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Efficient Derivation and Long Term Maintenance of Pluripotent Porcine Embryonic Stem-like Cells*

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ABSTRACT : Porcine embryonic stem (ES) cells have a great potential as tools for transgenic animal production and studies of regulation of differentiation genes. Although several studies showed successful derivation of porcine ES-like cells, these cells were not maintained long-term in culture. Therefore, this study was conducted to establish porcine pluripotent ES-like cells using *in vivo* fertilized embryos and to maintain these cells in long term culture. Porcine ES-like cells from *in vivo* embryos obtained by immuno-surgery or whole explant culture were successfully cultured for over 56 passages. Morphology of porcine ES-like cells was flat-shaped with a monolayer type colony. These cells stained for alkaline phosphatase throughout the culture. Furthermore, porcine ES-like cells reacted with antibodies against Oct-4, SSEA-1, SSEA-4, Tra-1-60, and Tra-1-81, which are typical markers of undifferentiated stem cells. To characterize the ability of porcine ES-like cells to differentiate into three germ layers, embryoid body formation was induced. After plating of these cells, porcine ES-like cells were spontaneously differentiated into various cell types of all three germ layers. In addition, porcine ES-like cells were successfully derived from IVF blastocysts in media containing human recombinant basic fibroblast growth factor. (**Key Words :** Porcine, Embryonic Stem Cells, Pluripotent, IVF, Basic Fibroblast Growth Factor)

INTRODUCTION

Embryonic stem (ES) cells are undifferentiated and pluripotent cells established either from intact blastocysts (Evans et al., 1981) or isolated inner cell masses (ICMs) (Martin, 1981; Thomson et al., 1998) of preimplantation embryos. ES cells have distinct characteristics such as self-renewal and pluripotency, differentiating into all three germ layer and germ cell lineages (Kondoh et al., 1999). The successful isolation of pluripotent stem cells from livestock has provided a powerful tool for genetic manipulation in agricultural species, the study of genetic regulation and function, and the generation of animal models for human diseases (Kokron et al., 1997; Shastry, 1998). Although few studies have examined the pluripotent stem cells in pigs and other domestic animals, stem-like cells have been isolated from various species (Dyce et al., 2004). The report about porcine ES cells and epiblast cell culture were started in 1990's. In previous studies, stem cells isolated from pigs are ES-like cells (Talbot et al., 1993b; Li et al., 2003; Li et al., 2004a) derived from the ICM of blastocysts and can differentiate *in vitro* into cells representative of endodermal, neuroectodermal and mesodermal tissues. Therefore, these porcine ES-like cells were pluripotent (Talbot et al., 1996; Talbot et al., 2001; Talbot et al., 2002, Xu et al., 2007). However, when porcine ES-like cells were previously isolated and characterized, the optimal culture conditions were not determined and, more importantly, they were not able to be maintained long term *in vitro*. Also, the most common methods to determine the characteristics of porcine ES-like cells were morphological analysis and some markers such as alkaline phosphatase (AP) activity and *oct-4* expression. However, various pluripotential markers used in mouse and human ES cells were not well characterized in porcine ES-like cells. Moreover, the culture condition used

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in porcine ES-like cells were mostly adapted from those used in mouse and human ES cells and also not optimized for efficient derivation and long term maintenance.

The ES cells of mouse and human can be basically cultured on murine fibroblast feeder layers with cytokine, leukemia inhibitory factor (LIF) and basic fibroblast growth factor (bFGF), respectively. Also, previous studies have developed a similar procedure for the establishment of porcine ES-like cells which consists of co-culture with feeder cells and cytokines. The most common supplements used to establish and maintain ES-like cells in pigs include LIF (Shim et al., 1997; Saito et al., 2003; Li et al., 2004a), bFGF (Li et al., 2003; Li et al., 2004a), stem cell factor (SCF) (Saito et al., 2003), and various combination of these supplements (Piedrahita et al., 1998; Li et al., 2003; Tsung et al., 2003), or no supplemental growth factors (Chen et al., 1999; Mueller et al., 1999). However, none of these studies were demonstrated in long term culture of porcine ES-like cells while maintaining undifferentiated state.

Derivation of ES cells from *in vitro* produced (IVP) embryos *in vitro* fertilization (IVF) or somatic cell nuclear transfer) has huge potential in stem cell biotechnology and as genetic analysis tools, as well as in animal biotechnology to produce transgenic animals. Also, outbreed large animal such as pigs would be a good model for the application of human ES cells in therapeutic purposes. However, ES cells derived from IVP embryos were scant study, since IVP embryos have fewer cell numbers and lower quality compared to *in vivo* embryos (Han et al., 1999). Therefore, it is required to develop efficient derivation and maintenance conditions to establish ES cells from IVP embryos.

In the present study, we attempted to establish and maintain pluripotent porcine ES-like cell lines derived from *in vivo* blastocysts and analyzed their characteristics using various pluripotent stem cell markers as well as differentiation markers. Also, we tried to develop an efficient culture condition for the derivation of porcine ES-like cells from *in vitro* fertilized embryos using various media conditions.

MATERIALS AND METHODS

All materials were obtained from Invitrogen-Gibco BRL (Carlsbad, CA, USA) unless otherwise stated. This study was conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, published by the Consortium for Developing a Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (1988).

Collection of porcine *in vivo* blastocysts

Porcine blastocysts were collected by flushing the uterus on day 8 of the estrus cycle (estrus = day 0) with phosphate buffered saline (PBS) containing 0.4% bovine serum albumin (BSA, Fraction V; Sigma, St. Louis, MO, USA) and 3% Antibiotic-Antimycotic.

Production of porcine *in vitro* fertilized blastocysts

Porcine blastocysts were produced using the methods described in a previous study (Lee et al., 2007). Cumulus-oocyte complexes were collected from prepubertal gilt ovaries and matured *in vitro*. Embryos at the four-cell stage were produced by culturing embryos for 2 days after *in vitro* fertilization (IVF). After removal of the zona pellucida with acid tyrode solution (Sigma), zona pellucida-free four-cell stage embryos were cultured to the blastocyst stage.

Porcine ES-like colony formation

In vivo produced blastocyst were cultured in porcine ES (PES) medium (50:50 mixture of Dulbecco's modified Eagle's medium (DMEM, low glucose) and Ham's F10 medium supplemented with 15% fetal bovine serum (FBS; collected and processed in Canada; Hyclone, Logan, UT, USA), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% MEM nonessential amino acids, 1% Antibiotic-Antimycotic containing cytokines, 40 ng/ml human recombinant SCF (hrSCF; R&D Systems, Minneapolis, MN, USA), 20 ng/ml human recombinant bFGF (hrbFGF; R&D Systems), and 10 ng/ml human recombinant LIF (hrLIF; Chemicon, Temecula, CA, USA). *In vivo* fertilized porcine embryos were cultured in DMEM/F10 (50% low glucose DMEM and 50% F10) or DMEM/F12 (50% high glucose DMEM and 50% F-12) with 15% FBS (Hyclone) or 20% Knockout serum replacement (KSR), 1% nonessential amino acids, 1.7 mM L-glutamine, 1% antibiotic-antimycotic, and 0.1 mM β-mercaptoethanol on a mytomycin-C (Roche, Mannheim, Germany) inactivated murine embryonic fibroblast (MEF) feeder layer. The culture medium supplemented with various combinations of cytokines, 1,000 units of hrLIF (Chemicon), 40 ng/ml hrSCF (R&D Systems), and 20 ng/ml hrbFGF (R&D Systems). Cultures were maintained in a humidified environment with 5% CO_2 in air at 37°C.

Porcine embryos were seeded whole, or inner cell mass cells were obtained by immuno-surgery and seeded. After 3-7 days, the compact ES-like colony was mechanically dissociated into several pieces and seeded on new feeder cells. The cultures were monitored daily and the culture medium was changed every other day. Porcine ES-like cells showing typical morphology were sub-passaged by physical separation. The colonies were expanded by routine passage every 5-7 days.

Embryoid body formation and *in vitro* differentiation

Evaluation of the differentiation of porcine ES-like cells

was conducted by spontaneously prolonged culture and in hanging drops as embryoid bodies (EBs). EBs were cultured in DMEM medium (high glucose) supplemented with 15% FBS (Hyclone), 2 mM L-glutamine, and 1% Antibiotic-Antimycotic without cytokines. After 5 days, the EBs were transferred to dishes and cultured for 5 days. Later, the EBs were dissociated by 0.25% trypsin-EDTA and replated on a 12-well plate.

Alkaline phosphatase (AP) activity

After culture plates were rinsed twice in PBS, cells were fixed in 4% formaldehyde in PBS for 15 min at room temperature. After washing, fixed cells were stained using nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3 indolylphosphate toluidine salt (BCIP) stock solution (Roche) in buffer solution for 30 min at room temperature.

Immunocytochemistry

Fixed cells were rinsed twice with BSA (1 mg/ml) in PBS (PBS/BSA). Endogenous peroxidase activity was quenched by incubating for 5 min with 0.3% hydrogen peroxide. Cells were then rinsed twice in PBS/BSA. A blocking solution of PBS/BSA with 10 goat serum (Sigma) was applied for 30 min. The cells were then incubated with one of the following primary antibodies: SSEA1 (Catalog Number MAB4301; Chemicon), SSEA4 (MAB4304; Chemicon), Tra 1-60 (MAB4360; Chemicon), Tra 1-81 (MAB4381; Chemicon), Oct-3/4 (sc-9081; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), neurofilament (MAB1615; Chemicon), cardiac troponin 1 (MAB3152; Chemicon), and α fetoprotein (V7049; Biomeda Corp., Foster City, CA, USA). Primary antibodies were diluted to 2 μg/μl for IgMs (SSEA1, Tra 1-60, and Tra 1-81) and 1 μg/μl for IgGs (SSEA4 and Oct-4) in PBS/BSA. Primary antibodies were applied to the samples and incubated at 4°C overnight. The next day, the samples were rinsed three times with PBS/BSA. Secondary antibodies were applied and incubated at room temperature for 1 h. The secondary antibody for all reactions was DakoCytomation LSAB 2-system HRP (Dako, Glostrup, Denmark). Samples were rinsed three times with BSA/PBS, and 3-3`diminobenzidine (DAB; Vector Laboratories, Burlingame, CA, USA) was used as the indicator.

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

Total RNA was isolated from cells using TRIZOL® reagent (Invitrogen-Gibco BRL) according to the manufacturer's instructions. First, 2 μg total RNA per 20 μl reaction mixture was converted to complementary cDNA using an M-MLV Reverse Transcriptase Kit (Epicentre Biotechnologies, Madison, WI, USA). The amplification reaction mixture contained 1 μl cDNA, 1 μl of each primer,

Table 1. Establishment and long-term maintenance of porcine embryonic stem-like cells derived from *in-vivo* blastocysts

Serial number of blastocysts seeded[a]	Initial culture methods	Attached to feeder cells[b]	Established porcine ES-like cell Line[c]	Final number of passages[d]
1	Immuno-surgery	○	×	ND
2	Immuno-surgery	○	○	>13[e] (pESC2*)
3	Immuno-surgery	×	×	ND
4	Immuno-surgery	○	○	>17[e] (pESC4*)
5	Whole explant	○	○	>56[f] (pESC5*)
6	Immuno-surgery	×	×	ND
7	Whole explant	×	×	ND
8	Whole explant	○	○	3[g] (pESC8*)
9	Immuno-surgery	○	○	>29[e] (pESC9*)
10	Whole explant	○	○	>24[e] (pESC10*)
11	Whole explant	○	×	ND
12	Whole explant	○	×	ND
13	Whole explant	×	×	ND
14	Whole explant	×	×	ND
15	Whole explant	×	×	ND
16	Whole explant	×	×	ND
Total		9/16 (56%)	6/9 (67%)	

[a] The blastocysts were collected at 8 days of pregnancy.

[b] Determined by firm attachment to the feeder cells for at least 2 days. Percentage of blastocysts seeded.

[c] Determined by outgrowth of inner cell mass cells and ability to survive after passage to new feeder cells. Percentage of blastocysts attached to feeder cell.

[d] Porcine ES-like cells were passaged by mechanical dissociation of the colonies and plated on freshly prepared feeder cells.

[e] Presently frozen. [f] Presently being cultured. [g] Differentiated and disappeared.

* pES name was according to serial number of blastocysts seeded. ○: Positive result. ×: Negative result. ND: Not determined.

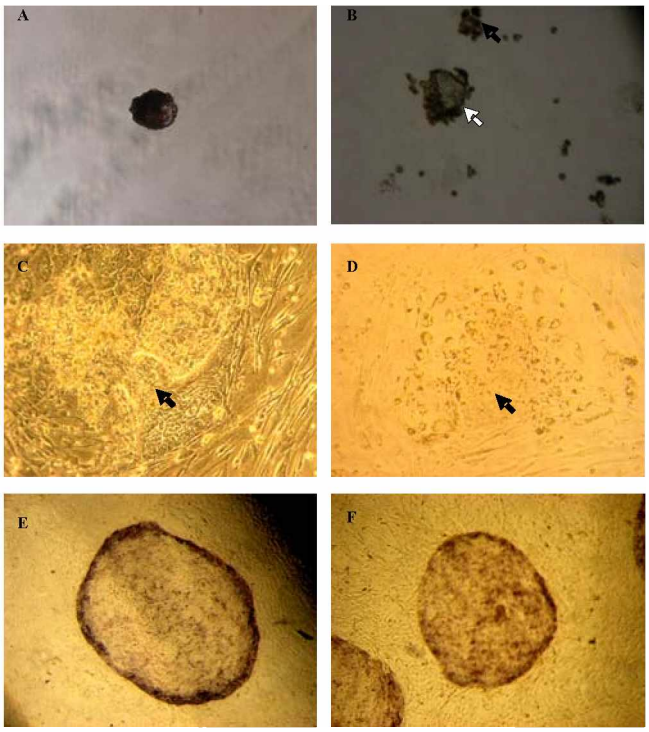

Figure 1. Isolation and morphology of porcine embryonic stem-like cells derived from *in vivo* produced blastocysts. (A) *In vivo* derived porcine blastocysts at 8 days (estrus = day 0; ×100). (B) Inner cell masses (black arrow) and trophoblst (white arrow) isolated by immuno-surgery (×200). The embryos and isolated inner cell masses were attached to mouse embryonic fibroblast feeder layer. (C), (D): A primary outgrowth of the attached embryos (C) and inner cell mass (D) was composed of trophoblast-like giant cells and inner cell mass-derived small cells (black arrows) (×100). (E), (F): Porcine ES-like cells in initial period of culture with typical flat, monolayered morphology and alkaline phosphatase activity (×40). The colonies were derived from whole embryo explants (C), (E) or inner cell masses isolated by immuno-surgery (D), (F).

10 μl of i-MaxII 2×PCR Master Mix (iNtRON Biotechnology, Seoul, Korea), and 7 μl distilled water. Thermal cycling conditions were 5 min at 94°C, then 35 cycles of 30 s at 94°C, 30 s at the 60°C, and 30 s at 72°C. After PCR, electrophoresis was performed on 1.0% agarose gel with ethidium bromide and visualized under ultraviolet illumination.

The primers used in this experiment were *oct-4* (5`-, AACGATCAAGCAGTGACTATTCG 3`- GAGTACAGGG TGGTGAAGTGAGG, 153 bp); *nanog* (5`- AATCTTCAC CAATGCCTGAG, 3`- GGCTGTCCTGAATAAGCAGA, 164 bp), and porcine *β-actin* (5`- GTGGACATCAGGAAG GACCTCTA, 3`- ATGATCTTGATCTTCATGGTGCT, 137 bp).

Karyotype analysis

Porcine ES-like colonies were cultured in PESC medium on 4-well plate without feeder layers. After 2days, the cells were incubated with 15 μl of colcemid (KARYO MAX® COLCEMID® Solution) for 24 h in a humidified environment of 5% CO_2 in air at 37°C. The cells were washed with PBS, trypsinized for single cell and then the pellets were treated with hypotonic solution (1% sodium citrate, Sigma) for 30 min at room temperature. The cells were fixed with Methanol-Acetic acid (1:1). And next, the cell suspension was put onto slide glass and broken by the drop of Methanol-Acetic acid. The slide with chromosome spreads were stained in 20% Giemsa staining solution (Sigma) for 5 min, rinsed, and observed under microscope of 1,000 magnifications with oil immersion.

RESULTS AND DISCUSSION

Derivation and long term culture of porcine ES-like cells

Sixteen *in vivo* produced porcine embryos were used to isolate ES-like cells (Table 1). All *in vivo* embryos were cultured with PES medium. Primary colonies were formed by 56% of the ICMs obtained using immuno-surgery or whole blastocysts. Six porcine ES-like cell lines out of 9 attached blastocysts were isolated from ICMs or whole blastocysts. Since the number of samples was low, it is difficult to determine whether the immuno-surgery or whole explant culture was better in initial culture of porcine ES-like cells. However, porcine ES-like cells were derived by both methods. Porcine ES-like cells in other studies were cultured from ICMs by immuno-surgery (Wianny et al., 1997; Chen et al., 1999) or the enzyme-treatment method (Li et al., 2003). In previous studies, isolation of ICMs was a very important step in the establishment of ES cells (Li et al., 2003; Li et al., 2004a). However, our results indicated that porcine ES-like cells isolated from intact blastocysts can be cultured. Primary colonies produced from immuno-surgery and whole embryo explants had similar morphology (Figure 1E and F). The porcine ES-like cells from whole explant cultures were successfully cultured for over 56 passages (Table 1). Whole embryos explant culture was also effective in derivation of porcine ES-like cells using *in vitro* fertilized blastocysts (Table 2).

All porcine ES-like cells were derived and cultured with inactivated murine embryonic fibroblasts (MEFs) as feeder cells, and the feeder layer is one of the most important factors affecting the derivation and culture of ES cells (Li et al., 2004b). MEFs are used to maintain ES cells from many animals (Brook et al., 1997; Amit et al., 2000; Li et al., 2004b), and every one to two passages, porcine ES-like cells were cultured in freshly prepared MEF, which is critical for the secretion of several cytokines (Smith et al., 1988; Li et al., 2003).

Attached blastocysts consisted of trophoblast-like giant cells, and compact clumps of small cells, which is

Table 2. Effect of culture media on the derivation of porcine embryonic stem-like cells from *in vitro* fertilized blastocysts

Medium	Number of blastocysts seeded	Number of Blastocysts attached (%)[a]	Number of colonies formed (%)[b]	Number of lines over passage 5 (%)[c]	Final number of passages
A	37	23 (62)	0 (0[d])	0 (0)	0
B	29	21 (72)	3 (11)	1 (33)	>15
C	42	28 (67)	2 (7)	2 (100)	>15
D	27	20 (74)	0 (0[d])	0 (0)	0
E	35	24 (69)	6 (25[e])	2 (33)	13
F	27	18 (67)	3 (5[d])	0 (0)	4
Total	197	134 (68)	14 (10)	5 (36)	

A: DMEM/F10 with 15% FBS. B: DMEM/F10 with 15% FBS, hrSCF, hrbFGF, and hrLIF. C: DMEM/F10 with 15% FBS and hrbFGF.
D: DMEM/F12 with 15% FBS. E: DMEM/F12 with 15% FBS and hrbFGF. F: DMEM/F12 with 20% KSR and hrbFGF.
[a] Percentage of blastocysts cultured.
[b] Percentage of blastocysts attached. Values with different superscripts (d or e) are significantly different ($p<0.05$).
[c] Percentage of colonies formed.
Experiments were repeated three times.

outgrowth of inner cell masses (Figure 1C and D). To avoid spontaneous differentiation by surrounding giant cells, the small cell clump was sub-cultured onto new MEF feeder cells. The morphology of most colonies was flat and monolayered, unlikely with typical mouse ES cell morphology but similar to that of human ES cells, and showed alkaline phosphatase (AP) activity (Figure 1E and F). We established five ES-like cell lines, excluding pESC8 because it was differentiated and disappeared after three passages. Four lines were frozen and one line (pESC5) was cultured for over 56 passages.

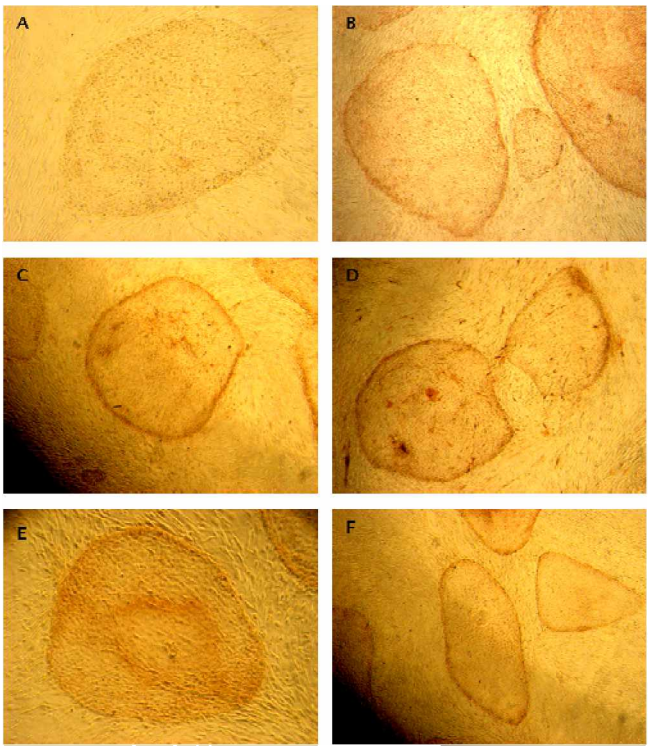

Figure 2. Expression of pluripotential stem cell markers in porcine embryonic stem-like cells. The general morphology of porcine ES-like cells were similar to that of human ES cells, which are large, flat and single layered. However, unlikely with mouse and human ES cells, the margins of porcine embryonic stem-like cells were more compact and had stronger staining activity than the inner cell portion. (A) Negative control lacking a primary antibody. Porcine ES cells were positive for OCT-4 (B), SSEA-1 (C), SSEA-4 (D), Tra1-60 (E), and Tra1-81 (F) at passage 8. (×40)

Characterization of porcine ES-like cells

Morphology and AP staining are the most common methods defining pluripotent ES cells. Cytochemical staining for AP is a marker of undifferentiated pluripotent ES and EG cells in pigs (Talbot et al., 1993a). The porcine ES-like cells derived from both *in vivo* and *in vitro* produced embryos were positive for AP staining (Figures 1E, F, and 6).

The pESC5 line was tested for the expression of undifferentiated and pluripotent stem cell markers, Oct-4, SSEA-1, SSEA-4, Tra-1-60, and Tra1-81 (Figure 2). Immunocytochemical analysis of porcine ES-like cells showed positive staining for Oct-4 (Figure 2B), SSEA-1 (Figure 2C), SSEA-4 (Figure 2D), Tra1-60 (Figure 2E), and Tra1-81 (Figure 2F) antibodies. Specificity for each antibody was tested in the absence of its respective primary antibody (Figure 2A). In previous studies, a few markers were tested in porcine ES-like cells, such as AP or Oct-4 (Li et al., 2003; 2004a,b). Our results were more similar to those of study with porcine EG cell lines, in terms of expression of SSEA-1 and -4 (Tsung et al., 2003). The antigens Tra-1-60 and Tra1-81 are expressed in human ES cells (Thomson et al., 1998; Reubinoff et al., 2000; Rose, 2002), but not expressed in mouse ES cells. In this study, we demonstrated that the antigens Tra-1-60 and Tra1-81 were expressed in porcine ES-like cells Therefore, these markers could be used as stem cell markers for porcine pluripotent cells. The expression of pluripotent cell related genes such as *oct-4* (Dyce et al., 2004; Kues et al., 2005) and *nanog* in porcine ES-like cells was assayed by RT-PCR (Figure 3). The *oct-4* and *nanog* mRNAs were expressed

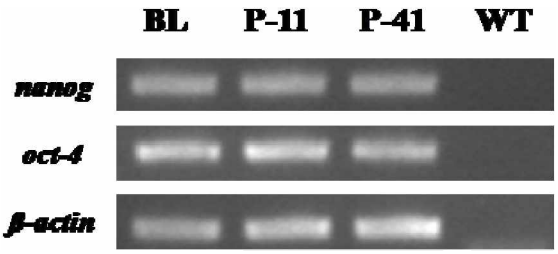

Figure 3. Expression of pluripotent marker genes in early (P-11) and late passage (P-41) porcine embryonic stem-like cells by RT-PCR. Transcripts of pluripotent marker genes, *oct-4* (153 bp) and *nanog* (164 bp), were detected in porcine ES-like cells. *β-actin* (137 bp), a house-keeping gene, served as an internal control. The porcine blastocysts (BL) were used as positive control. WT: water blank.

not only in cells of low passage (passage 11) but also in later passage (passage 41). Therefore, porcine ES-like cells could be maintained in long term without losing their pluripotency. To verify the stability of porcine ES-like cells maintained in log term culture, karyotype analysis was performed at 16th and 41st passages on the pESC5. They showed a normal karyotype with 38 chromosomes (Figure 4). Therefore, porcine ES-like cells could be maintained in long term without losing genetic stability. In previous studies, porcine ES-like cells were successful derived but hard to maintained in culture for long period (Li et al., 2003; Li et al., 2004a; Li et al., 2004b). In this study, we showed that porcine ES-like cells derived from *in vivo* embryos could be maintained in long term keeping their pluripotent marker expression and genetic stability.

The pESC5 line at passage 29 was trypsinized and cells were cultivated in hanging drops of medium containing 15% FBS without cytokines. After 5 days, pESC5 cells

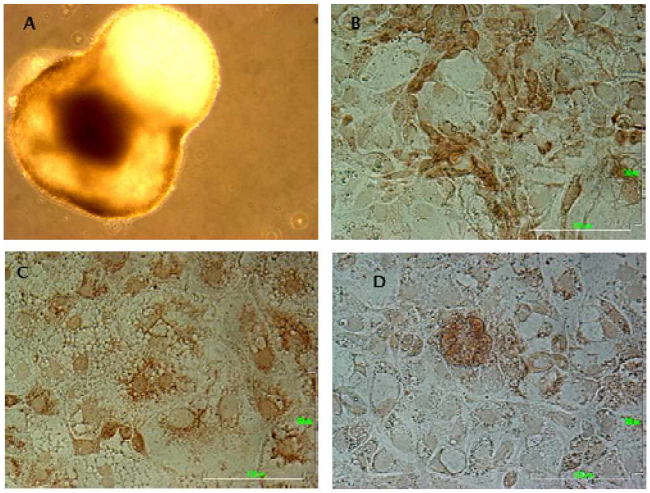

Figure 5. *In vitro* differentiation of porcine embryonic stem-like cells. (A) Cystic embryoid body (EB, ×100). After culture of dissociated cells from EBs, spontaneously differentiated cells were examined by immunocytochemical staining with antibodies specific for each three germ layer. The cells were positive for neurofilament (B) as an ectoderm marker, α-fetoprotein (C) as an endoderm marker, and cardiac troponin-1 (D) as a mesoderm marker (×400). Porcine ES-like cells were spontaneously differentiated over 25 days at passages 29.

formed embryoid bodies (EBs) and they were eventually differentiated into cystic EBs (Figure 5A). It is well known that EBs contains tissues from all three germ layers: endoderm, mesoderm, and ectoderm (Shigetoyo et al., 1976). Five days later, EBs were dissociated by trypsin-EDTA and re-plated on culture dishes. These cells then cultured to induce spontaneous differentiation for 15 days were stained for the marker of each three germ layers, as evidenced by immunohistochemical staining with

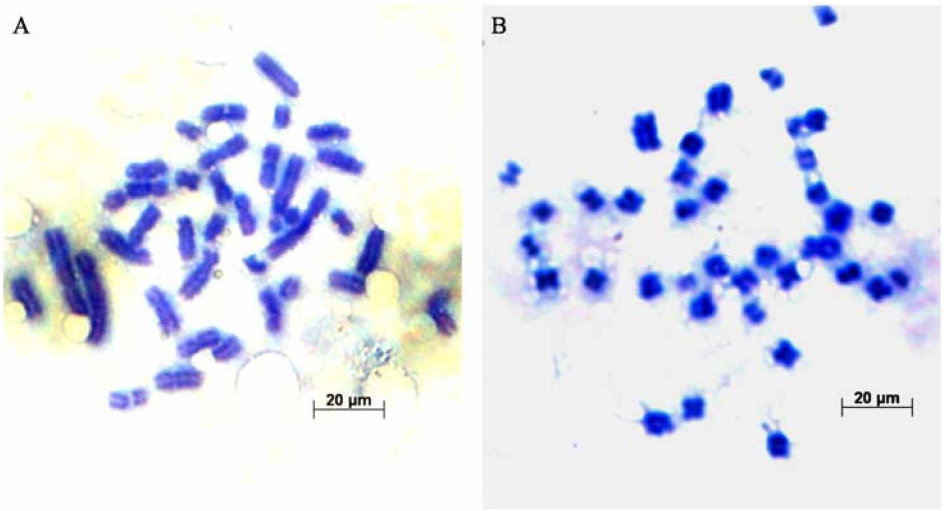

Figure 4. Chromosome spreads of porcine embryonic stem-like cells in long-term culture. Normal (38 chromosomes) spreads from a porcine ES-like cell line at passage 16 (A), and at passages 41 (B).

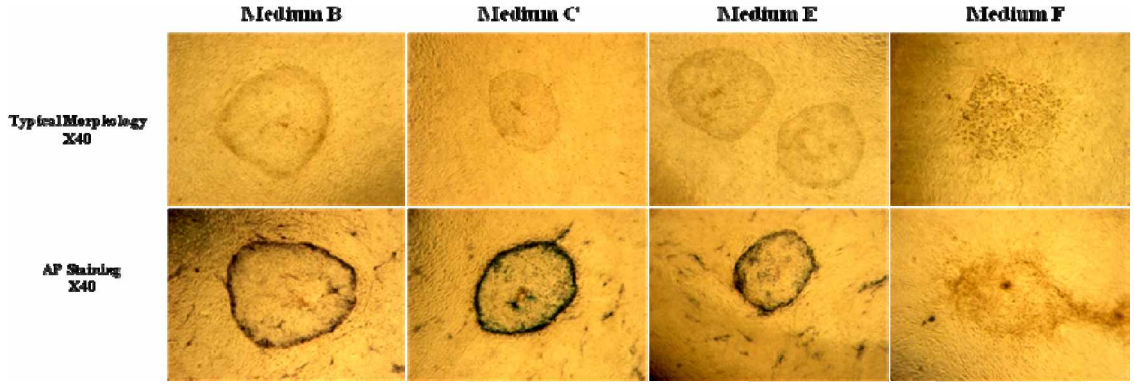

Figure 6. Morphology and alkaline phosphatase activity of porcine embryonic stem-like cells derived from *in vitro* fertilized blastocysts in different type of culture medium. The porcine ES-like cell lines derived from IVF embryos were established in media containing cytokines. The porcine ES-like colonies in medium B, C, and E showed compact shape and were positive for alkaline phosphatase (AP). However, colonies in medium F showed loose shape and were negative for AP. These colonies were gradually disappeared in passage 4. Medium B: DMEM/F10 with 15% FBS, hrSCF, hrbFGF, and hrLIF. Medium C: DMEM/F10 with 15% FBS and hrbFGF. Medium E: DMEM/F12 with 15% FBS and hrbFGF. Medium F: DMEM/F12 with 20% KSR and hrbFGF.

antibodies to neurofilament (ectoderm, Figure 5B), α-fetoprotein (endoderm, Figure 5C), and cardiac troponin I (mesoderm, Figure 5E). Therefore, porcine ES-like cells could differentiate into cells of three germ layers and demonstrated their ability to differentiation *in vitro*. These results were in consistent with other studies with porcine ES-like cells (Chen et al., 1999; Li et al., 2003; Li et al., 2004a; Li et al., 2004b).

Derivation of porcine ES-like cells from *in vitro* fertilized embryos

When isolating ES cells, the quality of blastocysts is one of the most important factors (Kim et al., 2007). Therefore, *in vivo* produced embryos showed better efficiency in derivation of ES cells than *in vitro* produced embryos, such as IVF and SCNT. This could be caused by lower in quality of the embryos in terms of total cell number and the ratio of ICM to trophectoderm (TE) cells than *in vivo* derived embryos (Machaty et al., 1998; Han et al., 1999). Moreover, isolating ICM cells by immuno-surgery could induce further damage to the embryos. Therefore, optimization of culture method and conditions is necessary for the derivation of ES cells by whole explant culture using *in vitro* produced embryos.

As shown in Table 2, 197 IVF porcine embryos were seeded as whole explant and cultured in medium A (DMEM/F10 with 15% FBS), B (PES medium; DMEM/F10 with 15% FBS, hrSCF, hrbFGF, and hrLIF), C (DMEM/F10 with 15% FBS and hrbFGF), D (DMEM/F12 with 15% FBS), E (DMEM/F12 with 15% FBS and hrbFGF), or F (DMEM/F12 with 20% KSR and hrbFGF). Since we speculated that porcine ES-like cell is more close to human ES cells than mouse ES cells in respect of general morphology and culture behavior. Therefore, we would like to compare the types of medium commonly used in porcine pluripotent cell culture (DMEM/F10) (Piedrahita et al., 1998) and in human ES cell culture (DMEM/F12) (Thomson et al., 1998). In addition, the cytokines used in pluripotent stem cell culture, (LIF, SCF, bFGF) were also assessed for their effects in derivation and culture of porcine ES-like cells.

While 68% of blastocysts were attached to feeder cells after 2 days, only 10% of the embryos formed primary colonies (Table 2). These results demonstrated the lower efficiency in derivation of porcine ES-like cells using IVF embryos in our previous study (Kim et al., 2007) than using *in vivo* produced embryos (Table 1). However, when comparing different type of media, all media containing basic fibroblast growth factor (bFGF) (medium B, C, E, F) showed better results than the other media (medium A, D). In human ES cells, bFGF supports undifferentiated cell growth without a conditioned medium (Xu et al., 2005; Dvorak et al., 2006; Levenstein et al., 2006). In addition, supplementation of FBS (medium D) showed better efficiency than KSR (medium E), which commonly used in human ES cell culture in xeno-free condition. Media B and C (DMEM/F10 based media) were suitable for the derivation and maintaining porcine ES-like cells from IVF embryos, although medium E (DMEM/F12 based medium) was the most efficient in colony formation. As a result, supplementation of FBS and bFGF in the medium could support the initial culture and establishment of porcine ES-like cells derived from IVF embryos. Even though their similarity to human ES cells, the porcine ES-like cells would be better in culture when used the medium designed for porcine pluripotent cell culture (DMEM/F10 based media). The morphology of porcine ES-like cells derived from IVF embryos was also round, flat and monolyered,

same porcine ES-like cells from *in vivo* embryos. These cell lines also showed AP activities (Figure 5). However, in medium F (with KSR and bFGF), the colonies were negative for AP and gradually disappeared at passage 4. Media containing bFGF produced primary colonies.

CONCLUSION

The results of this study indicated that porcine ES-like cells could be established not only from *in vivo* embryos but also from *in vitro* produced embryos by whole explant culture. Moreover, porcine ES-like cell lines from *in vivo* embryos could be maintained in long term (over 56 passages) without losing their pluripotency and differentiation ability. When using *in vitro* produced embryos, bFGF and FBS were needed to form pES-like colonies and maintain it over passage 5. The types of basic medium, DMEM/F10 or DMEM/F12, did not affect the formation and maintenance of IVF pES-like cells. Further studies are required to demonstrate their ability to differentiation *in vivo* system, such as teratoma formation and chimeric pig production.

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