Histone deacetylase inhibitor M344 significantly improves nuclear reprogramming, blastocyst quality, and in vitro developmental capacity of cloned pig embryos[1]

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ABSTRACT: M344 is a novel histone deacetylase inhibitor. There is no report on the effect of M344 treatment on the development of pig embryos after somatic cell nuclear transfer (SCNT). In the present study, we investigated the effect of M344 on the blastocyst formation rate in cloned embryos, acetylation level of histone H4 lysine 12 (AcH4K12), and the expression of pluripotency-related genes *Oct4*, *NANOG*, and *SOX2*. Our results indicated that treatment with 5 μ*M* M344 for 6 h improved the development of porcine embryos, in comparison with the untreated group (25.1% ± 5.0 vs. 10.9% ± 2.4; $P < 0.05$). Moreover, M344-treated embryos had increased average fluorescence intensity of AcH4K12 at the pseudo-pronuclear stage ($P < 0.05$). However, no differences exist in Oct4, NANOG, and SOX2 expression in M344-treated and untreated SCNT blastocysts. In evaluating the effect of M344 on in vivo development, 845 M344-treated embryos were transferred into 3 surrogates, 1 of whom became pregnant and developed 3 fetuses. These findings suggested that M344 elevated the level of histone acetylation, facilitated the nuclear programming, and subsequently improved the developmental competence of pig SCNT embryos.

Key words: histone acetylation, histone deacetylase inhibitors, M344, pig, somatic cell nuclear transfer

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INTRODUCTION

Somatic cell nuclear transfer (**SCNT**) has numerous roles to play in biomedical research and applications, such as animal production and biotechnology, derivation of patient-specific embryonic stem cells, xenotransplantation, and genetic conservation. However, the overall efficiency remains at 1 to 3% in any species (Estrada et al., 2007). The reasons for the unsatisfactorily low efficiency of this technique are unknown; however, functional studies have shown it is partly due to aberrant genomic methylation, histone modification, and gene expression in SCNT embryos (Kang et al., 2001; Wrenzycki et al., 2001; Xu et al., 2012). Hence, repair of abnormal epigenetic dysregulation may improve the success of animal cloning.

Recently, various histone deacetylase inhibitors (**HDACi**) have been used to try to improve the in vitro development of cloned embryos, such as suberoylanilide hydroxamic acid (Ono et al., 2010), oxamfaltin (Su et al., 2011), trichostatin A (**TSA**; Jeong et al., 2013), valproic acid (Kang et al., 2013; Sangalli et al., 2014), scriptaid (Whitworth et al., 2011; Liang et al., 2015), MGCD0103 (Jin et al., 2017), and PCI-24781 (Jin et al., 2016). M344, a novel HDACi with robust activity and relatively low toxicity compared with TSA, has been reported to inhibit the growth and induce apoptosis in a variety of cancer cells (Takai et al., 2006; Furchert et al., 2007). However, none of the past studies elucidated the effects of M344 on the developmental competence of SCNT embryos. Therefore, it was of interest to examine the effect of M344 treatment on the in vitro and in vivo

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development of porcine SCNT embryos, acetylation status of histone H4 lysine 12 (**H4K12**), apoptosis, and gene expression in M344-treated and untreated blastocysts.

MATERIALS AND METHODS

Unless otherwise specified, all chemicals and reagents used in this study were purchased from Sigma Chemical Company (St. Louis, MO). M344 was purchased from Selleck Chemicals (Houston, TX). This study was carried out in strict accordance with the guidelines for the care and use of animals of Yanbian University. All animal experimental procedures were approved by the Committee on the Ethics of Animal Experiments at Yanbian University.

Oocyte Retrieval and In Vitro Maturation

Porcine ovaries were collected at a local slaughterhouse and transported to the laboratory within 4 h at 31 to 35°C. Cumulus–oocyte complexes were aspirated from antral follicles (3–6 mm in diameter) using an 18-gauge needle. Cumulus–oocyte complexes were pooled and washed 3 times with HEPES-buffered NCSU-37 (North Carolina State University) medium containing 0.1% (wt/vol) polyvinyl alcohol (**PVA**). Cumulus–oocyte complexes with a compact cumulus cell mass were selected and cultured for 20 to 23 h at 38.5°C under 5% CO_2 in 95% humidified air in 4-well plates (Nunc A/S Plastfabrikation, Roskilde, Denmark). The maturation medium (NCSU-37) was supplemented with 10% pig follicular fluid (vol/vol), 0.6 m*M* cysteine, 1 m*M* dibutyryl cyclic adenosine monophosphate, and 0.1 IU/mL human menopausal gonadotropin (Teikokuzoki, Tokyo, Japan). Then, Cumulus–oocyte complexes were cultured without dibutyryl cyclic adenosine monophosphate and human menopausal gonadotropin for another 18 to 24 h.

Donor Cell Preparation

Porcine fetal fibroblast cells were isolated from a hybrid pig at Day 30 of gestation. After removal of the brain, intestines, and 4 limbs, the remaining tissues were cut into pieces and cultured at 38°C in an atmosphere of 5% CO_2 and 95% air in Dulbecco's modified Eagle medium (culture medium; Gibco, BRL, Grand Island, NY) containing 10% fetal bovine serum (culture medium; vol/vol; Gibco, BRL, Grand Island), 1 m*M* sodium pyruvate, and 100 IU/mL each of penicillin and streptomycin. When fibroblasts were at 90% confluence, they were trypsinized, rinsed, and subcultured into two 25-cm^2 cell culture flasks (Corning Inc., Kennebunk, MA) for further passaging. Nuclear donor cells for SCNT were derived from passages 4 to 8 and cultured in serum-starved medium [0.5% fetal bovine serum (vol/vol)] for 3 to 4 d.

Somatic Cell Nuclear Transfer

Nuclear transfer (**NT**) was performed as previously described by Yin et al. (2002). Matured oocytes with the first polar body were cultured in medium supplemented with 0.4 μg/mL demecolcine and 0.05 mol/L sucrose for 1 h. Treated oocytes were transferred to medium supplemented with 5 μg/mL cytochalasin B and 0.4 μg/mL demecolcine, and the protrusion was removed with a 15-μm inner diameter glass pipette. A single donor cell was placed into the perivitelline space of an enucleated oocyte and electrically fused using 2 direct pulses of 150 V/mm for 50 μs in 0.28 mol/L mannitol supplemented with 0.1 m*M* $MgSO_4$ and 0.01% (vol/vol) PVA. Fused couplets were cultured for 1 h in medium containing 0.4 μg/mL demecolcine before electro-activation. The reconstructed oocytes were activated by 2 direct pulses of 100 V/mm for 20 μs in 0.28 mol/L mannitol supplemented with 0.1 m*M* $MgSO_4$ and 0.05 m*M* $CaCl_2$. Activated eggs were cultured with 2 mmol/L 6-dimethylaminopurine in NCSU-37 medium for 4 h. Somatic cell NT embryos were cultured in NCSU-37 medium under paraffin oil on a plastic petri dish for 7 d at 38.5°C under 5% CO2 in 95% humidified air. Cleavage and blastocyst formation were evaluated at 48 and 148 h, respectively. To count the total cell numbers of blastocysts, they were collected on d 7, washed 3 times in 1% PVA-supplemented PBS (**PBS-PVA**), fixed with 4% paraformaldehyde in PBS (**PBS-PFA**) for 20 min, and placed on slides with a drop of mounting medium consisting of glycerol and PBS (9:1) containing 20 μg/mL Hoechst 33342. A cover slip was placed on top of the blastocysts. The number of nuclei was counted under an epifluorescent microscope (Nikon, Tokyo, Japan) equipped with a digital camera.

Immunodetection of Histone Acetylation

M344-treated and nontreated embryos were collected at the pseudo-pronuclear stage. Somatic cell NT embryos were washed 3 times in PBS-PVA and fixed with PBS-PFA for 30 min. These embryos were permeabilized using PBS containing 1% Triton X-100 for 30 min and then blocked in PBS containing 2% BSA for 1 h. Next, the embryos were incubated with primary anti-acetyl H4K12 antibodies (1:200; Upstate Biotechnology, Inc., Lake Placid, NY) at 4°C overnight. Goat anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was then applied for 1 h at room temperature. Cloned embryos were washed 3 times in PBS-PVA, and DNA was counterstained with 15 μg/

Table 1. List of primers used in this study[1]

Genes	Primer sequences (5′-3′)	Amplicon size, bp	Accession number
GAPDH	F: GTCGGTTGTGGATCTGACCT	207	NM_001206359.1
	R: TTGACGAAGTGGTCGTTGAG		
Oct4	F: AAGCAGTGACTATTCGCAAC	136	NM_001113060.1
	R: CAGGGTGGTGAAGTGAGG		
NANOG	F: TTCCTTCCTCCATGGATCTG	214	NM_001129971.1
	R:ATCTGCTGGAGGCTGAGGTA		
SOX2	F: CGCAGACCTACATGAACG	103	NM_001123197.1
	R: TCGGACTTGACCACTGAG		

[1]F = forward; R = reverse; GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

mL propidium iodide for 10 min. After a final wash in PBS-PVA, pig embryos were mounted on glass slides in a drop of 100% glycerol, gently compressed with a cover slip, and scanned by an epifluorescent microscope (Nikon) and images were captured and quantified using Nikon Instruments Software Basic Research.

Apoptosis Assays

Somatic cell NT blastocysts were washed 3 times in PBS-PVA and fixed with PBS-PFA for 30 min at room temperature. Then, porcine embryos were washed 3 times in PBS-PVA and permeabilized by incubation in 1% Triton X-100 in PBS for 1 h. Nuclear transfer embryos were washed once in PBS-PVA and incubated with a fluorescein-conjugated terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate nick end labeling (**TUNEL**) assay kit (In Situ Cell Death Detection Kit; Roche Diagnostics Deutschland GmbH, Mannheim, Germany) in the dark for 1 h at 38°C. Cloned blastocysts were stained with 25 μg/mL Hoechst 33342 to visualize the total cell number. Samples were mounted on glass slides with a drop of antifade mounting medium and analyzed using the epifluorescent microscope. At least 20 embryos were analyzed in 1 sample at once, and this experiment was repeated 6 times.

Reverse Transcription PCR

Expression of pluripotency-related *Oct4*, *NANOG*, and *SOX2* mRNA were investigated, together with *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) as an endogenous reference gene. Total RNA was extracted from at least 30 blastocysts in 1 sample at once using the Dynabeads mRNA DIRECT Kit (Life Technologies AS, Oslo, Norway) and the experiment was replicated 5 times. The RNA concentration was determined using a UV-Vis spectrophotometer (UV-2450; Shimadzu Corp., Tokyo, Japan). Complementary DNA was immediately synthesized from extracted RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The primers used for reverse transcription PCR analysis are shown in Table 1. The expected PCR product sizes of Oct4, NANOG, and SOX2 were 136, 214, and 103 bp, respectively. For PCR, platinum Taq DNA polymerase (Invitrogen) was added to the cDNA mixture (each cDNA sample was mixed with PCR mix containing 1x PCR buffer, 0.1 m*M* deoxyribonucleotide triphosphate mixture, 1.5 m*M* $MgCl_2$, and 0.25 μ*M* of each primer) and denatured. The mixture was subjected to PCR in a thermal cycler (T100 Thermal Cycle; Bio-Rad Laboratories, Inc., Hercules, CA). The PCR cycle was as follows: 95°C for 5 min followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s and a final extension of 72°C for 7 min. Polymerase chain reaction products were electrophoresed in 2% agarose gels. A Ready-Load 1000 bp DNA ladder (Invitrogen) was used as a molecular weight marker. Stained gels were imaged with a digital fluorescence recorder (GelDoc-It TS Imaging System; UVP, Upland, CA). The mRNA expression levels were determined by measuring the intensity of each band using ImageJ software (National Institutes of Health; https://imagej.nih.gov/ij/; Accessed 10 February 2016).

Embryo Transfer

For embryo transfer (**ET**), approximately 271 SCNT embryos per recipient were kept in manipulation medium, transported in a portable incubator, and loaded into a sterilized straw before transfer. Cloned embryos at the 2- to 4-cell stage were transferred into the oviducts of naturally cycling gilts on the first day of standing estrus. The recipients were sacrificed and fetuses were obtained on the 23rd day after transfer.

Statistical Analysis

Each experiment was repeated more than 3 times. The data are expressed as the mean values ± SEM. The data were analyzed using univariate ANOVA followed by Duncan's multiple range test using SPSS 16.0 (SPSS, Inc., Chicago, IL) statistical software. The average fluorescence intensity emitted by each individ-

Table 2. In vitro developmental competence of pig somatic cell nuclear transfer embryos with different concentrations of M344 for 24 h

Concentration, μ*M*	No. of embryos developed to [mean (SEM), %]			Mean total cell no. (SEM) in blastocysts
	No. of embryos cultured[1]	2 to 4 cell	Blastocyst	
0	245	215 [87.6 (1.7)]	24 [10.4 (3.1)][b]	39.2 (3.5)
5	237	208 [87.7 (0.8)]	40 [17.8 (2.9)][a]	39.8 (9.6)
50	210	183 [87.8 (2.4)]	23 [11.5 (1.9)][a,b]	41.3 (6.5)
500	206	176 [85.4 (2.6)]	0 (0)[c]	–

[a–c]Values within a column with different superscripts significantly differ ($P < 0.05$).

[1]Experiment was replicated more than 3 times.

ual nucleus was quantified using Nikon Instruments Software Basic Research. *P*-value < 0.05 was considered statistically significant.

RESULTS

In vitro developmental competence of pig NT embryos at various concentrations and durations of M344 treatment, acetylation level of H4K12 (**AcH4K12**) in pseudo-pronuclear stage, apoptosis in blastocysts, gene expression of pluripotency, and in vivo developmental capacity of M344-treated embryos were investigated in this study.

Effect of Various M344 Treatment Concentrations on Embryonic Development after Nuclear Transfer

The effect of different concentrations (0, 5, 50, and 500 μ*M*) of M344 on the development of porcine SCNT embryos was evaluated. As shown in Table 2, the rate of blastocyst formation was higher in the 5-μ*M* M344–treated group (Fig. 1A) than in the control group (17.8% ± 2.9 vs. 10.4% ± 3.1, respectively; $P < 0.05$). However, M344 treatment had no effect on cleavage rate (87.7% ± 0.8 vs. 87.6% ± 1.7) and total cell numbers per blastocyst (39.8 ± 9.6 vs. 39.2 ± 3.5).

Effect of Different M344 Treatment Durations on Development of Nuclear Transfer Embryos

Pig embryos were treated with 5 μ*M* M344 for 0, 6, 24, or 48 h after activation. The proportion of SCNT embryos that developed to the blastocyst stage was higher in the group treated with M344 for 6 h than in the control group (25.1% ± 5.0 vs. 10.9% ± 2.4, respectively; $P < 0.05$; Table 3). However, M344 treatment did not affect cleavage rate (87.8% ± 3.6 vs. 84.5% ± 2.6) and blastocyst quality, as determined by the mean number of cells per blastocyst (36.2 ± 5.9 vs. 37.7 ± 6.3; Fig. 1B).

Effect of M344 Treatment on Acetylation Level of Histone H4 Lysine 12 on Cloned Embryos

To clarify the mechanism for the improved developmental competence of SCNT embryos after M344 treatment, AcH4K12 in embryos at the pseudo-pronuclear stage was examined. The results (Fig. 2) showed that treatment of 5 μ*M* M344 for 6 h markedly increased the AcH4K12 in pig embryos when compared with the untreated embryos.

Effect of M344 Treatment on Apoptosis in Porcine Somatic Cell Nuclear Transfer Blastocysts

To determine whether the improvement in NT embryo development was reflected in blastocyst quality, the number of apoptotic cells was estimated by TUNEL assay. The percentage of apoptotic cells in blastocysts was remarkably lower in M344 treatment group than in control group (Fig. 3).

Gene Expression in Pig Nuclear Transfer-Derived Blastocysts

The expression of pluripotency-related *Oct4*, *NANOG*, and *SOX2* mRNA was investigated in cloned blastocysts. The results (Fig. 4) suggested that there were no differences in *Oct4*, *NANOG*, and *SOX2*

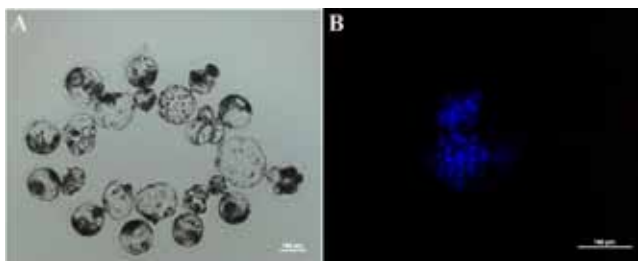

Figure 1. Effect of treatment with M344 on the development of pig somatic cell nuclear transfer (SCNT) embryos. (A) Day 7 porcine blastocysts derived from the M344-treated group. Original magnification, ×100. (B) An image of a Day 7 pig SCNT embryo stained with Hoechst 33342. Original magnification, ×200. Scale bar indicates 100 μm.

Table 3. In vitro development of porcine somatic cell nuclear transfer embryos with 5 μM M344 for different durations

Duration, h	No. of embryos developed to [mean (SEM), %]			Mean total cell no. (SEM) in blastocysts
	No. of embryos cultured[1]	2 to 4 cell	Blastocyst	
0	217	183 [84.5 (2.6)]	24 [10.9 (2.4)][b]	37.7 (6.3)
6	218	191 [87.8 (3.6)]	55 [25.1 (5.0)][a]	36.2 (5.9)
24	216	185 [85.8 (3.1)]	48 [22.2 (6.1)][a]	40.3 (7.2)
48	223	180 [81.0 (7.2)]	22 [9.8 (3.4)][b]	39.5 (6.2)

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

[1]Experiment was replicated more than 3 times.

mRNA expression between the M344-treated group and the untreated group.

Effect of M344 Treatment on In Vivo Developmental Competence of Embryos

To investigate the effect of M344 treatment on in vivo developmental capacity, cloned embryos were transferred to surrogates. Eight hundred fifteen M344-treated NT embryos were transferred into 3 surrogate sows (Table 4), 1 of whom became pregnant and 3 fetuses were obtained (Fig. 5).

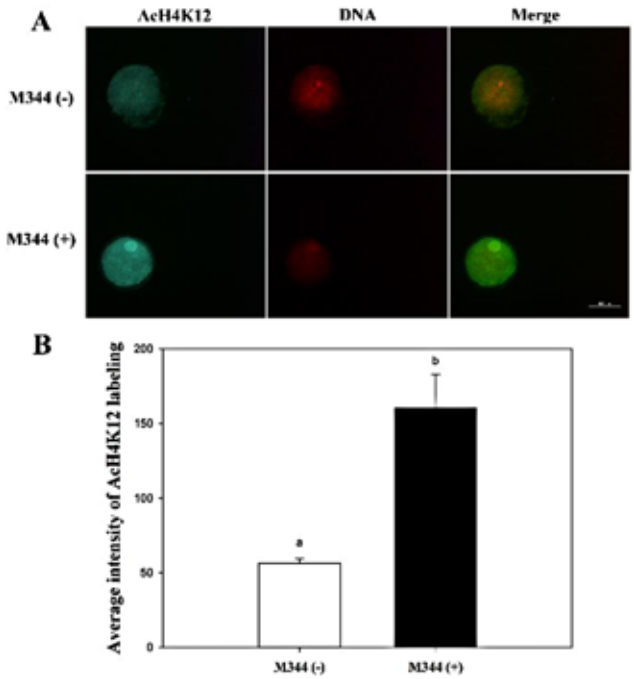

Figure 2. Evaluation of acetylation level of histone H4 lysine 12 (AcH4K12) in pseudo-pronuclear stage embryos. (A) M344-treated [M344 (+)] or nontreated [M344 (−)] pig embryos were labeled for AcH4K12 (green) and DNA (red). Merged images of AcH4K12 and DNA staining are with yellow showing overlapping patterns. Original magnification, ×200. Scale bar indicates 100 μm. (B) Average fluorescent intensity for AcH4K12 in pseudo-pronuclear stage was measured using Nikon Instruments Software Basic Research (Nikon, Tokyo, Japan). [a,b]Bars with different superscripts in each column indicate statistically significant differences ($P < 0.05$). At least 20 embryos were analyzed in 1 sample at once, and this experiment was repeated 8 times.

DISCUSSION

Due to the possibilities of companion and sport animal cloning, and also due to the increasing number of cloned farm animals, the topic of SCNT never ceases to be of public interest (Burgstaller and Brem, 2016). However, the efficiency of producing normal offspring remains low, owing partly to the incomplete reprogramming of SCNT embryos, including histone hypoacetylation and DNA hypermethylation. Su et al. (2011) have reported that histone acetylation plays a significant role in reprogramming and affects the development of NT embryo. Various studies have implied that HDACi can elevate the global levels of histone acetylation, and the treatment of reconstructed

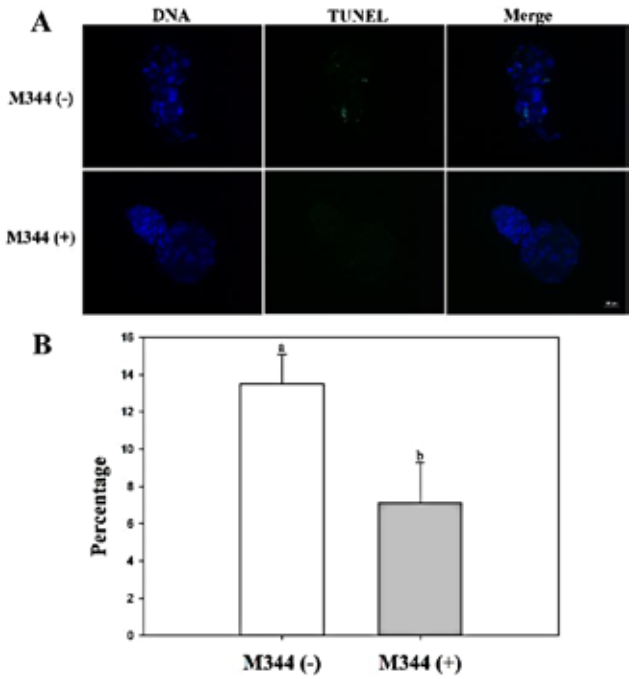

Figure 3. Representative apoptosis images in pig somatic cell nuclear transfer blastocysts. (A) Terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate nick end labeling (TUNEL) assay of blastocysts (green). Each sample was counterstained with Hoechst 33342 to visualize DNA (blue). Original magnification, ×200. Scale bar indicates 100 μm. (B) Percentage of apoptotic cells in cloned blastocysts. [a,b]Values with different superscripts in the same column were significantly different ($P < 0.05$). At least 5 blastocysts were analyzed in 1 sample at once, and this experiment was repeated 6 times.

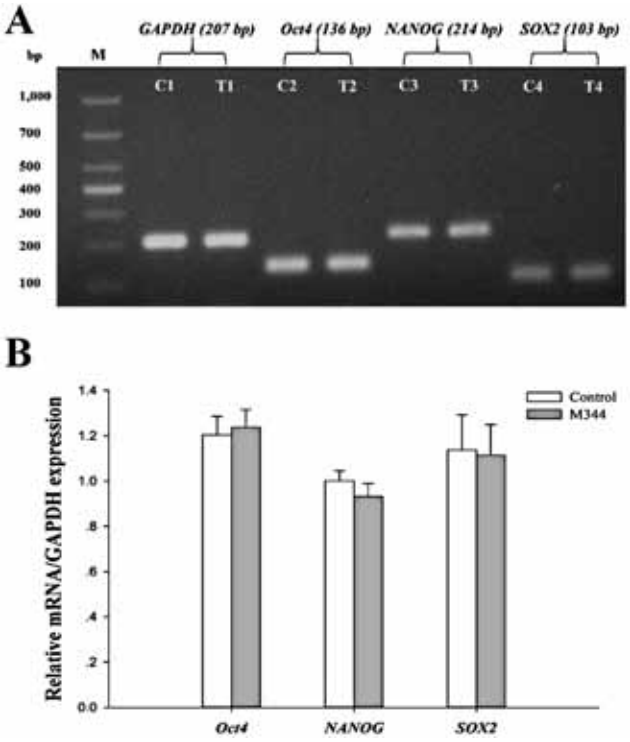

Figure 4. Reverse transcription PCR analysis. (A) *GAPDH*, *Oct4*, *NANOG*, and *SOX2* cDNA expression in control and M344 treatment groups. Lane M represents the 1,000-bp DNA marker; lanes C1, C2, and C3 represent the 0-μ*M* M344 treatment group; and lanes T1, T2, and T3 represent the 5-μ*M* M344 treatment group. (B) Comparison of mRNA expression levels (mean ± SEM) of *Oct4*, *NANOG*, and *SOX2* between untreated and M344-treated somatic cell nuclear transfer blastocysts. The experiment was replicated 5 times.

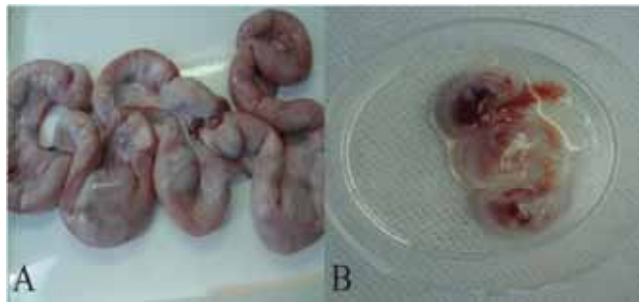

Figure 5. M344-treated pig somatic cell nuclear transfer embryos were transferred into a sow. The uterus of the sow (A) and the resulting fetuses (B) are shown at Day 23 of gestation.

embryos with HDACi has been reported to improve in vitro cloning efficiency (Jeong et al., 2013; Kang et al., 2013; Jin et al., 2016, 2017).

Histone acetylation induced by histone acetyltransferases is associated with gene transcription, whereas histone hypoacetylation induced by histone deacetylase (**HDAC**) activity is associated with gene silencing (Ropero and Esteller, 2007). M344 is a novel HDACi and an amide analog of TSA with substrate selectivity for HDAC6 (Heltweg et al., 2004). Yet the ability of M344 to improve cloning efficiency has not been reported. Therefore, we hypothesized that M344 treatment should increase the acetylation level of histones, leading to an "open chromatin" state. To address this hypothesis, we investigated the effect of M344 treatment on pre- and postimplantational development of porcine clones, AcH4K12, and pluripotent gene expressions in M344-treated embryos. Our results showed

Table 4. In vivo development of M344-treated porcine somatic cell nuclear transfer embryos

Recipient no.	No. of transferred embryos	Pregnancy status	No. of fetuses recovered (fetus collection day)
1	257	–	–
2	272	+	3
3	286	–	–

that albeit treatment with 5 μ*M* M344 for 6 h significantly improved in vitro development of pig NT embryos, the total number of cells in a blastocyst was similar within each group. Similar to a previous study, lower concentrations of M344 were more effective, with a dose of 10 μ*M* M344 producing the highest suppression of MCF-7 proliferation after 5 d (Yeung et al., 2012). This improvement of the developmental capacity may be due to HDACi-induced hyperacetylation, which plays many fundamental roles in cellular processes, one of them being crucial to cell proliferation (Verdone et al., 2006). In the current study, M344-treated SCNT embryos were transferred into 3 surrogate mothers and 3 fetuses were obtained. This finding was in line with previous studies (Jin et al., 2016, 2017).

Hyperacetylation of the N-terminal tails of histones H3 and H4 correlates with gene activation, whereas deacetylation mediates transcriptional repression (Strahl and Allis, 2000). Therefore, the mean fluorescent intensity of AcH4K12 in pig NT embryos at the pseudo-pronuclear stage was investigated. As shown in Fig. 2, the AcH4K12 signal was less than half in untreated embryos compared with the M344 treatment group. This finding is in line with our previous studies (Jin et al., 2016, 2017). It is not clear how HDACi can improve the animal cloning efficiency, but elevated histone acetylation by HDACi is important for nuclear programming.

Apoptosis is characterized by typical cell features such as membrane blebbing, chromatin condensation, and DNA fragmentation (Brill et al., 1999). Moreover, apoptosis occurs frequently during early embryonic development and has a marked impact on embryo development (Haouzi and Hamamah, 2009). Therefore, the apoptosis in pig M344-treated blastocysts and control blastocysts was explored. The TUNEL assay suggested that M344 treatment significantly inhibited the apoptosis in SCNT blastocysts and improved the quality of cloned embryos. The finding that HDAC treatment reduced cell death in the embryos further supports its effect on embryonic development as previously reported (Cui et al., 2011; Su et al., 2011; Liang et al., 2015).

During mammalian embryogenesis, early embryonic cells progressively differentiate from a pluripotent state into distinct cell lineages (Shi and Jin, 2010). Pluripotency describes a cell's ability to give rise to all the cells of an embryo and adult (Solter, 2006). Furthermore, pluripotency factors not only serve as markers of pluripotent cells but also are functionally important for pluripotency maintenance (Chen and Daley, 2008). *Oct4* is a member of the Pit-Oct-Unc transcription factor family; the expression of the gene encoding *Oct4* begins very early in the preimplantation embryo (Xing et al., 2009). *Oct4*-deficient mouse embryos develop into blastocysts lacking a pluripotent inner cell mass, which are unable to implant, suggesting an important role in pluripotent cell population in early embryos (Nichols et al., 1998). Aston et al. (2010) showed that lower expression of *Oct4* was found in bovine SCNT blastocysts than their in vitro–fertilized embryos. In this study, M344 treatment did not alter the expression of *Oct4*. However, previous studies demonstrated that valproic acid or scriptaid significantly enhanced in vitro development and *Oct4* expression of SCNT embryos (Miyoshi et al., 2010; Sangalli et al., 2014; Liang et al., 2015). *NANOG*, a homeodomain transcription factor, was the first protein identified as essential for both early embryo development and pluripotency maintenance in embryonic stem cells (Chambers et al., 2003; Mitsui et al., 2003). Forced expression of *NANOG* is sufficient to drive cytokine-independent self-renewal of undifferentiated embryonic stem cells (Chambers et al., 2003). Our results did not show a beneficial effect of M344 for the upregulated expression of *NANOG*. The data of Su et al. (2011) are consistent with our present results. *SOX2*, a member of the SoxB1 transcription factor family, is an important transcriptional regulator in pluripotent stem cells (Zhang and Cui, 2014). Experimental evidences suggest that *NANOG* functions to stabilize the pluripotent state (Chambers et al., 2007) whereas *SOX2* contributes to pluripotency primarily by regulating *Oct4* expression (Masui et al., 2007). As shown in Fig. 4, M344 supplementation did not alter the gene expression of *SOX2*. In contrast to other studies, TSA (Wang et al., 2011) or oxamflatin (Su et al., 2011) treatment increased the expression level of *SOX2*. Furthermore, the relative abundance of *SOX2* transcripts followed a pattern similar to *NANOG*, supporting previous findings (Magnani and Cabot, 2008). Taken together, M344 treatment did not significantly improve the quality of the pluripotent inner cell mass because there were no obvious differences in *Oct4*, *NANOG*, and *SOX2* mRNA expression.

In summary, this article has concentrated on the application of optimal conditions for M344 treatment on the in vitro and in vivo development of pig NT embryos. Based on our findings, treatment with 5 μ*M* M344 for 6 h produces positive effects on preimplantation development leading to improved SCNT blastocyst formation rates by elevating the histone acetylation, decreasing the death cells per blastocyst, and modulating gene expression related to development and pluripotency. In the future, the mechanism by which M344 improves cloning efficiency needs to be elucidate.

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