Enrichment and *In Vitro* Culture of Spermatogonial Stem Cells from Pre-Pubertal Monkey Testes

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Abstract Spermatogonial stem cells (SSCs) are essential for spermatogenesis throughout the lifespan of the male. However, the rarity of SSCs has raised the need for an efficient selection method, but little is known about culture conditions that stimulate monkey SSC proliferation *in vitro*. In this study, we report the development of effective enrichment techniques and *in vitro* culturing of germ cells from pre-pubertal monkey testes. Testis cells were analyzed by fluorescence-activated cell sorting techniques and were transplanted into the testes of nude mice to characterize SSCs. Thy-1-positive cells showed a higher number of colonies than the unselected control after xenotransplantation. Extensive colonization of monkey cells in the mouse testes indicated the presence of highly enriched populations of SSCs in the Thy-1-positive sorted cells. Furthermore, monkey testis cells were enriched by differential plating using extracellular matrix, laminin, and gelatin, and then cultured under various conditions. Isolation of monkey testicular germ cells by differential plating increased germ cell purity by 2.7-fold, following the combinational isolation method using gelatin and laminin. These enriched germ cells actively proliferated under culture conditions involving StemPro medium supplemented with bFGF, GDNF, LIF, and EGF at 37 °C. These results suggest that the enrichment and *in vitro* culture method proposed in the present study for harvesting a large number of functionally active monkey SSCs can be applied as the basis for efficient *in vitro* expansion of human SSCs.

Keywords Monkey spermatogonial stem cells · Enrichment · *in vitro* culture

1 Introduction

Spermatogonial stem cells (SSCs) are precursor cells for spermatogenesis that self-renew and regulate differentiation to maintain their population, and to produce

spermatozoa throughout the life of the male. In humans and other primates, A_{dark} and A_{pale} spermatogonia are undifferentiated spermatogonia that are considered to be stem cells for spermatogenesis [1–5]. However, it is difficult to distinguish morphological or biological differences between SSCs and other spermatogonia, since the number of SSCs is very low in the testis and little is known about their stem cell properties. Therefore, biological characteristics of SSCs need to be investigated for identification by efficient manipulations, such as functional or molecular assays.

Previous studies have applied several methods involving the extracellular matrix (ECM) and specific isolation techniques, such as fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS), to isolate rodent SSCs [6–8]. These methods have supported

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studies on efficient enriching of germ cells and developing enrichment techniques. Furthermore, the combination of functional and molecular assays has enabled many researchers to study and identify characteristics of stem cells. Currently, purification of SSCs is routinely accomplished by enrichment methods, followed by identification by functional assays to determine the activity of the cell population highly enriched for SSCs [6, 9, 10].

SSC functional assays, also known as transplantation assays, have been applied as a functional endpoint to identify stem cells in male reproductive studies to assess different strategies for SSC enrichment [11, 12]. However, in large animals, including bulls, goats, and boars, stem cell activity analyses by transplantation require difficult injection techniques and would completely deplete the germ cells of the recipients [13–15]. Thus, xenotransplantation of SSCs from other animals into immunodeficient mice has been generally applied for the analysis of stem cell activity [16, 17].

Although sequential methods of SSC enrichment and transplantation have been widely applied in rodents, these applications have not been available to provide a sufficient methodology for other species, such as non-human primates. In this study, we aimed to investigate the characteristics of undifferentiated spermatogonia, enhance SSC purity, and evaluate the *in vitro* culture conditions for germ cells—including SSCs—from pre-pubertal monkey testes.

2 Materials and methods

2.1 Donor testis cell collection

Five pre-pubertal (44 to 57-month-old) cynomolgus monkey were purchased from Genia Inc. (Seongnam, Gyeonggi-Do, Korea) for this study. All animal procedures were approved by the Animal Care and Use Committee of Chung-Ang University (IACUC no. 2015-00016) in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Donor testes were collected from pre-pubertal cynomolgus monkeys and placed in Dulbecco Phosphate-Buffered Saline (DPBS; Invitrogen, Grand Island, NY, USA) on ice until used. Testes were decapsulated and chopped with forceps and scissors. Testis tissues were digested with collagenase type IV (2 mg/mL) in DPBS at 37 °C for 30 min with periodic agitation. After digestion, testicular fragments were washed with DPBS and then incubated in a 4:1 solution of 0.25% trypsin in 1 mM EDTA (Invitrogen) and 7 mg/mL deoxyribonuclease I (DNase I; Roche, Basel, Switzerland) in DPBS at 37 °C for 5–10 min. Trypsin was inactivated

by the addition of fetal bovine serum (FBS; Hyclone, Logan, UT, USA) up to 10% of the total volume. Cell suspensions were filtered through a nylon mesh with 70 µm pores (BD Biosciences, San Jose, CA, USA) and centrifuged at 600×*g* for 7 min at 4 °C. The cell pellet was resuspended in basic medium (DMEM containing 10% FBS, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 100 U/mL penicillin, and 100 µg/mL streptomycin).

2.2 Flow cytometry and cell sorting

Testis cells were incubated with primary antibodies (1 µg/1 × 10⁶ cells) in PBS-S (DPBS containing 1% FBS, 10 mM HEPES, 1 mM pyruvate, 50 U/mL penicillin, 50 µg/mL streptomycin, and 1 mg/mL glucose) for 20 min at 4 °C. Primary antibodies used were phycoerythrin (PE)-conjugated mouse anti-human Thy-1 (BD Biosciences), mouse anti-human stage-specific embryonic antigen (SSEA-1; Santa Cruz Biotechnology, Dallas, TX, USA), mouse anti-human SSEA-4 (EMD Millipore, Billerica, MA, USA), PE-conjugated mouse anti-human c-Kit (Biolegend, San Diego, CA, USA), mouse anti-human E-cadherin (Santa Cruz Biotechnology), and mouse anti-human Ep-CAM (Santa Cruz Biotechnology). Isotype antibody controls used were normal mouse IgG1, FITC-conjugated normal rat IgG2a, and Alexa Fluor 488-conjugated normal mouse IgM (Santa Cruz Biotechnology). After incubation, cells were washed twice with PBS-S and then incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen), Alexa Fluor 546-conjugated goat anti-mouse IgG (Invitrogen), or Alexa Fluor 488-conjugated goat anti-mouse IgM (Invitrogen) secondary antibody for 20 min at 4 °C. Cells were washed twice with PBS-S and resuspended in PBS-S containing 1 µg/mL propidium iodide (PI). Flow cytometric analyses and cell sorting were performed using BD FACS Aria II (BD Biosciences, Center for Research Facilities, Chung-Ang University), according to manufacturer's instruction.

2.3 Enrichment and culture of undifferentiated spermatogonia

To remove erythrocytes and cellular debris from whole testis cells, 10 × 10⁶ isolated testis single cells were overlaid on 2 mL of a 30% Percoll solution and centrifuged at 600×*g* at 4 °C for 10 min, as previously described [16]. Cells from each layer were analyzed for the presence of promyelocytic leukemia zinc finger (PLZF)-positive cells. We confirmed that more PLZF-positive undifferentiated spermatogonia were found in the 30% Percoll bottom layer than cells in the upper layers. To increase the purity of undifferentiated spermatogonia, cell populations in the 30% Percoll bottom layer were used for subsequent

enrichment using differential plating. After Percoll separation, 10×10^6 cells suspended in 3 mL of media were incubated on a 60-mm plate pre-coated with 0.2% gelatin (overnight) or 20 $\mu\text{g/mL}$ laminin (20 min). For sequential selection, cells were initially incubated on gelatin plates overnight, and then cell population that were unbound or that loosely adhered to the gelatin-coated dish were incubated on a laminin-coated dish for 20 min. After incubation, each unbound and bound cell populations were harvested and counted for further analysis. The percentage of undifferentiated spermatogonia in each cell population was determined as follows:

$$\text{Percentage of undifferentiated spermatogonia} = (\text{Number of PLZF} - \text{positive cells} / \text{total number of DAPI} - \text{positive cells}) \times 100$$

Testis cells enriched for undifferentiated spermatogonia were maintained in serum-free medium (StemPro-34-based medium (Stempro), mouse serum-free medium (mSFM), rat serum-free medium (rSFM), and $2 \times$ rSFM with minor modifications [7, 18, 19]. Culture conditions at different temperatures were also investigated (34 and 37 °C). The growth factors used were human basic fibroblast growth factor (bFGF; BD Biosciences), human glial cell line-derived neurotrophic factor (GDNF; R&D Systems, Minneapolis, MN, USA), mouse leukemia inhibitory factor (LIF; Chemicon International, Temecula, CA, USA), and mouse epidermal growth factor (EGF; BD Biosciences).

2.4 Immunohistochemistry

Testis tissues were fixed in Bouin's solution and dehydrated by washing with a 70–100% ethanol gradient for 5 min each, followed by washing with xylene. After dehydration, tissues were embedded in paraffin wax, sectioned into 5 μm slices, and adhered to slides. Sections were then deparaffinized, followed by staining with hematoxylin and eosin (H&E). To prepare the immunostain, Paraffin-embedded sections were deparaffinized, rehydrated, and permeabilized with 0.1% Triton X-100. Sections were then blocked with 5% bovine serum albumin (BSA) for 30 min at room temperature. Then, sections were incubated with primary antibodies at 4 °C overnight. Primary antibodies included mouse anti-human PLZF (1:200; Calbiochem, Darmstadt, Germany), rabbit anti-human GFR α 1 (1:200; Santa Cruz Biotechnology), and rabbit anti-human VASA (1:200; Abcam, Cambridge, MA, USA). Sections were washed several times in DPBS and incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:200; Invitrogen) or Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:200; Invitrogen) secondary antibodies for 1 h at room temperature. Labeled sections

were washed 3 times in DPBS, followed by mounting with Vectashield mounting media containing DAPI (Vector Laboratories, Burlingame, CA, USA). After labeling, tissues were analyzed using a Nikon TE 2000 microscope with NIS Elements imaging software (Nikon, Tokyo, Japan).

2.5 Immunocytochemistry

Monkey undifferentiated spermatogonia were identified by PLZF-positive expression. Cells were fixed with 4% paraformaldehyde (PFA) for 30 min and permeabilized with 0.1% Triton X-100 in DPBS for 10 min at room temperature. Then, cells were blocked with DPBS containing 5% (w/v) BSA for 30 min at room temperature and incubated with mouse anti-human PLZF (1:200; Calbiochem) primary antibody at 4 °C overnight. The following day, cells were washed 3 times in DPBS and incubated with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (1:200; Invitrogen) for 1 h at room temperature. Labeled cells were washed 3 times in DPBS and counterstained, then mounted with Vectashield mounting media containing DAPI. To quantify undifferentiated spermatogonia, the percentage of PLZF-positive cells was determined by dividing the total number of labeled cells in 5 random microscopic fields by the total number of DAPI-positive cells in the same fields.

2.6 Transplantation

Immunodeficient BALB/c nude male mice (B&K Universal Ltd, Grimston, UK) were used as recipients to avoid the immune rejection response. The mice were treated with the chemotoxic drug busulfan (40 mg/kg) at 6 weeks of age to deplete endogenous spermatogenesis in the testes [20]. To visualize donor cells in the recipient testes after transplantation, donor cells were labeled with the PKH26 membrane linker dye, according to the manufacturer's instruction [16]. After labeling, donor cells were washed 3 times and PKH26-labeled cells were resuspended in basic medium. Approximately 10 μL of donor cell suspension was introduced into the seminiferous tubules of each recipient's testes through the rete testis 4–6 weeks after busulfan treatment, resulting in approximately 80% filling of the seminiferous tubules. Six weeks after transplantation, recipient testes were collected and analyzed for donor-derived colonies. Individual fluorescent tubules represented colonies generated from donor-derived SSCs [21, 22]. The number of colonies were counted by fluorescence microscopy. For the analysis of testes transplanted with monkey testis cells, the colony number was normalized to the number of colonies per 10^5 cells transplanted using the following equation:

$$\begin{aligned} \text{Colonies}/10^5 \text{ Cells Transplanted} \\ = \text{Number of Colonies} \\ \times 10^5 / \text{Number of Cells Transplanted} \end{aligned}$$

2.7 Statistical analysis

All statistical analyses were conducted using SPSS version 12 software (SPSS Inc., Mechanicsburg, PA, USA). Analysis of variance (ANOVA) was performed to test for differences between treatment groups. Significant differences between means were determined by Tukey's post hoc multiple comparisons test. Differences were considered significant if $p < 0.05$.

3 Results

3.1 Characterization of monkey testis cells

We analyzed testes from 4- to 6-year-old monkeys for the presence of the undifferentiated spermatogonia marker promyelocytic leukemia zinc finger (PLZF), also known as the zinc finger and BTB domain containing 16 (ZBTB16), at the beginning of the experiment. As expected, expression of PLZF was confined to cells on the basement membrane of the seminiferous tubules (Fig. 1A).

Whole testis cells were obtained from 4- to 6-year-old monkeys by enzymatic digestion and the number of cells were counted to determine cell viability in this cell population; the viability of the initial harvested cells was more than 90%.

To characterize pre-pubertal monkey testis cells, FACS and immunofluorescence analyses were performed. All subsequent FACS analyses were conducted using the forward scatter (G2) and live (PI-negative) cells (Fig. 1B). We probed the monkey testis cells with antibodies that were associated with undifferentiated spermatogonia markers, such as E-cadherin, EP-CAM, Thy-1, SSEA-1, SSEA-4, and the differentiating germ cell marker c-kit [5, 6, 10, 22–25]. The total testis cell population was first analyzed by FACS, using fluorescent antibodies. Although there was no positive expression of E-cadherin, EP-CAM, SSEA-1, and c-kit, the percentage of cells positive for Thy-1 and SSEA-4 expression were 4.5 and 5.1%, respectively (Fig. 1B).

Since only the total testis cell population showed Thy-1- and SSEA-4-positive marker expression, the two cell populations (Thy-1 and SSEA-4) were subsequently separated into two fractions based on marker expression and compared to the initial testis cells. The group of initial testis cells was stained with PLZF and showed $10.9 \pm 3.8\%$ PLZF expression, which was evidence that

only 11% of cells in the total initial testis cells were undifferentiated spermatogonia (Fig. 1C). Thy-1- and SSEA-4-positive and negative fractions were also stained with PLZF to confirm the purity of the undifferentiated spermatogonia. Immunofluorescence analyses of these cells revealed that a small number of PLZF-negative cells were present in every negative populations, whereas the majority of PLZF-positive cells was localized to the Thy-1-positive ($66.3 \pm 4.9\%$) and SSEA-4-positive ($59.4 \pm 9.4\%$) fractions (Fig. 1C). These results

Fig. 1 Characteristics of pre-pubertal monkey testis cells. **A** Hematoxylin and eosin (H&E) staining of donor testis (*left*). Immunohistochemical analysis of PLZF (*green*) within cross-sections of seminiferous tubules from pre-pubertal monkey testes (*right*). Histological sections were counterstained with DAPI (*blue*), indicating the nuclei of all cells in the section. Arrows indicate PGP 9.5-expressing germ cells. *Scale Bar* = 50 μ m. **B** Testis cells were sorted into two fractions (G1, G2) based on light-scattering properties by fluorescence-activated cell sorting (FACS) and PI-positive (*dead*) cells were excluded before the analysis (*top*). The cells in the G2 fraction, excluding the PI-positive cells, were analyzed to determine the percentage of cells positive for undifferentiated spermatogonia markers (E-cadherin, EP-CAM, Thy-1, SSEA-1, and SSEA-4), and the differentiating germ cell marker c-kit, compared with that of unselected controls ($n = 3$). The vertical lines represent the range of fluorescence in the controls. **C** Immunocytochemical detection of undifferentiated spermatogonia promyelocytic leukemia zinc finger (PLZF) in FACS-isolated cell fractions. The percentages of PLZF-positive cells were 10.9 ± 3.8 , 66.3 ± 4.9 , 3.6 ± 1.6 , 59.4 ± 9.4 , and $3.9 \pm 2.2\%$ in the unselected, Thy-1-positive, Thy-1-negative, SSEA-1-positive, and SSEA-4-negative populations, respectively. Values are depicted as the mean $\pm$ SEM, $n = 3$. Bars with different letters are statistically significant. (Color figure online)

demonstrated that undifferentiated monkey spermatogonia were positive for the Thy-1 and SSEA-4 markers.

3.2 Analysis of monkey spermatogonial stem cells by xenotransplantation

Based on the enrichment of PLZF expression, it was likely that undifferentiated spermatogonia and SSCs were enriched in the Thy-1- or SSEA-4-positive cell populations. To assess stem cell activity of pre-pubertal monkey testis cells isolated by FACS, using an anti-Thy-1 or anti-SSEA-4 antibody, we transplanted these cells, which were labeled with PKH26 membrane-linked red fluorescent dye, into the seminiferous tubules of immunodeficient nude mouse testes. These mice had any endogenous spermatogenesis ablated by treatment with busulfan (40 mg/kg of body weight) 6 weeks prior to transplantation. Recipient mice were transplanted each with Thy-1-positive or SSEA-4-positive testis cells or unselected testis cells.

Recipient mice were euthanized 1.5 months after transplantation to measure the activity of stem cells, and the PKH26-labeled colonies were observed under a fluorescent microscope. It appeared that the transplanted cells formed chains between the stem cells along the seminiferous tubule and were seen to organize into colonies (Fig. 2A–D).

We counted the number of PKH26-labeled colonies for each recipient testis. On average, 96.0 ± 5.1 colonies were formed per 10^5 viable cells transplanted in the unselected testis cell population. In contrast, 340.0 ± 140.0 and 257.1 ± 114.3 colonies in the Thy-1-positive and SSEA-4-positive cell population were formed, respectively. Only

Fig. 2 Germ cell transplantation of Thy-1-positive and SSEA-4-positive cells separated from monkey testis cells. **A–D** PKH26-labeled (*red*) unselected, Thy-1-positive, and SSEA-4-positive monkey testis cells formed colonies consisting of chains of cells in the recipient mouse testes. *Scale bar* = 200 μ m. **E** The number of colonies determined by PKH26-labeling was 96.0 ± 5.1 , 340.0 ± 140.0 , and 257.1 ± 114.3 in the unselected, Thy-1-positive, and SSEA-4-positive cell populations transplanted into the recipient mice, respectively. Values represent the mean $\pm$ SEM. Bars with different letters are statistically significant. (Color figure online)

the Thy-1-positive cell population yielded a significant increase for the number of colonies in the Thy-1-positive cell population of 3.5-fold ($340.0/96.0$), compared to the unselected testis cell population (Fig. 2E). Taken together, these results demonstrated that pre-pubertal monkey SSCs expressed Thy-1 and could be highly purified by FACS as a sorting technique.

SSC transplantation analysis was performed as a technique to determine the presence of male reproductive stem cells. This technique been used to evaluate their functional ability to self-renew and produce differentiated cells because each transplanted donor stem cell generates a colony during spermatogenesis [21, 26]. This

transplantation technique has also proven its application in other species, as well as in mice [5, 16, 27].

3.3 Enrichment of spermatogonial stem cells by differential plating

To gain purified, undifferentiated spermatogonia populations from testis cells, a Percoll gradient and differential plating methods, which were used for enrichment of neonatal pig testis cells in another study [16], were applied here with pre-pubertal monkey testis cells. We first performed the Percoll gradient separation using a density of 30% Percoll and the cells collected from the upper and bottom Percoll layer were stained with PLZF. However, each population gathered from either the upper or bottom Percoll layer were not enriched for undifferentiated spermatogonia (Fig. 3A–C). Therefore, differential plating was performed for the enrichment of spermatogonia from initial testis cells by selection using gelatin, laminin, or combination of gelatin and laminin. After differential plating, adherent and non-adherent cells were harvested and stained with PLZF, which verified the fraction of cell purity as 39.2 ± 3.6 , 39.2 ± 3.6 and $45.8 \pm 0.7\%$ for gelatin, laminin, or the combination of gelatin and laminin plates, respectively (Fig. 3D–G). The gelatin and laminin combination, among other treatments, showed the highest increase [2.7-fold (45.8/17.2)] in the number of PLZF-positive cells. Therefore, we performed gelatin and laminin combination plating for the enrichment of undifferentiated spermatogonia and the cells enriched were used for further investigation to evaluate *in vitro* culture conditions.

3.4 *In vitro* culture of monkey germ cells

To evaluate the maintenance of monkey germ cells *in vitro*, donor testis cells enriched for undifferentiated spermatogonia were cultured with several media, at several temperatures, and with several soluble factors for 1 week, then were harvested and counted. The germ cell number was defined as the number of PLZF-positive cells. We cultured donor testis cells with Stempro, mSFM, rSFM, or $2 \times$ rSFM for 1 week to examine the effect of media on germ cell maintenance *in vitro*. After 1 week of culture, the cells were counted and stained with PLZF. Culturing with Stempro, mSFM, rSFM, or $2 \times$ rSFM resulted in $2.9 \pm 0.3 \times 10^4$, $2.2 \pm 0.3 \times 10^4$, $1.7 \pm 0.3 \times 10^4$, and $2.9 \pm 0.2 \times 10^4$ testis cells, respectively (Fig. 4A). Total number of testis cells cultured with Stempro or $2 \times$ rSFM were significantly higher than that with the other media tested. However, the number of germ cells was greater in the cell population cultured with Stempro ($1.9 \pm 0.4 \times 10^4$ germ cells) than with the others (Fig. 4B). Although the number of total cultured cells decreased during the culture period, the proportion of germ cells increased to

65.5% (1.9/2.9) of testis cells in the Stempro culture system compared to that of the initial population (45.8%) for 1 week of cell culture.

To evaluate the effect of temperature on germ cell maintenance *in vitro*, monkey germ cells were analyzed for their *in vitro* growth pattern according to temperature (34 or 37 °C). The total number of testis cells cultured at 37 °C was significantly higher than those at 34 °C following 1 week of culture ($17.0 \pm 2.1 \times 10^3$ vs. $5.3 \pm 0.9 \times 10^3$ cells, respectively; Fig. 4C). In addition, the number of germ cells maintained at 37 °C was more than that under 34 °C condition ($3.2 \pm 0.7 \times 10^3$ vs. $0.5 \pm 0.1 \times 10^3$ cells, respectively; Fig. 4D). Therefore, the following experiments were carried out at a temperature of 37 °C.

Next, we observed the effect of various growth factors that have been reported to be synergistic in the growth of different stem cells [7, 18, 23, 28]. Monkey germ cells were maintained with a single factor or a combination of factors (bFGF, GDNF, LIF, or EGF) and counted after 1 week of culture. The number of testis cells was similar between treatment groups, excluding the treatments of bFGF and GDNF (Fig. 4E). There were significant differences in the number of germ cells in a group of 4 growth factors (Fig. 4F). Taken together, these results indicated that monkey germ cell proliferation was promoted under the culturing condition involving Stempro and the 4 growth factors (bFGF, GDNF, LIF, and EGF) at 37 °C.

4 Discussion

In rodents, undifferentiated spermatogonia are experimentally defined by using specific molecular markers *in vitro* [6, 22, 29–31] or by using a transplantation assay that is able to confirm the undifferentiated spermatogonia cells' functional ability for spermatogenesis *in vivo* [32, 33]. In contrast, little is known about SSCs in other species, especially, information about monkey SSCs is quite insufficient for further studies. Moreover, only a small number of researchers has investigated the properties of SSCs. In this study, we investigated the possibility of representative stemness antigens, including E-cadherin, EP-CAM, Thy-1, SSEA-1, and SSEA-4, being expressed in pre-pubertal monkey testes by FACS analysis, enrichment methods, and altered *in vitro* culture conditions to develop a defined stem cell culturing system.

Previous research has identified several molecules to confirm surface phenotypes of SSCs in rodents [6, 9, 30]. A few molecules used for the characterization of mouse SSCs have also been reported to be expressed in other species [7, 34–36], suggesting the existence of similar molecular characteristics between different species. Although these antigens are highly expressed in mice, there was a

Fig. 3 Immunofluorescence detection of PLZF in pre-pubertal monkey testis cells enriched after differential plating. **A–F** PLZF (green) expression in whole testis (initial), Percoll-selected, differential plating (gelatin, laminin, or combination of gelatin and laminin)-selected populations. The cells were counterstained with DAPI (blue). Scale bar = 50 μ m. **G** The percentages of PLZF-positive cells were 17.2 ± 3.3 , 14.3 ± 0.7 , 25.0 ± 3.4 , 30.5 ± 6.0 , 30.5 ± 1.0 , 45.8 ± 0.7 , 2.6 ± 0.4 , 5.1 ± 0.8 , and $3.7 \pm 0.4\%$ in the initial, Percoll upper, Percoll bottom, unbound (gelatin, laminin, and combination of gelatin and laminin), and bound population (gelatin, laminin, and combination of gelatin and laminin), respectively. Values represent the mean $\pm$ SEM, $n = 3$. Bars with different letters are statistically significant. (Color figure online)

difference in expression when comparing pre-pubertal monkeys to mice. Nevertheless, we confirmed the expression of Thy-1 and SSEA-4 as markers for undifferentiated spermatogonia in the pre-pubertal monkey testes. Furthermore, transplantation analyses showed that FACS-isolated Thy-1-positive and SSEA-4-positive testis cells were enriched for SSCs that have normal stem cell activity to colonize in the recipient mouse's testes. These results demonstrated that undifferentiated monkey spermatogonia have some conserved characteristics in different species, and these results were in agreement with those of previous reports [5, 22].

Previous studies identified that a laminin receptor shows affinity for Sertoli cells during several stages of germ cell development, including SSCs in mouse [30, 37]. However, this study showed that the negative selection by

combination plating using gelatin and laminin resulted in the highest enrichment for undifferentiated spermatogonia. Since in cynomolgus monkeys almost all gonocytes differentiate to spermatogonia, it can be difficult to find gonocytes within the testis 44–57 months after birth [38], the period termed as the pre-pubertal stage. In the mouse, gonocyte migration and differentiation into undifferentiated spermatogonia occurs between post-natal day 0 and 5 [39]. In the bull, gonocyte migration occurs at 4 weeks of age, and differentiation occurs between 8 weeks and 6 months of age [40]. Thus, during this time from 8 weeks to 6 months of age in the bull, the undifferentiated germ cells consist of a heterogeneous population of gonocytes and undifferentiated spermatogonia. On the basis of this timeline, it has been postulated that the SSC conversion and niche assembly occurs within this period of

Fig. 4 Verification of growth efficiency of monkey germ cells by *in vitro* culturing under various conditions. **A, B** Effects of the media on the maintenance of monkey germ cells. Monkey testis cells were cultured in various media, including Stempro, mSFM, rSFM, and $2 \times$ rSFM. The data represent the number of A total testis cells or B germ cells. **B A** significantly higher number of germ cells was observed in a culture group with Stempro ($1.9 \pm 0.4 \times 10^4$). **C, D** Effects of temperatures on *in vitro* culture of monkey germ cells. The number of testis cells and germ cells ($17.0 \pm 2.1 \times 10^3$ cells and $3.2 \pm 0.7 \times 10^3$ cells, respectively) cultured at 37°C were significantly greater than that for cells ($5.3 \pm 0.9 \times 10^3$ cells and $0.5 \pm 0.1 \times 10^3$ cells, respectively) cultured at 34°C . **E, F** Effects of growth factors on the maintenance of monkey germ cells. **F A** significantly higher number of germ cells was observed in the culture condition with all 4 growth factors ($5.0 \pm 1.1 \times 10^3$ cells). Values represent the mean $\pm$ SEM, $n = 3$. Bars with different letters are statistically significant

2–4 months of age [20]. Testes used in this study were obtained from 44- to 57-month-old monkeys, at a time where the spermatogonia appeared likely to have migrated to the basement membrane of the seminiferous tubules and some spermatogonia were settled in the stem cell niche (Fig. 1A). A majority of the germ cells showed a low affinity to laminin. According to this principle, we could provide an effective enrichment tool with this study.

Monkey germ cells were maintained more actively in Stempro medium supplemented with 4 growth factors (bFGF, GDNF, LIF, and EGF) at 37°C temperature than in other serum-free medium, including mSFM and rSFM. Stempro medium is known as a useful medium to maintain mouse and hamster germline stem cells long-term [19, 41]. Although it is unclear what components of Stempro medium enhanced germ cell proliferation for not only mice and hamsters germ cells, but also monkeys, the growth

requirements of monkey germ cells may be similar to those of hamster and mouse germ cells, but not identical.

As described herein, investigating characteristics of SSCs from monkey testes will provide the foundation for understanding functional stem cells and the development of an enrichment technique. In addition, an improved culturing system would clearly have the potential to allow for more advanced research on SSC self-renewal and its relationship with the stem cell niche in various species.

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Compliance with ethical standards

Conflicts of interest The authors have no financial conflicts of interest.

Ethical statement All animal procedures were approved by the Animal Care and Use Committee of Chung-Ang University (IACUC no. 2015-00016) in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and the Guide for Care and Use of Laboratory Animals.

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