

## Original Article

# Establishment of *In-Vitro* Culture System for Enhancing Production of Somatic Cell Nuclear Transfer (SCNT) Blastocysts with High Performance in the Colony Formation and Formation of Colonies Derived from SCNT Blastocysts in Pigs

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**ABSTRACT** Although somatic cell nuclear transfer (SCNT)-derived embryonic stem cells (ESCs) in pigs have great potential, their use is limited because the establishment efficiency of ESCs is extremely low. Accordingly, we tried to develop *in-vitro* culture system stimulating production of SCNT blastocysts with high performance in the colony formation and formation of colonies derived from SCNT blastocysts for enhancing production efficiency of porcine ESCs. For these, SCNT blastocysts produced in various types of embryo culture medium were cultured in different ESC culture medium and optimal culture medium was determined by comparing colony formation efficiency. As the results, ICM of porcine SCNT blastocysts produced through sequential culture of porcine SCNT embryos in the modified porcine zygote medium (PZM)-5 and the PZM-5F showed the best formation efficiency of colonies in  $\alpha$ -MEM-based medium. In conclusion, appropriate combination of the embryo culture medium and ESC culture medium will greatly contribute to successful establishment of ESCs derived from SCNT embryos.

**Keywords:** alpha-minimum essential medium, embryonic stem cells, pig, porcine zygotic medium, somatic cell nuclear transfer

## INTRODUCTION

The pigs have been considered as an ideal animal model suitable to human medical studies (Walters and Prather, 2013) because they have similar characteristics like humans in the aspect of anatomy, physiology, immunology, and genomics (Baek et al., 2017). Therefore, they have

been widely applied to applications such as drug efficacy evaluation (Singh et al., 2016), protein-based drug production (Whyte and Prather, 2011), xenotransplantation (Fischer et al., 2016), humanized models (Perleberg et al., 2018), and cell therapy (Rubessa et al., 2017). However, the specific applications such as drug screening, transgenic pig production or cell therapy requires very delicate

and precise manipulation at the cell level, and embryonic stem cells (ESCs) with self-renewal and pluripotency can be an alternative for these. Simultaneously, application range of pigs can be extended by establishing the ESCs derived from pig.

Genetic background of pigs which are classified with diverse breed are distinguished (Mujibi et al., 2018) and has not been widely studied. Therefore, porcine ESCs established from a variety of breed can be utilized as sources to compare specific genetic characteristics between breeds. To date, researchers have used embryos produced in *in-vivo* to generate porcine ESC lines and a few porcine ESC lines have been reported (Son et al., 2009; Cha et al., 2018). Although acquisition of high-qualified embryos is possible, approaches for obtaining *in-vivo*-derived embryos are costly, time-consuming and laborious (Vackova et al., 2007). As an alternative way, the usage of embryos produced in *in-vitro* through *in-vitro* fertilization (IVF) and somatic cell nuclear transfer (SCNT) methods have been attempted to generate porcine ESCs (Lee et al., 2015; Siriboon et al., 2015). However, in case of IVF procedure, polyspermy is routinely occurred (Wang et al., 2003), inducing abnormal embryonic development (Lee et al., 2016). On the other hand, SCNT embryos can be relatively and easily produced with oocytes and donor cells which are obtained from diverse breed of pigs compared to other methods. Therefore, ESCs derived from embryos produced through SCNT method have greater potential in the application than those through *in-vivo* and IVF.

To date, a variety of efforts have been conducted to establish ESCs derived from inner cell mass (ICM) of blastocysts in porcine species (Telugu et al., 2011; Jung et al., 2014; Hou et al., 2016), and successful establishment efficiency of porcine ESCs was dependent on age and source of porcine embryos, isolation methods of ICM from blastocysts, components of culture medium, and types of self-renewal-related cytokines and feeder cell layers (Vackova et al., 2007; Xue et al., 2016). However, although a variety of attempts have been widely conducted, the establishment efficiency of ESCs from porcine blastocysts is still and extremely low.

In the ESC establishment, formation of colonies derived from ICM of blastocysts and outgrowth of the colonies are very important steps. Especially, successful formation of colonies derived from ICM of blastocysts is dependent on the production of blastocysts with high performance in

the formation of colonies derived from ICM of blastocysts and the construction of microenvironment customized to formation of colonies derived from ICM of blastocysts. Therefore, *in-vitro* culture (IVC) systems stimulating production of blastocysts with high performance in the colony formation and successful formation of colonies derived from ICM of blastocysts are required for enhancing establishment efficiency of porcine ESCs.

Accordingly, we tried to identify the types of culture medium optimized to production of blastocysts with high performance in the formation of colonies from porcine SCNT embryos and formation of colonies derived from ICM of porcine SCNT blastocysts. For these, blastocysts were produced by culturing porcine SCNT embryos in various types of embryo culture medium and the produced porcine SCNT blastocysts in each embryo culture medium were cultured in different ESC culture medium. Subsequently, the optimal IVC media stimulating production of porcine SCNT blastocysts with high performance in the colony formation and colony formation were respectively determined by comparing formation efficiency of colonies derived from ICM of SCNT blastocysts.

## MATERIALS AND METHODS

### Animals

Fetuses derived from 13.5-day pregnant female ICR (DBL, Eumseong, Korea) mice were used as donors of mouse embryonic fibroblasts (MEFs) and porcine ovaries were collected 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.

### Oocytes collection and *in-vitro* maturation (IVM)

Aspiration of cumulus-oocyte complexes (COCs) from superficial follicles in the ovaries was performed using an 18-gauge needle. COCs that had multiple layers of unexpanded cumulus cells were collected 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). The washed COCs were cultured in a four-well culture dish (Nunc, Roskilde, Denmark)

containing 500  $\mu\text{L}$  of IVM medium consisting of medium-199 (M-199; Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) porcine follicular fluid, 1  $\mu\text{g}/\text{mL}$  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  $\mu\text{g}/\text{mL}$  kanamycin (Sigma-Aldrich), 10 IU/mL human chorionic gonadotropin (hCG; Intervet International BV, Boxmeer, Holland) and 80  $\mu\text{g}/\text{mL}$  follicle-stimulating hormone (FSH; Antrin R-10, Kyoritsu Seiyaku, Tokyo, Japan) at 39°C with 5%  $\text{CO}_2$ . After 22 h for IVM, the 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%  $\text{CO}_2$ .

### Preparation of donor cells

As donor cells, fetal fibroblasts from a newborn piglet 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. Synchronization of donor cells at the G0/G1 stage of the cell cycle was induced by contact inhibition for 72-96 h and detached with 0.05% trypsin-EDTA (Invitrogen). The dissociated donor cells were re-suspended 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, removal of cumulus cells from COCs was conducted mechanically by repeated pipetting in TLH supplemented with 0.4% (w/v) BSA and 0.1% (w/v) hyaluronidase (Sigma-Aldrich). Then, denuded oocytes were stained in calcium-free TLH supplemented with 0.4% (w/v) BSA and 5  $\mu\text{g}/\text{mL}$  Hoechst 33342 (Sigma-Aldrich) for 15 min at 39°C with 5%  $\text{CO}_2$ . The cumulus cell-free oocytes were washed twice with calcium-free TLH supplemented with 0.4% (w/v) BSA and transferred to a drop of calcium-free TLH supplemented with 0.4% (w/v) BSA and 5  $\mu\text{g}/\text{mL}$  cytochalasin B (Sigma-Aldrich) under mineral oil (Sigma-Aldrich). Subsequently, enucleation was conducted by aspirating the first polar body and metaphase II (MII) chromosomes from oocytes using a 17  $\mu\text{m}$  beveled pipette (Humagen, Charlottesville, VA) under an epifluorescence microscope (IX73; Olympus, Tokyo, Japan). A single cell was inserted into the perivitelline space of each enucleat-

ed oocyte. To fuse oocyte-cell couplets, they were placed on a fusion electrode chamber (NepaGene, Chiba, Japan) overlaid with 280 mM mannitol solution (Sigma-Aldrich) supplemented with 1  $\mu\text{M}$   $\text{CaCl}_2$  (Wako pure chemical industries, Osaka, Japan) and 50  $\mu\text{M}$   $\text{MgCl}_2$  (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  $\mu\text{s}$  using a cell fusion generator (LF101; NepaGene). After then, investigation of successful fusion of oocyte-cell couplets was conducted under a stereomicroscope and oocytes were incubated for 30 min in calcium-free TLH supplemented with 0.4% (w/v) BSA. To activate SCNT oocytes, SCNT oocytes were exposed to two DC pulse of 120 V/min for 60  $\mu\text{s}$  in a 280 mM mannitol solution supplemented 0.1 mM  $\text{CaCl}_2$  and 0.05 mM  $\text{MgCl}_2$ .

### *In-vitro* culture (IVC) medium used for culturing SCNT embryos

Supplementation of 0.34 mM trisodium citrate (Sigma-Aldrich), 2.77 mM myo-inositol (Sigma-Aldrich), and 10  $\mu\text{M}$   $\beta$ -mercaptoethanol (Sigma-Aldrich) into porcine zygote medium (PZM)-3 (herein referred to as the modified PZM-3) (Yoshioka et al., 2002) or -5 (herein referred to as the modified PZM-5) (Yamanaka et al., 2009) was conducted. Moreover, PZM-5F or -5K consists of the modified PZM-5 supplemented with 10% (v/v) FBS or 5% (v/v) knockout serum replacement (KSR; Invitrogen).

### Production of SCNT blastocysts

After electrical activation, the SCNT embryos incubated for 4 h in the modified PZM-3 or PZM-5 supplemented with 0.4  $\mu\text{g}/\text{mL}$  demecolcine (Sigma-Aldrich) and 2 mM 6-dimethylaminopurine (6-DMAP; Sigma-Aldrich) were washed with the modified PZM-3 or -5. Subsequently, the SCNT embryos washed with the modified PZM-3 were cultured for 168 h in 30  $\mu\text{L}$  of the modified PZM-3 droplets (10-15 embryos/droplet) covered with mineral oil at 39°C in 5%  $\text{CO}_2$ , 5%  $\text{O}_2$ , and 90%  $\text{N}_2$ . Moreover, the culture of SCNT embryos washed with the modified PZM-5 was conducted for 96 h in 30  $\mu\text{L}$  of the modified PZM-5 droplets (10-15 embryos/droplet) covered with mineral oil at 39°C in 5%  $\text{CO}_2$ , 5%  $\text{O}_2$ , and 90%  $\text{N}_2$ , and then the SCNT embryos cultured in the modified PZM-5 were additionally cultured for 72 h in 30  $\mu\text{L}$  of the modified PZM-5, -5F, or -5K droplets (10-15 embryos/droplet) covered with

mineral oil at 39°C in 5% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub>. Then, the SCNT blastocysts generated under each IVC condition were allocated to the following experiments.

### Preparation of MEF feeder cells

Pregnant mice at embryonic day 13.5 were sacrificed by cervical dislocation and uterus was 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 was minced with razor blades. Digestion of the minced tissues was conducted with 0.25% trypsin-EDTA (Welgene) for 10 min at 37°C. Subsequently, non-digested fibroblasts were removed using 70- $\mu$ m nylon mesh (SPL Life Sciences, Pocheon, Korea). The dissociated 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), and MEFs were cultured in MEF culture medium at 37°C under 5% CO<sub>2</sub> in a humidified air atmosphere until 90% confluency. The fresh culture medium was replaced at 2-day intervals. Confluent MEFs at passage 1 were inactivated in MEF culture medium containing 10  $\mu$ g/mL mitomycin C (Sigma-Aldrich) for 3 h at 37°C and dissociated with 0.05% trypsin-EDTA (Welgene). The inactivated MEFs

plated in four-well culture plate (SPL Life Sciences) coated with 0.1% (w/v) gelatin (Sigma-Aldrich) were used as a feeder layer.

### *In-vitro* outgrowth of inner cell mass (ICM) of SCNT blastocysts

The zona pellucida (ZP) of SCNT blastocysts were mechanically removed by insulin syringe under a stereomicroscope (Olympus). The ZP-free SCNT blastocysts were seeded on mitotically inactivated MEF feeder cells and cultured for 7 days in DMEM/Ham's F-10-, DMEM/Ham's F-12-, or  $\alpha$ -MEM-based medium at 37°C under an atmosphere of 5% CO<sub>2</sub> in air. DMEM/Ham's F-10-based medium consists of 1:1 low-glucose DMEM (LG-DMEM; Welgene):Ham's F-10 (Gibco; Carlsbad, CA) medium supplemented with 15% (v/v) heat-inactivated ES cell-screened FBS (Hyclone, Logan, UT), 0.2 mM  $\beta$ -mercaptoethanol (Gibco), 1% (v/v) nonessential amino acids (NEAA; Gibco), 20 ng/mL basic fibroblast growth factor (bFGF; PeproTech, Inc., Rocky Hill, NJ) and 1% (v/v) antibiotic-antimycotic solution. DMEM/Ham's F-12-based medium consists of DMEM/Ham's F-12 medium (Gibco) supplemented with 20% (v/v) KSR, 0.1 mM  $\beta$ -mercaptoethanol, 1% (v/v) NEAA, 20 ng/mL bFGF and 1% (v/v) antibiotic-antimycotic solution. Alpha-MEM-

**Table 1.** Determination of culture medium stimulating formation of colonies derived from ICM of blastocysts produced through culture of porcine SCNT embryos in the modified PZM-3

Type of culture medium	No. of SCNT blastocysts seeded	No. (%) <sup>d</sup> of	
		SCNT blastocysts attached to feeder cells	Colonies formed successfully from ICM
DMEM/Ham's F-10-based medium <sup>a</sup>	28	21 (75.00)	6 (21.43) <sup>e</sup>
DMEM/Ham's F-12-based medium <sup>b</sup>	29	19 (65.25)	1 (3.45) <sup>f</sup>
$\alpha$ -MEM-based medium <sup>c</sup>	30	26 (86.67)	9 (30.00) <sup>e</sup>

Model effects of treatments in each parameter, which is indicated as the *p* value, were 0.1679 and 0.0268 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.

Zona pellucida of blastocysts generated through culture of porcine SCNT embryos for 168 h in the modified PZM-3 were removed mechanically and subsequent cultured for 7 days on mitotically inactivated MEFs feeder cells in DMEM/Ham's F-10-based medium, DMEM/Ham's F-12-based medium, or  $\alpha$ -MEM-based medium.

<sup>a</sup>DMEM/Ham's F-10-based medium consists of DMEM/Ham's F-10 medium supplemented with 15% (v/v) FBS, 0.2 mM  $\beta$ -mercaptoethanol, 1% (v/v) NEAA, 20 ng/mL bFGF, and 1% (v/v) antibiotic-antimycotic solution.

<sup>b</sup>DMEM/Ham's F-12-based medium consists of DMEM/Ham's F-12 medium supplemented with 20% (v/v) KSR, 0.1 mM  $\beta$ -mercaptoethanol, 1% (v/v) NEAA, 20 ng/mL bFGF and 1% (v/v) antibiotic-antimycotic solution.

<sup>c</sup> $\alpha$ -MEM-based medium consists of  $\alpha$ -MEM medium supplemented with 10% (v/v) KSR, 0.05 mM  $\beta$ -mercaptoethanol, 1% (v/v) NEAA, 40 ng/mL EGF, 10  $\mu$ L/mL ITS, 1,000 U/mL mLIF, 20 ng/mL bFGF, and 1% (v/v) antibiotic-antimycotic solution.

<sup>d</sup>Percentage of the number of blastocysts seeded.

<sup>e,f</sup>Values in the same column with different superscript letters are significantly different, *p* < 0.05.

based medium consists of  $\alpha$ -MEM medium (Gibco) supplemented with 10% (v/v) KSR, 0.05 mM  $\beta$ -mercaptoethanol, 1% (v/v) NEAA, 40 ng/mL EGF (PeproTech, Inc.) 10  $\mu$ L/mL 100 $\times$  insulin-transferrin-selenium (ITS; Gibco), 1000 U/mL mouse leukemia inhibitory factor (mLIF; Chemicon International, Inc., Temecula, CA), 20 ng/mL bFGF, and 1% (v/v) antibiotic-antimycotic solution. The fresh culture medium was replaced daily during culture. Subsequently, attachment of SCNT blastocysts to MEF feeder cells and successful formation of colonies from ICM of SCNT blastocysts were monitored under an inverted microscope (CKX41; Olympus).

### Statistical analysis

All numerical data in each parameter were analyzed using SAS software (SAS Institute Inc., Cary, NY). Comparisons among treatment groups were performed using a generalized linear model (PROC-GLM) in the SAS package. A value of  $p < 0.05$  was taken to indicate a statistically significant difference.

## RESULTS

To determine culture medium stimulating formation of colonies derived from ICM of porcine SCNT blastocysts,

ZP-free porcine SCNT blastocysts produced in diverse embryo culture medium were seeded onto mitotically inactivated MEF feeder cells and cultured in three types of ESC culture medium such as DMEM/Ham's F-10-, DMEM/Ham's F-12- and  $\alpha$ -MEM-based medium for 7 days. As shown in Table 1, numerically the highest attachment efficiency of porcine SCNT blastocysts produced in the modified PZM-3 to feeder cells was detected when porcine SCNT blastocysts were cultured in  $\alpha$ -MEM-based medium. However, porcine SCNT blastocysts cultured in DMEM/Ham's F-10- and  $\alpha$ -MEM-based medium showed significantly higher efficiency in the colony formation than those in DMEM/Ham's F-12-based medium. In case of porcine SCNT blastocysts produced in the modified PZM-5 (Table 2), numerically the highest attachment efficiency of porcine SCNT blastocysts to feeder cells was observed when porcine SCNT blastocysts were cultured in  $\alpha$ -MEM-based medium. But, colony formation efficiency was significantly higher in porcine SCNT blastocysts cultured in DMEM/Ham's F-12- and  $\alpha$ -MEM-based medium than those in DMEM/Ham's F-10-based medium. As shown in Table 3, numerically the highest attachment efficiency of porcine SCNT blastocysts produced through sequential culture in the modified PZM-5 and the PZM-5F to feeder cells and significantly the highest colony forma-

**Table 2.** Determination of culture medium stimulating formation of colonies derived from ICM of blastocysts produced through culture of porcine SCNT embryos in the modified PZM-5

Type of culture medium	No. of SCNT blastocysts seeded	No. (%) <sup>d</sup> of	
		SCNT blastocysts attached to feeder cells	Colonies formed successfully from ICM
DMEM/Ham's F-10-based medium <sup>a</sup>	37	29 (78.38)	2 (5.41) <sup>e</sup>
DMEM/Ham's F-12-based medium <sup>b</sup>	38	25 (65.79)	9 (23.68) <sup>f</sup>
$\alpha$ -MEM-based medium <sup>c</sup>	40	33 (82.50)	13 (32.50) <sup>f</sup>

Model effects of treatments in each parameter, which is indicated as the  $p$  value, were 0.1634 and 0.0115 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.

Zona pellucida of blastocysts generated through culture of porcine SCNT embryos for 168 h in the modified PZM-5 were removed mechanically and subsequent cultured for 7 days on mitotically inactivated MEFs feeder cells in DMEM/Ham's F-10-based medium, DMEM/Ham's F-12-based medium, or  $\alpha$ -MEM-based medium.

<sup>a</sup>DMEM/Ham's F-10-based medium consists of DMEM/Ham's F-10 medium supplemented with 15% (v/v) FBS, 0.2 mM  $\beta$ -mercaptoethanol, 1% (v/v) NEAA, 20 ng/mL bFGF, and 1% (v/v) antibiotic-antimycotic solution.

<sup>b</sup>DMEM/Ham's F-12-based medium consists of DMEM/Ham's F-12 medium supplemented with 20% (v/v) KSR, 0.1 mM  $\beta$ -mercaptoethanol, 1% (v/v) NEAA, 20 ng/mL bFGF and 1% (v/v) antibiotic-antimycotic solution.

<sup>c</sup> $\alpha$ -MEM-based medium consists of  $\alpha$ -MEM medium supplemented with 10% (v/v) KSR, 0.05 mM  $\beta$ -mercaptoethanol, 1% (v/v) NEAA, 40 ng/mL EGF, 10  $\mu$ L/mL ITS, 1,000 U/mL mLIF, 20 ng/mL bFGF, and 1% (v/v) antibiotic-antimycotic solution.

<sup>d</sup>Percentage of the number of blastocysts seeded.

<sup>e,f</sup>Values in the same column with different superscript letters are significantly different,  $p < 0.05$ .

**Table 3.** Determination of culture medium stimulating formation of colonies derived from ICM of blastocysts produced through sequential culture of porcine SCNT embryos in the modified PZM-5 and the PZM-5F

Type of culture medium	No. of SCNT blastocysts seeded	No. (%) <sup>d</sup> of	
		SCNT blastocysts attached to feeder cells	Colonies formed successfully from ICM
DMEM/Ham's F-10-based medium <sup>a</sup>	44	28 (63.63)	7 (15.91) <sup>e</sup>
DMEM/Ham's F-12-based medium <sup>b</sup>	44	34 (77.27)	14 (31.82) <sup>e</sup>
$\alpha$ -MEM-based medium <sup>c</sup>	42	36 (85.71)	19 (45.24) <sup>f</sup>

Model effects of treatments in each parameter, which is indicated as the *p* value, were 0.0559 and 0.0013 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.

Zona pellucida of blastocysts generated through sequential culture of porcine SCNT embryos for 96 h in the modified PZM-5 and for 72 h in the PZM-5F consisting of the modified PZM-5 supplemented with 10% (v/v) FBS were removed mechanically and subsequent cultured for 7 days on mitotically inactivated MEFs feeder cells in DMEM/Ham's F-10-based medium, DMEM/Ham's F-12-based medium, or  $\alpha$ -MEM-based medium.

<sup>a</sup>DMEM/Ham's F-10-based medium consists of DMEM/Ham's F-10 medium supplemented with 15% (v/v) FBS, 0.2 mM  $\beta$ -mercaptoethanol, 1% (v/v) NEAA, 20 ng/mL bFGF, and 1% (v/v) antibiotic-antimycotic solution.

<sup>b</sup>DMEM/Ham's F-12-based medium consists of DMEM/Ham's F-12 medium supplemented with 20% (v/v) KSR, 0.1 mM  $\beta$ -mercaptoethanol, 1% (v/v) NEAA, 20 ng/mL bFGF and 1% (v/v) antibiotic-antimycotic solution.

<sup>c</sup> $\alpha$ -MEM-based medium consists of  $\alpha$ -MEM medium supplemented with 10% (v/v) KSR, 0.05 mM  $\beta$ -mercaptoethanol, 1% (v/v) NEAA, 40 ng/mL EGF, 10  $\mu$ L/mL ITS, 1,000 U/mL mLIF, 20 ng/mL bFGF, and 1% (v/v) antibiotic-antimycotic solution.

<sup>d</sup>Percentage of the number of blastocysts seeded.

<sup>e,f</sup>Values in the same column with different superscript letters are significantly different, *p* < 0.05.

tion efficiency were observed when porcine SCNT blastocysts were cultured in  $\alpha$ -MEM-based medium. In case of porcine SCNT blastocysts produced through sequential culture in the modified PZM-5 and the PZM-5K (Table 4), attachment efficiency of porcine SCNT blastocysts to feeder cells was significantly higher in DMEM/Ham's F-12- and  $\alpha$ -MEM-based medium than in DMEM/Ham's F-10-based medium. Although there was no significant difference in colony formation efficiency, numerically the highest colony formation efficiency showed when porcine SCNT blastocysts were cultured in  $\alpha$ -MEM-based medium. Entirely, regardless of the types of culture medium used for producing blastocysts from porcine SCNT embryos,  $\alpha$ -MEM-based medium showed the best formation efficiency of colonies derived from ICM of porcine SCNT blastocysts, indicating that  $\alpha$ -MEM-based medium should be necessarily used in the initial step for deriving successfully ESCs from porcine SCNT blastocysts.

Subsequently, for elucidating the type of embryo culture medium optimized to the production of porcine SCNT blastocysts with high performance in the colony formation, attachment and colony formation efficiency were compared among blastocysts produced through culture of porcine SCNT embryos in a variety of IVC conditions (Table 5). As the results, there were no significant differ-

ences in the attachment and colony formation efficiency among groups. Justly, numerically the highest attachment efficiency of porcine SCNT blastocysts to feeder cells was observed in blastocysts produced through culture of porcine SCNT embryos in the modified PZM-3. On the other hand, blastocysts produced through sequential culture of porcine SCNT embryos in the modified PZM-5 and the PZM-5F showed the numerically highest colony formation efficiency. These results demonstrate that sequential culture of porcine SCNT embryos in the modified PZM-5 and the PZM-5F is effective in the production of porcine SCNT blastocysts with high performance in the colony formation.

Based on these results, we could identify that culture of blastocysts produced through sequential culture of porcine SCNT embryos in the modified PZM-5 and the PZM-5F on the feeder cells in  $\alpha$ -MEM-based medium may be effective in the successful establishment of porcine SCNT embryo-derived ESCs.

## DISCUSSION

Here, as a first step toward developing novel system effectively establishing ESCs from SCNT embryos, we report IVC systems effective to production of blastocysts with

**Table 4.** Determination of culture medium stimulating formation of colonies derived from ICM of blastocysts produced through sequential culture of porcine SCNT embryos in the modified PZM-5 and the PZM-5K

Type of culture medium	No. of SCNT blastocysts seeded	No. (%) <sup>d</sup> of	
		SCNT blastocysts attached to feeder cells	Colonies formed successfully from ICM
DMEM/Ham's F-10-based medium <sup>a</sup>	46	26 (56.52) <sup>e</sup>	7 (15.21)
DMEM/Ham's F-12-based medium <sup>b</sup>	46	38 (82.61) <sup>f</sup>	13 (28.26)
$\alpha$ -MEM-based medium <sup>c</sup>	49	39 (79.59) <sup>f</sup>	14 (28.57)

Model effects of treatments in each parameter, which is indicated as the *p* value, were 0.0107 and 0.6610 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.

Zona pellucida of blastocysts generated through sequential culture of porcine SCNT embryos for 96 h in the modified PZM-5 and for 72 h in the PZM-5K consisting of the modified PZM-5 supplemented with 5% (v/v) KSR were removed mechanically and subsequent cultured for 7 days on mitotically inactivated MEFs feeder cells in DMEM/Ham's F-10-based medium, DMEM/Ham's F-12-based medium, or  $\alpha$ -MEM-based medium.

<sup>a</sup>DMEM/Ham's F-10-based medium consists of DMEM/Ham's F-10 medium supplemented with 15% (v/v) FBS, 0.2 mM  $\beta$ -mercaptoethanol, 1% (v/v) NEAA, 20 ng/mL bFGF, and 1% (v/v) antibiotic-antimycotic solution.

<sup>b</sup>DMEM/Ham's F-12-based medium consists of DMEM/Ham's F-12 medium supplemented with 20% (v/v) KSR, 0.1 mM  $\beta$ -mercaptoethanol, 1% (v/v) NEAA, 20 ng/mL bFGF and 1% (v/v) antibiotic-antimycotic solution.

<sup>c</sup> $\alpha$ -MEM-based medium consists of  $\alpha$ -MEM medium supplemented with 10% (v/v) KSR, 0.05 mM  $\beta$ -mercaptoethanol, 1% (v/v) NEAA, 40 ng/mL EGF, 10  $\mu$ L/mL ITS, 1,000 U/mL mLIF, 20 ng/mL bFGF, and 1% (v/v) antibiotic-antimycotic solution.

<sup>d</sup>Percentage of the number of blastocysts seeded.

<sup>e,f</sup>Values in the same column with different superscript letters are significantly different, *p* < 0.05.

**Table 5.** Comparison of attachment to feeder cells and formation of colonies among ICM of blastocysts produced through culture of porcine SCNT embryos in a variety of *in-vitro* culture conditions

Type of culture medium exposed to SCNT embryos		No. of SCNT blastocysts seeded	No. (%) <sup>a</sup> of	
For 96 h post-treatment of demecolcine and 6-DMAP	For 72 h post-culture for 96 h		SCNT blastocysts attached to feeder cells	Colonies formed successfully from ICM
PZM-3	PZM-3	30	26 (86.67)	9 (30.00)
PZM-5	PZM-5	40	33 (82.50)	13 (32.50)
	PZM-5F	42	36 (85.71)	19 (45.24)
	PZM-5K	49	39 (79.59)	14 (28.57)

Model effects of treatments in each parameter, which is indicated as the *p* value, were 0.8249 and 0.3598 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.

Zona pellucida of blastocysts generated through culture of porcine SCNT embryos for 168 h in each *in-vitro* culture condition were removed mechanically and subsequent cultured for 7 days on mitotically inactivated MEFs feeder cells in  $\alpha$ -MEM-based medium consisting of  $\alpha$ -MEM medium supplemented with 10% (v/v) KSR, 0.05 mM  $\beta$ -mercaptoethanol, 1% (v/v) NEAA, 40 ng/mL EGF, 10  $\mu$ L/mL ITS, 1,000 U/mL mLIF, 20 ng/mL bFGF, and 1% (v/v) antibiotic-antimycotic solution.

<sup>a</sup>Percentage of the number of blastocysts seeded.

high performance in the colony formation from SCNT embryos and formation of colonies derived from ICM of SCNT blastocysts. SCNT blastocysts with high performance in the colony formation could be produced by sequentially culturing SCNT embryos in the modified PZM-5 and the PZM-5F, and formation efficiency of colonies derived from ICM of SCNT blastocysts could be improved

by culturing SCNT blastocysts in  $\alpha$ -MEM-based medium. Accordingly, appropriate combination of these IVC systems will greatly contribute to successful establishment of ESCs derived from SCNT embryos.

Entirely, DMEM/Ham's F-12- and  $\alpha$ -MEM-based medium containing KSR showed higher colony formation efficiency in all blastocysts except for blastocysts produced

in the modified PZM-3 than DMEM/Ham's F-10-based medium containing FBS. These improvement of colony formation efficiency may result from the usage of FBS-free culture medium. FBS is known to have unknown factors inducing differentiation in extremely low concentration, whereas KSR is free from unknown factors inducing differentiation. Therefore, DMEM/Ham's F-10-based medium containing FBS will stimulate more actively differentiation of ICM derived from porcine SCNT blastocysts than their self-renewal for colony formation. On the other hands, DMEM/Ham's F-12- and  $\alpha$ -MEM-based medium containing KSR will stimulate more actively self-renewal of ICM derived from porcine SCNT blastocysts than their differentiation.

Because ESCs are derived from ICM of blastocysts (Bogliotti et al., 2018), blastocysts with good quality are required for successful establishment of ESCs. Generally, total number and hatching rate of the generated blastocysts are known as an indicator of quality of blastocysts (Cheong et al., 2015). Our study showed that hatching rate of blastocysts produced through sequential culture in the modified PZM-5 and the PZM-5F was significantly higher than those produced through culture in the others (data not shown). Therefore, sequential culture of SCNT embryos in the modified PZM-5 and the PZM-5F can be one of methods generating SCNT blastocysts with good quality, supported by the fact that they showed the best colony formation efficiency (Table 5).

Generally, bFGF signaling is required for the maintenance of undifferentiation status in porcine ESCs (Xue et al., 2016). Moreover, previous studies have shown that there is no LIF receptor in the ICM of porcine blastocysts (Hall et al., 2009; Hall and Hyttel, 2014). Despite the fact, our study showed that the usage of LIF-added ESC culture medium such  $\alpha$ -MEM-based medium induced higher colony formation efficiency than LIF-free ESC culture medium such as DMEM/Ham's F-10- and DMEM/Ham's F-12-based medium (Table 1-4). Accordingly, we speculate that LIF signaling are related to the maintenance of undifferentiated state in the ICM of porcine blastocysts through another pathway than receptor-mediated pathway.

In conclusion, we developed a SCNT embryo culture system for the production of blastocysts with high performance in the colony formation and a ESC establishment system for the formation of colonies derived from ICM of SCNT blastocysts. Moreover, appropriate combination of

the developed these systems will greatly contribute to the improvement of efficiency in the establishment of ESCs from porcine SCNT embryos.

## CONFLICTS OF INTEREST

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

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