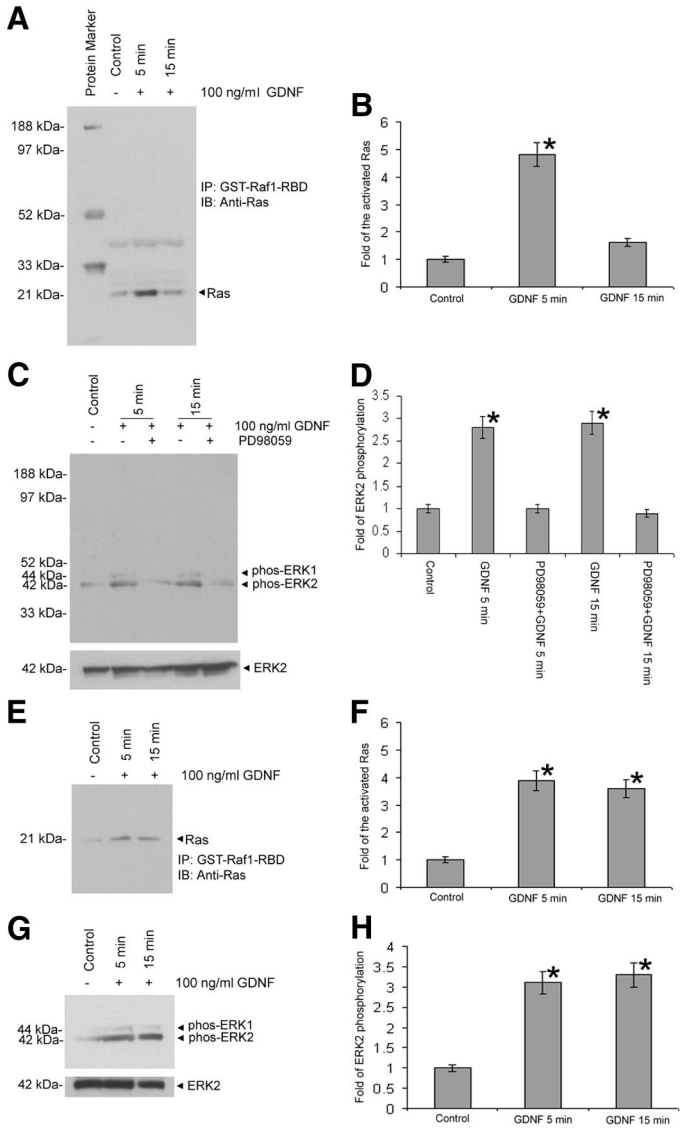

Figure 3. The Ras/ERK1/2 pathway is activated by GDNF in the C18-4 cells and type A spermatogonia. **(A):** Pull-down assay of the activated GTP-loaded Ras with a GST-fusion protein containing the RBD of Raf1 to affinity precipitate active Ras (GTP-Ras) from cell lysates of the C18-4 cells. Five hundred micrograms of cell lysates from the GDNF treated or untreated cells were incubated with GST-Raf1-RBD and an Immobilized Glutathione Disc, and half the pulled-down active Ras was detected by Western blotting using antibody to Ras. IP: immunoprecipitation; IB: immunoblotting. The molecular mass standards are shown on the left, and the results are representative of 3 independent experiments. **(B):** The GDNF-induced active Ras relative to the GDNF-untreated control (1.0). Statistically significant differences ($p < .05$) between the GDNF-treated and -untreated group were indicated by asterisks. **(C):** The C18-4 cells were serum-starved for 16 hours and then pretreated with or without PD98059 for 30 minutes. The cells were treated with 100 ng/ml GDNF for 5 and 15 minutes or without GDNF treatment. Fifty micrograms of total protein from the GDNF treated or untreated cells were resolved by SDS-PAGE and blotted with anti-phospho-ERK1/2 (upper panel). The same membrane was reprobed with anti-ERK2 and showed ERK2 expression in all lanes (lower panel). The molecular mass standards are shown on the left, and the results are representative of three independent experiments. **(D):** The GDNF-induced phosphorylation of ERK2 (42 kDa) relative to control (1.0) after normalization to the signal obtained with ERK2. Statistically significant differences ($p < .05$) between the GDNF-treated and-untreated group were indicated by asterisks. **(E):** Pull-down assay of the activated GTP-loaded Ras with a GST-fusion protein containing the RBD to affinity precipitate active Ras from cell lysates of the freshly isolated type A spermatogonia. Three hundred micrograms of cell lysates from the GDNF treated or untreated cells were incubated with GST-Raf1-RBD and an Immobilized Glutathione Disc, and half the pulled-down active Ras was detected by Western blotting using an antibody to Ras. IP: immunoprecipitation; IB: immunoblotting. The molecular weight is shown on the left, and the results are representative of 2 experiments. **(F):** The GDNF-induced active Ras relative to the GDNF-untreated control (1.0). Statistically significant differences ($p < .05$) between the GDNF-treated and-untreated groups were indicated by asterisks. **(G):** Type A spermatogonia of 6-day-old mice were serum-starved for 8 hours and then treated with 100 ng/ml GDNF for 5 and 15 minutes or without GDNF treatment. Eighty micrograms of total protein from the GDNF treated or untreated cells were resolved by SDS-PAGE and blotted with anti-phospho-ERK1/2 (upper panel). The same membrane was reprobed with anti-ERK2 and showed ERK2 expression in all lanes (lower panel). The molecular weights are shown on the left, and the results are representative of 2 experiments. **(H):** The GDNF-induced phosphorylation of ERK2 (42 kDa) relative to control (1.0) after normalization to the signal obtained with ERK2. Statistically significant differences ($p < .05$) between the GDNF-treated and -untreated group were indicated by asterisks.

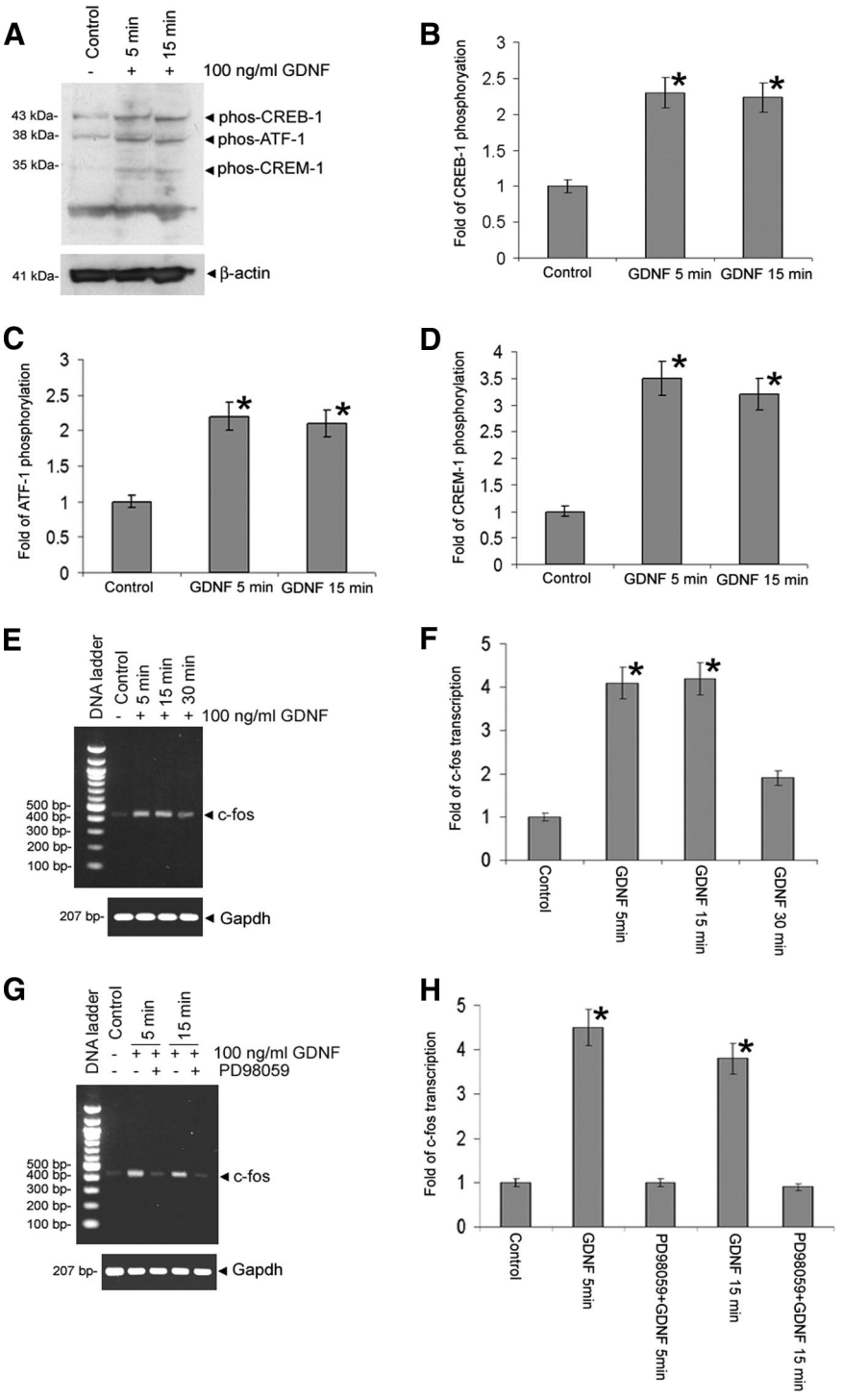

Figure 4. The phosphorylation of CREB-1, ATF-1, and CREM-1 and c-fos transcription are induced by GDNF in the C18-4 cells and the upregulation of c-fos transcription by GDNF is blocked by the inhibitor PD98059. **(A):** The phosphorylation of CREB-1 at Ser-133 and the CREB-related protein ATF-1 and CREM-1 are activated by GDNF in the C18-4 cells as shown by Western blots of 50 μg of total cell lysates with a specific anti-phospho-CREB1 antibody (upper panel). The same membrane was reprobed with anti-β-actin and demonstrated β-actin expression in all lanes (lower panel). The molecular weights are shown on the left, and the results are representative of 3 independent experiments. **(B–D):** The GDNF-induced phosphorylation of CREB-1 **(B)**, ATF-1 **(C)**, and CREM-1 **(D)** relative to control (1.0) after normalization to the signal of β-actin. Statistically significant differences ($p < .05$) between the GDNF-treated and -untreated group were indicated by asterisks. **(E):** RT-PCR analysis shows the time course of increase in c-fos mRNA induced by GDNF in C18-4 cells. The results are representative of 3 independent experiments. **(F):** The GDNF-induced c-fos mRNA relative to control (1.0) after normalization to the signal of Gapdh. Statistically significant differences ($p < .05$) between the GDNF-treated and -untreated group were indicated by asterisks. **(G):** RT-PCR analysis displays the c-fos mRNA in C18-4 cells pretreated with PD98059 for 30 minutes and then with GDNF treatment for 5 minutes and 15 minutes or without GDNF treatment. The results are representative of 3 independent experiments. **(H):** The GDNF-induced c-fos mRNA relative to control (1.0) after normalization to the signal of Gapdh. Statistically significant differences ($p < .05$) between the GDNF-treated and-untreated group were indicated by asterisks.

(Fig. 3G). Densitometric analyses showed significant differences for each of the experiments upon the addition of GDNF at the indicated time intervals (Fig. 3B, 3D, 3F, 3H).

GDNF Activates the Phosphorylation of Transcription Factors CREB-1, ATF-1, and CREM-1, and Enhances the Transcription of the Immediate-Early Gene c-fos in the C18-4 Cells

We next assessed whether the activation of the CREB/ATF-1 family transcription factors was induced by GDNF in the C18-4 cells using anti-phospho-CREB-1. This antibody is able to recognize the phosphorylation of the transcription factor CREB-1 at Ser-133 as well as ATF-1 and CREM-1. We found that GDNF activated the phosphorylation of CREB-1 at Ser-133, ATF-1, and CREM-1, as shown by Western blot analysis (Fig. 4A). We further explored whether GDNF could induce transcription of the immediate-early gene c-fos in the C18-4 cells. The c-fos mRNA was clearly enhanced in the C18-4 cells treated with GDNF for 5 and 15 minutes and declined at 30 minutes, but was still higher than the control as shown by semi-quantitative RT-PCR analysis (Fig. 4E). To confirm this result, we pretreated the C18-4

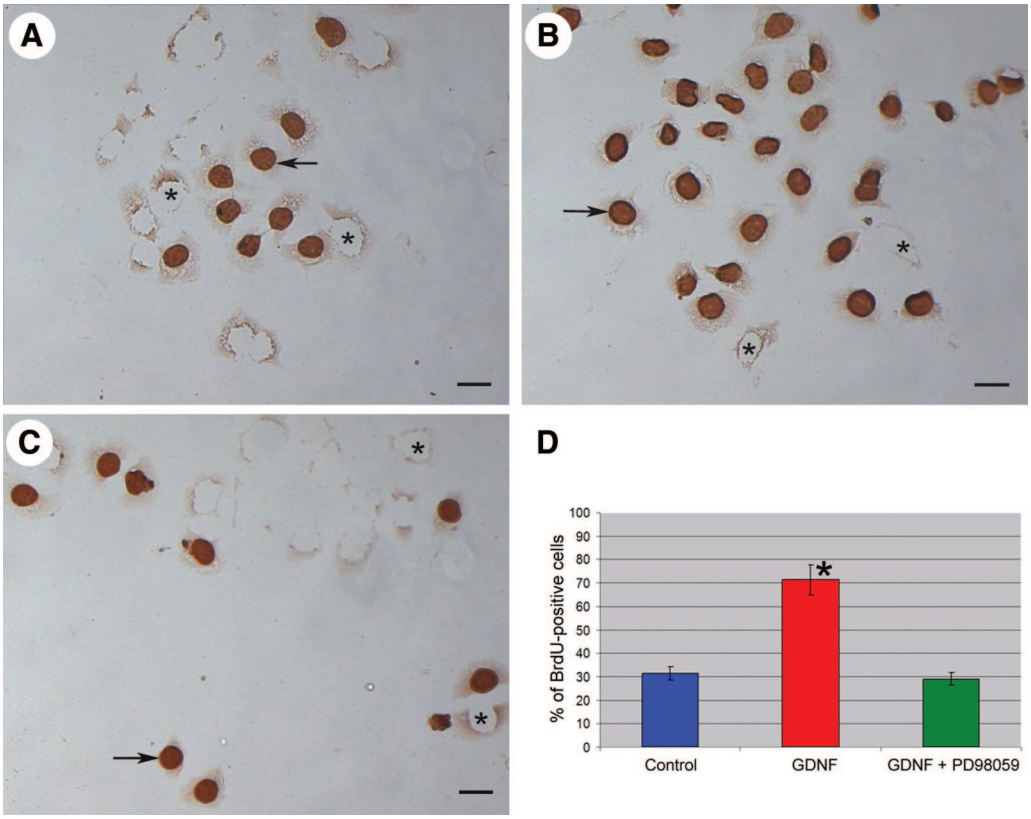

Figure 5. Inhibitor PD98059 blocks GDNF-induced BrdU incorporation in the C18-4 cells. The C18-4 cells were pretreated with or without PD98059 for 30 minutes, and then GDNF was added to the culture medium as described in the Materials and Methods. After 16 hours of culture, the cells were fixed in 4% paraformaldehyde and quenched with 3% hydrogen peroxide. DNA denaturation was achieved with 1 N hydrochloric acid, and enzymatic pretreatment was carried out with 0.1% trypsin. After permeabilization with Triton X-100 and blocking with goat serum, the cells were immunostained with anti-BrdU antibody, followed by incubation with the biotinylated second antibody and streptavidin-peroxidase, and detected by DAB chromogen. Immunocytochemical staining shows the BrdU incorporation in the C18-4 cells without GDNF treatment **(A)**, the cells with GDNF treatment **(B)**, and in the cells pretreated with PD98059 and then with GDNF treatment **(C)**, using anti-BrdU. The BrdU-positive cells are indicated by the arrows, and BrdU negative cells are denoted by asterisks. Scale bars in **A, B,** and **C** = 10 μm. **(D):** Percentage of the BrdU-positive cells in the C18-4 cells without or with GDNF treatment, or in the cells pretreated with PD98059 and then with GDNF stimulation. The data were presented as mean ± SEM of BrdU-positive cells out of 500 cells. The percentage of BrdU-positive cells is significantly increased ($p < .05$, as indicated by asterisk) in GDNF-treated cells compared to the untreated cells or the cells pretreatment with PD98059.

cells with the inhibitor PD98059. As anticipated, the upregulation of c-fos mRNA by GDNF in the C18-4 cells was completely blocked by the pretreatment with PD98059 (Fig. 4G, 4H). Densitometric analyses showed significant differences for each of the experiments upon the addition of GDNF at the indicated time intervals (Fig. 4B, 4C, 4D, 4F, 4H).

The Inhibitor PD98059 Blocks GDNF-Induced BrdU Incorporation in the C18-4 Cells

Cell proliferation of the C18-4 cells was assessed immunocytochemically using the BrdU incorporation assay, and the nuclei of cells in active DNA synthesis were stained with a BrdU monoclonal antibody. An increase in the BrdU-positive cells was observed in the GDNF-treated cells (Fig. 5B) with respect to the GDNF-untreated control (Fig. 5A), and Figure 5D showed 2.3-fold induction of the BrdU-positive cells by GDNF. Notably, the increase of the BrdU-positive cells induced by GDNF was completely abolished in the cells pretreated with the inhibitor PD98059 (Fig. 5C, 5D).

The Inhibitor PD98059 Blocks GDNF-Induced Metaphase Counts in the C18-4 Cells

Metaphase counting was also performed to evaluate whether GDNF uses the ERK1/2 pathway to promote cell cycle progression of the C18-4 cells. An increase in the metaphase counts (nuclei with condensed metaphase chromosomes) was observed in the GDNF-treated cells (Fig. 6B) compared to the GDNF-untreated control (Fig. 6A), and Figure 6D showed 3-fold induction of the metaphase counts by GDNF. Notably, the increase of the GDNF-induced metaphase counts was completely blocked in the cells pretreated with the inhibitor PD98059 (Fig. 6C, 6D).

GDNF Upregulates Cyclin A and CDK2 but Not Cyclin D1 Expression in the C18-4 Cells

We also tested how GDNF affects cell cycle G_1/S transition in the C18-4 cells by examining the expression changes of certain cell cycle regulators, including cyclin A, cyclin D1, and CDK2. As shown by Western blot analysis, the expression of cyclin A and CDK2 was upregulated in the C18-4 cells stimulated by GDNF for 6 and 24 hours (Fig. 6E). Furthermore, the upregulation of cyclin A and CDK2 expression induced by GDNF in the C18-4 cells was completely blocked by pretreatment with the inhibitor PD98059 (Fig. 6E, 6G, 6H). In contrast, no change of cyclin D1 expression was observed in the C18-4 cells with or without GDNF treatment (Fig. 6E).

The expression change and the subcellular localization of cyclin A in the C18-4 cells with or without GDNF treatment

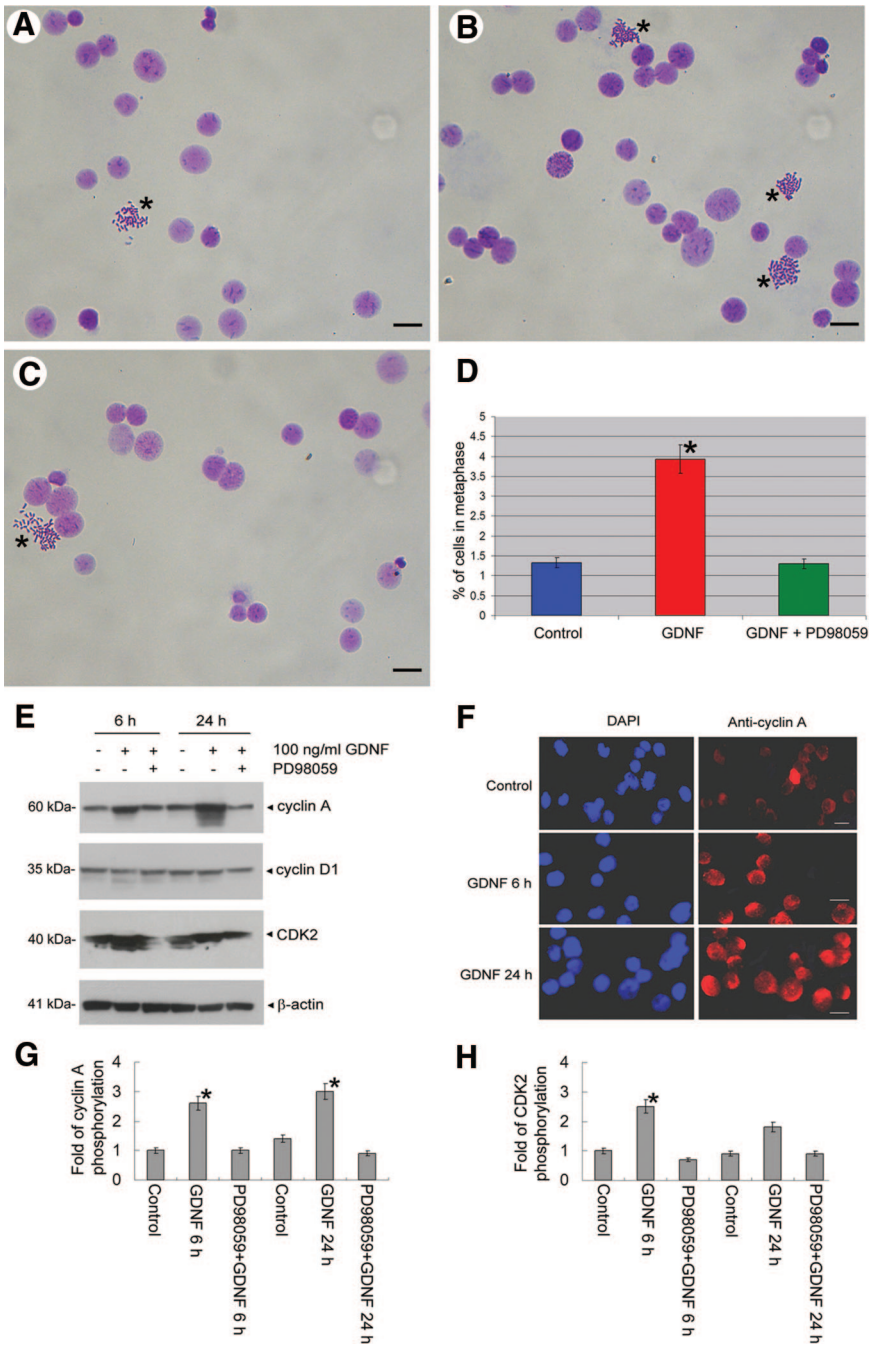

Figure 6. Inhibitor PD98059 blocks GDNF-induced metaphase counts in the C18-4 cells and the expression of cell cycle regulators, including cyclin A, cyclin D1, and CDK2 in the C18-4 cells with or without GDNF treatment. The C18-4 cells were cultured in the absence or constant presence of GDNF and/or PD98059 for 24 hours. The cells were stained with Giemsa solution, showing morphological characteristics in the C18-4 cells without GDNF treatment **(A)**, the cells with GDNF treatment **(B)**, and in the cells pretreated with PD98059 and then with GDNF treatment **(C)**. The asterisks indicate the condensed metaphase chromosomes typical of mitotic nuclei. Scale bars in **A, B,** and **C** = 10 μm. **(D):** Percentage of the cells in metaphase in the C18-4 cells without or with GDNF treatment, or in the cells pretreated with PD98059 and then with GDNF stimulation. The percentage of the cells in metaphase was statistically increased ($p < .05$, as indicated by asterisk) in GDNF-treated cells compared to the GDNF-untreated cells or the cells pretreated with PD98059. **(E):** Western blot analysis reveals an increase in the expression of cyclin A and CDK2 but not cyclin D1 in the C18-4 cells stimulated with GDNF for 6 and 24 hours. Inhibitor PD98059 blocked GDNF-induced cyclin A and CDK2 expression in these cells. The membranes were reprobed with anti-β-actin and showed β-actin expression in all lanes. The molecular weights are shown on the left, and the results are representative of 3 independent experiments. **(F):** Immunofluorescence analysis shows that an accumulation of cyclin A expression was induced by GDNF treatment for 6 and 24 hours in the nuclei of the C18-4 cells when compared to the cells without GDNF stimulation. Staining with DAPI (blue fluorescence) was used to show the cell nuclei. Scale bars = 10 μm. **(G–H):** The GDNF-induced expression of cyclin A **(G)** and CDK2 **(H)** relative to control (1.0) after normalization to the signal of β-actin. Statistically significant differences ($p < .05$) between the GDNF-treated and -untreated group were indicated by asterisks.

were further determined by immunofluorescence analysis. As shown in Figure 6F, GDNF induced a marked accumulation of cyclin A in nuclei of the C18-4 cells by GDNF treatment for 6 and 24 hours, respectively, compared to the GDNF-untreated control.

DISCUSSION

Spermatogonial stem cells (SSC) have recently assumed great significance in view of their unique characteristics. First, there is no intrinsic barrier to the genetic engineering of SSC using retrovirus, and once inserted, the foreign genes can be transmitted to subsequent generations [29]. Second, SSC self-renew throughout life and differentiate into spermatocytes, spermatids, and eventually sperm, and thus they can be used as a good model to elucidate the basic mechanisms of adult stem cell renewal versus differentiation. Third, recent evidence indicates that SSC from immature and adult mouse testis may acquire embryonic stem (ES) cell properties [30, 31]. These ES-like cells are able to differentiate into derivatives of the 3 embryonic germ layers [30, 31]. If this is true for human SSC, as proposed by Guan et al. [30], this would offer an alternative source of ES-like cells without the ethical issues involved in using discarded embryos. Finally, using SSC transplant [32, 33], patients suffering from cancer could retain their fertility after chemotherapy or irradiation therapy [34].

While there are reports showing that BMP4 induces spermatogonial differentiation via the Smad4/5 nuclear translocation [35] and SCF stimulates spermatogonial proliferation

through the activation of phosphoinositide 3-kinase [36–38] and the ERK pathway [22], little is known about the signaling pathways that lead to the self-renewal and differentiation of SSC. GDNF was originally characterized as a survival factor for midbrain dopaminergic neurons [39] and a potent trophic factor for both spinal motoneurons and central noradrenergic neurons [40, 41], and it functions as a morphogen for kidney development [42]. Over the past several years, a growing number of reports demonstrated that GDNF plays a pivotal role in regulating self-renewal of SSC [4, 8, 9, 17].

We have previously demonstrated that GDNF stimulates the proliferation of C18-4 cells, a murine SSC line [9]. We now report that GDNF/GFRα1/Ret activates the Ras/ERK1/2 pathway to stimulate DNA synthesis and proliferation in these cells. Furthermore, the activation of the Ras/ERK1/2 pathway by GDNF in the SSC line was confirmed in freshly isolated type A spermatogonia from 6-day-old mice. In the seminiferous epithelium of these immature mice, there are A single (As), A paired (Apr), and possibly some A aligned spermatogonia [43]. The A single and possibly the A paired are considered to be the SSC. About 50% of the cells are believed to be GFRα1 positive – these are the spermatogonial stem cells (As/Apr) [9, 14, 43–45]. It was also suggested recently that the A aligned may also serve as stem cells [46].

The C18-4 cell line was created by transfection of spermatogonial stem cells with a plasmid construct containing the SV40 large T antigen driven by a ponasterone A-inducible promoter [24]. However, after several passages, the germ cells escaped the hormonal control and continued to express the viral protein. We show in this report that the C18-4 cells still express the large T antigen and have conserved their germ-line stem cell properties over time. The molecular mechanism by which large T antigen promotes cell proliferation is not completely understood. However the consensus is that the large T protein binds to and inactivates p53 and Rb, and recent studies have confirmed this hypothesis (for reviews, see [47] and [48]). In particular, large T can bind all members of the pRb family, promoting the activation of the E2F family of transcription factors, thus inducing the expression of genes required for the entry into S-phase. Large T antigen does not stimulate Ras signaling, but the co-expression of large T antigen and an oncogenic mutant of Ras are necessary for cell transformation and tumor production [49]. Therefore, it is likely that the effects of large T antigen on the cell cycle are responsible for the basal proliferative state of the C18-4 cells and that the increase in proliferation induced by GDNF, which involves the activation of Ras, is independent from these mechanisms.

A major impediment to a better understanding of stem cell renewal in the testis has been the lack of specific markers that enable their distinction from more differentiated spermatogonia. Much progress in this area has been made during the past few years. GCNA1, Vasa, and Dazl are recognized as markers for male germ cells [50–52], and PCNA is a hallmark for proliferating spermatogonia [53]. In addition, Oct-4 is regarded as a marker for SSC [24, 54, 55]. GFRα1 and Ret are coreceptors for GDNF and are markers for spermatogonial stem cells, although Ret is also present in more differentiated A aligned spermatogonia [9, 17, 18]. Plzf has been characterized as a SSC specific transcriptional factor [56, 57] whereas c-kit is believed to be a marker for differentiating spermatogonia [22, 58, 59]. We found that the C18-4 cells expressed GCNA1, Vasa, Dazl, PCNA, GFRα1, Ret, Oct-4, and Plzf, suggesting that these cells are indeed phenotypically spermatogonial stem cells. Although c-kit mRNA was detected in the C18-4 cells, its protein was not present as demonstrated by immunocytochemical and Western blot analysis (data not shown). These results are consistent with our previous findings [24] and indicate that the C18-4 cells can be maintained in an undifferentiated state and can serve as a model for the spermatogonial stem cells.

We observed that GDNF stimulation led to an activation of the Ret phosphorylation at tyrosine 1,062 in the C18-4 cells. The tyrosine 1,062 in Ret represents a binding site for the docking protein Shc and is crucial for activation of the Ras/ERK pathway [60]. A common route to Ras activation is the recruitment of docking protein Shc by many receptor kinases, including Ret receptor tyrosine kinase [13]. We found that the phosphorylation of all 3 isoforms of Shc was activated in the C18-4 cells with GDNF stimulation and that GDNF induced the binding of the tyrosine phosphorylated Ret to Shc, indicating a direct association of Shc with Ret receptor. The adaptor protein Grb2 is a major link between the Ret receptor tyrosine kinase, Shc, and the Ras pathway [13, 60]. We discovered that GDNF also induced the binding of the tyrosine phosphorylated Ret to Grb2 in the C18-4 cells, reflecting that Grb2 is directly recruited to the activated Ret in these cells after GDNF stimulation [61].

The activated Ret engages docking protein Shc and adaptor protein Grb2, which results in the activation of the Ras/ERK pathway [62, 63]. We have previously shown using microarray analysis that the mRNA of Ras-related protein was upregulated in the GFRα1-positive spermatogonia treated with GDNF [9]. The 21 kDa monomeric G protein Ras is recognized as a major regulator for cellular growth and differentiation [19–21]. We thus tested whether Ras could be activated by GDNF/GFRα1/Ret. We detected an obvious and rapid increase of the active Ras in the C18-4 cells stimulated by GDNF. The activated Ras interacts with a variety of downstream effectors including Raf1. As expected from Ras activation, we also found a significant and rapid increase in the tyrosine phosphorylation of ERK1/2 in the C18-4 cells with GDNF stimulation. Significantly, the increase in tyrosine phosphorylation of ERK1/2 by GDNF stimulation can be completely blocked by pretreatment with PD98059, suggesting that GDNF/GFRα1/Ret activates the PD98059-sensitive ERK pathway.

ERK1/2 activation represents a significant signal for the regulation of cellular growth and proliferation. The activation of Ras/ERK1/2 pathway eventually results in the phosphorylation of specific transcription factors and induces transcription of the immediate-early genes in the nuclei [13]. The CREB/ATF-1 family is composed of CREB, CREM, and ATF-1, which constitutes a subfamily of β-Zip transcription factors, and these factors control eukaryotic gene transcriptional regulation [64, 65]. Knockout studies indicate that the disruption of CREB in mice severely impaired spermatogenesis and resulted in male infertility [66, 67], and CREM deficient mice show a reduction of cell proliferation and DNA synthesis upon partial hepatectomy [68]. Recently, it has been shown that CREB is present in proliferating spermatogonia but not more differentiated germ cells including spermatids and sperm in hydra [69], and a novel splice variant of human testis CREB is chiefly expressed in male germ cells [70]. An important downstream event of the ERK pathway is the phosphorylation of transcription factors CREB, ATF-1, and CREM [13, 71–73], and ERK1/2 regulates the phosphorylation of transcription factor CREB-1 [74]. This prompted us to assess whether GDNF could stimulate the phosphorylation of the members of CREB/ATF-1 family in the C18-4 cells. The phosphorylation of CREB-1 at Ser-133, ATF-1, and CREM-1, was activated by GDNF treatment in the C18-4 cells. The transcription factors CREB, ATF-1, and CREM are known to be a major regulator for immediate-early gene c-fos transcription [68, 72, 75]. We also found a significant increase in the transcription of c-fos in the C18-4 cells treatment with GDNF for 5 and 15 minutes and this remained at a high-level up to 30 minutes. As expected, the increase in c-fos

transcription by GDNF stimulation was completely blocked by pretreatment with the inhibitor PD98059, suggesting that GDNF uses the ERK1/2 pathway to motivate c-fos transcription. Considered together, these results demonstrate that GDNF stimulation of the C18-4 cells results in the activation of the Ras/ERK1/2 signaling cascades that stimulates the phosphorylation of transcription factors CREB-1, ATF-1, and CREM-1 and induces c-fos transcription.

GDNF stimulates cell cycle progress in SSC as indicated by our results showing that GDNF induced a marked increase in BrdU-positive cells and metaphase counts in the C18-4 cells. These results also reflect an essential role of GDNF in stimulating DNA synthesis, proliferation, and mitosis of these cells. Significantly, the inhibitor PD98059 completely blocked the GDNF-induced BrdU incorporation and metaphase figure, implicating that the ERK1/2 pathway is required for the GDNF-stimulated cell cycle progression in the C18-4 cells.

The cell cycle through the G_1/S checkpoint is regulated by multiple mitogenic signaling pathways, including Ras/ERK pathway. We then addressed how the GDNF/GFRα1/Ret/Shc/Ras/ERK1/2/CREB-1 plus CREM-1/c-fos pathway regulates the G_1/S transition in the C18-4 cells. Cyclin A is a key regulatory protein that is involved in control of the S-phase of the cell cycle and associates with CDK2 in mammalian cells. The rationale for examining the expression of cyclin A and CDK2 in the C18-4 cells treated with or without GDNF is that cyclin A and CDK2 function primarily in DNA replication at S-phase [76–78], and that the transcription factor CREB stimulates cyclin A transcription at G1/S-phase [79]. More importantly, a number of reports demonstrate that cyclin A is a target of c-fos in several cell types, including osteoblasts and chondrocytes [80, 81]. There are 2 types of cyclin A, cyclin A1 and A2, which exhibit distinct expression patterns during spermatogenesis. It has been suggested that cyclin A1 is highly expressed in pachytene spermatocytes and required for meiosis, while cyclin A2 is predominantly present in spermatogonia including SSC [22, 82–84]. Using immunohistochemistry, we found that cyclin A is expressed in a subpopulation of spermatogonia along the basement membrane in adult mouse testis (data not shown). Notably, cyclin A is also present in the C18-4 cells further indicating that these cells are SSC. It has been shown that c-fos upregulates cyclin A but not cyclin D1 and enhances CDK2 activity to accelerate S-phase entry in osteoblasts [80], and co-overexpression of CDK2 plus cyclin A and cyclin E rescued the G1/S block resulting from lack of Myc thus enabling the cells to enter mitosis [85]. We found that GDNF upregulated cyclin A expression in the nuclei of the C18-4 cells, and that GDNF induced a marked increase of cyclin A-associated CDK2 expression, which would facilitate the G1/S-phase transition and DNA synthesis in the C18-4 cells. Importantly, the upregulation of cyclin A and CDK2 induced by GDNF in these cells was blocked by pretreatment with the inhibitor PD98059, suggesting that ERK1/2 pathway is essential for the GDNF-induced G1/S transition in the C18-4 cells.

We chose to check the expression of cyclin D1, but not cyclin D2 or cyclin D3, in the C18-4 cells treated with or without GDNF because cyclin D1 was suggested to play a role in regulating the cell cycle through G_1/S-phase progression by the ERK1/2/cyclin D1 pathway [22, 86, 87], and more significantly, cyclin D1 is expressed only in proliferating spermatogonia during spermatogenesis and plays a role in spermatogonial proliferation, in particular during the G_1/S-phase transition [88]. Conversely, cyclin D2 is expressed at epithelial stage VIII when the A_{al} differentiate into A_1, as well as in spermatocytes and spermatids, but not in SSC, A_{pr}, or A_{al} spermatogonia and plays a role in spermatogonial differentiation [88, 89], while cyclin D3 is expressed in terminally differentiated Sertoli cells,

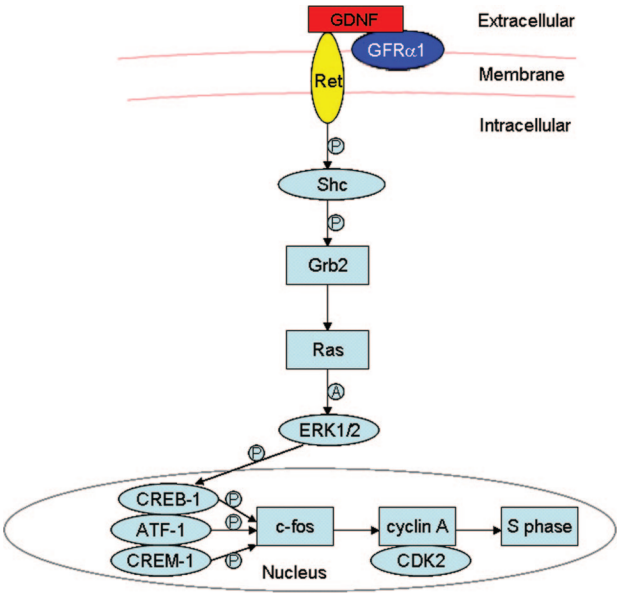

Figure 7. The schematic diagram demonstrates intracellular signaling events in the Ras/ERK1/2 pathway as well as the upstream and downstream cascades activated by GDNF in the C18-4 cells. "P" indicates "phosphorylate", and "A" denotes "activate".

Leydig cells, and spermatogonia in adult testis [88]. Although it was suggested that cyclin D1 plays a role in regulating the cell cycle through G_1/S-phase progression [22, 87, 88], we observed that there was no significant change of cyclin D1 expression in the C18-4 cells with or without GDNF stimulation, indicating that cyclin D1 is not involved in regulating the cell cycle progression from G_1 to S-phase induced by GDNF.

SUMMARY

We have demonstrated that GDNF stimulation of the C18-4 cells results in a series of signaling events in the activation of the Ras/ERK1/2 pathway. These include the phosphorylation of Ret tyrosine kinase, the phosphorylation of docking protein Shc and the recruitment of adaptor protein Grb2, the rapid activation of the Ras/ERK1/2 pathway, the phosphorylation of transcription factors CREB-1, ATF-1, and CREM-1, the induction in c-fos transcription, and the enhancement of cyclin A and CDK2 expression, which leads to accelerate S-phase entry in the C18-4 cells and promotes DNA synthesis and SSC proliferation (see Fig. 7). This study thus offers a novel insight into the dynamics of GDNF signaling in SSC and may shed light on the regulation of stem cell renewal vs. stem cell differentiation.

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Drs. M. Dym and M. Hofmann are co-senior authors.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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