

## Porcine Granulocyte-Macrophage Colony-Stimulating Factor Improves the *in vitro* Development of Cloned Porcine Embryos

Seong-Sung KWAK<sup>1</sup>), Seung-A CHEONG<sup>1</sup>), Yubyeol JEON<sup>1</sup>) and Sang-Hwan HYUN<sup>1</sup>)\*

<sup>1</sup>Laboratory of Veterinary Embryology and Biotechnology, College of Veterinary Medicine, Chungbuk National University, Cheongju 361-763, Republic of Korea

(Received 7 February 2012/Accepted 10 April 2012/Published online in J-STAGE 24 April 2012)

**ABSTRACT.** We examined the effects of porcine granulocyte-macrophage colony-stimulating factor (pGM-CSF) on the *in vitro* development of porcine embryos produced by somatic cell nuclear transfer (SCNT) for the first time. We evaluated the effects of pGM-CSF on SCNT-derived blastocyst formation and investigated gene expression. A total of 522 cloned embryos in 6 replicates were treated with 10 ng/ml pGM-CSF during *in vitro* culture (IVC). This treatment significantly ( $P < 0.05$ ) increased blastocyst formation and total cell number in blastocysts compared with the control (12.3% and 41.4 vs. 9.0% and 34.7, respectively). However, there was no effect on cleavage rate. The numbers of cells in the inner cell mass and trophectoderm were significantly higher in the pGM-CSF treatment group (6.0 and 43.0, respectively) compared with the control (4.4 and 31.9, respectively). Treatment with 10 ng/ml pGM-CSF significantly increased *POU5F1* and *Cdx2* mRNA expression in blastocysts. In addition, *Bcl-2*, *Dnmt1* and proliferating cell nuclear antigen (*PCNA*) mRNA expression were upregulated in blastocysts in the pGM-CSF supplemented group compared with the control. These results suggest that pGM-CSF improves the quality and developmental viability of porcine SCNT embryos by regulating transcription factor expression.

**KEY WORDS:** cytokine, embryo culture, embryo manipulation, embryonic development, swine.

doi: 10.1292/jvms.12-0050; *J. Vet. Med. Sci.* 74(9): 1095–1102, 2012

Recently, the pig has become a useful model for translational research. Despite significant improvements in culture conditions and techniques for porcine embryo research, the development of *in vitro*-produced embryos is still suboptimal, especially for embryos obtained from somatic cell nuclear transfer (SCNT). Because the preimplantation embryo is extremely sensitive to environmental factors, inadequate culture environments often lead to aberrant embryo development as evidenced by lower rates of blastocyst formation and lower total cell numbers in blastocysts [14]. In addition, cloned embryos have incomplete or incorrect epigenetic reprogramming [reviewed in 17, 35]. Therefore, modifications to the culture system that more closely mimic the *in vivo* microenvironment can potentially improve the development and reprogramming of porcine cloned embryos.

The growth and development of preimplantation embryos in the female reproductive tract can be regulated by various growth factors and cytokines [18]. These molecules include leukemia inhibitory factor (LIF) [1], insulin-like growth factor-I (IGF-I) [46], epidermal growth factor (EGF) [45], transforming growth factor beta (TGF- $\beta$ ) [16] and vascular endothelial growth factor [4]. Most of these growth factors and regulatory molecules are derived from or produced by the endometrium or embryo itself. These molecules mediate

signaling between the maternal tissues and the developing embryo and act on the pre- and post-implantation embryo to modulate developmental competence [18, 33].

Among these embryotrophic factors, granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that also affects the development of preimplantation embryos. GM-CSF is expressed during early pregnancy in epithelial cells lining the oviduct and uterus in humans [9], rodents [37] and other mammalian species [15, 32]. It enhances the development of preimplantation embryos *in vitro* and increases total cell numbers in the embryos of humans [41], mice [38], cows [13] and pigs [12, 26]. Human embryos exposed to recombinant human GM-CSF *in vitro* blastulate earlier, have increased cell numbers in both the inner cell mass (ICM) and trophectoderm (TE) with reduced apoptosis [42] and show improved ability to hatch and attach to the culture dish [41]. In mice, GM-CSF also increases blastocyst formation, hatching and subsequent attachment to the culture dish. GM-CSF stimulates glucose metabolism and increases the number of viable blastomeres by inhibiting apoptosis [38]. In previous research, GM-CSF inhibited the cellular stress response and apoptosis pathways to facilitate embryo growth and survival [10]. In bovine [13] and porcine [12, 26] embryos, GM-CSF also increases the percentage of embryos that develop to the blastocyst stage *in vitro*.

Many studies have investigated the physiological functions and beneficial effects of GM-CSF on the development of various animal embryos produced by parthenogenetic activation or *in vitro* fertilization; however, there is no report of the effects of GM-CSF on embryonic development or nuclear reprogramming of cloned embryos. Because of

\*CORRESPONDENCE TO: HYUN, S. H., Laboratory of Veterinary Embryology and Biotechnology (VETEMBIO), College of Veterinary Medicine, Chungbuk National University, 12 Gawshin dong Heungduk-gu, Cheongju 361-763, Republic of Korea.  
e-mail: shhyun@cbu.ac.kr

its positive influence in a number of previous studies, we hypothesized that porcine GM-CSF (pGM-CSF) may also be beneficial for the embryonic development of porcine cloned embryos under *in vitro* conditions. The developmental competence of porcine cloned SCNT embryos was examined in culture media in the presence and absence of pGM-CSF. Furthermore, total cell number, numbers of ICM and TE in blastocysts and the expression of various transcripts that are essential and related to reprogramming and apoptosis during embryo development (*POU5F1*, *Cdx2*, *Dnmt1*, *PCNA*, *Bax*, *Bcl-2* and *Caspase-3*) were investigated.

## MATERIALS AND METHODS

**Chemicals:** Unless otherwise indicated, all chemicals and reagents used in the present study were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, U.S.A.). In this study, we used recombinant porcine granulocyte-macrophage colony-stimulating factor (pGM-CSF) purchased as a lyophilized powder (R&D Systems Inc., Minneapolis, MN, U.S.A.).

**Oocyte collection and *in vitro* maturation:** Retrieval and IVM of porcine oocytes from local slaughterhouse-derived ovaries were performed as we described previously [4, 26]. Briefly, cumulus oocyte complexes (COCs) were aspirated using an 18-gauge needle attached to a 10 ml disposable syringe from superficial follicles 3 to 6 mm in diameters, pooled in 15 ml conical tubes and allowed to settle down as sediment for 5 min at 37°C. The supernatant was discarded, and the precipitate was resuspended with HEPES-buffered Tyrode's medium (TLH) containing 0.05% (w/v) polyvinyl alcohol (TLH-PVA) and observed under a stereomicroscope. Only compact COCs with  $\geq 3$  uniform layers of compact cumulus cells and homogenous cytoplasm were recovered from the collected fluid and washed three times in TLH-PVA. Approximately 50–60 COCs were transferred into each well of a 4-well Nunc dish (Nunc, Roskilde, Denmark) containing 500  $\mu$ l of culture medium (TCM-199; Invitrogen Corporation, Carlsbad, CA, U.S.A.) supplemented with 0.6 mM cysteine, 0.91 mM sodium pyruvate, 10 ng/ml epidermal growth factor, 75  $\mu$ g/ml kanamycin, 1  $\mu$ g/ml insulin, 10% (v/v) porcine follicular fluid (pFF), 10 IU/ml equine chronic gonadotropin (eCG) and 10 IU/ml hCG (Intervet, Boxmeer, Netherlands). For *in vitro* maturation (IVM), the selected COCs were incubated at 39°C in a humidified atmosphere of 5% CO<sub>2</sub> in 95% humidified air. After 21–22 hr of maturation with hormones, the COCs were washed twice in hormone-free IVM medium and then cultured in hormone-free IVM medium for an additional 18–19 hr.

**Donor cell preparation:** Donor cell preparation was performed same as described previously [4]. Fibroblasts were isolated from fetuses at day 40 of gestation. The head and other soft tissues were removed using iris scissors and watchmaker's forceps and discarded. After washing twice with DPBS (Invitrogen, Carlsbad, CA, U.S.A.), the carcass was minced with a surgical blade on a 100-mm culture dish. The minced fetal tissues were dissociated in DMEM (Invitrogen) supplemented with 0.1% (w/v) trypsin and 1 mM EDTA (In-

vitrogen) for 1–2 hr. Trypsinized cells were washed once by centrifugation at 300 $\times$ g for 10 min and subsequently seeded into 100-mm plastic culture dishes. Seeded cells were then cultured for 6–8 days in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen), 1 mM sodium pyruvate, 1% (v/v) nonessential amino acids (Invitrogen) and 10 mg/ml penicillin–streptomycin solution at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. After removing the unattached clumps of cells or explants, the attached cells were further cultured until confluent. Subculturing was done at intervals of 5–7 days by trypsinization for 2 min using 0.1% trypsin and 0.02% EDTA. The cells were then stored in freezing medium in liquid nitrogen after 2 passages. The freezing medium consisted of 70% (v/v) DMEM, 10% (v/v) DMSO and 20% (v/v) FBS. Prior to SCNT, the cells were thawed and subsequently cultured in 10% FBS with DMEM for 3–4 days until 80% confluence. The individual cells were retrieved from the monolayer by trypsinization for 1 min and subsequently used for SCNT.

**Micromanipulation for somatic cell nuclear transfer, fusion and activation:** After 40 hr of IVM, denuded oocytes were incubated for 5 min in a manipulation medium (calcium-free TLH-BSA) containing 5  $\mu$ g/ml Hoechst 33342 and then washed twice with fresh manipulation medium. The washed oocytes were transferred into a drop of manipulation medium containing 5  $\mu$ g/ml cytochalasin B. Oocytes were enucleated by aspirating the PB and MII chromosomes using a 16  $\mu$ m glass pipette (Humagen, Charlottesville, VA, U.S.A.). After enucleation using a fine injecting pipette, a 14–15  $\mu$ m trypsinized fetal fibroblast with a smooth cell surface was transferred into the perivitelline space of an enucleated oocyte. The couplets were equilibrated with 280 mM mannitol solution containing 0.001 mM CaCl<sub>2</sub> and 0.05 mM MgSO<sub>4</sub> [43] for 2–3 min and transferred to a fusion chamber containing two electrodes overlaid with mannitol solution. Membrane fusion was induced by applying an alternating current (AC) field of 2 V cycling at 1 MHz for 2 sec, followed by two pulses of 160 V/mm direct current (DC) for 60  $\mu$ sec using a cell fusion generator (LF101; Nepa Gene, Chiba, Japan). Fused oocytes were washed 3–4 times with TLH-BSA. After 1 hr, the oocytes were evaluated for membrane fusion under a stereomicroscope prior to activation. Reconstructed oocytes were activated with 2 pulses of 120 V/mm DC for 60  $\mu$ sec in 280 mM mannitol solution containing 0.1 mM CaCl<sub>2</sub> and 0.05 mM MgSO<sub>4</sub>. Following electrical activation, SCNT embryos were treated with 0.4  $\mu$ g/ml demecolcine and 5  $\mu$ g/ml cytochalasin B in porcine zygote medium (PZM)-3 for 4 hr [43]. SCNT embryos were washed three times in fresh IVC medium, transferred into 30  $\mu$ l IVC droplets of medium under mineral oil and then cultured at 39°C in a humidified atmosphere of 5% O<sub>2</sub>, 5% CO<sub>2</sub> and 90% N<sub>2</sub> for 7 days.

**Embryo evaluation and total cell count:** The day of SCNT was designated as day 0. The embryos were evaluated under a stereomicroscope for cleavage on day 2 (48 hr). Blastocyst formation was assessed at day 7 (168 hr) after SCNT. To determine the total cell number of blastocysts, at day 7, blastocysts were collected and washed in 1% (w/v) PBS-

Table 1. Primers used for gene expression analysis

Gene	Primer sequences	Product size (bp)	GenBank accession number
<i>PCNA</i>	F: 5'-CCTGTGCAAAAGATGGAGTG-3' R: 3'-GGAGAGAGTGGAGTGGCTTTT-5'	187	XM_003359883
<i>Dnmt1</i>	F: 5'-CCTCTATGGACGGCTTGAGT-3' R: 3'-GGTGCTGTCCAGGATGTTG-5'	185	NM_001032355
<i>POU5F1</i>	F: 5'-GCGGACAAGTATCGAGAACC-3' R: 3'-CCTCAAATCCTCTCGTTGC-5'	200	NM_001113060
<i>Cdx2</i>	F: 5'-GGAACCTGTGCGAGTGGATG-3' R: 3'-GCTCGGCCTTTCTCCGAATG-5'	168	CK_458871
<i>Bax</i>	F: 5'-TGCTCAGGATGCATCTACC-3' R: 3'-AAGTAGAAAAGCGCGACCAC-5'	199	XM_003127290
<i>Bcl-2</i>	F: 5'-AGGGCATTGAGTACCTGAC-3' R: 3'-CGATCCGACTCACCAATACC-5'	193	NM_214285
<i>Caspase-3</i>	F: 5'-CGTGCTTCTAAGCCATGGTG-3' R: 3'-GTCCACTGTCCGTCTCAAT-5'	186	NM_214131
<i>GAPDH</i>	F: 5'-GTCGGTTGTGGATCTGACCT-3' R: 3'-TTGACGAAAGTGGTCGTTGAG-5'	207	NM_001206359

BSA and stained with 10  $\mu\text{g/ml}$  Hoechst 33342 for 5 min. Following a final wash in PBS-BSA, embryos were fixed briefly in 4% paraformaldehyde in PBS. Then, the blastocysts were mounted on glass slides in a drop of 100% glycerol, squashed gently with a cover slip and observed under a fluorescence microscope (Nikon Corp., Tokyo, Japan) at  $\times 400$  magnification.

**Differential staining of blastocysts:** The quality of the blastocysts was assessed by the differential staining of inner cell mass (ICM) and trophectoderm (TE) cells according to a modified staining procedure [44]. Briefly, TE cells of blastocysts at 168 hr were stained with 100  $\mu\text{g/ml}$  fluorochrome propidium iodine after treatment with permeabilizing solution containing 1% (v/v) Triton X-100 ionic detergent. Blastocysts were then incubated in a second solution containing 100% ethanol (for fixation) at 4°C and bisbenzimidazole. Fixed and stained whole blastocysts were mounted and assessed for the number of cells present using epifluorescence microscopy (Leica, Germany) at  $\times 400$  magnification.

**Gene expression analysis by real-time PCR:** The expression of *POU5F1*, *Cdx2*, *Dnmt1*, *PCNA*, *Bax*, *Bcl-2* and *Caspase-3* mRNA in SCNT-derived blastocysts was analyzed by real-time PCR, as previously described [4]. Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.), according to the manufacturer's protocol, and the total RNA concentration was determined by measuring the absorbance at 260 nm. First-strand complementary DNA (cDNA) was prepared by subjecting 1  $\mu\text{g}$  of total RNA to reverse transcription using Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) and random primers (9-mers; Takara Bio. Inc., Shiga, Japan). To determine the conditions for logarithmic phase PCR amplification of target mRNA, 1  $\mu\text{g}$  aliquots were amplified using differing numbers of cycles. The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), was PCR-amplified to rule out the possibility of RNA degradation and to control for the variation in mRNA concentrations in the RT reaction.

A linear relationship between the PCR product band visibility and the number of amplification cycles was observed for the target mRNAs. The *GAPDH* and target genes were quantified using 40 cycles. The cDNA was amplified in a 20  $\mu\text{l}$  PCR reaction, which contained 1 U *Taq* polymerase (Intron Bio Technologies, Co., Ltd., Seongnam, South Korea), 2 mM dNTP mix and 10  $\mu\text{M}$  of each gene-specific primer. Quantitative real-time PCR was performed with 1  $\mu\text{l}$  cDNA template added to 10  $\mu\text{l}$  2 $\times$  SYBR Premix Ex Taq (Takara Bio Inc.) containing specific primers at a concentration of 10  $\mu\text{M}$  each. The reactions were carried out for 40 cycles, and the cycling parameters were as follows: denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. All oligonucleotide primer sequences are presented in Table 1. The fluorescence intensity was measured at the end of the extension phase of each cycle. The threshold value for the fluorescence intensity of all samples was set manually. The reaction cycle at which the PCR products exceeded this fluorescence intensity threshold was deemed the threshold cycle (Ct) in the exponential phase of the PCR amplification. The expression of each target gene was quantified relative to that of the internal control gene (*GAPDH*). The relative quantification was based on a comparison of Ct values at constant fluorescence intensity. The amount of transcript present was inversely related to the observed Ct, and for every 2-fold dilution in the amount of transcript, Ct was expected to increase by 1. The relative expression (R) was calculated using the equation  $R = 2^{-[\Delta\text{Ct}_{\text{sample}} - \Delta\text{Ct}_{\text{control}}]}$ . To determine a normalized arbitrary value for each gene, every obtained value was normalized to that of *GAPDH*. The experiments were repeated four times.

**Experimental design:** SCNT embryos were produced and then cultured in medium treated with or without 10  $\text{ng/ml}$  pGM-CSF for 7 days. In experiment 1, the effect of 10  $\text{ng/ml}$  pGM-CSF treatment during IVC on embryonic development was examined. In experiment 2, the effect of pGM-CSF treatment during IVC on blastocyst cell numbers especially cell

Table 2. Effect of pGM-CSF treatment during *in vitro* culture on embryonic development after somatic cell nuclear transfer of porcine oocytes

Group	No. of embryos cultured	No. (%) embryos developed to		Total cell number (n)**
		≥2 cells	Blastocyst *	
Control	258	171 (66.3 ± 2.8)	22 (9.0 ± 1.0) <sup>a)</sup>	34.7 ± 1.5 (13) <sup>a)</sup>
pGM-CSF	264	188 (72.3 ± 3.4)	33 (12.3 ± 0.9) <sup>b)</sup>	41.4 ± 2.2 (19) <sup>b)</sup>

\* Percentage of total cultured oocytes. \*\* Number of blastocysts examined.

a,b) Values with different superscripts within a column differ significantly ( $P < 0.05$ ).

The experiment was repeated six times. The data represent means ± SEM.

pGM-CSF: 10 ng/ml porcine granulocyte-macrophage colony-stimulating factor.

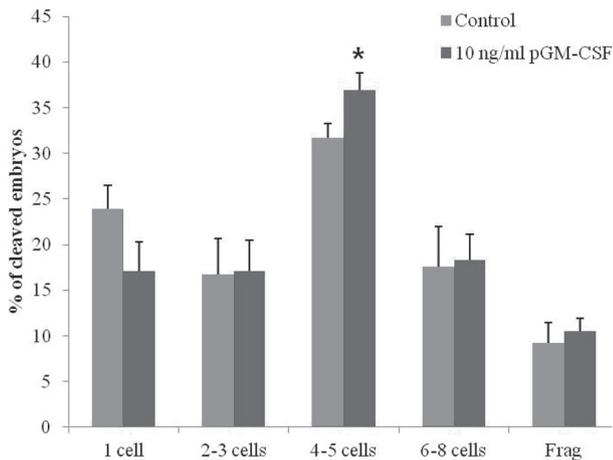


Fig. 1. Embryo cleavage pattern when 10 ng/ml pGM-CSF was supplemented during IVC at Day 2. There were no significant difference between the control and pGM-CSF treatment, but 10 ng/ml pGM-CSF treatment (\*) tended ( $P = 0.06$ ) to produce more 4-5-cell embryos than the control.

numbers of the ICM and TE was examined by differential staining. In experiment 3, the effect of pGM-CSF treatment on the mRNA expression of *POU5F1*, *Cdx2*, *Dnmt1*, *PCNA*, *Bax*, *Bcl-2* and *Caspase-3* in SCNT-derived blastocysts was analyzed.

**Statistical analysis:** Statistical analyses were carried out using SPSS 17.0 (SPSS Inc., Chicago, IL, U.S.A.). The experimental data were checked for homogeneity of variances across treatments by Levene's F-test and were analyzed by the Student's *t*-test. Data are presented as means ± SEM. Differences were considered to be significant, if the *P* value was less than 0.05.

## RESULTS

As shown in Table 2, the blastocyst formation rate and total cell number in blastocysts treated with 10 ng/ml pGM-CSF were significantly ( $P < 0.05$ ) higher than in control embryos (12.3% and 41.4 vs. 9.0% and 34.7, respectively). There was no difference in the cleavage rate between the groups ( $P > 0.05$ ). However, the cleavage pattern slightly differed between the two groups. At day 2, more embryos

Table 3. Effects of pGM-CSF on total cell number, cell numbers of the ICM and TE and the ICM to TE ratio

	Control	pGM-CSF
Total cells (14)**	36.3 ± 1.9	49.0 ± 4.8*
ICM cells	4.4 ± 0.3	6.0 ± 0.5*
TE cells	31.9 ± 1.8	43.0 ± 4.4*
ICM to TE ratio	0.14 ± 0.01	0.15 ± 0.01

The data represent means ± SEM. The experiment was replicated three times. ICM, inner cell mass; pGM-CSF, porcine granulocyte-macrophage colony-stimulating factor; TE, trophoblast.

\*  $P < 0.05$ .

\*\* Number of evaluated blastocysts for cell counting.

treated with pGM-CSF tended ( $P = 0.06$ ) to be higher at the 4–5-cell stage than the control embryos (Fig. 1). Blastocysts formed in the presence of pGM-CSF had significantly more ICM and TE cells and also more total cells than control embryos (Table 3 & Fig. 2). There was no effect of pGM-CSF on the ICM to TE ratio (Table 3).

The effect of pGM-CSF treatment during *in vitro* culture (IVC) on the expression of mRNA in SCNT blastocysts is shown in Fig. 3. Treatment with pGM-CSF during IVC promoted significantly higher ( $P < 0.05$ ) expression of *POU5F1*, *Cdx2*, and *Bcl-2* mRNA in SCNT-derived blastocysts. In addition, blastocysts produced in the presence of pGM-CSF had a tendency towards higher mRNA expression of *Dnmt1* ( $P = 0.09$ ) and *PCNA* ( $P = 0.92$ ). However, there was no effect of pGM-CSF on *Caspase-3* or *Bax* mRNA expression (Fig. 3).

## DISCUSSION

Our results implicate pGM-CSF as an embryotrophic factor in the development of porcine SCNT embryos. Treatment with pGM-CSF increased blastocyst formation and promoted the proliferation and viability of ICM and TE cells in blastocysts. This beneficial effect was mediated by regulating the expression of transcription factors such as *POU5F1*, *Cdx2*, *Bcl-2*, *Dnmt1* and *PCNA*.

We first investigated the effect of pGM-CSF on cloned embryo development *in vitro*, using 10 ng/ml pGM-CSF because a previous study [26] identified this as the optimal concentration for porcine IVF embryo development, in

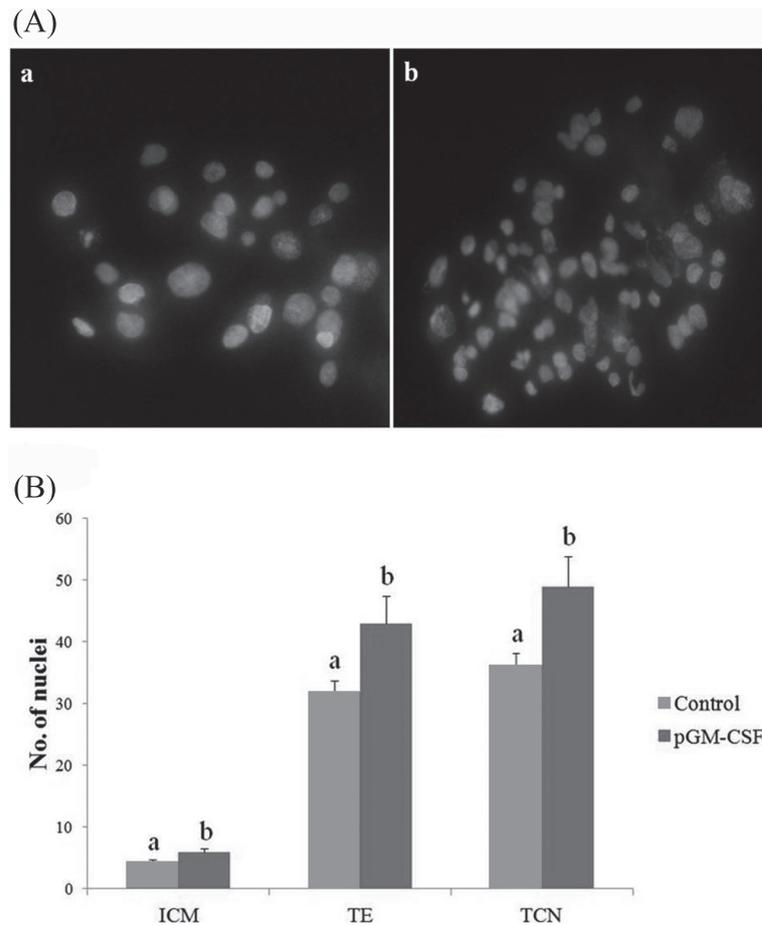


Fig. 2. Differentially stained day 7 porcine blastocysts. (A) The blue objects are the nuclei of ICM cells, and the red objects are the nuclei of TE cells. a: Differentially stained blastocyst produced with the control. b: Differentially stained blastocyst produced with the 10 ng/ml pGM-CSF treatment. The nuclei of blastocysts were photographed under magnification of 400 $\times$  and using a fluorescence microscope. (B) Cell numbers of blastocysts derived from treatment with or without pGM-CSF that developed *in vitro*. The numbers of ICM and TE cells and total cell numbers of blastocysts derived from treatment with 10 ng/ml pGM-CSF were significantly ( $P < 0.05$ ) higher than those of the control blastocysts. ICM, inner cell mass; TE, trophectoderm; TCN, total cell number.

agreement with previous reports for porcine parthenotes [12] and bovine embryos [13]. Supplementation with 10 ng/ml pGM-CSF during IVC improved blastocyst formation of cloned embryos and increased the total cell number of blastocysts. There was no significant difference in cleavage rate between the two groups, but cloned embryos in the presence of pGM-CSF had a tendency ( $P = 0.06$ ) to have more 4–5-cell stage embryos. This may have been due to its cytoprotective effects [20] in preimplantation embryos when they are cultured with pGM-CSF in early developmental stages.

Improved development of cloned embryos in the presence of pGM-CSF implies that the effect of GM-CSF is not limited to parthenogenetic or *in vitro*-fertilized embryos only. Here, treatment with pGM-CSF increased the proportion of

cloned embryos that became blastocysts as well as total cell number in blastocysts. The extent of these improvements was comparable to that reported for cloned embryos exposed *in vitro* to IGF-I [24] and VEGF [4]. Also, similar results were seen in previous studies conducted with GM-CSF in porcine IVF [26] and PA [12], bovine [13], human [41, 42] and mouse [38] embryos. Thus, it is clear that pGM-CSF treatment increases the viability of cloned embryos, allowing them to advance to the blastocyst stage of development.

The most evident effect of pGM-CSF in SCNT-derived blastocysts was an increase in the expression of *POU5F1* mRNA. The expression of *POU5F1* mRNA is essential for early development [8] and is involved in the self-renewal of undifferentiated embryonic stem cells [27]. Unlike in mice

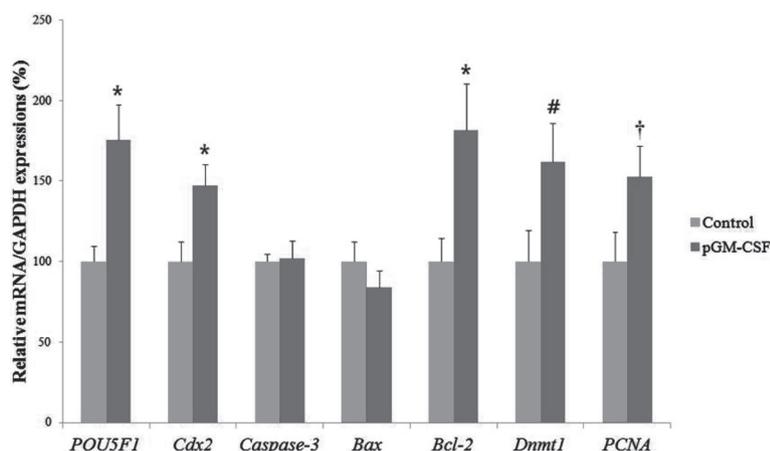


Fig. 3. Effect of pGM-CSF on gene expression in blastocysts. mRNA was quantified by real-time PCR from blastocysts cultured in control medium (5 blastocysts per one replicate) or medium with pGM-CSF and normalized to the expression of the reference gene, GAPDH. The transcripts analyzed were *POU5F1*, *Cdx2*, *Caspase-3*, *Bax*, *Bcl-2*, *Dnmt1* and *PCNA*. The experiment was replicated four times. Data are means  $\pm$  SEM. \* $P < 0.05$ ; # $P = 0.090$ ; † $P = 0.092$ .

[31], the pluripotency gene *POU5F1* is initially expressed in the ICM and TE of porcine preimplantation embryos [25]. In previous studies, expression of the pluripotency marker gene *POU5F1* was low in cloned embryos [5, 6]. The mechanisms underlying this presumably involve either accelerated cell division or diminished cell death. In this study, the observation that blastocysts from embryos treated with pGM-CSF had more ICM and TE cells than the control indicates the capacity of pGM-CSF to affect SCNT-derived blastocyst differentiation. Similarly, in human [42], mouse [22] and bovine [28] embryos, GM-CSF treatment during IVC results in more cells in the ICM of blastocysts. ICM cells are a crucial part of embryos, because they eventually give rise to the definite structure of the fetus. ICM cells are pluripotent; embryonic stem cells are derived from this population of cells. ICM cells in the preimplantation blastocyst stage appear to be a key parameter influencing implantation success and subsequent fetal size and health. Therefore, treatment with GM-CSF may improve the efficiency of transgenic pig production and also the establishment of SCNT-derived embryonic stem cells. Similarly, treatment with GM-CSF improves not only blastocyst formation *in vitro* but also posttransfer embryonic survival in murine [40] and bovine [28] embryos.

Treatment with pGM-CSF during IVC affected not only ICM cells but also TE cells and the expression of *Cdx-2* mRNA in cloned blastocysts. TE cells are restricted to the generation of trophoblast components of the placenta and play a supporting role in embryo development. GM-CSF increases the trophoblast proliferation rate in ovine [39] and bovine [30] embryos. Exposure of ovine and bovine embryos to GM-CSF *in vitro* increases their implantation potential through enhanced expression of the anti-luteotropic signal interferon- $\tau$  (IFN- $\tau$ ) in trophoblast cells [30, 39]. When cloning animals, low yield could possibly arise from

abnormal and/or poorly developed placentas. In cloned cows, trophoblast development during early stages of placentation is delayed [19]. Thus, GM-CSF treatment might help improve implantation of cloned porcine embryos.

We found that treatment with pGM-CSF during IVC increased the expression of transcription factor genes (*POU5F1*, *Cdx2*, *Bcl-2*, *Dnmt1* and *PCNA*) in blastocysts, except for *Bax* and *Caspase-3*. Modifying the culture conditions can modulate gene expression in mammalian cells and embryos [34, 47] and can also affect the epigenetic stage of the genome [21]. DNA methyltransferases 1 (*Dnmt1*) is involved in DNA methylation, cell proliferation [36] and nuclear reprogramming of cloned embryos [2]. In mice, cloned preimplantation embryos aberrantly express *Dnmt1*, and aberrant *Dnmt1* localization and expression may contribute to defects in DNA methylation and the developmental abnormalities seen in cloned mammals [11]. *PCNA* is an essential component of the DNA replication and repair machinery [23] and has been used as a parameter for the evaluation of developmental potential of bovine embryos [29]. Taken together, these results suggest that pGM-CSF treatment may influence nuclear reprogramming and the developmental potential of cloned embryos.

The *Bcl-2* family consists of proteins that play key roles in regulating apoptosis, and the *Bcl-2* gene is a well-known anti-apoptotic gene in oocytes and embryos [7]. Our findings are in agreement with a previous study that showed that GM-CSF upregulates *Bcl-2* expression in mouse embryos [3] and reduces apoptosis in embryos [3, 10, 42]. Changes in the expression of *Bcl-2* by pGM-CSF might inhibit the process of blastomere apoptosis in SCNT blastocysts. Thus, our data suggest that apoptosis in SCNT-derived blastocysts can be modulated by the addition of pGM-CSF and increase the viability and developmental competence of cloned embryos.

The molecular mechanisms by which pGM-CSF exerts

its beneficial effects on cloned embryo development are not clear. One possible mechanism of action is an anti-apoptotic effect via upregulation of *Bcl-2* mRNA through GM- $\alpha$  [42]. In mouse [38] and human [42] studies, preimplantation embryos have been shown to express only GM- $\alpha$  mRNA, not  $\beta$ c mRNA, throughout preimplantation development. It remains to be evaluated whether the effect of pGM-CSF on cloned embryos acts independently of the beta common ( $\beta$ c) subunit of the GM-CSF receptor. Therefore, further study will be needed to evaluate the expression of GM-CSF receptor in porcine preimplantation embryos.

In conclusion, we found that pGM-CSF helps to improve the quality and developmental ability of porcine SCNT embryos in terms of blastocyst rates, total cell number and especially the expression of *POU5F1* mRNA. We suggest that supplementation of IVC medium with pGM-CSF improves the developmental potential and nuclear reprogramming of porcine SCNT embryos. Moreover, pGM-CSF supplementation may improve the production of cloned pigs. These findings can potentially be applied to the establishment of SCNT-derived embryonic stem cells.

**ACKNOWLEDGMENTS.** This work was supported by a grant from the Next-Generation BioGreen 21 program (#PJ008121012011), Rural Development Administration, Republic of Korea

#### REFERENCES

- Anegon, I., Cuturi, M., Godard, A., Moreau, M., Terqui, M., F. M.-B. and Soulillou, J. 1994. Presence of leukaemia inhibitory factor and interleukin 6 in porcine uterine secretions prior to conceptus attachment. *Cytokine* **6**: 493–499. [Medline] [CrossRef]
- Armstrong, L., Lako, M., Dean, W. and Stojkovic, M. 2006. Epigenetic modification is central to genome reprogramming in somatic cell nuclear transfer. *Stem Cells* **24**: 805–814. [Medline] [CrossRef]
- Behr, B., Mooney, S., Wen, Y., Polan, M. L. and Wang, H. 2005. Preliminary experience with low concentration of granulocyte-macrophage colony-stimulating factor: a potential regulator in preimplantation mouse embryo development and apoptosis. *J. Assist. Reprod. Genet.* **22**: 25–32. [Medline] [CrossRef]
- Biswas, D., Jung, E., Jeung, E. and Hyun, S. 2011. Effects of vascular endothelial growth factor on porcine preimplantation embryos produced by *in vitro* fertilization and somatic cell nuclear transfer. *Theriogenology* **75**: 256–267. [Medline] [CrossRef]
- Boiani, M., Eckardt, S., Scholer, H. R. and McLaughlin, K. J. 2002. Oct4 distribution and level in mouse clones: consequences for pluripotency. *Genes Dev.* **16**: 1209. [Medline] [CrossRef]
- Bortvin, A., Eggan, K., Skaletsky, H., Akutsu, H., Berry, D. L., Yanagimachi, R., Page, D. C. and Jaenisch, R. 2003. Incomplete reactivation of Oct4-related genes in mouse embryos cloned from somatic nuclei. *Development* **130**: 1673. [Medline] [CrossRef]
- Boumela, I., Assou, S., Aouacheria, A., Haouzi, D., Dechaud, H., De Vos, J., Handyside, A. and Hamamah, S. 2011. Involvement of BCL2 family members in the regulation of human oocyte and early embryo survival and death: gene expression and beyond. *Reproduction* **141**: 549. [Medline] [CrossRef]
- Cauffman, G., Van de Velde, H., Liebaers, I. and Van Steirteghem, A. 2005. Oct-4 mRNA and protein expression during human preimplantation development. *Mol. Hum. Reprod.* **11**: 173. [Medline] [CrossRef]
- Chegini, N., Tang, X. and Dou, Q. 1999. The expression, activity and regulation of granulocyte macrophage-colony stimulating factor in human endometrial epithelial and stromal cells. *Mol. Hum. Reprod.* **5**: 459. [Medline] [CrossRef]
- Chin, P. Y., Macpherson, A. M., Thompson, J. G., Lane, M. and Robertson, S. A. 2009. Stress response genes are suppressed in mouse preimplantation embryos by granulocyte-macrophage colony-stimulating factor (GM-CSF). *Hum. Reprod.* **24**: 2997–3009. [Medline] [CrossRef]
- Chung, Y. G., Ratnam, S., Chaillet, J. R. and Latham, K. E. 2003. Abnormal regulation of DNA methyltransferase expression in cloned mouse embryos. *Biol. Reprod.* **69**: 146. [Medline] [CrossRef]
- Cui, X. S., Lee, J. Y., Choi, S. H., Kwon, M. S., Kim, T. and Kim, N. H. 2004. Mouse granulocyte-macrophage colony-stimulating factor enhances viability of porcine embryos in defined culture conditions. *Anim. Reprod. Sci.* **84**: 169–177. [Medline] [CrossRef]
- de Moraes, A. A. and Hansen, P. J. 1997. Granulocyte-macrophage colony-stimulating factor promotes development of *in vitro* produced bovine embryos. *Biol. Reprod.* **57**: 1060–1065. [Medline] [CrossRef]
- Duranthon, V., Watson, A. J. and Lonergan, P. 2008. Preimplantation embryo programming: transcription, epigenetics, and culture environment. *Reproduction* **135**: 141. [Medline] [CrossRef]
- Emond, V., MacLaren, L., Kimmins, S., Arosh, J., Fortier, M. and Lambert, R. 2004. Expression of cyclooxygenase-2 and granulocyte-macrophage colony-stimulating factor in the endometrial epithelium of the cow is up-regulated during early pregnancy and in response to intrauterine infusions of interferon. *Biol. Reprod.* **70**: 54. [Medline] [CrossRef]
- Gupta, A., Nancy, H., Bazer, F., Bustamante, L. and Jaeger, L. 1998. Beta transforming growth factors (TGF) at the porcine conceptus-maternal interface. Part I: Expression of TGF 1, TGF 2, and TGF 3 messenger ribonucleic acids. *Biol. Reprod.* **59**: 905. [Medline] [CrossRef]
- Han, Y.M., Kang, Y., Koo, D. and Lee, K. 2003. Nuclear reprogramming of cloned embryos produced *in vitro*. *Theriogenology* **59**: 33–44. [Medline] [CrossRef]
- Hardy, K. and Spanos, S. 2002. Growth factor expression and function in the human and mouse preimplantation embryo. *J. Endocrinol.* **172**: 221. [Medline] [CrossRef]
- Hashizume, K., Ishiwata, H., Kizaki, K., Yamada, O., Takahashi, T., Imai, K., Patel, O. V., Akagi, S., Shimizu, M. and Takahashi, S. 2002. Implantation and placental development in somatic cell clone recipient cows. *Cloning Stem Cells* **4**: 197–209. [Medline] [CrossRef]
- Huang, X., Choi, J. K., Park, S. R., Ha, Y., Park, H., Yoon, S. H., Park, H. C., Park, J. O. and Choi, B. H. 2007. GM-CSF inhibits apoptosis of neural cells via regulating the expression of apoptosis-related proteins. *Neurosci. Res.* **58**: 50–57. [Medline] [CrossRef]
- Jaenisch, R. and Bird, A. 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat. Genet.* **33**: 245–254. [Medline] [CrossRef]
- Karagenc, L., Lane, M. and Gardner, D. K. 2005. Granulocyte-macrophage colony-stimulating factor stimulates mouse blastocyst inner cell mass development only when media lack human serum albumin. *Reprod. BioMedicine Online* **10**: 511–518.

- [CrossRef]
23. Kelman, Z. 1997. PCNA: structure, functions and interactions. *Oncogene* **14**: 629. [Medline] [CrossRef]
  24. Kim, S., Lee, S. H., Kim, J. H., Jeong, Y. W., Hashem, M., Koo, O. J., Park, S. M., Lee, E. G., Hossein, M. and Kang, S. K. 2006. Anti apoptotic effect of insulin like growth factor (IGF) I and its receptor in porcine preimplantation embryos derived from *in vitro* fertilization and somatic cell nuclear transfer. *Mol. Reprod. Dev.* **73**: 1523–1530. [Medline] [CrossRef]
  25. Kuijk, E. W., Du Puy, L., Van Tol, H. T. A., Oei, C. H. Y., Haagsman, H. P., Colenbrander, B. and Roelen, B. A. J. 2008. Differences in early lineage segregation between mammals. *Dev. Dyn.* **237**: 918–927. [Medline] [CrossRef]
  26. Kwak, S. S., Jeung, S. H., Biswas, D., Jeon, Y. and Hyun, S. H. 2012. Effects of porcine granulocyte-macrophage colony-stimulating factor on porcine *in vitro*-fertilized embryos. *Theor. Biogenology* **77**: 1186–1197. [Medline] [CrossRef]
  27. Loh, Y. H., Wu, Q., Chew, J. L., Vega, V. B., Zhang, W., Chen, X., Bourque, G., George, J., Leong, B. and Liu, J. 2006. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat. Genet.* **38**: 431–440. [Medline] [CrossRef]
  28. Loureiro, B., Bonilla, L., Block, J., Fear, J. M., Bonilla, A. Q. and Hansen, P. J. 2009. Colony-stimulating factor 2 (CSF-2) improves development and posttransfer survival of bovine embryos produced *in vitro*. *Endocrinology* **150**: 5046–5054. [Medline] [CrossRef]
  29. Makarevich, A.V. and Markkula, M. 2002. Apoptosis and cell proliferation potential of bovine embryos stimulated with insulin-like growth factor I during *in vitro* maturation and culture. *Biol. Reprod.* **66**: 386. [Medline] [CrossRef]
  30. Michael, D. D., Wagner, S. K., Ocon, O. M., Talbot, N. C., Rooke, J. A. and Ealy, A. D. 2006. Granulocyte-macrophage colony-stimulating-factor increases interferon-tau protein secretion in bovine trophectoderm cells. *Am. J. Reprod. Immunol.* **56**: 63–67. [Medline] [CrossRef]
  31. Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H. and Smith, A. 1998. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95**: 379–391. [Medline] [CrossRef]
  32. O’Leary, S., Jasper, M., Warnes, G., Armstrong, D. and Robertson, S. 2004. Seminal plasma regulates endometrial cytokine expression, leukocyte recruitment and embryo development in the pig. *Reproduction* **128**: 237. [Medline] [CrossRef]
  33. O’Neill, C. 2008. The potential roles for embryotrophic ligands in preimplantation embryo development. *Hum. Reprod. Update* **14**: 275. [Medline] [CrossRef]
  34. Park, S. H., Park, S. B. and Kim, N. H. 2003. Expression of early development-related genes in bovine nuclear transferred and fertilized embryos. *Zygote* **11**: 355–360. [Medline] [CrossRef]
  35. Rideout, W. M., Eggan, K. and Jaenisch, R. 2001. Nuclear cloning and epigenetic reprogramming of the genome. *Science* **293**: 1093. [Medline] [CrossRef]
  36. Robertson, K. D. 2002. DNA methylation and chromatin-unraveling the tangled web. *Oncogene* **21**: 5361–5379. [Medline] [CrossRef]
  37. Robertson, S.A., Mayrhofer, G. and Seamark, R. 1992. Uterine epithelial cells synthesize granulocyte-macrophage colony-stimulating factor and interleukin-6 in pregnant and nonpregnant mice. *Biol. Reprod.* **46**: 1069. [Medline] [CrossRef]
  38. Robertson, S.A., Sjoblom, C., Jasper, M.J., Norman, R.J. and ark, R.F. 2001. Granulocyte-macrophage colony-stimulating factor promotes glucose transport and blastomere viability in murine preimplantation embryos. *Biol. Reprod.* **64**: 1206–1215. [Medline] [CrossRef]
  39. Rooke, J., Ewen, M., McEvoy, T., Entrican, G. and Ashworth, C. 2005. Effect of inclusion of serum and granulocyte-macrophage colony stimulating factor on secretion of interferon-tau during the *in vitro* culture of ovine embryos. *Reprod. Fertil. Dev.* **17**: 513–521. [Medline] [CrossRef]
  40. Sjöblom, C., Roberts, C. T., Wikland, M. and Robertson, S. A. 2005. Granulocyte-macrophage colony-stimulating factor alleviates adverse consequences of embryo culture on fetal growth trajectory and placental morphogenesis. *Endocrinology* **146**: 2142–2153. [Medline] [CrossRef]
  41. Sjöblom, C., Wikland, M. and Robertson, S. A. 1999. Granulocyte-macrophage colony-stimulating factor promotes human blastocyst development *in vitro*. *Hum. Reprod.* **14**: 3069–3076. [Medline] [CrossRef]
  42. Sjöblom, C., Wikland, M. and Robertson, S. A. 2002. Granulocyte-macrophage colony-stimulating factor (GM-CSF) acts independently of the beta common subunit of the GM-CSF receptor to prevent inner cell mass apoptosis in human embryos. *Biol. Reprod.* **67**: 1817–1823. [Medline] [CrossRef]
  43. Song, K., Hyun, S. H., Shin, T. and Lee, E. 2009. Post activation treatment with demecolcine improves development of somatic cell nuclear transfer embryos in pigs by modifying the remodeling of donor nuclei. *Mol. Reprod. Dev.* **76**: 611–619. [Medline] [CrossRef]
  44. Thouas, G.A., Korfiatis, N., French, A., Jones, G. and Trounson, A. 2001. Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. *Reprod. Biomed. Online* **3**: 25–29. [Medline] [CrossRef]
  45. Wei, Z., Park, K., Day, B. and Prather, R. 2001. Effect of epidermal growth factor on preimplantation development and its receptor expression in porcine embryos. *Mol. Reprod. Dev.* **60**: 457–462. [Medline] [CrossRef]
  46. Xia, P., Tekpetey, F. and Armstrong, D. 1994. Effect of IGF-I on pig oocyte maturation, fertilization, and early embryonic development *in vitro*, and on granulosa and cumulus cell biosynthetic activity. *Mol. Reprod. Dev.* **38**: 373–379. [Medline] [CrossRef]
  47. Zhou, W., Xiang, T., Walker, S., Farrar, V., Hwang, E., Findeisen, B., Sadeghieh, S., Arenivas, F., Abruzzese, R. V. and Polejaeva, I. 2008. Global gene expression analysis of bovine blastocysts produced by multiple methods. *Mol. Reprod. Dev.* **75**: 744–758. [Medline] [CrossRef]