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Long-Term Culture and Transplantation of Murine Testicular Germ Cells

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ABSTRACT: The objectives of this study were to develop an in vitro culture system to optimize germ cell proliferation and to measure the potential of the cultured germ cells to produce mature spermatozoa after transplantation into a recipient. Donor germ cells isolated from ROSA26 male mice were cultured with a STO feeder cell layer in Dulbecco's minimal essential medium (DMEM) supplemented with fetal bovine serum (FBS), stem cell factor, leukemia inhibitory factor, basic fibroblast growth factor, insulin-like growth factor 1, interleukin-11, L-glutamine, sodium pyruvate, 2-mercaptoethanol, murine oncostatin M, and platelet-derived growth factor. Donor germ cells formed colonies in the primary cultures after 8–21 days. These cultured colonies were maintained for 4 weeks or longer without subculture and proliferated for up to 8 passages over a period of 3 months. These colonies had alkaline phosphatase activity and incorporated 5-bromo-2′-deoxyuridine. These colonies were positive partially when screened with antibody for germ cell nuclear antigen and c-kit. Germ cells cultured with this supplemented medium showed enhanced colonization vs controls cultured with DMEM and FBS. Cultured germ cells from Rosa26 donors were transplanted into testes and were identified by X-gal staining and histological screening. The cells cultured in the supplemented medium colonized the tubules and initiated spermatogenesis in the recipient mice. This is an improved method for culturing germ cells and may be useful in gene therapy and the production of transgenic animals.

Key words: ROSA26 mice, germ cells, in vitro culture, testis, GCNA1, spermatozoa.

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Stem cells have the capacity both to self-renew and to generate differentiated progeny (Morrison et al, 1997). Therefore, the term ''stem cell'' is a functional definition. The spermatogonial stem cell is unique, since it is the only cell in the adult body that undergoes division and can contribute genes to subsequent generations. The spermatogonial stem cells present within the seminiferous tubules differ morphologically from primordial germ cells (PGCs) and are called prospermatogonia (McLaren and Durcov-Hills, 2001). In the adult testis, the first visible sign of differentiation of the stem cells is the formation of a pair of cells interconnected by an intercellular bridge (De Rooij et al, 1998).

Attempts to culture cells of the seminiferous tubules have met with limited success. Cultures of Sertoli cells, primarily derived from immature animals, have been used extensively to study mammalian Sertoli cell function (Jakubowiak et al, 1990). Germ cell cultures have been successful for only moderately long periods in nonmammalian species and are enhanced by the presence of Sertoli cells (Risley, 1990). Previous claims about germ cell lines that result in sperm production in culture have been seriously questioned (Rassoulzadegan et al, 1993). Limited successful culture and transfection of germ cells have been reported (Cooker et al, 1993). Mouse spermatogonial stem cells have been maintained in culture for approximately 4 months and these cultured stem cells were transplanted into recipient testes and successfully generated mouse spermatogenesis (Nagano et al, 1998). In vitro generation of spermatocytes and spermatids from telomerase-immortalized mouse type A spermatogonial cells in the presence of stem cell factors has been demonstrated (Feng et al, 2002). Spermatogonial stem cell transplantation is currently the only available functional assay for spermatogonial stem cells, as demonstrated by Brinster and Zimmerman (1994).

Cultures containing primarily Sertoli cells and gonocytes showed gonocytes undergoing proliferation and migration in culture at times that are in direct accordance and timing with proliferation and migration in vivo. Mouse PGCs have been cocultured on mitotically inactivated STO cells (Matsui et al, 1991; Resnick et al, 1992), and Nagano et al (1998) have reported that a STO feeder layer is critical to spermatogonial stem cell maintenance. The STO feeder cell layer may secrete some of the several growth factors essential for stem cell survival (Smith and Hooper, 1983). In other studies, primordial germ cells from day E 8.5 mouse embryos were main-

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tained in culture on feeder cells and in the presence of steel factor, leukemia inhibitory factor (LIF), and basic fibroblast growth factor (bFGF) for at least 20 passages (Godin and Wylie, 1991; Matsui et al, 1991; Resnick et al, 1992). A chicken embryonic germ (EG) cell line was generated by long-term culture of gonadal PGCs supplemented with human stem cell factor (hSCF), murine LIF (mLIF), bovine bFGF, human interleukin-11 (h-IL-11), and human insulin-like growth factor-1 (IGF-1, Park and Han, 2000). In addition, it was shown that these cells closely resembled undifferentiated embryonic stem cells (Park and Han, 2000). Some growth factors have been implicated in the regulation of in vitro spermatogonial survival and proliferation, including LIF, oncostatin M (OSM), vitamin A, and epidermal growth factor (Haneji et al, 1986; De Miguel et al, 1996, 1997).

The objective of the present study was to develop an in vitro culture system to characterize cellular proliferation of germ cells (putative spermatogonial stem cells) from murine testes. To test the efficacy of this system, cultured germ cells were transplanted into the testes of azoospermic recipients to assay for the presence of spermatogonial stem cells.

Materials and Methods

Experimental Animals

Protocols for the use of animals in these experiments were approved by the Washington State University Animal Care and Use Committee and in accord with National Institutes of Health Standards established by the Guidelines for the Care and Use of Experimental Animals. Donor testis cells and recipient mice were obtained from B6, 129-TgR (ROSA26-transgenic), and B6/129 from the Jackson Laboratory (Bar Harbor, Maine), respectively. ROSA26 mice are transgenic for the *Escherichia coli* β-galactosidase gene, which is expressed in all differentiating germ cells. The presence of ROSA26 germ cells can be detected by blue staining after incubation with X-gal. Animals were housed in a standard animal facility with free access to food and water.

Cell Collection

Donor testes from 4- or 6-week-old mice (Nagano et al, 1998) were enzymatically digested as described by O'Brien (1993) with modifications as described by McLean et al (2002). Briefly, testes were removed, immersed in Hank's buffer, and the tunica removed. Testes were transferred to tubes containing digestion medium, consisting of 0.5 mg/mL collagenase type IV (Sigma, St Louis, Mo), 0.25 mg/mL trypsin (Gibco BRL, Bethesda, Md), and 0.05 mg/mL DNase (Sigma) in Ca- and Mg-free Hanks buffer (pH 7.4). Testes were digested for 15 min by shaking at 33°C to dissociate tubules. The sample containing the tubule suspension was transferred to ice and the tubules were allowed to sediment for 5 minutes. The supernatant was removed, fresh digestion medium added, and the tubules digested for 15 minutes by shaking at 32–37°C. After the second digestion, the cell concentration was determined using a hemocytometer. The cell suspension was centrifuged again at 500 $\times$ *g* for 4 minutes and then resuspended in Enriched Krebs-Ringer Bicarbonate medium containing 0.03% trypan blue.

Culture of Mice Spermatogonial Stem Cells

Testicular germ cells, from 4- or 6-week-old mice, were cultured in 24-well plates (Falcon, San Jose, Calif) or in 6-well plates with stem cell culture medium consisting of Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah), 12 ng/mL mLIF (Sigma), 20 ng/mL bovine bFGF (Sigma), 0.04 ng/mL h-IL-11 (Sigma), 10 ng/mL hSCF (Sigma), 1 mM sodium pyruvate, 2 mM L-glutamine, 5.5×10^{-5} M 2-mercaptoethanol, 100 μg/mL streptomycin, 100 units/mL penicillin, 10 ng/mL OSM (Sigma), 1 ng/mL platelet-derived growth factor (PDGF; Sigma), and 15 ng/mL human IGF-I (Sigma).

The isolated germ cells from one testis were seeded into wells of the 6-well culture plates and incubated in a CO_2 incubator at 32°C until the stem cells had colonized as a primary culture (approximately 7–12 days). Mitomycin C-treated STO cells were used as feeder cells in culture. The feeder cells were seeded on the culture surface at a concentration of 5×10^6 cells/cm^2 for 1–2 days before testis cells were added. Medium was changed twice a week. For subculture, the colonies of cells were agitated by pipetting after trypsin treatment for 10 minutes at 37°C and harvested from the plate. The cells were centrifuged at 200 $\times$ *g* for 5 minutes and placed into 2 fresh plates with mitotically inactivated STO feeder cells. The cell colonies were passaged at an interval of 7 to 14 days on average. All culture experiments were repeated 3 times; each experiment comprised triplicate cultures. The size of colonies was determined using NIH Image 1.62 (Bethesda, Md) by measuring the blue areas in cultures.

β-Galactosidase Activity

Cultured germ cell colonies were stained with 5-bromo-4-chloro-3-indolyl β-galactoside (X-gal) to detect β-galactosidase activity to differentiate and identify germ cell colonies from feeder cells. To stain the cell colonies, medium was removed, the cell layer washed once with phosphate-buffered saline (PBS), fixed with 0.5% glutaraldehyde for 20 minutes in ice, washed twice with PBS, and then incubated with X-gal until blue color developed.

Alkaline Phosphatase Assay and Antibody Staining

Germ cell colonies were fixed for detection of alkaline phosphatase (AP) activity in 66% acetone/3% formaldehyde and then stained with naphthol/AS-MX-alkaline AP substrate (Sigma). For immunocytochemistry, cells were fixed in 3% paraformaldehyde in PBS. An antibody for GCNA1 (germ cell nuclear antigen 1), a gift from George Enders (University of Kansas, Kansas City, Kans), and an antibody to c-kit, a gift from Michael Skinner (Washington State University, Pullman, Wash), were used to detect the presence of GCNA1 and c-kit in cultures. Each antibody was diluted 1:100 in PBS and the subsequent steps were carried out using an avidin/biotin conjugate system. The reaction was stopped by adding 10 mM EDTA (pH 8.0).

PKH26 Labeling of Germ Cells and Separation

Collected germ cells were labeled with a PKH26 fluorescent staining kit (Sigma). The method of fluorescent staining (Horan et al, 1990) previously described was strictly followed except that volumes of the reagents in each staining step were all reduced proportionally by 1/16 in 1.5-mL tubes. Briefly, adherent cells were removed using enzymes and put into a single-cell suspension. Cells were centrifuged (400 $\times$ g) for 5 minutes into a loose pellet and then resuspended in 1 mL of diluent C. One milliliter of cells was added to 1 mL of 2$\times$ dye and the sample immediately mixed by gentle pipetting. The staining reaction was stopped after 5 minutes by adding an equal volume of serum. After centrifugation, the supernatant was removed and the cells were washed 3 times. The PKH26 fluorescence image was obtained with an excitation wavelength of 550 nm using a laser confocal microscope (Bio-Rad, Hercules, Calif). For the separation of pure germ cells, PKH26-labeled germ cells were separated according to the method of Chang et al (1992) with minor modifications. Germ cells were mixed with 300 μL of 10% Ficoll solution and gently added over 900 μL of 16% Ficoll solution in a 1.5-mL microcentrifuge tube. After centrifugation at 800 $\times$ g for 30 minutes, the germ cell-rich fraction located at the border layer between 10% and 16% Ficoll solutions was collected. This germ cell-rich fraction was diluted with medium 199 and centrifuged at 400 $\times$ g for 5 minutes.

Proliferation Assay

Germ cells were treated with 5-bromo-2′-deoxyuridine (BrdU) reagent for 1 hour at 37°C and rinsed twice with 1$\times$ PBS. The subsequent steps to detect stained cells were carried out according to the manufacturer's recommendation (Zymed Lab, South San Francisco, Calif). Germ cell colonies that had incorporated the BrdU were detected by anti-BrdU monoclonal antibody with the use of the peroxidase/diaminobenzidine system (Zymed Lab).

Transplantation of Cultured Germ Cells

Cultured donor germ cells from ROSA26 were transplanted into testes of B6/129 mice 4 weeks after treatment with busulfan (40 mg/kg). A midline incision was made in the recipient mouse abdomen and the testis exposed. A small hole was made in the efferent bundle and a glass needle containing 7 μL of the germ cell suspension was inserted into the rete testis. Both germ cells and feeder cells at a concentration of 10^7 cells/mL were injected into the seminiferous tubules via the rete testis. The testis was replaced and the animal allowed to recover. Recipient mice were sacrificed after 2 months by cervical dislocation and the testes removed. Testes were fixed in 4% paraformadehyde in PBS, pH. 7.4, for 1 hour on ice and washed twice in LacZ buffer (0.2 M sodium phosphate, pH 7.3; 2 mM $MgCl_2$; 0.02% NP-40; and 0.01% sodium deoxycholate) for 30 minutes. β-Galactosidase-positive cells were stained by incubating testes in LacZ stain solution (LacZ buffer containing 20 mM potassium ferricyanide, 20 mM potassium ferrocyanide, and 1 mg/mL X-gal).

Results

Spermatogonial Stem Cell Culture

To develop cultures of mice spermatogonial stem cells, we collected testicular cells from 4- or 6-week-old mice to be placed in culture according to Nagano et al (1998). Various growth factors were added to DMEM as described in the Methods section. After 1 week in culture, small colonies of cells developed, which continued to expand for 2 weeks (Figure 1A). Colonies were maintained in culture from 3 weeks (Figure 1B) to 2 months (Figure 1D). The cells of the colonies proliferated into more subcolonies formed after subculture (Figure 1E). Cell colonies cultured for 3 months were subcultured 8 times without any apparent morphological changes (Figure 1E and F).

Germ cells were also cultured in the presence of mitomycin-treated STO feeder cell layers. The number of germ cell colonies present in wells with STO feeder cells was greater than in wells without feeder cells (data not shown). After culturing for a month and after 3 subcultures, the colonies stained positively for AP (Figure 1G and H). The diameter of AP-stained cell colonies increased during culture (Figure 2B through D; arrow indicates AP-stained cells). In contrast, cell proliferation and colony formation did not occur with AP-negative cells. In addition, AP-positive colonies stained blue after incubation with X-gal, indicating that the cells originated from the testis of transgenic ROSA26 donor mice (Figure 2E). The negative control (Figure 2F), a Sertoli cell line (MSC-1), was not stained by AP or X-gal. The cells of the colonies did not pack strongly together in small nests, and it was not difficult to discern the individual component cells.

To investigate the proliferation of germ cells that expressed AP, testicular cells were cultured in 6-well plates and were exposed to AP staining during culture (Figure 2A through F). To determine whether all cell colonies derived from single germ cells, primary germ cells were separated by a modified Ficoll density centrifugation method (Chang et al, 1992). After enrichment by Ficoll density gradients, germ cells were labeled by PKH26, a fluorescent viable cell marker, to identify germ cells in developing colonies that derived from stained founder cells. PHK26 staining is maintained in daughter cells after multiple cell divisions so an accumulation of cells in culture from an individual founder can be monitored. The cell proliferation ratio was determined with the use of a confocal fluorescent laser microscope (Figure 3A through F). Most of the germ cells were distinguished by their size and characteristic pseudopods, and large nucleus (Figure 3A and B) (Kanatsu-Shinohara et al, 2003). Some cell proliferation was viewed on STO feeder layers after

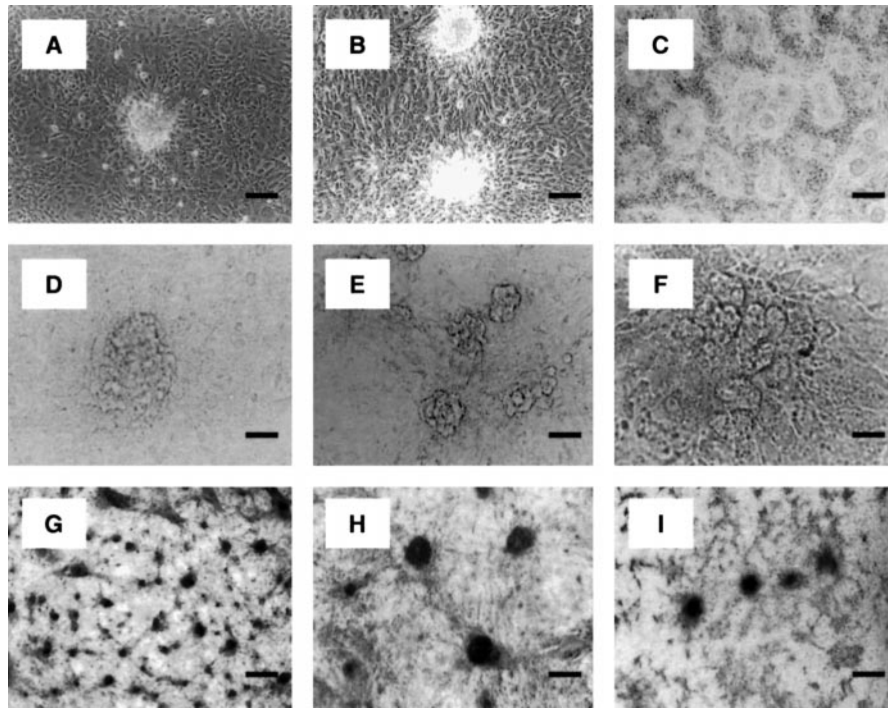

Figure 1. Mice spermatogonial stem cell culture. Appearance of 4-week-old mice (ROSA26) testes cells cultured on the STO feeders. **(A)** 14-day culture. **(B)** 21-day culture. **(C)** Appearance of 1-passage cultured cells after 2-week culture (1 month total culture time). **(D)** 2-month culture. **(E)** Culture passaged 8 times, cultured for 3 months total. **(F)** 3-month cultured colony at higher magnification (400×). **(G)** Alkaline phosphatase (AP) activity of 1-month culture. **(H)** Higher magnification of **G**. **(I)** AP staining of germ cell colonies passaged 3 times. Scale bar = 50 μm **(D)**, 33 μm **(A, B, C, E, H, I)**, 66 μm **(F)**. The experiments were repeated 3 times; each experiment comprised triplicate cultures.

culturing for 2 days (Figure 3C; arrow). These cells derived from single cells according to PHK26 staining and formed colonies during culture (Figure 3D through F).

Effects of Growth Factors on Stem Cell Proliferation

To identify the effects of stem cell medium, DMEM with FBS (control medium) and stem cell medium were used. Testis cells were collected from 4- and 6-week-old mice and cultured for 3 weeks, after which the colonies were stained by X-gal (Figure 4). The average number of colonies from 4-week-old mice with stem cell medium was 635 ± 34.2 (n = 3), in contrast to 113 ± 8.2 (n = 3) for 6-week-old mice with stem cell medium (Figure 3E).

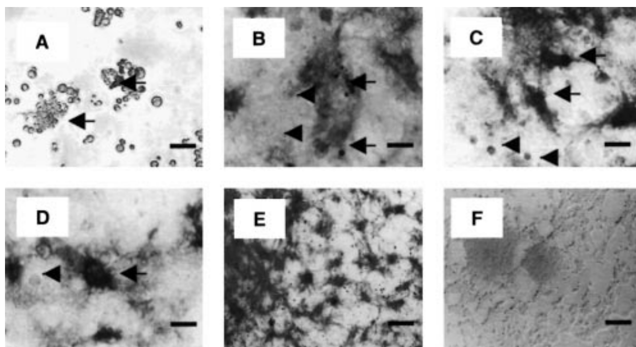

Figure 2. Detection of germ cell proliferation using alkaline phosphatase (AP) staining method. **(A)** Fresh cell suspension from 4-week-old mouse testis. **(B)** 1-day culture. **(C)** 2-day culture. **(D)** 7-day culture. **(E)** X-gal staining of cell colonies (after 12 days of culture); blue color indicates cells derived from the testis of transgenic donor mice. **(F)** Negative control Sertoli cell line, MSC-1. Arrows indicate cells with AP activity. Arrowheads indicate cells without AP activity. Scale bar = 17 μm **(F)**, 33 μm **(A, B, C, D, E)**.

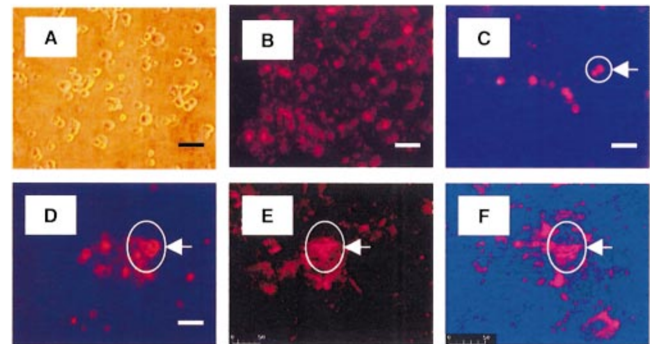

Figure 3. Identification of germ cell colonies derived from single germ cells. **(A)** Germ cells enriched by Ficoll density centrifugation method after PKH26 labeling of 4-week-old murine testis cells. **(B)** PKH26-stained cells viewed with fluorescent light (550 nm). **(C)** Germ cell proliferation viewed in white circle after 2-day culture period. **(D)** Cell colonies formed from **C** after 7-day culture period. **(E)** Proliferation of cells after 12-day culture period. **(F)** 21-day culture period. Scale bar = 33 μm. The experiments were repeated 3 times; each experiment comprised triplicate cultures. Arrows indicate cells in proliferation.

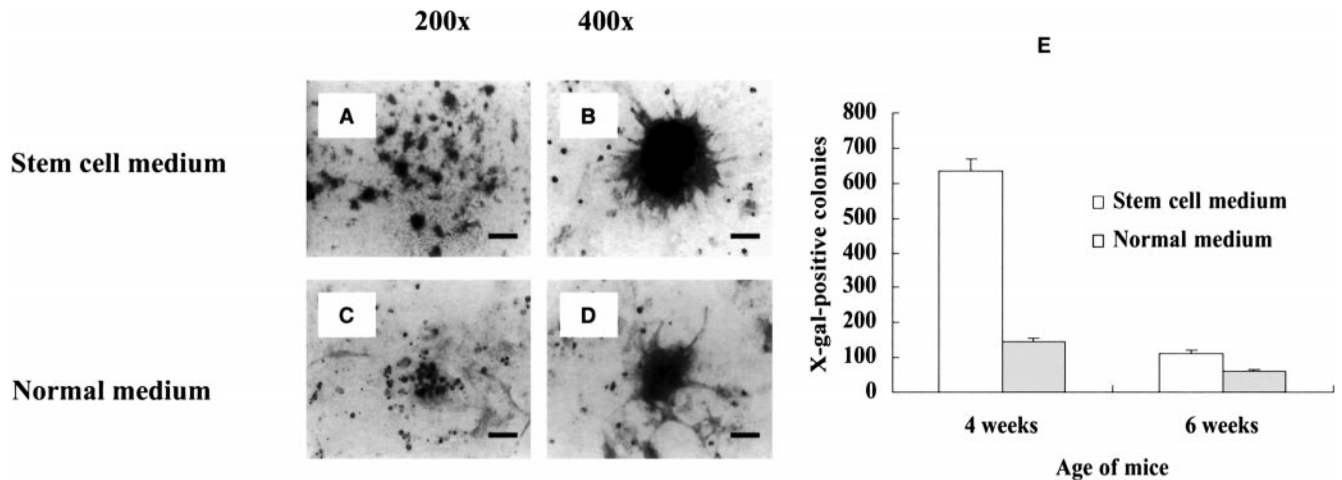

Figure 4. Effects of stem cell medium and age of mice on the ability of germ cells to survive and proliferate in culture. After 3 weeks of culture, the cultures were stained for β-galactosidase, and the number of X-gal-positive colonies was determined. **(A, B)** Cells cultured in stem cell culture medium. **(C, D)** Cells cultured in Dulbecco's minimal essential medium. **(E)** Effects of colony formation using the stem cell medium. Scale bar = 33 μm **(A, C)**, 66 μm **(B, D)**. Data are shown as mean number of colonies ± SEM. The experiments were repeated 3 times; each experiment comprised triplicate cultures.

However, the average number of colonies from 4-week-old mice with normal medium (DMEM) was 146 ± 11.9 (n = 3), in contrast to 59 ± 5.6 (n = 3) for 6-week-old mice with normal medium (DMEM). Cell colonies from 4-week-old mice were cultured in stem cell medium, and were 2 or 4 times larger than colonies treated with the control medium. The colony number was also significantly increased ($P < 0.05$). Cells from 6-week-old mice cultured in stem cell medium produced colonies that were only slightly increased in size over cell colonies obtained in the control medium. Furthermore, the number of stem cell colonies did not increase significantly when cells from 6-week-old mice were used.

To examine the effects of each growth factor in forming a stem cell colony, a BrdU proliferation assay system was used to determine whether cell colonies could proliferate, and whether their growth could be sustained in the presence of several growth factors (Figure 5) (Ellwart and Dormer, 1985; Gonchoroff et al, 1986; Fukuda et al, 1990). As shown in Figure 5A, the colony cultured in stem cell medium was stained positively with the BrdU assay. In the control medium, few cells were BrdU pos-

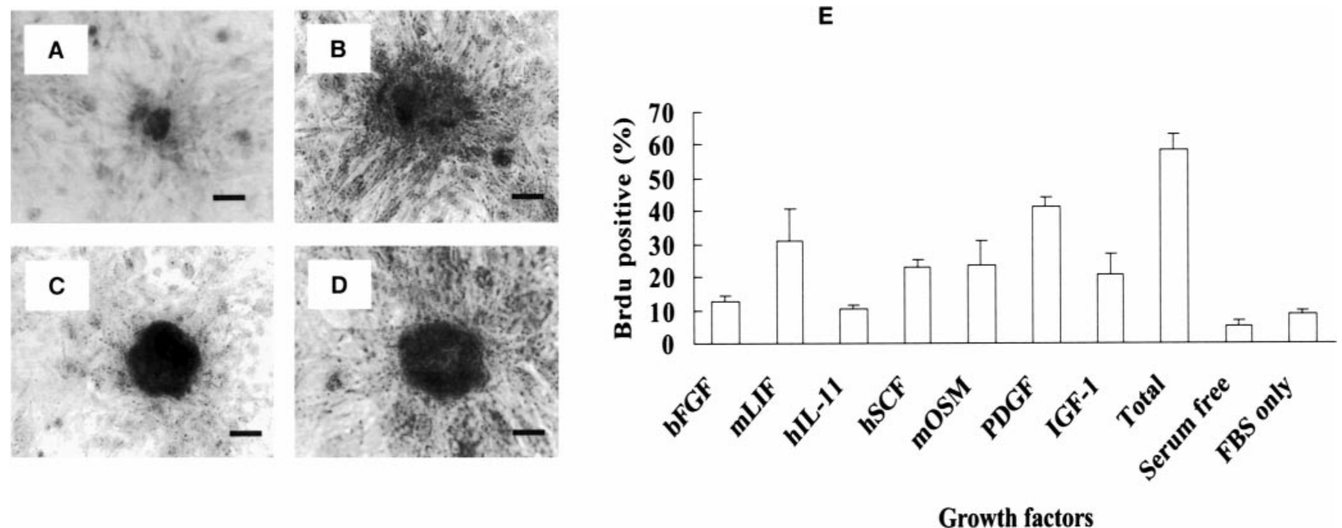

Figure 5. Proliferation assay using 5-bromo-2′-deoxyuridine (BrdU). **(A)** BrdU-positive cells (no hematoxylin counterstaining) of cell colony. **(B)** BrdU-positive cells in germ cell colony (normal medium). **(C)** BrdU-positive cells in the germ cell colony (stem cell medium). Note the greater percentage of BrdU-positive cells in **(C)**. **(D)** Outer space cells in the colony were stained. **(E)** Effects of several growth factors (basic fibroblast growth factor, murine leukemia inhibitory factor, human interleukin-11, human stem cell factor, murine oncostatin M, platelet-derived growth factor, insulin-like growth factor 1, serum free medium, fetal bovine serum only, and total medium). Scale bar = 33 μm **(A, B)**, 66 μm **(C, D)**. Data represent the mean ± SEM of one representative experiment. The experiments were repeated 3 times; each experiment comprised triplicate cultures.

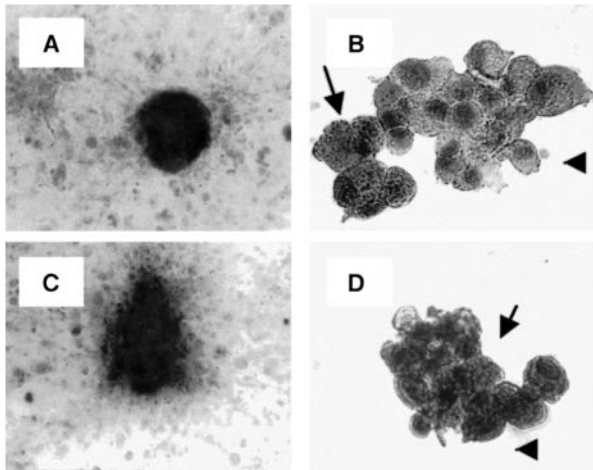

Figure 6. Antibody staining of c-kit and germ cell nuclear antigen 1 (GCNA1) within 4-week mouse testis cell colonies of ROSA26. **(A)** Anti-GCNA1 staining. **(B)** Dispersion of cell colony after GCNA1 staining. **(C)** Anti-c-kit staining. **(D)** Dispersion of cell colony after c-kit staining. Scale bar = 66 μm **(A, C)**, 165 μm **(B, D)**. The experiments were repeated 3 times; each experiment comprised triplicate cultures.

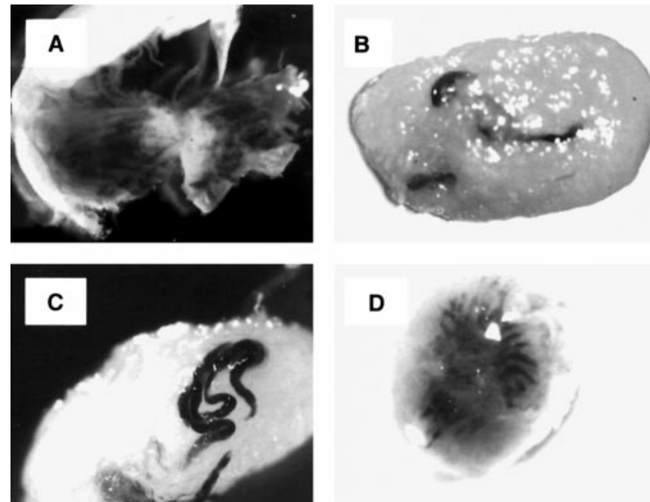

Figure 7. Transplantation of ROSA26 cultured testicular germ cells. **(A)** Recipient testis injected with ROSA26 testis cells cultured in stem cell culture medium for 4 weeks. Recipient mice were sacrificed and recipient testes stained with X-gal 2 weeks after germ cell injection. **(B)** Recipient testis injected with Rosa26 cells cultured in stem cell culture medium 1 month. **(C)** Recipient testis injected with Rosa26 cells cultured in stem cell culture medium 2 months. **(D)** Recipient testis injected with Rosa26 cells cultured in stem cell culture medium 2 months and passaged 3 times. **(B, C, D)** Recipient mice were sacrificed and recipient testes stained with X-gal 8 weeks after germ cell injection. Scale bar = 1 mm.

itive (Figure 5B), but when the total stem cell medium was used, almost all the cells were deeply stained. As shown in Figure 5D, cells were dividing on the periphery of the colonies. Testicular cells were also cultured in the presence of each factor separately (Figure 5E). BrdU-positive cells represented 31% of the cells in the colony in the mLIF medium and 44% in the PDGF medium but only 5%–7% in the DMEM.

Immunochemistry in Spermatogonial Stem Cell Colonies

We used antibodies to GCNA1 and to c-kit to determine if the cultured germ cells expressed these proteins. A few cells of the cultured germ cell colonies expressed GCNA1 and c-kit (differentiated spermatogonia marker; Kanatsu-Shinohara et al, 2003) (Figure 6). The rat IgM monoclonal antibody to GCNA1 has been used to identify dividing germ cells (Enders and May, 1994).

Transplantation of Cultured Spermatogonial Stem Cells

Germ cells from ROSA26 mice were cultured using several of the methods described, and the cells were transplanted into the recipient mice. After 2 weeks or 2 months, testes were collected from the recipient mice and stained with X-gal to determine the extent of spermatogenesis (Figure 7, Table). Cultured germ cells from 4-week-old Rosa 26 mice were transplanted into the testes of recipient mice. These germ cells were strongly stained with X-gal in the recipient mice after 2 weeks (Figure 7A) and after 2 months, respectively (Figure 7B). The number of testes colonized by germ cells maintained in the different culture methods was used to evaluate the

Spermatogenesis from cultured spermatogonial stem cells after transplantation ot recipient testes

Experiment	Culture Period (Week)	Stem Cell Medium	DMEM Medium	No. of Colonies/ Testes	Number of Testes Injected	Number of Testes Colonized
1	4	+	−	17.4 ± 7.4	14	9
2	4	−	+	5.1 ± 6.2	6	1
3*	2	+	−	4.7 ± 7.0	5	3
4	8	+	−	21.1 ± 5.4	6	3
5†	4	+	−	12.0 ± 4.1	5	2
6‡	12	+	−	23.7 ± 8.3	7	6

* Transplants from cryogenically preserved spermatogonial stem cell.
† Transplants from passage cultured spermatogonial stem cell (1 passage).
‡ Transplants from passage cultured spermatogonial stem cells (8 passage for 3 months). Number of individual colonies in each testis. Results were normalized to 10^5 cells injected/testis.

effectiveness of maintaining or stimulating spermatogonial stem cell proliferation (Table). At 2 months after transplantation, the number of testes colonized from experiment 1 (1-month culture) and experiment 4 (2-month culture) was 9 of 14 testes injected and 3 of 6 injected, respectively (Table). In addition, the number of testes colonized from experiment 5 (subculture) and experiment 6 (subculture) was 2 of 3 injected and 6 of 9 injected, respectively (Table). In contrast, the number of testes colonized from experiment 2 (control group) was 1 of 6 testes injected.

The most consistent germ cell colonization was detected in transplants from 3-month cultures, despite the fact that the donor germ cells were subcultured 8 times (Table). Lastly, germ cells that had been cultured for 4 weeks were cryopreserved for 1 month. Transplantation of cryopreserved cultured germ cells established donor-derived spermatogenesis in 3 testes of 5.

Discussion

Cells comprising the many organ systems are continuously lost through cell death or terminal differentiation. The lost cells are replaced by unique undifferentiated stem cells found within the organ. Stem cells are present in several organ systems including skin, intestinal epithelium, hematopoietic system, neuronal tissue, and reproductive tissue. Among these self-renewing tissue systems, hematopoiesis and spermatogenesis are the most mitotically active (Russell et al, 1990). Therefore, knowledge about the control of spermatogonial stem cell activity not only contributes to our understanding of reproductive biology but also has broad implications. Spermatogonial stem cells from mice were first cultured and identified by Nagano et al (1998). Previous studies with chicken germ cells identified several factors necessary for EG cell culture (Park and Han, 2000). The current study was performed to determine if similar results could be obtained using mouse spermatogonial stem cells with a stem cell in vitro culture technique.

These studies extend the studies of Nagano et al (1998) and demonstrate the formation of colonies of proliferating spermatogonial stem cells. In addition, the extent of colonization by cultured germ cells was affected by several growth factors. Identification of spermatogonial stem cell culture conditions for long-term culture, renewal, and differentiation could prove highly useful for the germ cell transplantation technique (Dirami et al, 1999). These results demonstrate that adult germ cells were able to proliferate and formed colonies resembleing EG cell lines developed using several growth factors. Kanatsu-Shinohara et al (2003) reported similar results, that most of these primary germ cell colonies consisted of compact clusters of cells with unclear borders but were distinct in appearance from embryonic stem (ES) cells. Each single testicular cell proliferated to form a colony of cells that resembled early-passage mouse EG cell colonies in that AP activity was present (Figure 1F). Mouse ES and EG cells share several morphological characteristics such as high levels of intracellular AP (Solter and Knowles, 1978; Polejaeva et al, 1997). We found that germ cell colonies cultured in the stem cell system described here retained AP expression during culture.

In our studies germ cell colonies started to appear within 12 days of culture in media containing several growth factors and STO feeder cells. The formation of cell colonies by cultured mouse germ cells has not been previously reported. In studies using PGCs conducted over a period of 7–21 days, PGCs gave rise to large multicellular colonies (Shamblott et al, 1998). Cultured embryonic stem cells from mice also give rise to the formation of colonies. High magnification of the mouse germ cell colonies showed that each single cell was attached to the colony, similar to a bunch of grapes (Figure 1F). The subculture method used in this study aimed to increase the number of stem cells, which do not have any known morphological characteristics. We established a successful long-term culture of testiscular germ cells in this study and the number of germ cells increased continuously during several subcultures.

We also observed a difference in the cell proliferation ratio between stem cell medium and control medium ($P < 0.05$) (Figure 4). This result indicated that several growth factors in the medium were more important in germ cell proliferation than DMEM with FBS. The percentage of cells incorporating BrdU in mLIF and PDGF cultures was 31% and 44%, respectively. Thus, these factors affect the proliferation of germ cells more than other factors. However, h-IL-11 in this study showed a BrdU activity lower than 10%, which was not much different from that of FBS. When several growth factors were added to the medium, the cells in the colonies continued to proliferate, and when these colonies were successfully transplanted into the recipient testis, spermatogenesis was detected in the testis after 2 months (Figure 7).

The growth factors required to allow pluripotent mouse EG cells to survive and divide are LIF, SCF, PDGF, IL-11, mOSM, IGF-1, and bFGF (Donovan, 1994; Shamblott et al, 1998). Sertoli cell-derived growth factors such as SCF, bFGF, and LIF play an important role for the survival and proliferation of PGCs, gonocytes, and stem cells (Hofmann et al, 2003). Although some somatic cells like Sertoli cells did not survive during long-term culture, germ cells need these growth factors for long-term survival (Kanatsu-Shinohara et al, 2003). As shown Figure 4, the colonies of germ cells in DMEM control cultures did not increase in size when compared to stem cell me-

dium culture. Therefore, some growth factors of stem cell medium such as PDGF and LIF play a role in germ cell proliferation in germ cell culture (Resnick et al, 1992; De Miguel et al, 1996). Also, the results suggest that the germ cell proliferation efficiency in stem cell medium was affected by combined effect of the growth factors (Figure 5E).

Germ cells from 4-week-old mice showed a higher level of proliferation than germ cells cultured from 6-week-old mice (Figure 1). This may be due to changes in the spermatogonial stem cell population during development. Spermatogonial stem cells increase in number in the testis in the mouse during neonatal development until adulthood (Shinohara et al, 2002). Therefore, the spermatogonial stem cell population is constantly changing. The spermatogonial stem cells in 4-week-old mice may still be forming niches when compared to older ages and thus more responsive to culturing. At 6 weeks postpartum, the population of spermatogonial stem cells may be set and have completely formed and are therefore less likely to be manipulated in culture.

Colonization of recipient testes injected with cultured germ cells was evaluated at 2 weeks and 8 weeks after injection. These evaluation times are selected to detect specific events associated with donor-derived colonization of the seminiferous tubules. Donor-derived colonies often merge within the seminiferous tubule during lateral expansion; therefore, 2-week evaluations allow for a precise indication of the total number of colonies formed compared to 8-week evaluations. The 8-week evaluations allow for an accurate determination of the extent of expansion of the donor-derived colonies by measuring the blue area. Transplantation of germ cells cultured using the stem cell medium colonized more recipient testes than controls (Table 1). This result indicates that spermatogonial stem cells are either proliferating in the culture system or the factors included in the culture system are preventing spermatogonial stem cell apoptosis. Further studies will determine if apoptosis is occurring in the control system to a greater extent than the stem cell culture system.

It has been reported that undifferentiated spermatogonial cells (stem cells) do not express c-kit except, perhaps, at only low levels (Shinohara et al, 1999, 2000). A small number of cells in the colonies was positive for GCNA1 (Figure 6B, arrow). However, most cells in the colonies were negative for GCNA1 (Figure 6B, arrowhead). We used an antibody to c-kit to determine if germ cell colonies had differentiated. Germ cell cultures were enzymatically digested to obtain a single-cell suspension. Most cells from these cultures were negative for c-kit staining (Figure 6D, arrowhead). A small number of cells from these cultures weakly stained for c-kit (Figure 6D, arrow). Although the culture medium in these experiments included SCF, which is the ligand for c-kit, the morphology of the cells did not change and the growth of the cells in the colonies did not change compared to stem cell medium without SCF (data not shown). Perhaps these colonies contained both differentiated and undifferentiated spermatogonial cells.

The long-term cultured germ cells may be more easily used to manipulate the germline genome to make gene-targeted transgenic mice than ES/EG cells. Spermatogonial stem cells are present throughout the lifetime of the animal, unlike ES/EG cell lines, which are available only during the embryonic period. ES/EG cell lines are also totipotent and differentiate into tissues with diverse phenotypes by forming teratomas (Kanatsu-Shinohara et al, 2003). Therefore, these long-term cultured germ cells will be useful for investigating the differentiation of germ cells and provide the tools for genetic modification in the germ line.

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