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Effects of *in vitro* Culture Period of Reconstructed Embryos and Genetic Background of Feeder Cells on Establishment of Embryonic Stem Cells Derived from Somatic Cell Nuclear Transfer Blastocysts in Pigs

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ABSTRACT The establishment of porcine embryonic stem cells (ESCs) from porcine somatic cell nuclear transfer (SCNT) blastocysts is influenced by *in vitro* culture day of porcine reconstructed embryo and feeder cell type. Therefore, the objective of the present study was to determine the optimal *in vitro* culture period for reconstructed porcine SCNT embryos and mouse embryonic fibroblast (MEF) feeder cell type for enhancing colony formation efficiency from the inner cell mass (ICM) of porcine SCNT blastocysts and their outgrowth. As the results, porcine SCNT blastocysts produced through *in vitro* culture of the reconstructed embryos for 8 days showed significantly increased efficiency in the formation of colonies, compared to those for 7 days. Moreover, MEF feeder cells derived from outbred ICR mice showed numerically the highest efficiency of colony formation in blastocysts produced through *in vitro* culture of porcine SCNT embryos for 8 days and porcine ESCs with typical ESC morphology were maintained more successfully over Passage 2 on outbred ICR mice-derived MEF feeder cells than on MEF feeder cells derived from inbred C57BL/6 and hybrid B6CBAF1 mice. Overall, the harmonization of porcine SCNT blastocysts produced through *in vitro* culture of the reconstructed embryos for 8 days and MEF feeder cells derived from outbred ICR mice will greatly contribute to the successful establishment of ESCs derived from porcine SCNT blastocysts.

Keywords: embryonic stem cells, genetic background of feeder cells, *in-vitro* culture period, pig, somatic cell nuclear transfer

INTRODUCTION

The pigs are considered as useful biomedical models and embryonic stem cells (ESCs) can self-renew and differentiate into three germ layers (Cha et al., 2018; Zhang et al., 2019). Therefore, ESCs derived from the inner cell

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mass (ICM) of porcine blastocysts have been applied in numerous applications, such as drug screening, cell therapy, and transgenic pig production (Kim and Hyun, 2016; Baek et al., 2017; Choi et al., 2019). To date, researchers have been tried to establish ESCs derived from porcine blastocysts for active uses of ESCs and efforts have been mainly performed using blastocysts produced *in vivo* due to higher quality of blastocysts produced *in vivo* than those of produced *in vitro* (Son et al., 2009; Nakamura et al., 2017; Lee et al., 2018). However, procedures for *in vivo* generation of porcine blastocyst are laborious, costly, and time-consuming (Han et al., 2019). Therefore, for establishment of porcine ESCs, the usages of *in vitro* produced porcine blastocysts through *in vitro* fertilization (IVF) or somatic cell nuclear transfer (SCNT) have been preferred to overcome obstacles which encountered during the production of *in vivo*-derived blastocysts (Hou et al., 2016). Despite these efforts, the establishment efficiency of ESCs derived from blastocysts generated from *in vitro* produced porcine embryos remains very low.

Various factors, including blastocyst age, feeder cell type, ICM isolation method, and culture medium, have been examined to improve ESC establishment methods (Brevini et al., 2007; Vackova et al., 2007; Haraguchi et al., 2012). A previous study reported that ICM derived from blastocysts cultured *in vitro* for 7 days maintained pluripotency (Tan et al., 2012). However, late growth and degeneration were observed in colonies derived from ICM of early-stage blastocyst (Chen et al., 1999). By contrast, ICM derived from blastocysts cultured *in vitro* for longer than 9 days did not exhibit pluripotency (Tan et al., 2012), and rapid differentiation was observed in ICM of late-stage blastocyst (Wianny et al., 1997). These results suggest that an appropriate blastocyst stage should be determined for the successful establishment of ESC.

Feeder cells are routinely used as an attachment matrix for ESC establishment and maintenance (Llames et al., 2015; Siriboon et al., 2015) because they secrete unknown factors that support proliferation and inhibit differentiation (Yang et al., 2016; Li et al., 2017). Because these unknown factors secreted from feeder cells differ according to the genetic background of feeder cells (Talbot et al., 2012), preferred genetic background of feeder cells is dependent on ESC lines (Brevini et al., 2012; Park et al., 2015; Yang et al., 2016). Therefore, optimization of genetic background of feeder cell is also essential for successful establishment of ESC.

Based on these knowledge, determination of appropriate blastocyst stage and genetic background of feeder cells is required for enhancing establishment efficiency of ESCs derived from porcine SCNT blastocysts. The objective of the present study was to establish a culture system by determining the appropriate blastocyst stage and genetic background of feeder cell to enhance the establishment efficiency of ESCs derived from porcine SCNT blastocysts. For these, attachment to feeder cells and colony formation were monitored in ICM of porcine SCNT blastocysts cultured *in vitro* for 7 or 8 days. Porcine SCNT blastocysts showing the highest feeder cell attachment and colony formation efficiency were then cultured in mouse embryonic fibroblast (MEF) feeder cells derived from three mouse strains, and the optimal strain of MEF feeder cell was determined by comparing the efficiencies in the attachment of porcine SCNT blastocysts to feeder cells and the formation of colony derived from ICM of porcine SCNT blastocysts.

MATERIALS AND METHODS

Animals

As mouse embryonic fibroblast (MEF) donors, fetuses derived from 13.5-day pregnant female ICR (DBL, Eumseong, Korea) crossed with male ICR (DBL), C57BL/6 (Nara Biotech, Seoul, Korea) crossed with male C57BL/6 (Nara Biotech), and C57BL/6 (Koatech, Pyongtaek, Korea) crossed with male CBA (Koatech) mice were used. Porcine ovaries were obtained from prepubertal gilts at a local abattoir. All animal housing, handling, and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (IACUC approval no. KW-170131-1) and conducted according to the Animal Care and Use Guidelines of Kangwon National University.

Retrieval of cumulus-oocyte complexes (COCs) and *in vitro* maturation (IVM)

In the ovaries, COCs were aspirated from superficial follicles using an 18-guage needle. Next, COCs with multiple layers of unexpanded cumulus cells were justly selected and washed three times in HEPES-buffered Tyrode's medium (TLH) supplemented with 0.05% (w/v) polyvinyl alcohol (PVA; Sigma-Aldrich, St. Louis, MO). Culture of the

washed COCs in a four-well culture dish (Nunc, Roskilde, Denmark) containing 500 μL of IVM medium consisting of medium-199 (M-199; Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) porcine follicular fluid, 1 μg/mL insulin (Sigma-Aldrich), 10 ng/mL epidermal growth factor (EGF, Sigma-Aldrich), 0.6 mM cysteine (Sigma-Aldrich), 0.91 mM pyruvate (Sigma-Aldrich), 75 μg/mL kanamycin (Sigma-Aldrich), 10 IU/mL human chorionic gonadotropin (hCG; Intervet International BV, Boxmeer, Holland) and 80 μg/mL follicle-stimulating hormone (FSH; Antrin R-10, Kyoritsu Seiyaku, Tokyo, Japan) was conducted for 22 h at 39°C with 5% CO_2. Subsequently, the *in vitro* maturated COCs were washed three times with hormone-free IVM medium and additionally cultured in hormone-free IVM medium for 20 h at 39°C with 5% CO_2.

Preparation of donor cells

As donor cells, a newborn piglet-derived fetal fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) with the nutrient mixture F-12 (Invitrogen) and 15% (v/v) fetal bovine serum (FBS, Invitrogen) until a complete monolayer of cells. Subsequently, the donor cells were synchronized at the G0/G1 stage of the cell cycle by inducing contact inhibition for 72-96 h and dissociated with 0.05% trypsin-EDTA (Invitrogen). The dissociated donor cells were resuspended in TLH supplemented with 0.4% (w/v) bovine serum albumin (BSA; Sigma-Aldrich) before nuclear transfer.

Generation of somatic cell nuclear transfer (SCNT) embryos

After IVM, cumulus cells were removed from COCs by repeated pipetting in TLH supplemented with 0.4% (w/v) BSA and 0.1% (w/v) hyaluronidase (Sigma-Aldrich). Subsequently, the cumulus cell-free oocytes were stained in calcium-free TLH supplemented with 0.4% (w/v) BSA and 5 μg/mL Hoechst 33342 (Sigma-Aldrich) for 15 min at 39°C with 5% CO_2 and washed twice with calcium-free TLH supplemented with 0.4% (w/v) BSA. The washed oocytes were transferred to a drop of calcium-free TLH supplemented with 0.4% (w/v) BSA and 5 μg/mL cytochalasin B (Sigma-Aldrich) under mineral oil (Sigma-Aldrich). Next, the first polar body and metaphase II (MII) chromosomes of oocytes were aspirated using a 17 μm beveled pipette (Humagen, Charlottesville, VA) under an epifluorescence microscope (IX73; Olympus, Tokyo, Japan) for generation of enucleated oocytes, and a single donor cell was inserted into the perivitelline space of each enucleated oocyte. After then, oocyte-cell couplets were placed on a fusion electrode chamber (NepaGene, Chiba, Japan) overlaid with 280 mM mannitol solution (Sigma-Aldrich) supplemented with 1 μM $CaCl_2$ (Wako pure chemical industries, Osaka, Japan) and 50 μM $MgCl_2$ (Wako pure chemical industries) and exposed to an alternating current of 2 V cycling at 1 MHz for 2 sec, and two pulses of 175 V/mm direct current (DC) for 30 μs using a cell fusion generator (LF101; NepaGene). Successful fusion of oocyte-cell couplets was investigated under a stereomicroscope and incubation of fused oocyte-cell couplets was conducted for 30 min in calcium-free TLH supplemented with 0.4% (w/v) BSA. After 30 min of incubation, SCNT oocytes were activated by exposing to two DC pulse of 120 V/min for 60 μs in a 280 mM mannitol solution supplemented 0.1 mM $CaCl_2$ and 0.05 mM $MgCl_2$.

In vitro culture of SCNT embryos

For culturing SCNT embryos, 0.34 mM trisodium citrate (Sigma-Aldrich), 2.77 mM myo-inositol (Sigma-Aldrich), and 10 μM β-mercaptoethanol (Sigma-Aldrich) were supplemented into porcine zygote medium (PZM)-5 described previously (Yamanaka et al., 2009) (herein referred to as the modified PZM-5). Moreover, 10% (v/v) FBS was supplemented into the modified PZM-5 (herein referred to as the PZM-5F). The electrically activated SCNT embryos were incubated for 4 h in the modified PZM-5 supplemented with 0.4 μg/mL demecolcine (Sigma-Aldrich) and 2 mM 6-dimethylaminopurine (6-DMAP; Sigma-Aldrich). After washing with the modified PZM-5, the washed SCNT embryos were cultured for 96 h in 30 μL of the modified PZM-5 droplets (10-15 embryos/droplet) covered with mineral oil at 39°C in 5% CO_2, 5% O_2, and 90% N_2 and then the SCNT embryos were additionally cultured for 72 h or 96 h in 30 μL of the modified PZM-5F droplets (10-15 embryos/droplet) covered with mineral oil at 39°C in 5% CO_2, 5% O_2, and 90% N_2. Subsequently, the produced SCNT blastocysts were allocated to the following experiments.

Preparation of MEF feeder cells

Uterus obtained from pregnant mice at embryonic day 13.5 were transferred to a Petri dish containing Dulbecco's phosphate-buffered saline (DPBS; Welgene Inc., Daegu,

Korea). Organs of fetuses were discarded, and the remainder of fetal tissues were minced with razor blades. Then, the minced tissues were digested with 0.25% trypsin-EDTA (Welgene) for 10 min at 37°C. Non-digested fibroblasts were discarded using 70-μm nylon mesh (SPL Life Sciences, Pocheon, Korea), and the digested MEFs were washed with MEF culture medium consisting of DMEM (Welgene) supplemented with 10% (v/v) heat-inactivated FBS (Welgene) and 1% (v/v) antibiotic-antimycotic solution (Welgene). Subsequently, the purified MEFs were cultured in MEF culture medium at 37°C under 5% CO_2, until 90% confluency, and the fresh culture medium was changed at 2-day intervals. To inhibit proliferation of MEFs, confluent MEFs at Passage 1 were inactivated in MEF culture medium containing 10 μg/mL mitomycin C (Sigma-Aldrich) for 3 h at 37°C and detached with 0.05% trypsin-EDTA (Welgene). The inactivated MEFs were plated in four-well culture plate (SPL Life Sciences) coated with 0.1% (w/v) gelatin (Sigma-Aldrich) and provided as a feeder layer.

In vitro outgrowth of ICM of SCNT blastocysts

The three types of culture medium were used to induce formation of colonies derived from ICM of SCNT blastocysts. Alpha-MEM-based medium consists of α-MEM medium (Gibco, Carlsbad, CA) supplemented with 10% (v/v) knockout serum replacement (KSR; Invitrogen), 0.05 mM β-mercaptoethanol (Gibco), 1% (v/v) nonessential amino acids (NEAA; Gibco), 40 ng/mL EGF (PeproTech, Inc., Rocky Hill, NJ), 10 μL/mL 100x insulin-transferrin-selenium (ITS; Gibco), 1000 U/mL mouse leukemia inhibitory factor (mLIF; Chemicon International, Inc., Temecula, CA), 20 ng/mL basic fibroblast growth factor (bFGF; PeproTech, Inc.,), and 1% (v/v) antibiotic-antimycotic solution. DMEM/Ham's F-10-based medium consists of 1:1 low-glucose DMEM (LG-DMEM; Welgene):Ham's F-10 (Gibco) medium supplemented with 15% (v/v) heat-inactivated ES cell-screened FBS (Hyclone, Logan, UT), 0.2 mM β-mercaptoethanol, 1% (v/v) NEAA, 20 ng/mL bFGF and 1% (v/v) antibiotic-antimycotic solution. Mixture medium consists of a 50:50 mixture of μ-MEM-based medium and DMEM/Ham's F-10-based medium.

To induce colony formation derived from ICM of porcine SCNT blastocysts, the zona pellucida (ZP) of SCNT blastocysts was mechanically removed by insulin syringe under a stereomicroscope (Olympus), and ZP-free SCNT blastocysts were cultured for 7 days on mitotically inactivated MEF feeder cells derived from fetuses of outbred ICR, inbred C57BL/6, or hybrid B6CBAF1 mice in α-MEM-based medium at 37°C under an atmosphere of 5% CO_2 in air (Passage 0). The fresh culture medium was replaced daily. Subsequently, the outgrown ICM were dissociated mechanically using glass capillary pipettes, and the detached clumps were reseeded and cultured on fresh mitotically inactivated MEF feeder cells for 4 days in mixture medium at 37°C under an atmosphere of 5% CO_2 in air (Passage 1). Under the same condition as Passage 1, the following subculture was conducted. Formation of colonies from ICM of SCNT blastocysts and morphology of colonies were observed under an inverted microscope (CKX41; Olympus).

Statistical analysis

The Statistical Analysis System (SAS) software (SAS Institute Inc, Cary, NY) was used for analyzing all numerical data in each parameter. Comparisons among treatment groups were conducted using a generalized linear model (PROC-GLM) in the SAS package. The less than 0.05 of p value was regarded as a statistically significant difference.

RESULTS

Firstly, to determine the optimal embryo culture period to produce porcine SCNT blastocysts leading effectively to formation of colonies from ICM of blastocysts, the reconstructed porcine SCNT embryos were *in vitro* cultured for 7 or 8 days. Then, SCNT blastocysts were cultured for 7 days on MEF feeder cells derived from ICR mice. As shown in Table 1, *in vitro* culture period of reconstructed embryos had no significant effect on attachment efficiency of porcine SCNT blastocyst. However, colony formation was significantly improved in the ICM of porcine SCNT blastocysts cultured *in vitro* for 8 days, compared to those cultured for 7 days.

Subsequently, to elucidate the effects of MEF feeder cell type on the formation and outgrowth of colonies derived from the ICM of porcine SCNT blastocysts, porcine SCNT blastocysts generated through *in vitro* embryo culture for 8 days were further cultured for 7 days on MEF feeder cells derived from outbred ICR, inbred C57BL/6, and hybrid B6CBAF1 mice, respectively. As the results, numerically the highest efficiency of attachment and colony formation was observed in outbred ICR mice (Table 2).

Table 1. Comparison of attachment to feeder cells and formation of colonies between blastocysts produced by *in vitro* culture of porcine SCNT embryos for 7 and 8 days

In-vitro culture day of porcine SCNT embryos	No. of SCNT blastocysts seeded	No. (%)[c] of	
		SCNT blastocysts attached to feeder cells	Colonies formed successfully from ICM
7 days[a]	62	46 (74.19)	23 (37.10)[d]
8 days[b]	56	40 (71.43)	31 (55.36)[e]

Model effects of treatments in each parameter, which is indicated as the p value, were 0.7385 and 0.0473 in the number of blastocysts attached to feeder cells and the number of colonies formed successfully from ICM.

PZM: Porcine zygote medium, ICM: Inner cell mass.

[a]SCNT blastocysts were generated through sequential culture of porcine SCNT embryos for 96 h in the modified PZM-5 and for 72 h in the PZM-5F.

[b]SCNT blastocysts were generated through sequential culture of porcine SCNT embryos for 96 h in the modified PZM-5 and for 96 h in the PZM-5F.

Zona pellucida of generated blastocysts were removed mechanically and subsequent cultured for 7 days on mitotically inactivated MEF feeder cells derived from outbred ICR mice in α-MEM-based medium.

[c]Percentage of the number of SCNT blastocysts seeded.

[d,e]Values in the same column with different superscript letters are significantly different, $p < 0.05$.

Table 2. Determination of genetic background of feeder cells stimulating *in-vitro* maintenance of colonies formed from ICM of blastocysts produced through *in vitro* culture of porcine SCNT embryos for 8 days

Types of genetic background of feeder cells	No. of SCNT blastocysts seeded	No. (%)[a] of		
		SCNT blastocysts attached to feeder cells	Colonies formed successfully from ICM	Colonies cultured over Passage 2
ICR	90	57 (63.33)	26 (28.89)	3 (3.33)
C57BL/6	72	44 (61.11)	18 (25.00)	0 (0.00)
B6CBAF1	71	43 (60.56)	20 (28.17)	0 (0.00)

Model effects of treatments in each parameter, which is indicated as the p value, were 0.9286, 0.8501 and 0.09 in the number of blastocysts attached to feeder cells, the number of colonies formed successfully from ICM, and the number of colonies cultured over Passage 2.

PZM: Porcine zygote medium, ICM: Inner cell mass.

Zona pellucida of SCNT blastocysts generated through sequential culture of porcine SCNT embryos for 96 h in the modified PZM-5 and for 96 h in the PZM-5F were removed mechanically and subsequent cultured for 7 days on mitotically inactivated MEFs feeder cells derived from outbred ICR, inbred C57BL/6, and hybrid B6CBAF1 mice in α-MEM-based medium.

[a]Percentage of the number of SCNT blastocysts seeded.

Notably, among 26 colonies formed from the ICM of porcine SCNT blastocysts cultured on MEF feeder cells derived from outbred ICR mice, three colonies with well-defined boundaries and flattened morphology (Fig. 1) were successfully outgrown and consistently maintained over Passage 2 (Table 2). However, long-term maintenance of colonies outgrown from the ICM of porcine SCNT blastocyst was unsuccessful on MEF feeder cells derived from outbred ICR mice. On the other hand, outgrown colony maintained over Passage 2 was not observed from the ICM of porcine SCNT blastocysts cultured on MEF feeder cells derived from inbred C57BL/6 or hybrid B6CBAF1 mice (Table 2). Thus, MEF feeder cells derived from outbred ICR mice were optimal for the formation and outgrowth of colonies derived from the ICM of porcine SCNT blastocysts.

Thus, the formation and outgrowth of colonies derived from the ICM of porcine SCNT blastocysts can be effectively stimulated by *in vitro* culture of porcine SCNT embryos for 8 days and subsequent culture of the resulting porcine SCNT blastocysts on MEF feeder cells derived from outbred ICR mice. These findings represent an essential step toward the establishment of ESCs from porcine SCNT blastocyst.

DISCUSSION

Successful derivation of ESCs from porcine SCNT blastocysts depends on the quality of porcine SCNT blastocysts and the harmonization of cellular niche derived

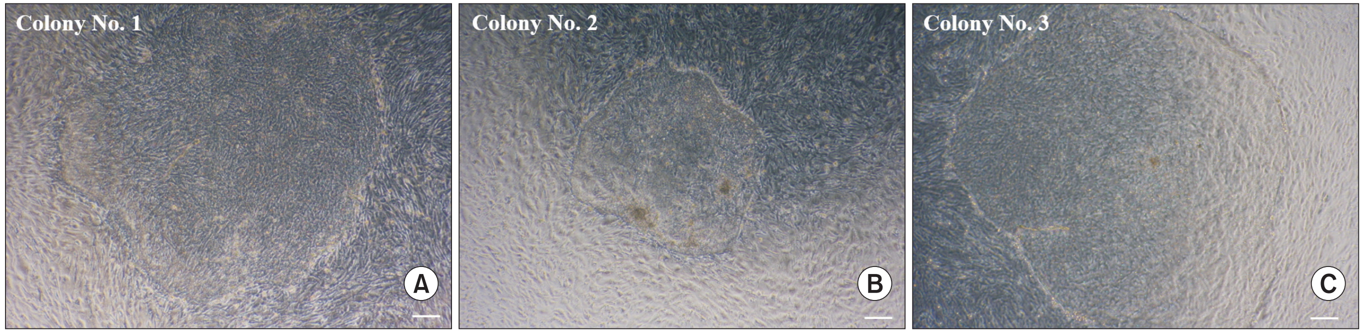

Fig. 1. Morphologies of *in vitro* maintained colonies over Passage 2 post-formation of colonies from inner cell mass (ICM) of blastocysts produced through *in vitro* culture of porcine somatic cell nuclear transfer (SCNT) embryos for 8 days. Blastocysts were generated through sequential culture of porcine SCNT embryos for 96 h in modified PZM-5 medium and for 96 h in PZM-5F. Zona pellucida-free porcine SCNT blastocysts were cultured for 7 days on mitotically inactivated mouse embryonic fibroblast (MEF) feeder cells derived from outbred ICR mice in α-MEM-based medium. Outgrowth colonies were mechanically dissociated into several clumps, which were then cultured for 4 days in the mixture medium, and subculture was conducted under the same conditions. All colonies subcultured until Passage 2 showed well-defined boundaries and flattened morphology (A-C). Scale bar, 200 μm.

from feeder cells with ICM of porcine SCNT blastocysts. Therefore, we attempted to develop a culture technique for producing blastocysts with enhanced efficiency in the colony formation from reconstructed porcine SCNT embryos and determine the genetic origin of cellular niche stimulating formation and proliferation of colonies from ICM of porcine SCNT blastocysts. As the results, the best formation efficiency and maintenance of colonies were observed when porcine SCNT blastocysts produced *in vitro* for 8 days were cultured on cellular niche derived from outbred ICR MEFs. Accordingly, this study demonstrates that the quality of porcine SCNT blastocysts can be upgraded through *in vitro* culture for 8 days and the genetic origin of cellular niche supporting adaptation of ICM derived from porcine SCNT blastocysts to *in-vitro* environment is genetic background derived from outbred ICR mouse.

The number of cells in blastocysts affect not only embryo quality but also formation of colonies derived from ICM in the generation of ESCs. A previous study reported that lengthening the *in vitro* culture period of reconstructed embryo resulted in an increase in the total cell number of porcine SCNT blastocysts (Tan et al., 2012). In our results, formation efficiency of colonies derived from ICM was significantly enhanced in porcine SCNT blastocysts produced through *in vitro* culture of the reconstructed porcine SCNT embryos for 8 days, compared to those for 7 days (Table 1). Therefore, these improvement of efficiency in the formation of colonies from porcine SCNT blastocysts may result from higher total cell number of porcine SCNT blastocysts produced through *in vitro* embryo culture for 8 days than those for 7 days.

Generally, feeder cells regulate self-renewal of ESCs by secreting cytokines or unknown factors produced by feeder cells (Nii et al., 2014; Guo et al., 2018), which are transported by microtubules to extracellular fluid (Li et al., 2004). Thus, the status of microtubules in feeder cells can be used as a selective marker for quality of feeder cell. Previous research demonstrated that porcine embryonic fibroblast (PEFs) and MEF feeder cells increasing efficiency of attachment and colony formation in the establishment of ESCs from porcine blastocysts showed microtubules with long length, widespread distribution, and increased number, compared to STO feeder cells reducing efficiency of attachment and colony formation in the establishment of ESCs from porcine blastocysts (Li et al., 2004). As shown in Table 2 and Fig. 1, compared to MEF feeder cells from inbred C57BL/6 and hybrid B6CBAF1 mice, MEF feeder cells derived from outbred ICR mice showed higher attachment efficiency and colony formation, and stronger *in vitro* maintenance of colonies with typical porcine ESC morphology over Passage 2. Therefore, we suggest that the higher competence of MEF feeder cells derived from outbred ICR mice may result from strong or continuous secretion of cytokines or unknown factors which may result from microtubule characteristics including higher abundance, longer length, and more widespread distribution in the cytoplasm of ICR mice-derived MEF feeder cells.

In conclusion, as a step toward developing a novel sys-

tem for ESC establishment, we developed a culture technique for producing blastocysts with enhanced efficiency in the colony formation from reconstructed porcine SCNT embryos and stimulating colony formation of ICM derived from porcine SCNT blastocysts. A combination of these techniques will contribute greatly to improving the efficiency of the establishment of ESCs derived from porcine SCNT blastocysts.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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AUTHOR CONTRIBUTIONS

NRH and SB designed carried out most of the experiments in this research. YL and JL generated SCNT blastocysts. Moreover, JIY and EL analyzed and discussed the results. The manuscript was written by NRH and STL who supervised the research.

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