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Akt mediates self-renewal division of mouse spermatogonial stem cells

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Spermatogonial stem cells have unique properties to self-renew and support spermatogenesis throughout their lifespan. Although glial cell line-derived neurotrophic factor (GDNF) has recently been identified as a self-renewal factor for spermatogonial stem cells, the molecular mechanism of spermatogonial stem cell self-renewal remains unclear. In the present study, we assessed the role of the phosphoinositide-3 kinase (PI3K)-Akt pathway using a germline stem (GS) cell culture system that allows in vitro expansion of spermatogonial stem cells. Akt was rapidly phosphorylated when GDNF was added to the GS cell culture, and the addition of a chemical inhibitor of PI3K prevented GS cell self-renewal. Furthermore, conditional activation of the myristoylated form of Akt-Mer (myr-Akt-Mer) by 4-hydroxy-tamoxifen induced logarithmic proliferation of GS cells in the absence of GDNF for at least 5 months. The myr-Akt-Mer GS cells expressed spermatogonial markers and retained androgenetic imprinting patterns. In addition, they supported spermatogenesis and generated offspring following spermatogonial transplantation into the testes of infertile recipient mice, indicating that they are functionally normal. These results demonstrate that activation of the PI3K-Akt pathway plays a central role in the self-renewal division of spermatogonial stem cells.

KEY WORDS: Spermatogenesis, Testis, Stem cells, Transplantation, Microinsemination, Mouse

INTRODUCTION

Spermatogonial stem cells are unique in their self-renewing potential. Although they are present in low numbers in the testis (0.02-0.03% of the total testis cell population) (Tegelenbosch and de Rooij, 1993; Meistrich and van Beek, 1993), these cells divide continuously and produce sperm throughout life in males. Spermatogonial stem cells can produce progenitor cells that undergo terminal differentiation, while maintaining the ability to remain undifferentiated. The control of this process plays a crucial role in spermatogenesis. For example, stem cells can proliferate rapidly when the testis is damaged by chemicals or radiation, whereas under physiological conditions, these cells divide slowly to produce both stem cells and progenitor cells (de Rooij and Russell, 2000). The process of self-renewal is strongly influenced by Sertoli cells, which interact closely with germ cells throughout spermatogenesis.

Although little was known previously about the molecular mechanisms of spermatogonial stem cell self-renewal, it has recently been shown that glial cell line-derived neurotrophic factor (GDNF) is involved in stem cell regulation (Meng et al., 2000). GDNF is secreted from Sertoli cells, and ablation of GDNF by gene targeting reduces the self-renewal of spermatogonial stem cells, whereas GDNF overexpression in transgenic mice stimulates the proliferation of undifferentiated spermatogonia. Based on these findings, we recently replicated the self-renewal of spermatogonial stem cells in vitro; the addition of GDNF to testis cell cultures triggered self-renewal of spermatogonial stem cells in vitro (Kanatsu-Shinohara et al., 2003b). The cells proliferated logarithmically to 10^{85}-fold over 2 years, and produced offspring after transplantation into the seminiferous tubules of infertile animals (Kanatsu-Shinohara et al., 2005c). These studies establish that GDNF is a crucial self-renewal factor for spermatogonial stem cells.

In this study, we analyzed the self-renewal mechanism in spermatogonial stem cells using in vitro cultured spermatogonial stem cells (germline stem, or GS cells). The advent of this cell culture system has allowed us to amplify low numbers of spermatogonial stem cells to large populations, which has facilitated molecular and biochemical analyses of self-renewal division. We found that the self-renewal of GS cells is mediated by the phosphoinositide-3 kinase (PI3K)-Akt pathway, and that this process can be replicated by activating Akt in the absence of GDNF.

MATERIALS AND METHODS

Cell culture

GS cells were established from wild-type DBA/2 mice. In some experiments, we used GS cells derived from the transgenic mouse line C57BL6/Tg14(act-EGFP-OsbY01) bred into the DBA/2 background (designated Green) (Kanatsu-Shinohara et al., 2003b).

Testis cells were collected by two-step enzymatic digestion using collagenase and trypsin (Ogawa et al., 1997), and cell culturing was performed according to the previously published protocol (Kanatsu-Shinohara et al., 2003b). The growth factors used were 20 ng/ml mouse epidermal growth factor (EGF; BD Biosciences, Franklin Lakes, NJ), 10 ng/ml human basic fibroblast growth factor (bFGF; BD Biosciences) and 15 ng/ml recombinant rat GDNF (R&D Systems, Minneapolis, MN). Where indicated, GDNF was replaced with 4-hydroxy-tamoxifen (4OHT) (Sigma, St Louis, MO). Cells were cultured in the presence of 1% fetal bovine serum (JRH Biosciences, Lenexa, KS). LY294002 was used at 33 μM, whereas PD098059 was used at 25 μM (both from Calbiochem, Tokyo, Japan). Transfection was performed using the electroporation method, as described (Kanatsu-Shinohara et al., 2005a).

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Cell cycle analysis
Cultured cells were concentrated onto silane-coated glass slides, and fixed with 70% ethanol at 4°C overnight. Fixed cells were incubated with staining solution (50 μg/ml propidium iodide, 0.09% sodium azide, and 500 μg/ml RNase A in phosphate-buffered saline) at room temperature for 15 minutes. The DNA content was analyzed by laser scanning cytometry (LSC101; Olympus, Tokyo, Japan).

TUNEL assay
After 5 days in culture, the cells were washed, concentrated on glass slides, fixed in 4% paraformaldehyde at room temperature for 1 hour and incubated in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate). The cells were labeled using the in situ cell death detection kit: tetramethylrhodamine (TMR) red (Roche Applied Science, Mannheim, Germany) following the manufacturer's protocol. The slides were counterstained with DAPI, mounted, and analyzed under a fluorescent microscope.

Transplantation procedure
Approximately 1.5×10^4 cells were introduced into the seminiferous tubules of 4- to 10-week-old WBB6F1-W/W^v (W/W) recipient mice, which are congenitally infertile (Geissler et al., 1988). Microinjection was performed by the efferent duct injection method (Ogawa et al., 1997). Because the recipient mice were not histocompatible with the transplanted cells, they were treated with anti-CD4 antibody to induce tolerance to the donor cells (Kanatsu-Shinohara et al., 2003a). The Institutional Animal Care and Use Committee of Kyoto University approved all of the animal experimentation protocols.

Reverse transcriptase-polymerase chain reaction (RT-PCR)
Total RNA was isolated from the cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized using Superscript II (RNase H- reverse transcriptase, Invitrogen). PCR was performed using specific primers (5′-AGCCTAACAGCCACCAAACC-3′ and 5′-TGAATTCTCAGCGGCATG-3′ for *Taf4b*, 5′-AGAGAAGCC-GTATCAGTGCAC-3′ and 5′-CAATCTGTCTCCACCTTCAGC-3′ for *Zfp42*, 5′-GCCTCATTGGAGGAATTCC-3′ and 5′-AGATGCTTGAGA-GCCTCCAC-3′ for *Neurog3*, and 5′-GATCACCCACACTGATGTGG-3′ and 5′-ATGACGAACACGCCTCTCTC-3′ for *Ccnd2*). The remaining primers were previously described (Kanatsu-Shinohara et al., 2005c).

Real-time PCR analysis of *Neurog3* gene expression
To examine the changes in *Neurog3* expression, quantitative comparisons were made by normalizing *Neurog3* expression values to that of hypoxanthine phosphorybosyl transferase using Light Cycler and Light Cycler FastStart DNA Master Plus SYBR Green I (Roche Applied Science). PCR conditions were 95°C for 10 minutes, followed by 40 cycles at 95°C for 5 seconds, 61°C for 10 seconds and 72°C for 12 seconds.

Combined bisulfite restriction analysis (COBRA)
The degree of methylation of the imprinted region was assessed by COBRA using specific primers (Kanatsu-Shinohara et al., 2005c). The PCR products were digested with restriction enzymes that recognize sequences containing the CpG motif.

Analysis of testes
Donor cell colonization was analyzed by UV fluorescence microscopy. For histological analysis, the recipient testes were also fixed in 10% formalin, and processed for paraffin sectioning. All sections were stained with hematoxylin and eosin.

Flow cytometry
The primary antibodies used were: rat anti-EpCAM (G8.8) and mouse anti-SSEA-1 (MC-480; Developmental Studies Hybridoma Bank, University of Iowa), rat anti-human α6-integrin (CD49f) (GoH3), biotinylated hamster anti-rat β1-integrin (CD29) (Ha2/5), biotinylated rat anti-CD9 antigen (KMC8) and APC-conjugated rat anti-mouse c-kit (CD117) (2B8) (all from BD Biosciences). APC-conjugated goat anti-rat-IgG (Cedarlane Laboratories, Ontario, Canada), APC-conjugated streptavidin (BD Biosciences) and Alexa Fluor-633-conjugated goat anti-mouse IgM (Molecular Probes, Eugene, OR) were used as the secondary antibodies. The stained cells were analyzed using the FACSCalibur™ system (BD Biosciences), as described (Shinohara et al., 1999).

Western blot analysis
Samples were separated by SDS-PAGE, and transferred to Hybond-P membranes (Amersham Biosciences, Little Chalfont, UK). The primary antibodies used were: polyclonal rabbit anti-Akt, Akt-P (Ser 473), Akt-P (Thr 308), GSK3β-P (Ser 9), p44/42 mitogen-activated protein kinase (MAPK) (1/2)-P (Thr 202/Tyr 204), cyclin D2, cyclin E2 (Cell Signaling, Danvers, MA), and cyclin A (Upstate Biotechnology, Lake Placid, NY). Monoclonal mouse anti-cyclin B1 (V152) and anti-cyclin D3 (DCS22) (Cell Signaling) were also used. Peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were used as the secondary antibodies (Cell Signaling).

Microinsemination
Microinsemination was performed as described previously (Kimura and Yanagimachi, 1995). Embryos that reached the two-cell stage after 24 hours in culture were transferred to the oviducts of day-1 pseudopregnant Institute of Cancer Research (ICR) female mice. Fetuses that were retrieved on day 19.5 were raised by ICR foster mothers. The presence of the transgene was determined by PCR using *Neo*-specific primers (5′-ATGGGATCGG-CCATTGAACAAG-3′ and 5′-TCAGAAGAACTCGTCAAGAAGGC-3′).

Statistical analyses
All statistical analyses were performed using the Student's *t*-test.

RESULTS
Inhibition of PI3K-Akt signaling prevents GS cell self-renewal
GS cells are critically dependent upon the GDNF for proliferation, such that removal of GDNF from the culture medium results in growth cessation. Whereas the number of GS cells increased to 340% in the presence of GDNF for 6 days, it decreased to 40% of input cells in the absence of GDNF when the cells were cultured on mouse embryonic fibroblasts (MEFs) (Fig. 1A). Likewise, GS cells proliferated to 300% in the presence of GDNF, whereas 110% of the input cell number could be recovered in the absence of GDNF in feeder-free culture conditions on laminin-coated plates (Kanatsu-Shinohara et al., 2005b) (Fig. 1A,B). The low recovery rate was possibly because of apoptosis and limited growth of GS cells in the absence of GDNF. Apoptotic cells were observed after 4 to 6 days, as detected by TUNEL staining (Fig. 1C), and removal of GDNF induced a decrease in the proportion of cells in the S phase and an increase in the proportion of cells in the G1 phase (Fig. 1D).

To determine the signaling pathway for self-renewal division of GS cells, we first examined the effects of pharmacological inhibitors of the PI3K and MAPK pathways, both of which are activated by GDNF-c-ret signaling. When GS cells were cultured in the presence of PD098059 (MEK-specific inhibitor) (Burdon et al., 1999), the GS cells continued to proliferate logarithmically at inhibitory concentrations (2.5 to 25 μM), and the growth rate and morphologies of these GS cells were comparable to untreated cells (Fig. 2A). However, when GS cells were cultured in the presence of LY294002 (PI3K-specific inhibitor), the growth of GS cells was significantly inhibited and the number of cells did not increase after 6 days of culture (Fig. 2A). The cell recovery rate was progressively reduced from ~10 μM LY294002, and effectively blocked at 33 μM LY294002. Cell cycle analysis revealed that LY294002 treatment, but not PD098059 treatment, increased the proportion of cells in the G1 phase (Fig. 2B). Interestingly, this inhibition of GS cell growth was reversible, and GS cell colonies resumed proliferation after the removal of LY294002 (Fig. 2C). Neither LY294002 nor PD098059

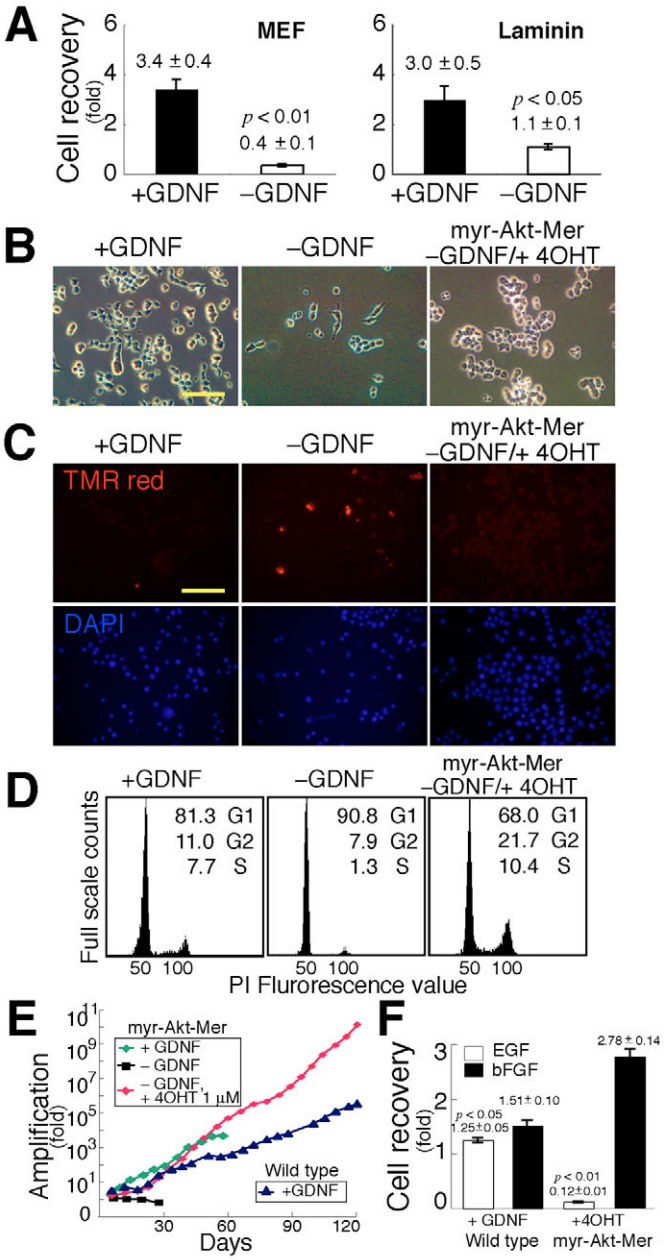

Fig. 1. Effect of GDNF on GS cell proliferation. (**A**) Effect of GDNF on cell recovery after 6 days in culture on MEFs (left) or laminin (right) (mean±s.e.m.; *n*=5). (**B**) Appearance of wild-type (left and center) and myr-Akt-Mer (right) GS cells, 6 days after passage on laminin. (**C**) Apoptosis of GS cells in the absence of GDNF. Apoptotic cells were detected by TMR red staining. Apoptotic cells were observed when GDNF was removed from the culture medium. (**D**) Analysis of cell cycle distribution, 5 days after passage on laminin. Significantly more cells are in the S phase in the presence of GDNF or 4OHT. (**E**) Growth curve for myr-Akt-Mer GS cells that were maintained by 4OHT on laminin. (**F**) Effect of EGF and bFGF on cell recovery after 5 days of culture on laminin (mean±s.e.m.; *n*=5). Whereas the wild-type GS cells can grow in the presence of EGF, myr-Akt-Mer GS cells are unable to respond to EGF. Scale bars: in B,C, 100 μm.

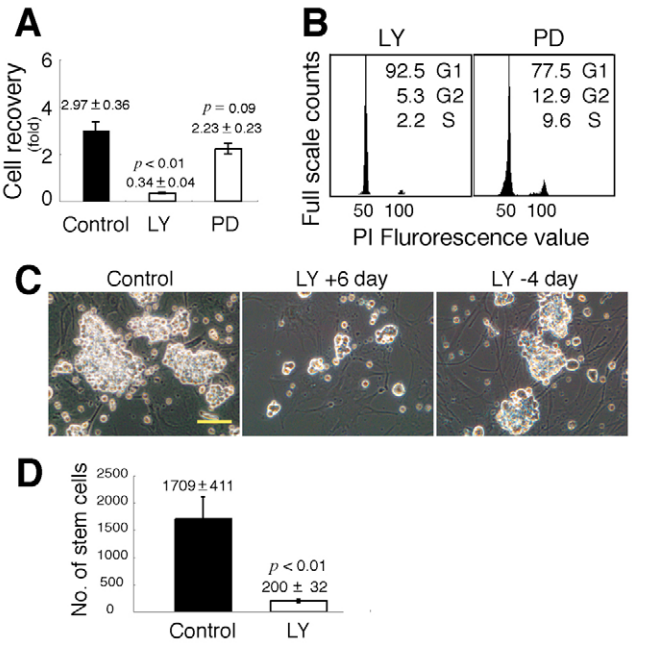

Fig. 2. Inhibition of GDNF-induced proliferation by LY294002. Cells were cultured in the presence of 33 μM LY294002 or 25 μM PD98059. (**A**) LY294002 decreases cell recovery after 6 days in culture on MEFs (mean±s.e.m.; *n*=5). (**B**) Analysis of cell cycle distributions, 5 days after passage on laminin in the presence of 33 μM LY294002 (left) or 25 μM PD98059 (right). Significantly more of the cells are in the G1 phase in the presence of LY294002. (**C**) Appearance of GS cells that were cultured on MEFs in the presence of 33 μM LY294002. Whereas control untreated cells form large colonies within 6 days (left), only small colonies are found in the presence of LY294002 (middle). However, these small colonies reinitiate growth after removal of LY294002 and form large colonies, 4 days after supplementation with fresh medium (right). (**D**) Quantification of spermatogonial stem cells in culture by germ cell transplantation. The same number of GS cells (2.5×10^5 cells per six-well plate) was cultured on MEFs for 6 days with or without 33 μM LY294002, and the total number of spermatogonial stem cells was quantified by transplanting 5×10^4 cells into the seminiferous tubules of infertile recipient testes. The total numbers of stem cells were estimated by multiplying the total cell recovery by the stem cell concentration in the injected cell suspension. Results from three independent experiments (mean±s.e.m.; *n*=9 for control, and *n*=13 for LY294002). Scale bar: in C, 100 μm.

had any apparent effect on GS cell differentiation, as they did not induce the expression of c-kit, a marker of differentiated spermatogonia (data not shown). These results suggest that the PI3K pathway plays a crucial role in the self-renewal of GS cells.

We also performed western blot analysis of the GS cells. In these experiments, GS cells were cultured on laminin-coated dishes in the absence of GDNF for 4 to 6 days, and changes in the phosphorylation patterns were examined upon the addition of GDNF. The phosphorylation of several molecules, including PTEN, Stat3 and cdk4, was not changed significantly by the addition of GDNF (data not shown). However, Akt and MAPK underwent dramatic changes in phosphorylation upon the addition of GDNF, and a slight but clear increase in phosphorylated Gsk3β was also observed (Fig. 3A-C). We also examined the expression of cyclin molecules after addition of GDNF. The changes in cyclin expression were different depending on their types. We were not able to detect cyclin A after 23 hours, whereas cyclin E2 was induced by GDNF (Fig. 3D). By contrast, the changes in cyclin B and cyclin D were relatively modest. This finding agrees with the previous observation that cyclin D is expressed in both proliferating and resting

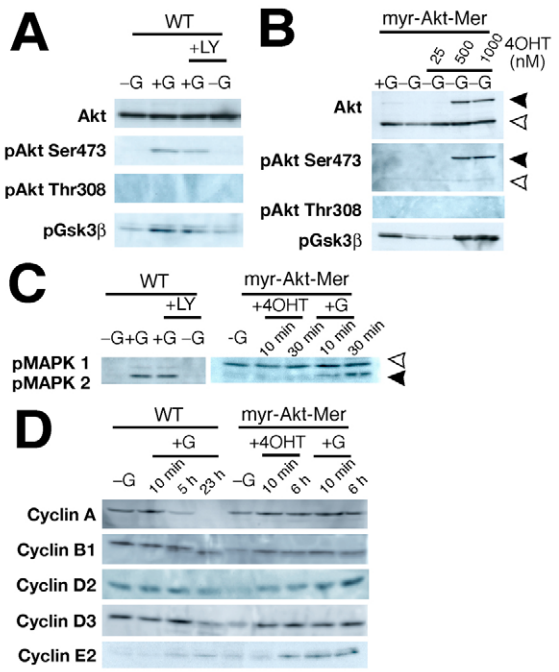

Fig. 3. GDNF signaling in wild-type and myr-Akt-Mer GS cells. (**A**) Phosphorylation of Akt and Gsk3β in wild-type GS cells. GS cells on laminin were starved for 4 days, and then left untreated or treated with GDNF. Where indicated, cells were also incubated for 45 minutes with 33 μM LY294002 before the addition of GDNF. (**B**) Phosphorylation of Akt and Gsk3β in myr-Akt-Mer GS cells. The myr-Akt-Mer GS cells on laminin were cultured with GDNF or indicated concentrations of 4OHT for 6 days. White and black arrowheads represent endogenous Akt and myr-Akt-Mer, respectively. (**C**) Phosphorylation of MAPK after GDNF or 1 μM 4OHT stimulation. (**D**) Expression of cyclin molecules after GDNF or 1 μM 4OHT stimulation.

spermatogonia (Beumer et al., 2000). Phosphorylation of Akt at Ser 473 was detected as early as 10 minutes after the addition of GDNF (Fig. 3A). Although this signal became weaker after 1 hour, it had not disappeared after 3 days (data not shown). Phosphorylation of Akt at Thr 308 was not detected, suggesting that activation of Akt through Ser 473 is involved in GS cell self-renewal.

Proliferation of GS cells transfected with myr-Akt-Mer–expressing plasmid

To further confirm that Akt is functionally involved in the self-renewal of GS cells, GS cells were transfected with an expression plasmid encoding the myristoylated (myr)-Akt-Mer protein, which is a fusion protein consisting of the active form of Akt and the modified ligand-binding domain of the estrogen receptor (Mer), followed by the internal ribosome entry site linked to enhanced green fluorescent protein (IRES-EGFP) (Watanabe et al., 2006). The enzymatic activity of myr-Akt-Mer was activated by the addition of the ligand of Mer, 4OHT, but was not activated in the absence of 4OHT. A previous study has shown that conditional activation of this molecule by 4OHT maintains the pluripotency and growth of embryonic stem (ES) cells (Watanabe et al., 2006). Although the expression level of the transgene was slightly lower than that of the endogenous gene (Fig. 3B), myr-Akt-Mer–expressing GS cells that were cultured in the presence of 4OHT retained the capacity to proliferate, and the morphology of these cells was indistinguishable from that of cells cultured with GDNF (Fig. 1B). We did not observe significant differences between 4OHT and GDNF in cyclin expression level (Fig. 3D). The addition of 4OHT also rescued the apoptosis that was induced by the absence of GDNF (Fig. 1C).

Although the myr-Akt-Mer GS cells proliferated slightly faster than wild-type GS cells (Fig. 1E), both types of cells could be passaged at 1:2 to 1:3 after 5 to 6 days on laminin-coated plates. Interestingly, the proliferation of myr-Akt-Mer GS cells was limited on MEFs, with the cell population multiplying only 2.6-fold in 27 days. Moreover, when we examined the effects of individual cytokines on proliferation, the myr-Akt-Mer cells grew in the bFGF+4OHT but not in the EGF+4OHT condition, suggesting that EGF and bFGF have different roles in promoting GS cell growth (Fig. 1F). This is in contrast to wild-type GS cells, which can grow in either bFGF+GDNF or EGF+GDNF (Kanatsu-Shinohara et al., 2005b). The growth of myr-Akt-Mer GS cells did not significantly increase by raising the 4OHT concentration, and these cells underwent apoptosis at 10 μM, probably because of non-specific effect of 4OHT. The addition of both GDNF and 4OHT also did not act synergistically to improve the growth of GS cells. Indeed, although the addition of 4OHT increased the phosphorylation of Akt at Ser in myr-Akt-Mer GS cells, it did not increase the phosphorylation of MAPK in myr-Akt-Mer GS cells (Fig. 3B,C). These results suggest that Akt alone does not fully mimic the effect of GDNF. Nevertheless, Akt-transfected GS cells continued to proliferate actively on laminin-coated dishes in the absence of GDNF (Fig. 1E). We did not observe the development of ES-like cells in these GS cell cultures (Kanatsu-Shinohara et al., 2004).

Phenotypic and functional analysis of cultured cells

We next examined the phenotypic characteristics of the myr-Akt-Mer GS cells. When the cultured cells were examined for the expression of cell surface markers by flow cytometry (Fig. 4A), they expressed several spermatogonial markers, including β1- and α6-integrins, CD9 and EpCAM. C-kit, which is a marker for differentiated spermatogonia (Yoshinaga et al., 1991), was weakly expressed. However, the cultured cells never expressed stage-specific embryonic antigen 1 (SSEA-1; Fut4 – Mouse Genome Informatics), which is a marker of primordial germ cells (PGCs) and ES cells. RT-PCR analysis also showed the spermatogonial phenotypes of the cultured cells (Fig. 4B). Several spermatogonial markers, including *Pou5f1* (Oct-4), *Zfp42* (Rex-1), *Ret* (c-ret), *Ddx4* (Mvh), *Zbtb16* (PLZF) and *Taf4b* (TAF4b), were expressed in the cultured cells, whereas the expression of *Nanog*, which is a marker of pluripotent cells, was not detected (Chambers and Smith, 2004). These phenotypic features of myr-Akt-Mer GS cells are generally similar to those of wild-type GS cells cultured in the presence of GDNF (Kanatsu-Shinohara et al., 2005c). However, we observed slight upregulation of c-kit, a marker for differentiated spermatogonia (Yoshinaga et al., 1991), in Akt-Mer GS cells in the presence of 4OHT (Fig. 4A), and expression of *Neurog3* (ngn3) was upregulated when GDNF or 4OHT were removed from culture medium (Fig. 4C). Interestingly, upregulation of *Neurog3*, but not c-kit, was similarly observed in wild-type cells in the absence of GDNF.

We also examined the epigenetic properties of the myr-Akt-Mer GS cells. When the methylation statuses of differentially methylated regions (DMRs) in two paternally methylated regions [*H19* and *Meg3 IG* (Gtl2 – Mouse Genome Informatics)] and two maternally methylated regions (*Igf2r* and *Peg10*) were examined, COBRA

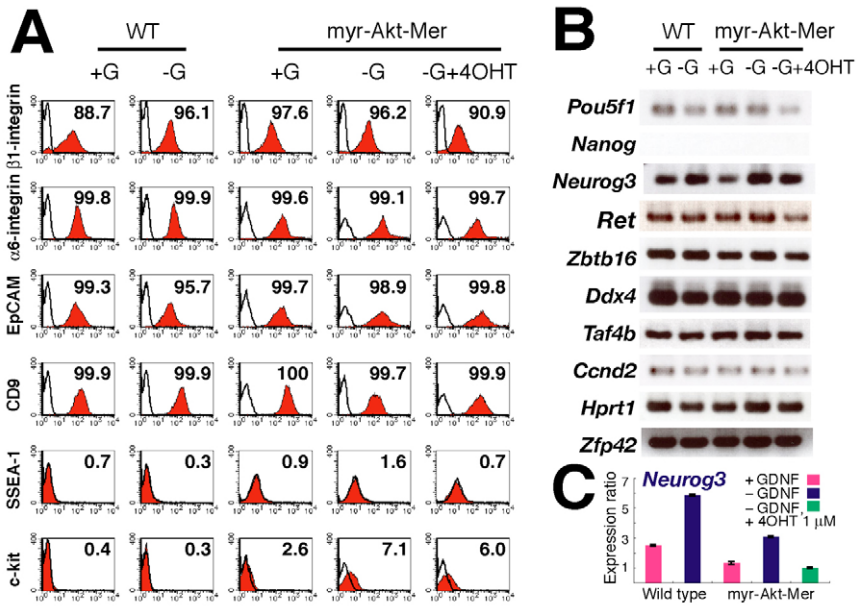

Fig. 4. Phenotypic analysis of GS cells. (**A**) Characterization of cell surface antigens by flow cytometry. Red shading, specific antibody; unshaded, unstained control. (**B**) RT-PCR analysis of GS cells. GS cells on laminin were cultured for 9 days in the indicated conditions. (**C**) Suppression of *Neurog3* transcript expression by GDNF/4OHT identified by real-time PCR analysis (mean±s.e.m.; *n*=3). Expression of *Neurog3* was enhanced in both wild-type and myr-Akt-Mer GS cells in the absence of GDNF.

showed that the DMRs in the cultured cells were methylated in *H19* and *Meg3 IG*, but not in *Igf2r* and *Peg 10*. This androgenetic imprinting pattern did not change when GDNF or 4OHT was removed from the culture medium (Fig. 5).

To determine whether the culture cells had spermatogonial stem cell activity, a spermatogonial transplantation technique was used. Transplanted spermatogonial stem cells can colonize in empty seminiferous tubules and reinitiate and maintain long-term spermatogenesis (Brinster and Zimmermann, 1994). In the first set of experiments, GS cells from Green mouse were cultured in the presence of LY294002 for 6 days, and the cultured cells were microinjected into the seminiferous tubules of infertile W/W mice. W/W mice lack differentiating germ cells because of mutations in the *c-kit* genes (Geissler et al., 1988), and can serve as recipients for germ cell transplantation. Two months after transplantation, donor-derived germ cell colonies were counted under UV light. The number of stem cells recovered was lower (5-28%) in the culture with LY294002 than in the control culture (Fig. 2D). However, normal complete spermatogenesis was detected in the recipient testes by histological analysis, indicating that LY294002 treatment does not compromise the differentiation capacity (data not shown).

We then examined, using the spermatogonial transplantation technique, whether myr-Akt-Mer GS cells that had been maintained with 4OHT retain spermatogonial stem cell activity. After 166 days of in vitro culturing with 4OHT (25 nM to 1 μM), during which time the myr-Akt-Mer GS cells expanded 5.3×10^{10}-fold in the absence of GDNF, the cells were microinjected into the seminiferous tubules of W/W mice. When the testes were placed under UV light, weak EGFP-positive colonies were detected, indicating colonization by the cultured cells (Fig. 6A). Histological sections of the recipient testes showed colonization and differentiation of the transplanted cells, whereas differentiation appeared to continue only up to the round spermatid stage and no elongated spermatids or mature sperm were observed (Fig. 6B). Nonetheless, spermatogenesis continued for at least 8 months after transplantation, confirming the continuous proliferation and differentiation of spermatogonial stem cells. Teratoma formation was not observed for any of the recipient testes. These transplantation experiments demonstrate that the myr-Akt-Mer GS cells have spermatogonial stem cell activities.

Germline transmission of myr-Akt-Mer–expressing GS cells

Finally, to confirm that the germ cells developed from myr-Akt-Mer GS cells are functionally normal, we used in vitro microinsemination (Kimura and Yanagimachi, 1995). The seminiferous tubules of the recipient animals were mechanically dissociated by repeated pipetting, 87 days after spermatogonial

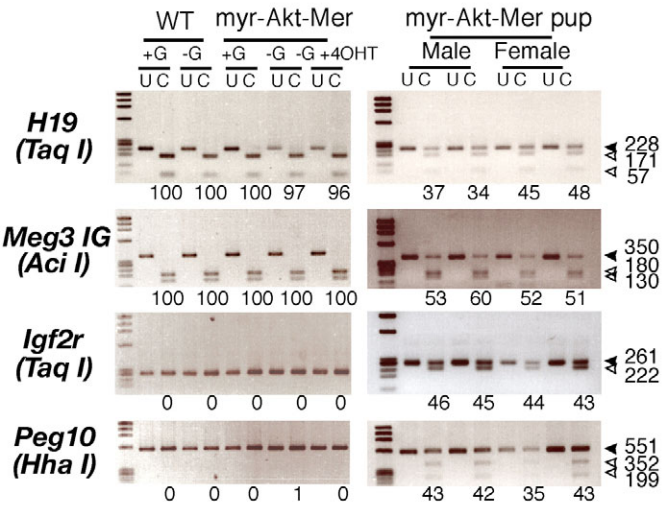

Fig. 5. COBRA of wild-type and myr-Akt-Mer GS cells (left) and offspring derived from myr-Akt-Mer GS cells (right). GS cells on laminin were cultured for 6 days in the indicated conditions. The PCR products of each DMR region were digested with the indicated restriction enzymes with recognition sequences containing CpG in the original unconverted DNA. Black and white arrows indicate the sizes of the unmethylated and methylated DNA fragments, respectively. Levels of percentage methylation, as estimated by the intensity of each band, are indicated below the gels. C, cleaved; U, uncleaved.

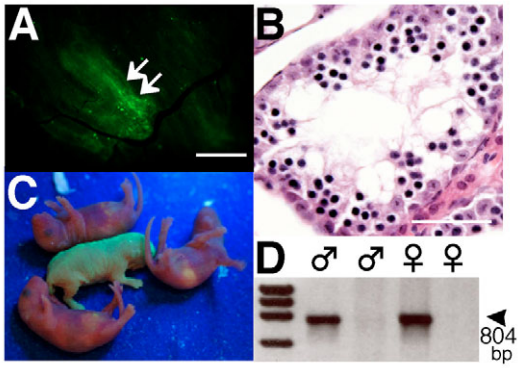

Fig. 6. Germline transmission of myr-Akt-Mer GS cells. (**A**) A recipient testis with EGFP fluorescence in the GS cell-derived germ cell colonies. Arrows indicate the green fluorescence of donor cell colonization. (**B**) Histological appearance of a recipient testis that underwent germ cell transplantation showing round spermatids. (**C**) Offspring derived from the microinjection of oocytes with round spermatids, showing fluorescence. (**D**) PCR analysis of tail DNA samples from the offspring. The *Neo* transgene is detected in two of the four offspring. Scale bars: in A, 500 μm; in B, 50 μm.

transplantation. Round spermatids were picked by micromanipulation, and microinjected into oocytes derived from C57BL/6×DBA/2 F1 females. In all, 26 eggs were cultured for 24 hours, and 24 embryos developed normally into the two-cell stage. These embryos were transferred into the oviducts of two pseudopregnant ICR host mothers. Six embryos implanted successfully in the uteri, and four offspring were born, two males and two females (Fig. 6C). The average body and placental weights of the offspring were within the normal ranges. Two offspring contained the transgene (Fig. 6D), and one of the offspring showed strong EGFP fluorescence under UV light, which confirmed the donor origin (Fig. 6C). These offspring grew up to be normal adults, and COBRA of the offspring revealed normal imprinting patterns (Fig. 5).

DISCUSSION

Studies on tissue-specific stem cells have been hampered by their low numbers and the lack of methods for identification and manipulation. Spermatogonial stem cells provide a valuable opportunity to study self-renewal, as it is possible to collect high numbers of stem cells for molecular characterization and genetic manipulation (Kanatsu-Shinohara et al., 2006). Whereas many signaling pathways possibly maintain the biology of the stem cells, the PI3K-Akt pathway has been directly implicated in the self-renewal of ES cells, which is another cell type that has germline potential. Several recent studies have shown that the activation of PI3K plays a role in the proliferation as well as maintenance of the undifferentiated state in ES cells (Jirmanova et al., 2002; Paling et al., 2004; Watanabe et al., 2006). However, ES cells are different from tissue-specific stem cells in their proliferation and differentiation patterns, which raises the possibility that self-renewing machinery may be different between these two systems (Oatley et al., 2006).

In the spermatogenic system, GDNF was the first molecule that was found to be involved in the self-renewal of spermatogonial stem cells (Meng et al., 2000). However, the downstream signals that mediate this effect remain unclear. GDNF activates a diverse range of molecules upon binding to the c-ret-GFRα complex, including PLC-γ, Shc and the Grb2, Grb7 and Grb10 adaptor proteins, and these molecules eventually lead to activation of the PI3K-Akt and/or MAPK pathways (Arighi et al., 2005). Of these pathways, our study shows that the PI3K-Akt pathway plays a central role in the self-renewal of spermatogonial stem cells. Interestingly, the PI3K-Akt pathway also plays important roles in the differentiating spermatogonia. In vitro studies showed that the PI3K-Akt pathway mediates the signal from the c-kit receptor (Feng et al., 2000; Dolci et al., 2001). Moreover, in vivo study confirmed that the failed PI3K binding to the c-kit receptor decreases Akt activation and induces arrest of spermatogenesis, leaving only spermatogonial stem cells (Blume-Jensen et al., 2000). Thus, these results collectively show that the signals from different receptors are commonly mediated by the PI3K-Akt pathway to regulate the survival and differentiation of spermatogonia from different developmental stages.

In the current study, the growth characteristics and phenotypes of the myr-Akt-Mer–transfected GS cells were somewhat different from those of wild-type GS cells induced by GDNF. This raised a possibility that Akt signaling mediates only the proliferation of GS cells, rather than cell renewal. Indeed, it is impossible to distinguish proliferation of progenitors and stem cells by in vitro experiments, because spermatogonial stem cells are identified by functional assay, not by their proliferative capacity. However, this concern was resolved by the result of the transplantation experiments, which showed that Akt-activated cells had stem cell activity. Therefore, we have reason to believe that Akt may not fully replace the c-ret signals but at least mediate stem cell self-renewal. From this viewpoint, the upregulation of c-kit in some of the myr-Akt-Mer GS cells may suggest that loss of GDNF signaling triggered differentiation in spermatogonial stem cells but that Akt activation alone was not strong enough to prevent all of them from differentiating. Whereas the activation of the MAPK pathway may cooperate to enhance the biological activity of Akt, it is also possible that GDNF signals through neural cell adhesion molecule (NCAM) to activate Src-like kinase Fyn and focal adhesion kinase (Paratcha et al., 2003). Because NCAM is expressed in spermatogonia (Orth and Jester, Jr, 1995), further studies are required to examine whether c-ret is the only receptor to mediate the effect of GDNF and whether other c-ret downstream molecules are involved in the self-renewal process.

GS cells have several other unique characteristics (e.g. stable karyotype, shortening telomeres) that may also be governed by the PI3K-Akt pathway (Kanatsu-Shinohara et al., 2005c). In particular, recent studies have revealed a close relationship between the PI3K-Akt pathway and pluripotency. Activation of the PI3K-Akt pathway in PGCs not only promotes the proliferation in vivo, but also enhances their conversion into teratoma or pluripotent embryonic germ (EG) cells (Kimura et al., 2003; Moe-Behrens et al., 2003). Whereas PGCs give rise to EG cells when cultured in the presence of stem cell factor (SCF), leukemia inhibitory factor (LIF) and bFGF (Matsui et al., 1992; Resnick et al., 1992), similar multipotent GS (mGS) cells can be derived from postnatal testes when cultured in the presence of GDNF, LIF, bFGF and EGF (Kanatsu-Shinohara et al., 2004). Because both GDNF and SCF can activate the PI3K-Akt pathway, we anticipated that strong activation of myr-Akt-Mer by 4OHT or costimulation with GDNF might enhance the frequency of mGS cell development, which occurs at a low frequency in wild-type testis cultures (1 in 1.5×10^7 testis cells). However, in the current study, we did not observe mGS cell formation, suggesting that the conversion of germline lineage cells into pluripotent cell types appears to be mediated by distinct mechanisms, which may depend on the stage of germline development.

Whereas the transplantation study showed that activation of Akt supported stem cell self-renewal, it is unclear why the differentiation of the transplanted cells was incomplete. Although infertility was rescued successfully by in vitro microinsemination, the myr-Akt-Mer GS cells stopped differentiation at the round spermatid stage even after 8 months. At present, we can only speculate that myr-Akt-Mer protein was slightly activated by cross-reactivity with unknown endogenous steroid ligands in the testis, and that this interfered with spermiogenesis. Interestingly, a recent study has shown that the expression of dominant-negative c-ret molecules in transgenic animals results in reductions in numbers of round and elongated spermatids (Jain et al., 2004). Our study may provide additional evidence that the level of c-ret signaling has an impact in the later stages of spermatogenic cell differentiation.

The current study reveals the molecular machinery of the GDNF signaling pathway in spermatogonial stem cell self-renewal. Besides GDNF, several molecules, such as PLZF, Bcl6b, FGFR2 and TAF4b, have now been implicated in spermatogonial stem cell self-renewal (Buaas et al., 2004; Costoya et al., 2004; Falender et al., 2005; Oatley et al., 2006). Their functional interactions with GDNF signaling will be the next focus of study, which will be useful for dissecting this unique process.

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