Asian-Aust. J. Anim. Sci.
Vol. 22, No. 2 : 187 - 193
February 2009

www.ajas.info

# Isolation and *In vitro* Culture of Pig Spermatogonial Stem Cell*

**Su Young Han, Mukesh Kumar Gupta, Sang Jun Uhm and Hoon Taek Lee****
Department of Animal Biotechnology, Bio-Organ Research Center, Konkuk University
1, Hwayang-dong, Gwangjin-gu, Seoul 143701, Korea

**ABSTRACT :** The present study identified the favorable conditions for isolation, enrichment and *in vitro* culture of highly purified, undifferentiated pig spermatogonial stem cell (SSC) lines that proliferate for long periods of time in culture. The colonies displayed morphology similar to miceSSC and were positive for markers of SSC (PGP9.5), proliferating germ cell (PigVASA), pre-meiotic germ cell (DAZL) and pluripotency (OCT4, SSEA-1, NANOG, and SOX2) based on immuno-cytochemistry and RT-PCR. The purity of these colonies was confirmed by negative expression of markers for sertoli cell (GATA4 and SOX9), peritubular myoid cell (α-SMA), differentiating spermatogonial and germ cells (c-KIT). The colonies could be maintained with undifferentiated morphology for more than two months and passaged more than 8 times with doubling time between 6-7 days. Taken together, we conclude that pigSSC could be successfully isolated and cultured *in vitro* and they possess characteristics similar to miceSSC. (**Key Words :** Spermatogonial Stem Cell, SSC, Pluripotency, Long Term Culture, Pig)

## INTRODUCTION

Spermatogonial stem cells (SSC) are small self-renewing subpopulation of type A spermatogonia found in the basal compartment of seminiferous tubules. They form the foundation of spermatogenesis and are required for the continuous production of sperm through a balance between SSC self-renewal and differentiation in adult testis (Hofmann, 2008). When transplanted into the seminiferous tubules of an infertile male, they can establish donor-derived spermatogenesis and produce spermatozoa that transmit the donor haplotype to progeny (Dobrinski, 2006). In addition, when cultured in the appropriate conditions, they can acquire pluripotency and differentiate into derivatives of the three embryonic germ layers (Guan et al., 2006) including sperm (Hong et al., 2004). Development of a method for isolation and *in vitro* culture of SSC that can maintain self-renewal or can differentiate into germ cells therefore, provides a uniquely valuable approach for the study, preservation and manipulation of male fertility and for tissue regeneration in mammalian species. The ability to genetically manipulate, and transplant these SSC further provides an unique opportunity to modify the germline and therefore, has tremendous potential for transgenesis in species wherein embryonic stem cells are not available and somatic cell nuclear transfer and reprogramming pose several problems (Dobrinski and Travis, 2007).

In recent years, much attention has been paid to isolate, cultivate and maintain the SSC *in vitro*. First pioneered in rodents, SSC has now been isolated in several mammalian species including primates (Kanatsu-Shinohara et al., 2008b; Guan et al., 2006; Dobrinski, 2006; Ryu et al., 2005). However, culture method that effectively promotes the *in vitro* proliferation of SSC has not been well established for pigs, despite their increasing importance as a model species for biomedical researches, medicine, industry, and xenotransplantation. Dirami et al. (1999) could isolate purified type A spermatogonia in pigs but ~50-70% of them lost viability within 120 h of *in vitro* culture. More recently, Goel et al. (2007) showed the *in vitro* culture of gonocytes, a primitive germ cell (PGC), for up to 7 days. These cells formed focal three dimensional colonies and expressed SSEA-1 protein which is a marker for embryonic stem (ES) cells. However, these colonies were not maintained in culture for longer period of time. Luo et al. (2006) established a method of enriching spermatogonial cells using protein gene product 9.5 (PGP9.5) as a marker. The PGP9.5 enriched cells contained SSC and could be maintained in culture for two weeks without losing their

* This work was supported by grants from Biogreen21, RDA, Korea.

* Corresponding Author: Hoon Taek Lee. Tel: +82-2-4503675, Fax: +82-2-4578488, E-mail: htl3675@konkuk.ac.kr

Received June 4, 2008; Accepted August 23, 2008

PGP9.5 expression. However, the first report of porcineSSC probably appeared in year 2006 wherein Cheng and Feng (2006) successfully isolated SSC in minipigs. But these minipigSSC could also not be maintained in culture for more than 10 days.

Here, we describe a simple and reproducible protocol for the derivation and maintenance of pigSSC cell lines that proliferate for long periods of time in culture. We isolated primary cultures of pigSSC cell lines from freshly isolated testicular tubules and studied the formation of cell colonies and their capacity to express SSC and pluripotent markers. These SSC are now available for further study and *in vitro* manipulation.

## MATERIALS AND METHODS

All chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise specifically indicated.

### Animals and collection of testes

All animal procedures were approved and performed under the guidelines of the Konkuk University Animal Care and Experimentation Committee. Neonatal testes were collected from 5-10 days-old crossbred (Landrace×Duroc× Yorkshire) piglets in a local pig farm and transported to the laboratory, within 2 h of collection, on ice in Dulbecco's modified Eagle medium (DMEM; GibcoBRL, Grand Island, NY) supplemented with 1% penicillin-streptomycin (GibcoBRL).

### Cell preparation and enrichment

Upon arrival of testes to laboratory, they were washed several times with calcium- and magnesium- free Dulbecco's phosphate buffered saline (DPBS; GibcoBRL). Tunica albuginea and visible connective tissues were then removed and seminiferous tubules were mechanically dissociated using scissors and forceps. Single cell suspensions were then prepared either by sequential enzymatic digestion (Nagano et al., 1998) or by a non-enzymatic mechanical method. For enzymatic digestion method, dissociated seminiferous tubules were sequentially incubated at 37°C with collagenase (1 mg/ml, Type IV) for 15 min followed by hyaluronidase (1 mg/ml) for 10 min. Tissues were then washed two times with DMEM medium supplemented with 0.25% (w/v) trypsin and 1 mM EDTA (GibcoBRL) for 5 min at 37°C. The dispersed cells were then washed twice in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS; GibcoBRL) to stop the enzymatic digestion and were filtered through 70 μ and 40 μ nylon meshes to remove the myoid and sertoli cells. For non-enzymatic mechanical method, seminiferous tubular cells were dissociated by repeated vigorous pipetting and passing through a 10 ml hypodermic syringe fitted with a 22G needle. The cells were then filtered through 70 μ and 40 μ nylon meshes and incubated overnight at 37°C in DMEM supplemented with 10% (v/v) FBS in humidified atmosphere of 5% $CO_2$ in air. At the end of incubation period, cells settled at the bottom of culture dishes were collected for further culture. Total cell number counting and cell viability were determined by mixing the samples 1:1 with 0.4% Trypan Blue and counting live (Trypan Blue excluding) and dead cells with a hemocytometer.

Enrichment of SSC was done either by discontinuous Percoll density gradient (van Pelt et al., 1996) or differential plating method (Rodriguez-Sosa et al., 2006). For Percoll density gradient method, 2 ml of cell suspension containing ~$1\times10^7$ cells was layered on the top of 15, 30, 45, and 60% Percoll gradients and centrifuged at 800 g for 30 min. Cells located at the interface of different gradient layers were then collected and washed two times with DMEM supplemented with 10% (v/v) FBS. For differential plating method, cell suspensions were plated on 0.1% Gelatin-coated 60 mm petridishes (Falcon BD, NJ, USA) at a concentration of ~$1\times10^7$ cells/dish and incubated at 37°C in humidified atmosphere of 5% $CO_2$ in air. After 16 h of incubation, floating cells were collected for further culture.

### *In vitro* culture

For *in vitro* culture, SSC-enriched cells ($1\times10^5$ cells/well) were plated on 0.1% Gelatin coated 4-well multidish (Falcon BD) with or without mitomycin C-treated STO cell feeder layer and cultured at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Culture medium consisted of high glucose DMEM (GibcoBRL) supplemented with 15% (v/v) FBS (Hyclone, Logan, UT), 1% penicillin-streptomycin (GibcoBRL), 1% L-glutamine, 2 mM 2-mercaptoethanol, 1% nonessential amino acids, 1,000 U of leukemia inhibitory factor (LIF; Chemicon, Temecula, CA), and 10 ng/ml human recombinant glial cell line-derived neurotrophic factor (GDNF; R&D, Minneapolis, MN). Culture medium was changed every day. After 6 to 7 days of culture, SSC-derived colonies were briefly treated with 0.05% (w/v) trypsin and 1 mM EDTA and colonies were individually picked and reseeded on nonproliferative mitomycin C-treated STO cells in fresh medium. In case of feeder-free culture system, LIF- and GDNF-free culture media was conditioned on nonproliferative mitomycin C-treated STO cell layer for 24 h, filtered and then used after addition of LIF and GDNF.

### Alkaline phosphatase (AP) activity

For assessing the AP-activity in putative SSC, the cells were fixed with 4% paraformaldehyde and stained histochemically using an AP-staining kit (Sigma) following the manufacturer's protocol. The AP-activity was estimated

**Table 1.** Details of primer pairs used for reverse transcriptase-polymerase chain reaction (RT-PCR)

| Gene name | Primer sequence (5' to 3') | GenBank accession number | Annealing temperature (°C) | Amplicon size (bp) |
|---|---|---|---|---|
| GAPDH | TCATTGACCTCCACTACATGGTCT<br>AGGCTGTTGTCATACTTCTCATGG | AF017079 | 57 | 322 |
| OCT4 | CTACCTCTACTGTCACGTCGGCTA<br>CACCATATCGGGGTGACTGATATT | AJ251914 | 57 | 289 |
| NANOG | ACATCCTGAACCTTAGCTACAA<br>AACATAGTTGTTGAGCTGGCTA | DQ447201 | 57 | 341 |
| PGP9.5 | GAGATGCTGAACAAAGTGCTG<br>CATGGTTCACCGGAAAAGG | AY459531 | 56 | 526 |
| GATA4 | TCTCGATATGTTTGATGACTTCTC<br>GTCTTCGATTTGTTAAGGTTCTTG | AY115491 | 56 | 378 |
| SOX9 | CCTAATCTCGATATGTTTGATGAC<br>GTCTTCGATTTGTTAAGGTTCTTG | NM_213843 | 56 | 383 |
| SOX2 | AATGCCTTCATGGTGTGGT<br>CGGGGCCGGTATTTATAAT | DQ400923 | 60 | 203 |
| PigVASA | AAAATAGTGAATTAGACCCAGACC<br>TTCCACAAGAATAGTGTCGTATTT | AY626785 | 56 | 332 |
| c-KIT | AACTCATCTGTATCACCGTTTGGA<br>TTGTTTCCATTTATCTCCTCGACA | AB250963 | 60 | 302 |
| SCF | GTAATAGGAAGGCCTCAGATTCCA<br>TATGGAACAGCTTCCGCTAACATA | NM_214269 | 60 | 381 |
| LIF-r | GGAAACAAAACTTTCTGAGATTCC<br>AGGGTCCAGACTGAGATGAGTTAC | SSU97364 | 60 | 269 |
| α-SMA | AATGGCTCTGGGCTCTGTAAG<br>CTTTTCCATGTCGTCCCAGT | DQ400922 | 60 | 219 |
| VIMENTIN | CAGGATGAGATTCAGAACATGAAG<br>AAGGCACTTGAAAGCTATTTCTTG | DQ190948 | 60 | 307 |
| DAZL | AGATTTTGTCCCCTTACTTCAGTG<br>GGCTCAGTACTTGTCTCTTTCTCC | NM_010021 | 60 | 221 |

by visual analysis of the stained cells (Ju et al., 2008).

**Immunocytochemical staining**

Immunocytochemical analysis for SSC and pluripotent markers was performed as we described earlier (Ju et al., 2008). Briefly, cells and colonies were fixed with 4% PFA and methanol, and incubated with appropriate dilutions of mouse monoclonal antibodies against PGP9.5 (1:100, BIOMOL International), OCT4 (1:50, ES cell marker sample kit, Chemicon International), SSEA-1 (1:50, ES cell marker sample kit, Chemicon International) and NANOG (1:300, Abcam). Cells were incubated in 1:500 normal goat serum (NGS; Vector Laboratories) for 1 h to block nonspecific binding. Primary antibodies were localized with FITC-conjugated IgG second antibody, mounted on VECTASHIELD mounting medium containing 1.5 μg/ml DAPI (Vector Laboratories) and then observed under Olympus FLUOVIEW FV1000 confocal microscope.

**Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis**

Total RNA was isolated by Trizol reagent (Invitrogen, Carlsbad, CA). Contaminating genomic DNA was eliminated using DNaseI. The cDNA was synthesized by RT-Premix (AccuPowerR RT-Premix, Bioneer, Daejon, Korea) according to the manufacturer's instruction. cDNA amplification was carried out in a total volume of 20 μl using PCR Premix (AccuPowerR PCR-Premix, Bioneer) according to the manufacturer's instruction. Nucleotide sequences were obtained from GenBank and primer pairs were designed by Primer3 program. The details of primers used for RT-PCR is shown in Table 1. The amplification profile consisted of hotstart at 94°C for 5 min followed by following three steps: 94°C for 30 s (denaturation), annealing temperature (Table 1) for 30 s (annealing), and 72°C for 45 s (extension). After 35 amplification cycles, the samples were retained at 72°C for 7 min to ensure complete strand extension. PCR products were run on 1% agarose gels and visualized by ethidium bromide staining.

## RESULTS AND DISCUSSION

The SSC comprise only 0.03% of all germ cells in adult testis and their isolation is often hindered by the presence of spermatogonial cells at different stages of differentiation (Aponte et al., 2005). Furthermore, in our preliminary experiments, we found that the proportion of PGP9.5- and DBA-positive cells in the basal compartment of 5 days to 9

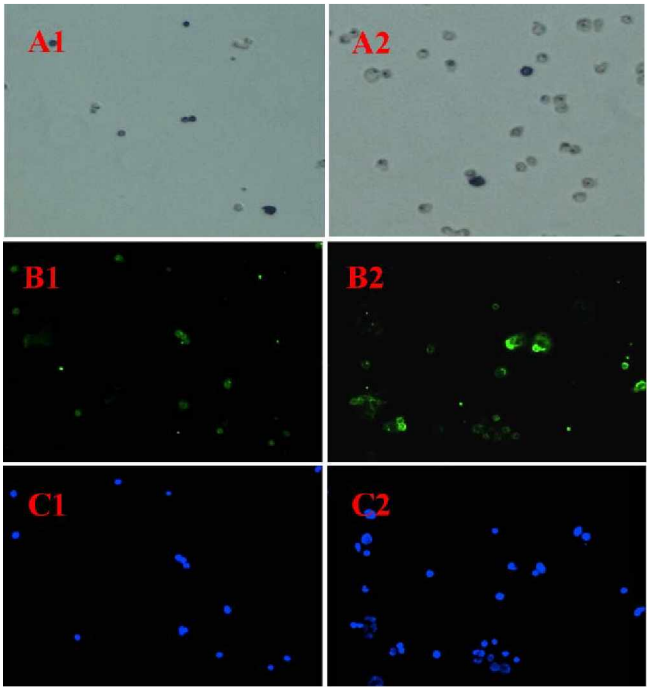


**Figure 1.** Staining for alkaline phosphatase activity (A) and PGP9.5 expression (B) in pig semineferous tubular cells enriched for pig spermatogonial stem cells by Percoll density gradient (Lane 1) or differential plating (Lane 2) method. C: Fluorescent DAPI nuclear staining.

week old neonatal pig testes decreased with increasing age (Data not shown). Therefore, to increase the purity of isolated SSC, we preferred to use testes from 5-10 days-old neonatal piglets in which seminiferous tubules primarily contained gonocytes, sertoli cells and myoid cells beside SSC.

**Isolation and enrichment of pigSSC**

Identification and isolation of SSC had been difficult due to their rarity in testis and lack of SSC-specific cell surface markers. Various methods such as differential plating, velocity sedimentation, elutriation, discontinuous gradient, Hoechst 33342 and rhodamine123 side population, magnetic-activated cells sorting (MACS) and fluorescence activated cells sorting (FACS) have been employed to isolate SSC in different species (van Pelt et al., 1996; Shinohara et al., 2000; Luo et al., 2006; Rodriguez-Sosa et al., 2006). However, MACS and FACS methods could not be applied to pigs due to lack of SSC-specific cell surface antibodies. In our study, when Percoll density gradient method was used for enrichment of pigSSC, 95.94±0.60% and 83.62±4.24% of cells in 45-60% and 30-45% Percoll layers, respectively were positive for PGP9.5 (Figure 1) while cells in other layers primarily contained somatic cells. When these cells were analyzed for AP-activity, 51.20±14.29% of cells in 45-60% Percoll layer were AP-positive (Figure 1) while less than 18% of cells were AP-positive in all other layers. Therefore, cells at 45-60% Percoll layer were presumably more enriched with SSC and hence, were used for further culture.

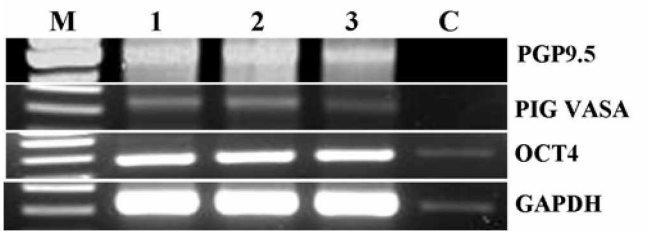


**Figure 2.** RT-PCR analysis for the expression of PGP9.5, PigVASA and OCT4 in pig seminiferous tubular cells enriched for pig spermatogonial stem cells by discontinuous Percoll density gradient (Lane 1) or differential plating (Lane 2), pig spermatogonial stem cell colony (Lane 3) and *in vitro* fertilized pig blastocyst (Lane C). GAPDH was used as control. M: 100 bp molecular marker.

We also evaluated the differential plating method for the isolation of pigSSC. Results showed that 94.02±5.70% of cells, enriched by differential plating method, were positive for PGP9.5 expression (Figure 1). The viability of isolated cells was more than 95% in both Percoll density gradient and differential plating methods. However, the latter method yielded greater number of presumptive pigSSC cells per testis than the former method (Figure 1). The RT-PCR analysis of SSC-enriched cells showed no difference in the expression of PGP9.5, PigVASA and OCT4 genes (Figure 2).

***In vitro* culture and growth characteristics of pigSSC**

Because pigSSC can replicate in the seminiferous tubules of mouse after transplantation, we hypothesized that the growth factors required for pigSSC self-renewal may be similar to those of miceSSC (Dobrinski et al., 2000). Therefore, we chose the miceSSC culture system for culturing pigSSC (Guan et al., 2006). The culture system was partially modified based on our preliminary experiments.

Under our culture system, the pigSSC increased in number within 2-4 days as single, paired or clustered cells while the differentiated cells began to die. After 4 days of culture, the pigSSC cells formed mulberry-shaped small colony with a distinct boundary from feeder layer: contaminating somatic cells grew as spindle shape or fibroblast-like cells. The colonies grew bigger with time and their average diameter reached 110±4.5 μm by 6-7 days of culture. By 10 days of culture, colonies had three dimensional ES-cell colony like morphology (Figure 3A). These pigSSC colonies could be sub-cultured for 3-8 passages with a doubling time between 6 to 7 days.

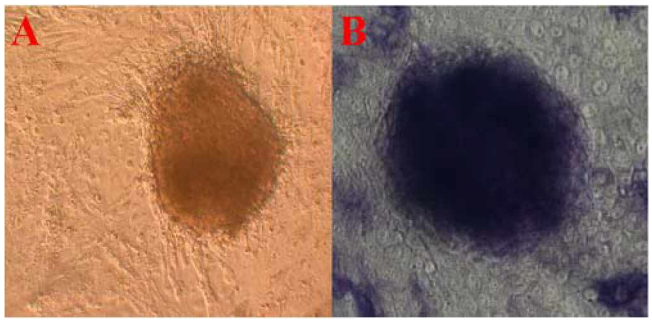


**Figure 3.** Morphology (A) and positive alkaline phosphatase activity (B) in pig spermatogonial stem cell colony.

However, maximum passage number of pigSSC colonies varied with the method of their isolation and enrichment (Table 2). When pigSSCs were isolated by non-enzymatic mechanical method, greater number of colonies formed than any other method. However, these colonies could not be maintained in culture for more than three passages. On the contrary, pigSSCs isolated by enzymatic method could be sub-cultured for seven passages. Combination of enzymatic method of pigSSC isolation with differential plating method of enrichment not only yielded greater number of colonies but also could be sub-cultured for 8 times or more and maintained in culture for more than 2 months. Percoll density gradient method of enrichment yielded large sized colonies but these colonies could not be sub-cultured for more than three times.

We also evaluated the culture characteristics of pigSSCs under feeder-free condition using conditioned medium (Table 2). We observed that, there were no apparent differences in the colony formation and growth characteristics of pigSSC colonies cultured in the presence or absence of STO cell feeder layer. However, we could never sub-culture the pigSSC colonies for more than four passages under feeder-free condition. This might suggest that continuous secretion of some unknown factors by feeder cells is important for proliferation and self-renewal of pigSSC.

**Purity of pigSSC**

The SSC isolated from seminiferous tubules is often contaminated by differentiating spermatogonial cells, sertoli cells and peritubular myoid cells. Most SSC culture systems are known to contain a mixture of testicular cells with about 1.33% SSC (Aponte et al., 2005). Although we enriched the pigSSC by differential plating or Percoll density gradient method, it was still containing few contaminating cells. Therefore, to further maximize the purity, we individually picked the putative pigSSC colonies and used them for sub-culture. To confirm the purity of these putative pigSSC colonies, we isolated mRNA from these colonies and performed RT-PCR with primer pairs specific for PGP9.5 to identify SSC (Luo et al., 2006), PigVASA to identify proliferating primordial germ cells (Luo et al., 2006), DAZL to identify premeiotic germ cells (Seligman and Page, 1998), c-KIT to identify differentiating spermatogonial and germ cells (Aponte et al., 2005), α-smooth muscle actin (α-SMA) to identify peritubular myoid cells (Honaramooz et al., 2007), and GATA-binding protein4 (GATA4; (Honaramooz et al., 2007)) and SOX9 (Mizukami et al., 2008) to identify sertoli cells. As expected, discrete bands were obtained for PGP9.5, PigVASA and DAZL amplicons while no amplicon bands could be detected for c-KIT, α-SMA, GATA4 and SOX9 suggesting that the our pigSSC colonies were highly purified (Figure 4). The pigSSC colonies were also negative for mRNA expression of c-KIT ligand (stem cell factor or SCF) as has been reported for bovineSSC (Oatley et al., 2004). Interestingly, pigSSC colonies were also positive for VIMENTIN transcript, which is known to be expressed in pig gonocytes (Goel et al., 2007) and sheep pre-spermatogonial cells (Steger and Wrobel, 1994). Immunofluorescence study further confirmed the expression of PGP9.5 proteins in these pigSSC colonies (Figure 5).

**Expression of pluripotent markers in pigSSC colonies**

Earlier studies, in mice, provide evidences that SSC can acquire pluripotency upon *in vitro* culture (Guan et al., 2006; Kanatsu-Shinohara et al., 2008a). Therefore, to investigate the pluripotency of our pigSSC cell lines, we immunocytochemically analyzed them for AP-activity, and expression of SSEA-1, NANOG and OCT-4 proteins that

**Table 2.** Isolation, enrichment and culture methods for pig spermatogonial stem cells and their effect on passage number

| Isolation method | Enrichment method | Feeder layer | Culture medium | Passage number |
|---|---|---|---|---|
| Mechanical digestion | - | STO | DMEM+15% FBS+LIF+GDNF | 3 |
| Enzymatic digestion | - | STO | DMEM+15% FBS+LIF+GDNF | 7 |
| Enzymatic digestion | Differential plating | STO | DMEM+15% FBS+LIF+GDNF | 8 |
| Enzymatic digestion | Discontinuous Percoll density gradient | STO | DMEM+15% FBS+LIF+GDNF | 3 |
| Mechanical digestion | - | - | STO-conditioned medium | 4 |
| Enzymatic digestion | - | - | STO-conditioned medium | 3 |
| Enzymatic digestion | Differential plating | - | STO-conditioned medium | 3 |
| Enzymatic digestion | Discontinuous Percoll density gradient | - | STO-conditioned medium | 3 |

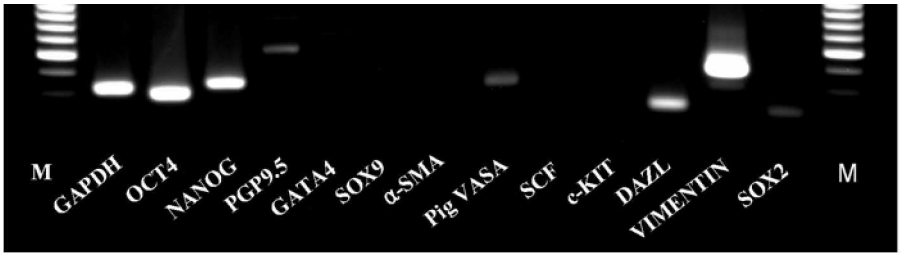


**Figure 4.** RT-PCR analysis of pig spermatogonial stem cell for the expression of markers of spermatogonial stem cell (PGP9.5), proliferating germ cell (Pig VASA), pluripotency (OCT4, NANOG, and SOX2), sertoli cell (GATA4 and SOX9), peritubular myoid cell (α-SMA), differentiating spermatogonial cell and germ cell (c-KIT), c-KIT ligand (SCF), deleted in azoospermia-like (DAZL) and VIMENTIN. GAPDH was used as control. M: 100 bp molecular marker.

characterize undifferentiated stem cells (Figure 5). OCT4 and NANOG are transcriptional factors for regulating self-renewal and pluripotency and are highly expressed in ES cells of different species including pig (Brevini et al., 2007). On the other hand, SSEA-1 is a biochemical marker for ES cells in mice and is expressed in PGCs of pig (Takagi et al., 1997). In neonatal pig testis, SSEA-1 and NANOG are expressed in gonocytes (Goel et al., 2008) while OCT4 is reported to be expressed in undifferentiated spermatogonia (Luo et al., 2006). Our analyses revealed that pigSSC were similar to the established pluripotent ES and SSC cells in mice, not only in morphology but also in the expression of specific cell markers for pluripotency. The colonies stained positive for AP-activity and were presumably undifferentiated (Figure 3B). They also showed expression for SSEA-1, NANOG and OCT4 proteins (Figure 5). The RT-PCR assay further confirmed the expression of OCT4, NANOG and SOX2 in pigSSC colonies (Figure 3). SOX2, a transcriptional factor required for self-renewal of mice ES-cells, has been reported to be expressed in miceSSC (Shi et al., 2006). Taken together, these results suggest that our pigSSC cell lines are undifferentiated.

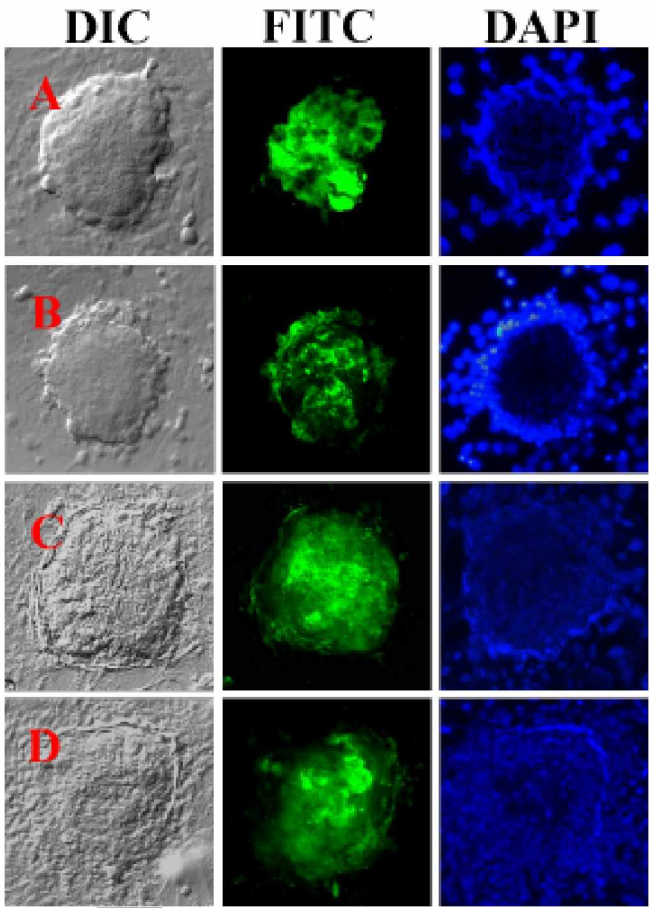


**Figure 5.** Immunocytochemistry of pig spermatogonial stem cell colony for PGP9.5 (A), OCT4 (B), SSEA-1 (C) and NANOG (D) proteins as observed under confocal microscope. Primary antibody against the proteins of interest were captured by FITC-conjugated secondary antibody and counterstained with DAPI nuclear stain.

In summery, we isolated highly purified primary cultures of pigSSC cell lines from freshly isolated testicular tubules. These cell colonies displayed morphology similar to that reported previously for miceSSC and were positive for SSC markers such as PGP9.5, PigVASA and DAZL and were negative for sertoli cell marker (GATA4 and SOX9), peritubular myoid cell marker (α-SMA), and differentiating spermatogonial and germ cells (c-KIT). These colonies also stained positive for AP-activity and expressed pluripotent markers such as OCT-4, NANOG, SSEA-1 and SOX2. The pigSSC colonies could be cultured for more than two months and sub-cultured for more than 8 passages with doubling time between 6 to 7 days. In conclusion, the present study identified the favorable conditions for isolation, enrichment and *in vitro* culture of highly purified undifferentiated pigSSC cell lines that proliferate for long periods of time in culture. These pigSSCs possessed characteristics similar to those reported for miceSSCs. Our technique will provide an important starting point for further purification and characterization of pigSSC. In future, long-term pigSSC culture will be useful for studying mechanism of spermatogenesis and has important implications in pig transgenesis.

## REFERENCES


Aponte, P. M., M. P. van Bragt, D. G. de Rooij and A. M. van Pelt. 2005. Spermatogonial stem cells: characteristics and experimental possibilities. Apmis, 113:727-742.

Brevini, T. A., S. Antonini, F. Cillo, M. Crestan and F. Gandolfi. 2007. Porcine embryonic stem cells: Facts, challenges and hopes. Theriogenol. 68(Suppl 1):S206-213.

Cheng, G. and S. T. Feng. 2006. Studies on spermatogonial stem cells cultured *in vitro* of Wuzhishan Mini Porcine. Sheng Wu Gong Cheng Xue Bao, 22:689-693.

Dirami, G., N. Ravindranath, V. Pursel and M. Dym. 1999. Effects of stem cell factor and granulocyte macrophage-colony stimulating factor on survival of porcine type A spermatogonia cultured in KSOM. Biol. Reprod. 61:225-230.

Dobrinski, I., M. R. Avarbock and R. L. Brinster. 2000. Germ cell transplantation from large domestic animals into mouse testes. Mol. Reprod. Dev., 57:270-279.

Dobrinski, I. 2006. Advances and applications of germ cell transplantation. Hum Fertil (Camb), 9:9-14.

Dobrinski, I. and A. J. Travis. 2007. Germ cell transplantation for the propagation of companion animals, non-domestic and endangered species. Reprod. Fertil. Dev., 19:732-739.

Goel, S., M. Sugimoto, N. Minami, M. Yamada, S. Kume and H. Imai. 2007. Identification, isolation, and *in vitro* culture of porcine gonocytes. Biol. Reprod. 77:127-137.

Goel, S., M. Fujihara, N. Minami, M. Yamada and H. Imai. 2008. Expression of NANOG, but not POU5F1, points to the stem cell potential of primitive germ cells in neonatal pig testis. Reproduction.

Guan, K., K. Nayernia, L. S. Maier, S. Wagner, R. Dressel, J. H. Lee, J. Nolte, F. Wolf, M. Li, W. Engel and G. Hasenfuss. 2006. Pluripotency of spermatogonial stem cells from adult mouse testis. Nature 440:1199-1203.

Hofmann, M. C. 2008. Gdnf signaling pathways within the mammalian spermatogonial stem cell niche. Mol. Cell Endocrinol.

Honaramooz, A., S. O. Megee, R. Rathi and I. Dobrinski. 2007. Building a testis: formation of functional testis tissue after transplantation of isolated porcine (*Sus scrofa*) testis cells. Biol. Reprod. 76:43-47.

Hong, Y., T. Liu, H. Zhao, H. Xu, W. Wang, R. Liu, T. Chen, J. Deng and J. Gui. 2004. Establishment of a normal medakafish spermatogonial cell line capable of sperm production *in vitro*. Proc. Natl. Acad. Sci. USA. 101:8011-8016.

Ju, J. Y., C. Y. Park, M. K. Gupta, S. J. Uhm, E. C. Paik, Z. Y. Ryoo, Y. H. Cho, K. S. Chung and H. T. Lee. 2008. Establishment of stem cell lines from nuclear transferred and parthenogenetically activated mouse ocytes for therapeutic cloning. Fertil. Steril. 89(Suppl 5):1314-1323.

Kanatsu-Shinohara, M., J. Lee, K. Inoue, N. Ogonuki, H. Miki, S. Toyokuni, M. Ikawa, T. Nakamura, A. Ogura and T. Shinohara. 2008a. Pluripotency of a single spermatogonial stem cell in mice. Biol. Reprod. 78:681-687.

Kanatsu-Shinohara, M., T. Muneto, J. Lee, M. Takenaka, S. Chuma, N. Nakatsuji, T. Horiuchi and T. Shinohara. 2008b. Long-term culture of male germline stem cells from hamster testes. Biol. Reprod. 78:611-617.

Luo, J., S. Megee, R. Rathi and I. Dobrinski. 2006. Protein gene product 9.5 is a spermatogonia-specific marker in the pig testis: application to enrichment and culture of porcine spermatogonia. Mol. Reprod. Dev. 73:1531-1540.

Mizukami, T., Y. Kanai, M. Fujisawa, M. Kanai-Azuma, M. Kurohmaru and Y. Hayashi. 2008. Five azacytidine, a DNA methyltransferase inhibitor, specifically inhibits testicular cord formation and Sertoli cell differentiation *in vitro*. Mol. Reprod. Dev. 75:1002-1010.

Nagano, M., M. R. Avarbock, E. B. Leonida, C. J. Brinster and R. L. Brinster. 1998. Culture of mouse spermatogonial stem cells. Tissue Cell 30:389-397.

Oatley, J. M., J. J. Reeves and D. J. McLean. 2004. Biological activity of cryopreserved bovine spermatogonial stem cells during *in vitro* culture. Biol. Reprod. 71:942-947.

Rodriguez-Sosa, J. R., H. Dobson and A. Hahnel. 2006. Isolation and transplantation of spermatogonia in sheep. Theriogenol. 66:2091-2103.

Ryu, B. Y., H. Kubota, M. R. Avarbock and R. L. Brinster. 2005. Conservation of spermatogonial stem cell self-renewal signaling between mouse and rat. Proc. Natl. Acad. Sci. USA. 102:14302-14307.

Seligman, J. and D. C. Page. 1998. The Dazh gene is expressed in male and female embryonic gonads before germ cell sex differentiation. Biochem. Biophys. Res. Commun. 245:878-882.

Shi, Y. Q., Q. Z. Wang, S. Y. Liao, Y. Zhang, Y. X. Liu and C. S. Han. 2006. *In vitro* propagation of spermatogonial stem cells from KM mice. Front Biosci. 11:2614-2622.

Shinohara, T., K. E. Orwig, M. R. Avarbock and R. L. Brinster. 2000. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. Proc. Natl. Acad. Sci. USA. 97: 8346-8351.

Steger, K. and K. H. Wrobel. 1994. Immunohistochemical demonstration of cytoskeletal proteins in the ovine testis during postnatal development. Anat. Embryol. (Berl). 189: 521-530.

Takagi, Y., N. C. Talbot, C. E. Rexroad, Jr. and V. G. Pursel. 1997. Identification of pig primordial germ cells by immunocytochemistry and lectin binding. Mol. Reprod. Dev. 46:567-580.

van Pelt, A. M., A. R. Morena, F. M. van Dissel-Emiliani, C. Boitani, I. C. Gaemers, D. G. de Rooij and M. Stefanini. 1996. Isolation of the synchronized A spermatogonia from adult vitamin A-deficient rat testes. Biol. Reprod. 55:439-444.