Journal of Reproduction and Development, Vol. 61, No 2, 2015

—Original Article—

Cell-free extract from porcine induced pluripotent stem cells can affect porcine somatic cell nuclear reprogramming

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Abstract. Pretreatment of somatic cells with undifferentiated cell extracts, such as embryonic stem cells and mammalian oocytes, is an attractive alternative method for reprogramming control. The properties of induced pluripotent stem cells (iPSCs) are similar to those of embryonic stem cells; however, no studies have reported somatic cell nuclear reprogramming using iPSC extracts. Therefore, this study aimed to evaluate the effects of porcine iPSC extracts treatment on porcine ear fibroblasts and early development of porcine cloned embryos produced from porcine ear skin fibroblasts pretreated with the porcine iPSC extracts. The Chariot^{TM} reagent system was used to deliver the iPSC extracts into cultured porcine ear skin fibroblasts. The iPSC extracts-treated cells (iPSC-treated cells) were cultured for 3 days and used for analyzing histone modification and somatic cell nuclear transfer. Compared to the results for nontreated cells, the trimethylation status of histone H3 lysine residue 9 (H3K9) in the iPSC-treated cells significantly decreased. The expression of *Jmjd2b*, the H3K9 trimethylation-specific demethylase gene, significantly increased in the iPSC-treated cells; conversely, the expression of the proapoptotic genes, *Bax* and *p53*, significantly decreased. When the iPSC-treated cells were transferred into enucleated porcine oocytes, no differences were observed in blastocyst development and total cell number in blastocysts compared with the results for control cells. However, H3K9 trimethylation of pronuclear-stage-cloned embryos significantly decreased in the iPSC-treated cells. Additionally, *Bax* and *p53* gene expression in the blastocysts was significantly lower in iPSC-treated cells than in control cells. To our knowledge, this study is the first to show that an extracts of porcine iPSCs can affect histone modification and gene expression in porcine ear skin fibroblasts and cloned embryos.

Key words: H3K9 trimethylation, Nuclear reprogramming, Porcine induced pluripotent stem cells (iPSC) extracts, Somatic cell nuclear transfer (SCNT)

(**J. Reprod. Dev. 61:** 90–98, 2015)

Somatic cell nuclear transfer (SCNT) is one of the main methods for the production of cloned animals and nuclear reprogramming. However, its efficiency remains low, and it is generally understood that nuclear reprogramming of donor cells is essential for successful SCNT. Reprogramming is defined as the conversion of one cell type into another or as the loss/gain of some properties. Many studies have reported the importance of reprogramming donor cells in SCNT. To successfully induce embryonic development, various methods have been used to reprogram cells, including treatment with a histone deacetylase inhibitor (HDACi) and a DNA methyltransferase inhibitor (DNMTi).

Histone methylation, acetylation (especially at histone H3 lysine residue 9), and DNA methylation are the most important epigenetic mechanisms for donor cell reprogramming [1]. Several chemicals, such as trichostatin A (TSA) [2], 6-(1,3-dioxo-1H, 3H-benzo[de] isoquinolin-2-yl)-hexanoic acid hydroxyamide (Scriptaid) [3], valproic acid (VPA) [4], sodium butylate [5], suberoylanilide hydroxamic acid (SAHA) [6], m-carboxycinnamic acid bishydroxamide (CBHA) [7] and oxamflatin [8] have been used as HDACis to improve embryonic development. These chemicals have been observed to improve the development of cloned embryos to the blastocyst stage in porcine [9] and mouse [10] models. Additionally, to reduce DNA methylation levels, 5-aza-2'-deoxycytidine (5-aza-dC) has been widely used as a DNMTi. Treatment with 5-aza-dC has been observed to reduce DNA methylation of donor cells [11]. Moreover, treatment with both HDACi and DNMTi has been reported to improve embryonic development by increasing histone acetylation and decreasing DNA methylation [12]. These data suggest that epigenetic modification is important for donor cell reprogramming.

Several recent studies have shown that various extracts obtained from mammalian and *Xenopus laevis* oocytes and embryonic stem cells can induce cellular and nuclear reprogramming in various mammalian somatic cells. In a mouse study, treatment of mitotic *Xenopus* egg extracts reduced H3K9 trimethylation and DNA methylation levels and also facilitated successful induced pluripotent stem cell (iPSC) production [13]. Bui *et al.* [14] reported that treatment of somatic cells with GV-stage oocyte extracts induced H3K9 acetylation and reduced H3K9 trimethylation. Furthermore, treatment with embryonic

Received: June 18, 2014
Accepted: November 10, 2014
Published online in J-STAGE: January 24, 2015
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stem cell (ESC) extracts induces histone modifications at pluripotent gene promoters such as *Oct4* and *Nanog* [15]. In addition, Cho *et al*. [16] reported that both DNA methylation status and histone modification patterns (H3K4 and H3K27 trimethylation) of *Oct4* and *Nanog* promoter regions changed following treatment with ESC extracts. Although the mechanisms underlying such changes remain undefined, data obtained from some of the studies described above suggest that the extracts of undifferentiated cells have the ability to enhance embryonic development and reprogramming.

Takahashi and Yamanaka [17] reported that infection of four transcription factors, called Yamanaka factors, induced cellular and nuclear reprogramming and resulted in the generation of induced pluripotent stem cells (iPSCs). iPSCs and ESCs share similar properties, such as morphology, gene expression, chromatin modification, teratoma formation, chimera production, and germ line transmission [18]. However, no studies have reported the reprogramming of somatic cells using iPSC extracts. Therefore, we hypothesized that extracts derived from porcine iPSCs would affect reprogramming in somatic cells and SCNT embryos.

In this study, we investigated whether porcine iPSC extracts could modulate the reprogramming of porcine ear skin fibroblasts and affect the reprogramming of SCNT embryos cloned from donor cells treated with porcine iPSC extracts.

Materials and Methods

Cells

Porcine ear skin fibroblasts (EFs) were derived from a 10-day-old Massachusetts General Hospital (MGH) major histocompatibility complex (MHC) inbred miniature pig (MGH pig). Porcine EFs were cultured in GlutaMAX^{TM} (Gibco, Grand Island, NY, USA) with 15% FBS (HI-FBS, Gibco), 1% penicillin-streptomycin (Gibco), and 4 mM L-glutamine at 38.5 C with 5% CO_2. The second to fifth passages of porcine EFs were utilized in the present study. Porcine iPSCs were obtained from Dr. Kwon [18], and cells were cultured as described in Kwon *et al.* with slight modifications [19]. Briefly, porcine iPSCs were cultured in DMEM/F12 supplemented with 10% knockout serum replacement (KSR, Gibco), 10% FBS, 1% penicillin-streptomycin solution, 2 mM L-glutamine (Gibco), 1% nonessential amino acids (NEAAs, Gibco), 1 µM β-mercaptoethanol and 1,000 unit/ml leukemia inhibitory factor (LIF; Sigma, St. Louis, MO, USA) at 38.5 C with 5% CO_2.

Porcine iPSC extracts

To prepare iPSC extracts, cells were washed twice in PBS and lysed with a ProteoJET^{TM} Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas, Pittsburgh, PA, USA) according to the manufacturer's instructions. The lysate was sedimented at 20,000 × *g* for 15 min at 4 C to pellet the coarse material. Concentration of extracts was 6 mg/ml. The supernatant was aliquoted and stored at –80 C.

Treatment of porcine iPSC extracts

We mixed 10 µg/ml of iPSC extracts containing an ATP-regenerating system (1 mM ATP, 10 mM creatine phosphate, 25 µg/ml creatine kinase, 100 µM GTP (Sigma) and 1 mM nucleotide triphosphate (NTP; Roche, South San Francisco, CA, USA)) with the protein-delivery reagent Chariot^{TM} (Active Motif, Carlsbad, CA, USA) and incubated them for 30 min at room temperature (RT) as described by the manufacturer. Porcine EFs (70–80% confluency) from the second to fifth passages were washed twice in cold Ca^{2+}- and Mg^{2+}-free PBS, combined with the extract-Chariot mixture and then incubated for 2 h in a 5% CO_2 incubator at 38.5 C. After incubation, the extract-Chariot mixture was removed, and EFs were cultured in ES cell media (DMEM, 15% FBS, 0.1 mM β-mercaptoethanol, 1% nonessential amino acid and 1% penicillin/streptomycin solution, supplemented with 1,000 unit/ml recombinant LIF) for 3 days. Transfection efficiency of the Chariot reagents was determined using β-galactosidase staining, as described by the manufacturer.

Donor cell preparation

The cells treated with porcine iPSC extracts and control EFs were cultured to confluency for synchronization to the G0/G1 stage and washed twice in PBS and suspended via 0.05% trypsin-EDTA (Gibco) treatment. Cells were then incubated in DMEM containing 0.5% FBS for 1 h until cell injection.

Collection and maturation of porcine oocytes

Porcine ovaries were obtained from a local slaughterhouse. Cumulus oocyte complexes (COCs) were aspirated from 2–6 mm antral follicles using a 10 ml syringe with an 18-gauge needle. COCs that had even cytoplasms and were surrounded by compact cumulus cells were collected and washed twice in TL-HEPES. The collected COCs were transferred to TCM-199 medium supplemented with 10% porcine follicular fluid (pFF), 0.1% polyvinyl alcohol, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 75 µg/ml penicillin G and 50 µg/ml streptomycin, 0.57 mM cysteine, 0.5 µg/ml LH, 0.5 µg/ml FSH and 10 ng/ml epidermal growth factor (EGF; Sigma). This mixture was then incubated for 40–42 h at 38.5 C in a 5% CO_2 incubator.

Nuclear transfer

Following *in vitro* maturation, oocytes were denuded with 0.1% hyaluronidase. Metaphase II stage oocytes from which cumulus cells were removed were stained with 10 µg/ml Hoechst 33342 (Sigma) for 5 min at 39 C. Stained oocytes were transferred into droplets of TCM-199 containing 5 µg/ml cytochalasin B (Sigma), and then enucleation was performed with a 14-µm (internal diameter) glass pipette by aspirating the first polar body and surrounding cytoplasm under UV light. The donor cells were transferred into the perivitelline space of enucleated oocytes. Oocyte-donor cell couplets were equilibrated in fusion medium (0.3 M D-mannitol, 1.0 mM $CaCl_2 \cdot 2H_2O$, 0.1 mM $MgCl_2 \cdot 6H_2O$, and 0.5 mM HEPES). Oocyte activation and fusion were performed by applying direct current twice (140 V/mm and 50 µsec) at the same time. After fusion, fused couplets were cultured in PZM-3 media (108 mM NaCl, 10 mM KCl, 0.35 mM KH_2PO_4, 0.4 mM $MgSO_4 \cdot 7H_2O$, 25.07 mM $NaHCO_3$, 0.2 mM sodium pyruvate, 2 mM Ca-(lactate)$_2 \cdot 5H_2O$, 1 mM L-glutamine, 5 mM hypotaurine, 20 ml/l BME-essential amino acids, 10 ml/l MEM nonessential amino acids, 0.05 mg/ml gentamicin, and 30 mg/ml fatty acid-free BSA) covered with paraffin oil under 5% CO_2, 5% O_2 and 90% N_2 at 38.5 C for 6 days.

Table 1. Primers and amplification conditions used for real-time PCR

Genes	Primer sequence	Size (bp)	Amplification conditions
Bax-a	F : CCTTTTGCTTCAGGGTTTCA R :ATCCTCTGCAGCTCCATGTT	165	95 C for 5 sec, 63 C for 13 sec, 72 C for 15 sec, 45 cycles
Bcl-xl	F : GTTGACTTTCTCTCCTACAAG R : GGTACCTCAGTTCAAACTCAT	193	95 C for 5 sec, 63 C for 13 sec, 72 C for 15 sec, 45 cycles
Sirt6	F : CACTCCCCATCTCTTGCCTA R : GCAAGCCTCTATTGCCTGTC	116	95 C for 5 sec, 55 C for 13 sec, 72 C for 15 sec, 45 cycles
Gcn5	F : CGAGTTGTGCCGTAGCTGTGA R : ACCATTCCCAAGAGCCGGTTA	96	95 C for 5 sec, 56.6 C for 13 sec, 72 C for 15 sec, 45 cycles
Suv39h1	F : AAGGATGCAGTGTGTGTTGC R : CCTGTTCGCGGATCTTTTTA	98	95 C for 5 sec, 57 C for 13 sec, 72 C for 15 sec, 45 cycles
Jmjd2b	F : TCACCAGCCACATCTACCAG R : GATGTCCCCACGCTTCAC	68	95 C for 10 sec, 57 C for 13 sec, 72 C for 15 sec, 45 cycles
p53	F : CGAACTGGCTGGATGAAAAT R : CTGCCAGGGTAGGTCTTCTG	145	95 C for 10 sec, 57 C for 13 sec, 72 C for 15 sec, 45 cycles
β-actin	F : CATCACCATCGGCAACGAGC R : TAGAGGTCCTTGCGGATGTC	150	95 C for 5 sec, 55 C for 13 sec, 72 C for 15 sec, 35 cycles

Immunostaining

Fused oocytes were washed three times in PBS containing 0.3% PVP (PBS/PVP), fixed in ice-cold 4% paraformaldehyde in PBS for 30 min at RT and then washed three times in PBS/PVP. Fused oocytes were permeabilized with PBS containing 0.2% Triton X-100 for 30 min at RT. After being washed again, fused oocytes were blocked in 4% bovine serum albumin (BSA) for 30 min at RT. The primary antibodies anti-acetyl H3K9 (ab10812; Abcam, Cambridge, MA, USA) and trimethyl H3K9 (NBP1-30141, Novus Biologicals, Littleton, CO, USA) were diluted 1:100 in 4% BSA and incubated for 30 min at RT. The secondary antibody Alexa Fluor 488-labeled goat anti-rabbit IgG (Invitrogen, Grand Island, NY, USA) was diluted 1:100 in 4% BSA and incubated for 20 min at RT. Mounting and nuclear staining were performed with Vectashield (Vector Laboratories, Burlingame, CA, USA) containing 4',6-diamidino-2-phenylindole (DAPI). DAPI and green signals were observed using Olympus IX71 microscope (Olympus, Japan) with an exposure time of 400 msec. Intensity was measured by manually outlining the nucleus on the display using the ImageJ software (National Institutes of Health, NIH).

Real-time PCR

Total RNAs of porcine EFs (control cells) and iPSC extract-treated cells (iPSC-treated cells) were isolated using an RNeasy Plus Mini Kit (Qiagen, Hamburg, Germany), and cDNA was synthesized using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen). Total RNA isolation and cDNA synthesis for blastocysts were performed using a FastLane Cell cDNA Kit (Qiagen). Each procedure was performed with one blastocyst according to the manufacturer's instructions. The mRNA expression levels were quantified using a Rotor-Gene SYBR Green PCR Kit (Qiagen), and amplifications were performed using a Rotor-Gene 6000 real-time rotary analyzer (Corbett, San Francisco, CA, USA). Amplification conditions and primer designs are described in Table 1. The expression levels were calculated manually using the delta-delta CT methods. *Beta-actin* was used as an internal control.

TUNEL assay

Blastocysts were washed three times in PBS containing 0.3% PVP (PBS/PVP) and then fixed with 4% paraformaldehyde for 1 h at RT. After the cells were washed twice, they were permeabilized with PBS containing 0.5% Triton X-100 for 30 min at RT and then washed twice in PBS/PVP. Blastocysts were incubated for 1 h at 37 C in the dark with TMR red reagent (Roche). Blastocysts were washed again in PBS/PVP for 5 min. Mounting and nuclear staining were performed with Vectashield containing DAPI.

Western blotting

Control cells and iPSC-treated cells were harvested with a scraper. Cells were washed three times with PBS. Lysis was performed using RIPA Buffer (Thermo, South Logan, UT, USA) as described by the manufacturer. Briefly, cells were washed with PBS and lysed using RIPA Buffer. Lysates were sedimented at 12,000 rpm for 1 min, and supernatants were retained. Samples consisting of 20 µg of the supernatant were loaded onto a 12% SDS gel and transferred to an Immobilon®-P PVDF membrane (Millipore, Billerica, MA, USA) at 20 V for 45 min using a Trans-Blot® Turbo™ Transfer System (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% skim milk for 30 min at RT. Acetylated H3K9 (Abcam), trimethylated H3K9 (Novus Biologicals) and TBP antibodies (ab818, Abcam) were incubated for 1 h at RT and used as primary antibodies at a dilution of 1:4000 in the blocking solution. Anti-mouse IgG and anti-rabbit IgG (Sigma) were incubated for 1 h at RT and used as secondary antibodies at a dilution of 1:5000 in blocking solution. All blots were incubated for 5 min using Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare, Pittsburgh, PA, USA) and developed using X-ray films (Kodak, Rochester, NY, USA).

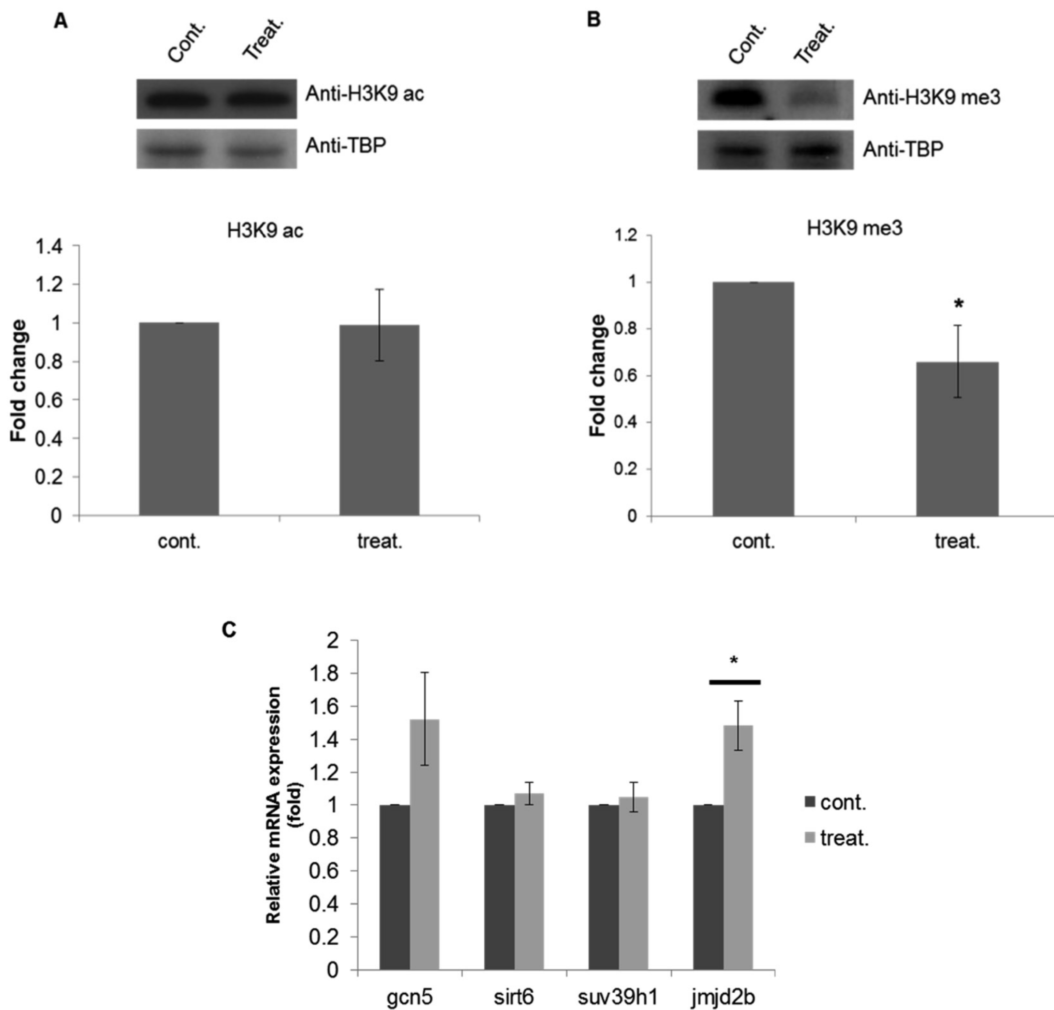

Fig. 1. Effect of treatment with iPSC extracts on levels of H3K9 modification in porcine EFs. Western blotting analysis of (A) H3K9 acetylation (H3K9 ac) and (B) H3K9 trimethylation (H3K9 me3) after 3 days of treatment with iPSC extracts. (C) Quantitative PCR analysis of H3K9-specific enzymes (*Gcn5*, acetyltransferase; *Sirt6*, deacetylase; *Suv39h1*, methyltransferase; *Jmjd2b*, demethylase). * $P < 0.05$. The experiments were replicated 10–15 times.

Band intensities were calculated using the ImageJ software.

Statistical analysis

Differences in histone H3K9 modification, gene expression and blastocyst apoptosis between control cells and iPSC-treated cells were analyzed by two-way Student's *t*-test. Embryo development data were analyzed by χ^2 test. The results were considered significant when P values were less than 0.05.

Results

Porcine iPSC extracts reduced H3K9 trimethylation in porcine EFs

We estimated the efficiency of the Chariot™ reagent using β-galactosidase staining, as described by the manufacturer (Supplementary Fig. 1: online only). Then, to estimate the effects of treatment with iPSC extracts on histone H3K9 epigenetic modifications, we treated EFs with the iPSC extracts and observed H3K9 acetylation and trimethylation at 3 days after treatment. We found that H3K9 trimethylation levels were significantly ($P < 0.05$) lower in iPSC-treated cells than in control cells, whereas H3K9 acetylation levels were not affected (Fig. 1A and B).

Since H3K9 epigenetic modifications can be regulated by specific enzymes, such as acetyltransferase, methyltransferase, deacetylase and demethylase [20–22], we tested the mRNA expression levels of H3K9-specific enzymes. We observed that *Gcn5* (acetyltransferase), *Sirt6* (deacetylase), and *Suv39h1* (methyltransferase) were not affected by treatment with iPSC extracts but that *Jmjd2b* (demethylase) was significantly ($P < 0.05$) increased in iPSC-treated cells (Fig. 1C).

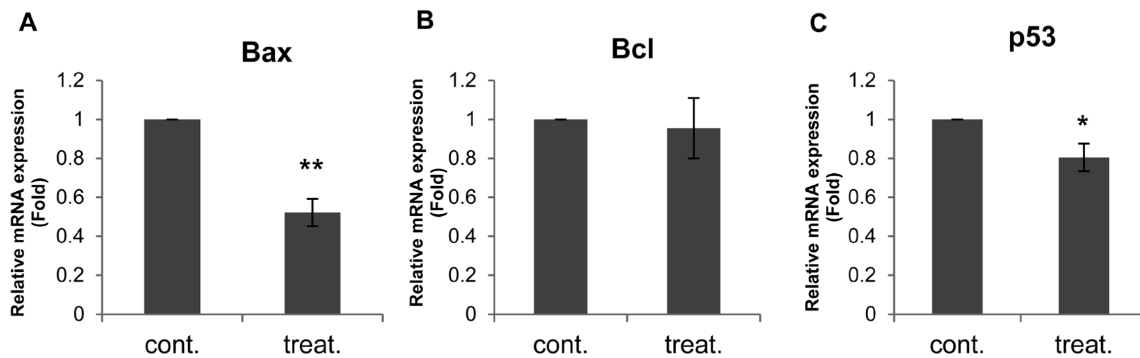

Fig. 2. Effect of treatment with iPSC extracts on expression of apoptosis-related genes. (A) Quantitative PCR analysis of cells treated with iPSC extracts for the proapoptotic gene *Bax*, (B) antiapoptotic gene *Bcl* and (C) tumor suppress gene *p53*. * $P < 0.01$; ** $P < 0.05$. The experiments were replicated 10–15 times.

Porcine iPSC extracts reduced expression of apoptosis-related genes in porcine EFs

Because H3K9 trimethylation levels are linked to *p53*-induced apoptosis [23], we tested the transcription levels of pro- (*Bax* and *p53*) and anti-apoptotic genes (*Bcl*) in iPSC-treated cells. We found that *Bax* and *p53* were significantly ($P < 0.01$ and $P < 0.05$) reduced in iPSC-treated cells (Fig. 2A and C), whereas *Bcl* did not differ between the control and iPSC-treated cells (Fig. 2B).

Porcine iPSC extracts did not affect in vitro development of SCNT embryos

To estimate embryonic development when using donor cells treated with iPSC extracts for SCNT, we observed the rates of fusion, cleavage, and blastocysts. As seen in Table 2, no differences were observed in the rates of fusion (86.2 and 86.6%) and cleavage (87.1 and 86.6%). Additionally, no significant differences were observed in the blastocyst rates (28.4 and 23.4%) for the control and iPSC-treated cells. Total cell numbers in blastocysts did not differ between the control and iPSC-treated cells (41.2 ± 11.5 and 43.8 ± 10.7). Additionally, no differences were observed in apoptosis rates between the cells (6.0 ± 5.8 and 4.6 ± 3.5), as seen in Table 3; however, the rates of blastocysts with higher distributions of apoptotic cells (> 10%) tended to be lower when compared with those of control cells (20% and 7.1%).

Table 2. Development of porcine embryos cloned from donor cells treated with the iPSC extracts

Group	No. of oocytes	No. (%) of oocytes fused	No. (%) of embryos developed to	
			≥ 2 cell	Blastocysts
Control	225	194 (86.2)	169 (87.1)	55 (28.4)
Treatment	232	201 (86.6)	174 (86.6)	47 (23.4)

The experiments were replicated 5 times in each group.

H3K9 trimethylation levels remained lower in SCNT embryos derived from iPSC-treated cells

Although treatment with the iPSC extracts reduced H3K9 trimethylation in control cells, we considered that the features described above would also be observed in SCNT embryos derived from iPSC-treated cells; therefore, we tested H3K9 modification in SCNT embryos. The results of this study indicated that H3K9 acetylation in pronuclear-stage SCNT embryos was not affected by iPSC cells (Fig. 3A and B), but H3K9 trimethylation was significantly lower in SCNT embryos derived from iPSC-treated cells than in control cells. In particular, the H3K9 trimethylation levels were significantly lower at 1 h and 4 h after fusion (Fig. 3C and D).

Porcine iPSC extracts reduced the expression of apoptosis-related genes in cloned blastocysts

We tested expression levels of apoptosis-related genes in blastocysts using quantitative real-time PCR. As seen in Fig. 4, the expression of *Bax* was significantly ($P < 0.05$) lower in blastocysts derived from iPSC-treated cells than from control cells (Fig. 4A). The expression of *p53* was also lower ($P < 0.05$) in iPSC-treated cells than in control cells (Fig. 4C), although no difference was observed for the expression of *Bcl* (Fig 4B).

Table 3. Apoptosis in porcine blastocysts cloned from donor cells treated with the iPSC extracts

Group	No. of blastocysts	Total cells	No. (%) of apoptotic cells (mean ± SE)	Rate of apoptosis	
				0–10%	> 10%
Control	55	41.2 ± 11.5	2.2 ± 1.9 (6.0 ± 5.8)	44 (80.0%)	11 (20.0%)
Treatment	42	43.8 ± 10.7	1.9 ± 1.2 (4.6 ± 3.5)	39 (92.9%)	3 (7.1%)

The experiments were replicated 5 times in each group.

Fig. 3. H3K9 modifications in porcine SCNT embryos derived from donor cells treated with iPSC extracts. (A) Immunostaining of H3K9 acetylation at 4 h after fusion in porcine SCNT embryos. (B) Fold changes in H3K9 acetylation levels at indicated times after fusion. (C) Immunostaining of H3K9 trimethylation at 4 h after fusion in porcine SCNT embryos (D) Fold changes of H3K9 trimethylation levels at indicated times after fusion. * $P < 0.01$; ** $P < 0.05$. More than 20 embryos/group were analyzed. Bar = 20 µm.

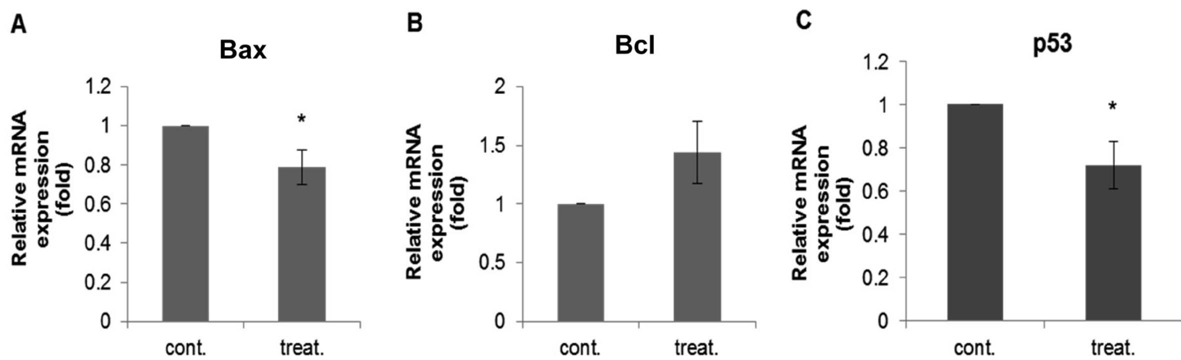

Fig. 4. Pro- and antiapoptotic gene expression in porcine SCNT blastocysts derived from iPSC extract-treated donor cells. Quantitative PCR analysis of the (A) proapoptotic gene *Bax*, (B) antiapoptotic gene *Bcl* and (C) tumor suppressor gene *p53* in porcine SCNT blastocysts derived from donor cells treated with iPSC extracts. * $P < 0.05$. The experiments were replicated 10–15 times.

Discussion

Previous studies have reported the use of extracts from cells that have obtained stemness, including *Xenopus* eggs [13, 24, 25], mammalian oocytes [14, 26–28] and ESCs [15, 16, 29–32], but we found no prior reports indicating the use of iPSC extracts. In this study, we present the first evidence that treatment with iPSC extracts affects histone modification in somatic cells and SCNT embryos.

Previous studies have also indicated that extracts from undifferentiated cells can affect epigenetic changes, such as DNA methylation, histone modification, and especially acetylation and trimethylation of H3K9 [29]. H3K9 acetylation has been reported as one of the most important histone modification markers, which plays a role in gene activation [33]. Conversely, both DNA methylation and H3K9 trimethylation are important as silencers of transcriptional gene expression [34–36]. Moreover, it is known that H3K9 trimethylation is necessary for formation of heterochromatin by heterochromatin protein 1 (*HP1*) recruitment [37]. These results indicate that undifferentiated (stemness) cells may potentially affect chromatin remodeling and modulation of gene expression. Since iPSCs and ESCs share similar properties, we hypothesized that iPSC extracts might also be able to affect histone modification of somatic cells and SCNT embryos.

Changes in H3K9 trimethylation were observed in porcine somatic cells (porcine ear fibroblasts) treated with porcine iPSC extracts at 3 days after treatment, whereas no differences were observed in H3K9 acetylation. H3K9 trimethylation was approximately 40% lower than that for the control group, which was similar to results reported when *Xenopus* egg extracts or GV-stage pig oocyte extracts were used to treat targeted somatic cells [13, 14]. In this latter study, H3K9 acetylation levels were also enhanced by treatment with the extracts [38], but this was not observed in our study. These apparently conflicting results could be due several reasons, including methodological differences, extracts concentrations or differences in cell types.

Histone modifications, including acetylation, methylation (lysine and arginine), phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination and proline isomerization, are regulated by various enzymes [34–36]. *PCAF/Gcn5* has been identified as an acetyltransferase acting on lysine residues 9, 14 and 18 of histone H3, which is a transcriptional coactivator in gene expression [39]. Conversely, deacetylase sirtuin 6 (*sirt6*) functions as a deacetylase on lysine residue 9 of histone H3 [40]. Chen *et al.* [41] reported that H3K9 trimethylation is specifically regulated by suppressor of variegation 3-9 homolog 1 (*suv39h1*), the human ortholog of the Drosophila Su(var)3-9 histone methyltransferase, and that Jumonji domain containing 2B (*jmjd2b*) acts specifically as a demethylase on lysine residue 9 of histone H3 [42]. Therefore, we evaluated how changes affected those enzymes. Our results indicated that mRNA levels of *Jmjd2b* were significantly reduced when iPSC extracts were used to treat somatic cells. Although the expression of acetyltransferase *Gcn5* mRNA was higher than in control cells, the observed difference was not significant. These data indicated that treatment with iPSC extracts modulated the trimethylation level of H3K9 by regulating its specific enzyme.

p53 is a major sensor of cellular stresses, and its activation influences cell fate decisions. The biological outcomes of tumor suppressor *p53* activation, such as cell cycle arrest, apoptosis and senescence, are linked to many molecules and various pathways [43]. On the other hand, Li *et al*. [23] reported that knockdown of *jmjd2b* expression by small interfering RNA (siRNA) in cancer cells inhibited cell proliferation and/or induced apoptosis and elevated the expression of *p53* by disrupting the balance in histone H3K9 methylation. Moreover, *p53* has also been linked to human somatic cell reprogramming [44–47]. Generally, the proapoptotic gene *Bax* and antiapoptotic gene *Bcl* are known as major *p53*-dependent apoptosis pathway molecules, involved in transcriptional regulation by *p53* [48]. In this study, we compared the expression of the *p53*, *Bax* and *Bcl* genes between non-treated cells and iPSC-treated cells. Quantitative PCR data indicated that the expression levels of *p53* and *Bax* were significantly lower than those of the control, whereas *Bcl* was no different. Protein levels of *p53* were also reduced by treatment with iPSC extracts (data not shown). These results indicate that treatment with iPSC extracts may promote H3K9 trimethylation levels by upregulation of *jmjd2b*, thereby leading to inhibition of the apoptosis genes *p53* and *Bax* and regulation of somatic cell nuclear reprogramming.

Cloned embryos exhibit higher levels of histone methylation and DNA methylation than fertilized embryos [49, 50]. This causes the existing methylation in donor cells to not fully erasure. Bru *et al.* [15] reported that reprogramming of somatic cells occurs early and rapidly following treatment with ESC extracts. In fertilized embryos, high levels of tri- and dimethylation of histone H3 lysine residue 9 have been observed in the maternal pronucleus, while the opposite trend has been observed in the paternal pronucleus [51]. In mouse cloned embryos, the levels of H3K9 trimethylation and acetylation persisted for 3 h after fusion, with demethylation and deacetylation occurring 6 h after fusion [52]. In this study, we investigated the effects of donor cells treated with porcine iPSC extracts on histone H3K9 acetylation and H3K9 trimethylation of pronuclear-stage SCNT embryos. Our results indicated that the levels of H3K9 trimethylation and acetylation increased in SCNT embryos from both control and iPSC cells during the initial 4 h after fusion. Acetylation of H3K9 in SCNT embryos from iPSC cells was not significant, whereas trimethylation of H3K9 remained at low levels compared with the control during the initial 4 h after fusion. Moreover, H3K9 trimethylation levels were significantly reduced, by 50%, when compared with control cells. These data suggested that the histone modification status of donor cells treated with iPSC extracts, especially H3K9 trimethylation, was conserved in SCNT embryos.

Previous studies have reported that the use of donor cells treated with extracts from undifferentiated cells of various species enhanced embryonic development, blastocyst quality and offspring birth rate. The cytoplasmic extracts of mouse germinal vesicle-stage (GV) oocytes promoted somatic cell reprogramming, cloned embryo development, blastocyst quality and the production of cloned offspring in the mouse [53]. Additionally, the use of GV oocyte cytoplasmic extracts to treat porcine somatic cells increased total cell numbers in cloned blastocysts and enhanced nuclear reprogramming in the pig [14]. The treatment of donor cells with bovine mature oocyte extracts improved the quality and development of cloned blastocysts in the bovine [28]. Likewise, reprogramming of somatic cells with *Xenopus* oocyte extracts before SCNT improved the birth rate of live

offspring in sheep and increased blastocyst formation in pigs [54, 55].

In this study, we investigated the effects of donor cells treated with porcine iPSC extracts on the *in vitro* development of porcine SCNT embryos. Our results demonstrated that donor cells treated with porcine iPSC extracts did not promote *in vitro* development of porcine SCNT embryos. However, the results of TUNEL analysis revealed a reduction in blastocytes with a high apoptotic index (≥ 10% apoptotic cells) when donor cells were treated with the porcine iPSC extracts. Our investigation of the expression of apoptosis-related genes in SCNT blastocyst embryos showed that it was similar to that in cells treated with porcine iPSC extracts. The proapoptotic genes *Bax* and *p53* showed significantly reduced expression in SCNT blastocysts derived from donor cells treated with iPSC extracts than in the control cells. Therefore, we inferred that although treatment of donor cells with porcine iPSC extracts may not be sufficient to improve subsequent development of porcine SCNT embryos, treatment with porcine iPSC extracts can regulate down the proapoptotic genes *Bax* and *p53* in cloned blastocyst embryos. Also, the results regarding gene expression suggested that a low level of histone methylation in donor cells treated with porcine iPSC extracts can modify the reprogramming dynamics of *Bax* and *p53* during the preimplantation embryo development.

To our knowledge, this study is the first to show that porcine iPSC extracts have the potential to enhance nuclear reprogramming of somatic cells, which is similar to the case for other undifferentiated cells, including embryonic stem cells, eggs, and mammalian oocytes. We also observed that the levels of H3K9 trimethylation of porcine EFs and early SCNT embryos can be modified by treatment with porcine iPSC extracts. Further studies will need to be performed to ascertain the effect of porcine iPSC extracts on full-term development following transfer of porcine SCNT embryos.

Acknowledgments

This work was carried out with the support of the "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ009333022014)," Rural Development Administration, Republic of Korea.

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