Establishment and in vitro culture of porcine spermatogonial germ cells in low temperature culture conditions

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Received 20 February 2013; received in revised form 5 August 2013; accepted 17 August 2013
Available online 24 August 2013

Abstract The objective of this study was to establish a porcine spermatogonial germ cell (pSGC) line and develop an in vitro culture system. Isolated total testicular cells (TTCs) from 5-day-old porcine testes were primary cultured at 31, 34, and 37 °C. Although the time of colony appearance was delayed at 31 °C, strong alkaline phosphatase staining, expressions of pluripotency marker genes such as OCT4, NANOG, and THY1, and the gene expressions of the undifferentiated germ cell markers PLZF and protein gene product 9.5 (PGP9.5) were identified compared to 34 and 37 °C. Cell cycle analysis for both pSGCs and feeder cells at the three temperatures revealed that more pSGCs were in the G2/M phase at 31 °C than 37 °C at the subculture stage. In vitro, pSGCs could stably maintain undifferentiated germ cell and stem cell characteristics for over 60 days during culture at 31 °C. Xenotransplantation of pSGCs to immune deficient mice demonstrated a successful colonization and localization on the seminiferous tubule basement membrane in the recipient testes. In conclusion, pSGCs from neonatal porcine were successfully established and cultured for long periods under a low temperature culture environment in vitro.

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Introduction

Spermatogenesis is a complex process that includes mitosis, meiosis, and morphological changes in germ cells to produce spermatids.
male gametes. Spermatogonial stem cells (SSCs), the male germline stem cells in the testes, are located at the start point of spermatogenesis, and they have the ability to balance self-renewal and unipotency to generate differentiating germ cells capable of simultaneous production of mature sperm (Aponte et al., 2005; Clermont, 1972; Meistrich, 1993; van Pelt et al., 1996). Since the 1956 report of the presence of stem cells in adult testes (Oakberg, 1956), SSCs have been isolated and identified in mice (Nagano et al., 1998), rats (Ryu et al., 2007), porcine (Goel et al., 2007), and humans (Liu et al., 2011). In addition, transplanting isolated mouse SSCs into sterile mice testes results in successful spermatogenesis; thus, isolated SSCs have the ability to generate spermatogenesis in recipients (Brinster and Zimmermann, 1994). Furthermore, the generation of transgenic spermatozoa and animals using foreign gene-introduced SSCs has been intensively studied using in vivo transplantation and in vitro organ culture methods (Nagano et al., 2001; Ryu et al., 2007; Sato et al., 2011).

Besides the production of transgenic spermatozoa and animal production, studies using in vitro maintained spermatogonial stem cells are important to identify the mechanism of spermatogenesis in mammals, facilitate research on male infertility, and disseminate the genetics of animal production. To reach these goals using SSCs, a large number of SSCs must be maintained under in vitro culture conditions. SSCs in mice have been cultured on mitomycin C-treated mouse embryonic fibroblasts (MEFs) and long-term culture (4–5 months) and self-renewal of SSCs have been reported in the presence of added glial cell line-derived neurotrophic factor (GDNF), epidermal growth factor (EGF), and basic fibroblast growth factor 2 (FGF2) in the presence of fetal bovine serum (FBS) (Kanatsu-Shinohara et al., 2003). Furthermore, the same group showed the successful culture of mice SSCs under serum and feeder-free conditions using Stempro-34 medium containing GDNF, FGF2, and EGF (Kanatsu-Shinohara et al., 2011). In addition, porcine SSC-like cells have been isolated and cultured using the same Stempro-34 medium with additional growth factors, although proliferation stopped after nine passages (Kuijik et al., 2009).

Mammalian testicular temperature is maintained constantly lower than that of body temperature. Hyperthermia studies of rat testes have revealed the stage-specific nature of germ cell degeneration (Chowdhury and Steinberger, 1970) and apoptosis of pachytene spermatocytes (Lue et al., 1999; Yin et al., 1997). In addition, culture of human testis tissue fragments at 31 and 37 °C for 22 h demonstrated that DNA synthesis is significantly lower at 37 °C than at 31 °C, although the number of spermatogonia and resting primary spermatocytes is not significantly different (Nakamura et al., 1987). In view of the extended functional changes due to heat stress of germ cells, we hypothesized that the optimal temperature for porcine SSC culture might be an important factor to control proliferation and maintain porcine SSC characteristics in vitro.

Although the specific and accurate species-specific markers for SSC have been disputed, identifying SSC-specific markers is very important to isolate and characterize SSCs. In addition, cell type-specific markers can be used to monitor whether SSCs maintain their characteristics during in vitro culture or whether they have initiated cellular differentiation into functional spermatozoa. Mouse SSCs express Stra8, Neurog3, promyelocytic leukemia zinc finger (PLZF), Ret, and OCT4, but NANOG and teratocarcinoma derived growth factor 1 (TDGF1) are not expressed (Kanatsu-Shinohara et al., 2011). Esr3 is expressed in rat SSCs (Hamra et al., 2004). The lectin Dolichos biflorus agglutinin (DBA) and PGP9.5 (ubiquitin C-terminal hydrolase L-1) are strongly expressed in bull SSCs (Herrid et al., 2007). PGP9.5 has been reported as a marker of porcine spermatogonial cells (Luo et al., 2006), and NANOG is expressed in most DBA and ZBTB16-positive gonocytes in porcine testes, while POU5F1 is not expressed in the same cells (Goel et al., 2008). Although markers for SSCs have been developed by many researchers, the disputations for species-dependent marker genes still remain. However, if porcine spermatogonial cells express these putative marker proteins, these cells could be putative porcine spermatogonial stem cells.

In the present study, we examined the effect of various culture temperatures on porcine SSC colony formation, SSC characteristics, and marker expression to establish the porcine spermatogonial germ cells in vitro culture condition.

Materials and methods

pSGC derivation and in vitro culture

Testes were collected from 5-day-old crossbred piglets (Landrace × Large White Yorkshire) from Sam-Woo breeding farm located in Yang Pyung, Korea. The decapsulated testes were weighed, and five-fold volumes (v/w) of enzyme A containing 0.5 mg/ml type collagenase IV from Clostridium histolyticum (Sigma-Aldrich, St Louis, MO, USA, C5138), 0.01 mg/ml DNAse I (Sigma-Aldrich, DN25), 0.1 mg/ml soybean trypsin inhibitor (Gibco, Carlsbad, CA, USA, 17075-029), and 0.1 mg/ml hyaluronidase (Sigma-Aldrich, H6254) were added for 10 min at room temperature (RT). The testes were washed with phosphate buffered saline (PBS); then the five-fold volume (v/w) of enzyme B in the original testes weighed (5 mg/ml collagenase IV, 0.01 mg/ml DNAse I, and 0.1 mg/ml soybean trypsin inhibitor) was added for 10 min; then, the tissues were washed with PBS at RT. The testes were meshed using 40 μm nylon mesh, and red blood cells (RBCs) were eliminated using RBC lysis buffer (Sigma-Aldrich, R7757). The isolated 2 × 10^5 cells were seeded onto each 0.2% (w/v) gelatin-coated well of 12-well plates and incubated at 31, 34, or 37 °C in 5% CO₂. Stempro-34 medium (Gibco, 10640-019) was used for all processes, including the derivation and culture of porcine SGCs. The medium was modified from that of the previous report (Kanatsu-Shinohara et al., 2011), and supplemented with insulin–transferrin–selenium (ITS; 25 μg/ml, 100 μg/ml, or 30 nM, Gibco, 41400-045), 6 mg/ml glucose (Sigma-Aldrich, G6152), 2 mM l-glutamine (Gibco, 25030), 1% NEAA solution (Gibco, 11140), 1% vitamin solution (Gibco, 11120), 100 U/ml Penicillin/Streptomycin (Gibco, 15140), 1 mM sodium pyruvate (Gibco, 11360), 0.1 mM vitamin C (Sigma-Aldrich, A4403), 1 μg/ml lactic acid (Sigma-Aldrich, L1750), 30 ng/ml estradiol (Sigma-Aldrich, E2758), 60 ng/ml progesterone (Sigma-Aldrich, P7756), 0.2% bovine serum albumin (BSA, Sigma-Aldrich, A4403), 1% knockout serum replacement (Gibco, 10828), 20 ng/ml mEGF (Millipore, Millpore, Billerica, MA, USA, EA140), 10 ng/ml bFGF (Peprotech, Rocky Hill, NJ, USA, AF-100-18B), 10 ng/ml GDNF (R&D...
systems, Minneapolis, MN, USA, 512-GF-010), and $10^3$ U/ml leukemia inhibitory factor (Millipore, EA140). During the subculture when the feeder cells were confluent, the total cells containing porcine spermatogonial germ cell (pSGC) colonies and feeder cells were moved to new gelatin-coated tissue culture plates every 7 or 8 days using 0.005% trypsin-EDTA (Gibco, 25300-054). Then $2 \times 10^6$ cells were seeded onto 0.2% (w/v) each gelatin-coated well of new 12-well plates.

Alkaline phosphatase (AP) staining

AP staining was performed using a CBA-300 AP staining kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer’s instructions. Briefly, pSGCs were fixed in fixation solution, washed three times with PBS, and incubated with AP staining solution for 10–20 min. The AP solution was removed, cells were washed with PBS, and the AP stained cells were observed by microscopy.

Isolation of porcine spermatogonial cells, testicular somatic cells, pSGC colony and feeder cell

Based on a previous study by Kim et al. (2010), porcine spermatogonial cells and testicular somatic cells were separated, that isolated total testicular cells (TTCs) by using the method described in pSGC derivation and in vitro culture section were applied to 20–40% Percoll solution, and centrifuged at 600 x g for 10 min at 4 °C. The cells from the middle layer were moved to a 0.2% gelatin-coated dish and incubated for 3 h. The cells were divided into attached and floating cells for feeder and spermatogonial germ cells, respectively (Kim et al., 2010).

To isolate only the pSGC colony from the polymerase chain reaction (PCR), western blot, immunocytochemistry and cell cycle analysis, pSGC colonies that contained the culture media in the dish were gently aspirated and released several times using 1 ml pipette; subsequently, the supernatant was moved to a 0.2% gelatin-coated dish and incubated for 3 h in order to eliminate gelatin-bound somatic cells. After incubation, the supernatant was moved to a fresh tissue culture dish, and washed three times with PBS. Then the cells were moved to a 0.2% gelatin coated dish and incubated for 3 h. The supernatant was aspirated, and the gelatin-bound cells were washed three times with PBS. In order to eliminate gelatin-unbound cells. Finally, 0.005% trypsin-EDTA was treated, and single feeder cells were washed three times with PBS. The cells were applied for additional analysis.

Reverse transcriptase (RT)-PCR and semiquantitative Real-Time PCR

Total RNA was extracted from the pSGC colony, TTCs of 5-day-old porcine testes, and porcine muscle using Trizol (Life Technologies, Grand Island, NY, USA). The extracted RNA was treated with DNase (New England BioLabs, Ipswich, MA, USA, M0303S) for 30 min and inactivated enzyme activities using 0.2 M EDTA (Sigma-Aldrich, E6635) solution for 10 min. cDNA was synthesized from 1 μg of total RNA using a RT-PCR Premix kit (iNtRON, Seongnam, South Korea, 25081). PCR amplification was performed using 30 cycles for 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. The primer sets used to amplify the genes identified in this study are described in Supplementary Table 1.

Relative levels of OCT4, NANOG, PLZF, and PG9.5 mRNA expressions were estimated in the duplicate samples via fluorescence; further, they were quantified using an iQ Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The reaction was initiated in a total volume of 20 μl containing 10 ng cDNA and 1pM primers in a reaction buffer containing iQ SYBR Green Supermix (Bio-Rad Laboratories, 170-8880). All of the cycle threshold (Ct) values were normalized against β-actin. The results were expressed as a target gene expression relative to a control gene expression. PCR amplification was performed using 40 cycles for 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. The same primer sets were used in real time PCR, as indicated in Supplementary Table 1.

Western blotting

Total protein from pSGC colonies and porcine tissues was isolated using a Proprep kit (iNtRON, 17081) according to the manufacturer’s instructions. Fifty micrograms of protein from each sample was separated by 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, 162-0177). The membranes were blocked in 5% non-fat milk and incubated for 1 h at RT with dilutions of primary antibodies for each protein as follows: anti-rabbit human PGP9.5 (1:5000 dilution; AbD Serotec, Oxford, UK, 7863-0504), anti-rabbit human PLZF (1:2000 dilution; Santa Cruz Biotechnology, SC-22839), anti-mouse human OCT3/4 (1:1000 dilution; Santa Cruz Biotechnology, SC-5279), and anti-mouse chicken β-actin (1:5000 dilution; Santa Cruz Biotechnology, SC-47778). After washing the membranes three times with Tris buffered saline-Tween, goat anti-rabbit IgG-HRP (1:5000 dilution, Santa Cruz Biotechnology, SC-2004) to PG9.5 and PLZF and goat anti-mouse IgG-HRP (1:5000 dilution, Santa Cruz Biotechnology, SC-2031) to OCT3/4 and β-actin were added for 2 h at RT, and protein expression was confirmed using enhanced chemiluminescence.

Histology, immunocytohistochemistry, and histochemistry

Cells and colonies were washed three times with PBS and fixed in 4% paraformaldehyde for 10 min; then the cells and colonies were treated with PBS containing 0.1% Triton X-100 for 10 min in order to permeabilize the membranes as well as to detect different porcine SSC markers. The cells were blocked with 2% BSA-PBS for 30 min at room temperature, and incubated for 1 h at RT with a dilution of each of the following primary antibodies: PG9.5 (1:1000 dilution), PLZF (1:100 dilution), anti-goat human DAZL (1:100 dilution, Santa Cruz Biotechnology, SC-27333), anti-goat mouse GATA4 (1:100 dilution, Santa Cruz Biotechnology, SC-1237), anti-goat mouse luteinizing hormone receptor (LHR, 1:50 dilution; Santa Cruz Biotechnology, SC-26341), and D. biflorus agglutinin (DBA, 1:100 dilution; US Biological, Marblehead, MA, USA, D8085-30). Then, the cells
were washed three times with PBS and incubated for 1 h at RT with Alexa Fluor® 568 Rabbit Anti-Goat IgG (Life Technologies, A-11079) to DAZL, GATA4 and LHR, and Alexa Fluor® 568 Donkey Anti-Rabbit IgG (Life Technologies, A-11042) to PGP9.5 and PLZF. The 4′,6-diamidino-2-phenylindol (DAPI; Sigma-Aldrich, D9542) was added to 1 μg/ml concentration for 10 min in order to identify the nuclei in the samples. Finally, a mounting solution (DAKO, Carpinteria, CA, USA, S3025) was used to fix the pSGCs and colonies.

Bouin’s solution (Sigma-Aldrich, HT10132) was used to fix the testes overnight at 4 °C for immunohistochemistry of the 5- and 180-day-old porcine testes samples. The testes were subsequently washed in 70–100% (v/v) ethanol, embedded in paraffin, sliced into a 6-μm thick sections using a microtome (Thermo, Barrington, IL, USA), and mounted onto glass slides. The nuclei and cytoplasm were stained with hematoxylin and eosin (H&E). To localize PGP9.5 expressing cells in the testes, fixed testes were embedded in a Tissue-Tek optimal cutting temperature (O.C.T.) compound (Sakura Finetechnical, Tokyo, Japan, 4583) and frozen at −70 °C. Samples were cut into a 6-μm thick section at −20 °C and then stained with a 1:1000 dilution of PGP9.5 antibody as well as with Alexa Fluor® 568 Donkey Anti-Rabbit IgG secondary antibody.

**Karyotype analysis**

The pSGCs were incubated with 100 μl of colcemid solution (Irvine Scientific, Santa Ana, CA, USA, 9311) for 3 h at 31 °C, and the cells were treated with 1% citrate. Then, the cells were lysed and fixed in a methanol:glacial acetic acid (3:1) solution. The formation of the G-band was identified on each chromosome.

**Transplantation of in vitro cultured pSGCs into germ cell depleted, immune deficient mice testes**

Busulfan (Sigma-Aldrich, B2635) was used at a concentration of 40 mg/kg to eliminate endogenous testicular cells in 4-week-old recipient BALB/c nude mice (Orient Bio, Seongnam, South Korea). pSGCs in passage 5 (40 days) were isolated and digested with 0.005% trypsin-EDTA and labeled with 32 × 10^6 M PKH26 Red Fluorescent Membrane Linker Dye (Sigma-Aldrich, P9691) for 5 min. Then, PKH26 stained donor cells were washed three times with Dulbecco’s Modified Essential Medium (DMEM) and resuspended in DMEM containing 10% fetal bovine serum (FBS) and 0.001% Trypan blue was used as an indicator of the injection into recipient seminiferous tubules. An aliquot of 1 × 10^5 cells/10 μl PKH26 labeled donor cells was injected into each recipient testis, which resulted in up to 80% filling of the recipient seminiferous tubules. To clarify whether unbound PKH26 dye can bind to endogenous cells in seminiferous tubules treated with busulfan, the exact same concentration of free PKH26 dye, 32 × 10^6 M, was injected to the busulfan-treated mouse testes. Both pSGCs and free PKH26 dye injected recipient testes were recovered 8 weeks after transplantation and PKH26 positive colonies and localization of pSGCs in seminiferous tubules were detected by fluorescence microscopy.

**Statistical analysis**

One-way ANOVA was performed for the calculation of colony numbers and real time PCR results using GraphPad Prism 4® (La Jolla, GA, USA) for Windows XP; Tukey’s multiple comparison test was used for making comparisons among groups. Cell proliferation rate was analyzed using an unpaired t-test with Welch’s correction analysis of variance. All data from the figures are expressed as means ± standard deviation (s.d). The null hypothesis was rejected when the probability was P < 0.05.

**Results**

**Histological comparison between 5- and 180-day-old porcine testes**

Five- and 180-day-old porcine testes were compared and analyzed to identify the SGC population using H&E staining and immunohistochemistry (Fig. 1). The 5-day-old porcine testes showed full formation of seminiferous tubules. However, the tubules had not fully matured, and the interstitial area was wider than the seminiferous cord area (Fig. 1A). In the 180-day-old testes, the seminiferous tubules were fully developed and also contained many developed germ cell types, including spermatogonial cells, spermatocytes, spermatids, and elongated spermatids (Fig. 1A). To identify the location and population of spermatogonial cells, PGP9.5 expression was assessed in 5- and 180-day-old porcine testes using cryopreserved testis sections. PGP9.5 expressing cells were near the lumen of the seminiferous cord in neonatal porcine testes. However, the PGP9.5 expressing germ cells were near the basement membrane of the tubule (Fig. 1B). Based on these data, 5-day-old testes were selected to further derive male germ cells because testes of this age contained a limited number of other germ cell types and furthermore, most germ cells were PGP9.5 positive.

**Isolation of porcine TTCs and identification of spermatogonial specific marker expressing cells among all cells**

TTCs were isolated from 5-day-old testes and immunocytochemistry was performed using antibodies for germ cells and...
spermatogonial specific marker proteins to identify the spermatogonia. Among the cells, PGP9.5 was expressed strongly in the cytoplasm of germ cells, and the size of these cells was larger than that of the PGP9.5 negative cells (Fig. 2). Some putative germ cells also expressed PLZF and DAZL in the nucleus, which are pre-meiotic germ cell

**Figure 1**  Histological comparison of 5- and 180-day-old porcine testes. (A) Hematoxylin and eosin staining. SG, spermatogonia; STC, spermatocyte; ESTD, elongated spermatid; and SC, Sertoli cell. (B) Immunolocalization of the PGP9.5 protein. Left panel shows the expression of PGP9.5 protein, middle panel demonstrates location of nuclei stained by DAPI, and right panel shows the merged images of both PGP9.5 expression and nuclei. Arrows indicate porcine spermatogonial stem cells.
Figure 2  Immunocytochemistry of specific markers for spermatogonial germ cells and somatic cells. TTCs were freshly isolated from 5-day-old porcine testes without a specific separation process and cultured in vitro. PGP9.5, PLZF, and DAZL are undifferentiated spermatogonial germ cell markers. GATA4 and LHR are Sertoli and Leydig cell markers, respectively. The left panel shows the expression of specific marker proteins, the middle panel demonstrates the location of the nuclei stained by DAPI, and the right panel illustrates the merged images. Arrows indicate localization of marker genes with the nucleus.
markers; the size of the PLZF and DAZL positive cells was similar to that of the PGP9.5 positive cells (Fig. 2). The population of these three marker expressing cells showed a similar ratio in all testicular cells. In addition, GATA4 and LHR (Sertoli and Leydig cell markers, respectively) protein expression was observed in a variety of other cells besides germ cells (Fig. 2).

Derivation of pSGC at different temperatures

The isolated porcine spermatogonial cells were seeded on mitomycin C treated mouse embryonic fibroblast (MEF), STO, JK1 and porcine testicular somatic cells. The TTCs contained spermatogonial and somatic cells, which were also seeded on a gelatin-coated cell culture dish. Although pSGC colonies were not detected on mitomycin C treated feeder cells, pSGC colonies appeared in the total testicular cell (TTC) culture. Based on this result, TTCs containing spermatogonial germ cells were used for pSGC derivation. Three different temperatures were tested to identify morphology and colony formation for the first 7 days. Colony formation (>50 μm) was observed after 3, 4, and 5 days of culture at 31, 34 and 37 °C, respectively (Figs. 3A and B). Colonies were round and more compactly shaped at 31 °C than at 37 °C (Fig. 3A). After 5 days at 37 °C and 7 days at 34 °C, the colonies became amorphous in shape; at 31 °C, the colonies remained round and compact (Fig. 3A). Total cell growth rate and the number of colonies at different temperatures showed that cell number doubled at days 3, 4, and 5.5 at 37, 34 and 31 °C, respectively, and cell growth was faster at 37 °C than at other temperatures (Fig. 3B). Interestingly, the colonies first appeared at the time the cell number doubled (Figs. 3A and B). A significant increase in colony number was observed at 34 and 31 °C after the first colony appeared, but the number of colonies did not increase until day 7 at 37 °C (Fig. 3C). After the passages, the pSGC colonies did not appear at 37 and 34 °C; however, pSGCs were re-colonized at the 31 °C culture condition. The pSGC colonies were maintained at 31 °C over 8 passages with compact and round morphology, and the numbers of colonies were also increased.

A cell cycle analysis of both pSGC and feeder cells was performed on the day the first colony appeared as well as on the day of subculture in order to identify the possible reasons for different temperature effects on pSGC colony formation. Following the purified method mentioned in Materials and method section Isolation of porcine spermatogonial cells, testicular somatic cells, pSGC colony and feeder cell, the successful isolation of the pSGC colonies was determined by gentle aspiration and releasing using 1 ml pipette, however a few single cells, whether these cells were from the feeder cells or colonies, were also identified (Supplementary Fig. 1A). Subsequent gelatin negative selection of pSGC colonies to restrict the feeder cell contamination showed that most of the isolated pSGCs were positive to anti-PGP9.5 antibody by immunocytochemistry (Supplementary Fig. 1B).

The days involved with the first colony formation at 31, 34, and 37 °C were days 5, 4, and 3, respectively; the days of subculture at 31, 34, and 37 °C were days 8, 6, and 4, respectively (Fig. 4A). At the colony formation stage, the percentage of the G2/M phase of both pSGCs and feeder cells at 37 °C was significantly lower than 31 and 34 °C, whereas no significance was observed between 31 and 34 °C (Fig. 4B). The percentage of the G0/G1 phase of feeder cells at 37 °C was significantly increased compared to the 31 and 34 °C at the colony formation stage (Fig. 4B). The percentage of the G0/G1 phase of pSGCs showed no significance among the temperatures, whereas the percentage of the G0/G1 phase of feeder cells at 37 °C was significantly higher than 31 and 34 °C at the time of subculture (Fig. 4B). The G2/M phase cells of both pSGCs and feeder cells at 37 °C were significantly lower than 31 °C, and G2/M phase pSGCs in 34 °C located in between 31 and 37 °C (Fig. 4B). To assess the proliferation differences between pSGCs and somatic feeder cells at the three different temperatures, cell proliferation assay was performed. The proliferation of feeder cells in 34 and 37 °C was significantly higher than that in 31 °C (Fig. 4C). However, the proliferation of pSGCs did not show a significant difference among the temperatures (Fig. 4C).

Characterization of the pSGCs at the three different temperatures

AP staining was performed to identify the stem cell characteristics and compare the established pSGCs from different temperatures. At day 7 of culture, all three pSGC cultures were positive for AP staining although AP staining intensity was different among temperatures; AP was strongly, intermediately, and weakly stained at 31, 34, and 37 °C, respectively (Fig. 5A). In addition to identifying stem cell marker expression in the pSGCs at the three different culture conditions, RT-PCR was performed using primer sets for PGP9.5, PLZF, GATA4, OCT4, and NANOG. PGP9.5 and PLZF mRNA expression was relatively higher at 31 than at 34 and 37 °C (Fig. 5B). Expression of GATA4 and LHR was not observed in any of the colonies from the three different culture conditions (Fig. 5B). Among the tested markers, OCT4 and NANOG mRNA were strongly expressed at 31 °C, whereas minimal or null expression was observed at 34 and 37 °C (Fig. 5B). To obtain a quantitative mRNA expression data, real-time PCR was performed. Relative levels of mRNA expressions of OCT4, NANOG, PLZF and PGP9.5 among the three different temperatures along with TTCs were compared; expressions of OCT4, NANOG, PLZF and PGP9.5 were significantly increased in the 31 °C culture environment (Fig. 5C). Western immunoblots were performed with anti-rabbit human PGP9.5 and PLZF and anti-mouse human OCT4. Because of a lack of cross-reactivity between human and porcine proteins, the expression of other marker protein antibodies could not be used in this study. The immunoblots also showed strong expression of PGP9.5, PLZF, and OCT4 in colonies maintained at 31 °C (Fig. 5D). Cells from the 31 °C culture were selected for further characterization after determining stem cell marker expression.

Characterization of long-term cultured pSGCs

Although the proliferation of pSGCs was slower at 31 °C than that at 34 and 37 °C, PGP9.5, PLZF, NANOG, and OCT4 were strongly expressed (Figs. 5B, C and D). To determine whether this marker gene expression could be maintained for a longer culture period, stem cell marker gene expression in 1 and 8 passage cultured pSGCs was compared to mRNA extracted from porcine muscle and TTCs from 5-day-old testes. As shown
in Fig. 6A, PGP9.5, PLZF, kit, OCT4, NANOG, and THY1 expression was identified in both SGCs and TTCs regardless of passage (Fig. 6A). In addition, PGP9.5, PLZF, OCT4, NANOG, and THY1 were expressed at a relatively stronger intensity than that of TTCs at both passages 1 and 8 (60 days of culture), whereas weaker expression was evident in SGCs than in total testicular cells (Fig. 6A). The expression of myosin heavy chain (MYH) 1, a muscle specific marker, only appeared in mRNA extracted from muscle (Fig. 6A). In support of these RT-PCR data, Western immunoblots for PGP9.5, PLZF, and OCT4 in total protein extracted from pSGCs at passage 8 showed that these proteins were still expressed in cells from the longer culture at 31 °C (Fig. 6B). In addition, immunocytochemistry for PGP9.5 and DBA in colonies from passage 8 also showed expression of these proteins in pSGC colonies, whereas no expression of GATA4 and LHR was found (Fig. 6C). In addition, the results of karyotyping analysis indicated that in vitro cultured pSGCs exhibited 38 XY normal chromosomes (Fig. 6D).
Figure 4  Cell cycle analysis of both porcine spermatogonial germ cell (pSGC) colonies and feeder cells at the time the first colony appeared and was subcultured. (A) Days 5, 4, and 3 are the times the first colony appeared, and days 8, 6, and 4 are the times of pSGC subculture at 31, 34 and 37 °C, respectively. M1: G0/G1, M2: S, M3: G2/M. (B) Statistical analysis from five different cell cycle analyses. (C) Cell proliferation assay of both pSGCs and testicular somatic cells. These experiments were repeated five times, and the results were expressed as the means ± s.d. P < 0.05 was considered as being statistically significant.
Transplantation of in vitro cultured pSGCs into immune deficient mice testes

PKH26 stained cultured pSGCs were transplanted into the testes of busulfan-treated immune deficient mice that had been depleted of both testicular germ cells and somatic cells to identify the pSGC spermatogonia-like potential in recipient testes. Transplanted pSGCs colonized the recipient testes at 8 weeks post-transplantation (Fig. 7A left), and PKH26 stained pSGCs were present in the seminiferous tubules of recipient testes (Fig. 7A center). Moreover, an alignment formation of the pSGCs was also identified in the sections of the testes showed that the transplanted pSGCs had successfully migrated and settled on the basement of the tubules (Fig. 7A Right). Injection of PKH26 dye only to busulfan-treated immune deficient mice did not show any endogenous cell-bound fluorescent sensitivities in the whole testes and seminiferous tubules (Fig. 7B).

Discussion

Among testicular germ cells, A single (As) spermatogonia can be differentiated as A paired (Apr) and A aligned (Aal) spermatogonia, and these cells are referred to as SSCs (de Rooij and Russell, 2000). The SSC population accounts for <4% of all spermatogenic cells in adult testes (Meachem et al., 2001). Furthermore, the very few SSCs in adult testes and the lack of information on specific markers for SSCs in different species have made it difficult to prepare pure populations of SSCs (Tegelenbosch and de Rooij, 1993). However, prior to onset of spermatogenic differentiation in neonatal and prepubertal testes, the seminiferous tubules contain a relatively larger number of SSCs. Therefore, enrichment of SSCs from immature testes is more efficient than that from adult testes. The histological comparison between 5- and 180-day-old testes showed that spermatogonia were the unique germ cells in 5-day-old porcine testes, whereas various stages of differentiating spermatogenic germ cells were identified in 180-day-old porcine testes (Fig. 1A). In addition, expression of the porcine spermatogonial cell marker, PGP9.5, was also observed in 5-day-old porcine testes with high frequency (Fig. 1B). Therefore, 5-day-old porcine testes should be an optimal source for preparing SGCs to study these cells and spermatogenesis.

In this study, localization of the SSC population was determined by PGP 9.5 expression. PGP 9.5, also called ubiquitin C-terminal hydrolase L-1, is expressed in neuronal cells and testes, and specific expression of PGP9.5 was identified in spermatogonia of day 8 and 16 mice, and PGP9.5 expression has also been identified in Sertoli cells of adult mice (Kon et al., 1999). PGP9.5 expression has also been reported in cattle (Herrid et al., 2007; Wrobel et al., 1996), monkeys (Tokunaga et al., 1999), and porcine (Luo et al., 2006), whereas its expression was localized to gonocytes and spermatogonia. Based on these findings, PGP9.5 was used as a marker for identifying porcine spermatogonia in 5-day-old porcine testes; only spermatogonia were successfully identified, suggesting that PGP9.5 can be used as a marker of SSCs in porcine.

Mammalian testicular temperature is lower than body temperatures to protect spermatogonial cells from heat damage. In this study, three different culture temperatures of 31, 34, and 37 °C were applied to derive pSGCs. The different culture temperatures showed comparable pSGC colony formation, and mRNA and protein expression (Figs. 3–5). Among the three conditions, AP strongly stained cells at 31 °C rather than at 34 and 37 °C, and more intensive PGP9.5 mRNA expression was also detected at 31 °C than that at other temperatures. Interestingly, the pre-meiotic marker, PLZF, and the pluripotency markers, OCT4 and NANOG mRNA, were also strongly expressed at 31 °C than those at 34 and 37 °C. These data suggest that the lower temperature for pSGC derivation and culture may provide an optimal environment to maintain spermatogonial characteristics. In support of this hypothesis, when mice spermatogenic cells were cultured under a 37 °C condition, the expression of many proliferation and differentiation-related genes was inhibited and c-kit gene mutation was identified, whereas mRNA and protein expression levels of c-kit and PI3-kinase in spermatogonial cells increased at a 32 °C culture temperature compared to those at 37 °C (Zhu et al., 2012). In addition, cultures of human testicular tissue fragments also showed decreased DNA synthesis at 37 °C compared to that at 31 °C (Nakamura et al., 1987).

Besides the molecular characteristics, morphological differences were also compared among the cultures at different temperatures. Round and embryonic body shaped colonies that appeared on day 5, and their morphology were maintained for a longer period at 31 °C. However, the morphology of the round-shaped colonies on day 3 changed dramatically at 37 °C and the degree of morphological changes in the colonies at 34 °C was intermediate between that at 31 and 37 °C. In addition, the number of colonies increased under the 31 and 34 °C culture conditions, but no increase in colony number was observed at 37 °C. These data clearly demonstrate that an appropriate culture temperature is important to maintain pSGC colony formation and to increase the number of colonies.

A cell cycle analysis was performed with both pSGC and feeder cells from cells cultured at the three different temperatures to determine the reason for the different gene expression levels and morphological changes in pSGC. At the time initial colonies appeared, a significant number of pSGCs at 31 and 34 °C were in the G2/M phase than those at 37 °C; however, a significant number of pSGCs were in the G2/M phase at 31 °C than that at 37 °C at the subculture stage. These results indicate that pSGCs grew slowly at 31 °C but that the ability of DNA synthesis for proliferation was maintained over the long term. Culturing at a reduced temperature during the early stage of mesenchymal stem cell culture is more beneficial than culturing at 37 °C, as anti-apoptotic heat shock proteins are upregulated, pro-apoptotic proteins are downregulated, and the levels of reactive oxide species, nitric oxide, and lipofuscin decrease (Stolzing and Scutt, 2006; Stolzing et al., 2006). In MEB5 neural stem cell cultures, the apoptotic cell population is significantly lower at 32 °C than that at 37 °C in the absence of EGF (Saito et al., 2010). In Chinese hamster ovary cells, culture temperatures <37 °C suppress cell growth but cell viability remains high for long-term culture (Yoon et al., 2003). Presently, cell cycle arrest was prevented under the 31 °C culture condition, suggesting that a low temperature is useful and beneficial to derive and maintain pSGCs in vitro.

OCT4, NANOG, and THY1 are referred to as stem cell pluripotency markers. NANOG is expressed in porcine male
germ cells, but OCT4 is not expressed in gonocytes of neonatal testes (Goel et al., 2008). However, OCT4 expression is detected in 2 week cultures of PGP9.5 positive porcine spermatogonia from 10-week-old testes (Luo et al., 2006). Nevertheless, OCT4 expression in mouse gonocytes and undifferentiated SSCs has been reported (Pesce et al., 1998; Tadokoro et al., 2002), and the expression of OCT4 is essential to maintain mice spermatogonial stem cells in vitro and retain their self-renewal property (Dann et al., 2008). OCT4 and NANOG expression has also been detected in in vitro cultured bovine male germ cells, and transplantation of these cells results in stable colonization in the testes of recipient immune

A

31°C  34°C  37°C

B

PGP9.5  PLZF  GATA4  LHR  OCT4  NANOG  GAPDH

C

OCT4  NANOG

D

PLZF  PGP9.5  OCT4  Beta-actin
deficient mice (Fujihara et al., 2011). THY1 expression has been reported in undifferentiated spermatagonia and SSCs in rodent and non-human primates. In addition, THY1 positive human male germ cells that express OCT4 and NANOG, and stained with AP are maintained in vitro for 1 week, and transplanting these cells into immune deficient nude mice does not cause tumor formation over at least 6 months (Kobayashi et al., 2009). THY1 positive cells reportedly express VASA and the spermatogonial specific transcription factor PLZF in bovine male germ cells (Reding et al., 2010). Taken together, these results suggest that the expression of the pluripotency markers OCT4, NANOG, and THY1 is required to maintain spermatogonial germ cell in vitro, and that the porcine male germ cell markers are very conserved with other species but that the expression patterns of these markers need to be elucidated at specific spermatogenesis developmental stages. In the present study, pSGCs cultured at 31 °C expressed PGP9.5, PLZF, OCT4 and NANOG; however, pSGCs cultured at 34 and 37 °C expressed only PGP9.5 and PLZF, suggesting that the 31 °C culture condition may be an optimal environment to maintain porcine spermatogonial germ cells.

Unlike mouse SSC cultured in vitro, long-term culture of porcine male germ cells has not been successfully conducted (Goel et al., 2008; Luo et al., 2006), and in vitro cultured porcine male germ cells have been maintained for only up to 1–3 weeks. However, the present study showed stable maintenance of colonized pSGCs expressing both SSC and pluripotency marker genes over 60 days at 31 °C. Furthermore, long-term cultured pSGCs bound lectin DBA (Fig. 6C). Lectin DBA binding to germ cells is an indicator of the presence of germ cells (Goel et al., 2007) and germ cells with strong DBA binding are more primitive than those that weakly bind. Therefore, our results suggest that the low temperature culture condition may not only be suitable for the expression of pSGC molecular markers but is also important to maintain stem cell characteristics.

Stem cells are typically cultured on feeder cells. We tried to derive and culture our SSCs on MEFs; however, no germ cell colonies were detected on MEFs at the three temperature conditions. pSGC colonies appeared only on porcine testicular somatic cells in Stempro 34 medium, suggesting that pSGCs may require signals from CD34 positive testicular cells for proliferation and maintenance of stem cell characteristics. Supporting this hypothesis, CD34 positive testicular stromal cells support long-term expansion of adult spermatogonial and progenitor cells (Kim et al., 2008) and spermatogonial stem cells can be proliferated and remain undifferentiated on bone marrow stromal cells (Xu et al., 2006). Additionally, bovine male germ cells have also been successfully cultured in vitro for 1.5 months on bovine testicular somatic cells as a feeder, based on an unknown factor from the testicular somatic cells (Fujihara et al., 2011). In the present study, pSGCs were cultured over 60 days with maintaining of both pluripotency and undifferentiated germ cell marker gene expressions. However detachment of pSGC colonies from the feeder cells and reduced colony formation after subculture was observed over 70 days, and cultures were eventually limited. This may be due to the aging of feeder cells, and the aged feeder cells could not provide sufficient survival environment for pSGCs. Although the biochemical and cellular characteristics in between freshly isolated feeder cells and aged feeder cells have not been compared in this study, aged niches inhibited cell proliferation and initiated the loss of stemness marker gene expression in human embryonic and muscle stem cells (Carlson and Conboy, 2007). Therefore, identification and development of suitable feeder cell characteristics for pSGCs will be important to maintain undifferentiated spermatogonial germ cells for long-term culture.

As male germ line stem cells can colonize recipient mouse testes (Brinster and Zimmermann, 1994), transplantation of isolated male germ cells into recipient mouse testes has been performed in various animal species including rodents and large animals (Clouthier et al., 1996; Dobrinski et al., 1999, 2000; Izadyar et al., 2003; Nagano et al., 2001; Ogawa et al., 1999). Although non-rodent derived donor germ cells cannot complete spermatogenesis in recipient testes, colonization, localization to the basement membrane, and germ cell proliferation are conserved among different species (Dobrinski, 2005). Therefore, xenotransplantation of spermatogonial germ cells provides a valuable bioassay for stem cell potential of isolated and in vitro cultured germ cells (Dobrinski, 2005; Dobrinski et al., 1999, 2000; Fujihara et al., 2011; Izadyar et al., 2003). In agreement with these previous studies, transplanted pSGCs in the present study also formed colonies and localized to the basement membrane in recipient seminiferous tubules. Therefore, the combination of the xenotransplantation results and the expression of pluripotency stem cell markers in the cultured pSGCs data strongly suggest that the pSGCs developed in this study have early stage spermatogonial and stem cell characteristics.

In the current study, the PKH26 fluorescent cell linker was used to track the transplanted pSGCs. PKH26 stably incorporates a yellow-orange fluorescent dye into the lipid regions of the cell membrane (Wallace et al., 2008). PKH26-labeled cells have been successfully identified in tissue subjected to standard paraffin embedding and sectioning; yet, such methods risk the loss of intensity by utilizing clearing agents that may partially extract membrane lipid and lipophilic dyes (Wallace et al., 2008). Histological studies regarding tissues containing cells labeled with lipophilic membrane dyes have typically been carried out on frozen sections. In this study, the recipient mouse testes transplanted PHK26 labeling pSGCs was sectioned in frozen. The PKH26 signal became dilute after several cell divisions; however, the pSGC proliferation rate was

![Figure 5](image-url) Characterization of porcine spermatogonial germ cell (pSGC) under different temperature culture conditions at the time of subculture. (A) Alkaline phosphatase (AP) staining of pSGC colonies. (B) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of pSGCs with undifferentiated spermatogonial germ cell markers (PGP9.5 and PLZF), pluripotent markers (OCT4 and NANOG), Sertoli cell marker (GATA4), and Leydig cell marker (luteinizing hormone receptor, LHR). GAPDH was used as the loading control. (C) Real time RT-PCR analysis of pSGCs from three different culture conditions. 5-Day testes indicate TTCs from 5-day porcine testes. Results were expressed as the means ± s.d. P < 0.05 was considered as being statistically significant. TTC: total testicular cells. (D) Western immunoblot analysis of pSGCs using PLZF, PGP9.5, and OCT4 antibodies. Beta-actin was used as the protein loading control. RT-PCR, real time RT-PCR and western immunoblot were repeated five times, with similar results in each instance.
not fast in the testes of the recipient mouse. Consequently the fluorescent sensitivity of PKH26 might be observed 8 weeks after transplantation. Therefore, the PKH26 is a useful tool for in vivo cell tracking studies, particularly when the labeled cells are to be followed for periods longer than a few weeks (Rieck, 2003). In addition, the injected free PKH26 dye did not incorporate into the endogenous cells; only transplanted cells showed PKH26 fluorescence. This data suggests that injected free PKH26 may flow out from the seminiferous tubules before cells in the seminiferous tubules are recovered from busulfan treatment; hence, non-specific incorporation of free PKH26 to the endogenous cells might be ruled out with this system.

Figure 7  Transplantation of red fluorescence labeled porcine spermatogonial germ cell (pSGC) into seminiferous tubules of germ cell-depleted immune deficient mice. Donor pSGCs from passage 4 (30 days in culture) were labeled with PKH26, and injected into busulfan-treated recipient nude mice. The testes were recovered after 2 months, and the location of the pSGCs was determined in (A, left) whole testes and (A, center) seminiferous tubules. (A, right) Frozen-dissected recipient testes were analyzed to determine the location of pSGCs in the seminiferous tubule. Asterisks indicate PKH26-labeled pSGCs in the seminiferous tubules. (B) Only PKH26 dye was injected into recipient mouse testes, and recovered after 8 weeks.

Figure 6  Characterization of long-term cultured porcine spermatogonial germ cells (pSGCs) under the 31 °C culture condition. (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of pSGCs from passage 1 and passage 8 (60 days old), porcine muscle, and TTCs from 5-day-old testes with undifferentiated spermatogonial germ cell markers (PGP9.5 and PLZF), pluripotent markers (OCT4, NANOG, and THY1), and germ cell marker (kit). Myosin heavy chain (Myh) 1 was used as the negative control, and GAPDH was used as the loading control. (B) Western immunoblot analysis of pSGCs from passage 8 compared with cells from muscle, TTCs from 5-day-old testes using PLZF, PGP9.5, and OCT4 antibodies. Beta-actin was used as the protein loading control. (C) Immunocytochemistry of pSGC colonies from passage 8 with PGP9.5, luteinizing hormone receptor (LHR) and GATA4 antibodies. The lectin-DBA binding assay was performed to identify cells with primitive characteristics. The left panel shows expression of specific marker proteins, the middle panel demonstrates the location of nuclei stained by DAPI, and the right panel shows the merged images of both markers and nuclei. (D) Karyotype of pSGCs from the passage 8 culture. TTC: total testicular cells. RT-PCR and western immunoblot were repeated five times, with similar results in each instance.
Conclusions

Porcine spermatogonial germ cells were successfully established and cultured in vitro under low temperature conditions (31°C) in Stempro 34 medium. Among the three different temperature culture conditions (31, 34 and 37°C), pSGC showed strong AP staining and stable expression of OCT4, NANOG, THY1, and SSC markers, as well as PGP9.5 and PLZF under the 31°C culture condition. The in vitro long-term culture study revealed that the morphology of pSGC and the expression of markers did not change over 60 days, and strong lectin DBA binding was also detected. Transplantation of pSGCs to the testes of immune deficient mice revealed the migration of pSGCs to the seminiferous tubule basement membrane. Colony formation and alignment of pSGCs were also detected. This achievement is a key advancement for a clinical approach to male infertility, production of transgenic animals mediated male germ cells, and studying spermatogenic meiosis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2013.08.008.

Acknowledgment

This study was supported by grant PJ009535 from the Next-Generation BioGreen 21 Program, Rural Development Administration, Republic of Korea.

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