FGF2 mediates mouse spermatogonial stem cell self-renewal via upregulation of *Etv5* and *Bcl6b* through MAP2K1 activation

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**SUMMARY**

Fibroblast growth factor 2 (FGF2) and glial cell line-derived neurotrophic factor (GDNF) are required to recapitulate spermatogonial stem cell (SSC) self-renewal in vitro. Although studies have revealed the role of the GDNF signaling pathway in SSCs, little is known about how FGF2 is involved. In the present study, we assessed the role of the FGF2 signaling pathway using a mouse germline stem (GS) cell culture system that allows in vitro expansion of SSCs. Adding GDNF or FGF2 induced phosphorylation of MAPK1/3, and adding the MAP2K1 inhibitor PD0325901 reduced GS cell proliferation and MAPK1/3 phosphorylation. Moreover, GS cells transfected with an activated form of *Map2k1* not only upregulated *Etv5* and *Bcl6b* gene expression, but also proliferated in an FGF2-independent manner, suggesting that they act downstream of MAP2K1 signaling to drive SSC self-renewal. Although GS cells transfected with *Map2k1*, *Etv5* or *Bcl6b* showed normal spermatogonial markers, transplanting GS cells expressing *Bcl6b* into infertile mouse testes resulted in the formation of a germ cell tumor, suggesting that excessive self-renewal signals causes tumorigenic conversion. These results show that FGF2 depends on MAP2K1 signaling to drive SSC self-renewal via upregulation of the *Etv5* and *Bcl6b* genes.

**KEY WORDS:** Spermatogonia, Self-renewal, FGF2

**INTRODUCTION**

Spermatogonial stem cells (SSCs) have the unique ability to undergo self-renewal division and to support spermatogenesis throughout life. Although SSCs are present in low numbers in the testis (0.02-0.03% of the total testis germ cell population) (Meistrich and van Beek, 1993; Tegelenbosch and de Rooij, 1993), they support spermatogenesis by undergoing self-renewal divisions to maintain themselves and produce progenitor cells that undergo terminal differentiation. Importantly, the SSC division pattern is influenced by the surrounding microenvironment. For example, SSCs increase in number rapidly when the testis is damaged by chemicals or radiation, whereas they divide slowly to produce both stem cells and progenitor cells under physiological conditions (de Rooij and Russel, 2000). Sertoli cells are thought to be the major regulator of SSC self-renewal because they are the only somatic cells that directly interact with germ cells. However, much remains unknown about how the microenvironment regulates self-renewal division and its molecular machinery.

Previous studies implicated glial cell line-derived neurotrophic factor (GDNF) in SSC self-renewal (Meng et al., 2000). GDNF is secreted from Sertoli cells, and transgenic mice that overexpress GDNF accumulate undifferentiated spermatogonia and seminomatous tumors in the seminiferous tubules (Meng et al., 2000; Meng et al., 2001), whereas decreased GDNF levels in heterozygous knockout mice cause hypospermatogenesis and depletion of spermatogonia, leading to infertility. Based on these in vivo observations, an in vitro SSC culture system was developed. Adding cytokines, including GDNF and fibroblast growth factor 2 (FGF2), induces SSCs to increase in number exponentially for at least 2 years (Kanatsu-Shinohara et al., 2005a). The cultured cells, designated germline stem (GS) cells, produced offspring after transplantation into the seminiferous tubules of infertile animals (Kanatsu-Shinohara et al., 2003b). The successful in vitro expansion of SSCs allowed us to collect a large number of SSCs for biochemical and molecular biological analyses to study the influence of various cytokines on SSC self-renewal.

Using this culture technique, we and others previously found that inhibiting the phosphoinositide 3-kinase–AKT pathway abrogates SSC self-renewal and survival in cells cultured with GDNF and FGF2 (Braydich-Stolle et al., 2007; Lee et al., 2007; Oatley et al., 2007). The inhibition was reversible and cells resume proliferation upon removal of the inhibitors. Moreover, we also found that the GDNF signal can be mimicked by activating AKT. In these experiments, GS cells stably transfected with activated AKT1 (Akt-GS cells) proliferated with FGF2 alone for at least 4 months, and reinitiated spermatogenesis and produced normal offspring after transplantation into the seminiferous tubules (Lee et al., 2007). However, AKT activation alone did not induce cell proliferation, suggesting that molecules downstream of the FGF2 signal collaborate with AKT and play important roles in SSC self-renewal.

In contrast to GDNF, little is known about the role of FGF2 in SSC self-renewal. FGF2 is secreted from various cell types in the testis, including Sertoli cells, Leydig cells and differentiating germ cells (Han et al., 1993; Mullaney and Skinner, 1992). Several recent studies have demonstrated the involvement of FGF2 in spermatogonial proliferation. For example, human patients with specific activating FGF receptor mutations have an increased number of mutant sperm, which is thought to be caused by increased clonal expansion of mutant spermatogonia (Goriely et al., 2005). Moreover,
in vivo transduction of FGF4 enhances the regeneration of spermatogenesis after testicular damage (Yamamoto et al., 2002). These beneficial effects of FGF molecules observed in vivo appear to be mediated by direct action on spermatogonia because adding FGF2 significantly improves germ cell survival in vitro (van Dessel-Emilianni et al., 1996). However, the molecules involved in FGF2 signaling and how they collaborate with GDNF for GS cell proliferation have remained elusive.

In this study, we analyzed the role of FGF signaling in GS cell self-renewal. We found that FGF2 induces MAP2K1 phosphorylation and that activation of the MAP2K1 pathway upregulates expression of En5 and Bcl6b, both of which are thought to play crucial roles in SSC self-renewal. GS cells transfected with any of the MAP2K1-ETV5-BCL6B signaling pathway molecules proliferated in the absence of FGF2. Moreover, we also observed that Bcl6b overexpression produced germ cell tumors. These results underscore the importance of MAP2K1 signaling for driving SSC self-renewal.

MATERIALS AND METHODS
Cell culture and transfection
GS cells were established from 8-day-old DBA/2 pups (Japan SLC, Shizuoka, Japan) or from the transgenic mouse line C57BL/6Tg14(Act-EGFP-OsbY01) that was bred into a DBA/2 background (designated Green) (Kanatsu-Shinohara et al., 2003b). GS cell culture was conducted as previously described using StemPro-34 SFM (Invitrogen, Carlsbad, CA, USA) (Kanatsu-Shinohara et al., 2003b). The growth factors used were 10 ng/ml human FGF2 and 15 ng/ml recombinant rat GDNF (both from Peprotech, London, UK). For inhibitor studies, PD0325901 (4 µM; Stemgent, San Diego, CA, USA) or PD98059 (Calbiochem, Tokyo, Japan) were added at the time of cell plating. We selected maximum doses that maintained attachment of mouse embryonic fibroblasts (MEFs) to culture dishes (PD0325901, ~12 µM; PD98059, ~200 µM). To passage the cells in a 6-well culture plate, 0.5 ml 0.25% trypsin in 1 mM EDTA was added, and the cells were incubated for 4 minutes at 37°C. To stop the trypsin activity, 0.5 ml Iscove’s modified Dulbecco’s medium supplemented with 2% fetal calf serum (FCS) (Thermo Fisher Scientific, Waltham, MA, USA) was added. Dissociated cells were triturated by repeated pipetting using P1000 pipette tips and were then centrifuged for 5 minutes. Cell number was determined at each passage. The number of cells seeded was 3.0 × 10³ in a 6-well culture plate.

For lentiviral transfection, cDNAs encoding Map2k1 (MEK-DD), in which two Raf1/MAPKKK-dependent regulatory phosphorylation sites were substituted by aspartic acid residues (S218D/S222D) (Addgene, Cambridge, MA, USA), En5, Lhx1 (both from Open Biosystems, Huntsville, AL, USA) and Bcl6b (gift from Dr T. Tokuhisa, Chiba University, Japan) were cloned into the CSIIL-EF-RES2-Paro vector (a gift from Dr H. Miyoshi, RIKEN BRC, Tsukuba, Japan). For the short hairpin RNA (shRNA)-mediated gene knockdown (KD), KD vectors for En5 (TRCN0000054783 and TRCN0000054786), Bcl6b (TRCN0000084593, TRCN0000084595, TRCN0000084596 and TRCN0000084597) and EGFP (SHC005) (Open Biosystems) were used. Lentiviruses were produced by transfecting 293T cells, as described previously (Kanatsu-Shinohara et al., 2008), and an equal volume of virus supernatant was mixed for infection. All infection experiments were performed in 6-well plates using 3 × 10⁵ GS cells on mitomycin C-treated MEFs. The virus titer of the mixed supernatant was measured using the In Situ Cell Death Detection Kit, TMR Red (Roche Applied Science, Mannheim, Germany) following the manufacturer’s protocol. The stained cells were analyzed using the FACSCalibur system (BD Biosciences, Franklin Lakes, NJ, USA).

Histology and immunohistochemistry
For staining of frozen sections, tissue samples were fixed in 4% paraformaldehyde for 2 hours, embedded in Tissue-Tek OCT compound and processed for cryosectioning. Paraffin sections were prepared by fixation in 10% neutral-buffered formalin. Sections were stained with Hematoxylin and Eosin. Sections were viewed under an inverted microscope (DP70, Olympus, Tokyo, Japan). All immunohistological images were acquired by confocal laser-scanning microscopy (FV1000-D, Olympus).

The primary antibodies used were: monoclonal mouse anti-human MAGEA4, polyclonal rabbit anti-human ALPP (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal rabbit anti-human phospho-MAPK1/3 (Thr202/Tyr204; Cell Signaling Technology, Danvers, MA, USA), monoclonal rat anti-mouse KIT (ACK2), monoclonal rat anti-mouse CDH1 (DECM-A-1) (both from eBioscience, San Diego, CA, USA), and monoclonal rat anti-mouse EPCAM (G8.8; BioLegend, San Diego, CA, USA). The primary antibodies were detected by Alexa Fluor 647-conjugated goat anti-rat IgG, Alexa Fluor 568-conjugated goat anti-rabbit IgG or Alexa Fluor 568-conjugated goat anti-mouse IgG (all from Invitrogen). Hoechst 33342 (Sigma) was used for counterstaining.

For quantification of phosphorylated MAPK1/3 in the testis, cells expressing each marker in 15 tubules were counted.

Western blot analysis
We used SDS-PAGE to separate cell lysates, which were then transferred to Hybond-P membranes (Amersham Biosciences, Little Chalfont, UK) using standard procedures. The primary antibodies used were polyclonal rabbit anti-human phospho-MAPK1/3 (Thr202/Tyr204) and monoclonal mouse anti-MAP2K1 (MEK1, 61B12), and peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (all from Cell Signaling Technology) were used to detect primary antibodies.

Reverse transcription PCR (RT-PCR)
Total RNA was recovered from cultured cells using Trizol following the manufacturer’s protocol (Invitrogen). First-strand cDNA was produced using a Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). PCR conditions were 94°C for 5 minutes, then 30 cycles of 94°C for 30 seconds, 60°C for 1 minute and 72°C for 1 minute, followed by 72°C for 4 minutes. For real-time PCR analyses, transcript levels were normalized to those of Hprt using a StepOnePlus Real-Time PCR system and Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). PCR conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. PCR primers are listed in supplementary material Table S1.

Flow cytometry
Dissociated cultured cells were suspended in PBS containing 1% FCS at 1 × 10⁶ cells/100 µl. The primary antibodies used were: monoclonal rat anti-mouse EPCAM (G8.8; BioLegend), monoclonal mouse anti-mouse FUT4 (MC-480; eBioscience), monoclonal rat anti-human ITGA6 (CD49f; GoH3), biotinylated monoclonal hamster anti-rat ITGB1 (CD29; Ha2/5), biontinated monoclonal rat anti-mouse CD9 (KMC8) and monoclonal rat anti-mouse KIT (CD117; 2B8) (all from BD Biosciences). Allophycocyanin (APC)-conjugated goat anti-rat IgG or APC-conjugated streptavidin (BD Biosciences) were used to detect the primary antibodies. The stained cells were analyzed using the FACSCalibur system.

Germ cell transplantation
Approximately 2 × 10⁶ cells were injected into the seminiferous tubules of 4- to 6-week-old WBB6F1/W-W^{+} (designated W) mice (Japan SLC) via the efferent duct. Testis cells from primary recipients were dissociated 2 months after transplantation by two-step enzymatic digestion using collagenase and trypsin for serial transplantation (Ogawa et al., 1997). Single cells were suspended in 12-16 µl injection medium, which consisted of 0.1% Triton X-100, 0.1% sodium citrate). The cells were labeled using the In Situ Cell Death Detection Kit, TMR Red (Roche Applied Science, Mannheim, Germany) following the manufacturer’s protocol. The stained cells were analyzed using the FACSCalibur system (BD Biosciences, Franklin Lakes, NJ, USA).

Flow cytometry

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining
Cultured cells were washed three times with phosphate-buffered saline (PBS) and suspended at 2 × 10⁷ cells/ml in 100 µl PBS. For fixation, an equal volume of 4% paraformaldehyde was added to the cells for 1 hour at room temperature, followed by incubation in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate). The cells were labeled using the In Situ Cell Death Detection Kit, TMR Red (Roche Applied Science, Mannheim, Germany) following the manufacturer’s protocol. The stained cells were analyzed using the FACSCalibur system (BD Biosciences, Franklin Lakes, NJ, USA).
of Dulbecco’s modified Eagle’s medium with 10% FCS. Approximately 4 μl of the cell suspension was microinjected into each testis of a secondary W recipient. Recipient animals were treated with anti-CD4 antibody to avoid rejection of allogeneic donor cells (GK1.5; gift from Dr T. Honjo, Kyoto University, Kyoto, Japan) as described previously (Kanatsu-Shinohara et al., 2003a). The Institutional Animal Care and Use Committee of Kyoto University approved all animal experimentation protocols.

Analysis of recipient testes
Recipient mice were sacrificed and their testes were recovered at 6-8 weeks post-transplantation to quantify germ cell colonies. Testes were exposed to UV light, and the fluorescent donor cell clusters were defined as colonies when they occupied the entire basal surface of the tubule and were at least 0.1 mm in length.

Statistical analysis
Results of three to four experiments are presented as mean±s.e.m. Data were analyzed with Student’s t-test. Effects of cytokines in real-time PCR or western blot analyses were determined using ANOVA followed by Tukey’s HSD. P<0.05 was considered significant.

RESULTS
Activation of the MAP2K1 pathway in GS cells
GS cells require both GDNF and FGF2 for continuous proliferation (Oatley and Brinster, 2008). Spermatogonia freshly isolated from the testes failed to form germ cell colonies in the absence of FGF2 or GDNF (Fig. 1A). Germ cell colonies were established only when both cytokines were added to the culture medium (Fig. 1A). These cytokines are also important for maintaining GS cells because removing FGF2 or GDNF led to increased apoptosis and cessation of growth in established GS cells (Fig. 1B,C).

Although MAP kinase is one of the major signaling molecules that acts downstream of FGF signaling, we showed previously that GS cell proliferation is not influenced by PD98059, a MAP2K1 inhibitor, at concentrations that inhibit embryonic stem (ES) cell growth (Lee et al., 2007). However, immunohistological staining of the testis showed that phosphorylated MAPK1/3 is found not only in 63/721 (8.7%) of EPCAM+ or 11/423 (2.6%) of KIT+ spermatogonia but also in 3/68 (4.4%) of CDH1+ undifferentiated spermatogonia, which suggested that its activation plays a role in spermatogonia proliferation (Fig. 1D).

In accordance with these results, we found that adding PD0325901, a novel and more specific MAP2K1 inhibitor, significantly reduced GS cell proliferation at lower concentrations (Fig. 1E,F). Whereas control cells could expand ~400% during 6 days on MEFs, the number of cells recovered during the same period was reduced in the presence of PD0325901 in a dose-dependent manner. GS cell proliferation was also inhibited at high PD98059 concentrations (50-150 μM) (Fig. 1E). These results suggest the involvement of MAP2K1 in SSC self-renewal and...
survival. We examined MAPK1/3 phosphorylation by western blotting to confirm the effect of PD0325901 on MAP2K1 activity. After culturing GS cells on laminin-coated dishes for 3 days without cytokines, the cells were restimulated with FGF2 or GDNF. Although adding either FGF2 or GDNF induced MAPK1/3 phosphorylation (Fig. 1G), this phosphorylation by exogenous cytokines was suppressed by PD0325901.

We performed germ cell transplantation experiments to examine the effect of PD0325901 on SSCs (Brinster and Zimmermann, 1994). Transplanted SSCs colonized empty seminiferous tubules and established spermatogenesis for the long term. GS cells were cultured in the presence of PD0325901 (4 μM) for 6 days, and ~2×10^5 cells were transplanted into the seminiferous tubules of congenitally infertile W mice (Fig. 1H). The numbers of colonies generated by PD0325901-treated and control cells in four experiments were 1.6±0.2×10^2 and 1.6±0.3×10^3 colonies/10^5 cells, respectively (n=18). Although GS cell number changed 3.9-fold in control cultures, the cell number did not increase in PD0325901-treated cultures during this period. Therefore, this result suggested that PD0325901 inhibited the net SSC increase by suppressing MAP2K1.

Substitution of the FGF2 signal by a constitutively active MAP2K1
To gain insight into the mechanism of MAP2K1 action, we produced green GS cells that stably overexpressed the constitutively active form of MAP2K1 (MEK-DD) by lentivirus-mediated gene delivery. The morphology of MEK-DD-transfected GS cells (MEK-GS) was indistinguishable from that of parental wild-type green GS (WT-GS) cells (Fig. 2A). Real-time PCR and western blot analyses confirmed increased expression of the transgene (Fig. 2B and supplementary material Fig. S1). RT-PCR and flow cytometric analyses showed that the cells expressed normal levels of spermatogonial markers without the expression of ES cell markers such as FUT4 or Nanog, both of which are expressed in ES-like multipotent germline stem (mGS) cells (Kanatsu-Shinohara et al., 2004) (Fig. 2C,D). Western blot analyses showed changes in MAPK1/3 phosphorylation after transfection (Fig. 2E,F). In WT-GS cells, FGF2 consistently induced stronger MAPK1/3 phosphorylation than GDNF. However, compared with WT-GS cells, MEK-GS cells showed a 7.4±1.3- and 4.6±1.0-fold increase in MAPK1 and MAPK3 phosphorylation, respectively, when cultured without exogenous cytokines (n=4).

Fig. 2. Substitution of FGF2 with MEK-DD.
(A) Appearance of GS cells transduced with MEK-DD. No significant difference was noted to parental control cells. MEK-GS cells could proliferate without FGF2. (B) Western blot analysis of MAP2K1 expression. (C) Flow cytometric analysis of MEK-GS cells cultured on MEFs. Green lines indicate the control. (D) RT-PCR analyses of MEK-GS cells. Samples were collected after culturing GS cells on laminin for 6 days with GDNF and FGF2. (E) MAPK1/3 phosphorylation by cytokine stimulation. Cells were starved for 3 days on laminin and the samples were collected 30 minutes after cytokine stimulation. MEK-GS cells showed strong MAPK1/3 phosphorylation even in the absence of cytokine stimulation. (F) Quantification of phosphorylated MAPK1/3 expression (n=4). Error bars indicate s.e.m. (G) Cumulative growth curve of MEK-GS cells cultured under different cytokine stimulation conditions. MEK-GS cells proliferated with GDNF alone, albeit at a slower rate than with FGF2 and GDNF. Cultures were maintained on MEFs. (H) Culture of MEK-GS cells with GDNF. WT-GS cells stopped proliferating after several passages. Cultures were maintained on MEFs. F, FGF2; G, GDNF. Scale bar: 50 μm.
Although co-stimulation by FGF2 and GDNF further enhanced MAPK1/3 phosphorylation in MEK-GS cells, we did not observe increased MAPK1/3 phosphorylation when either FGF2 or GDNF was added to the culture.

To examine the effect of MAP2K1 overexpression on GS cell proliferation, we cultured MEK-GS cells under different cytokine stimulations. Despite the increased phosphorylation levels, the speed of MEK-GS cell proliferation was comparable to that of control WT-GS cells when they were cultured with both FGF2 and GDNF. These cells could be passaged at a ratio of 4:1 to 6:1 every 5-6 days (Fig. 2G). However, we noted that MEK-GS cells continued to proliferate for more than 8 weeks with only GDNF supplementation, during which time the total cell number increased by ~3.0×10^5-fold (Fig. 2H). The expression level of MAP2K1 did not change significantly, at least at the two points measured during culture (supplementary material Fig. S2). Because MEK-GS cells could expand more than 1×10^5-fold during the experimental period by co-stimulation with FGF2 and GDNF, this suggests that the FGF2 signal provides a stronger stimulus than that of MEK-DD. By contrast, MEK-GS cells cultured only with FGF2 stimulation gradually ceased proliferating and could not be maintained for longer than 4-5 weeks. Similarly, WT-GS cells could not be maintained without FGF2 stimulation after passages (Fig. 2H). These results show that MAP2K1 activation partly substitutes for FGF2 stimulation.

**Identification of target genes that are regulated by MAP2K1**

We conducted RT-PCR on WT-GS and MEK-GS cells to identify the target genes involved in MEK-DD-induced proliferation. After cytokine starvation, we collected both cell types and compared the expression levels of candidate transcription factors known to be expressed in spermatogonia (Oatley and Brinster, 2008). Whereas Neurog3 and Zbtb16 expression levels decreased slightly, MEK-GS cells showed enhanced *Etv5*, *Bcl6b* and *Lhx1* expression when the cells were cultured under cytokine-free conditions (Fig. 3A). This suggests that these three genes are upregulated as a result of MAP2K1 stimulation.

We quantitatively analyzed the expression of these genes in WT-GS cells cultured under different cytokine stimulation conditions. Real-time PCR analyses showed that expression of the three genes was upregulated strongly by FGF2 treatment (Fig. 3B). GDNF also increased their expression, but the expression level was weaker and no statistically significant difference was found. However, GDNF and FGF2 synergistically enhanced the expression of these three genes compared with control samples cultured without cytokines. Although the values for *Etv5* and *Bcl6b* were significantly higher than those induced by FGF2 alone, the increase was not statistically significant for *Lhx1*. Inhibiting MAP2K1 with PD0325901 downregulated their expression in WT-GS cells cultured in the presence of GDNF and FGF2 (Fig. 3C). These results suggest that the MAP2K1 signal is necessary and sufficient for *Etv5*, *Bcl6b* and *Lhx1* gene expression.

To examine their function, we next produced GS cells that stably overexpress each of these candidate genes. We transduced green GS cells with lentivirus vectors expressing *Etv5*, *Bcl6b* or *Lhx1*. The appearance of the transfectants did not differ significantly from that of WT-GS or MEK-GS cells (Fig. 3D). RT-PCR and flow cytometric analyses did not show any significant changes in spermatogonia marker expression (Fig. 3E,F). Additionally, these cells did not express ES cell markers such as *Nanog* and FUT4. Although the growth of these transfectants did not apparently change when they were cultured with both GDNF and FGF2, GS cells expressing either *Etv5* (*Etv5*-GS) or *Bcl6b* (*Bcl6b*-GS) proliferated without FGF2 in a manner similar to MEK-GS cells (Fig. 3G). By contrast, GS cells expressing *Lhx1* (*Lhx1*-GS) were unable to proliferate under the same culture conditions. This occurred despite higher *Lhx1* expression in *Lhx1*-GS cells compared with MEK-GS cells (Fig. 3H). As expected from the MEK-GS cell proliferation pattern, Etv5- or Bcl6b-GS cells stopped proliferating when they were cultured with FGF2 alone (data not shown). The expression levels of *Etv5* and *Bcl6b* did not change significantly at the two points measured during culture (supplementary material Fig. S2). These results suggest that *Etv5* and *Bcl6b*, but not *Lhx1*, act downstream of the MAP2K1 signal to drive GS cell proliferation.

**Regulation of Bcl6b expression by Etv5**

The growth rate of Bcl6b-GS and Etv5-GS cells was comparable to that of MEK-GS cells when they were cultured with GDNF alone. Moreover, real-time PCR analysis showed that expression of *Ret*, a component of the GDNF receptor, did not differ significantly among the three GS cell transfectants (Fig. 4A). These results suggest that the level of GDNF sensitivity is comparable among the transfectants and that both *Etv5* and *Bcl6b* act downstream of GDNF signaling. Therefore, we examined the relationship between these molecules by evaluating *Etv5* and *Bcl6b* mRNA expression levels in each transfectant.

Real-time PCR showed differential *Etv5* and *Bcl6b* expression levels. *Bcl6b* was expressed 4.5-fold more strongly in Etv5-GS cells than in WT-GS cells, whereas the *Etv5* expression level did not change significantly in Bcl6b-GS cells (Fig. 4B,C). Considering that both genes were upregulated in MEK-GS cells, these results suggest that MAP2K1 activation induced *Etv5* gene expression, which then upregulated *Bcl6b*. To corroborate this hypothesis, we used shRNA to examine the effect of gene depletion on proliferation. Depletion of target mRNA was confirmed using WT-GS cells (Fig. 4D). When the shRNA was transfected into the GS cell transfectants, inhibition of *Etv5* decreased the proliferation of MEK-GS cells, and the recovery of cells after 6 days of culture was 56.3% of that of control cells (Fig. 4E). The transfection did not have a significant effect on *Etv5*-GS or *Bcl6b*-GS cells. By contrast, inhibition of *Bcl6b* by shRNA suppressed MEK-GS and *Etv5*-GS cell proliferation to 48.0% and 53.2% of that of control cells, respectively. However, the same treatment did not have significant effects on Bcl6b-GS cells, possibly owing to *Bcl6b* overexpression in these cells. Taken together, these results show that *Etv5* and *Bcl6b* are necessary for GS cell proliferation and that *Bcl6b* acts downstream of *Etv5*.

**Tumorigenic potential of Bcl6b-GS cells**

To confirm whether the transplanted cells maintain normal SSC activities, the cultured cells were microinjected into the seminiferous tubules of W mice at three different time points during culture without FGF2. WT-GS cells that were cultured with both GDNF and FGF2 were used as a control. To test the effect of the transgenes during in vivo colony regeneration, some of the recipient testes were recovered at 2 months post-transplantation and serially transplanted into secondary recipient testes after an analysis of colony numbers under UV light (Fig. 5A). The testes of other recipients were recovered at 3 months for histological analyses.

When the testes were placed under UV light, EGFP-positive colonies were detected, indicating SSC activity of the cultured cells (Fig. 5B, Table 1). Analyses of germ cell colony number in the primary recipients not only confirmed the increase in total SSC numbers for all types of transfectant, but also showed significantly...
increased colony formation of MEK-GS and Bcl6b-GS cells (Fig. 5C). The doubling times of SSCs (GS cells in parentheses) in WT-GS, MEK-GS, Etv5-GS and Bcl6b-GS cells were 3.2 (2.8), 5.0 (5.0), 17.6 (5.3) and 4.8 (5.0) days, respectively (Table 1). Although the colonization levels of Etv5-GS cells varied significantly during culture, this was within the range of previous results with WT-GS cells (Kanatsu-Shinohara et al., 2005b). Serial transplantation experiments did not show significant differences in the number of secondary colonies generated from the transplantation of primary recipient testis cells (Fig. 5C), suggesting that the degree of SSC self-renewal is not significantly influenced by the transgene in vivo.

By contrast, colony morphology analyses suggested abnormal germ cell differentiation from the GS cell transfectants. Although recipient testes with WT-GS cells showed colonies of normal appearance that were filled with vertically differentiating germ cells, MEK-GS cells spread on the basement membrane and few cells differentiated adluminally (Fig. 5D). Cells in the Etv5-GS cell recipients differentiated vertically, but the degree of differentiation appeared to be limited compared with that of WT-GS cells. Bcl6b-GS cells produced colonies with irregular clumps in 4/6 (66.7%) testes (Fig. 5D). These colonies showed irregular EGFP fluorescence and some of the recipient testes were enlarged. Consistent with these observations, histological sections of the recipient testes showed that the donor cells had limited differentiation ability (Fig. 5E). Whereas we observed normal spermatogenesis from parental WT-GS cells, MEK-GS and Etv5-GS cells differentiated only up to spermatocytes. Although no apparent spermatids could be found in the histological sections of the Bcl6b-GS cell recipients, RT-PCR analyses showed expression of Prm1, a haploid-specific marker (Fig. 5F).
Histological sections of the Bcl6b-GS cell recipient testes revealed that the transplanted cells produced an unclassified type of intratubular germ cell neoplasia (Fig. 5G). Immunohistological staining showed that these tumors expressed MAGEA4, which is a marker for seminoma (Aubry et al., 2001). However, ALPP, a marker for seminoma and embryonal carcinoma, was not expressed in these tumors (Fig. 5H), suggesting that they are of a different character to those produced by GS cells upon expressing active Hras1 (Ras-GS cells) or Cnd2/Ccnd1 (cyclin-GS cells) (Lee et al., 2009). Despite the extensive growth, abnormal cells were not found in the interstitial tissue, suggesting that they are benign tumors.

DISCUSSION

The SSC self-renewal machinery has been an enigma. Although previous studies have demonstrated the essential role of GDNF in SSC self-renewal (Meng et al., 2000), little is known about the mechanism of FGF2 action. Moreover, despite the discovery of several transcription factors implicated in SSC self-renewal, much remains unknown about how these transcription factors are regulated by cytokine signals.

We found that MAP2K1 was activated by both FGF2 and GDNF signals, which led to Etv5 and Bcl6b expression. Recent studies have shown the involvement of Etv5 and Bcl6b in spermatogenesis. Etv5 is expressed not only in the germline but also in Sertoli cells. Mice with a targeted Etv5 deletion undergo the first wave of spermatogenesis, but they subsequently show progressive germ cell depletion and a Sertoli cell-only syndrome (Chen et al., 2005). Whether the defect was caused by Sertoli or germ cell dysfunction was unclear in the initial study, but germ cell transplantation experiments later showed that Etv5 is important in both germ cells and Sertoli cells and that Etv5-deficient SSCs have a limited ability to colonize seminiferous tubules of wild-type mice (Morrow et al., 2007; Tyage et al., 2009). By contrast, Bcl6b is not expressed in Sertoli cells, and the involvement of this gene in SSCs was discovered by microarray analyses of cultured spermatogonia stimulated with GDNF. Male mice with a Bcl6b targeted deletion are fertile, indicating that it is dispensable for spermatogenesis (Oatley et al., 2006). However, the animals show focal degeneration of spermatogenesis in the testis and reduced litter size. In vitro experiments have shown by transplantation that transient siRNA oligonucleotide treatment reduces the number of SSCs (Oatley et al., 2006). Because the increase in cell number was not measured during culture in that study, determining whether these genes are involved in SSC survival or self-renewal was not possible.

An important finding of our study is the replacement of the FGF2 signal with MAP2K1, ET5V and BCL6B. FGF2-indepedent proliferation of the three types of transfected GS cells suggests that the FGF2 signal plays a pivotal role in sustaining Etv5 and Bcl6b expression in GS cell cultures. MEK also upregulated Lhx1, but this gene did not have such an effect, despite the increased expression. These results were somewhat unexpected because Etv5 and Bcl6b were upregulated by GDNF in previous studies and their expression was inhibited by an AKT inhibitor (Oatley et al., 2006). Based on these observations, the authors suggested that Etv5 and Bcl6b are downstream GDNF/AKT signaling targets, but the possibility of FGF2-mediated regulation was not investigated. By contrast, although GDNF tended to increase Etv5 and Bcl6b mRNA expression levels in our study, the difference was not significant. In fact, our analyses showed that FGF2 exerted a stronger effect on the induction of these genes than GDNF. Moreover, a MAP2K1 inhibitor, but not an AKT inhibitor, suppressed their expression in our cultures (our unpublished observations).

One possibility that might account for the differences is the timing of sample collection. Whereas Oatley et al. showed that these genes are upregulated 2 hours after GDNF administration following 18 hours of cytokine deprivation (Oatley et al., 2006), we analyzed samples 30 minutes after GDNF administration following 3 days of cytokine deprivation. Longer starvation might have reduced the background signals in our analyses. Another important factor is the genetic background. Whereas previous studies were based on C57BL/6 (B6) mice, we used DBA/2 mice. GS cells are easily derived from the latter strain and proliferate for at least 2 years without significant changes in SSC activity.
(Kanatsu-Shinohara et al., 2005a), whereas those from B6 mice exhibit an almost 10-fold decrease in SSC activity by 14-18 months and do not proliferate under the current culture conditions (Kanatsu-Shinohara et al., 2003b; Schmidt et al., 2011). Although both GDNF and FGF2 activated the MAP2K1 pathway, augmentation of GDNF signaling by GFRA1 is thought to be necessary to derive SSC cultures from B6 mice (Oatley and Brinster, 2008). Therefore, it is possible that SSCs with different genetic backgrounds altered the balance between the two signaling pathways, and these genes might be induced more predominantly by GDNF in a B6 background.

Our study might provide a clue as to the synergistic nature of cytokines in GS cells. GS cells could be established only in the presence of GDNF and FGF2, but why the cells required two cytokines for self-renewal remains unclear. A previous study showed that germ cells in Etv5 knockout mice have reduced Ret expression (Tyagi et al., 2009). Ret mRNA expression was ~60% of that in wild-type testes at postnatal day 4, and it progressively decreased to 10% by day 24. Therefore, reduced GDNF signaling caused the gradual decrease in SSCs in the Etv5 knockout mice. Because FGF2 induces stronger MAPK1/3 phosphorylation than GDNF, Activated MAP2K1 induces the expression of Etv5, which subsequently upregulates Bcl6b expression. Bcl6b overexpression results in germ cell tumor formation after spermatogonial transplantation. Because Ret expression is downregulated in Etv5 knockout mice (Tyagi et al., 2009), Etv5 is probably one of the factors that sustains Ret expression. Etv5 upregulation by FGF2 activation might contribute to the synergistic effect of FGF2 and GDNF. Scale bars: 1 mm in B; 400 μm in D; 20 μm in E,G,H.
modulated the expression of other genes that act oppositely to Bcl6b. These possibilities need to be examined in future experiments.

Another striking observation in this study was the tumorigenesis in the Bcl6b-GS cells. Bcl6b is a homolog of the Bcl6 oncogene, but its oncogenic activity has not been reported. Bcl6b is thought to act as a transcriptional repressor that associates with BCL6 and recruits S1N3A histone deacetylase. We previously showed that transplantation of Ras-GS cells or cyclin-GS cells produces ALPP-expressing seminomas (Lee et al., 2009). Both HRAS1 and cyclin mutations have been implicated in germ cell tumor development in humans (Goriely et al., 2009). These tumors are similar to Bcl6b-induced tumors in that they develop only in vivo. Therefore, the testicular microenvironment provides additional signals for their transformation. Such signals from the testicular microenvironment might also have obscured the effect of the transgenes during serial transplantation. Interestingly, these Ras- or cyclin-induced tumors are distinct in histology from those observed in the current study, as they lacked ALPP expression, suggesting that they developed via a different mechanism. Although no reports are available for germ cell tumors expressing Bcl6b, MAP2K1 has been suggested to be involved in human germ cell tumors (Goriely et al., 2009). Our results lend support to the notion that excessive stimulation of spermatogonia proliferation can cause germ cell tumors, and our novel experimental system might be useful for elucidating the pathogenic mechanism of germ cell tumor development.

In contrast to GDNF, little is known about how FGFR2 contributes to SSC self-renewal. Our study provides an important clue to understanding the action of FGFR2 in SSCs. Several questions need to be addressed in future experiments. First, although Etv5 and Bcl6b overexpression could replace FGFR2 signaling, the cell growth rate was somewhat slower than that of WT-GS cells, suggesting that additional molecules are involved in GS cell proliferation. Comparing Ras-GS cells with Akt-GS or MEK-GS cells might also provide a clue to understanding how self-renewal and differentiating divisions are differentially regulated. Second, we still do not know how MAP2K1 upregulates Etv5 or Bcl6b transcription. Third, it is necessary to determine how other transcription factors interact with ET5 and BCL6B. Whereas Etv5 and Bcl6b are inducible genes, Zbtb16 and Pou5f1 are thought to be regulated by AKT and are not responsive to GDNF stimulation. Understanding these points is the next important goal towards increasing our knowledge of SSC self-renewal.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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Table 1. SSC expansion during long-term culture of WT-GS, MEK-GS, Etv5-GS and Bcl6b-GS cells

<table>
<thead>
<tr>
<th>Transfectant</th>
<th>Days to transplant* (passage)</th>
<th>Colonies/testis</th>
<th>Colonies/10^5 GS cells</th>
<th>Stem cells/10^5 GS cells²</th>
<th>Increase in GS cell number³ (fold)</th>
<th>Increase in stem cell number⁴ (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-GS</td>
<td>0 (0)</td>
<td>4.0±1.2</td>
<td>200.0±57.7</td>
<td>2000.0</td>
<td>1.0×10³</td>
<td>1.2×10³</td>
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<tr>
<td>28 (5)</td>
<td>4.7±0.9</td>
<td>233.3±45.9</td>
<td>2333.3</td>
<td>583.3</td>
<td>6.8×10⁴</td>
<td>1.9×10⁴</td>
</tr>
<tr>
<td>45 (8)</td>
<td>1.2±0.4</td>
<td>58.3±20.0</td>
<td>583.3</td>
<td>583.3</td>
<td>6.8×10⁴</td>
<td>1.9×10⁴</td>
</tr>
<tr>
<td>MEK-GS</td>
<td>0 (0)</td>
<td>7.0±2.5</td>
<td>350.0±125.8</td>
<td>3500.0</td>
<td>5.2×10³</td>
<td>7.7×10³</td>
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<tr>
<td>27 (5)</td>
<td>4.7±1.7</td>
<td>233.8±131.1</td>
<td>2333.3</td>
<td>3583.3</td>
<td>3.0×10⁵</td>
<td>3.1×10⁵</td>
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<tr>
<td>91 (16)</td>
<td>7.2±1.6</td>
<td>358.3±81.1</td>
<td>3583.3</td>
<td>351100.8</td>
<td>1625.0</td>
<td>1625.0</td>
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<tr>
<td>Etv5-GS</td>
<td>0 (0)</td>
<td>3.2±2.0</td>
<td>162.5±100.8</td>
<td>1625.0</td>
<td>4.8×10¹</td>
<td>8.0×10¹</td>
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<tr>
<td>28 (5)</td>
<td>5.3±0.7</td>
<td>266.3±33.3</td>
<td>2663.3</td>
<td>250.0</td>
<td>3.8×10²</td>
<td>5.9×10¹</td>
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<tr>
<td>45 (8)</td>
<td>0.5±0.3</td>
<td>25.0±17.1</td>
<td>250.0</td>
<td>250.0</td>
<td>3.8×10²</td>
<td>5.9×10¹</td>
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<tr>
<td>Bcl6b-GS</td>
<td>0 (0)</td>
<td>6.3±1.2</td>
<td>3167.6±51.1</td>
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<td>2.6×10⁵</td>
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<td>64 (11)</td>
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<td>92 (16)</td>
<td>11.1±2.8</td>
<td>5583.3±139.3</td>
<td>5583.3</td>
<td>5583.3</td>
<td>3.2×10⁵</td>
<td>8.6×10⁵</td>
</tr>
</tbody>
</table>

Values are means±s.e.m. of results from at least four recipient testes. WT-GS cells were cultured with both GDNF and FGFR2, and the others were cultured without FGFR2. In each experiment, 2×10^5 cells were microinjected into the seminiferous tubules of infertile recipient testis.

*The number of days from initiation of culture to transplantation.

It is assumed that 10% of transplanted stem cells can colonize the testis (Kanatsu-Shinohara et al., 2010).

The increase in GS or stem cell number from the first transplantation.

Increment in GS cell number at indicated time point/(stem cells/10^5 GS cells at first transplantation).
Spermatogonia self-renewal


<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<td>Bcl6b</td>
<td>ACTCCTCCGACGTGCTTAGC</td>
<td>GGCCCCGGAAATTTGGAATAG</td>
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<td>Ccna1</td>
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<td>Lhx1</td>
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<td>Prm1</td>
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<td>Ret</td>
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<td>Sohlh1</td>
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