Discussion

In this study, we examined the SP phenotype of a pure spermatogonia population *in vitro* using GS cell culture, and we transplanted GS cells cultures into testes so that we could follow SP phenotypic changes occurring in response to the seminiferous tubule microenvironment. Although the analysis of SP cells in the testis can be complicated by contaminating Leydig stem cells, which also exhibit an SP phenotype [27], we were able to exclude these cells from our analyses because we gated only EGFP-expressing germ cells developed from transplanted GS cells. Although some of our GS cell cultures contained SP cells and we consistently found SP cells *in vivo* after transplantation of WT or *H-RasV12*-expressing GS cells, SP cells from these sources were not enriched in SSCs. Thus, the SP phenotype does not appear to be a reliable criterion for defining SSCs both *in vitro* or *in vivo*.

Previous studies on the feasibility of SSC enrichment via the SP phenotype have yielded inconsistent results. Whereas three studies demonstrated significant SSC enrichment in both pup and adult testes [9, 10, 12], two other studies failed to show SSC enrichment in the SP population [6, 25]. EPCAM-expressing undifferentiated spermatogonia from adult testis were also reported to contain SP cells, but no transplantation was conducted to confirm SSC enrichment in that study [11]. In the present study, SP cells appeared stochastically *in vitro*, but consistently *in vivo* 3–4 months after transplantation, which was when the donor cells were undergoing steady spermatogenesis. Consistent SP cell development was also observed after transplantation of *H-RasV12*-expressing GS cells. These results confirm previous reports that SP cells are produced not only by Leydig cells but also by germ cells [6, 9–12, 25]. However, despite the consistent development of SP cells in seminiferous tubules, transplantation of SP cells did not enrich the SSCs or cancer stem cell population in the recipient testes.

At present, it is difficult to reconcile the contradictory results of this and some of the previous studies; however, it was pointed out that the use of cryptorchid testes as SSC donor in some studies may have influenced SSC biology in those studies [10]. Hoechst 33342 toxicity might also contribute to the inconsistency of the SP phenotype [25]. However, we did not note any differences between cryptorchid and WT testes with regard to SP phenotype, and we confirmed that cell sorting using Hoechst 33342 at the experimental concentration of 4 μg/ml had no apparent toxic effects, at least at the time of transplantation, by trypan blue staining. Although further studies are needed to explain the various experimental discrepancies, our results indicate that the SP phenotype is not practically useful for identifying SSCs in GS cell culture. Moreover, our failure to observe SSC enrichment after GS cell transplantation calls into question the biological significance of the SP phenotype. Although it is possible that GS cells are culture-adapted/modified germ cells, they reinitiate normal spermatogenesis after transplantation, and we observed consistent SP cell development, as other studies have reported. As previously suggested [28], caution is necessary when an SP separation method is used for SSC identification *in vivo*.

Our results also provide evidence for SP-phenotypic stochasticity of GS cells. The SP phenotype was not only unstable, but was also interconvertible with the non-SP phenotype. We previously demonstrated a similar phenotypic reversibility in SSCs [29]; in GS cell cultures, SSCs exhibited nontraditional phenotypes and stochastically expressed KIT, a marker of differentiating spermatogonia and early spermatocytes. The KIT^+ and KIT^- GS cells had comparable levels of SSC activity, and spermatogenesis occurred after transplantation of either population [29]. Although another group recently reported that rat SSCs differentiate in a stochastic manner [30], their findings were distinct from ours with regard to the reversibility of SSC fate. Whereas SSCs in the rat study were irreversibly committed to differentiation and died synchronously, the KIT phenotype in our previous study was interconvertible: KIT^+ cells turned into KIT^- cells without losing SSC activity [29]. In this context, the reversibility of the SP phenotype in the present study is reminiscent of the reversibility of KIT expression, confirming the fluctuation of the SSC phenotype *in vitro*.

SP phenotypic reversibility has also been reported in hematopoietic stem cells (HSCs). SP cells in bone marrow are remarkably enriched in HSCs [7, 8]. Although SP cells were initially thought to differentiate unidirectionally into non-SP cells, quantitative transplantation studies have revealed that both SP and non-SP cells exhibit similar levels of HSC activity [31]. Moreover, the non-SP and SP phenotypes are reversible *in vivo* after transplantation. Although SP cells developed only inconsistently in our GS cell cultures, they develop consistently in hematopoietic systems. Although the lack of HSC culture systems prevents direct comparison with the spermatogenic system, these studies showed that the SP phenotype does not specify all HSCs. In fact, the SP phenotype is not the only unstable characteristic of HSCs; HSCs also exhibit dynamic changes in the expression of CD34 [32], a marker for activated HSCs that is not expressed in mitotically quiescent HSCs. It is possible that SSCs might have a similarly flexible phenotype and do not necessarily show fixed marker expression.

The findings of the present study extend our previous study and provide additional evidence that SSCs exhibit different phenotypes in different biological microenvironments. We speculate that the stochastic changes observed in GS cells *in vitro* are attributable to excessive stimulation of SSC self-renewal. Indeed, SSCs *in vivo* are constantly exposed to differentiation triggers, while the differentiation of SSCs *in vitro* is probably inhibited by their continuous exposure to GDNF. Although a recent study suggested that SSCs differentiate stochastically *in vivo* during steady-state spermatogenesis [33], we do not know whether SSC commitment occurs in the same manner *in vitro*. Comparison of *in vivo* and *in vitro* phenotypes may provide clues to the mechanisms by which SSCs are triggered to self-renew or differentiate and will further enhance our understanding on SSC fate commitment and its regulation.

Acknowledgments

We thank Ms Y Ogata for technical assistance. Financial support for this research was provided by the Genome Network Project, Japan Science and Technology Agency (CREST), Program for Promotion of Basic and Applied Researches for Innovation in Bio-orientated Industry, the Ministry of Health, Labour, and Wel-

fare of Japan, Senri Life Science Foundation, Astellas Foundation for Research on Metabolic Disorders, and the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan.

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BIOLOGY OF REPRODUCTION 84, 97–105 (2011)
Published online before print 15 September 2010.
DOI 10.1095/biolreprod.110.086462

Serum- and Feeder-Free Culture of Mouse Germline Stem Cells[1]

Mito Kanatsu-Shinohara,[3] Kimiko Inoue,[4] Narumi Ogonuki,[4] Hiroko Morimoto,[3] Atsuo Ogura,[4] and Takashi Shinohara[2,3,5]

Department of Molecular Genetics,[3] Graduate School of Medicine, Kyoto University, Kyoto, Japan
The Institute for Physical and Chemical Research (RIKEN),[4] Bioresource Center, Ibaraki, Japan
Japan Science and Technology Agency (CREST),[5] Kyoto University, Kyoto, Japan

ABSTRACT

Spermatogonial stem cells (SSCs) undergo self-renewal divisions to support spermatogenesis. Although several in vitro SSC culture systems have been developed, these systems include serum or fibroblast feeders, which complicate SSC self-renewal analyses. Here, we developed a serum- and feeder-free culture system for long-term propagation of SSCs. In addition to the SSC self-renewal factors, including glial cell line-derived neurotrophic factor, supplementation with fetuin and lipid-associated molecules was required to drive SSC proliferation in vitro. Cultured cells proliferated for at least 6 mo at a rate comparable to that of serum-supplemented cultured cells. However, germline potential was reduced under serum- and feeder-free conditions, as indicated by a lower SSC frequency after germ cell transplantation. Nevertheless, the cultured cells completed spermatogenesis and produced offspring following spermatogonial transplantation into seminiferous tubules of infertile mice. This culture system provides a basic platform for understanding the regulation of SSC fate commitment in vitro and for improving SSC culture medium.

culture, developmental biology, gametogenesis, self-renewal, Sertoli cells, serum, spermatogenesis, testis, transplantation

INTRODUCTION

Spermatogonial stem cells (SSCs) undergo self-renewal division and support spermatogenesis. A single SSC can produce two SSCs or two differentiating cells [1]. The two types of division are thought to occur with equal frequency, thereby maintaining a constant SSC population size while producing many differentiating cells. SSC identification has been challenging, both because of the difficulty in distinguishing SSCs from other committed, undifferentiated spermatogonia and because of the lack of SSC-specific markers. However, this problem was resolved by development of a germ cell transplantation technique [2] in which transplanted SSCs reinitiate long-term spermatogenesis after microinjection into the seminiferous tubules of infertile mice, which can then produce offspring by mating with wild-type females.

The estimated number of SSCs in the testis is very small, because SSCs comprise 0.02–0.03% of the total testis germ cell population [3, 4]. However, the SSC frequency changes significantly according to the levels of the SSC self-renewal factor glial cell line-derived neurotrophic factor (GDNF) [5]. GDNF overexpression induced the accumulation of undifferentiated spermatogonia in *Gdnf* transgenic mice, whereas decreased GDNF levels in heterozygous *Gdnf* knockout mice resulted in hypospermatogenesis and male infertility. Based on this in vivo finding, a long-term SSC culture system was developed in which SSCs proliferated in the presence of GDNF supplemented with epidermal growth factor (EGF) or basic fibroblast growth factor (FGF2) on mitomycin C-treated mouse embryonic fibroblasts (MEFs) in the presence of fetal bovine serum (FBS) [6]. The cultured cells, deemed germline stem (GS) cells, proliferated as spermatogonia in vitro but reinitiated spermatogenesis following transplantation into seminiferous tubules. GS cells maintained fertility after 2 yr of consecutive culture [7], whereas embryonic stem (ES) cells lost germline potential after several months [8, 9]. GS cells have been used to produce knockout mice after homologous recombination [10]. Moreover, they have pluripotency potential and convert into ES-like multipotent GS (mGS) cells [11].

The initial GS cell culture protocol was subsequently simplified and improved. Although GS cells proliferated more rapidly than SSCs in vivo (doubling every 2–3 days) [6, 7], the serum and fibroblast feeder cells used for GS cell culture prevented SSC self-renewal analyses. When propagated without MEFs, GS cells proliferated successfully over 6 mo on laminin-coated plates and retained fertility [12]. Moreover, GS cells could be maintained in suspension without feeder or laminin support [13]. Although GS cells proliferated more slowly in suspension than on MEFs (doubling every 4–5 days), the cells expressed spermatogonial markers and produced offspring following spermatogonial transplantation. This result was unexpected, because ES cells readily differentiate and lose germline potential in suspension. Thus, GS cells have a relatively flexible requirement for structural support in maintaining their stem cell potential.

Conversely, the effect of serum in GS cell culture is not well understood. Generally, serum varies from batch to batch and often induces stem cell differentiation. Although bone morphogenic protein (BMP) is important for derivation, maintenance, and proliferation of ES cells, only a minute amount of BMP in serum induced differentiation when leukemia-inhibitory factor (LIF) was removed [14]. Moreover, neural stem cells initiated differentiation following the addition of serum to the medium [15]. One study demonstrated an inhibitory role of serum for SSCs and showed the feasibility of serum-free culture on LIF-secreting STO (S, Sandoz inbred mouse; T, 6-thioguanine-

[1]Supported by the Genome Network Project; Japan Science and Technology Agency (CREST); Program for Promotion of Basic and Applied Researches for Innovation in Bio-orientated Industry; the Ministry of Health, Labor, and Welfare; Senri Life Science Foundation; Novartis Foundation (Japan) for the Promotion of Science; Astellas Foundation for Research on Metabolic Disorders; and the Ministry of Education, Culture, Sports, Science, and Technology (MEXT).
[2]Correspondence: Takashi Shinohara, Department of Molecular Genetics, Graduate School of Medicine, Kyoto University, Yoshida Konoe, Sakyo-ku, Kyoto 606-8501, Japan. FAX: 81 75 751 4169;
e-mail: takashi@mfour.med.kyoto-u.ac.jp

Received: 3 June 2010.
First decision: 29 June 2010.
Accepted: 30 August 2010.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

resistant; O, ouabain-resistant) embryonic fibroblasts [16]. However, throughout that study, serum was continuously used to neutralize trypsin [17], which complicated the interpretation of results. We also developed another serum-free culture system for GS cells using MEFs [12], but we could not exclude the possibility that despite extensive washing, residual serum in MEFs might have caused GS cell proliferation. In fact, when GS cells were transferred onto laminin-coated plates in the same culture medium, they failed to propagate, and proliferation ceased. Serum was also necessary when we derived cultured SSCs from embryos or hamster testes [18, 19]. However, the component in the serum that caused GS cell self-renewal remained unknown.

In the present study, we report, to our knowledge, the first successful culture of SSCs under serum- and feeder-free conditions. Using these conditions, the cells proliferated for 6 mo and reinitiated spermatogenesis after germ cell transplantation. However, the cells demonstrated a lower SSC frequency, suggesting that serum enhanced the self-renewal division. Nevertheless, the development of a new GS cell culture system may be useful for understanding the self-renewal machinery and the biological potential of these cells.

MATERIALS AND METHODS

Cell Culture

The GS cells were established from the transgenic mouse line B6-*TgR(ROSA26)26Sor* (The Jackson Laboratory) bred into a DBA/2 background [6]. For primary culture of gonocytes, we used 0-day-old pups in ICR background (Japan SLC). Gonocytes were enriched by gelatin selection and cultures initiated as described previously [6]. For primary culture of spermatogonia, we used 8-day-old pups in DBA/2 background (Japan SLC). Spermatogonia were collected by using a magnetic bead selection technique with biotinylated rat anti-mouse CD9 antibody (KMC8; BD Bioscience) and streptavidin-conjugated Dynabeads (Invitrogen) as previously described [20]. After selection, 2–3 $\times$ 10^5 cells were plated in six-well plates coated with laminin (20 μg/ml; BD Biosciences).

Medium A (original GS cell medium) was made by supplementing StemPro-34 serum-free medium (Invitrogen) with 20 ng/ml of mouse EGF, 10 ng/ml of human FGF2, and 15 ng/ml of rat GDNF (all from PeproTech EC) as described previously [6]. This medium contained 1% FBS (Thermo Fisher Scientific). Medium B was modified from medium A by replacing serum and bovine serum albumin (BSA; ImmunO, fraction V; MP Biomedicals) with 3 mg/ml of lipid-rich BSA (Albumax II; Invitrogen), 1 mg/ml of fetuin (Sigma), 1:100 Lipid Mixture 1 (Sigma), and 1:1000 Lipoprotein-Cholesterol Concentrate (MP Biomedicals). Cells were cultured on laminin-coated dishes at 37°C under of 5% CO_2 in air. The cells were passaged by incubation with 0.25% trypsin for 5 min. Trypsin reaction was stopped by adding two volumes of medium B. In some experiments, we also used sphingosine kinase inhibitor 2 (SKI II; 10 μM; Cayman Chemical) at the time of cell plating. When indicated, heat-inactivated FBS or charcoal-treated FBS (Thermo Fisher Scientific) was added to the medium as a control. Sera from sheep, horse, goat, rabbit, and pig (all from Invitrogen) were used after heat inactivation for 30 min at 55°C. The cells were cryopreserved after adding 10% dimethyl sulfoxide (DMSO; Sigma).

For laminin adhesion assays, 2 $\times$ 10^5 cells/well were plated in six-well plates coated with laminin and incubated overnight at 37°C. Adherent cells were recovered by trypsin digestion after washing the plates twice with PBS as previously described [21].

Flow Cytometry

The primary antibodies used were mouse anti-FUT4 (MC-480; Chemicon), rat anti-mouse EPCAM (G8.8), rat anti-human ITGA6 (CD49f) (GoH3), biotinylated hamster anti-rat ITGB1 (CD29) (Ha2/5), biotinylated rat anti-mouse CD9 (KMC8), and rat anti-mouse KIT (CD117) (2B8) (all from BD Biosciences). Allophycocyanin (APC)-conjugated goat anti-rat immunoglobulin G (Cedarlane Laboratories) and APC-conjugated streptavidin (BD Biosciences) were used to detect the primary antibodies. Antibodies were used at 5 μg/ml.

Transplantation Procedure

For germ cell transplantation, cultured cells were microinjected into the seminiferous tubules of WBB6F1-W/W^v (W) mice via an efferent duct (Japan SLC). Approximately 80–90% of the tubules were filled in each recipient testis. To avoid rejection of donor cells, the recipient mice were treated with anti-CD4 antibody (GK1.5) to induce tolerance to the donor cells [22]. The Institutional Animal Care and Use Committee of Kyoto University approved all of the animal experiments.

Analysis of Recipient Testes

The donor cell colonization levels were determined by staining the recipient testes for the LacZ gene product, β-galactosidase with X-gal (Wako Pure Chemical Industries), as previously described [23]. Tubules that stained blue were counted under a stereomicroscope. Colonies were defined as germ cell clusters longer than 0.1 mm that occupied the entire circumference of the tubule. For histological analyses, the samples were embedded in paraffin blocks and processed for sectioning. All sections were 12 μm in thickness and were stained with hematoxylin and eosin.

Analysis of Gene Expression

Total RNA was isolated using TRIzol reagent (Invitrogen), and first-strand cDNA was produced by Superscript II (RNase H^- reverse transcriptase; Invitrogen). RT-PCR was performed using the specific primers listed in Supplemental Table S1 (available online at www.biolreprod.org). To quantify mRNA expression by real-time PCR, we used a StepOnePlus real-time PCR system and *Power* SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's recommendations. The transcript levels were normalized to those of *Hprt*. The PCR conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The experiments were performed on six independent samples, and each PCR reaction was run in triplicate.

Combined Bisulfite Restriction Analysis

Genomic DNA was treated with sodium bisulfite, which deaminates unmethylated cytosines to uracils but does not affect 5-methylated cytosines. Using this template, we amplified the indicated differentially methylated regions (DMRs) by PCR with the specific primers listed in Table S1. The PCR products were digested with the indicated restriction enzymes, which had recognition sequences containing CpG in the original unconverted DNA. The intensity of the digested bands was assessed using Image Gauge software (Fuji Photo Film).

Karyotype Analysis

Cultured cells were harvested, treated with 75 mM KCl for 15 min, and fixed with methanol/acetic acid (3:1). Metaphase spreads were prepared using standard procedures, and the slides were stained with Hoechst 33258 (Sigma).

Microinsemination

Spermatozoa were collected by mechanically dissociating the seminiferous tubule segments of fresh or frozen recipient testes [24]. These cells were microinjected into C57BL/6 $\times$ DBA/2 (BDF1) oocytes using a piezo-driven micropipetter (PrimeTech) [25]. Embryos at the 2-cell stage after 24 h in culture were transferred to the uteri of ICR recipient females.

Statistical Analyses

Results are presented as the mean ± SEM. Data were analyzed by Student *t*-test.

RESULTS

Requirement of Fetuin in Feeder-Free Culture of GS Cells

The GS cells changed their morphology when they were transferred onto laminin-coated plates (Fig. 1A). Original GS cell culture medium (medium A) contained serum. However, removal of serum prevented GS cells from attaching to the laminin-coated plates in the next passage, and proliferation ceased. The failure to attach to laminin was not ameliorated by reducing the trypsin concentration or by using other proteases, such as type I collagenase or dispase, suggesting that destruction of cell surface molecules during passage is not

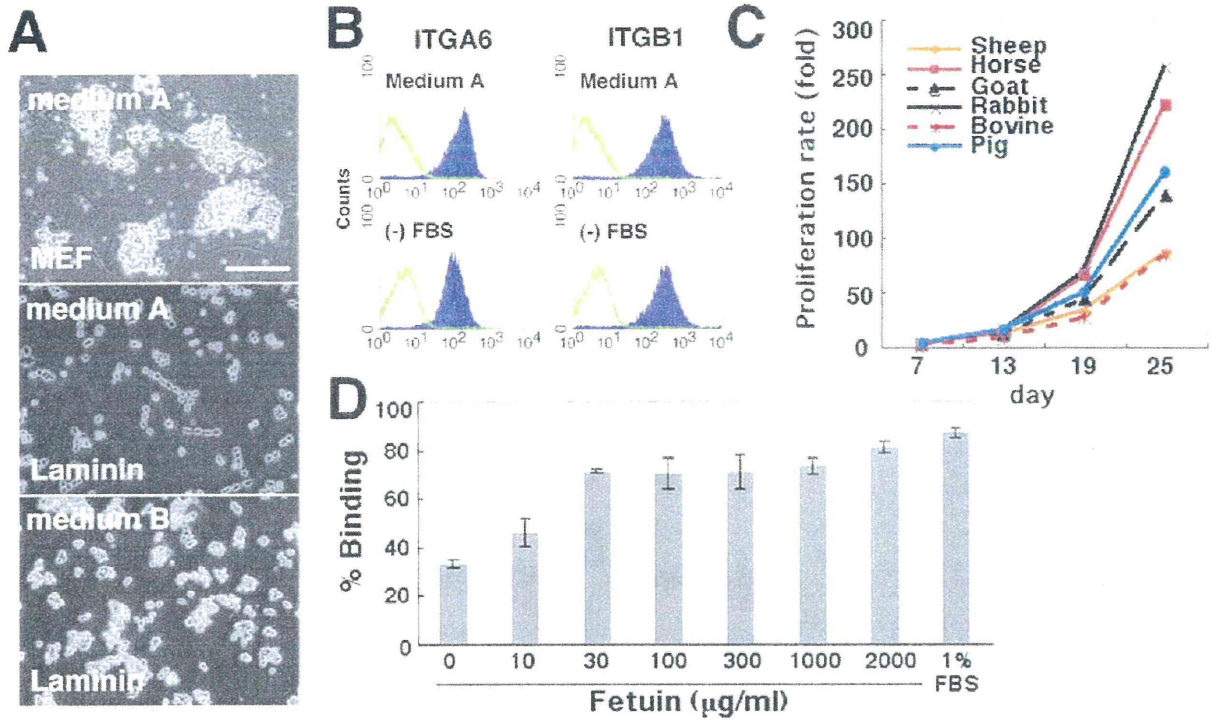

FIG. 1. GS cells cultured under serum- and feeder-free conditions. **A**) Morphology of GS cells under different culture conditions. **Top**) GS cells on MEFs with 1% FBS. Note the morula-like morphology. **Middle**) GS cells on laminin with 1% FBS. Note the chain-type colonies. **Bottom**) GS cells on laminin without serum. Note the clump-like colonies. **B**) Flow cytometric analysis of ITGB1 and ITGB6 expression after removing serum from medium A. Although cells were detaching from the laminin, no significant changes in ITGB1 and ITGA6 were noted. Green lines indicate controls. **C**) Effects of serum from various animal species on GS cell proliferation. Each serum type was added at a concentration of 1%. **D**) Promotion of GS cell attachment to laminin by fetuin supplementation. GS cells were plated overnight on laminin (20 μg/ml) in the presence of different concentrations of fetuin (0–2 mg/ml). After washing twice with PBS, the plates were treated with trypsin to recover adherent cells. Results are from three independent experiments (n = 3). Bar = 100 μm (**A**).

responsible for growth inhibition. Flow cytometric analyses did not reveal significant changes in the expression levels of ITGB1 and ITGA6, which comprise laminin receptor (Fig. 1B). Conversely, no species-specific serum requirement was observed for GS cell culture: Sera (at 1%) from sheep, horse, goat, rabbit, and pig maintained GS cells for at least 2 wk on laminin-coated plates without noticeable changes (Fig. 1C). Furthermore, charcoal/dextran-treated FBS, which is reduced in hormonal or growth factor content, maintained GS cells at a comparable efficiency (data not shown).

Because only a small amount of serum was necessary for proliferation, we hypothesized that the effective components must be present as a significant proportion of the serum. Supplementation with fetuin, a predominant glycoprotein comprising approximately 45% of the total FBS protein [26], allowed GS cell attachment to laminin under serum-free conditions. In the present study, GS cells from ROSA26 mice were incubated overnight on laminin-coated dishes with different concentrations of fetuin (0–2 mg/ml) in serum-free medium, and the cells were analyzed for attachment efficiency (Fig. 1D). GS cells attached to laminin efficiently when fetuin was added at concentrations of greater than 30 μg/ml: 70–80% of the cells bound to the laminin after overnight culture, which was comparable to the effect of 1% serum. Increasing the fetuin concentration to 2 mg/ml did not improve attachment.

Enhanced Proliferation of GS Cells by Lipid Supplementation

Although GS cells adhered to laminin-coated plates following fetuin supplementation, the cells failed to grow after two to three passages, despite increasing the concentrations of cytokines or adding soluble GDNF receptor α-1. This suggests that enhanced cytokine signaling alone does not support GS cell proliferation. We then studied the effect of the lipid components by replacing the lipid-poor albumin (ImmunO, fraction V) in the original GS cell culture medium (medium A) with lipid-rich BSA (Albumax II) and adding other lipid-associated components in the medium, such as high-density lipoprotein (HDL), cholesterol, and lipid mixtures. In the improved medium, the colony morphology changed from flat-type to morula-like clumps, and cell proliferation was stimulated (Fig. 2, A and B). The enhanced amplification ratio following lipid supplementation suggests that lipids are an important component of the serum. Because sphingosine kinase has been involved in regulation of cell proliferation, differentiation, senescence, and apoptosis in stem cells and their progenitors by producing bioactive sphingosine 1-phosphate [27], we tested the effect of SKI II, a specific inhibitor of sphingosine kinase, on GS cell proliferation. Addition of SKI II significantly suppressed GS cell proliferation within 5 days in 1% serum-containing medium (medium A) (Fig. 2C).

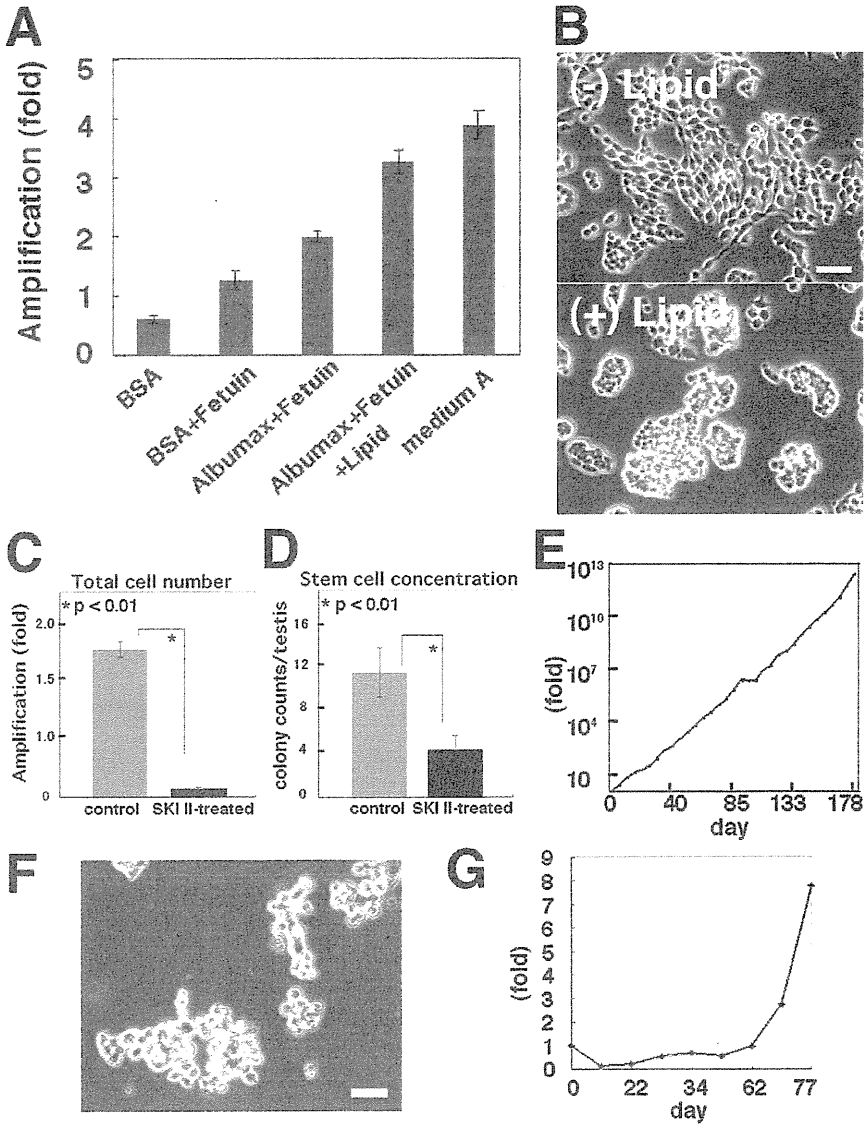

FIG. 2. Improvement of GS cell proliferation by lipid supplementation under serum- and feeder-free conditions. **A**) Effect of Albumax II and lipid supplementation on GS cell proliferation (n = 8). Cells were cultured on laminin-coated plates for 6 days. Note the improved growth of GS cells with fetuin and lipid supplementation. **B**) Effect of lipid supplementation on GS cell morphology on laminin-coated plates. **Top**) GS cells on laminin without lipid supplementation. Note the epithelial morphology. **Bottom**) GS cells on laminin with lipid supplementation. Note the clump-like morphology. **C** and **D**) Decreased cell recovery (**C**) and SSC expansion (**D**) after 5 days of culture with SKI II (n = 16 for cell recovery, n = 8–10 for SSC number). SSC numbers were determined by germ cell transplantation. The same numbers of cells were cultured on laminin in 1% serum-supplemented medium (medium A) with or without SKI II for 5 days. The number of SSCs was quantified by multiplying the total cell recovery by the SSC concentration, as estimated by colony counting after germ cell transplantation. Results are from three independent experiments. **E**) Growth curve for GS cells. Cells were maintained for 178 days, and the total number of cells was counted at each passage. **F**) Morphology of GS cells established by culturing newborn testis cells in medium B. **G**) Growth curve for GS cells from neonatal testes during culture initiation. Bar = 50 μm (**B** and **F**).

We confirmed the inhibition of SSC proliferation by transplanting cells that had been cultured for 5 days in the presence of SKI II into the seminiferous tubules of W mice (Fig. 2D). The inhibitor treatment suppressed the SSC number; the SKI II-treated group exhibited only 40% of the SSCs observed in the DMSO-treated controls. These results indicate that sphingosine-mediated signaling is necessary for SSC proliferation.

The cytokine requirements of the GS cells remained unaltered in the improved medium (medium B), and the cells were trypsinized and passaged at a ratio of 4:1 to 6:1 every 5–6 days. Overall, the cells expanded by a factor of 1.9×10^{12}-fold during 178 days in vitro, with 32 passages (Fig. 2E). The colony morphology of cells was somewhat different from those cultured in the serum (Figs. 1A and 2B), which resembled chains of spermatogonia during early phases of germ cell colonies in vivo (Fig. 1A) [23]. However, the colonies in the serum- and feeder-free culture conditions (medium B) did not form chains but, instead, occurred in clumps. The morphological change was not irreversible, and the cells exhibited a typical morula-like appearance when plated on MEFs in medium A. The improved medium was also useful for culturing SSCs from other mouse strains, such as BDF1 or ICR. In addition, we were able to establish serum- and feeder-free GS cultures using primary germ cells of 0- or 8-day-old pup testes, indicating that the culture medium was useful for initiating and maintaining GS cell cultures (Fig. 2, F and G). The cells were cryopreserved after adding 10% DMSO to the serum-free culture medium. We did not observe mGS cells at any point during the entire culture period [11].

Phenotypic Characterization of GS Cells under Serum- and Feeder-Free Conditions

We next examined the phenotype of the cultured cells. Despite morphological differences, flow cytometric analyses demonstrated that the cultured cells expressed normal SSC markers, including ITGB1 and ITGA6, EPCAM, and CD9 (Fig. 3A). The cells also weakly expressed KIT, suggesting that most cells were undifferentiated spermatogonia. However, the

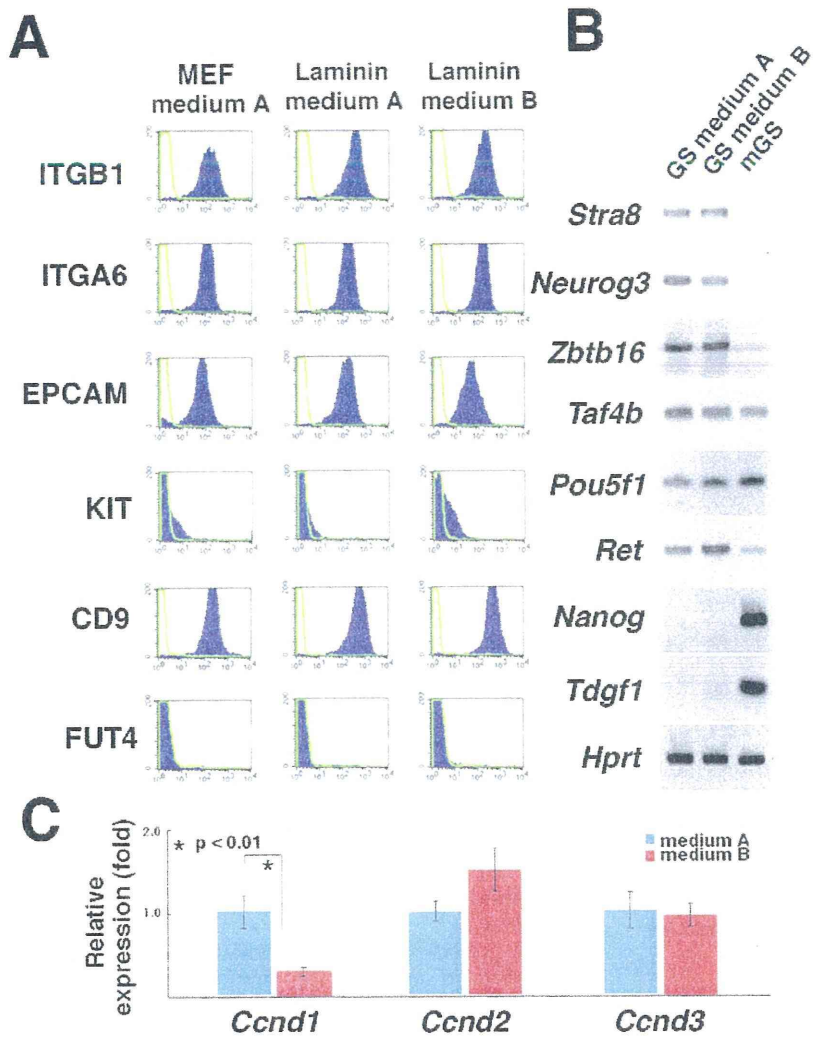

FIG. 3. Phenotypic analyses of GS cells cultured under serum- and feeder-free conditions. **A**) Flow cytometric analysis of cell surface marker expression. Green lines indicate controls. **B**) RT-PCR analyses. Gene-specific primers for each gene were used to amplify cDNA from GS cells cultured under different conditions. **C**) Real-time PCR analysis of cyclin D gene expression. Cells were cultured on laminin under the indicated conditions. The values are normalized to *Hprt* expression levels in GS cells cultured in medium A.

cells did not express FUT4 (SSEA-1), a marker of primordial germ cells (PGCs) and ES cells. This overall pattern of marker expression was unaltered regardless of serum or MEFs. The RT-PCR analyses confirmed normal spermatogonia marker expression: the GS cells expressed *Stra8*, *Neurog3*, *Zbtb16*, *Taf4b*, *Pou5f1*, and *Ret*, but not *Nanog* or *Tdgf1*, both of which are strongly expressed in ES cells (Fig. 3B). Thus, the cultured cells maintained a normal spermatogonial phenotype.

We previously showed that GS cells had a normal chromosome number and a stable DNA methylation pattern even after long-term in vitro culture [7]. Consistent with those observations, cytogenetic analysis by Hoechst 33258 staining in the present study revealed that the cultured cells had a normal karyotype after 6 mo of continuous culture (Fig. 4A). In addition, combined bisulfite restriction analysis of DNA methylation patterns on imprinted genes showed that the DNA methylation at DMRs of two paternally imprinted genes (*H19* and *Meg3 IG*) and a maternally imprinted gene (*Peg10*) displayed typical androgenetic DNA methylation patterns, including hypermethylation of *H19* and *Meg3 IG* DMRs and hypomethylation of *Peg10* DMRs (Fig. 4B). Taken together, these results indicate that the cultured cells retained normal genetic and epigenetic properties of spermatogonia during in vitro culture.

Functional Analyses of GS Cells under Serum- and Feeder-Free Conditions

To confirm the increase in SSC number, we transplanted the cultured cells into seminiferous tubules at three different time points during 172 days of continuous culture (Table 1). In these experiments, at least three recipients received donor cell transplantation. Two months after transplantation, the recipients were killed, and their testes were examined for donor cell colonization by LacZ staining (Fig. 5A). Assuming that 10% of the SSCs were able to colonize seminiferous tubules [23], the SSC concentration of the transplanted cells was calculated as 0.4–1.0% (average, 0.6%); the SSC frequency in traditional GS cell cultures is usually 1–2% [28]. A direct comparison between the GS cell cultures in medium A and medium B showed that the GS cells cultured with serum produced 230.3 colonies per 10^5 cells whereas those cultured without serum produced 98.2 colonies per 10^5 cells, suggesting that certain serum components enhanced the SSC frequency (Fig. 5B). Given the significant impact of the cyclin D expression pattern on SSC frequency [28], we also analyzed the expression levels of cyclin D genes (Fig. 3C). Although we did not observe significant differences in *Ccnd2* and *Ccnd3* expression, *Ccnd1* was significantly downregulated under serum- and feeder-free

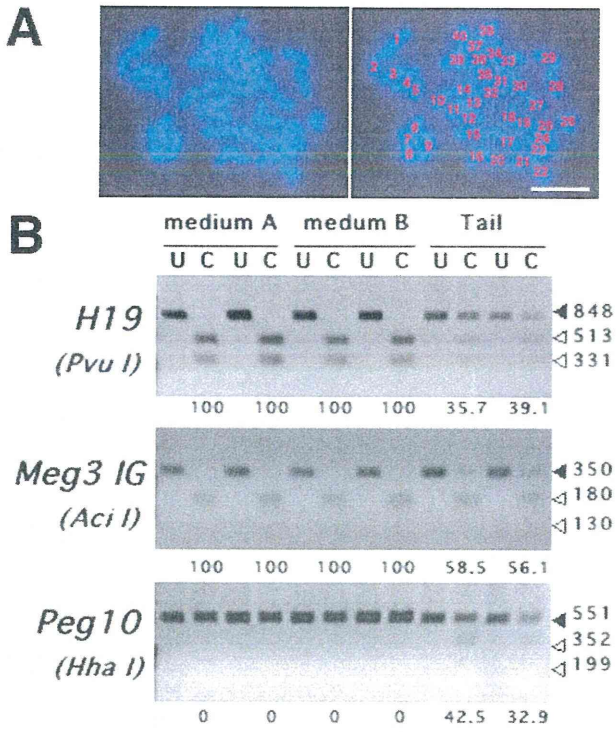

FIG. 4. Genetic and epigenetic characterization of GS cells under serum- and feeder-free conditions. **A**) Metaphase spread of GS cells. The two images are the same; the right image indicates that 40 chromosomes are in this cell. Original magnification ×100. **B**) Combined bisulfite restriction analysis of GS cells. Open arrowheads indicate the size of methylated DNA. Closed arrowheads indicate the size of unmethylated DNA. The enzymes used to cleave each locus are indicated in parentheses. C, cleaved; U, uncleaved. The numbers at the bottom of each gel indicate the percentage of digested DNA/undigested DNA (i.e., C/U).

conditions, suggesting that abnormal *Ccnd1* levels played a role in the reduced SSC frequency. However, transplantation analyses confirmed a consistent increase in SSC number during in vitro culture. Cultured SSCs expanded by 3.4×10^9-fold during 132 days, whereas the total cells increased by 1.3×10^9-fold. The doubling time of SSCs was 4.1 days. Histological analyses of the recipient testes confirmed normal spermatogenesis in the seminiferous tubules (Fig. 5C).

Finally, to test the fertility of these germ cells, we used intracytoplasmic sperm injection (ICSI), a technique commonly used to treat infertility in animals and humans [25, 29]. In the first set of experiments, GS cells were cultured for 78 days, and one of the mice was killed at 108 days after transplantation for cryopreservation of testes. The testes were frozen for 113 days before ICSI. In the second set of experiments, GS cells were cultured for 124 days, and one of the recipient mice was killed at 175 days after transplantation for ICSI without cryopreservation. Spermatogenic cells were recovered mechanically by repeated pipetting of the seminiferous tubules. Spermatozoa, identified by their unique morphology, were microinjected into oocytes from BDF1 females using a piezo-driven microinjector (Fig. 5D). Of the 67 embryos formed, 31 (46.3%) developed to 2-cell stage (Table 2) and were transferred into the uteri of three pseudopregnant female mice. One offspring resulted from the sperm derived from fresh recipient testes, indicating the fertility of the donor GS cells (Fig. 5E). However, the efficiency of offspring production was apparently reduced in this experiment, because in our previous experiments using serum-supplemented culture medium [12, 13], 22–74% of the constructed embryos underwent implantation and 15–50% developed to term. We were unable to obtain offspring using sperm from the frozen-thawed testes.

DISCUSSION

Serum plays important roles in SSC culture. We used serum in our original GS cell culture medium, because spermatogenic cells cultured without serum readily reaggregated with somatic cells to form cell clumps, preventing germ cell proliferation. Conversely, high concentrations of serum stimulated proliferation of testicular somatic cells and interfered with the germ cell growth. We resolved this dilemma by decreasing the serum concentration to 1%, which limited the proliferation of testicular somatic cells while providing factors necessary for germ cell responses to self-renewal factors. A similar strategy was used to develop culture systems for PGCs/gonocytes and hamster SSCs [18, 19], although a lower serum concentration (0.04%) was sufficient for promoting germ cell proliferation. To our knowledge, the active components in serum have yet to be identified.

In the present study, we focused on cell attachment and proliferation to improve the culture medium. At serum concentrations of less than 0.04%, the cells did not adhere to laminin-coated plates and did not proliferate. Because GS cells can proliferate in suspension [13], attachment is not an absolute requirement for GS cell proliferation, but GS cells proliferate more actively when attached to a matrix or to feeder cell layer. Serum contains many factors, such as fibronectin or vitronectin, that facilitate cell attachment. In particular, SSCs have a strong preference for laminin over other extracellular matrix substrates [28]. Although GS cells can attach to artificial substrate, such as poly-L-lysine, cell proliferation is not stimulated [21]. In the present study, fetuin, a major component of bovine serum, allowed GS cell attachment to laminin under serum-free conditions. In general, total serum protein concentration gradually increases during development, but the fetuin concentration is significantly higher in fetuses than in adults

TABLE 1. SSC expansion in serum- and feeder-free culture.

Experiment[a]	Days to transplant (passage)[b]	Colonies/ 10^5 GS cells[c]	Increase in cell no. (fold)[d]	Increase in stem cell no. (fold)[d]
1	40 (7)	37.5 ± 21.7		
2	74 (14)	37.5 ± 12.5	260	260
3	172 (30)	102.1 ± 31.4	1.3×10^9	3.4×10^9

[a] In each experiment, $4.0–8.0 \times 10^3$ cells were microinjected into the seminiferous tubules of infertile recipient testis.
[b] The number of days from initiation of culture to transplantation.
[c] Values are mean ± SEM; results from four to six recipient testes for each transplantation.
[d] The increase in the total cell or stem cell number from the initial transplantation (experiment 1).

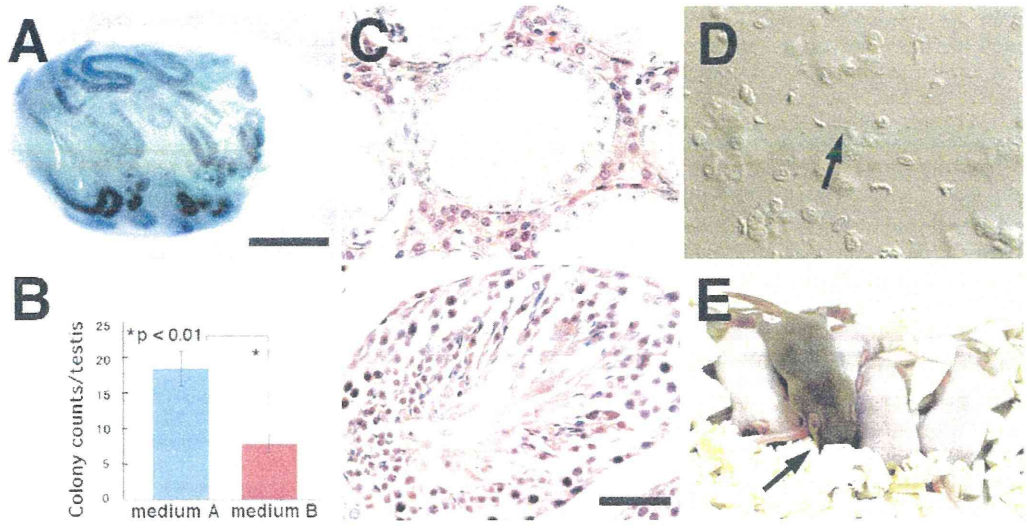

FIG. 5. Production of offspring from cultured cells by germ cell transplantation. **A**) LacZ staining of a recipient testis, indicating the presence of the donor cell maker. Blue stretches of tubules indicate the presence of donor cell-derived germ cell colonies. **B**) Decreased SSC activity of GS cells cultured under serum- and feeder-free conditions. Results are from three separate experiments. Approximately 8×10^3 cells were injected into each testis (n = 19–21). **C**) Histological appearance of recipient testis showing normal spermatogenesis. **Top**) Nontransplanted W recipient testis. **Bottom**) Normal-appearing spermatogenesis in testis transplanted with GS cells cultured under serum- and feeder-free conditions. **D**) Spermatozoon (arrow) recovered from recipient testis. **E**) Offspring (arrow) derived from spermatozoa produced from transplanted GS cells. Bars = 1 mm (**A**) and 50 μm (**C**).

[30]. Fetuin has positive effects on the growth and differentiation of various cells in culture [30], and it promotes cell attachment, by an unknown mechanism, depending on the cell type. We previously demonstrated that GS cells attached to laminin via integrins; our current results suggest that this interaction may involve additional factors.

Fetuin supplementation was effective for GS cell attachment to laminin under serum- and feeder-free conditions, but it was not sufficient for maintaining cell proliferation. Lipid signaling is another important process mediated by serum, and the inhibition of GS cell proliferation by SKI II indicates that sphingosine-mediated signaling was necessary for GS cell proliferation. Therefore, we added lipid components or HDL mixtures and lipid-rich BSA (Albumax II) to the GS cell culture medium. Albumin, a major serum component, functions as a carrier protein for thyroid and other steroid hormones and binds to fatty acids and other unknown metabolites. A critical role has been demonstrated for BSA in cultures of ES cells and other stem cells, including SSCs [16, 31, 32]. Albumax II contains free fatty acids (~54%), lysophosphatidylcholine (~17%), triacylglycerides (~15%), phosphatidylcholine (~8%), phosphatidic acid (~3%), cholesterol (~1%), and sphingomyelin (~1%) [32]. These additions to the medium, combined with fetuin, allowed GS cell proliferation in the absence of serum. Although our results indicated that sphingosine was necessary for proliferation, we currently do not know how sphingosine influenced GS cells; it is important to study whether it directly stimulated proliferation of GS cells or influenced apoptosis. Because addition of sphingosine to the culture medium was not sufficient to drive GS cell proliferation (unpublished observation), sphingosine likely interacts with other lipid components in the serum or Albumax II. The identity of other lipid components must be further investigated.

TABLE 2. ICSI using spermatozoa recovered from recipient mice.

Type of testis	No. of cultured embryos	No. of cleaved (%)	Embryo transfer[a]	Implantation (%)	Offspring (%)
Fresh	15	9 (60.0)	9	1 (6.7)	1 (6.7)
Freeze-thawed	52	22 (42.3)	22	1 (1.9)	0 (0.0)
Total	67	31 (46.3)	31	2 (3.0)	1 (1.5)

[a] Embryos were transferred after culturing 24 h in vitro.

An unexpected finding from the present study was the low SSC frequency under serum- and feeder-free culture conditions. Generally, 1–2% of GS cells exhibit SSC activity, as calculated from the colony number [28], and the presence of feeder cells did not significantly change this frequency. However, the SSC frequency in the present study was significantly lower (0.4–1.0%). Given that the cultured cells expanded at comparable levels in serum-free and serum-containing media, these results suggest reduced efficiency of self-renewing divisions in our serum-free medium. However, we did not detect significant changes in spermatogonial phenotype based on marker analyses, and we were able to obtain one normal offspring. Changes in SSC frequency were evident only in functional transplantation assays.

Presently, little is known about the mechanism of SSC fate commitment. Although GDNF levels apparently influence fate commitment in vivo, factors that influence SSC frequency in vitro have remained unknown for a long time. However, we recently found that cyclin D expression had a significant impact on SSCs activity [28], such that *Ccnd1* overexpression impairs the SSC activity of GS cells whereas GS cells overexpressing *Ccnd2* can proliferate without self-renewal factors and retain SSC activity. Our current results indicate that serum also contains one or more factors that may influence SSC fate commitment and cyclin D expression. Importantly, *Ccnd1* expression under serum- and feeder-free conditions was significantly downregulated compared with that under serum-supplemented conditions. Because *Ccnd1* overexpression also impaired SSC homing [28], it seems that *Ccnd1* levels must be

maintained within an appropriate range for successful SSC homing. Although the link between cyclin D expression and SSC self-renewal awaits confirmation by further experiments, our finding suggests that SSC fate commitment is not entirely stochastic [33, 34] but, instead, may be influenced by environmental factors, such as serum. The culture system reported here may be useful for studying the effect of various test molecules on SSC self-renewal and differentiation.

Although we developed serum- and feeder-free culture conditions for GS cells, additional improvements in the medium are still necessary. Culture conditions are being developed for SSCs from other animal species [19, 35–38], but their growth in vitro is still reduced compared with that of mouse GS cells. This exposes a critical concern regarding the use of this technology for genetic modification of the germline: Slow growth will limit transfection efficiency and in vitro drug selection. We also must further define the culture medium. Although GS cells can proliferate faster in StemPro medium than in other media, StemPro may contain numerous factors that are not well optimized for GS cells. Establishing defined culture conditions for GS cells is apparently important for understanding the molecular requirements of the SSC self-renewal machinery and providing important information on stem cell niche/microenvironments in vivo. Hopefully, our culture system will stimulate future developments toward completely defined culture conditions for GS cells.

ACKNOWLEDGMENT

We thank Ms. Y. Ogata for technical assistance.

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CHAPTER TWO

Germline Modification Using Mouse Spermatogonial Stem Cells

Mito Kanatsu-Shinohara *and* Takashi Shinohara

Contents

Abstract

Spermatogonial stem cells (SSCs) in the testes are a new target for germline modification. With the development of an *in vitro* culture system and spermatogonial transplantation technique, SSCs can now be manipulated and used as an

Department of Molecular Genetics, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Methods in Enzymology, Volume 477
ISSN 0076-6879, DOI: 10.1016/S0076-6879(10)77002-6

20111000 2B (2/2)

別紙１

厚生労働科学研究費補助金

創薬基盤推進研究事業

精子幹細胞を用いた創薬モデルラット作成技術の開発

平成２１年度～平成２３年度　総合研究報告書

研究代表者　田中　敬

平成２４（２０１２）年　５月

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alternative to embryonic stem cells for knockout mice production. The genetic and epigenetic stability of SSCs provide new possibilities for the application of germline mutagenesis in a wide range of animals.

1. Introduction

Spermatogonial stem cells (SSCs) provide a foundation for spermatogenesis. SSCs, which constitute a fraction of spermatogonia in the testes, self-renew and differentiate to produce sperm throughout adult life. While female germline cells stop proliferating during the fetal period and isolation of oocyte/eggs is limited, male germ cells can be isolated and expanded in number. These advantages suggest that SSCs are a valuable target for germline modification.

Recently, several SSC manipulation techniques have been developed. First, a breakthrough was made in the establishment of a germ cell transplantation technique. When dissociated donor testicular cells are injected into seminiferous tubules of infertile recipient testes lacking endogenous spermatogenesis, they colonize, differentiate into sperm, and produce normal offspring (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). Spermatogenesis also occurs with cryopreserved SSCs and with SSCs in xenogeneic host. Rat SSCs undergo spermatogenesis in immunodeficient nude mouse testis and produce normal offspring, indicating a significant flexibility of spermatogenesis (Clouthier *et al.*, 1996; Shinohara *et al.*, 2006).

Second, a long-term SSC culture technique was established. In 2000, glial cell line-derived neurotrophic factor (GDNF) was found to induce spermatogonial proliferation (Meng *et al.*, 2000). Homozygous GDNF knockout mice die perinatally, whereas heterozygous knockout mice exhibit reduced spermatogenesis and eventually become infertile due to germ cell depletion. In contrast, GDNF-transgenic mice possess clumps of undifferentiated spermatogonia, suggesting that GDNF stimulates the self-renewal division of SSCs. Considering this finding, our group succeeded in the long-term culture of SSCs in 2003, and we designated these cells as germline stem (GS) cells (Kanatsu-Shinohara *et al.*, 2003a). In the presence of GDNF, GS cells produce uniquely shaped germ cell colonies. Although GS cells were originally established from neonatal testis, similar cells were subsequently established from adult testis, demonstrating that GS cells can be derived from SSCs at various stages (Kanatsu-Shinohara *et al.*, 2004a; Kubota *et al.*, 2004; Ogawa *et al.*, 2004). GS cells can be used to produce transgenic and knockout animals through genetic transduction and drug selection (Kanatsu-Shinohara *et al.*, 2005c, 2006a). Because the transgene is transmitted to half of the haploid cells, the efficiency of transgenesis is about

50%, and it is 5–10 times higher than that achieved by conventional methods using eggs or oocytes (Nagano *et al.*, 2001). Moreover, the frequency of homologous recombination is comparable to that achieved in embryonic stem (ES) cells (Kanatsu-Shinohara *et al.*, 2006a). Most importantly, GS cells possess a very stable germline potential, retain a normal karyotype and DNA methylation patterns, and produce normal fertile offspring even after 2 years of culture (Kanatsu-Shinohara *et al.*, 2005b). This is in contrast to ES cells, which often change DNA methylation patterns and lose their germ cell potential due to trisomy (Liu *et al.*, 1997; Longo *et al.*, 1997). Thus, SSCs may serve as a new target for animal transgenesis, which may provide an alternative to ES cells.

We also found that the developmental potential of SSCs is not limited to spermatogenesis. Although primordial germ cells (PGCs), the fetal precursors of SSCs, can give rise to ES-like pluripotent cells (Matsui *et al.*, 1992; Resnick *et al.*, 1992), germline cells were believed to be fully committed to the germline by the middle of gestation and that such ES-like potential was missing from postnatal germ cells (Labosky *et al.*, 1994). Unexpectedly, however, ES-like cells rarely appear in GS cell cultures of neonatal testes during culture initiation (Kanatsu-Shinohara *et al.*, 2004a). These cells, referred to as multipotent GS (mGS) cells, not only differentiate into somatic cells, but also differentiate into germ cells. Several groups have reported the derivation of similar pluripotent/multipotent cells from mouse and human postnatal testes, including adults (Golestaneh *et al.*, 2009; Guan *et al.*, 2006; Kossack *et al.*, 2009). Although the origin of these pluripotent/multipotent cells is unclear, we recently discovered that GS cells may be converted directly into mGS cells *in vitro* (Kanatsu-Shinohara *et al.*, 2008a). mGS cells behave like ES cells and are capable of producing knockout animals in a manner similar to ES cells (Takehashi *et al.*, 2007b). Although the efficiency of establishing mGS cells (1 of 30 testes) is low and needs improvement, these results suggest an alternative use for SSCs in germline modification.

In this chapter, we describe the methods associated with the SSC culture technique. Although several groups have reported alternative SSC culture methods (Guan *et al.*, 2006; Kubota *et al.*, 2004; Seandel *et al.*, 2007), our method allows for the genetic selection and production of knockout mice from SSCs.

2. Establishing and Maintaining a GS Cell Culture

GS cell culture may be initiated from both neonatal/pup and adult testes (Kanatsu-Shinohara *et al.*, 2003a, 2004a; Kubota *et al.*, 2004; Ogawa *et al.*, 2004), but establishment is quicker and more efficient using neonatal/

pup testes. Immature testes are useful because the germ cell/somatic cell ratio is relatively high, and germ cells are easily separated from somatic cells based on their differential ability to attach to a gelatin-coated dish; therefore, antibody-mediated purification is not necessary. Furthermore, immature germ cells proliferate more actively than adult germ cells. In contrast, one must purify SSCs from adult testes because only 0.02–0.03% of all germ cells in the testis are stem cells (Meistrich and van Beek, 1993; Tegelenbosch and de Rooij, 1993). In both cases, removing as many somatic cells as possible is advisable.

The efficiency of establishing GS cells is affected by the mice strain. While DBA/2, ICR, and C57BL/6 × DBA/2F1(B6D2F1) are efficient, C57BL/6 is less efficient. GS cells can also be established from ddy, C3H, A, and AKR with variable efficiencies. Here, we describe a protocol for establishing a GS cell culture from P0-3 testes of DBA/2 or the ICR strain.

2.1. GS cell culture medium

GS cell culture medium is prepared by modifying commercial medium (StemPro®-34 serum-free medium (SFM); Invitrogen, Carlsbad, CA). Although other conditions with defined medium can also support SSC proliferation, a modified StemPro®-34 medium currently provides the most efficient proliferation of GS cells in our laboratory.

SSC proliferation is maintained by a combination of several cytokines. GS cell culture medium contains GDNF, and fibroblast growth factor-2 (FGF2). Although epidermal growth factor (EGF) was included in the original protocol, it is dispensable, and GS cell proliferation can be maintained with GDNF + FGF2 only. Leukemia inhibitory factor (LIF) enhances colony formation from gonocytes, while it is dispensable for spermatogonia culture (Kanatsu-Shinohara *et al.*, 2007). It is also dispensable for the maintenance of GS cells.

Complete GS cell culture medium is prepared by adding several factors to basal medium (Table 2.1). Basal medium is made by adding 16 components to StemPro®-34 SFM, followed by filtration through a 0.22-μm bottle-top filter. It can be stored in a refrigerator for at least 3 weeks. The six additives listed in Table 2.2 should be added before use to make the complete medium. Complete medium with growth factors can be stored in a refrigerator for up to 3 days.

2.2. Step 1. Dissociation of testis cells

Dissolve collagenase (#C5138; Sigma, St. Louis, MO) at 1 mg/ml and deoxyribonuclease (DNase, #DN25; Sigma) at 7 mg/ml in Hanks' balanced salt solution (HBSS) and filter. Isolate the testes from the mice, and remove

Table 2.1 Composition of basal medium for GS cell culture

Component	Catalogue #	Final concentration	Volume	Aliquots
Insulin	Nacalai Tesque[a] #19251-24	25 μg/ml	500 μl	Dissolve 100 mg/3.8 ml DDW + 0.2 ml 1 *N* HCl; store at −20 °C
Transferrin	Sigma #T1147	100 μg/ml	1 ml	Dissolve 100 mg/2 ml DDW; store at −20 °C
Putrescine	Sigma #P7505	60 μM	500 μl	Dissolve 96.7 mg/10 ml DDW; store at −20 °C
Sodium selenite	Sigma #S1382	30 nM	500 μl	Dissolve 5.2 mg/1000 ml DDW; store at −20 °C
D-(+)-glucose	Sigma #G7021	6 mg/ml	3g	Dissolve in 10 ml DDW and add all
Pyruvic acid	Sigma #P2256	200 μg/ml	100 mg	
DL-Lactic acid	Sigma #L4263	1 μl/ml	500 μl	
Bovine albmin	MP Biomedicals[b] #810661	5 mg/ml	2.5 g	
L-Glutamine	Sigma #G7513	2 mM	5 ml	100×; store at −20 °C
2-Mercaptoethanol	Sigma #M3148	5×10^{-5} M	5 ml	5×10^{-3} M
MEM vitamin solution	Invitrogen #11120-052		5 ml	Store at 4 °C
Nonessential amino acids	Invitrogen #11140-050		5 ml	Store at 4 °C
Ascorbic acid	Sigma #A4544	10^{-4} M	500 μl	Dissolve 17.6 mg/ml DMSO; use immediately
d-Biotin	Sigma #B4501	10 μg/ml	500 μl	Dissolve 10 mg/ml DMSO; use immediately
β-Estradiol	Sigma #E2758	30 ng/ml	750 μl	Dissolve 1 mg/ml ethanol; add 49 ml sterile medium. Store at −20 °C
Progesterone	Sigma #P8783	60 ng/ml	1.5 ml	Dissolve 1 mg/ml ethanol;, add 49 ml sterile medium. Store at −20 °C

StemPro®-34 SFM (Invitrogen #10639) is modified by addition of the following components. The amounts to be added to 500 ml StemPro®-34 SFM are shown.

[a] Nacalai Tesque, Inc., Kyoto, Japan.

[b] MP Biomedicals, Inc., Irvine, CA.

Table 2.2 Components added to basal medium immediately before use

Component	Catalog number	Final	Volume	Aliquot
StemPro®-34 supplement	Invitrogen #10639		20 μl	50× Supplement is supplied with StemPro®-34SFM.
Mouse EGF	BD Biosciences 354010	20 ng/ml	2 μl	Optional; Dissolve 100 mg/10 ml PBS+BSA; store at −20 °C
Human FGF2	Peprotech Inc.[a] #100-18B	10 ng/ml	5 μl	Dissolve 10 mg/5 ml PBS+BSA; store at −20 °C
Rat GDNF	Peprotech Inc.[a] #450-51	15 ng/ml	15 μl	Dissolve 10 mg/10 ml PBS+BSA; store at −20 °C
FBS	Hyclone[b] #SH30396.03	1%	10 μl	
ESGRO (murine LIF)	Millipore[c] #ESG1107	10^3 units/ml	10 μl	Optional; enhances GS cell establishment when added to the initiation of neonatal testis culture. Dissolve 10^6 units/10 ml PBS+BSA; store at −20 °C

The amounts to be added to 1 ml basal medium are shown.
[a] Peprotech Inc. Rocky Hill, NJ.
[b] Hyclone Laboratories, Inc. South Logan, UT.
[c] Millipore, Billerica, MA.

the tunica with fine forceps in cold HBSS. Wash two to three times with HBSS and transfer the tissue to 1–2 ml of collagenase and incubate at 37 °C for 15 min. Agitate the tube several times during the incubation. Wash twice with HBSS and add 0.8 ml of 0.25% trypsin + 0.2 ml of DNase, shake the tube several times to dissociate the seminiferous tubules, and incubate at 37 °C for 10 min. Add 5 ml of Iscove's modified Dulbecco's medium (IMDM) + 2% fetal bovine serum (FBS) and repeat pipetting until the cells are dissociated. Centrifuge and remove the supernatant.

2.3. Step 2. Initiation of GS cell culture

Dissolve 1 g gelatin in 500 ml phosphate-buffered saline (PBS) and autoclave to make a 0.2% gelatin solution. Coat a 12-well culture plate with the 0.2% gelatin/PBS and incubate at room temperature for more than 20 min. Remove the gelatin solution, suspend the cells in complete culture medium, and transfer them to a gelatin-coated culture plate. The density should be $\sim 2 \times 10^5$ cells/0.8 ml medium per well of a 12-well culture plate. Incubate in 5% CO_2 at 37 °C overnight.

Many cells attach to the plate after the overnight incubation, but a significant number of germ cells, as distinguished by their large size and characteristic pseudopod, remain floating. The floating cells should be transferred to a second culture plate after vigorous pipetting (use P1000-pipette tips, 10–15 times; Gilson, Middleton, WI). The second culture plate does not need to be treated with gelatin. Very few germ cells are left on the original gelatin-coated plate, and cells transferred to secondary plates are relatively germ cell-enriched (Fig. 2.1A).

Three to four days later, remove half of the culture medium and add the same volume. Within 1 week, the transferred cells will proliferate and spread on the bottom of the well; round proliferating cells will form germ cell colonies on top of the flat cell layer. Most of these primary colonies consist of compact clusters of cells with unclear borders.

The timing of the first passage depends on colony growth, but between 10 and 14 days after culture initiation (DIV, days *in vitro*) is recommended. Wash twice with PBS, add 0.25% trypsin, and incubate at 37 °C for 4 min. Add IMDM + 2% FBS to stop the reaction. Replate at 1× dilution. The colonies will grow to their original size in about 10 days, when the cells are passed again (1/2× dilution).

2.4. Step 3. Maintenance

After the third or fourth passage, the cells should be transferred onto mitomycin-C treated mouse embryonic fibroblasts (MEFs) (Fig. 2.1B). MEF feeders should be prepared according to the conventional ES cell

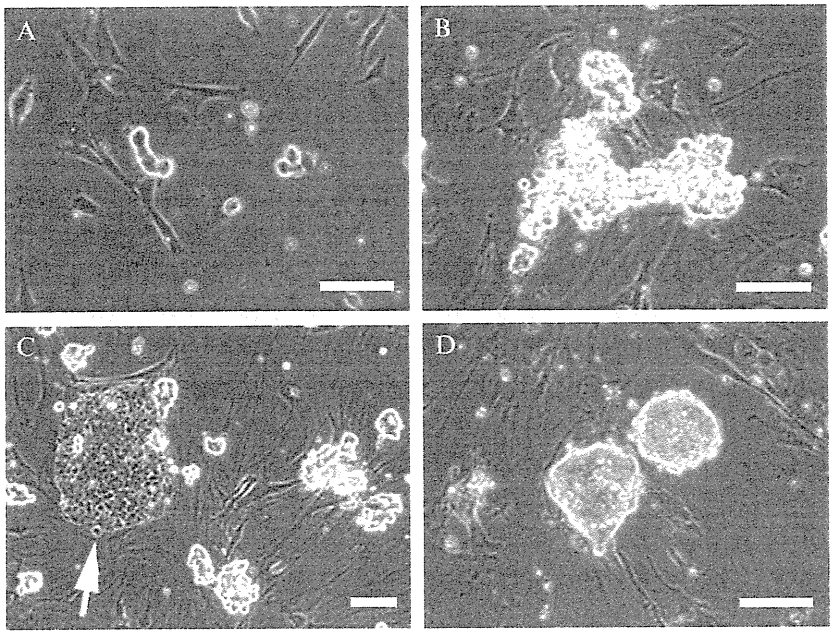

Figure 2.1 Culture appearances. (A) Gonocytes at 2 days *in vitro*. Gonocytes have large cell bodies and attach loosely to the plate, whereas fibroblasts and other somatic cells attach strongly to the plate. (B) Established GS cells. GS cell colonies produce a grape-like cluster. (C) Epiblast-like cell sheet, which appears in GS cell cultures. An epiblast-like cell sheet (arrow) is often observed when mGS cells appear in the culture. While distinguishing clearly between GS cell and mGS cell colonies is difficult, this is a clear sign indicating that mGS cells are starting to appear in the culture. However, this structure disappears after mGS cells are established, and whether it is an mGS cell precursor or a differentiated mGS cell state that exists only transiently is unclear. (D) mGS cells have similar appearances to ES cells. Bar = 100 μm.

culture method, except they should be plated at a lower density (7.5×10^4 per well in 6-well culture plates). Briefly, treat MEF with 10 μg/ml mitomycin-C (#M053; Sigma; dissolve in PBS at 2 mg/4 ml and filter) for 2 h at 37 °C and dissociate with trypsin. Suspend the MEF cells in Dulbecco's modified Eagle's medium (DMEM) + 10% FBS and plate onto a gelatin-coated culture dish. MEF feeders should be used within 10 days (it is not necessary to change medium). Immediately before transferring the GS cells remove the medium from the MEF feeders and wash once (or twice) with PBS.

GS cell growth becomes stable after about 30 DIV. The established GS cells should be plated at a density of 3×10^5 cells/well in 6-well culture plates. Cultures should be passed every 4–6 days depending on proliferation. The medium should be changed every 3 days (half medium change). Established GS cells continue to proliferate for more than 2 years without losing stem cell activity.