Development of a high-yield technique to isolate spermatogonial stem cells from porcine testes

Min Hee Park · Ji Eun Park · Min Seong Kim · Kwon Young Lee ·
Hye Jin Park · Jung Im Yun · Jung Hoon Choi · Eun song Lee ·
Seung Tae Lee

Received: 10 March 2014 / Accepted: 1 June 2014 / Published online: 18 June 2014
© Springer Science+Business Media New York 2014

Abstract

Purpose To date, the methods available for isolating spermatogonial stem cells (SSCs) from porcine testicular cells have a low efficiency of cell separating. Therefore, we tried to develop a novel isolation technique with a high-yield cell separating ability to isolate SSCs from porcine testes.

Methods We confirmed the presence of SSCs by measuring alkaline phosphatase (AP) activity and SSC-specific gene expression in neonatal porcine testis-derived testicular cells. Subsequently, the isolation of SSCs from testicular cells was performed using different techniques as follows: differential plating (DP), double DP, Petri dish plating post-DP, magnetic-activated cell sorting (MACS), and MACS post-DP. Positive AP staining was used to assess and compare the isolation efficiency of each method.

Results Petri dish plating post-DP resulted in the highest isolation efficiency. The putative SSCs isolated using this method was then further characterized by analyzing the

expression of SSC-specific genes and -related proteins, and germ cell-specific genes. OCT4, NANOG, EPCAM, THY1, and UCHL1 were expressed transcriptionally, and OCT4, NANOG, SOX2, TRA-1-60, TRA-1-81, and PLZF were expressed translationally in 86 % of the isolated SSCs. In contrast, no difference was observed in the percentage of cells expressing luteinizing hormone receptor (LHR), a Leydig cell-specific protein, or GATA4, a Sertoli cell-specific protein, between SSCs and negative control cells. In addition, transcriptional expression of VASA, a primordial germ cell-specific marker, and DAZL, a premeiotic germ cell-specific marker, wasn't and was detected, respectively.

Conclusions We successfully developed a novel high-yield technique to isolate SSCs from porcine testes to facilitate future porcine SSC-related research.

Keywords Porcine · Spermatogonial stem cells · Testis · Cell sorting

Capsule Petri dish plating post-DP is a high-yield technique that isolates SSCs from porcine neonatal testis.

M. H. Park · M. S. Kim · H. J. Park · S. T. Lee
Department of Animal Life Science, Kangwon National University,
Chuncheon 200-701, South Korea

J. E. Park · S. T. Lee
Division of Applied Animal Science, Kangwon National University,
Chuncheon 200-701, South Korea

K. Y. Lee · J. I. Yun · J. H. Choi · E. song Lee
College of Veterinary Medicine and Institute of Veterinary Science,
Kangwon National University, Chuncheon 200-701, South Korea

S. T. Lee (✉)
Department of Animal Life Science and Division of Applied Animal
Science, Kangwon National University, Dongsangdae 2-#105-1,
Chuncheon 200-701, South Korea
e-mail: stlee76@kangwon.ac.kr

Introduction

During embryogenesis, primordial germ cells (PGCs) originated from the proximal epiblast experience migration from the base of the allantois to the genital ridges via the hindgut mesentery [1]. Subsequently, the migrated PGCs are differentiated into gonocytes in the genital ridge and, in the neonatal testis after birth, gonocytes are migrated into the basement membrane of seminiferous tubule and differentiated into spermatogonial stem cells (SSCs) [2, 3].

The SSCs, which self-renew in seminiferous tubules, are undifferentiated male germ cells that can differentiate into sperm cells via spermatogenesis [2, 4–8]. They therefore play an important role in transferring genetic information to the next generation [8–11]. Unipotent SSCs transformed under specific niche conditions can acquire pluripotency and

Fig. 1 Experimental design

differentiate into specialized tissue cells [5, 7, 12–14]. Therefore, they are regarded as a useful tool for treating male infertility [15, 16] and allow the development of individual cell-based therapies without the risk of immune rejection [17].

Although many model species are available, porcine SSCs are of great interest because they can be propagated permanently in pigs with a valuable genetic background [18]. In addition, transgenic pigs can be generated to produce disease-specific models; xenotransplantation can also be performed [19–21]. However, when performing studies using porcine SSCs, collecting SSCs from a variety of testicular cells is important to obtain precise results [3, 22, 23]. Nevertheless, the isolation techniques

developed previously are not optimal due to toxicity and a poor separating efficiency [3, 22, 23]. We therefore developed a high-yield method to isolate SSCs from porcine testes by analyzing and comparing the characteristics of SSCs isolated using different methods.

Materials and methods

Animals

Crossbred (Landrace×Yorkshire) or purebred (Yorkshire×Yorkshire) piglets were kindly donated by Gumbo Inc.

(Wonju, Korea). Testes were harvested from 1- to 4-day-old male piglets through routine castration surgery performed at a local farm (Gumbo Inc.). All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (IACUC approval No. KW-131106-1), and were performed according to the Animal Care and Use Guideline of Kangwon National University.

Isolation of testicular cells from neonatal porcine testes

The tunica albuginea and epididymis were removed from the testes transferred to our laboratory in ice-cold Dulbecco's phosphate-buffered saline (DPBS; Welgene Inc., Daegu, Korea) containing 1 % (w/v) antibiotic–antimycotic solution (Welgene) within 1 h. Subsequently, the seminiferous tubules were digested using 0.1 % (w/v) type IV collagenase (Worthington Biochemical, Lakewood, CA) in Dulbecco's modified Eagle's medium (DMEM; Welgene) at 37 °C for 15 min. The fragmented seminiferous tubules were incubated sequentially and separately at 37 °C for 10 min in DMEM supplemented with 0.1 % (w/v) hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) and 0.25 % trypsin–EDTA (Welgene). The dispersed cells were washed twice in DMEM supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA), and then filtered through a 70- μ m nylon mesh (SPL, Pocheon, Korea) to remove myoid and Sertoli cells. Erythrocytes were removed by incubation at room temperature for 10–15 min in red blood cell lysis buffer (Sigma-Aldrich). The isolated testicular cells were then counted using a hemocytometer.

Alkaline phosphatase (AP) staining

Cells were fixed with 4 % (v/v) paraformaldehyde (Junsei Chemical Co., Ltd., Chuo-ku, Japan) and rinsed twice with

DPBS. Fixed cells were then stained with alkaline phosphatase solution consisting of 0.1 M Tris buffer (pH 8.2) supplemented with 0.2 mg/ml naphthol AS-MX phosphate, 2 % (v/v) dimethyl formamide, and 1 mg/ml Fast Red TR salt (all from Sigma-Aldrich) for 90 min. Stained cells were then washed twice with DPBS, and the percentage of the positively stained cells was counted using a hemocytometer.

Experimental design

The presence of SSCs within the dissociated testicular cells was confirmed by measuring AP activity and SSC-specific gene expression. To determine how to sort SSCs from other testicular cells more efficiently, SSCs were isolated in the following ways: differential plating (DP), double DP, Petri dish plating post-DP, magnetic-activated cell sorting (MACS), and MACS post-DP. DP was performed by collecting floating cells after incubating 5×10^6 testicular cells plated on 0.1 % (w/v) gelatin-coated 100-mm Petri dishes in DMEM supplemented with 10 % (v/v) heat-inactivated FBS for 16 h at 37 °C. Double DP consisted of DP repeated twice. For Petri dish plating post-DP, floating cells were collected after plating 1×10^6 cells isolated from DP on a 35-mm Petri dish and incubating them overnight at 37 °C in DMEM supplemented with 15 % (v/v) heat-inactivated FBS, 0.1 mM β -mercaptoethanol, 1 % (v/v) nonessential amino acid (NEAA), 1 mM sodium pyruvate, 2 mM L-glutamine (all from Invitrogen, Carlsbad, CA, USA), 1 % (v/v) antibiotic–antimycotic solution (Welgene), 1,000 units/ml mouse leukemia inhibitory factor (LIF; Chemicon International, Inc., Temecula, CA, USA), and 10 ng/ml glial cell-derived neurotrophic factor (GDNF; R&D Systems, Inc., Minneapolis, MN, USA). MACS was performed in accordance with the instruction manual of the MACS system (Invitrogen) using anti-mouse CD90 (EXBIO, Vestec, Czech Republic) and biotin-conjugated anti-mouse IgG1 (Abcam, Cambridge, UK).

Table 1 Oligonucleotide primers and PCR cycling conditions

Genes	GenBank number	Primer sequence		Size (bp)	Temp (°C)
		Sense (5'>3')	Anti-sense (5'>3')		
<i>GAPDH</i>	NM_001206359.1	AGGGCTGCTTTAACTCTGGCAA	GATGGTGATGGCCTTTCCATTG	180	60
<i>OCT4</i>	NM_001113060.1	CGCGAAGCTGGACAAGGAGA	CAAAGTGAGCCCCACATCGG	151	60
<i>NANOG</i>	NM_001129971.1	AACCAAACCTGGAACAGCCAGAC	GTTTCCAAGACGGCCTCCAAAT	158	60
<i>EPCAM</i>	NM_214419.1	CAATGCAGGGTCTACAGGCTGG	TGCATCTCGCCCATCTCCTTT	154	60
<i>THY1</i>	NM_001146129.1	GTGCTCTTGGGCACTGTGGG	TCTTGCTGGAGATGCTGGGC	178	60
<i>UCHL1</i>	NM_213763.2	TCCGGAAGACAGAGCAAAATGC	TCCGGAAGACAGAGCAAAATGC	150	60
<i>VASA</i>	AY626785.1	CCACAACGGGTGGTTTTGGA	ATGGCCCCGATGCTTCTGAT	195	60
<i>DAZL</i>	XM_003358321.2	ACAGTGGCCTGCTGGGGAAC	TGTGGGCCATTTCCAGAGGA	153	60

Temp Temperatur

Table 2 Primary and Secondary antibody

Antibody name	Catalog number	Company	Application	Dilution rate
Mouse anti-CD90 IgG1	10-652	Abcore	MACS	1:100
Biotin Rat anti-Mouse IgG1	ab99604	Abcam	MACS	1:100
Rabbit anti-Oct-3/4 IgG	Sc-9081	Santa cruz biotechnology	FACS	1:100
Rabbit anti-Sox2 IgG	AB5603	Chemicon International, Inc.	FACS	1:100
Rabbit anti-PLZF IgG	Sc-22839	Santa cruz biotechnology	FACS	1:100
Goat anti-LHR IgG	Sc-26341	Santa cruz biotechnology	FACS	1:100
Goat anti-GATA-4 IgG	Sc-1237	Santa cruz biotechnology	FACS	1:100
Rabbit anti-Nanog IgG	Sc-33759	Santa cruz biotechnology	FACS	1:100
Rabbit anti-TRA-1-60 IgG	MAB4360	Millipore	FACS, ICC	1:100
Rabbit anti-TRA-1-81 IgG	MAB4381	Millipore	FACS, ICC	1:100
Alexa Fluor-488® Chicken anti-Rabbit IgG	A-21441	Molecular probes	FACS, ICC	1:100
Alexa Fluor-568® Donkey anti-Goat IgG	A-11057	Molecular probes	FACS	1:100

MACS Magnetic-Activated Cell Sorting, FACS Fluorescence-Activated Cell Sorting, ICC Immunocytochemistry

antibodies. MACS post-DP was performed by exposing cells isolated by DP to the MACS system. Subsequently, the efficiency of each method was evaluated by measuring the percentage of SSCs with AP activity. The expression of SSC-specific genes and -related proteins, and germ cell-specific genes in SSCs was then quantified in cells isolated using the method showing the highest efficiency. The experimental design is shown in Fig. 1.

mRNA extraction, cDNA synthesis, and polymerase chain reaction (PCR)

Total mRNA was extracted from cells using the Dynabeads mRNA Direct™ Kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. Next, cDNA was synthesized from the prepared mRNA using the ReverTra Ace qPCR RT Master Mix with gDNA remover kit (Toyobo, Osaka, Japan). Subsequently, i-MAX™II DNA Polymerase (iNtRON, Seongnam, Korea) was used to identify gene expression using the primers listed in Table 1. PCR amplification was performed using the following conditions: 4 min at 94 °C for initial denaturation, followed by 34 cycles of 30 s at 94 °C, 45 s at the appropriate annealing temperature for each primer, and 1 min at 72 °C; the final extension was then performed at 72 °C for 10 min. The PCR products were separated by electrophoresis on 1.5 % (w/v) agarose gels (Promega, Madison, WI, USA), stained with Redsafe™ (iNtRON), and visualized using the Gel Doc™ XR+ imaging system (Bio-Rad Laboratories, Hercules, CA, USA).

Flow cytometry

Isolated SSCs were fixed with 4 % (v/v) paraformaldehyde for 15 min and washed with ice-cold DPBS. Fixed cells in DPBS

containing 2 % (v/v) heat-inactivated FBS and 0.01 % (v/v) Triton X-100 (Sigma-Aldrich) were stained for 2 h at 4 °C with anti-rabbit OCT3/4 (SSC-specific marker; Santa Cruz Biotechnology, Dallas, TX, USA), anti-rabbit NANOG (SSC-specific marker; Santa Cruz Biotechnology), anti-rabbit SOX2 (SSC-specific marker; Chemicon), anti-rabbit PLZF (SSC-specific marker; Santa Cruz Biotechnology),

Fig. 2 Existence of SSCs in testicular cells derived from neonatal porcine testes. Testes derived from neonatal male porcine were enzymatically dissociated and the presence of SSCs in dissociated testicular cells was identified by conducting alkaline phosphatase (AP) staining and analysis of SSC-specific genes expression through reverse transcription-polymerase chain reaction (RT-PCR). As the results, there were observed testicular cells stained positively for AP activity (arrow, **a**) and showing the expression of *OCT4* (151 bp), *NANOG* (158 bp), *EPCAM* (154 bp), *THY1* (178 bp) and *UCHL1* (150 bp) (**b**). *GAPDH* (180 bp) was used as an internal control. $n=3$. Scale bars represent 200 μ m

Fig. 3 Comparison of isolation efficiency of SSCs from neonatal porcine testicular cells according to different SSC isolation techniques. Putative SSC populations were retrieved from testicular cells derived from neonatal porcine testes by each SSC isolation method and stained for alkaline phosphatase (AP) activity. The SSCs retrieved by Petri dish plating post-DP method showed significantly the highest percentage of SSCs stained positively by AP staining. All data shown are means $\pm$ SD of five independent experiments. * $p<0.05$, n=3

anti-rabbit TRA-1-60 (SSC-specific marker; Millipore, Billerica, MA, USA), anti-rabbit TRA-1-81 (SSC-specific marker; Millipore), anti-goat GATA4 (Sertoli cell-specific marker; Santa Cruz Biotechnology), and anti-goat luteinizing hormone receptor (LHR; Leydig cell-specific marker; Santa Cruz Biotechnology) antibodies. Primary antibodies were then detected using Alexa Fluor 488 chicken anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) or Alexa Fluor 568 donkey anti-goat IgG (Molecular Probes) antibodies for 2 h at 4 °C. Detailed information regarding the antibodies and dilutions used is shown in Table 2. The stained cells were rinsed twice with DPBS and then sorted using a FACSCalibur (Becton, Dickinson and Co., Franklin Lakes, NJ, USA); data were then analyzed using the BD CellQuest Pro software (Becton, Dickinson and Co.).

Immunocytochemistry

The SSCs fixed with 4 % (v/v) paraformaldehyde for 15 min were rinsed twice with ice-cold DPBS. Next, the cells were incubated for 2 h at 4 °C with anti-rabbit TRA-1-60 and TRA-1-81 antibodies, respectively, and Alexa Fluor 488 chicken anti-rabbit IgG antibody was applied and incubated for 2 h at 4 °C for detecting primary antibodies. After washing twice with ice-cold DPBS, the stained cells was counterstained with Mounting medium for fluorescence with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) and observed under fluorescence microscope (BX53, Olympus, Tokyo, Japan).

Statistical analysis

The statistical analysis of all the numerical data shown in each experiment was performed using the Statistical Analysis System (SAS) program. Moreover, in detecting a significance of the main effects through variance (ANOVA) analysis in the SAS package, each treatment was compared by the least-square or DUNCAN method and the less than 0.05 of p value was regarded as significant differences.

Results

Identification of SSCs in testicular cells derived from neonatal porcine testes

To determine whether the testicular cells retrieved from the seminiferous tubules of neonatal porcine testes included SSCs, SSC-specific AP activity and gene expression were assessed. AP-positive SSCs were detected (Fig. 2a), and individual testicular cells also expressed SSC-specific genes including *OCT4*, *NANOG*, *EPCAM*, *THY1*, and *UCHL1*

Fig. 4 Reverse transcription-polymerase chain reaction (RT-PCR) analysis for the expression of SSC- and germ cell-specific genes in porcine SSCs isolated by Petri dish plating post-DP method. Isolated SSCs showed successfully transcriptional expression of *OCT4* (151 bp), *NANOG* (158 bp), *EPCAM* (154 bp), *THY1* (178 bp) and *UCHL1*

(150 bp) (SSC-specific marker genes) (a), with *DAZL* (153 bp) (premeiotic germ cell-specific marker gene) (b). However, any transcripts derived from *VASA* (195 bp) (primordial germ cell-specific marker gene) weren't detected (b). *GAPDH* (180 bp) was used as an internal control. n=3

Fig. 5 Translational expression of SSC-specific genes in porcine SSCs isolated by Petri dish plating post-DP method. The yield of SSCs stained positively with antibodies detecting SSC-specific proteins was measured by flow cytometry analysis. The expression of OCT4, NANOG, SOX2, TRA-1-60, TRA-1-81 and PLZF was identified in isolated SSCs (**a**) and at least 95 % or more of SSCs showed positivity against OCT4, NANOG, SOX2, and PLZF expression. Whereas, the expression of TRA-1-60 and TRA-1-81 was detected in at least 86 % or more of SSCs and the percentage of SSCs stained positively by TRA-1-60 and TRA-1-81 antibodies was significantly lower than those of the other markers. Moreover, localization of TRA-1-60 and TRA-1-81 on SSC surface membrane was identified by immunocytochemistry (**b**). All data shown are means $\pm$ SD of three independent experiments. Negative control means the unstained SSCs. Scale bar is 40 μ m. *–*** $p < 0.05$

(Fig. 2b). These results suggest that SSCs coexisted with testicular cells that were dissociated from neonatal porcine testes.

Development of a high-yield method to isolate putative SSCs from testicular cells

Next, putative SSCs were isolated from testicular cells using different techniques to determine the method that achieved the highest percentage of putative SSCs, as described in the Materials and methods (Fig. 1). The efficiency of each isolation method was then determined by calculating the percentage of putative SSCs that were AP-positive. As shown in

Fig. 3, the highest isolation efficiency (96.50 ± 1.20 %) was obtained with the Petri dish plating post-DP method, whereas the MACS method showed the lowest isolation yield (36.67 ± 6.43 %). Moreover, the direct (58.86 ± 7.51 %), DP (79.76 ± 5.20 %), and double DP (68.87 ± 2.71 %) methods all achieved a significantly higher efficiency than the MACS method, while the direct (without sorting process) and double DP methods showed a significantly lower yield than the DP method. However, the isolation efficiency of the MACS post-DP method (43.45 ± 14.01 %) was comparable to the direct, double DP, and MACS methods. These results suggest that the Petri dish plating post-DP method could be used as an effective

technique to isolate putative SSCs from testicular cells and obtain a high yield.

Characterization of putative SSCs isolated from testicular cells using the high-yield isolation method

To determine whether the putative SSCs isolated using the developed Petri dish plating post-DP method were truly SSCs, the expression of SSC-specific genes and -related proteins, and germ cell-specific genes were analyzed. The putative SSCs expressed *OCT4*, *NANOG*, *EPCAM*, *THY1*, and *UCHL1* mRNAs (Fig. 4a), along with OCT4, NANOG, SOX2, TRA-1-60, TRA-1-81, and PLZF proteins (Fig. 5a). Importantly, at least 86 % of the putative SSCs showed positivity for OCT4 (95.92 ± 5.41 %), NANOG (96.34 ± 2.09 %), SOX2 (99.12 ± 0.86 %), TRA-1-60 (86.05 ± 2.26 %), TRA-1-81 (87.35 ± 5.26 %) and PLZF (95.23 ± 0.53 %), and localization of TRA-1-60 and TRA-1-81 was identified on surface membrane of SSCs (Fig. 5b). In contrast, no significant difference was observed in the expression of LHR (6.98 ± 1.13 %), a Leydig cell-specific marker [24], or GATA4 (6.68 ± 1.77 %), a Sertoli cell-specific marker [24], between the putative SSCs and unstained putative SSCs (6.01 ± 0.07 %; negative control; Fig. 6). Moreover, in the putative SSC population, no transcriptional expression of *VASA*, a primordial germ cell-specific marker, was detected and *DAZL*, a premeiotic germ cell-specific marker, was expressed transcriptionally (Fig. 4b). These results suggest that most of the putative SSCs isolated from testicular cells by the Petri dish plating post-DP method were composed of only SSCs, without contamination by Leydig or Sertoli cells. Therefore, this method was demonstrated to be a high-yield technique to isolate SSCs from neonatal porcine testes.

Discussion

This study introduced a novel method to isolate porcine SSCs without contamination with any other testicular cells. Analyses revealed that 96.5 and 86.05 % or more of the putative SSCs isolated using the Petri dish plating post-DP method showed AP activity (Fig. 3) and expressed SSC-specific proteins (OCT4, NANOG, SOX2, TRA-1-60, TRA-1-81, and PLZF), respectively (Fig. 5a). Importantly, the Leydig and Sertoli cell-specific markers LHR and GATA4, respectively, were not detected (Fig. 6), suggesting that the putative SSCs isolated included no contamination by Leydig or Sertoli cells; therefore, the novel method successfully separated these cells. Accordingly, these results demonstrate that the Petri dish plating post-DP method might be a high-yield technique to effectively isolate SSCs from neonatal porcine testes.

Fig. 6 The percentage of Leydig and Sertoli cells coexisted in porcine SSCs isolated by Petri dish plating post-DP method. The isolated SSCs were stained with Leydig cell-specific LHR and Sertoli cell-specific GATA4 antibodies and the yield of Leydig and Sertoli cells were analyzed by flow cytometry. As the results, the percentage of cells showing positivity against LHR and GATA4 antibodies wasn't significantly different from those of the unstained SSCs (negative control). All data shown are means $\pm$ SD of three independent experiments

In this study, the cells retrieved by the Petri dish plating post-DP method showed transcriptional expression of *DAZL* (Fig. 4b) and translational expression of *PLZF* (Fig. 5a), with no transcriptional expression of *VASA* (Fig. 4b). These results demonstrate that the isolated cells aren't primordial germ cells expressing *VASA* and are undifferentiated SSCs, supported by the fact that undifferentiated type A spermatogonia simultaneously express translationally *DAZL* and *PLZF* in mouse [25]. Moreover, co-expression of these marker proteins in pig should be requested further for identifying obviously identity of the putative SSCs isolated from testis.

OCT4, NANOG, SOX2, and PLZF have been reported to be markers expressed specifically in SSCs, regardless of the species [24, 26–28]. However, no reports to date have described the expression of TRA-1-60 and TRA-1-81 in SSCs. Therefore, we investigated the expression and localization of TRA-1-60 and TRA-1-81 in porcine SSCs to determine whether TRA-1-60 and TRA-1-81 were SSC-specific proteins. As shown in Fig. 5, most SSCs derived from porcine testes expressed TRA-1-60 and TRA-1-81 on cell surface membrane, suggesting that these proteins could be used as additional SSC-specific markers.

Various methods such as discontinuous Percoll gradient, DP and MACS for isolation and enrichment of SSCs have been used to isolate SSC in diverse species [3, 23, 29]. When Han et al. used Percoll density gradient method for purification of porcine SSCs, yield of purified SSCs were 95.94 ± 0.60 % in 45–60 % Percoll layers, whereas 51.20 ± 14.29 % of SSCs in 45–60 % Percoll layer justly showed AP activity. In the usage of DP method for isolation of porcine SSCs [3],

85.00±3.00 % of cells were identified as SSCs. However, to sort SSCs using MACS have never been reported in pig. In our study, Petri dish plating post-DP method showed the highest isolation efficiency (96.50±1.20 %), which was higher than efficiency reported in previous studies. Accordingly, we emphasize that Petri dish plating post-DP method is the best technique in the isolation of SSCs from porcine testis.

As shown in Figs. 3 and 5, over 95 % of the putative SSCs isolated using the Petri dish plating post-DP method exhibited AP activity and expressed OCT4, NANOG, SOX2, and PLZF, suggesting that this might be the most effective method for isolating pure SSCs from porcine testes, which contain a variety of cell types. In contrast, a significantly lower percentage of putative SSCs expressed TRA-1-60 and TRA-1-81 compared with other SSC-specific proteins, which might have resulted from the differentiation of some SSCs during the isolation process. This result is supported by previous reports in which the expression of TRA-1-60 and TRA-1-81 was regulated during the differentiation of stem cells [30–32].

In conclusion, the efficient isolation of SSCs from testicular cells in porcine testis tissue was achieved using the Petri dish plating post-DP method described in this study. This novel method will help overcome the barriers of previous inefficient systems used to isolate SSCs and thus could make a significant contribution to future porcine SSC-related research. Nevertheless, this isolation method should be evaluated further by isolating SSCs from the testes of other species.

Acknowledgments This research was supported by Agricultural Biotechnology Development Program (IPET112015-4), Ministry of Agriculture, Food and Rural Affairs, Republic of Korea.

References

- McLaren A. Primordial germ cells in the mouse. *Dev Biol.* 2003;262(1):1–15.
- Brinster RL. Germline stem cell transplantation and transgenesis. *Science.* 2002;296:2174–6.
- Yang Y, Honaramooz A. Efficient purification of neonatal porcine gonocytes with Nycodenz and differential plating. *Reprod Fertil Dev.* 2011;23:496–505.
- Dym M. Spermatogonial stem cells of the testis. *PNAS.* 1994;91:11287–9.
- De Rooij DG. Stem cells in the testis. *Int J Exp Pathol.* 1998;79:67–80.
- De Rooij DG. Proliferation and differentiation of spermatogonial stem cells. *Reproduction.* 2001;121:347–54.
- Aponte PM, van Bragt MP, De Rooij DG, van Pelt AM. Spermatogonial stem cells: characteristics and experimental possibilities. *APMIS.* 2005;113:727–42.
- Oatley JM, Brinster RL. Regulation of spermatogonial stem cell self-renewal in mammals. *Annu Rev Cell Dev Biol.* 2008;24:263–86.
- Kanatsu-Shinohara M, Ogonuki N, Inoue K, Miki H, Ogura A, Toyokuni S, et al. Long-term proliferation in culture and germline transmission of mouse male germline stem cell. *Biol Reprod.* 2003;69:612–6.
- Kanatsu-Shinohara M, Inoue K, Lee J, Yoshimoto M, Ogonuki N, Miki H, et al. Generation of pluripotent stem cells from neonatal mouse testis. *Cell.* 2004;119:1001–12.
- Kanatsu-Shinohara M, Toyokuni S, Shinohara T. Genetic selection of mouse male germline stem cells in vitro: offspring from single stem cells. *Biol Reprod.* 2005;72:236–40.
- Hofmann MC. Gdnf signaling pathways within the mammalian spermatogonial stem cell niche. *Mol Cell Endocrinol.* 2008;288:95–103.
- Caires K, Broady J, McLean D. Maintaining the male germline: regulation of spermatogonial stem cells. *J Endocrinol.* 2010;205:133–45.
- Oatley JM, Brinster RL. The germline stem cell niche unit in mammalian testes. *Physiol Rev.* 2012;92:577–95.
- Kubota H, Brinster RL. Technology insight: in vitro culture of spermatogonial stem cells and their potential therapeutic uses. *Nature.* 2006;2:99–108.
- Vlajković S, Cukuranović R, Bjelaković MD, Stefanović V. Possible therapeutic use of spermatogonial stem cells in the treatment of male infertility: a brief overview. *Sci World J.* 2012;2012:374151.
- Daley GQ, Scadden DT. Prospects for stem cell-based therapy. *Cell.* 2008;132:544–8.
- Nowak-Imialek M, Kues W, Carnwath JW, Niemann H. Pluripotent stem cells and reprogrammed cells in farm animals. *Microsc Microanal.* 2011;17:474–97.
- Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, et al. Production of α -1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science.* 2008;295:1089–92.
- Lavitrano M, Busnelli M, Cerrito MG, Giovannoni R, Manzini S, Vargiolu A. Sperm-mediated gene transfer. *Reprod Fertil Dev.* 2006;18:19–23.
- Prather RS, Shen M, Dai Y. Genetically modified pigs for medicine and agriculture. *Biotechnol Genet Eng Rev.* 2008;25:245–66.
- Goel S, Sugimoto M, Minami N, Yamada M, Kume S, Imai H. Identification, isolation, and in vitro culture of porcine gonocytes. *Biol Reprod.* 2007;77:127–37.
- Han SY, Gupta MK, Lee SJ, Uhm HT. Isolation and in vitro culture of pig spermatogonial stem cell. *AJAS.* 2009;22:187–93.
- Lee WY, Park HJ, Lee R, Lee KH, Kim YH, Ryu BY, et al. Establishment and in vitro culture of porcine spermatogonial germ cells in low temperature culture conditions. *Stem Cell Res.* 2013;11:1234–49.
- Hao J, Yamamoto M, Richardson TE, Chapman KM, Denard BS, Hammer RE, et al. Sohlh2 knockout mice are male-sterile because of degeneration of differentiating type A spermatogonia. *Stem Cells.* 2008;26(6):1587–97.
- Luo J, Megee S, Rath R, Dobrinski I. 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.* 2006;73:1531–40.
- Luo J, Megee S, Rath R, Dobrinski I. Asymmetric distribution of UCH-L1 in spermatogonia is associated with maintenance and differentiation of spermatogonial stem cells. *J Cell Physiol.* 2009;220:460–8.
- Goel S, Fujihara M, Minami N, Yamada M, Imai H. Expression of NANOG, but not POU5F1, points to the stem cell potential of primitive germ cells in neonatal pig testis. *Reproduction.* 2008;135:785–95.
- Ryu BY, Kubota H, Avarbock MR, Brinster RL. Conservation of spermatogonial stem cell self-renewal signaling between

- mouse and rat. *Proc Natl Acad Sci U S A*. 2005;102(40): 14302–7.
30. International Stem Cell Initiative, Adewumi O, Aflatoonian B, Ahrlund-Richter L, Amit M, Andrews PW, et al. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat Biotechnol*. 2007;25:803–16.
31. Schopperle WM, DeWolf WC. The TRA-1-60 and TRA-1-81 human pluripotent stem cell markers are expressed on podocalyxin in embryonal carcinoma. *Stem Cells*. 2007;25:723–30.
32. Gafni O, Weinberger L, Mansour AA, Manor YS, Chomsky E, Ben-Yosef D, et al. Derivation of novel human ground state naive pluripotent stem cells. *Nature*. 2013;504:282–6.