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In Vitro Developmental Competence of Porcine SCNT Embryos is improved by m-Carboxycinnamic Acid Bishydroxamide, Histone Deacetylase Inhibitor

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ABSTRACT

Differentiated nuclei can experimentally be returned to an undifferentiated embryonic status after nuclear transfer (NT) to unfertilized metaphase II (MII) oocytes. Nuclear reprogramming is triggered immediately after somatic cell nucleus transfer (SCNT) into recipient cytoplasm and this period is regarded as a key stage for optimizing reprogramming. In a recent study (Dai *et al.*, 2010), use of m-carboxycinnamic acid bishydroxamide (CBHA) as a histone deacetylase inhibitor during the *in vitro* early culture of murine cloned embryos modifies the acetylation status of somatic nuclei and increases the developmental competence of SCNT embryos. Thus, we examined the effects of CBHA treatment on the *in vitro* preimplantation development of porcine SCNT embryos and on the acetylated status of histone H3K9 on cloned embryos at the zygote stage. We performed the three groups SCNT: SCNT (NT), CBHA treatment at the porcine fetus fibroblast cells (PFFs) used as donor cells prior to SCNT (CBHA-C) and CBHA treatment at the porcine SCNT embryos during the *in vitro* early culture after oocyte activation (CBHA-Z). The PFFs were treated with a 15 μM of CBHA (8 h) for the early culture and the porcine cloned embryos were treated with a 100 μM concentration of CBHA during the *in vitro* early culture (10 h). Cleavage rates and development to the blastocyst stage were assessed. No significant difference was observed the cleavage rate among the groups (82.6%, 76.4% and 82.2%, respectively). However, the development competence to the blastocyst stage was significantly increased in CBHA-Z embryos (22.7%) as compared to SCNT and CBHA-C embryos (8.6% and 4.1%)($p<0.05$). Total cell numbers and viable cell numbers at the blastocyst stage of porcine SCNT embryos were increased in CBHA-Z embryos as compared to those in CBHA-C embryos ($p<0.05$). Signal level of histone acetylation (H3K9ac) at the zygote stage of SCNT was increased in CBHA-Z embryos as compared to SCNT and CBHA-C embryos. The results of the present study suggested that treatment with CBHA during the *in vitro* early culture (10 h) had significantly increased the developmental competence and histone acetylation level at the zygote stage.

(Key words : Porcine SCNT embryos, m-Carboxycinnamic acid bishydroxamide, Histone deacetylase inhibitor)

INTRODUCTION

The first cloned animal "Dolly" was successfully produced from the somatic cell nuclear transfer (SCNT) technique using cultured somatic cells as nuclear donors. This technology has widely been applied to various mammalian species for agricultural, biotechnological and medical purposes (Cibelli *et al.*, 1998; Wakayama *et al.*, 1998; Baguisi *et al.*, 1999; Polejaeva *et al.*, 2000; Shin *et al.*, 2002; Chesne *et al.*, 2002; Galli *et al.*, 2003; Zhou *et al.*, 2003; Lee *et al.*, 2005; Li *et al.*, 2006).

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However, the developmental efficiency of SCNT still remains low. The reasons may be attributed to incomplete or inappropriate reprogramming of the transferred nuclear genome (Campbell *et al*., 2007). In general, transplantation of somatic cell nucleus into recipient cytoplasm environment undergoes reprogramming processes including histone modification and DNA methylation, which refer to erasure and remodeling of epigenetic marks (Reik *et al*., 2001; Jouneau *et al*., 2003). The status of epigenetic can be returned from a differentiated cell status into an undifferentiated status. This remodeling may be modified the histone acetylation or methylation, phosphorylation, ubiquitination and sumoylation (Shi and Yang, 2007). Many studies have indicated that remodeling occurring immediately after SCNT is involved in reprogramming efficiency (Yang *et al*., 2007). During the remodeling process, morphological modifications occurs in the nucleus of donor cells including nuclear envelop breakdown (NEBD), premature chromosome condensation (PCC), dispersion of nucleoli, and then reestablish new ones for a relatively short time (Zuccotti *et al*., 2000; Wrenzycki *et al*., 2001).

In the mammalian cells, the chromatin structure can be inherited from paternal or maternal genomic status. Heterochromatin has a highly condensed chromatin, resulted in being silenced in genes (Misteli *et al.,* 2007). Gene silencing may be associated with repressed chromatin structure signed by histone hypoacetylation (Jenuwein *et al*., 2001; Bhaumik *et al*., 2007), because it can affect gene transcription by making silent chromatin structures and interrupting transcription factor binding. Acetylation of histone is regulated by balance of both histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities (Kikuchi and Fujimoto, 1973; Csordas 1990). In the previous studies, several epigenetic remodeling drugs such as the histone deacetylase inhibitor (HDACi) trichostatin A (TSA) (Kishigami *et al*., 2006; Zhang *et al*., 2007), Scriptaid (Van Thuan *et al*., 2009; Zhao *et al*., 2009), sodium butyrate (Shi *et al*., 2003; Das *et al*., 2010), valproic acid (VPA) (Costa-Borges *et al*., 2010; Miyoshi *et al*., 2010), suberoylanilide hydroxamic acid (SAHA) (Ono *et al*., 2010), oxamflatin (Su *et al*., 2011) and m-carboxycinnamic acid bishydroxamide (CBHA) (Dai *et al*., 2010) have been used to increase the developmental competence of SCNT embryos. In addition, it was reported that the HDACi can cause a marked increase in histone acetylation and enhance the developmental competence of SCNT embryos.

Several studies have been carried out to evaluate the use of HDACis on somatic cell nuclear transfer. These include the treatment with HDACis either before (i.e., of the nuclear donor cells) or after (early embryos) somatic cell nuclear transfer. As a result, different group have found a significant improvement of embryonic preimplantation developmental rates (Li *et al.,* 2006; Shi *et al.,* 2008; Costa-Borge *et al.,* 2011) and blastocyst quality (Iager *et al.,* 2008; Cui *et al.,* 2011). Furthermore, an improvement of full-term development has also been reported in mice and miniature pigs regarding production of cloned animals using HDACis such as TSA (Kishigami *et al.,* 2006) and Scriptaid (Zhao *et al.,* 2009). Zhao and coworkers (2010) also tested the effect of TSA and Scriptaid in domestic pigs. These authors found that TSA improved clone efficiency but the treatment resulted in the death of three out of four live born piglets after birth. On the other hand, Scriptaid was effective in increasing the development potential of somatic cell nuclear transfer embryos (Zhou *et al.,* 2003). Nonetheless, little is known about the effect of HDACis on postimplantation development of large animals. The long gestation period and high costs of maintenance are points that contribute to make these data scare for species such as cattle. Moreover, detrimental effects of the use of HDACis have also been shown, especially when somatic cells or embryos are exposed to high doses of these drugs (Enright *et al.,* 2003).

Therefore, herein we carried out a series of experiments to test whether the treatment of nuclear donor cell or reconstructed oocytes with m-carboxycinnamic acid bishydroxamide (CBHA) as a histone deacetylase inhibitor would affect the pre-implantation development of cloned domestic pigs. We hypothesized that the association with drug targeting histone acetylation could alter chromatin configuration and turn it more amenable to be reprogrammed. CBHA was chosen as a more amenable treatment because of the effect on cellular viability. Based on the hypothesis, acetylation status of histone 3 at lysine residue 9 (H3K9ac) and the quality of porcine SCNT embryos (total cell numbers and apoptotic rates in blastocyst) in *in vitro* fertilized (IVF), SCNT, CBHA treatment to the PFFs used as nuclear donor cell prior to SCNT (CBHA-C) and CBHA treatment to SCNT embryos during the *in vitro* early culture (CBHA-Z) were evaluated.

MATERIALS AND METHODS

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

Oocyte Collection and *In Vitro* Maturation

Ovaries of prepubertal gilts were collected at a local slaughterhouse and transported to the laboratory in phosphate-buffered saline (PBS) at 39℃ within 2 h. The ex-

tra tissue from ovaries was trimmed off and washed twice in PBS at 39℃. Cumulus-oocyte complexes (COCs) were aspirated from 2 to 5 mm antral follicles with a 18-gauge injection needle attached to a 10 ml syringe. The follicular fluid was allowed to settle for 3 min and diluted with Tyrode's lactate-HEPES (TL-HEPES) buffer [114.0 mM NaCl, 3.2 mM KCl, 0.34 mM NaH_2PO_4, 60% syrup Na-lactate (w/w), 0.5 mM $MgCl_2 \cdot 6H_2O$, 10.0 mM HEPES, 0.2 mM Na-pyruvate, 12.0 mM sorbitol, 2.0 mM $NaHCO_3$, 2.0 mM $CaCl_2 \cdot 2H_2O$, 0.294 g/l gentamycin, 0.065 g/l penicillin G, and 0.1 g/l poly vinyl alcohol (PVA)] at 39℃. Only COCs surrounded with at least three layers of compact cumulus cells and an uniform cytoplasm were selected for *in vitro* maturation. Selected oocytes were washed three times in Brilliant Cresyl Blue (BCB) medium [Dulbecco's phosphate-buffered saline (D-PBS) supplemented with 4 mg/ml BSA and 13 mM of BCB]. The COCs were incubated for 90 min with 5% CO_2 at 39℃ in a humidified atmosphere. After BCB treatment for 90 min, only BCB stained oocytes were selected for *in vitro* maturation. The COCs were incubated in maturation medium [Tissue culture medium-199 (TCM-199, Gibco Life Technologies Inc., Paisley, UK) supplemented with 10% PVA, 3.05 mM D-glucose, 0.91 mM Na- pyruvate, 0.57 mM cysteine, 75 μg/ml penicillin, 50 μg/ml streptomycin, 10 ng/ml Epidermal growth factor (EGF), 1 μg/ml Follicle-stimulating hormone (FSH) and 5 μg/ml Luteinizing hormone (LH)] supplemented with 5 μg/ml of cycloheximide (CHXM) for 16 h to synchronize meiotic maturation (Ye *et al*., 2002). After retrieval of CHXM, COCs were washed three times and cultured in maturation medium in the 4-well Nunc dish (Nunc, Roskilde, Denmark) covered with mineral oil for 30~32 h with 5% CO_2 at 39℃ in a humidified atmosphere.

Preparation of Porcine Fetal Fibroblast Cells

A 30-day old Berkshire fetus was obtained from a slaughterhouse. The porcine fetus was transported on ice to the laboratory and washed in Ca^{2+}-, Mg^{2+}-free D-PBS containing 0.1% (v/v) penicillin/streptomycin. After the internal organs and head were removed, they were chopped using sterilized scissors in D-PBS containing 0.25% (v/v) trypsin/EDTA. Cells obtained from fetus were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 1.25% MEM-nonessential amino acids, 1.25% β-mercaptoethanol, 1% penicillin/streptomycin supplemented with 10% FBS at 39℃ in 5% CO_2. Until the use, primary cultured cells were stored in liquid nitrogen (LN_2). Cells were thawed and cultured until growing to 80~90% confluence. After being cultured until passage 4~5, cells were synchronized at G0 phase for 3~5 days in DMEM supplemented with 0.1% fetal bovine serum (Gibco) prior to use as donor cell.

Pathenogenetic Activation

After 46~48 h of oocyte maturation, oocytes were placed into 500 μl of TL-HEPES buffer containing 0.1 % hyaluronidase in a 15 ml conical tube and then vortexed for 5 min in order to remove the cumulus cells. Denuded oocytes at metaphase II (MII) with the first polar body were selected for oocyte activation and placed in chamber composing of two platinum electrodes 0.2 mm apart. And then oocytes were activated in the fusion medium [0.3M mannitol containing 0.1 mM $MgSO_4$, 0.5 mM $CaCl_2$, 3 mg/ml bovine serum albumin (BSA fatty acid free)]. Oocytes were exposed to two electrical DC pulses of 1.25 KV/cm for 30 sec using a multiporator (Eppendorf, Germany). Activated oocytes were subsequently incubated for 5 h in porcine zygote medium 5 (PZM5) [108.0 mM NaCl, 10.0 mM KCl, 0.35 mM KH_2PO_4, 0.40 mM $MgSO_4 \cdot 7H_2O$, 25.07 mM $NaHCO_3$, 0.20 mM Na-pyruvate, 2.0 mM Ca-(lactate)$_2 \cdot 5H_2O$, 2.0 mM L-Glutamine, 5.0 mM hypotaurine, 50 μg /ml Gentamicin, 20 ml/l BME amino acids, 10 ml/l MEM amino acids, 3 mg/ml BSA (fatty acid free)] supplemented with 2 μg/ml of CHXM and 0.5 μg/ml cytochalasin B (CB). These parthenogenetic embryos were transferred and cultured into fresh PZM5 containing 20 ml/l BME amino acids, 10 ml/l MEM-nonessential amino acids, 3 mg/ml BSA at 5% CO_2 and 5% O_2 at 39 ℃ in a humidified atmosphere. Cleavage rates and blastocyst rates were evaluated on the day 2 and 7, respectively.

***In Vitro* Fertilization (IVF)**

After 46~48 h of oocyte maturation, cumulus cells surrounding oocytes were removed by pipetting and then washed three times in fertilization medium (mTBM) [113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 11.0 mM Glucose, 5.0 mM Na-pyruvate, 0.57 mM Cysteine, 20.0 mM Trisma, 100 IU/ml Penicilline G, 2 mg/ml Streptomycine sulfate] supplemented with 2 mg/ml bovine serum albumin (BSA; fatty acid free). Groups of 15 COCs were placed in pre-equilibrated 50 μl drop of fertilization medium (mTBM) covered with mineral oil. After fresh semen was washed once by centrifugation at 900 g for 5 min at 37℃, spermatozoa were resuspended in mTBM supplemented with 2 mM Caffeine and 2 mg/ml BSA. 50 μl of this suspension was added to the fertilization drop containing the oocytes, giving a final concentration of 1×10^5 sperm/ml. Following 6 h of fertilization, oocytes were washed and cultured in PZM5 medium supplemented with 20 ml/l BME amino acids, 10 ml/l MEM-nonessential amino acids,

3 mg/ml BSA at 39℃ in 5% CO_2, 5% O_2 in air. On day 2, cleaved embryos were transferred into fresh PZM5 medium. Embryos were incubated until the blastocyst stage.

Somatic Cell Nuclear Transfer SCNT

After porcine COCs were treated with CHXM to induce meiotic synchronization for 16h, the COCs were further maturated for 17~18 h in the maturation medium. COCs were placed into 500 μl of TL-HEPES buffer containing 0.1% hyaluronidase in a conical tube and then vortexed for 5 min to remove cumulus cells. Denuded oocytes were stained in 25 mM HEPES buffered maturation medium containing 1 μg/ml Hoechst 33342 for 10 min. Stained oocytes were transferred into a drop of 25 mM HEPES buffered manipulation medium containing 5 μg/ml of CB with mineral oil. For oocyte enucleation, AI-TI spindle containing a little cytoplasm was aspirated using a 20~25 μm of glass micropipette (Lee & Campbell, 2006). Successful enucleation was checked by a brief exposure to UV light in the aspirated karyoplast. The enucleated oocytes were cultured in the maturation medium with 5% CO_2 at 39 ℃ in a humidified atmosphere until the transfer of donor cells and cell fusion. After being cultured for 12 h, a single donor cell was transferred into the perivitelline space of the oocyte through the hole which previously made for enucleation. The reconstructed oocytes were exposed to two DC pulses of 1.25 KV/cm for 30 sec using a multiporater (Eppendorf, Germany). Fused oocytes were then incubated in PZM5 plus 20 ml/l BME amino acids, 10 ml/l MEM amino acids, 3 mg/ml BSA (fatty acid free) with 5% CO_2 and 5% O_2 at 39℃ in a humidified atmosphere.

CBHA Treatment Following Oocyte Activation

m-Carboxycinnamic acid bishydroxamide (CBHA, Calbiochem, 382148) as a histone deacetylase inhibitor (HDACi) was dissolved in dimethly sulfoxide (DMSO) to adjust a stock solution of 100 mM and stored at −80 ℃. Working solution was freshly prepared just before use. For determination of the optimal concentration of exposure to CBHA, porcine embryos were treated with PZM5 medium containing the concentrations of 0 μM, 1 μM, 20 μM, 100 μM, 200 μM or 300 μM of CBHA for 10 h with 5% CO_2 and 5% O_2 at 39℃ in a humidified air. The optimal concentration of CBHA was used for further SCNT experiment. After 10 h of incubation with CBHA, the embryos were cultured in PZM5 medium until the blastocyst stage at 39℃ in 5% CO_2 and 5% O_2 in humidified air. To determine optimal concentration of exposure to CBHA to donor cells, porcine fetal fibroblast cells were exposed to DMEM at the concentrations of 0 μM, 5 μM, 10 μM, 15 μM or 20 μM of CBHA for 8h. The optimal concentration of exposure to CBHA to donor cell was determined by the cell viability using trypan blue staining and immunostaining of histone acetylation.

Immunocytochemistry for Histone Acetylation

Porcine embryos were fixed in fixation solution [4% paraformaldehyde (PFA) diluted in PBS, pH 7.4, freshly prepared] for 1 h at 4℃ and washed three times for 15 min per wash in PBS-T [PBS containing 0.1% Tween 20, 0.05% NaAzide]. The embryos were permeabilized with 1% Triton X-100 in PBS for 30 min at room temperature (RT) and then washed three times for 15 min in PBS-T. These embryos were blocked with the Blocking solution [5% BSA in PBS-T] for overnight at 4℃ and then washed three times. Afterward, the samples were incubated with the primary antibody of H3K9ac (Histone H3 Lys-9 acetylation, 1:300, Upstate, USA) for 1 h at 37℃ and washed three times. They were incubated with Alexafluor 488-conjugated secondary antibody (1:150, Invitrogen, USA) for 1 h at RT in the dark and washed three times for 15 min per wash. The samples were counterstained with 10 μg/ml propidium iodide (PI) in PBS for 15 min and then washed three times for 15 min per wash. The samples were mounted on glass slides with a drop of VECTASHIELD® (Vector Laboratories Inc). Images were captured using a fluorescence microscope (LEICA DM 2500).

After treatment of CBHA, porcine fetal fibroblast cells (PFFs) were placed onto a cover slip by centrifuge (2050 centrifuge, Centurion Scientific Ltd, UK) and fixed in fixation solution for 15 min and then washed three times in PBS-T [0.1% Tween 20 and 0.05% NaAzide in PBS]. Fixed cells were blocked with the blocking solution for 1 h at RT. These samples were incubated in the primary antibody H3K9ac (Histone H3 Lys-9 acetylation, 1:250, Milipore, USA) at 4℃ overnight. They were incubated with the secondary antibody (Alexafluor 488-goat anti-rabbit IgG (1:150, Invitrogen) for 1 h at RT in the dark. The samples were stained with 0.5 μl/ml of PI in PBS for 10 min and then washed three times. The samples were mounted on glass slides with a drop of VECTASHIELD® (Vector Laboratories Inc). Images were captured using a fluorescence microscope (LEICA DM 2500).

Apoptotic Assay of SCNT Embryos

In Situ Cell Death Detection Kit (F. Hoffmann-La Roche Ltd, Switzerland) was used for apoptosis assays. According to the manual instruction, porcine blastocysts on the day 7 were fixed in fixation solution for 1 h at 4℃ and then washed three times in PBS. The em-

bryos were permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate in PBS for 2 min at 4℃ and then washed three times. Permeabilized embryos were labeled with the TUNEL reaction mixture for 60 min at 37℃ in the dark and washed three times. Finally, DNA was stained with 5 μl/ml of Hoechst 33342 for 10 min at 39℃. Embryos were mounted on glass slides with a drop of VECTASHIELD® (Vector Laboratories, Inc) and covered with coverslip. Images were captured using a fluorescence microscope (LEICA DM 2500).

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA). A probability of $p<0.05$ was considered to be statistically significant.

RESULTS

Determination of the Optimal Concentration of Exposure to CBHA through *In Vitro* Development of Parthenogenetic Embryos

In order to determine the optimal concentration of m-carboxycinnamic acid bishydroxamide (CBHA) as a histone deacetylase inhibitor (HDACi) exposed to porcine SCNT embryos during *in vitro* early development (10 h) after oocyte activation, the parthenogenetically active porcine embryos were treated with 0 μM, 1 μM, 20 μM, 100 μM, 200 μM or 300 μM for 10 h, respectively. As shown in Table 1, there was no significant difference in the cleavage rates on the day 2 among all concentrations of CBHA. However, the *in vitro* pre-implantation development to the blastocyst stage on the 7 day was significantly higher in 1 μM, 20 μM and 100 μM (20.9%, 18.5% and 20.9%) treated embryos than in 0 μM, 200 μM and 300 μM (15.8%, 10.4% and 11.0%) treated embryos ($p<0.05$). In addition, the total cell number of porcine parthenogenetic blastocysts was increased in 100 μM of CBHA treatment (32.5±2.2) as compared to the other groups. Considering the blastocyst rates and total cell number of the blastocysts, 100 μM was determined to the optimal concentration of exposure to CBHA as a histone deacetylase inhibitor (HDACi) to porcine SCNT embryos for further experiment.

Determination of the Optimal Concentration of Exposure to CBHA through Culture of Porcine Fetus Fibroblast Cells (PFFs)

In this experiment, we carried out to determine optimal concentration of exposure to CBHA as a histone deacetylase inhibitor (HDACi) to porcine fetal fibroblast cells (PFFs) used as nucleus donor cell before somatic cell nuclear transfer. The PFFs were treated with various concentrations of CBHA at 0 μM, 5 μM, 10 μM, 15 μM or 20 μM for 8 h, respectively. The number of survival cells was detected by incorporation of trypan blue staining. The results from these studies are represented in Table 2. There was no difference in the cell viability between all groups after CBHA treatment for 8 h (89.7±3.1, 89.5±2.9, 89.2±2.9, 83.0±2.2 and 76.1±4.2). However, 20 μM of CBHA treatment to the PFFs showed lower survival rate as compared to the other groups (0 μM, 5 μM, 10 μM and 15 μM). In the experiment, 15 μM was chosen for the optimal concentration of exposure to CBHA as the histone deacetylase inhibitor (HDACi) to porcine fetal fibroblast cells as nucleus donor celss for further SCNT experiments.

Determination of the Optimal Period of Exposure to CBHA to PFFs for being Used as Donor Cells

To determine the optimal period of exposure to CBHA as the histone deacetylase inhibitor (HDACi), to

Table 1. Effect of different concentrations of CBHA treatment on *in vitro* development of porcine parthenogenetic embryos

Concentrations of CBHA(μM)	No. of oocytes	No. (%) of cleaved embryos	No. (%) of blastocysts	No. of cells /blastocyst (mean±SEM)
0	152	133 (87.5)	21 (15.8)[b]	27.6±4.3
1	151	129 (85.4)	27 (20.9)[a]	22.9±3.6
20	150	135 (90.0)	25 (18.5)[a]	31.7±7.1
100	150	134 (89.3)	28 (20.9)[a]	32.5±2.2
200	145	125 (86.2)	13 (10.4)[b]	18.9±7.1
300	151	127 (84.1)	14 (11.0)[b]	20.6±8.7

[a~c] Values with the same superscript within a column are significantly different ($p<0.05$).

Table 2. Effect of CBHA treatment on cell viability of porcine fetal fibroblast cells

Concentration (μM)	Cell viability (mean±SEM)
0	89.7±3.1
5	89.5±2.9
10	89.2±2.9
15	83.0±2.2
20	76.1±4.2

The number of survival cells was detected by trypan blue staining.

the porcine fetus fibroblast cells (PFFs) used as nucleus donor cell for SCNT, the PFFs were cultured in histone deacetylase inhibitor CBHA for 0 h, 2 h, 4 h, 6 h, 8 h or 10 h, respectively. The histone acetylation status of H3K9ac after treatment of CBHA was assessed using an antibody specific (Alexafluor 488-goat anti-rabbit IgG, 1:150) to the acetylated form of H3K9. As shown in Fig. 1, the intensity of H3K9ac staining in the PFFs was induced after treatment of 2 h with CBHA. However, the intensity of H3K9ac staining in the PFFs was significantly elevated after treatment of CBHA for 8 h. In the experiment, 8 hours was chosen for the optimal period of exposure to CBHA as the histone deacetylase inhibitor (HDACi) for further studies.

Effect of CBHA Treatment to Either Nuclear Donor Cell or Reconstructed Oocytes on the *In Vitro* Development of Porcine SCNT Embryos

In order to assess whether nucleus donor cells or reconstructed oocytes treated with CBHA as the histonedeacetylase inhibitor (HDACi) affects the *in vitro* preimplantation development of porcine SCNT embryos, the cloned embryo were divided into two groups: SCNT embryos (SCNT-C) reconstructed by using CBHA-treated donor cells (15 μM of CBHA, 8 h) and SCNT embryos (CBHA-Z) treated with CBHA during the early culture (100 μM of CBHA, 10 h). The results from these studies are represented in Table 3. There was no significant difference in the cleavage rates on the day 2 between all groups (SCNT, CBHA-C and CBHA- Z; 82.6%, 76.4% and 82.2%, respectively). However, the *in vitro* development to the blastocyst stage in the IVF and CBHA-Z embryos (19.7% and 22.7%) was significantly increased as compared to the SCNT and CBHA-C embryos (8.6% and 4.1%) ($p<0.05$). The reasons why CBHA-Z embryos shows greater *in vitro* development to the blastocyst stage may benefit morphological modifications such as nuclear envelop breakdown (NEBD), premature chromosome condensation (PCC), dispersion of nucleoli and then reestablish new ones for the zygote stage.

Status of Histone Acetylation in Porcine CBHA-C and CBHA-Z Cloned Embryos

To confirm the epigenetic modification of the porcine SCNT embryos following the CBHA treatment to either nuclear donor cell or reconstructed oocytes, we examined the the intensity of histone 3 at lysine residue 9 (H3K9ac) at the zygote stage by immunostaining. As shown in Fig. 2, there was the significant difference in the intensity of H3K9ac between CBHA-C and CBHA-Z cloned embryo groups ($p<0.05$). The intensity of H3K9

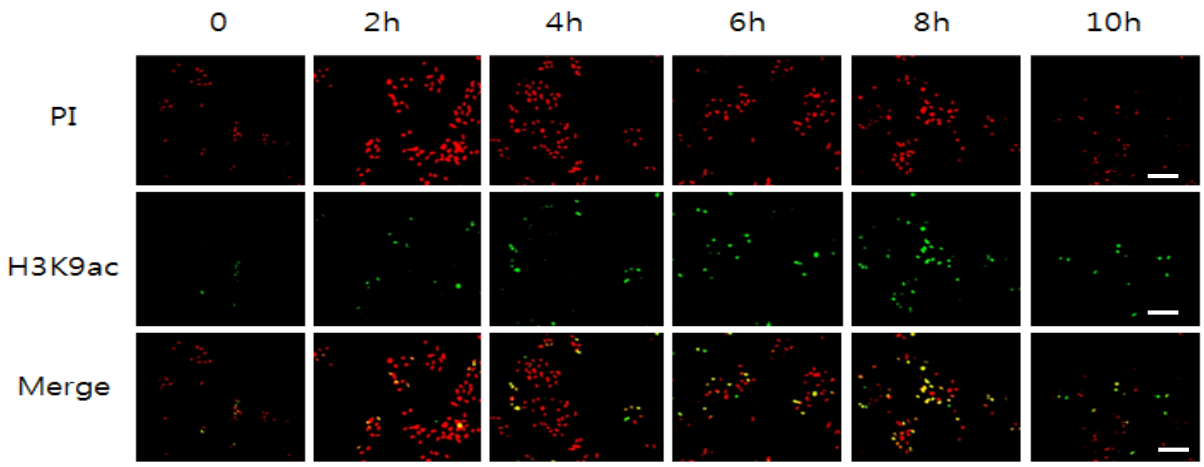

Fig. 1. Histone acetylation status of H3K9 in PFFs treated with CBHA using immunostaining. Example of images obtained when visualizing H3K9ac of PFFs immunostained after treatment of CBHA for 0, 2, 4, 6, 8 of 10 h. Red : PI stained nuclei, Green : Alexaflour 488-labeled H3K9ac. Scale bar=50 μm.

Table 3. Effect of CBHA treatment to nuclear donor cell or reconstructed oocytes on *in vitro* development of porcine SCNT embryos

Treatment	No. of oocytes	No. (%) of fused NT embryos	No. (%) of cleaved NT embryos	No. (%) of blastocysts
IVF	409	-	208 (50.9)	41 (19.7)[a]
NT	157	155 (98.7)	128 (82.6)	11 (8.6)[b]
CBHA-C	200	191 (95.5)	146 (76.4)	6 (4.1)[b]
CBHA-Z	124	118 (95.2)	97 (82.2)	22 (22.7)[a]

[a,b] Values with the same superscript within a column are significantly different (p<0.05).
IVF: *In vitro* fertilized embryos
NT: SCNT embryos reconstructed by using no CBHA-treated donor cells and treated with no CBHA during the early culture
CBHA-C: SCNT embryos reconstructed by using CBHA-treated donor cells
CBHA-Z: SCNT embryos treated with CBHA during the early culture
Cleavage percentage: No. cleaved SCNT embryos / No. fused SCNT embryos
Cleavage percentage of IVF: No. Cleaved embryos / No. oocytes
Blastocyst percentage: No. blastocysts / No. cleaved embryos

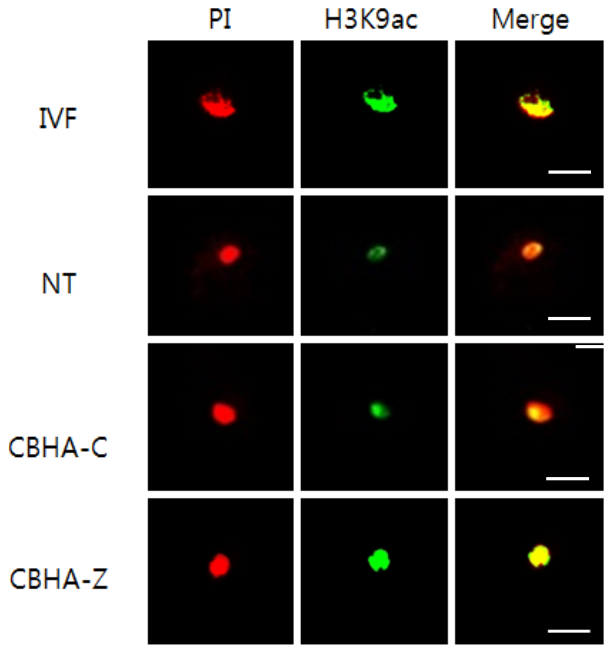

Fig. 2. Level of histone acetylation (H3K9ac) of SCNT embryos at the zygote stage on CBHA treatment to either nuclear donor cell or reconstructed oocytes. Examples of images obtained when visualizing H3K9ac of IVF, SCNT, CBHA-C (SCNT embryos reconstructed by using CBHA-treated donor cells), CBHA-Z (SCNT embryos treated with CBHA during the early culture) at the zygote stage were shown Green: Alexa flour 488-labeled H3K9ac, Rred: PI stained nuclei. Scale bar=50 μm.
IVF: *In vitro* fertilized embryos
NT: SCNT embryos reconstructed by using no CBHA-treated donor cells and treated with no CBHA during the early culture
CBHA-C: SCNT embryos reconstructed by using CBHA-treated donor cells
CBHA-Z: SCNT embryos treated with CBHA during the early culture

ac in the CBHA-Z cloned embryos was significantly increased in SCNT and CBHA-C cloned embryos (p< 0.05). In addition, the H3K9ac level of CBHA-C cloned embryos at the zygote stage was similar to that of the IVF embryos. This result indicated that the treatment of CBHA as the histone deacetylase inhibitor to porcine SCNT zygotes gives more benefit epigenetic modifications than to nuclear donor cell in terms of histone acetylation status.

Quality of Porcine SCNT Blastocysts Following CBHA Treatment to Either Nuclear Donor Cell or Reconstructed Oocytes

To assess the quality of porcine SCNT blastocysts following CBHA treatment to either nuclear donor cell or reconstructed oocytes, we examined to measure the apoptotic cells and the total cell number of blastocysts using TUNEL assay. As shown in results of Table 4. and Fig. 3, the total cell number of SCNT blastocysts was significantly increased in CBHA-Z blastocysts as compared to that of CBHA-C blastocysts (41.0 vs. 27.0) (p<0.05). The rate of apoptotic cells in the SCNT blastocysts (46.7%) and the CBHA-C blastocysts (63.0%) was significantly increased as compared to those in CBHA-Z blastocysts (29.3%) (p<0.05). These results indicated that the quality of CBHA-Z blastocysts improves the CBHA treatment for reconstructed oocytes.

DISCUSSION

Nuclear reprogramming of differentiated somatic cells into a totipotent embryonic status by somatic cell nuclear transfer (SCNT) was firstly introduced by the

Table 4. Effect of CBHA treatment to nuclear donor cell or reconstructed oocytes on apoptosis of blastocyts derived from porcine SCNT embryos

Treatment	No. Cells /blastocyst	No. (%) of apoptotic cell /total blastocyst cell
IVF	20	3 (15.0)
NT	30	14 (46.7)
CBHA-C	27	17 (63.0)
CBHA-Z	41	12 (29.3)

IVF: *In vitro* fertilized embryos
NT: SCNT embryos reconstructed by using no CBHA-treated donor cells and treated with no CBHA during the early culture
CBHA-C: SCNT embryos reconstructed by using CBHA-treated donor cells
CBHA-Z: SCNT embryos treated with CBHA during the early culture

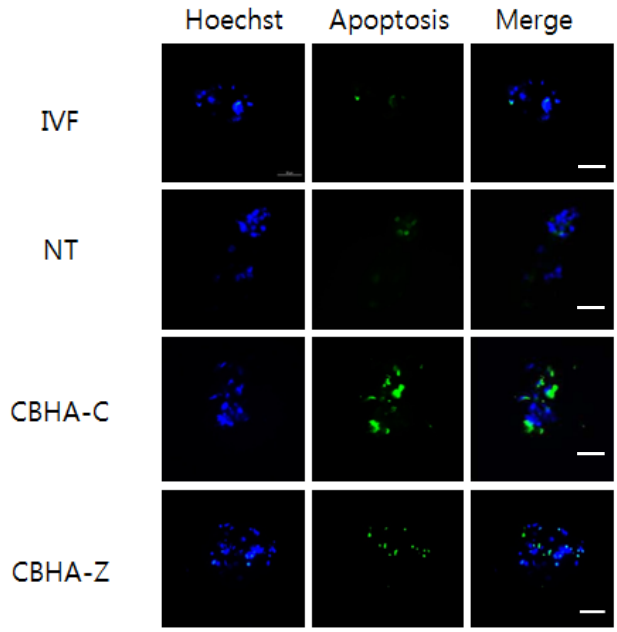

Fig. 3. TUNEL assay in porcine blastocysts of CBHA-C and CBHA-Z embryos at the blastocyst stage. The total cell numbers and viable cell numbers in the blastocyst stage of porcine SCNT embryos were increased in CBHA-Z cloned embryos as compared to CBHA-C cloned embryos based on TUNEL assay. Blue: Hoechst 33342, Green: TUNEL. Scale bar=50 μm.
IVF: *In vitro* fertilized embryos
NT: SCNT embryos reconstructed by using no CBHA-treated donor cells and treated with no CBHA during the early culture
CBHA-C: SCNT embryos reconstructed by using CBHA-treated donor cells
CBHA-Z: SCNT embryos treated with CBHA during the early culture.

birth of "Dolly" the sheep. However, the efficiency of SCNT is still low (Vajta *et al.*, 2007) due to the aberrant and incomplete reprogramming of the transferred nuclear genome (Campbell *et al.*, 2007; Gurdon and Melton, 2008). The nuclear reprogramming events following SCNT occur mostly at an epigenetic level and one of the epigenetic pathways related to chromatin structure is the global level of acetylation of the nuclear histones (Yang *et al.*, 2007). In this sense, for facilitating nuclear reprogramming of SCNT, many studies have focused on histone modifications such as methylastion, acetylation, ubiquitination and phosphorylation. In particular, histone acetylation and deacetylation during the chromatin remodeling are the important epigenetic modification on the chromatin structure and a nuclear reprogramming process (Kishigami *et al.*, 2006). The acetylation of histone occurs on the lysine residues of histone dependent on the introduction of an acetyl group (Kouzarides, 2007; Surani *et al.*, 2007; Wang *et al.*, 2007). Increasing histone acetylation levels induce the relaxation of chromatin structure and help to access transcriptional factors to DNA or histone (Hebbes *et al.*, 1988; Hong *et al.*, 1993; Lee *et al.*, 1993; Zlatanova *et al.*, 2000). On the contrary, the deacetylation of histone is induced by histone methylation and established the repressed chromatin structures (Eberharter and Becker, 2002).

In this sense, SCNT embryos have been treated with histone deacetylase inhibitors (HDACis) to increase the histone acetylation level and to improve the epigenetic remodeling ability of somatic cells. In the resent report, the treatment of m-carboxycinnamic acid bishydroxamide (CBHA), one of HDACi family, was improved the efficiency of murine SCNT (Dai *et al.*, 2010). However, it is not still reported whether CBHA treatment can improve the development of porcine SCNT embryos. Therefore, we investigated the ability of CBHA to improve nucleus reprogramming of the transferred donor nuclei and *in vitro* developmental competence of porcine SCNT embryos to the blastocyst stage. We treated nuclear donor cells or cloned zygotes with CBHA to evaluate their effect on pre-implantation development of porcine SCNT embryos. Our results showed that porcine SCNT embryo treated with 100 μM of CBHA (10 h) immediately after spontaneous cell fusion and oocyte activation have significantly increased the global acetylation level (acetylation on histone H3 at lysine 9 (H3K9ac)) of the transferred donor nuclei and improved the *in vitro* development and the quality of porcine SCNT embryos as compared to cloned embryos reconstructed by using the nuclear donor cells treated with 15 μM of CBHA for 8 h.

In the previous studies, pretreatment with DNA demethylation agent such as 5-aza-2′-deoxycytidine re-

sulted in reduction of the general methylation levels in transferred donor cell but did not affect the developmental competence of SCNT embryos to the blastocyst stage (Enright *et al*., 2005). Trichostatin A (TSA) as a widely known HDACi improves the levels of acetylated histones (Yoshida *et al*., 1990). In this experiment, the treatment of CBHA (CBHA-C; 15 μM, 8 h) to the donor cell prepared from porcine fetal fibroblasts before introduction of donor cells increased the level of histone acetylation. Interestingly, little or no improvement of the development and formation to blastocysts was observed.

Our results also showed that porcine SCNT embryos (CBHA-Z) treated with 100 μM of CBHA for the early culture (10 h) immediately after oocyte activation were improved the *in vitro* developmental competence to the blastocyst stage. It was previously reported that CBHA treatment at the zygote stage improves the cloning efficiency of the *in vivo* and *in vitro* development to blastocyst stage and potential for full-term (Dai *et al*., 2010). In addition, the treatment of TSA to porcine SCNT embryo after oocyte activation promoted the blastocyst rates *in vitro* (Zhao *et al*., 2010).

We hypothesized that CBHA treatment for the early culture (10 h) soon after spontaneous cell fusion and oocyte activation can lead to structural changes in the chromatin for binding the transcription factors. As regards histone modifications, the level of acetylation on histone H3 at lysine 9 (H3K9ac) in the porcine cloned embryos at the zygote stage was induced significantly higher CBHA-Z group than that in CBHA-C group. The intensity of H3K9ac in the CBHA-Z group showed similar to that of porcine IVF embryos. According to the results of Zhao *et al.* (2010), Scriptaid, one of the histone deacetylase inhibitors (HDACi) also showed very similar results in porcine cloned embryos. The reasons why HDACi induces greater development of SCNT embryos may lead to increase histone acetylation and decrease DNA methylation, which are important for development (Cervoni and Szyf, 2001). The evaluation for blastocyst quality is related to the total cell number as well as the rate of survived cells in blastocysts. So to speak, increasing the apoptotic rate in the blastocysts means the poor quality of blastocysts (Yu *et al*., 2007). In this study, the total cell number of blastocyst derived from CBHA-Z group was significantly increased as compared to that of the NT group and CBHA-C group, with quite similar to that of murine SCNT embryos (Ono *et al*., 2010).

We have established that treatment of CBHA as the histone deacetylase inhibitor (HDACi) to increase the level of histone acetylation (H3K9ac) in the transferred somatic nuclei of porcine SCNT embryo at the zygote stage and the *in vitro* development to the blastocyst stage in CBHA-Z group. In addition, we also confirmed that the blastocysts derived from CBHA- Z group possesses greater cell numbers and good quality in terms of survival cell number.

REFERENCES

1. Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM, Godke RA, Gavin WG, Overström EW, Echelard Y (1999): Production of goats by somatic cell nuclear transfer. Nature Biotech 17:456-461.
2. Betthauser J, Forsberg E, Augenstein M, Childs L, Eilertsen K, Enos J, Forsythe T, Golueke P, Jurgella G, Koppang R, Lesmeister T, Mallon K, Mell G, Misica P, Pace M, Pfister-Genskow M, Strelchenko N, Voelker G, Watt S, Thompson S, Bishop M (2000): Production of cloned piglets from *in vitro* systems. Nature Biotech 18:1055-1059.
3. Bhaumik SR, Smith E, Shilatifard A (2007): Covalent modifications of histones during development and disease pathogenesis. Nat Struct Mol Biol 14: 1008-1016.
4. Campbell KH, Fisher P, Chen WC, Choi I, Kelly RD, Lee JH, Xhu J (2007): Somatic cell nuclear transfer: Past, present and future perspectives. Theriogenology 68:214-231.
5. Campbell KH, McWhir J, Ritchie WA, Wilmut I (1996): Sheep cloned by nuclear transfer from a cultured cell line. Nature 380:64-66.
6. Campbell KHS, Alberio R, Choi I, Fisher P, Kelly RDW, Lee JH, Maalouf W (2005): Cloning: eight years after dolly. Reprod Dom Anim 40:256-268.
7. Cervoni N, Szyf M (2001): Demethylase activity is directed by histone acetylation. J Biol Chem 276: 40778-40787.
8. Chesne P, Adenot, PG, Viglietta C, Baratte M, Boulanger L, Renard JP (2002): Cloned rabbits produced by nuclear transfer from adult somatic cells. Nature Biotech 20:366-369.
9. Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de León, FA, Robl JM (1998): Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science 280:1256-1258.
10. Csordas A (1990): On the biological role of histone acetylation. Biochem J 265:23-38.
11. Costa-Borges N, Santalo J, Ibanez E (2010): Comparison between the effects of valproic acid and trichostatin a on the *in vitro* development, blastocyst quality, and full-term development of mouse

somatic cell nuclear transfer embryos. Cell Reprogram 12:437-446.
12. Costa-Borges N, Gonzalez S, Santaló J, Ibáñez E (2011): Effect of the enucleation procedure on the reprogramming potential and developmental capacity of mouse cloned embryos treated with valproic acid. Reproduction 141:789-800.
13. Cui XS, Xu YN, Shen XH, Zhang LQ, Zhang JB, Kim NH (2011): Trichostatin a modulates apoptotic-related gene expression and improves embryo viability in cloned bovine embryos. Cell Reprogram 13:179-89.
14. Dai X, Hao J, Hou XJ, Hai T, Fan Y, Yu Y, Jouneau A, Wang L, Zhou Q (2010): Somatic nucleus reprogramming is significantly improved by m-carboxycinnamic acid bishydroxamide, a histone deacetylase inhibitor. J Biol Chem 285: 31002-10.
15. Das ZC, Gupta MK, Uhm SJ, Lee HT (2010): Increasing histone acetylation of cloned embryos, but not donor cells, by sodium butyrate improves their *in vitro* development in pigs. Cell Reprogram 12:95-104.
16. Ding X, Wang Y, Zhang D, Wang Y, Guo Z, Zhang Y (2008): Increased preimplantation development of cloned bovine embryos treated with 5-aza-2 9-deoxycytidine and trichostatin A. Theriogenology 70:622-630.
17. Eberharter A, Becker PB (2002): Histone acetylatio: a switch between repressible and permissive chromatin. Second in review series on chromatin dynamics. EMBO Reports 3:224-229.
18. Enright BP, Kubota C, Yang X, Tian XC (2003): Epigenetic characteristics and development of embryos cloned from donor cells treated by trichostatin A or 5-aza-2'-deoxycytidine. Biol Reprod 69: 896-901.
19. Enright BP, Sung LY, Chang CC, Yang X, Tian XC (2005): Methylation and acetylation characteristics of cloned bovine embryos from donor cells treated with 5-aza-2'-deoxycytidine. Biol Reprod 72: 944-948.
20. Galli C, Lagutina I, Crotti G, Colleoni S, Turini P, Ponderato N, Duchi R, Lazzari G (2003): Pregnancy: a cloned horse born to its dam twin. Nature 424:635.
21. Gurdon JB, Melton DA (2008): Nuclear reprogramming in cells. Science 322:1811-1815.
22. Hebbes TR, Thorne AW, Crane-Robinson C (1988): A direct link between core histone acetylation and transcriptionally active chromatin. EMBO J 7:1395-1402.
23. Hong L, Schroth GP, Matthews HR, Yau P, Bradbury EM (1993): Studies of the DNA binding properties of histone H4 amino terminus. Thermal denaturation studies reveal that acetylation markedly reduces the binding constant of the H4 "tail" to DNA. J Biol Chem 268:305-314.
24. Iager AE, Ragina NP, Ross PJ, Beyhan Z, Cunniff K, Rodriguez RM, Cibelli JB (2008): Trichostatin a improves histone acetylation in bovine somatic cell nuclear transfer early embryos. Cloning Stem Cells 10:371-379.
25. Jenuwein T, Allis CD (2001): Translating the histone code. Science 293:1074-80.
26. Jouneau A, Renard JP (2003): Reprogramming in nuclear transfer. Curr Opin Genet Develop 13:486-491.
27. Kikuchi H, Fujimoto D (1973): Multiplicity of histone deacetylase from calf thymus. FEBS Letters 29:280-282.
28. Kishigami S, Mizutani E, Ohta H, Hikichi T, Thuan NV, Wakayama S, Bui HT, Wakayama T (2006): Significant improvement of mouse cloning technique by treatment with trichostatin A after somatic nuclear transfer. Biochem Biophys Res Commun 340:183-189.
29. Kouzarides T (2007): Chromatin modifications and their function. Cell 128:693-705.
30. Lee BC, Kim MK, Jang G, Oh HJ, Yuda F, Kim HJ, Hossein MS, Kim JJ, Kang SK, Schatten G, Hwang WS (2005): Dogs cloned from adult somatic cells. Nature 436:641.
31. Lee DY, Hayes JJ, Pruss D, Wolffe AP (1993): A positive role for histone acetylation in transcription factor access to nucleosomal DNA. Cell 72: 73-84.
32. Li Z, Sun X, Chen J, Liu X, Wisely SM, Zhou Q, Renard JP, Leno GH, Engelhardt JF (2006): Cloned ferrets produced by somatic cell nuclear transfer. Develop Biol 293:439-448.
33. Maalouf WE, Liu Z, Brochard V, Renard JP, Debey P, Beaujean N, Zink D (2009): Trichostatin A treatment of cloned mouse embryos improves constitutive heterochromatin remodeling as well as developmental potential to term. BMC Develop Biol 9:11.
34. Meng Q, Polgar Z, Liu J, Dinnyes A (2009): Live birth of somatic cell-cloned rabbits fellowing trichstatin A treatment and cotransfer of parthenogenetic embryos. Cloning Stem Cells 11:203-208.
35. Misteli T (2007): Beyond the sequence: cellular organization of genome function. Cell 128:787-800.
36. Miyoshi K, Mori H, Mizobe Y, Akasaka E, Ozawa A, Yoshida M, Sato M (2010): Valproic acid enhances *in vitro* development and Oct-3/4 expression of miniature pig somatic cell nuclear transfer embryos. Cell Reprogram 12:67-74.
37. Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda

K, Awata T, Hanada H, Perry AC (2000): Pig cloning by microinjection of fetal fibroblast nuclei. Science 289:1188-1190.
38. Ono T, Li C, Mizutani E, Terashita Y, Yamagata K, Wakayama T (2010): Inhibition of class IIb histone deacetylase significantly improves cloning efficiency in mice. Biol Reprod 83:929-937.
39. Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, Colman A, Campbell KH (2000): Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 7:86-90.
40. Reik W, Dean W, Walter J (2001): Epigenetic reprogramming in mammalian development (Review). Science 293:1089-1093.
41. Shi LH, Miao YL, Ouyang YC, Huang JC, Lei ZL, Yang JW, Han ZM, Song XF, Sun QY, Chen DY (2008): Trichstatin A (TSA) improves the development of rabbit intraspecies cloned embryos, but not rabbit-human interspecies cloned embryos. Develop Dynam: An Official Publication of the American Association of Anatomists 237:640-648.
42. Shin T, Kraemer D, Pryor J, Liu L, Rugila J, Howe L, Buck S, Murphy K, Lyons L, Westhusin M (2002): A cat cloned by nuclear transplantation. Nature 415:859.
43. Shi, Yang (2007): Taking epigenetics center stage. Cell 128:639-640.
44. Shi W, Hoeflich A, Flaswinkel H, Stojkovic M, Wolf E, Zakhartchenko V (2003): Induction of a senescent-like phenotype does not confer the ability of bovine immortal cells to support the development of nuclear transfer embryos. Biol Reprod 69: 301-309.
45. Su J, Wang Y, Li Y, Li R, Li Q, Wu Y, Quan F, Liu J, Guo Z, Zhang Y (2011): Oxamflatin significantly improves nuclear reprogramming, blastocyst quality, and *in vitro* development of bovine SCNT embryos. PLoS One 6:23805.
46. Surani MA, Hayashi K, Hajkova P (2007): Genetic and epigenetic regulators of pluripotency. Cell 128: 747-762.
47. Svensson K, Mattsson R, James TC, Wentzel P, Pilartz M, MacLaughlin J, Miller SJ, Olsson T, Eriksson UJ, Ohlsson R (1998): The paternal allele of the H19 gene is progressively silenced during early mouse development: the acetylation status of histones may be involved in the generation of variegated expression patterns. Development 125: 61-69.
48. Vajta G, Zhang Y, Macha´ty Z (2007) Somatic cell nuclear transfer in pigs: recent achievements and future possibilities. Reprod Fertil Develop 19:403-423.
49. Van Thuan N, Bui HT, Kim JH, Hikichi T, Wakayama S, Kishigami S, Mizutani E, Wakayama T (2009): The histone deacetylase inhibitor scriptaid enhances nascent mRNA production and rescues full-term development in cloned inbred mice. Reproduction 138: 309-317.
50. Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R (1998): Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394: 369-374.
51. Wang F, Kou Z, Zhang Y, Gao S (2007): Dynamic reprogramming of histone acetylation and methylation in the first cell cycle of cloned mouse embryos. Biol Reprod 77:1007-1016.
52. Wrenzycki C, Wells D, Herrmann D, Miller A, Oliver J, Tervit R, Niemann H (2001): Nuclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. Biol Reprod 65:309-317.
53. Wu X, Li Y, Li GP, Yang D, Yue Y, Wang L, Li K, Xin P, Bou S, Yu H (2008): Trichostatin A improved epigenetic modifications of transfected cells but did not improve subsequent cloned embryo development. Anim Biotech 19:211-224.
54. Yang X, Smith SL, Tian XC, Lewin HA, Renard JP, Wakayama T (2007): Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. Nature Genet 39:295-302.
55. Yoshida M, Kijima M, Akita M, Beppu T (1990): Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. J Biol Chem 265:17174-17179.
56. Yu Y, Ding C, Wang E, Chen X, Li X, Zhao C, Fan Y, Wang L, Beaujean N, Zhou Q, Jouneau A, Ji W (2007): Piezo-assisted nuclear transfer affects cloning efficiency and may cause apoptosis. Reproduction 133:947-954.
57. Zhang Y, Li J, Villemoes K, Pedersen AM, Purup S, Vajta G (2007): An epigenetic modifier results in improved *in vitro* blastocyst production after somatic cell nuclear transfer. Cloning Stem Cells 9:357-363.
58. Zhao J, Ross JW, Hao Y, Spate LD, Walters EM, Samuel MS, Rieke A, Murphy CN, Prather RS (2009): Significant improvement in cloning efficiency of an inbred miniature pig by histone deacetylase inhibitor treatment after somatic cell nuclear transfer. Biol Reprod 81:525-530.
59. Zhou Q, Renard JP, Le Friec G, Brochard V, Beaujean N, Cherifi Y, Fraichard A, Cozzi J (2003): Generation of fertile cloned rats by regulating oocyte activation. Science 302:1179.
60. Zlatanova J, Caiafa P, Van Holde K (2000): Linker histone binding and displacement: versatile mecha-

nism for transcriptional regulation. FASEB J 14: 1697-1704.
61. Zuccotti M, Garagna S, Redi CA (2000): Nuclear transfer, genome reprogramming and novel opportunities in cell therapy. J Endocrin Invest 23:623-629.

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