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Effects of Suspension Culture on Proliferation and Undifferentiation of Spermatogonial Stem Cells Derived from Porcine Neonatal Testis

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ABSTRACT

Despite many researches related with *in-vitro* culture of porcine spematogonial stem cells (SSCs), adherent culture system widely used has shown a limitation in the maintenance of porcine SSC self-renewal. Therefore, in order to overcome this obstacle, suspension culture, which is known to have numerous advantage over adherent culture, was applied to the culture of porcine SSCs. Porcine SSCs retrieved from neonatal testes were suspension-cultured for 5 days or 20 days, and characteristics of suspension-cultured porcine SSCs including proliferation, alkaline phosphatase (AP) activity, and self-renewal-specific gene expression were investigated and compared with those of adherent-cultured porcine SSCs. As the results, the suspension-cultured porcine SSCs showed entirely non-proliferative and significantly higher rate of AP-positive cells and expression of self-renewal-specific genes than the adherent-cultured porcine SSCs. In addition, long-term culture of porcine SSCs in suspension condition induced significant decrease in the yield of AP staining-positive cells on post-day 10 of culture. These results showed that suspension culture was inappropriate to culture porcine SSCs, because the culture of porcine SSCs in suspension condition didn't stimulate proliferation and maintain AP activity of porcine SSCs, regardless of culture periods.

(Key words : Pig, Suspension culture, Spermatogonial stem cells, Proliferation, Undifferentiation)

INTRODUCTION

Spermatogonial stem cells (SSCs) can self-renew infinitely in the basal compartment of seminiferous tubules (Caires *et al*., 2010; De Rooij, 2001; Yang and Honaramooz, 2011) and produce functional sperm through spermatogenesis (Caires *et al*., 2010; De Rooij, 1998 2001; Dym, 1994). Moreover, with transferring useful genetic information to the next generation (Kanatsu-Shinohara, 2003 2004 2005; Oatley and Brinster, 2008), SSCs acquiring pluripotency by specific signals can differentiate into derivatives of the three embryonic germ layers (Caires *et al*., 2010; De Rooij, 1998 2001 2006; Li *et al*., 2010; Oatley and Brinster, 2012). Therefore, SSCs can be utilized usefully for the research, preservation and manipulation of male reproduction (Kubota and Brinster, 2006; Vlajković *et al*., 2012), cell-based therapies (Daley and Scadden, 2008), and tissue regeneration (Mineault and Batra, 2006).

Pigs have received great attention as an animal model for a variety of human diseases due to the physiological and genomic similarities with human (Aigner *et al*., 2010; Meurens *et al*., 2012; Fan and Lai, 2013). But, to date, generation of pig model with the specific human diseases has been not easy in spite of lots of the related researches (Aigner *et al*., 2010; Meurens *et al*., 2012). Accordingly, porcine SSCs have been regarded as an alternative making it possible to overcome this hurdle. Through culture, differentiation and engineering of porcine SSCs, genetic resources of valuable pigs possessing high performance in the economic traits can be eternally preserved and improved production of transgenic pigs can be possible using transgenic sperms differentiated from porcine SSCs engineered by knock-in or -out of the specific genes.

Generally, *in-vitro* culture of porcine SSCs have been conducted in adherent culture system using mitotically

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inactivated feeder cell- (Lee *et al.*, 2013; Han *et al.*, 2009; Yang and Honaramooz, 2011) or extracellular matrix protein-coated culture plate (Luo, 2006 2009). However, adherent culture system haven't showed effective maintenance of porcine SSC self-renewal for a short as well as long term and lots of researches have been progressed actively in order to overcome this barricade.

Although they can be justly used in cells adapted to suspension condition and a few other cell lines that are non-adhesive, suspension culture have a lot of advantages over adherent culture. The cells are uniformly bathed in the culture medium containing nutrient ingredients, external manipulation of components is possible, and handling of cells and cellular aggregates is rendered easy. Accordingly, we tried to identify the potential of suspension culture as culture system maintaining effectively self-renewal of porcine SSCs and develop suspension culture system by investigating proliferation and alkaline phosphatase (AP) activity of porcine SSCs suspension-cultured for a short and long term.

MATERIALS AND METHODS

Animal

Retrieval of testes from crossbred (Landrace×Yorkshire) or purebred (Yorkshire×Yorkshire) male piglets (from 1- to 4-day-old), kindly donated from Gumbo Inc. (Wonju, Korea), were conducted through routine castration surgery. The Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (IACUC approval No. KW-131106-1) approved all animal experimental procedures, which were conducted according to the Animal Care and Use Guideline of Kangwon National University.

Isolation of SSCs from Porcine Testes

Testes in ice-cold Dulbecco's phosphate-buffered saline (DPBS; Welgene Inc., Daegu, Korea) supplemented with 1% (v/v) antibiotic-antimycotic solution (Welgene) were transferred from a local farm (Gumbo Inc.) to our laboratory within 1 hour. The tunica albuginea and epididymis were removed from testis and the seminiferous tubules were digested by incubating at 37℃ for 15 min in high glucose Dulbecco's modified Eagle's medium (DMEM; Welgene) including 0.1% (w/v) type IV collagenase (Worthington Biochemical, Lakewood, CA). Dissociation of the fragmented seminiferous tubules were sequentially conducted at 37℃ for 10 min in a mixture of high glucose DMEM supplemented with 0.1% (w/v) hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) and 0.25% trypsin-EDTA (Welgene), respectively. Subsequently, myofibroblasts and Sertoli cells were eliminated from the dissociated cells through filtration using a 70-μm nylon mesh (SPL, Pocheon, Korea), and erythrocytes were removed from the dissociated and filtered cells by incubating for 15 min at room temperature in red blood cell lysis buffer (Sigma-Aldrich). 5×10^6 of isolated testicular cells plated on 0.1% (w/v) gelatin(Sigma-Aldrich)-coated 100-mm Petri dishes (SPL) were incubated for 16 hours in high glucose DMEM supplemented with 10% (v/v) heat-inactivated FBS (Welgene) and 1% (v/v) antibiotic-antimycotic solution at 37℃, and then the suspended SSCs were collected and counted using a hemocytometer.

Adherent Culture of Porcine SSCs

Isolated 4×10^5 porcine SSCs were plated on 2×10^5 STO feeder cells inactivated mitotically by the treatment of 10 μg/ml mitomycin C (Sigma-Aldrich), and cultured for 6 days in modified mouse embryonic stem cell culture medium (ESCCM) consisting of high glucose DMEM supplemented with 15% (v/v) heat-inactivated FBS, 0.1 mM β-mercaptoethanol (Gibco®, MD, USA), 1% (v/v) NEAA (Gibco®), 2 mM L-glutamine (Gibco®), 1% (v/v) antibiotic-antimycotic solution, 1,000 units/ml mouse leukemia inhibitory factor (mLIF; Chemicon International, Inc., Temecula, CA, USA), and 10 ng/ml glial cell-derived neurotrophic factor (GDNF; R&D Systems, Inc., Minneapolis, MN, USA) at 37℃ under 5 % CO_2 in a humidified air atmosphere. Replacement of fresh culture medium was conducted at 2 day intervals and the cultured porcine SSCs were counted using a hemocytometer.

Suspension Culture of Porcine SSCs

Isolated 5×10^5 porcine SSCs were inoculated in round tube (SPL) containing 1 ml modified mouse ESCCM and cultured for 5 or 20 days without any medium replacement at 37℃ under 5% CO_2 in a humidified air atmosphere. Then, the cultured porcine SSCs were collected and counted using a hemocytometer.

AP Staining

After fixing cells with 4% (v/v) paraformaldehyde (Junsei Chemical Co., Ltd., Chuo-ku, Japan), washing was conducted two times with DPBS and AP solution consisting of 0.1 M Tris buffer (pH 8.2) supplemented with 0.2 mg/ml naphthol AS-MX phosphate (Sigma-Aldrich), 2% (v/v) dimethyl formamide (Sigma-Aldrich), and 1 mg/ml Fast Red TR salt (Sigma-Aldrich) was treated for 90 min. Subsequently, the stained cells were rinsed two times with DPBS and the percentage of po-

Table 1. Oligonucleotide primers and PCR cycling conditions

Genes	GenBank number	Primer sequence		Size (bp)	Temp[a] (℃)
		Sense (5′>3′)	Anti-sense (5′>3′)		
GAPDH	NM_001206359.1	AGGGCTGCTTTTAACTCTGGCAA	GATGGTGATGGCCTTTCCATTG	180	60
OCT4	NM_001113060.1	CGCGAAGCTGGACAAGGAGA	CAAAGTGAGCCCCACATCGG	151	60
NANOG	NM_001129971.1	AACCAAACCTGGAACAGCCAGAC	GTTTCCAAGACGGCCTCCAAAT	158	60
EPCAM	NM_214419.1	CAATGCAGGGTCTACAGGCTGG	TGCATCTCGCCCATCTCCTTT	154	60
THY1	NM_001146129.1	GTGCTCTTGGGCACTGTGGG	TCTTGCTGGAGATGCTGGGC	178	60
UCHL1	NM_213763.2	TCCGGAAGACAGAGCAAAATGC	TCCGGAAGACAGAGCAAAATGC	150	60

[a] Temp=Temperature.

sitively stained cells was calculated using a hemocytometer.

Real-Time Polymerase Chain Reaction (PCR)

The extraction of total mRNA from cells was conducted using the Dynabeads mRNA Direct™ Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Reverse transcription of the extracted mRNA was performed using ReverTra Ace qPCR RT Master Mix with gDNA remover kit (Toyobo, Osaka, Japan). Subsequently, the 7,500 Real time PCR system (Applied Biosystems, Foster City, CA, USA) was used for PCR amplification and a Real-time PCR Master Mix (Toyobo) for quantifying the expression of specific genes. PCR specificity was identified by analyzing melting curve data and the specific genes expression was normalized by comparison to the *GAPDH* transcriptional level. Relative mRNA level was calculated as $2^{-\Delta Ct}$, where Ct=the threshold cycle for target amplification and $\Delta Ct=Ct_{target\ gene}$ (specific genes for each sample)$-Ct_{internal\ reference}$ (*GAPDH* for each sample). General information and sequences of primers designed with cDNA sequences derived from GenBank for pig and by Primer3 software (Whitehead Institute/MIT Center for Genome Research) are shown in Table 1.

Statistical Analysis

Statistical analysis using the Statistical Analysis System (SAS) program was conducted in all the numerical data presented in each experiment. When a significance of the main effects through variance (ANOVA) analysis in the SAS package was detected, comparison among each treatment was conducted by the least-square or DUNCAN method. Moreover, p values less than 0.05 were regarded as indicative of significant differences.

RESULTS

Effects of Suspension Culture on Self-Renewal of SSCs Derived from Porcine Neonatal Testis

In order to evaluate whether suspension culture can support maintenance of self-renewal in porcine SSCs or not, proliferation and AP activity of suspension-cultured porcine SSCs were analyzed and compared with those of adherent cultured porcine SSCs. As shown in Fig. 1, adherent cultured porcine SSCs showed significantly higher proliferation rate than suspension-cultured porcine SSCs. Furthermore, proliferation rate of adherent cultured porcine SSCs was more than 1, indicating proliferation of porcine SSCs, whereas proliferation rate of suspension-cultured porcine SSCs was less than 1, indicating non-proliferation or of porcine

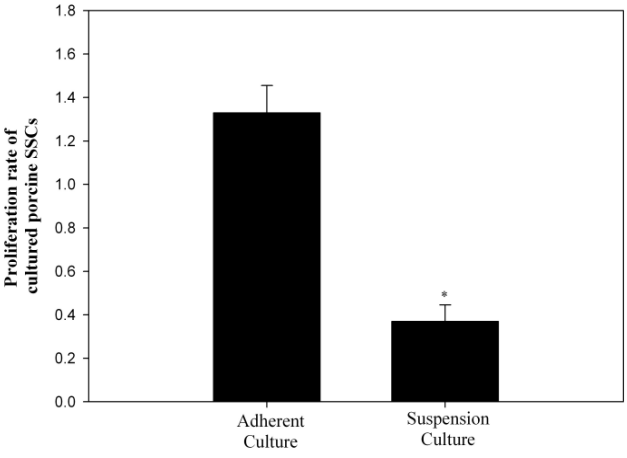

Fig. 1. Effects of suspension culture on proliferation of porcine spermatogonial stem cells (SSCs). In the modified mouse ESCCM, porcine SSCs retrieved from neonatal testis were adherent cultured for 6 days on STO feeder cells and suspension-cultured for 5 days in the round tube, respectively. Subsequently, cellular proliferation was calculated by dividing initial number of plated SSCs into total number of cultured SSCs. As the results, suspension-cultured porcine SSCs showed significantly lower proliferation rate than adherent cultured porcine SSCs. Error bars represent standard deviation (SD). n=3. $^{*}p<0.05$.

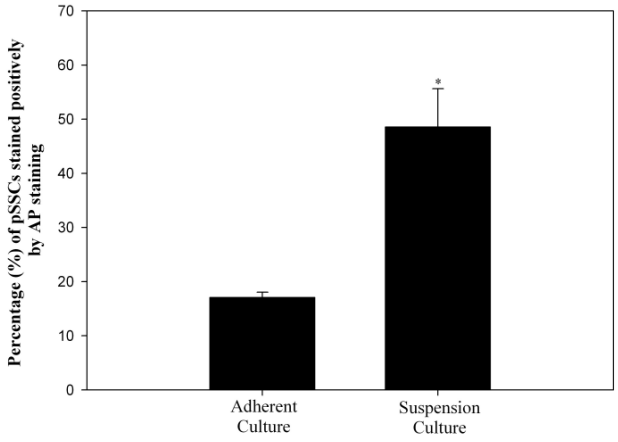

Fig. 2. Effects of suspension culture on alkaline phosphatase (AP) activity of porcine spermatogonial stem cells (pSSCs). In the modified mouse ESCCM, porcine SSCs retrieved from neonatal testis were adherent cultured for 6 days on STO feeder cells and suspension-cultured for 5 days in the round tube, respectively. Subsequently, for measuring AP activity, the percentage of the positively AP stained cells was counted using a hemocytometer. Suspension-cultured porcine SSCs showed significantly higher percentage of AP-positive cells than adherent cultured porcine SSCs. Error bars represent standard deviation (SD). n=3. $^{*}p<0.05$.

SSCs. In case of AP activity, suspension-cultured porcine SSCs showed significantly higher yield of positively stained porcine SSCs than adherent cultured porcine SSCs (Fig. 2). Moreover, significantly higher transcriptional expression of self-renewal-specific genes, *OCT4*, *NANOG*, *EPCAM*, *THY1* and *UCHL1*, than adherent cultured porcine SSCs was detected in suspension-cultured porcine SSCs (Fig. 3). From these results, we could identify that suspension culture of porcine SSCs was effective to maintain undifferentiation of, but not to stimulate proliferation of porcine SSCs.

Effects of Long-Term Suspension Culture on Self-Renewal of SSCs Derived from Porcine Neonatal Testis

Subsequently, in order to investigate whether porcine SSCs cultured for a long term in suspension condition keep on maintaining self-renewal characteristics or not, proliferation and AP activity in porcine SSCs were analyzed at 5 day intervals during 20 days of suspension culture, respectively. Entirely, suspension-cultured porcine SSCs showed non-proliferation and significant decrease of proliferation rate were observed from 10 days of culture (Fig. 4). Moreover, significantly the highest percentage of AP-positive cells was detected in porcine SSCs suspension-cultured for 5 days and yield of porcine SSCs stained positively by AP staining was decreased according to elongation of culture period (Fig. 5). These results demonstrated that

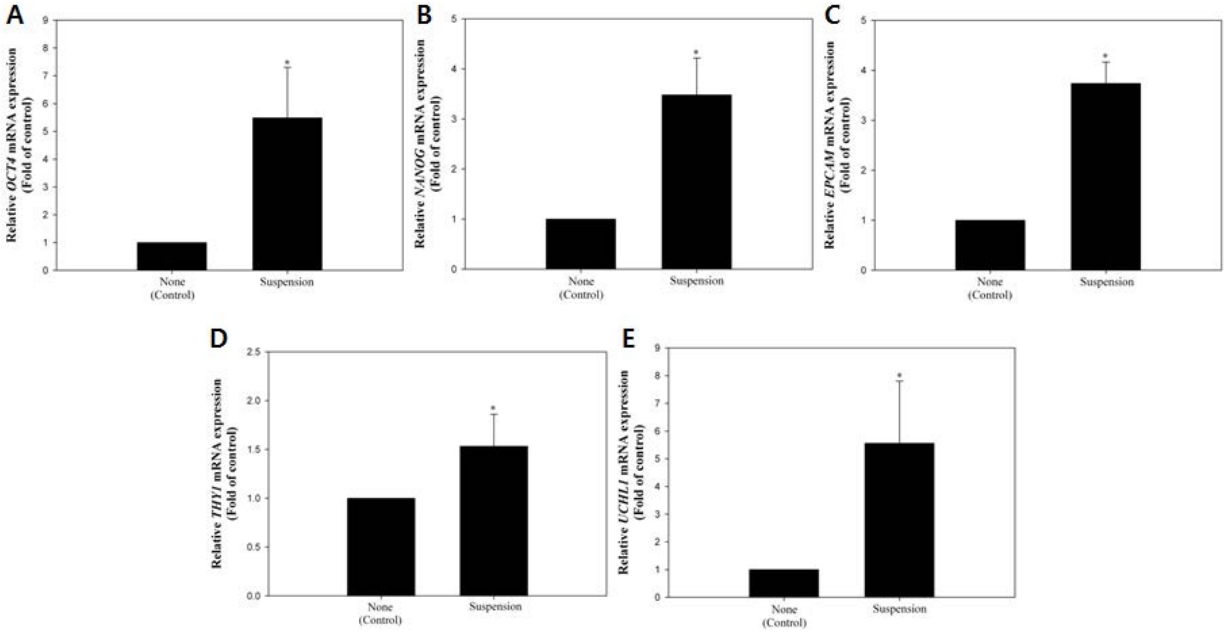

Fig. 3. Effects of suspension culture on transcriptional expression of self-renewal-specific genes in porcine spermatogonial stem cells (pSSCs). In the modified mouse ESCCM, porcine SSCs retrieved from neonatal testis were adherent cultured for 6 days on STO feeder cells and suspension-cultured for 5 days in the round tube, respectively. Subsequently, transcriptional levels of self-renewal-specific genes were analyzed by real-time PCR. As the results, suspension-cultured porcine SSCs showed significantly higher transcriptional level of *OCT4* (A), *NANOG* (B), *EPCAM* (C), *THY1* (D) and *UCHL1* (F) than adherent cultured porcine SSCs. Error bars represent standard deviation (SD). n=3. $^{*}p<0.05$.

proliferation and undifferentiation of porcine SSCs didn't be stimulated and maintained in spite of exposing porcine SSCs to suspension condition for a long term. Accordingly, we confirmed that suspension culture system was inappropriate to maintain self-renewal of porcine SSCs in *in-vitro* culture.

DISCUSSION

In this study, suspension culture entirely showed negative effects on proliferation of porcine SSCs and maintenance of porcine SSC self-renewal, indicating that suspension culture can't be used appropriately to proliferate and maintain undifferentiated porcine SSCs. However, interestingly, as shown in Fig. 2 and 3, AP activity and transcriptional expression of self-renewal-specific genes in porcine SSCs cultured for short time (5 days) were significantly increased by suspension culture, compared to adherent culture, indicating that undifferentiation of porcine SSCs for a short term was maintained better by suspension culture than by adherent culture. This may be resulted from the absence of extracellular signaling inducing differentiation into the specific lineage cells (De Rooij, 2006; Mineault and Batra, 2006; Lawson *et al*., 1999) in suspension condition. In the process of suspension culture, because no supporting cells like feeder cells usually used in the culture of embryonic stem cells are used, supply of differentiation factors, which may be produced in supporting cells, won't be happened. However, in case of adherent culture, because porcine SSCs were cultured on STO feeder cells, differentiation factors will be secreted from STO feeder cells and greatly stimulate differentiation of porcine SSCs. Accordingly, necessity of suspension culture system should be strongly requested in the porcine SSCs.

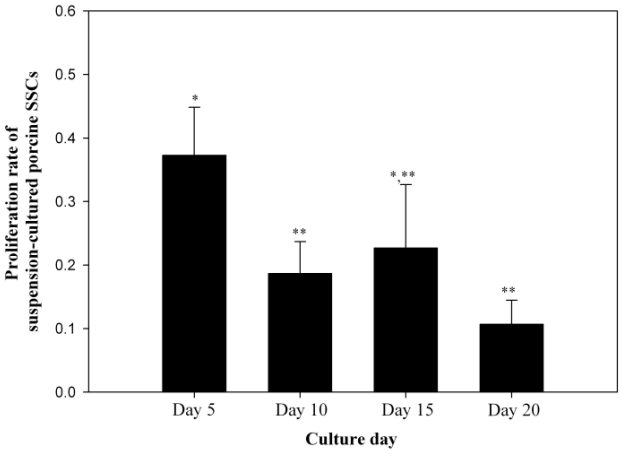

Fig. 4. Effects of long-term suspension culture on proliferation of porcine spermatogonial stem cells (pSSCs). Porcine SSCs isolated from neonatal testis were suspension-cultured for 5, 10, 15 and 20 in the round tube, respectively. Subsequently, cellular proliferation was calculated by dividing initial number of plated SSCs into total number of cultured SSCs. Porcine SSCs suspension-cultured for 5 days showed significantly the highest proliferation rate and significant decrease of proliferation rate in the suspension-cultured porcine SSCs was observed post-10 days of culture. Error bars represent standard deviation (SD). n=3. $^{*,**}p<0$.

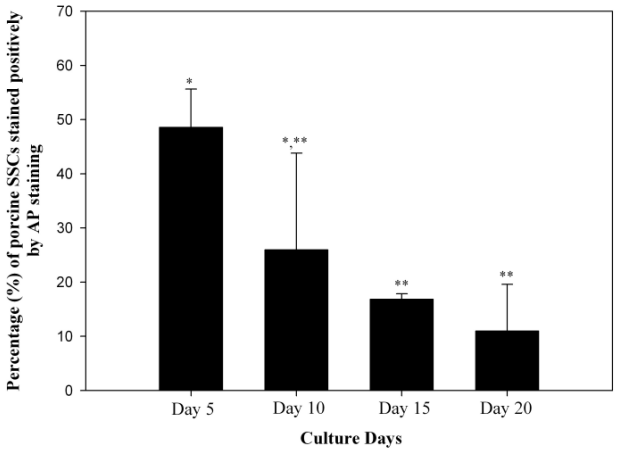

Fig. 5. Effects of long-term suspension culture on alkaline phosphatase (AP) activity of porcine spermatogonial stem cells (pSSCs). Porcine SSCs isolated from neonatal testis were suspension-cultured for 5, 10, 15 and 20 in the round tube, respectively. Subsequently, for measuring AP activity, the percentage of the positively AP stained cells was counted using a hemocytometer. Porcine SSCs suspension-cultured for 5 days showed significantly the highest percentage of AP-positive cells and decrease of yield of cells stained positively by AP staining in the suspension-cultured porcine SSCs was observed post-10 days of culture. Error bars represent standard deviation (SD). n=3. $^{*,**}p<0.05$.

As shown in Fig. 5, decrease of AP activity, a self-renewal-specific marker in porcine SSCs (Han *et al*., 2009; Lee *et al*., 2013), in the long-term suspension-cultured porcine SSCs was observed. This may be induced by quiescence of the suspension-cultured porcine SSC proliferation. In the previous reports, quiescence of the cellular proliferation representatively induced two events inside the cells as following: differentiation (De Rooij, 2001) and programmed cell death (De Rooij, 1998; Meng *et al*., 2002; Shinohara *et al*., 1999; Woodle and Kulkarni, 1998). Especially, quiescence of the cellular proliferation is very important in the process of stem cell differentiation (De Rooij, 1998). Cell differentiation is usually accompanied by irreversible cell-cycle exit, and regulatory molecules related with cell-cycle exit are exquisitely regulated during differentiation process. With the onset of differentiation, occurrence of the cell-cycle exit in G1 phase is mediated through up-regulating CDK inhibitor proteins,

activating the RB protein family and inactivating E2F proteins (Cobrinik, 1993; Heuvel S van den, 2005). As well, various differentiation inducers such as p21WAF1, p27KIP1, p130 and E2F4 proteins play a pivotal role in cell cycle arrest and differentiation induction (Liu *et al*., 2003; Litovchick *et al*., 2007; Polyak *et al*., 1994; Weinberg and Denning, 2002). Accordingly, studies on regulatory mechanism of the above mentioned regulatory molecules in the suspension-cultured porcine SSCs should be additionally conducted in order to overcome a limitation of suspension culture.

In addition, as shown in Fig. 4, total number of suspension-cultured porcine SSCs was gradually decreased according to elongation of culture time. This may be induced by apoptosis resulted from quiescence of the cellular proliferation. Moreover, this prediction was indirectly supported by identifying increase of dead cells within the suspension-cultured porcine SSCs according to elongation of culture time (not shown date). Additionally, for direct certification, we need to investigate ratio of apoptotic cells within the suspension-cultured porcine SSCs in the following.

In conclusion, if proliferation of porcine SSCs in the suspension condition can be stimulated by combining the specific factors stimulating cellular proliferation (Pennock and Wang, 2003), extracellular matrix (ECM) proteins activating cellular downstream signaling or ECM-coated microbeads mimicking cellular adhesion with suspension culture system (Barrila *et al*., 2010; Geckil *et al*., 2010), adverse problems of suspension culture presented in the study will be able to be overcome successfully. For these, additional studies for advancing suspension culture system of porcine SSCs should be consistently conducted in the future and successful development of suspension culture system supporting *in-vitro* culture of porcine SSCs will greatly contribute to conducting a variety of researches using porcine SSCs.

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