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Effects of Trichostatin A and 5-aza-2'deoxycytidine on Nuclear Reprogramming in Pig Cloned Embryos

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ABSTRACT

Low efficiency of somatic cell nuclear transfer (SCNT) is attributed to incomplete reprogramming of transfered nuclei into oocytes. Trichostatin A (TSA), histone deacetylase inhibitor and 5-aza-2'deoxycytidine (5-aza-dC), DNA methylation inhibitor has been used to enhance nuclear reprogramming following SCNT. However, it was not known molecular mechanism by which TSA and 5-aza-dC improve preimplantation embryo and fetal development following SCNT. The present study investigates embryo viability and gene expression of cloned porcine preimplantation embryos in the presence and absence of TSA and 5-aza-dC as compared to embryos produced by parthenogenetic activation. Our results indicated that TSA treatment significantly improved development. However 5-aza-dC did not improve development. Presence of TSA and 5-aza-dC significantly improved total cell number, and also decreased the apoptotic and autophagic index. Three apoptotic-related genes, *Bak*, *Bcl-xL*, and Caspase 3 (*Casp3*), and three autophagic-related genes, *ATG6*, ATG8, and lysosomal-associated membrane protein 2 (*LAMP2*), were measured by real time RT-PCR. TSA and 5-aza-dC treatment resulted in high expression of anti-apoptotic gene *Bcl-xL* and low pro-apoptotic gene Bak expression compared to untreated NT embryos or parthenotes. Furthermore, LC3 protein expression was lower in NT-TSA and NT-5-aza-dC embryos than those of NT and parthenotes. In addition, TSA and 5-aza-dC treated embryos displayed a global acetylated histone H3 at lysine 9 and methylated DNA H3 at lysine 9 profile similar to the parthenogenetic blastocysts. Finally, we determined that several DNA methyltransferase genes Dnmt1, Dnmt3a and Dnmt3b. NT blastocysts showed higher levels Dnmt1 than those of the TSA and 5-aza-dC blastocysts. Dnmt3a is lower in 5-aza-dC than NT, NTTSA and parthenotes. However, Dnmt3b is higher in 5-aza-dC than NT and NTTSA. These results suggest that TSA and 5-aza-dC positively regulates nuclear reprogramming which result in modulation of apoptosis and autophagy related gene expression and then reduce apoptosis and autophagy. In addition, TSA and 5-aza-dC affects the acetylated and methylated status of the H3K9.

(Key words : TS, 5-aza-dC, Autophagy, Apoptosis, H3K9, SCNT)

INTRODUCTION

Porcine somatic cell nucleus transfer (SCNT) is a tool useful both for basic research and biomedicine. In biomedicine SCNT using pigs as a source for human xenotransplantation or as models for certain diseases SCNT is the only practical way to produce targeted genetic modifications in pigs (Lai *et al*., 2002; Vajta *et al*., 2007).

Although cloning has been successful in many species, the overall rate of success has remained quite low, in the range of 1-5% for both amphibians and mammals (Hao *et al*., 2003; Yang *et al*., 2007). The inefficiency of cloning may be attributed to multiple factors, including artificial activation conditions (Cheong *et al*., 2002; Zhu *et al*., 2002; Ziecik *et al*., 2005; Koo *et al*., 2008), the SCNT procedure (Miyoshi *et al*., 2000; Du *et al*., 2007), inappropriate or incomplete nuclear reprogramming following SCNT (Lee *et al*., 2006), and also the conditions of embryo transfer (Koo *et al*., 2009b). Many different approaches have been taken to try to improve success, including treatment of donor cells or their nuclei with chemical agents or cellular extracts, employing different methods of depleting the recipient oocyte of its genome, employing different stages of oocyte recipient, employing different cell cycle stages of donor cell, employing different types of donor cells, and employing serial nuclear transfer, including the pro-

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duction of embryonic stem cells, which are then used for a second round of cloning. Some of these efforts have met with modest success, but in general they have not yielded a major increase in efficiency.

Pevious studies have demonstrated that epigenetic modifications such as DNA methylayion and histone acetylation are abnormally reprogrammed in SCNT embryos during early development (Kang *et al*., 2001). Recent molecular analysis of cloned embryos have revealed abnormal epigenetic modifications such as DNA methylation and histone modifications (Santos *et al*., 2003; Ohgane *et al*., 2004; Wee *et al*., 2006).

Moreover, the low success rate is believed to be associated with epigenetic errors, including abnormal histone modification. In early-stage porcine embryos, the pattern of histone H3 lysine 9 (H3K9) methylation closely mimics that of DNA methylation. Histone acetylation is known as one of the epigenetic modification (Grunstein *et al*., 1997; Turner *et al*., 2002). Hyperacetylation of histones increases the access of some transcription factors to nucleosomes (Lee *et al*., 1993; Li, 2002). Previous studies have reported that DNA methylation and histone H3 lysine 18 acetylation are reasonable markers of nuclear reprogramming, correlating with the developmental potential of SCNT embryos (Santos *et al*., 2003; Li *et al*., 2008). Moreover, increase in the histone acetylation level in donor cells and/or SCNT embryos improves their developmental competence (Enright *et al*., 2003; Wee *et al*., 2007). Thus, prevention of abnormal gene expression and epigenetic modifications is necessary to improve the success rate of animal cloning.

In natural reproduction, relatively low levels of DNA methylation exist in male and female gametes, which are further demethylated during early development (Oswald J *et al*., 2000; Wade PA *et al*., 2002). Recently, found that histone hyperacetylation induced by trychostatin A (TSA), a potent inhibitor of histone deacetylase, increased embryonic gene expression during porcine embryogenesis (Rybouchkin A *et al*., 2006). These findings clearly showed that histone acetylation is involved in embryonic gene expression. Previously, 5-aza-2′-deoxycytidine (5-aza-dC, DNAmethyltransferase inhibitor) and trichostatin A (TSA, histone deacetylase inhibitor) were separately used on cloned mice embryos and bovine donor cells to improve cloning efficiency (Nervi *et al*., 2001; Enright BP *et al*., 2003; Kishigami S *et al*., 2006).

Trichostatin A (TSA) is a well-known histone-deacetylase inhibitor, which enhances the pool of acetylated histones (Yoshida *et al*., 1990) and DNA demethylation (Hattori *et al*., 2004). TSA can remarkably reduce abnormal DNA hypermethylation depending on the origins of transferred nuclei and their genomic regions (Kishigami *et al*., 2006). Recent studies shown that TSA has increased the rate of development to the blastocyst stage and improved blastocysts quality through the induction of hyperacetylation in cloned mouse (Rybouchkin *et al*., 2006; Ding *et al*., 2008), bovine (Cervera *et al*., 2009) and, porcine (Zhang *et al*., 2007; Shi *et al*., 2008) and rabbit (Xiang *et al*., 1996) embryos. The DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine (5-aza-dC) is widely used to regulate the DNA methylation (Enright BP *et al*., 2005).

Apoptosis may play an important role in protecting the embryo from embryotoxic conditions by removing damaged cells (Woo *et al*., 1998; Warner *et al*., 1998). Recent reports have shown that several members of two major families of apoptotic genes, the Bcl-2 family, and the caspase family, were expressed in preimplantation stage embryos (Juriscova *et al*., 1998; Gozuacik *et al*., 2004). The Bcl-2-related gene products are known to act at a distal step in the apoptotic pathway, and may either suppress (Bcl-2, Bcl-xL) or promote (Bak, Bax) the induction of apoptosis.

Type II cell death or autophagic cell death is characterized by the presence of abundant autophagic vacuoles that engulf bulk cytoplasm and cytosolic organelles, such as mitochondria and endoplasmic reticulum, with subsequent degradation by the cells'own lysosomal system (Yu *et al*., 2004). The proteins encoded by the autophagy-related genes (ATG) are required for the formation of autophagic vesicles (Pattingrs *et al*., 2005; Suzuki *et al*., 2007; Xie *et al*., 2007). ATG5 is associated with the ATG12-ATG16 complex through a ubiquitin-like system. ATG6/Beclin-1 functions in cooperation with the class III PI3-kinase signaling complex to positively control formation of the autophagic vacuole (Petiot *et al*., 2000; Kihara *et al*., 2001). Microtubule-associated protein light chain 3 (LC3/ATG8) is converted to a membrane-conjugated form by ATG4, while ATG9 is an integral membrane protein required for the formation of the double membrane vesicle (Kirisako *et al*., 2000; Noda *et al*., 2000; Baehrecke *et al*., 2005). Lysosomal-associated membrane protein 2 (LAMP2) is a ubiquitous lysosomal membrane protein that is highly expressed in normal human pancreatic tissue and is required for the proper fusion of lysosomes with autophagosomes in the late stage of the autophagic process (Fortunato *et al*., 2009). However, the role of autophagy in the embryos is still poorly understood.

The objective of this research was to investigate to the pattern of autophagy which modulates of apoptosis and autophagy by TSA and 5-aza-dC treatment in pig SCNT embryos.

MATERIALS AND METHODS

In Vitro Porcine Oocyte Maturation and Parthenogenic Activation

Prepubertal porcine ovaries were collected from a local abbatoir and transported to the laboratory at 25℃ in Dulbecco's phosphate-buffered saline (PBS) supplemented with 75 mg/ml penicillin G and 50 mg/ml streptomycin sulfate. Cumulus-oocyte complexes (COC) were aspirated from follicles of 3~6 mm in diameter with an 18-gauge needle and disposable 10 ml syringe. The COC were washed three times with Hepes-buffered Tyrode's medium containing 0.1% (w/v) polyvinyl alcohol (Hepes-TL-PVA). Each group of 50 COC was matured in 500 ul tissue culture medium (TCM)-199 (with Earle's salts; Gibco; Grand Island, NY, USA) supplemented with 0.57 mM cysteine (Sigma, St. Louis, MO), 10 ng/ml EGF, (Sigma), 100 IU/ml PMSG (Sigma) and 10 IU/ml hCG (Sigma) under paraffin oil at 39℃ for 44 h. Following maturation, cumulus cells were removed by pipetting in the presence of 1 mg/ml hyaluronidase for 2~3 min. Oocytes were activated for parthenogenesis with 50 uM Ca^{2+} ionophore A23187 for 5 min. After 3 h of culture in porcine zygote medium 3 (PZM3) medium containing 7.5 mg/ml cytochalasin B (CB, Sigma), embryos were washed three times in PZM 3 medium with 0.4% (w/v) BSA and cultured in the same medium for 48 h at 39℃ in an atmosphere of 5% CO_2 and 95% air.

Donor Ear Fibroblast Culture and Preparation

Ear fibroblast cells were cultured from the ear biopsy of an adult female Holstein. The ear biopsy was washed several times in phosphate-buffered saline (PBS) and sliced into 1-mm pieces. Explants were transferred into a 100-mm tissue culture dish containing Dulbecco's modified Eagle's medium (DMEM) +10 % FBS and then cultured in the incubator under the same conditions as for *in vitro* maturation. When confluence was achieved after approximately 10 days in culture, cells were trypsinized, and the resulting pellet was resuspended in the above culture medium and subcultured until use.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Assay

Approximately 10 blastocysts were washed three times in dPBS (pH 7.4) containing 1 mg/ml PVA (dPBS/PVA) and then fixed in 3.7% paraformaldehyde in dPBS /PVA for 1 h at room temperature. After fixation, embryos were washed in dPBS/PVA and permeabilized by incubation in 0.5% Triton X-100 for 1 h at room temperature. They were then washed twice in dPBS/PVA and incubated with fluorescein-conjugated dUTP and the terminal deoxynucleotidyl transferase enzyme (*In Situ* Cell Death Detection Kit, Roche, Mannheim, Germany) in the dark for 1 h at 37℃. Nuclei were counterstained with 50 μg/ml RNase A in 40 μg/ml propidium iodide (PI) for 1 h at 37℃, after which the embryos were washed in PBS/PVA, mounted with slight coverslip compression, and examined by confocal microscopy.

For the other experiments, embryos were fixed in 3.7% paraformaldehyde in PBS for 1 h at RT and stained with 40 μg/ml PI for 1 h at 37℃ to label the nuclei. Total cell numbers were counted under a fluorescence microscope (Olympus, Tokyo, Japan).

Nuclear Transfer

Oocytes were enucleated in Hepes-TCM199 medium containing 7.5 mg/ml cytochalasin B, which was overlaid with warm mineral oil. Enucleation of oocytes was performed by the blind method (without the visualization of DNA by exposure to UV light). The zonae pellucidae were partially dissected with a fine glass needle and the first polar body (PB) and a small amount of cytoplasm containing metaphase-II chromosomes were pushed out by squeezing the oocyte with the holding pipette and the glass needle. After enucleation, the enucleated oocytes were incubated at 38.5℃ in 5% CO_2 in air until subsequent microinjection of the donor nucleus. A single intact donor cell was injected into the perivitelline space and placed adjacent to the recipient cytoplasm.

Terminal Deoxynucleotidyl Transferasemediated dUTP Nick-End Labeling (TUNEL) Assay

Blastocysts were washed three times in PBS (pH 7.4) containing polyvinylalcohol (PVA, 1 mg/ml), followed by fixation in 3.7% paraformaldehyde in PBS for 1 h at room temperature (RT). After fixation, embryos were washed in PBS/PVA and permeabilized by incubation in 0.3% Triton X-100 for 1 h at room temperature. Embryos were then washed twice in PBS/ PVA and incubated with fluorescein-conjugated dUTP and the terminal deoxynucleotidyl transferase enzyme (*in situ* Cell Death Detection Kit, Roche; Mannheim, Germany) in the dark for 1 h at 37℃. After counterstaining with 50 mg/ml RNase A in 40 mg/ml propidium iodide (PI) for 1 h at 37℃ to label all nuclei, embryos were washed in PBS/PVA, mounted with slight coverslip compression and examined under a confocal microscope. In other experiments, embryos were fixed in 3.7% paraformaldehyde in PBS for 1 h RT, stained with 40 mg/ml PI for 1 h at 37℃ to label all nuclei and total cell numbers were counted under a fluorescence microscope

(Olympus, Tokyo, Japan).

Immunofluorescence Staining

To determine the expression of autophagy (LC3) protein, porcine embryos were washed in Dulbecco's phosphate-buffered saline (dPBS) containing polyvinyl alcohol (PVA, 1 mg/ml), fixed for 20 min in 3.7% paraformaldehyde in dPBS, and permeabilized with 0.2% Triton X-100 in dPBS for 30 min at room temperature. And then fixed embryos were incubated with rabbit polyclonal LC3 (ab58610-100, abcam) antibody for 1 h and then with FITC-labeled secondary antibody (Sigma). Hoechst 33342 was used to stain the nuclei. Slides were examined using laser-scanning confocal microscopy, which was performed using a Leica DM IRB equipped with a krypton-argon ion laser for the simultaneous excitation of fluorescence for protein and DNA.

Immunostaining and Quantification of Fluorescence Intensity

Oocytes and embryos at different stages of development were fixed with 3.7% paraformaldehyde (PFA; EMS, Hatfield, Pennsylvania) in PBS supplemented with polyvinyl alcohol (PVA) for 1 h at RT. They were then washed in PBS containing 0.1% Tween 20 for 1 h and permeabilized with 0.5% Triton X100 in PBS for 30 min at RT. The samples were then blocked with PBS-PVA supplemented with 1% BSA at 4℃ overnight. Afterward, the embryos were incubated with the primary antibody anti-acetyl histone H3 (lys 9) (AcH3K9; Millipore Ibe´rica, Madrid) and anti-methyl histone H3 (lys 9) (MeH3K9; Millipore Ibe´rica, Madrid) for 1 h at 38.5 ℃. After washing the embryos twice with PBSPVA-BSA, they were incubated with Alexa fluor 647 goat anti-rabbit IgG (Invitrogen, Barcelona, Spain) for 1 h at RT (darkness) and then washed twice with PBSPVA-BSA. The samples were mounted on slides with mounting medium containing 40,6-diamidino-2-phenylindole (DAPI; ProLong Gold antifade reagent with DAPI; Invitrogen, Barcelona, Spain). The total fluorescence intensity emitted by each individual nucleus was measured on merged images, after background subtraction, by the Leica Confocal Software (LCS Version Lite) and averaged per embryo by the ratios of AcH3K9 and MeH3-K9 to DAPI DNA signals.

Real Time Reverse Transcription Polymerase Chain Reaction (Real Time RT-PCR)

Embryos were washed in Ca^{2+}- and Mg^{2+}-free PBS, snap frozen in liquid nitrogen and stored at −70℃. Messenger RNA was extracted using the Dynabeads mRNA Direct Kit (Dynal Asa, Oslo, Norway) according to the manufacturer's instructions. Synthesis of cDNA was achieved by reverse transcription of the RNA by using the Oligo (dT) 12～18 primer and the superscript reverse transcriptase enzyme (Invitrogen Co., Grand Island, NY, USA). The mRNA expression was then detected by real time RT-PCR with specific primer pairs (Table 1). The PCR reactions were performed according to the instructions of the real-time PCR machine manufacturer (DNA Engine Opticon 2 fluorescence detection system, MJ Research, Waltham, MA, USA). The threshold cycle (Ct) value represents the cycle number at which sample fluorescence rise statistically significantly above the background. The reactions were conducted according to the protocol of the DyNAmo SYBR green qPCR kit containing modified Tbr DNA polymerase, SYBR Green, optimized PCR buffer, 5 mM $MgCl_2$ and d-NTP mix including dUTP (Finnzymes Oy, Espoo, Finland). PCR was performed as follows: denaturation program (95℃ for 10 min), amplification and quantification program repeated 40 times (94℃ for 10 s, 55 or 60 ℃ for 30 s, 72℃ for 30 s with a single fluorescence measurement), melting curve program (65～95℃, with a heating rate of 0.2℃ / s and continuous fluorescence measurement) and finally a cooling step to 12℃. Fluorescence data were acquired after the extension step during PCR reactions containing SYBR Green. Thereafter, PCR products were analyzed by generating a melting curve. Since melting curves are sequence-specific, non-specific PCR products could be distinguished from specific ones. The crossing point (CP) must be determined for each transcript to generate the mathematical model. CP is defined as the point at which the fluorescence rises appreciably above the background fluorescence. The relative quantification of gene expression was analyzed by the 2-DDCt method. In all experiments, GAPDH mRNA was used as an internal standard.

Trichostatin A (TSA) Treatment

For TSA treatment, chemically-activated reconstructed oocytes were cultured in PZM3 medium containing 5 nM TSA for 26 h and then further cultured in PZM3 medium for another 24 or 142 h at 39℃ in a humidified atmosphere of 5% CO_2 in air.

5-aza-2'-deoxycytidine (5-aza-dC) Treatment

For 5-aza-dC treatment, chemically-activated reconstructed oocytes were cultured in PZM3 medium containing 10 nM 5-aza-dC for 72 h and then further cultured in PZM3 medium for another 24 or 142 h at 39 ℃ in a humidified atmosphere of 5% CO_2 in air.

Statistical Analysis

The general linear models (GLM) procedure embedded within the statistical analysis system (SAS User's

Table 1. List of primers used for quantitative real-time RT-PCR

Genes	Accession no.	Primer sequence	Product size
Casp3	NM_214131	F: CTAAGCCATGGTGAAGAAGG R: TCGCCAGGAATAGTAACCAG	267
Bcl-xL	AF216205	F: ACTGAATCAGAAGCGGAAAC R: AAAGCTCTGATACGCTGTCC	249
Bak	AJ001204	F: CTAGAACCTAGCAGCACCAT R: CGATCTTGGTGAAGTACTC	151
Atg6	NM_001044530	F: AGGAGCTGCCGTTGTACTGT R: CACTGCCTCCTGTGTCTTCA	189
Atg8	NM_001190290	F: CCGAACCTTCGAACAGAGAG R: AGGCTTGGTTAGCATTGAGC	206
Lamp2	XM_001926458	F: CACCCACTCCAAAGGAAAAA R: GGTTGTCGTTTTTCACAGCA	246
Dnmt1	NM_001032355	F: GTGTGGCGTTTGTGAGGTTTGTCA R: ATTGGGACACCTTCTCTCTTGGCA	126
Dnmt3a	NM_001097437.1	F: ACTGAGAAGCCCAAGGTCAAGGA R: TGGCACATTCCTCCGATGAAGAGA	162
Dnmt3b	NM_001162404	F: AGTGTGTGAGGAGTCCATTGCTGT R: GCTTCCGCCAATCACCAAGTCAAA	133
GAPDH	AF017079	F: GGGCATGAACCATGAGAAGT R: AAGCAGGGATGATGTTCTGG	230

(F: forward. R: reverse)

Guide) was used to analyze the data from all experiments. Significant differences were determined using Tukey's Multiple Range Test with $p<0.05$ considered significant. A paired Student's *t*-test was used to compare relative gene expression.

RESULTS

Effect of TSA and 5-aza-dC Treatment on Porcine SCNT Embryos Viability

TSA and 5-aza-dC was added in PZM3 medium containing 0.4% BSA, and the effect of TSA and 5-aza-dC on embryo development was determined. The SCNT embryos were treated with 5 nM TSA for 26 h and 10 nM 5-aza-dC for 72h after activation. The development rates of porcine SCNT two-cell embryos were not significantly different compared to other groups (Fig. 1A), but significantly increased in cultures treated with 5 nM TSA for 26 hours compared to SCNT embryos cultured without TSA. However 5-aza-dC not improved development than SCNT embryos (Fig. 1B).

Effect of TSA and 5-aza-dC on Blastocyst Cell Number and Programmed Cell Deth of Porcine Nucleus Transfer Embryos

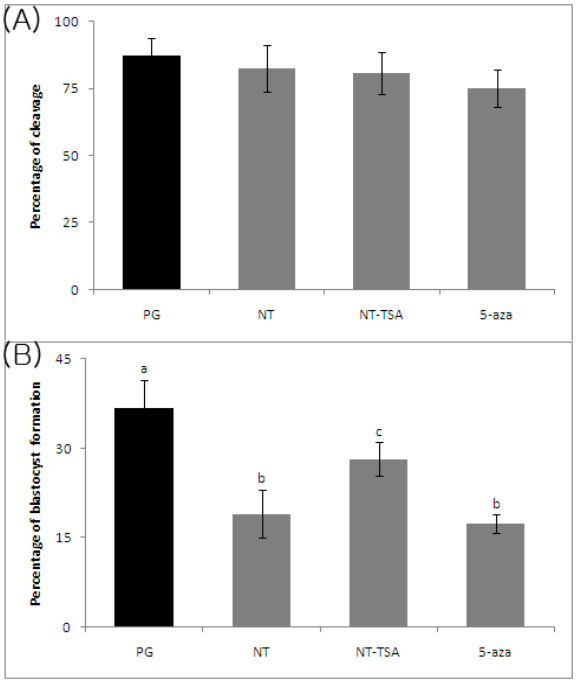

Fig. 1. The effect of TSA and 5-aza-dC treatment on the development at 2-cell stage (A) and blastocyst stage (B). The TSA treatments : 5 nM for 26 hr and 5-aza-dC treatment : 10 nM for 72 hr. $^{a\sim c}$ significant difference compared with the NT ($p<0.05$).

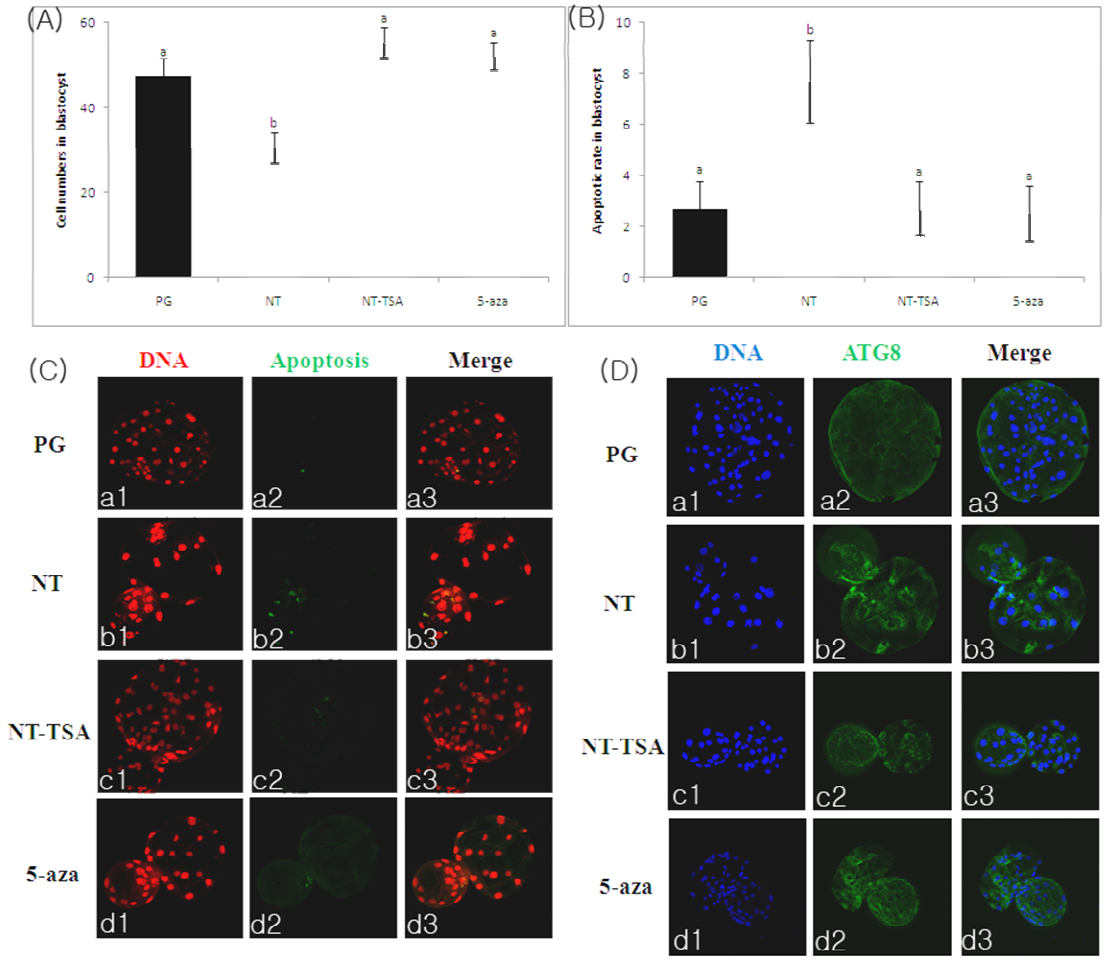

Fig. 2. The effect of TSA and 5-aza-dC treatment on the total cell numbers (A) and apoptotic rate (number of apoptotic cells/number of total cells) in blastocysts (B). Laser scanning confocal microscopic images of total nuclei (red), fragmented DNA (green) (C) and LC3 protein (D) in porcine blastocysts (400×). Blue, chromatin (Da1, Db1, Dc1 and Dd1); green, LC3 protein stained by FITC-conjugated secondary antibody for anti-LC3 (Da2, Db2, Dc2 and Dd2).

Blastocysts were stained with PI to label all nuclei and counted under a fluorescence microscope (Olympus, Tokyo, Japan). At day 7, the mean cell number in blastocysts derived from treatment with 5 nM TSA and 10 nM SCNT embryos were significantly higher than in the SCNT embryos (Fig. 1A). The degree of apoptotic index (fragmented cell number/total cell number) in blastocysts derived from cultures supplemented with 5 nM TSA and 10 nM 5-aza-dC were lower than in the *in vitro*-derived NT embryos. However, apoptotic index was not significantly different in PA, NTTSA and 5-aza-dC blastocysts (Fig. 3B). To determine whether TSA reduces apoptosis, blastocysts were stained with PI to label all nuclei and by TUNEL to label apoptotic nuclei, and were analyzed by epifluorescent and confocal microscopy (Fig. 3C). We examined the localization of LC3 expression in the porcine blastocysts derived from the PA, NT, NTTSA and 5-aza-dC embryos. Following addition of 5 nM TSA and 10 nM 5-aza-dC to the culture medium, the amount of LC3 protein was significantly decreased compared to that of the NT embryos. However, LC3 protein was not significantly different in PA and SCNT blastocysts (Fig. 3D).

Quantification Via Real-Time RT-PCR of the mRNA Expression Programmed Cell Deth (PCD) in Porcine Blastocysts

The relative amounts of apoptotic- related genes of Casp3, Bcl-xL, Bak mRNA expression in blastocysts cultured with TSA and 5-aza-dC were measured by real time RT-PCR. To normalize the RT-PCR reaction efficiency, porcine GAPDH was used as an internal standard. There were 10 embryos per treatment group and each experiment was repeated four times, with three

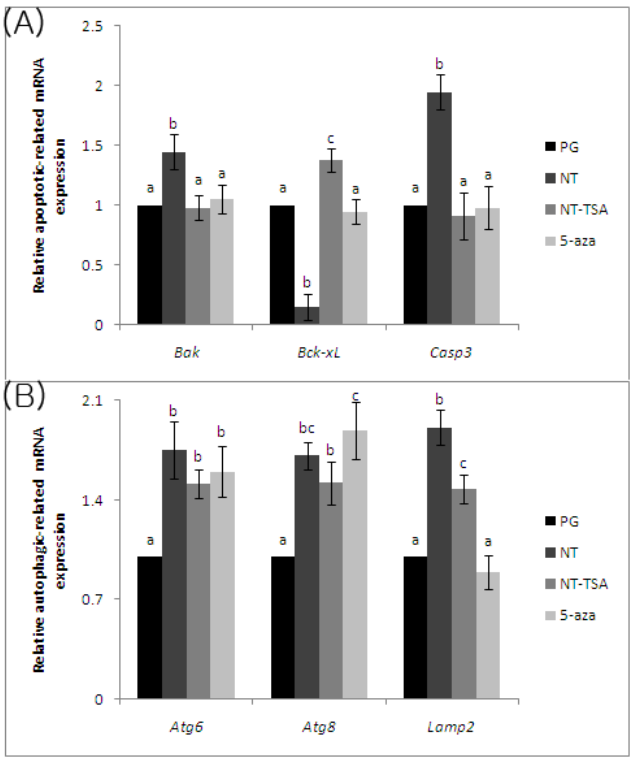

Fig. 3. The effect of TSA and 5-aza-dC treatment on the apoptotic- (A) and Autophagic (B) related gene expression analyzed by real time RT-PCR. Gapdh was used as the internal standard.

replicates. Treatment with TSA and 5-aza-dC did not affect the mRNA expression of Bak and casp3 compared to PG, However it is significantly (p<0.01) decreased mRNA expression level of Bak, Casp3 and Bcl-xL compared to SCNT (p<0.01 Fig. 3A).

5 nM TSA and 10 nM 5-aza-dC modulates mRNA expression of autophagic-related genes in porcine PG and NT developing *in vitro*, the relative amounts of *ATG6, ATG8* and *Lamp2* mRNA expression in blastocysts cultured in PZM3 containing 0.4% BSA medium supplemented with 5 nM TSA and 10 nM 5-aza-dC were measured by real time RT-PCR. Treatment with 5 nM TSA significantly decreased mRNA expression levels of *ATG6, ATG8* and *Lamp2* in the blastocysts. However, treatment with 10 nM 5-aza-dC decreased *Lamp2* only (p<0.05, p<0.01 Fig. 2b).

Effect of TSA and 5-aza-dC Treatment on the Acetlation and Methylation Status of Histon H3K9 at the Blastocysts

To determine whether TSA and 5-aza-dC can increase acetylation levels, we examined the localization of acH3K9 and meH3K9 levels in the porcine blastocysts derived from the PG, NT, NT-TSA and 5-aza-dC embryos. Following addition of 5 nM TSA and 10 nM 5-aza-dC to the culture medium, the amount of acetylation levels was significantly increased compared to that of the NT embryos (Fig. 4A, C). Also, methylation levels were significantly decreased compared to NT blastocysts (Fig. 4B, D).

Quantification Via Real-Time RT-PCR of the mRNA Expression DNA Methyltransferase (DNMT) in Porcine Blastocysts

To investigate whether 5 nM TSA and 10 nM 5-aza-dC can modulate mRNA expression of DNMT1, DNMT3a and DNMT3b genes in porcine PG and NT developing *in vitro*, the relative amounts of DNMT1, DNMT3a and DNMT3b mRNA expression in blastocysts cultured in PZM3 containing 0.4% BSA medium supplemented with 5 nM TSA and 10 nM 5-aza-dC were measured by real time RT-PCR. Treatment with 5 nM TSA significantly decreased mRNA expression levels of DNMT1 in the blastocysts. However treatment with 10 nM 5-aza-dC was DNMT1 and DNMT3a decreased compared to SCNT (p<0.05, p<0.01 Fig. 5).

DISCUSSION

Mammalian cloning by SCNT often results in gestational or neonatal failure with a low percentage of successful live births from reconstructed embryos (Rideout *et al*., 2001). The low efficiency of SCNT is caused by several factors. Particularly, an important factor that may affect the successful development of NT embryos is the epigenetics of reconstructed embryos, such as DNA methylation, chromatin remodeling, imprinting, X chromosome inactivation, telomere maintenance and epignetic modification (Shi W *et al*., 2003). Low levels of histone acetylation patterns in embryos derived by NT are known to be related to the low efficacy of NT generating live offspring (Wee *et al*., 2006).

Therefore the HDAC inhibitor TSA and DNA methyltransferase inhibitor 5-aza-dC was suggest to be used a recovery of aberrant nuclear reprogramming following nuclear transfer. In facts, TSA has been increased the rate of development to the blastocyst stage and improved blastocysts quality through induce hyperacetylation incloned mouse (Kishigami *et al*., 2006; Rybouchkin *et al*., 2006), bovine and porcine (Cervera *et al*., 2009; Zhang *et al*., 2007) and rabbit (Shi *et al*., 2008) embryos. In the present study we treated porcine cloned embryos with the concentration of 5 nM of TSA and 10 nM 5-aza-dC, consistent with previous report (Cervera *et al*., 2009). In this study, we demonstrated that TSA

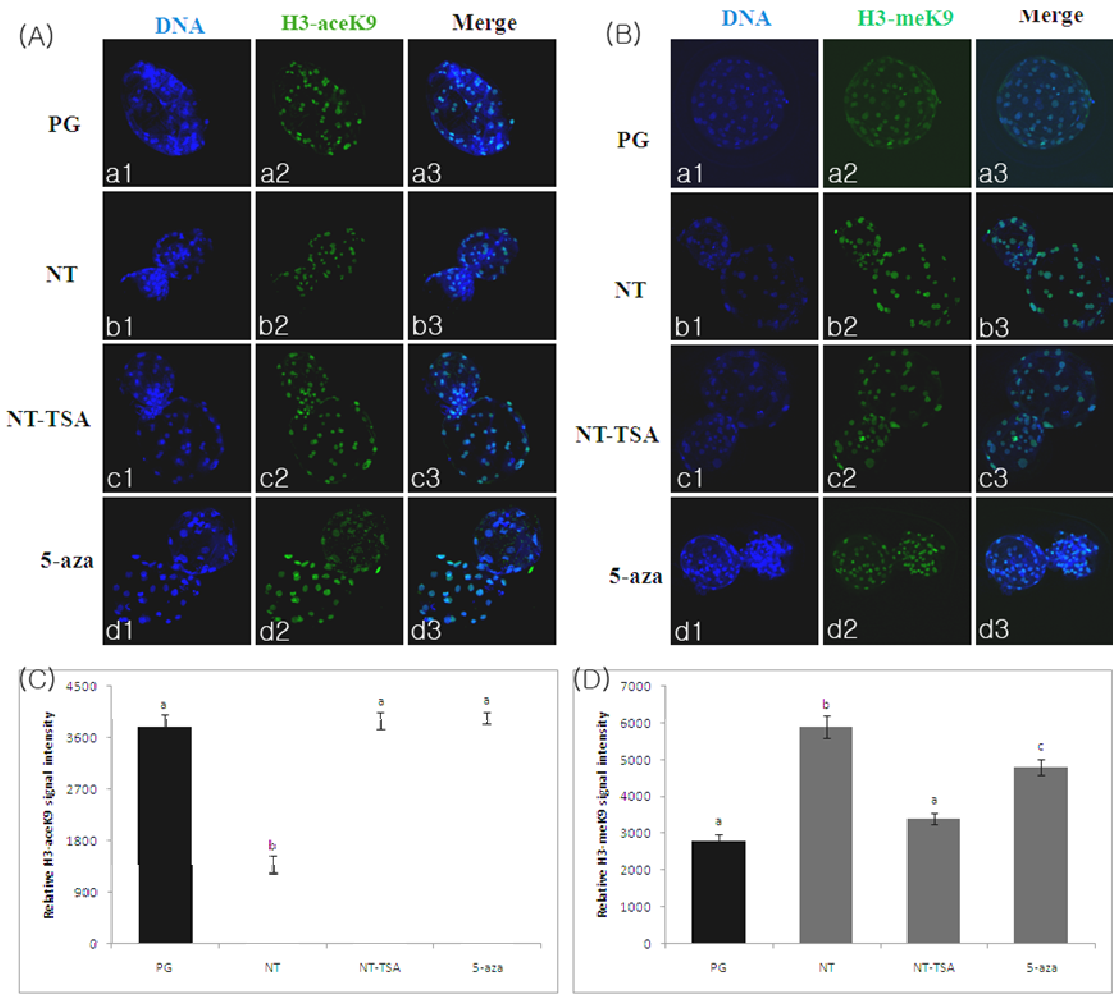

Fig. 4. Laser scanning confocal microscopic images of H3K9 acetylation (A) and H3K9 methylation (B) in porcine blastocysts (400×). Blue, chromatin (a1, b1, c1 and d1); green, H3K9 levels stained by FITC-conjugated secondary antibody (a2, b2, c2 and d2) (A, B). Relative levels of H3K9 actylation (C) and methylation (D) in PG, SCNT, NT-TSA and 5-aza-dC blastocysts ($p<0.05$).

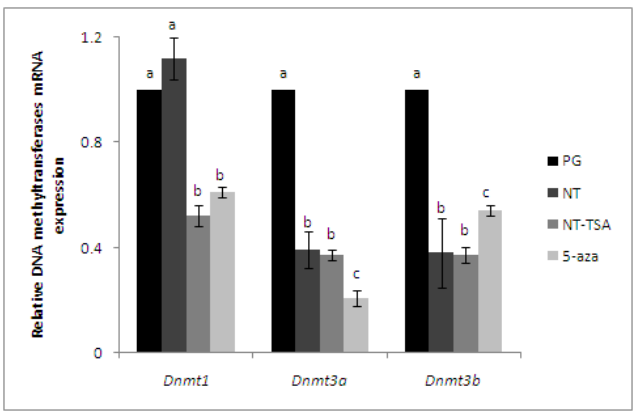

Fig. 5. Quantification via real-time RT-PCR of the mRNA expression of DNA methyltransferase (DNMT1, DNMT3a and DNMT3b) in PG, SCNT, NT-TSA and 5-aza-dC porcine embryos at blastocysts stage. *Gapdh* was used as the internal standard.

affected rates of development of the blastocyst stage. Also TSA and 5-aza-dC treatment affected of cell numbers, apoptosis, and autophagy in the blastocyst stage of SCNT.

In our study, treatment with 5 nM TSA and 10 nM 5-aza-dC increase the cell number and decrease apoptosis in the blastocyst stage following SCNT. We found that addition of TSA and 10 nM 5-aza-dC significantly decreased the percentage of apoptotic TUNEL-labeled nuclei in the blastocysts of SCNT. Apoptosis is another criterion of quality to eliminate cells with nuclear or chromosomal abnormalities. Apoptotic cell death occurs in pre-implantation mammalian embryos and plays an important role in embryo development. The increase of apoptosis in NT blastocysts is also correlated with a decrease in the total cell number. In the present study, NT embryos showed a higher apoptotic index than par-

thenote group.

The molecular mechanism by which TSA and 5-aza-dC affects apoptosis in the mammalian NT embryo is not clear at present. Our examination of the mRNA expression levels of apoptosis related genes during blastocyst development *in vitro* support the idea that TSA and 5-aza-dC reduces apoptosis. During embryogenesis, the Bcl-2 family controls programmed cell death. The Bcl-2 gene family is known to include anti-apoptotic and pro-apoptotic subgroups, and the Bcl-xL (*Bcl2l2*) gene functions to protect against apoptosis (Shen XH. 2006). In contrast, another group of highly conserved genes are positive regulators of apoptosis, these include the *Bak* and *Bax* proteins that lead to the release of cytochrome c and activation of the caspase cascade.

Autophagy, a process by which eukaryotic cells degrade and recycle macromolecules and organelles, has an important role in the cellular response to stress. In the present study, we first found the autophagy in porcine NT embryos subjected to TSA and 5-aza-dC treatment. Our results showed that NT embryos that were cultured in the presence of TSA and 5-aza-dC in PZM3 medium significantly reduced the expression of Microtubule-associated protein light chain 3 (LC3), which is a marker for the presence of autophagosomes. The addition of TSA and 5-aza-dC also reduced the expression of autophagic-related genes *ATG6, ATG8* and *Lamp-2*. Previous observations and the present study suggest that treatment of TSA may also lead to autophagy in porcine preimplantation NT embryos.

Improved epigenetic reprogramming may account for the beneficial outcomes brought about by TSA in SCNT. Using immunofluorescence, we found that TSA could greatly elevate the levels of histone acetylation in cloned embryos. The nuclear remodeling process is accompanied by dynamic changes in histone acetylation levels in cloned embryos. Generally, histone acetylation is associated with an open chromatin state and thus gives transcriptional factors access to the DNA templates (Wee *et al*., 2006). Increasing acetylation levels may improve reprogramming of donor genomes (Rybouchkin *et al*., 2006). In fact, improved acetylation reprogramming contributes greatly to increased developmental efficiency caused by TSA treatment in SCNT embryos. Our result seems to support this notion by showing low levels of H3K9 acetylation in cloned porcine embryos. This abnormality in histone acetylation reprogramming could be alleviated by HDAC inhibition. In porcine SCNT embryos, levels of histone acetylation were greatly increased by TSA and 5-aza-dC treatment. A family of structurally related proteins termed DNA (cytosine-5) methyltransferases (DNMTs) has been identified that catalyze the production and modulate the dynamics of mammalian patterns of global genomic DNA methylation. DNMT1 is the most abundantly expressed methyltransferase, and is thought to be largely responsible for maintaining methylation patterns through DNA replication.

In conclusion, in this study, we demonstrated that histone deacetylase inhibitor TSA can improve the viability of porcine somatic cell cloned embryos by increasing cell numbers and preventing apoptotic and autophagic cell death. However, the effect of treating embryos with 5-aza-dC in the development of cloned embryos is not increased. The anti-apoptotic effect of TSA and 5-aza-dC seems to be positively linked to anti-apoptotic mRNA *Bcl-xL* expression, and also increased expression levels of the autophagic-related genes *ATG6, ATG8* and *Lamp2* in porcine preimplantation stage embryos.

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