

Improvement in the blastocyst quality and efficiency of putative embryonic stem cell line derivation from porcine embryos produced *in vitro* using a novel culturing system

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Received May 28, 2014; Accepted March 26, 2015

DOI: 10.3892/mmr.2015.3634

Abstract. Porcine embryonic stem cells (pESCs) have great potential for application in translational biomedical research, including xenotransplantation and disease models. Obtaining high-quality blastocysts is the most important factor in the isolation and colonization of primary ESCs and the establishment of ESC lines. In pigs, *in vitro*-derived blastocysts have a limited cell number compared to *in vivo*-derived blastocysts and show an indefinite inner cell mass, which may result in failure to establish pESC lines. In the present study, the effects of resveratrol (RES), granulocyte-macrophage colony stimulating factor (GM-CSF) and β -mercaptoethanol (β -ME) on the quality of blastocysts and the efficiency of colony derivation were investigated for the establishment of ESCs. A novel culturing system was developed in which $2\ \mu\text{M}$ RES was added to the oocyte *in vitro* maturation (IVM) medium, and 10 ng/ml pGM-CSF and $10\ \mu\text{M}$ β -ME were added to embryo *in vitro* culture (IVC) medium. This novel system showed significantly more parthenogenetic activation (PA) blastocysts ($54.5\pm 1.8\%$ vs. $43.4\pm 1.2\%$; $P<0.05$) and *in vitro* fertilization (IVF) blastocysts ($36.9\pm 3.3\%$ vs. $26.2\pm 2.9\%$; $P<0.06$) at day seven as compared with that in the control system. The PA and IVF blastocysts from the novel system showed a significantly greater hatching rate ($P<0.05$) and greater cell numbers (55.1 ± 2.0 vs. 45.6 ± 2.0 ; $P<0.05$ and 78.9 ± 6.8 vs. 58.5 ± 7.2 ; $P<0.06$, for PA and IVF, respectively) at day seven compared to that in the control system. After seeding on feeder cells, the

PA blastocysts produced by the novel system showed a significantly increased rate of attachment ($28.8\pm 3.9\%$ vs. $17.2\pm 2.4\%$; $P<0.062$). Finally, two putative pESC lines from PA embryos produced by the novel system and one by the control system were established. In conclusion, the novel system improved blastocyst quality and increased the derivation efficiency of putative pESC lines from porcine PA and IVF embryos produced *in vitro*.

Introduction

Embryonic stem cell (ESC) lines are able to renew without differentiation and exhibit pluripotency *in vitro* under optimal conditions over a long period of time (1). Since ESC lines were first established from the mouse inner cell mass (ICM) in 1981 (2), attempts to isolate human ESCs have been successfully performed (1). In addition, numerous studies have attempted to establish ESC lines from other species, including sheep (3), hamster (4), mink (5), rabbit (6), pig (7), cattle (8), rat (9) and horse (10). However, to date, authentic ESCs have only been established from the rat (11,12).

Compared to other animals, pigs are a favored animal model for translational research regarding humans due to their similarities in size and physiology (13). There is also great potential for using genetically modified pigs as organ donors for xenotransplantation and as a model for human diseases (14). Putative porcine ESC (pESC) lines were first established in 1990 using porcine blastocysts expanded *in vivo* for 7-9 days (15). Although embryos produced *in vivo* are of high quality compared to embryos produced *in vitro*, this approach is more expensive and laborious. Therefore, it would be economically desirable to use blastocysts created *in vitro*. However, to date, only few putative ESC lines have been established in farm animals from material produced *in vitro* (16).

The production of porcine blastocysts *in vitro* was first attempted in 2000 (17). However, it has remained challenging to establish pESCs from porcine blastocysts produced *in vitro*, as there is often no obvious ICM, or only a few cells are present in porcine embryos (16). One of the reasons for this difficulty is an incomplete *in vitro* production system for porcine embryos compared to the *in vivo* environment. Therefore, numerous attempts have been made to improve the conditions of *in vitro*

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Key words: *in vitro* maturation, *in vitro* culture, porcine embryonic stem cells, resveratrol, porcine granulocyte-macrophage colony stimulating factor, β -mercaptoethanol

systems to the level of what occurs *in vivo*. Traditionally, M199 has been used as a common medium for *in vitro* maturation (IVM) in most laboratories (18). Funahashi *et al.* (19,20) found that the cytoplasmic maturation of porcine oocytes is significantly affected by the maturation medium. Supplementation of the maturation medium with cystein enhances the amount of glutathione in porcine oocytes and improves the formation of the maternal pronucleus (MPN) after sperm penetration (21). For *in vitro* culture (IVC), porcine zygote medium-5 (PZM5) had been developed by Yoshioka *et al.* (22) and has been used in numerous studies (23) as a replacement for porcine zygote medium-3 (PZM3), the traditional medium for IVC (24). However, the developmental competence of *in vitro* culture systems was still below that observed *in vivo*.

The aim of the present study was to examine the effects of resveratrol (RES), granulocyte-macrophage colony stimulating factor (GM-CSF) and β -mercaptoethanol (β -ME) on the quality of blastocysts and the efficiency of colony derivation in the establishment of ESCs. To obtain porcine blastocysts derived *in vitro*, an *in vitro* fertilization (IVF) and parthenogenetic activation (PA) were performed. The control system used M199 media for IVM and PZM3 for IVC. By contrast, the novel system used M199 media with 2 μ M RES for IVM and PZM5 with 2 μ M RES, 10 ng/ml of porcine GM-CSF (pGM-CSF) and 10 μ M β -ME for IVC, while the control system had no additives. To characterize putative pESCs from PA embryos derived by the novel and control systems, various parameters were compared, including typical morphology, alkaline phosphatase (AP) activity analysis, embryoid body (EB) formation and gene expression analysis (OCT4, SOX2, Nanog, NESTIN, BLBP, C.actin, SOX17, GATA6 and PECAM).

Materials and methods

Chemicals. All chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) unless stated otherwise.

***In vitro* maturation (IVM) of porcine oocytes.** Ovaries of gilts were collected from a commercial abattoir. Porcine cumulus-oocyte complexes (COCs) with follicular fluid were aspirated from 3- to 6-mm antral follicles using a 10-ml syringe and an 18-gauge needle. COCs were recovered under a stereoscopic microscope (SZ51; Olympus, Tokyo, Japan) and washed in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered Tyrode's medium containing 0.05% (w/v) polyvinyl alcohol (TLH-PVA). Subsequently, ~60-70 COCs from the control group were cultured in a four-well dish (Nunc, Roskilde, Denmark) containing 500 μ l tissue culture medium 199 (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 26 mM sodium bicarbonate, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 10 ng/ml epidermal growth factor, 0.5 IU/ml porcine luteinizing hormone, 0.5 IU/ml porcine follicle-stimulating hormone, 10% (v/v) pFF, 75 μ g/ml penicillin-G and 50 μ g/ml streptomycin at 39°C under 5% CO₂ in air. After 22 h, COCs were replaced in the same medium without hormone supplements and then cultured for 20 h under the same conditions. COCs from the treatment group were cultured in the same medium with 2 μ M RES. After 42 h of IVM, matured oocytes were selected and used

for production of IVF and PA embryos. In the control system, M199 media was used for IVM, while the novel system used M199 media with 2 μ M RES for IVM.

***In vitro* fertilization (IVF) of porcine oocytes.** For IVF, liquid semen supplied weekly from the Veterinary Service Laboratory (Department of Livestock Research, Yong-in, Korea) and was maintained at 17°C for 5 days prior to use. After IVM, a denuded oocyte was co-incubated with sperm for 20 min at 39°C in a humidified atmosphere of 5% CO₂ and 95% air. After co-incubation, attached sperm was removed from the zona pellucida (ZP) by pipetting. Oocytes were then washed three times and incubated in modified Tris-buffered medium (mTBM) without sperm for 5-6 h at 39°C in a humidified atmosphere of 5% CO₂ and 95% air. Thereafter, zygotes were washed three times with embryo culture medium and cultured in PZM3 or modified PZM5. The control system used PZM3 for IVC, while the novel system used PZM5 with 2 μ M RES, 10 ng/ml pGM-CSF and 10 μ M β -ME for IVC. The embryos with culture medium were covered with pre-warmed mineral oil and incubated at 39°C for seven days under a humidified atmosphere of 5% O₂, 5% CO₂ and 90% N₂.

Parthenogenetic activation (PA) of oocytes. Oocytes were activated with two pulses of 120 V/mm of DC for 60 μ s in 280 mM mannitol solution containing 0.01 mM CaCl₂ and 0.05 mM MgCl₂. For PA, oocytes that reached MII stage after 42 h of IVM were activated. Following electrical activation, PA embryos were treated with 2 mM 6-dimethylaminopurine, 0.4 μ g/ml demecolcine and 5 μ g/ml of cytochalasin B in PZM3 for 4 h. PA embryos were then washed three times in fresh IVC medium, transferred into 30- μ l microdrops of IVC medium in mineral oil and then cultured at 39°C in a humidified atmosphere of 5% O₂, 5% CO₂ and 90% N₂ for seven days. The control system used PZM3 for IVC, while the novel system used PZM5 with 2 μ M RES, 10 ng/ml pGM-CSF and 10 μ M β -ME.

Embryo evaluation and total cell count. Embryos were evaluated for cleavage under a stereomicroscope on day two. Blastocyst formation on day seven after PA or IVF was classified according to the degree of expansion and hatching status: Early blastocyst (small blastocyst with a blastocoel equal to or less than half of the embryo volume), expanded blastocyst (large blastocyst with a blastocoel greater than half of the embryo volume or blastocyst with a blastocoel completely filling the embryo) and hatched blastocyst (hatching or already hatched blastocyst). To determine the total number of blastocysts on day seven, all blastocysts were collected, washed in phosphate-buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA) and stained with 10 μ g/ml of Hoechst-33342 for 5 min. After washing again in PBS-BSA, embryos were fixed in PBS containing 4% paraformaldehyde. Then, the blastocysts were mounted on glass slides in a drop of glycerol, covered gently with a cover slip and observed under a fluorescence microscope (Nikon Corp., Tokyo, Japan). This study was approved by the committee on the ethics committee on the ethics of animal experiments (Chungbuk National University, Cheongju, Republic of Korea; Permit no. CBNUA-584-13-01).

Table I. Primer sequences for gene expression analysis.

Gene	Primer sequences (5'-3')	Product size (bp)	GenBank accession number
<i>CDX2</i>	F: GGAACCTGTGCGAGTGGATG R: GCTCGGCCTTTCTCCGAATG	168	CK_458871
<i>BAX</i>	F: TGCCTCAGGATGCATCTACC R: AAGTAGAAAAGCGCGACCAC	199	XM_003127290
<i>BAK</i>	F: GCGGAAAACGCCTATGAGTA R: GCAGTGATGCAGCATGAAGT	189	XM_001928147
<i>Bcl-2</i>	F: AGGGCATTCAAGTGACCTGAC R: CGATCCGACTCACCAATACC	193	NM_214285
<i>Sirt1</i>	F: GGACAGTTCCAGCCATCTCT R: CCTCGTACAGCTTCACAGTCA	200	NM_001145750
<i>OCT4</i>	F: GCGGACAAGTATCGAGAACC R: CCTCAAAATCCTCTCGTTGC	200	NM_001113060
<i>Nanog</i>	F: AGCAACCAAACCTGGAACAG R: TGGGTACCGCAGTACTTTGA	209	NM_001129971
<i>SOX2</i>	F: CTGCAGTACAACCTCCATGACCA R: CATGCTGATCATGTCCCGTA	216	NM_001123197
<i>NESTIN</i>	F: GCCCACAATAGATTGGTATTT R: AGCATCTTTACAGCGACAGTC	201	TC295480
<i>BLBP</i>	F: TTGGTGATGTGGTTGCTGTT R: TCACATTTTCCACCTCCACA	174	TC241634
<i>C.actin</i>	F: CAGGTATTGCTGATCGCATGCA R: ATTTGCGG GGACGATGGA	201	TC270296
<i>PECAM</i>	F: CATTTCCAA AGTCAGCAGCA R: ATCATCATGCCTCCCTTCTG	171	TC238302
<i>GATA6</i>	F: CAGGAA ACG AAA ACCTAAGAGCAT R: TTCTCGGGATTAGCGCTCTC	201	TC238300
<i>SOX17</i>	F: CGCACGGAGTTTGAACAATA R: CAGACGTCGGGGTAGTTACAG	167	TC248504
<i>GAPDH</i>	F: GTCGGTTGTGGATCTGACCT R: TTGACGAAGTGGTCGTTGAG	207	NM_001206359

Gene expression analysis of blastocysts and putative pESCs by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from blastocysts using TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. Complementary DNA (cDNA) was prepared by subjecting 0.8 μ g of total RNA to reverse transcription using Moloney murine leukemia virus (MMIV) reverse transcriptase (Invitrogen Life Technologies) and random primers (9-mers; Takara Bio Inc., Otsu, Japan). Then, quantitative real-time PCR was performed on MX3000P machine (Stratagene-Agilent Technologies, Waldbronn, Germany) with 1 μ l cDNA template added to 10 μ l 2X SYBR Premix Ex Taq (Takara Bio Inc.) containing specific primers. The reactions were performed over 40 cycles, and the cycling parameters were as follows: Denaturation at 95°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 30 sec. All primer sequences are presented in Table I. The expression levels of each target gene were quantified relative to those of

GAPDH. Relative quantification was based on a comparison of threshold cycle (Ct) at constant fluorescence intensity. Relative mRNA expression (R) was calculated using the equation, $R=2^{-[\Delta Ct \text{ sample}-\Delta Ct \text{ control}]}$. To determine a normalized arbitrary value for each gene, every obtained value was normalized to that of GAPDH. Experiments were repeated at least three times.

The cDNA from pESCs was amplified in a 20- μ l PCR reaction containing 10 pmol forward and reverse primers (iNtRON Biotechnology, SungNam., Korea), 2 units Taq polymerase (iNtRON Biotechnology), 10X PCR buffer (iNtRON Biotechnology), 5 pmol desoxyribonucleotide triphosphate mixtures (iNtRON Biotechnology) and template (cDNA), on a PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA, USA). The PCR reactions were performed for 30 cycles with the following conditions: Denaturation for 30 sec at 95°C, annealing for 30 sec at 57°C and extension for 30 sec at 72°C. The reaction products were analyzed on a 1.5%

Table II. Effects of the novel system on embryonic development in porcine embryos following parthenogenetic activation.

Groups	Embryos cultured (n)	Embryos developed to		Total cell number in blastocysts, n (N) ^a
		≥2 cells, n (%)	Blastocysts, n (%)	
Control	649	562 (86.2±0.8) ^b	280 (43.4±1.2) ^b	45.6±2.0 (34) ^b
Novel system	561	499 (88.5±0.6) ^c	313 (54.5±1.8) ^c	55.1±2.0 (58) ^c

Values are expressed as the mean ± standard error of the mean (n=7). ^aN, number of blastocysts examined. ^{b,c}Values with different superscripts within a column differ significantly (P<0.05).

Table III. Effects of the novel system on embryonic development after *in vitro* fertilization.

Groups	Embryos cultured (n)	Embryos developed to		Total cell number in blastocysts, n (N) ^a
		≥2 cells, n (%)	Blastocysts, n (%)	
Control	375	267 (70.9±1.4)	94 (26.2±2.9) ^b	58.5±7.2 (11) ^b
Novel system	237	172 (73.4±3.6)	87 (36.9±3.3) ^c	78.9±6.8 (18) ^c

Values are expressed as the mean ± standard error of the mean (n=3, 4). ^aN, number of blastocysts examined. ^{b,c}Values with different superscripts within a column differ significantly (P<0.05).

agarose gel (Pronadisa, Laboratorios Conda SA, Madrid, Spain) pre-stained with ethidium (Sigma-Aldrich Chemical Company, St. Louis, MO, USA). The gels were scanned by a Gel Doc 2000 apparatus (Bio-Rad Laboratories, Hercules, CA, USA) and compared to a 100-bp ladder of DNA (iNtRON Biotechnology).

Preparation of mouse embryonic fibroblasts as the feeder cell layer. The feeder cell layer was prepared from ICR mice (DBL, Eumseong, Republic of Korea) at pregnancy day 13, when the mice were sacrificed and the fetuses recovered. Fetal heads, internal organs and legs were removed, after which the remaining tissues were minced in PBS and centrifuged at 357 x g for 3 min at least twice until mouse embryonic fibroblasts (MEFs) were obtained. MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Carlsbad., CA., USA) with 10% fetal bovine serum (Gibco-BRL), 1% non-essential amino acids (Gibco-BRL), 1% glutamine (Gibco-BRL), 0.1 mM β-mercaptoethanol (Gibco-BRL) and 1% antibiotics-antimycotics (Gibco-BRL) (growth medium) at 37°C under 5% CO₂ in air. MEFs were passaged 2-3 times and then inactivated with 10 μg/ml mitomycin C (Roche, Basel, Switzerland) for 2-2.5 h for use as culture pig blastocysts. Inactivated MEFs were plated at a density of 5x10⁵ cells/ml in a four-well dish coated with 0.5% gelatin in growth medium.

Derivation and culture of putative pESC lines. Stem cell culture medium consisted of low-glucose DMEM/F10 (Gibco-BRL) containing 1% non-essential amino acids, 1% glutamine, 0.1 mM β-mercaptoethanol and 1% antibiotics-antimycotics with 4 ng/ml basic fibroblast growth factor (Invitrogen Life Technologies) and 10% FBS. Inactive feeder cells were replaced

by stem cell culture medium 2 h prior to blastocyst plating. Blastocysts were removed from the zona pellucida using 0.5% protease. For plating, blastocysts were washed three times in stem cell culture medium and plated onto the feeder cell layer. The plating efficiency of primary cultures was determined by scoring the number of attached colonies after 48 h. Medium was changed daily, and cells were passaged manually every 7-10 days under phase contrast microscopy (DMI 4000B, Leica Microsystems, Wetzlar, Germany).

AP activity detection and in vitro differentiation. After removal of the medium, pESCs were washed with PBS three times and then fixed with PBS containing 4% paraformaldehyde for 5 min at room temperature. AP activity was then assayed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate solution (Roche). To confirm the *in vitro* differentiation of putative pESCs, cells were harvested and washed to remove feeder cells. Putative pESCs were transferred into DMEM supplemented with 10% FBS without bFGF for 14 days of suspension.

Statistical analysis. Statistical analyses were performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Percentages and rates (e.g., cleavage, blastocyst formation and number of nuclei) were compared by one-way analysis of variance followed by Duncan's multiple range test. All values are expressed as the mean ± standard error of the mean.

Results

Effect of the novel system on blastocyst quality. In the first set of experiments, the novel system was evaluated regarding subsequent embryonic development following PA and IVF.

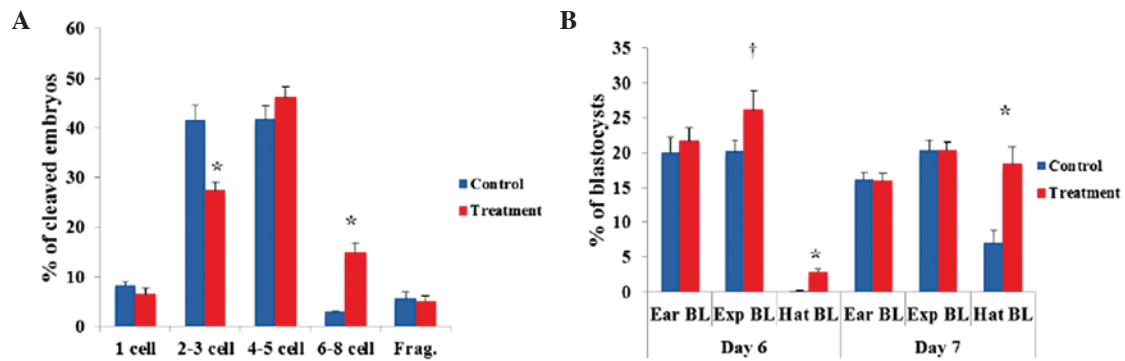


Figure 1. Effects of the novel system on the cleavage and blastocyst patterns after PA. (A) Cleavage pattern at day 2 after PA. (B) Blastocyst pattern after PA. The data are expressed as the mean \pm standard error of the mean of three replicates ($^*P < 0.05$, $^\dagger P = 0.074$). PA, parthenogenetic activation; BL, blastocyst; Ear, early; Exp, expanded; Hat, hatched.

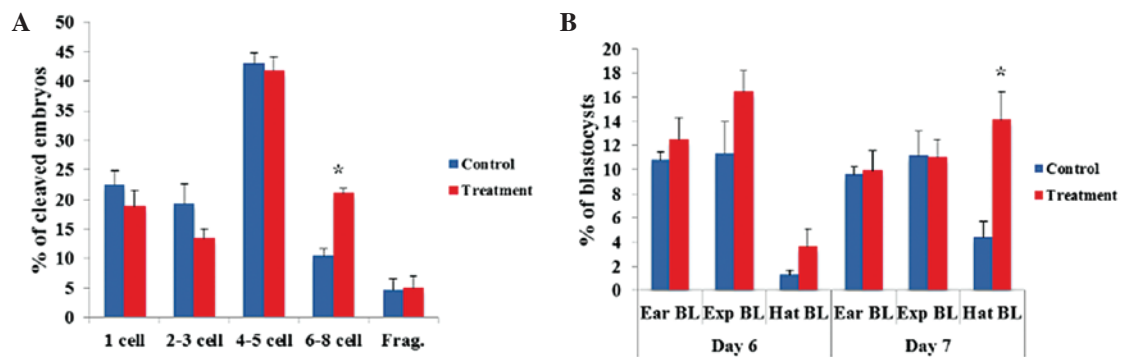


Figure 2. Effects of the novel system on cleavage and blastocyst patterns after *in vitro* fertilization. (A) Cleavage pattern at day 2 after PA. (B) Blastocyst pattern after PA. The data are expressed as the mean \pm standard error of the mean of three replicates ($^*P < 0.05$). PA, parthenogenetic activation; BL, blastocyst; Ear, early; Exp, expanded; Hat, hatched.

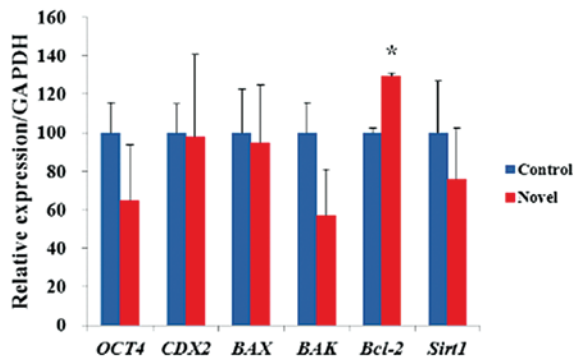


Figure 3. mRNA expression levels of *OCT4*, *CDX2*, *BAX*, *BAK*, *Bcl-2* and *Sirt1* in blastocysts following parthenogenetic activation following novel *in vitro* maturation system. The data are expressed as the mean \pm standard error of the mean of three replicates ($^*P = 0.067$).

The blastocyst formation rates were significantly greater ($P < 0.05$) in the novel system ($54.5 \pm 1.8\%$) compared to those in the control system ($43.4 \pm 1.2\%$) in PA (Table II). The total numbers of blastocysts also tended to be greater ($P < 0.05$) in the novel system (55.1 ± 2.0) compared to those in the control system (45.6 ± 2.0). In the IVF experiment, the blastocyst formation rate and total number of blastocysts were significantly greater ($P < 0.06$) in the novel system ($36.9 \pm 3.3\%$ and 78.9 ± 6.8 , respectively) compared to those in the control system

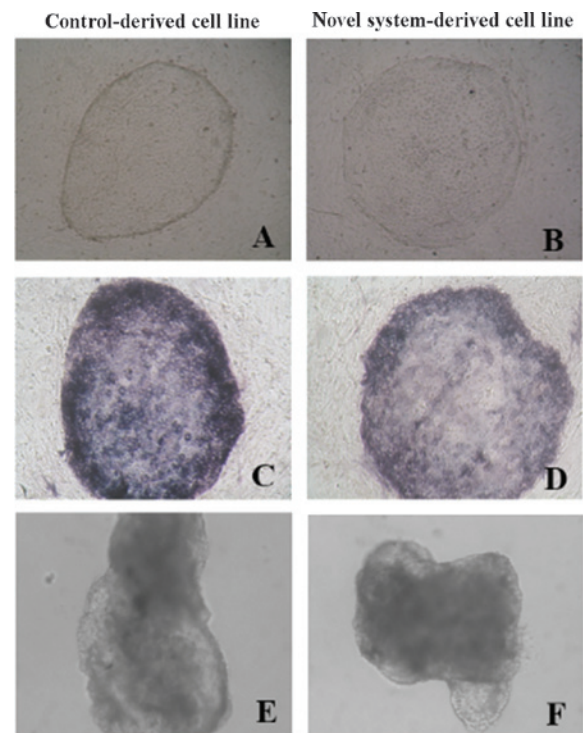


Figure 4. Derivation of putative pESC lines from blastocysts following parthenogenetic activation. (A and B) Typical morphology of putative pESCs; (C and D) alkaline phosphatase activity analysis; (E and F) embryoid body formation (magnification, x50). pESC, porcine embryonic stem cell.

Table IV. Effects of the novel system on the attachment rate of porcine blastocysts.

IVP	Group	Blastocysts attached, n (%)			Average (mean \pm SEM)
		Trial no. 1	Trial no. 2	Trial no. 3	
PA	Control	2/10 (20.0)	4/21 (19.0)	2/16 (12.5)	17.2 \pm 2.4
	Novel system	5/14 (35.7)	6/21 (28.6)	6/27 (22.2)	28.8 \pm 3.9 ^a
IVF	Control	3/15 (20.0)	1/21 (7.7)	-	13.9 \pm 8.7
	Novel system	10/18 (55.6)	1/7 (14.3)	-	35.0 \pm 29.2

^aP=0.062. IVF, *in vitro* fertilization; PA, parthenogenetic activation; SEM, standard error of the mean; IVP, *in vitro* production.

Table V. Effects of the novel system on the colonization rate of porcine blastocysts.

IVP	Group	No. (%) of blastocyst colonized		Average (mean \pm SEM)
		Trial no. 1	Trial no. 2	
PA	Control	0/10 (0)	1/21 (4.8)	2.4 \pm 3.4
	Novel system	4/14 (21.4)	1/21 (4.8)	13.1 \pm 11.7
IVF	Control	0/15 (0)	1/7 (14.3)	7.2 \pm 10.1
	Novel system	5/18 (27.8)	1/12 (7.7)	17.8 \pm 14.2

IVF, *in vitro* fertilization; PA, parthenogenetic activation; SEM, standard error of the mean; IVP, *in vitro* production.

Table VI. Association between the quality and attachment efficiency of *in vitro* fertilization blastocysts.

Group	Blastocysts attached, n (%)					Average (mean \pm SEM)
	Trial no. 1	Trial no. 2	Trial no. 3	Trial no. 4	Trial no. 5	
Early blastocysts	0/6 (0)	0/3 (0)	0/5 (0)	1/5 (20.0)	-	5.0 \pm 5.0 ^a
Expanding blastocysts	1/2 (50.0)	1/2 (50.0)	2/5 (20.0)	4/7 (57.1)	4/4 (100)	55.4 \pm 12.9 ^b
Hatched blastocysts	2/3 (66.7)	2/3 (66.7)	1/1 (100)	4/5 (80.0)	-	78.4 \pm 7.9 ^b

^{a,b}Values with different superscripts within a column differ significantly (P<0.05). SEM, standard error of the mean.

(26.2 \pm 2.9% and 58.5 \pm 7.2, respectively) (Table III). Regarding the cleavage pattern, there were significantly more 6- to 8-cell PA embryos and fewer 2- to 3-cell PA embryos in the novel system compared to the control system (Fig. 1A). However, no significant differences were observed in the cleavage patterns of 4- to 5-cell PA embryos. Regarding the blastocyst formation patterns on days 6 and 7, the number of hatched PA blastocysts was significantly (P<0.05) greater in the novel system than that in the control system (Fig. 1B). However, there were no significant differences in the numbers of early and expanding PA blastocysts. In the IVF experiment, there were significantly more 6- to 8-cell IVF embryos produced by the novel system compared to the control system (Fig. 2A). The hatched IVF blastocyst formation rates on day 7 were significantly higher in the novel system than those in the control system (P<0.05) (Fig. 2B).

To examine the expression of pluripotency- and apoptosis-associated genes, the mRNA expression levels of *Oct4*, *Cdx2*, *Bax*, *Bak*, *Bcl-2* and *Sirt1* in blastocysts from each system were examined (Fig. 3). The blastocysts derived from the novel system showed significantly (P<0.067) higher mRNA expression levels of *Bcl-2* compared to those from the control system. No significant differences were observed for the other transcripts.

Effect of the novel system on the attachment and outgrowth of blastocysts. As it is difficult to isolate ICM cells from intact porcine blastocysts produced *in vitro*, whole blastocysts were plated directly onto mouse embryonic fibroblasts. Following plating, the attachment rates were higher in blastocysts produced by the novel system (28.8 \pm 3.9% in PA and 35.0 \pm 29.2% in IVF) compared to those in the control system (17.2 \pm 2.4% in PA and

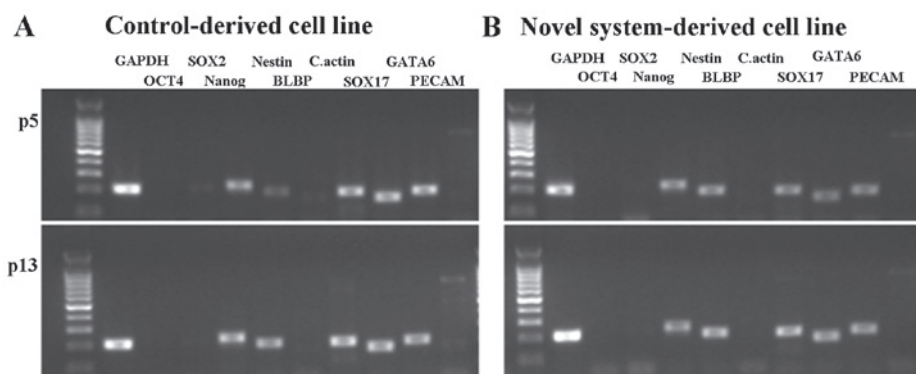


Figure 5. Reverse transcription-polymerase chain reaction analysis of pESCs. (A) Control system-derived pESC line. (B) Novel system-derived pESC line. *GAPDH*, *OCT4*, *SOX2*, *Nanog*, *NESTIN*, *BLBP*, *C.actin*, *SOX17*, *GATA6* and *PECAM* were determined. pESC, porcine embryonic stem cell.

13.9±3.9% in IVF) (Table IV). Furthermore, the colonization rate was higher in the novel system (13.1±11.7% in PA and 17.8±14.2% in IVF) (Table V). Two putative pESC lines were derived from PA blastocysts using this novel system and one putative pESC line using the control system. The association between blastocyst quality and attachment efficiency was also analyzed. The numbers of expanding and hatched blastocysts (55.4±12.9% and 78.4±7.9%, respectively) were significantly greater than those of early blastocysts (5.0±5.0%) (Table VI).

Generation and characterization of porcine pluripotent cell lines. Putative pESC lines were derived from PA embryos created by the novel and control systems. For characterization, various parameters were compared between putative pESC lines from PA embryos derived by the two systems, including typical morphology, AP activity analysis, EB formation and gene expression analysis (*OCT4*, *SOX2*, *Nanog*, *NESTIN*, *BLBP*, *C.actin*, *SOX17*, *GATA6* and *PECAM*). All of the cell lines showed similar primary colonies with typical morphology and AP activity, as shown in Fig. 4A-D. The differentiation potentials of these cell lines into EBs were subsequently assessed. As shown in Fig. 4E and F, the two cell lines showed the potential to undergo EB formation following five days of culture. When EBs were cultured onto plates coated with 0.1% gelatin, a variety of differentiated cells were observed after 2 weeks (data not shown). The expression of *Nanog* was confirmed in the two cell lines, whereas *OCT4* and *SOX2* were weakly expressed or not expressed at all (Fig. 5). The three genes known to be involved in differentiation (ectoderm markers *NESTIN* and *BLBP*; mesoderm marker *C.actin*; endoderm markers *SOX17* and *GATA6*) were expressed in all cell lines.

Discussion

Blastocyst quality is one of the most important factors in implantation as well as in pregnancy as is indicated by the presence of distinct and large ICMs in high-quality blastocysts vs. poor-quality blastocysts (25,26). For the establishment of ESCs from the ICM of expanded and hatched blastocysts in an *in vitro* system, initial blastocyst quality is crucial. The present study established a novel system using a modified culture medium treated with resveratrol, pGM-CSF

and β -ME, which improved blastocyst quality and subsequently enhanced the colonization rates of putative pESCs.

Several studies have attempted to enhance the quality of blastocysts in an *in vitro* system in IVM as well as IVC. According to a previous study by our group, treatment with 2.0 μ M resveratrol during IVM improved the developmental potentials of PA and IVF porcine embryos by increasing intracellular glutathione levels, decreasing reactive oxygen species (ROS) and regulating gene expression during oocyte maturation due to the anti-oxidative effect of resveratrol (27). Treatment with pGM-CSF in the IVC medium also improved the developmental potential of porcine IVF embryos (28). In addition, the supplementation of IVC medium with β -ME was able to increase the developmental competence of porcine PA embryos (29). β -ME was also able to increase intracellular glutathione levels and reduce damage to the embryo caused by ROS, thus improving embryo development. The present study confirmed the positive combination effect of the above reagents, showing that the novel system, by adding RES during IVM and RES, pGM-CSF, β -ME during IVC, produced more blastocysts with higher quality in PA and IVF embryos.

Furthermore, the novel system produced embryos with significantly higher expression levels of *Bcl-2* mRNA when compared with the control system. *Bcl-2* is well known to inhibit apoptosis in oocytes and embryos, while *Bax* is a pro-apoptotic gene (30). During the development of the early embryo, apoptosis can cause embryonic damage, including embryo arrest and developmental failure (31). In *in vitro* conditions, environmental stresses such as oxidative stress can also lead to apoptosis (32,33). Therefore, the expression levels of anti-apoptotic genes suggested that the DNA damage occurring under *in vitro* conditions was significantly reduced in the novel system.

In vivo-derived blastocysts have markedly higher attachment and colony outgrowth rates than their *in vitro*-derived counterparts, which show an indistinct ICM in most of the embryos. Thus, most pESC lines have been established from *in vivo*-derived embryos (34). As shown by the results of the present study, due to the beneficial effects of the novel system, the embryos demonstrated better attachment rates and primary colony formation competence than control embryos. In human ESC derivation, blastocysts with a blastocoel size equal to or larger than half of the embryo volume had a higher rate

of establishing ESC cell lines (35). In the present study, the expanding and hatched blastocysts showed a significantly higher attachment rate than blastocysts at an early stage, which was expected. As the novel system produced significantly more hatched blastocysts at day 7 compared to the control system, a greater number of opportunities was available to establish putative pESC cell lines from *in vitro*-derived porcine embryos.

Putative pESCs from PA embryos derived by the novel system showed certain pluripotent characteristics, including alkaline phosphatase activity, embryoid body formation and *Nanog* expression. However, the low or absent expression of *Oct4* and *Sox2* along with the expression of three germ layer markers (*Nestin*, *C.actin*, *Sox17* and *Gata6*) showed that these cell lines lost a certain amount of their pluripotency and may have been partially differentiated. In a recent study on putative pESC derivation, *Oct4* and *Nanog* expression were detected (36), and *Oct4*, *Nanog* and *Sox2* expression was reported in another study (37). The pluripotent gene expression profile may have been affected by the different culture conditions. Therefore, it is presumed that the differences in gene expression observed in the present study are due to the different culture conditions and that this reflects the myriad of difficulties faced in the derivation of porcine ESCs. Thus, further studies are required.

In conclusion, the novel system using RES during IVM and RES, β -ME, pGM-CSF during IVC improved the quality of blastocysts and increased the derivation efficiency of putative pESC cell lines from *in vitro*-derived porcine embryos. Therefore, the present study suggested that this novel system in combination with techniques for establishing ESCs provides a more efficient approach to produce bona fide pESC as compared with traditional systems. The method established in the present study will help improve *in vitro* systems for producing porcine embryos as well as stem cell research on domestic animals.

Acknowledgements

This study was supported, in part, by a grant from the Cooperative Research Program for Agriculture Science & Technology Development (no. PJ011288), the Rural Development Administration and the National Research Foundation of Korea Grant funded by the Korean Government (nos. NRF-2012R1A1A4A01004885, NRF-2013R1A2A2A04008751).

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