The Effects of 5-Aza-2'-Deoxycytidine and Trichostatin A on Gene Expression and DNA Methylation Status in Cloned Bovine Blastocysts

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Abstract

We previously found that treatment of both donor cells and early cloned embryos with combination of 5-aza-2'-deoxycytidine (5-aza-dC) and trichostatin A (TSA) significantly improve the *in vitro* and full-term development of nuclear transfer (NT) bovine embryos. To investigate how this treatment improved the epigenetic reprogramming of somatic cell nuclei, we compared the expression levels of DNA methylation-, chromatin structure-, and development-related genes in *in vitro* fertilized (IVF group), NT (C-NT group), and 5-aza-dC and TSA-treated NT (T-NT group) single blastocyst using quantitative real-time PCR. We also compared the DNA methylation status of *satellite 1* among three groups using bisulfite sequencing analysis and combined bisulfite restriction analysis (COBRA). There were significantly lower levels of *DNMT1*, *DNMT3b*, *HDAC2*, and *IGF2* transcripts in T-NT blastocysts than in C-NT blastocysts, whereas the relative abundance of *OCT4* and *SOX2* mRNA was significantly increased in T-NT blastocysts compared to C-NT blastocysts. In addition, the treatment also reduced the DNA methylation levels of NT blastocysts on *satellite 1* sequence. It is likely that TSA may act synergistically with 5-aza-dC to exert such modifications in gene expression and DNA methylation, subsequently enhancing developmental potential (*in vitro* and full-term) of treated cloned embryos.

Introduction

SOMATIC CELL NUCLEAR TRANSFER (SCNT) is a promising technology with potential applications in transgenic research, species preservation, livestock propagation, human xenotransplantation, disease models, and therapeutic cloning. But it is plagued by low efficiency; most cloned embryos die during pre- and postimplantation development *in vitro* and *in vivo*, and the few that develop to term present a high incidence of abnormalities, including obesity, short life span, prolonged gestation, dystocia, fetal edema, and hydramnios, respiratory problems, severe placental deficiency, oversized organs [i.e., large offspring syndrome (LOS)], and perinatal death (Farin et al., 2006; Yang et al., 2007b; Young et al., 1998). It is generally believed that the low efficiency of somatic cloning is mostly attributed to incorrect or incomplete nuclear reprogramming of the differentiated somatic cells, which results in faulty epigenetic modifications such as aberrant DNA methylation and histone modification.

To improve cloning efficiency, several epigenetic re-modeling drugs such as DNA methylation inhibitor 5-aza-2'-deoxycytidine (5-aza-dC) (Ding et al., 2008; Enright et al., 2005; Tsuji et al., 2009), histone deacetylase inhibitor (HDACi), trichostatin A (TSA) (Beebe et al., 2009; Costa-Borges et al., 2010; Ding et al., 2008; Himaki et al., 2010; Lager et al., 2008; Kishigami et al., 2006; Li et al., 2008a; Maalouf et al., 2009; Meng et al., 2009; Rybouchkin et al., 2006; Shi et al., 2008; Zhang et al., 2007), valproic acid (Costa-Borges et al., 2010; Miyoshi et al., 2010), scriptaid (Van Thuan et al., 2009; Zhao et al., 2009, 2010), and sodium butyrate (Das et al., 2010; Shi et al., 2003; Yang et al., 2007a) have recently been tested to improve the developmental competence of cloned embryos. For instance, two independent studies showed that TSA treatment improves the ability of mouse SCNT oocytes to develop into blastocyst and progress to full term, thereby significantly improving mouse cloning efficiency (Kishigami et al., 2006; Rybouchkin et al., 2006).

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Our previous study showed that treatment of both donor cells and early cloned embryos with a combination of 10 nM 5-aza-dC and 50 nM TSA significantly improved the ability to develop into blastocyst (Ding et al., 2008). Furthermore, our recent work showed that this treatment also significantly enhanced the development of NT bovine embryos *in vivo*, thereby dramatically increasing the cloning efficiency (number of surviving calves at 60 days of birth/number of recipient cows) from 2.6% to 13.4% (Wang et al., 2011).

Accumulated evidences have shown that these epigenetic remodeling agents can significantly improve the *in vitro* and full-term development of cloned embryos; however, the molecular mechanisms behind them remain unknown. To investigate how this treatment improves the epigenetic reprogramming of somatic cell nuclei, and how it affects gene expression and DNA methylation in NT embryos, we compared the expression levels of DNA methylation-related genes in *in vitro* fertilized (IVF group), NT (C-NT group), and 5-aza-dC and TSA-treated NT (T-NT group) blastocysts using quantitative real-time polymerase chain reaction (PCR). We also compared the DNA methylation status of *satellite 1* among three groups using bisulfite sequencing analysis and COBRA analysis.

Materials and Methods

All chemicals were obtained from the Sigma-Aldrich Company (St. Louis, MO, USA) unless specifically indicated otherwise. Disposable, sterile plasticware was purchased from Nunclon (Roskilde, Denmark).

The entire experimental procedure was approved by the Animal Care Commission of the College of Veterinary Medicine, Northwest A&F University, P.R. China.

Nuclear donor cells preparation and treatments

Fibroblast cell cultures were established from the ear skin of a 1-week-old female Holstein calf. Briefly, the ear notch was recovered and cleaned and the hair was removed. The ear notch was then rinsed four times with phosphate-buffered saline (PBS), and minced into pieces (1 mm³) using sterile scissors in a 60-mm Petri dish. The small tissue pieces were cultivated for 1–2 weeks in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 1 mM sodium pyruvate, 100 IU/mL penicillin, and 100 mg/mL streptomycin. Fibroblast cells at 90% confluence were trypsinized, rinsed, and recultivated to three new 60-mm Petri dishes for further passaging. Nuclear donor cells for SCNT were derived from passage 2 to 5 and cultured in serum-starved medium (0.5% FBS) for 2 days. For the T-NT group, cells were first treated with 10 nM 5-aza-dC for 60 h alone and subsequently with a combination of both 10 nM 5-aza-dC and 50 nM TSA for 12 h before used for SCNT. The 5-aza-dC and TSA were dissolved in DMSO and prepared as a 1000-fold concentrated stock solution. These stock solutions were added to the culture medium at a 1:1000 dilution according to experimental procedure.

Oocyte collection and *in vitro* maturation (IVM)

Ovaries from slaughtered mature cattle were collected from a local abattoir and transported to the laboratory within 4 h in a thermos bottle with sterile saline at 25–30°C. Cumulus–

oocyte complexes (COCs) were aspirated from antral follicles with a diameter of between 2 and 8 mm with a 12-gauge needle attached to a 10-mL syringe, then recovered in PBS supplemented with 5% (v/v) FBS. Only oocytes surrounded by a minimum of three cumulus cell layers and with evenly granulated cytoplasm were selected. They were washed in PBS supplemented with 5% (v/v) FBS, and cultured for 20 h in bicarbonate-buffered tissue culture medium 199 (TCM-199, Gibco) supplemented with 10% (v/v) FBS, 1 µg/mL 17 β-estradiol, and 0.075 IU/mL human menopausal gonadotropin (HMG) in 95% humidified air with 5% CO₂ at 38.5°C.

Nuclear transfer, fusion, activation, and culture of cloned embryos

Nuclear transfer, fusion, activation of reconstructed embryos, and culture of cloned embryos were performed as described previously (Wang et al., 2010). Briefly, after IVM for 20 h, COCs were vortexed for 3 min in PBS supplemented with 0.1% bovine testicular hyaluronidase in 1.5-mL centrifuge tubes to disperse the cumulus cells. Oocytes with a first polar body and evenly granulated ooplasm were selected and stained with 10 µg/mL Hoechst 33342 for 10 min for enucleation. The first polar body and the small amount of surrounding cytoplasm was removed from the oocytes with a glass pipette (inner diameter, 20 µm) in PBS microdrops supplemented with 7.5 µg/mL cytochalasin B (CB) and 10% FBS. The expelled cytoplasm was examined under ultraviolet radiation to confirm that the nuclear material had been removed. Two groups of cells (treatment with or without TSA and 5-aza-dC) were used for donor cells. A single donor cell was placed in the perivitelline space of the enucleated oocytes. The oocyte–cell couplet was sandwiched between a pair of platinum electrodes connected to a micromanipulator in microdrops of Zimmermann's fusion medium, and a double electrical pulse of 35 V for 10 µsec was applied for oocyte–cell fusion (Liu et al., 2007). Reconstructed embryos were kept in SOFaa containing 5 µg/mL cytochalasin B for 2 h until activation. The mSOF medium was prepared according to the formulae as described previously (Takahashi and First, 1992) and supplemented with 8 mg/mL of bovine serum albumin, 1% MEM nonessential amino acid solution, and 2% BME essential amino acid solution. Successfully reconstructed embryos were activated in 5 µM ionomycin for 4 min followed by 4-h exposure to 1.9 mM dimethylamino-pyridine (DMAP) in SOFaa. After activation, embryos were cultured in mSOF medium. For T-NT group embryos, reconstructed embryos were cultured in mSOF medium supplemented with a combination of 10 nM 5-aza-dC and 50 nM TSA for 12 h and followed by 10 nM 5-aza-dC alone for 60 h. After treatment, embryos were washed twice with mSOF, and cultured in 50 µL drops of mSOF medium supplemented with 8 mg/mL bovine serum albumin (BSA) in a humidified atmosphere with 5% CO₂ in air at 38.5°C. Droplets of 50 µL mSOF were prepared in a 35-mm cell culture dish under mineral oil and equilibrated for 2 h before loading of embryos (10 embryos/microdrop). Embryos were transferred into new media droplets on day 3 of culture (day 0 being the day when embryos were reconstructed). Subsequent *in vitro* development to two-cell and blastocysts stages was monitored at 48 and 168 h of culture, respectively (0 h being the time embryos were transferred to mSOF).

In vitro fertilization

IVF was carried out as previously described (Nagao et al., 1995) with some modifications. Briefly, frozen-thawed spermatozoa were washed in Brackett and Oliphant (BO) medium supplemented with 6 mg/mL BSA and 20 μ g/mL heparin and centrifuged twice at 500 $\times$ g for 5 min. A 50 μ L sperm suspension (a concentration of 2×10^6 spermatozoa/mL) was added to 20–25 COCs in a 50 μ L microdrop of the BO medium supplemented with 6 mg/mL BSA and 20 μ g/mL heparin under mineral oil. After 20 h of *in vitro* fertilization, cumulus cells and redundant spermatozoa were dispersed from the oocytes with PBS supplemented with 0.1% bovine testicular hyaluronidase. Oocytes were washed twice with mSOF, and cultured in drops of 50 μ L mSOF medium supplemented with 8 mg/mL BSA in a humidified atmosphere with 5% CO₂ in air at 38.5°C. Droplets of 50 μ L mSOF were prepared in a 35-mm cell culture dish under mineral oil and equilibrated for 2 h before loading of embryos (10 embryos/microdrop). Culture medium was replaced by transferring embryos into fresh mSOF medium droplets at 72 h of culture. Subsequent *in vitro* development to two-cell and blastocyst stages were monitored at 48 and 168 h of culture, respectively (0 h being the time embryos were transferred into mSOF).

Reverse transcription

A single high-quality blastocyst was used per sample, and five to eight embryos were used for each group. Embryos were collected on day 7 postactivation/fertilization (Fig. 1) and treated with Cells-to-Signal™ Kit (Ambion Co., Austin, TX, USA) according to the manufacturer's protocol with some modifications. Briefly, each single blastocyst was washed twice with PBS and transferred to 15 μ L Cell-to-Signal Lysis Buffer. The sample was shaken gently for 4 min to lyse embryos. Finally, the samples were treated with RNA-free Dnase I (Invitrogen, Carlsbad, CA, USA) to digest the genomic DNA.

The RT reaction was performed using the M-MLV RT included in the Cells-to-Signal Kit under the following conditions. The reaction mixture (20 μ L), which contained 4 μ L embryo lysate, 4 μ L dNTP Mix, 2 μ L Oligo (dT) Primers, 2 μ L RT Buffer, 1 μ L M-MLV RT, 1 μ L RNase Inhibitor, and 6 μ L nuclease-free water, was assembled on ice. The reactions

were then mixed gently and centrifuged briefly to collect the contents at the bottom of the vessel. The reaction mixture was incubated at 42°C for 30 min for reverse transcription, followed by 95°C for 10 min to inactivate the reverse transcriptase.

Quantitative real-time PCR

The mRNA levels were quantified on a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) using SYBR Premix Ex Taq™ II (TaKaRa, Japan). The primer sequences for all genes were synthesized according to previous reports (Iager et al., 2008; Katz-Jaffe et al., 2009; Lin et al., 2008; McGraw et al., 2003) (Supplementary Table 1; see online supplementary data at www.liebertonline.com). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was initially used as housekeeping reference gene. In agreement with previously studies, *GAPDH* was found to be consistently expressed in bovine IVF, NT, and treated NT blastocyst, and was therefore used as the internal control gene (Smith et al., 2007). All primers used were optimized to ensure similar reaction efficiencies (96–98%) between target genes and *GAPDH*. cDNA was diluted such that threshold cycles were similar between the target genes and *GAPDH*. Reactions were performed in Low Tube Strips (Bio-Rad). Each reaction mixture (20 μ L) contained 2 μ L cDNA template, 10 μ L SYBR Premix Ex Taq II (2 $\times$), 0.8 μ L both PCR forward and reverse primers (10 μ M), and 6.4 μ L dH₂O. Thermal cycling conditions were 95°C for 1 min, followed by 40 PCR cycles of 95°C for 5 sec, 57°C or 60°C (Supplementary Table 1) for 30 sec, and 72°C for 30 sec. The melting protocol was a step cycle starting at 65°C and increasing to 95°C with 0.5°C/5-sec increments. Transcripts were quantified in three replicates for each sample and calculated relative to the transcription in every sample of the housekeeping gene, *GAPDH* (endogenous control). The specificity of the PCR reaction was confirmed by gel electrophoresis on a 2.5% agarose gel and by a single peak in the melt curve. As negative controls, dH₂O replaced cDNA in the real-time reaction tubes.

The results of RT-PCR are presented as the C_T value, defined as the threshold cycle number of PCRs at which the amplified product was first detected. The $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) was used for relative

FIG. 1. Representative photographs of day 7 bovine blastocysts (40 $\times$) obtained by IVF (A), SCNT (B), and SCNT combined 5-aza-dC and TSA treatment (C).

quantification of target gene expression levels using the following formula:

$$\Delta C_T = C_{T(\text{target gene})} - C_{T(\text{GAPDH})}, \text{ and}$$

$$\Delta\Delta C_T = \Delta C_{T(\text{sample})} - \Delta C_{T(\text{IVF})}.$$

The relative level of mRNA was calculated as $2^{-\Delta\Delta C_T}$. The mean of all samples within the NT and treated NT groups was determined, and values for each gene are reported relative to the IVF mean (IVF = 1).

Bisulfite sequencing analysis

A single high-quality blastocyst on day 7 postactivation/fertilization was used per sample, and 6, 11, and 13 embryo samples were used for IVF, C-NT, and T-NT groups, respectively. Embryo DNA isolation and bisulfite conversion were achieved using the EZ DNA Methylation-Direct™ Kit (Zymo Research, Irvine, CA, USA) in accordance with the instruction manual with minor modifications. Briefly, each single blastocyst was washed twice with PBS and transferred to a digestion mixture (20 μ L), which contained 10 μ L M-digestion Buffer (2 $\times$), 9 μ L dH₂O, and 1 μ L Proteinase K. After incubation at 50°C for 3 h, the 20- μ L sample was added to 130 μ L CT Conversion Reagent in a PCR tube. The tubes were placed in the thermal cycle (MJ Research, Waltham, MA, USA) for 8 min at 98°C for DNA denaturation and 3.5 h at 64°C for bisulfite conversion. Modified DNA was then desalted, purified, and finally eluted with 15 μ L Elution Buffer. Subsequently, Bisulfite Sequencing PCR (BS-PCR) was immediately carried out using 2 μ L modified DNA per PCR run or modified DNA stored at -80°C avoiding freeze thawing. The primers to amplify part of the *satellite I* (211 bp) was synthesized as described previously (Kang et al., 2001) (Supplementary Table 1). The Hot Start DNA polymerase Zymo Taq premix (Zymo Research) was used in BS-PCR. Each reaction mixture (50 μ L) contained 25 μ L Zymo Taq premix, 2 μ L modified DNA, 21 μ L dH₂O and 1 μ L of both forward and reverse primers. PCR was performed with a thermal cycler (MJ Research) using the following program: 95°C for 4 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 46°C for 30 sec, extension at 72°C for 20 sec, and a final extension at 72°C for 7 min. PCR products were resolved on 2.5% agarose gels to confirm the specific amplification of the product by size and then gel-purified using the TIANGel Midi Purification Kit (Tiangen, China). Purified fragments were subcloned into pMD18-T vectors (TaKaRa). The clones confirmed by PCR were selected for DNA sequencing (BGI, China). Three independent amplification experiments were performed for each sample. We sequenced three or four clones from each independent set of amplification and cloning, so there were a total of 9 to 12 clones for each sample. Bisulfite sequencing data and C-T conversion rates were analyzed by BIQ Analyzer software (Bock et al., 2005). To ensure high data quality, sequences that had a C-T conversion rate below 95% were excluded. Methylation data from bisulfite sequencing were analyzed by computing the percentage of methylated CpGs of the total number of CpGs.

Combined bisulfite restriction analysis (COBRA)

Half of all gel-purified PCR products used for bisulfite sequencing analysis from the three replicates of each sample

was pooled and digested with restriction enzyme Taq^a I (NEB, Beverly, MA, USA) for 3 h at 65°C. The digested fragments were electrophoresed on 3% agarose gels. During the sodium bisulfite treatment, unmethylated cytosine residues were converted to thymine, whereas methylated cytosine residues were retained as cytosine. The restriction sites (5'-TCGA-3' and 5'-AGCT-3') can be cleaved if CpG dinucleotides are methylated, but not if they are unmethylated. Therefore, in the mixed population of the resulting PCR fragments, the ratio of band intensity of digested fractions to the combined intensities of both digested and nondigested fractions reflects the level of DNA methylation. Band intensity was calculated using the ChemiDoc image analyzer and Quantity One software (Bio-Rad).

Statistical analysis

Data are presented as mean $\pm$ SEM. The levels of gene expression and DNA methylation among the three groups were tested by one-way analysis of variance and LSD test using the SPSS 13.0 software. Differences were considered significant at $p < 0.05$.

Results

Effect of combination treatment of donor cells and early embryos with 5-aza-dC and TSA on the development of bovine cloned embryos in vitro

Developmental rates to the cleavage and day 7 blastocyst stages of the embryos were analyzed from four replicates (Table 1). Combination treatment of both donor cells and early embryos with 5-aza-dC and TSA had no effect on cleavage, but the ability of cloned embryos to develop into day 7 blastocysts significantly improved after the treatment ($38.34\% \pm 1.19$ vs. $25.98\% \pm 2.42$, $p < 0.05$).

Relative expression levels of 14 genes among IVF, C-NT, and T-NT blastocysts

The relative abundance of the 14 selected genes in IVF, C-NT, and T-NT single blastocysts is presented in Figure 2. The expression levels for *DNMT1*, *DNMT3b*, *HDAC2*, and *IGF2* in T-NT blastocysts were significantly lower than those in C-NT blastocysts. The relative abundance of development-related genes *OCT4* and *SOX2* was significantly higher in T-NT blastocysts than in C-NT blastocysts. There were lower

TABLE 1. EFFECT OF THE 5-AZA-DC AND TSA TREATMENT ON *IN VITRO* DEVELOPMENT COMPETENCE OF BOVINE CLONED EMBRYOS^a

Group	Number of embryos Cultured	No. (mean % $\pm$ SEM) of embryos developed to $\geq$ two-cell on day 2	No. (mean % $\pm$ SEM) of embryos developed to blastocyst on day 7
IVF	226	179 (79.52% $\pm$ 2.03) ^b	81 (35.89% $\pm$ 1.63) ^b
C-NT	208	154 (74.09% $\pm$ 0.82) ^c	54 (25.98% $\pm$ 2.42) ^c
T-NT	216	168 (77.70% $\pm$ 1.18) ^{bc}	83 (38.34% $\pm$ 1.19) ^b

^aFour replicates were performed. Numbers in parentheses are cleavage rates and blastocyst rates, whereas other numbers are the total number of embryos of four replicates among three groups.

^bValues with different superscript within columns are significantly different from each other ($p < 0.05$).

FIG. 2. Relative expression levels of DNA methylation- (A), chromatin structure- (B), development- (C) related genes and imprinted genes (D) in day 7 IVF (open bar), C-NT (gray bars), and T-NT (black bar) single blastocysts. Different superscripts differ significantly ($p < 0.05$); $n = 5$ to 8.

expression levels of *DNMT3a*, *HDAC1*, and *IGF2R* in both C-NT and T-NT blastocysts than in IVF blastocysts. The expression levels of *HDAC3*, *HDAC7*, *NANOG*, *CDX2*, and *H19* were not significantly different among the three groups.

DNA methylation status analyzed by bisulfite sequencing

Bisulfite sequencing results are shown in Figure 3 and Table 2. Methylation of the *satellite I* sequence of IVF blastocysts ($18.86\% \pm 4.57$) and T-NT blastocysts ($29.03\% \pm 6.41$) was lower than that of C-NT blastocysts ($55.54\% \pm 7.17$).

Overall DNA methylation profiles analyzed by COBRA

To confirm that the bisulfite sequencing results from a limited number of templates reflect the overall methylation status for the *satellite I* region in embryos, we performed COBRA analysis using restriction enzymes *Taq*^a I on the same bisulfite-treated PCR amplification products that were used for bisulfite sequencing. As shown in Figure 4, it was obvious that most T-NT blastocysts (9 from 13) show minor cutting by *Taq*^a I, like the IVF embryos, whereas most C-NT blastocysts (9 from 11) show severe cutting by *Taq*^a I. The ratio of band intensity of digested fractions to the combined

intensities of both digested and nondigested fractions showed that the DNA methylation level of *satellite I* was significantly lower in T-NT blastocysts ($37.38\% \pm 7.96$) and IVF blastocysts ($26.00\% \pm 4.31$) compared with that of C-NT blastocysts ($63.55\% \pm 6.41$) (Table 2), which is consistent with the bisulfite sequencing results.

Discussion

5-Aza-dC is an inhibitor of DNA methylation. It prevents DNA methylation by inhibiting the activity of DNMT enzymes. It has been reported that treatment of donor cells (Enright et al., 2005) or early NT embryos (Tsuji et al., 2009) with 5-aza-dC does not increase the developmental potential of cloned embryos. Several histone deacetylase inhibitors (HDACi) such as trichostatin A, valproic acid, scriptaid, and sodium butyrate, have previously been tested as a means to improve SCNT efficiency in various species, including piglets (Beebe et al., 2009; Das et al., 2010; Li et al., 2008a; Zhang et al., 2007; Zhao et al., 2010), NIH miniature pigs (Himaki et al., 2010; Zhao et al., 2009), miniature pigs (Miyoshi et al., 2010), mice (Costa-Borges et al., 2010; Maalouf et al., 2009; Rybouchkin et al., 2006; Van Thuan et al., 2009), rabbits (Shi et al., 2008; Yang et al., 2007a), and cattle (Ding et al., 2008;

FIG. 3. Methylation profiles of 12 CpGs in the *satellite I* region analyzed by bisulfite sequencing. Unfilled (white) and filled (black) circles represent unmethylated and methylated CpGs, respectively. Horizontal lines of circles represent one separate clone that was sequenced (9 to 12 for each sample). Lollipop diagrams were generated by BIQ Analyzer software (Bock et al., 2005). For each sample, the methylation data were analyzed by computing the percentage of methylated CpGs of the total number of CpGs.

Iager et al., 2008; Wee et al., 2007). To date, the effect of the HDACi on the developmental competence has been thoroughly studied, whereas there are few investigations that examined how HDACi enhance the epigenetic remodeling ability of somatic cell nuclei in SCNT embryos.

In the present study, we evaluated the expression patterns of DNA methylation-, chromatin structure-, and

development-related genes in blastocysts developed from both donor cells and SCNT oocytes treated with or without 5-aza-dC and TSA. The DNA methylation status of the *satellite I* region was also examined in three types of embryos. We found that the treatment significantly reduced mRNA expression of *DNMT1*, *DNMT3b*, *HDAC2*, and *IGF2* in day 7 cloned bovine blastocysts, whereas mRNA levels of two

TABLE 2. METHYLATION LEVELS OF INDIVIDUAL BLASTOCYSTS IN THE SATELLITE I REGION ANALYZED BY BISULFITE SEQUENCING AND COBRA

Group	No. of embryos examined	Methylation levels examined by bisulfite sequencing (mean % $\pm$ SEM)	Methylation levels examined by COBRA (mean % $\pm$ SEM)
IVF	6	18.86% $\pm$ 4.57 ^a	26.00% $\pm$ 4.31 ^a
C-NT	11	55.54% $\pm$ 7.17 ^b	63.55% $\pm$ 6.41 ^b
T-NT	13	29.03% $\pm$ 6.41 ^a	37.38% $\pm$ 7.96 ^a

^{a,b}Values with different superscript within columns are significantly different from each other ($p < 0.05$).

development-related genes (*NANOG*, *SOX2*) were significantly higher in the 5-aza-dC and TSA-treated NT group (T-NT blastocysts) than the untreated NT group (C-NT blastocysts). We also demonstrated that 5-aza-dC and TSA treatment significantly reduced the DNA methylation level of the *satellite I* region in SCNT blastocysts.

Enright et al. (2005) reported that treatment of donor cells with 5-aza-dC alone does not increase the developmental potential of cloned embryos. We also found that donor cells treated with 5-aza-dC alone resulted in a nonsignificant increase in SCNT blastocyst rate and quality. However, when both 5-aza-dC and TSA were used, the preimplantation developmental competence of cloned bovine embryos was significantly improved (Ding et al., 2008). The study also showed that the DNA methylation level was significantly reduced and the histone acetylation level was increased in cloned two-cell embryos after treatment of donor cells or cloned embryos with both 5-aza-dC and TSA. Therefore, we inferred that there might be an interaction between 5-aza-dC and TSA. Perhaps the decreased methylation level and increased acetylation level affect the chromatin structure, facilitate nucleus reprogramming, and subsequently enhance the developmental competence of cloned embryos.

DNA methylation is a key epigenetic factor that modifies and regulates the chromatin structure. It also plays a crucial role in sustaining genomic stability, activating or suppressing gene expression, maintaining genomic imprinting, regulating X-chromosome inactivation, and silencing repetitive elements (Lan et al., 2010). Aberrant promoter hypermethylation in tumor suppressor genes enhances carcinogenesis, and the nucleoside analogue 5-aza-dC has been widely used as a DNA demethylation agent against several types of cancer. It was found by Xiong et al. (2005) that TSA not only modifies histone acetylation but also potentially induces DNA demethylation. Although our previous study showed that combination treatment of both 5-aza-dC and TSA decreases global DNA methylation levels in cloned embryos, the effect of combination treatment with both agents on DNA methyltransferase activity in cloned embryo is not understood. The present study showed that mRNA levels of *DNMT1* and *DNMT3b* are significantly lower in treated NT blastocysts than in untreated NT blastocysts. TSA treatment also significantly reduced mRNA expression of *DNMT3b* in cloned mouse blastocysts (Li et al., 2008b). 5-aza-dC is a DNA demethylation agent, which prevents DNA methylation by inhibiting the activity of DNMT enzymes (Oka et al., 2005). TSA induces DNA demethylation through down-regulation of *DNMT3b* mRNA and protein expression (Xiong et al., 2005). Thus, abnormally high levels of *DNMT3b* transcripts in SCNT embryos may be suppressed by 5-aza-dC and TSA. NT blastocysts (treated or untreated) had significantly lower expression levels of *DNMT3a*, another *de novo* DNA methyltransferase, compared with IVF blastocysts, confirming previous result (Beyhan et al., 2007).

We used bisulfite sequencing and COBRA assays to further investigate whether 5-aza-dC and TSA treatment affects DNA methylation levels of cloned embryos. Most treated NT embryos (9 out of 13), like the IVF embryos, showed a significantly lower DNA methylation levels of the *satellite I* sequence than nontreated NT embryos. The 5-aza-dC and TSA treatment seem to result in a "correcting" effect on the DNA

FIG. 4. Methylation status of individual blastocysts in the *satellite I* region analyzed by COBRA using restriction enzymes *TaqI*. The enzymes only cut the fragments when the specific CpG site was methylated. Marker is indicated on the side.

methylation status of the *satellite 1* region. A previous study reported that the methylation levels of *satellite 1* in NT bovine blastocysts were reduced when the donor cells were treated with TSA (Wee et al., 2007). Our previous study also found that the treatment can decrease global DNA methylation levels in early cloned embryos (Ding et al., 2008). Down-regulated DNA methylation on the global level or the *satellite 1* region in treated NT embryos may be due to the reduced *DNMT1* and *DNMT3b* expression. The activity of DNMT enzymes (*DNMT1* and *DNMT3b*) may be inhibited by the 5-aza-dC and (or) TSA.

Deacetylation of the lysines in the tails of the core histones is controlled by the actions of HDACs enzymes. In general, histone deacetylation suppresses gene transcription. TSA inhibits the activity of HDACs by chelation of zinc atoms within their catalytic sites (Finnin et al., 2001; Imai et al., 2000). Inhibition of the deacetylases results in an increase in the global acetylation of histones, which could alleviate transcriptional suppression by facilitating chromatin remodeling and relieving methylated CpG sites (Jones et al., 1998; Nan et al., 1998). It is well accepted that increased acetylation levels by TSA results in a change in the chromatin structure so that proteins like RNA polymerases can gain access to the DNA and begin transcription (Van Thuan et al., 2009). TSA may act synergistically with 5-aza-dC to reactivate DNA methylation-silenced genes, and subsequently enhance the developmental potential of cloned embryos. We found that the expression level of HDAC2 was reduced in the 5-aza-dC and TSA treated NT blastocysts.

OCT4 is a vital regulator of pluripotency that is important for maintaining ICM cell fate and pluripotency of ES cells. Two studies have demonstrated that the expression level of *OCT4* was downregulated in cloned bovine blastocysts compared to their IVF counterparts (Aston et al., 2010; Beyhan et al., 2007). We found similar results. However, 5-aza-dC and TSA treated cloned blastocysts shared the same *OCT4* expression profile as the IVF embryos. It seems that the treatment exerts a "correcting" effect on *OCT4* expression in cloned bovine embryos. *SOX2* is another pivotal regulator of pluripotency that can act synergistically with *OCT3/4* to activate *OCT-SOX* enhancers, which regulate the expression of *Nanog*, *OCT3/4*, and *SOX2* itself (Masui et al., 2007). In this study, we found the 5-aza-dC and TSA treatment increased the expression levels of *SOX2* in the cloned bovine blastocysts. The expression of *OCT4* and *SOX2* may be restricted to or higher in the ICM of preimplantation bovine embryos, and both pluripotency-related genes are important for maintaining ICM cell fate; therefore, the upregulated expression of these two genes in the treated NT blastocysts may be associated with the higher ICM:TE ratio. We reported previously that the number of ICM cells was higher in treated NT blastocyst compared to controls (Ding et al., 2008).

Insulin-like growth factor 2 (*IGF2*), an imprinted gene, is expressed on only one of the two chromosome homologues in a parent-of-origin-dependent manner regulated primarily by DNA methylation in imprinting control regions (ICRs). Abnormally high expression levels of *IGF2* were found in multiple tissues of deceased cloned calves suffering from LOS (Yang et al., 2005), and were found in the livers of mouse ES clones (Humphreys et al., 2002). In deceased cloned transgenic calves suffering from LOS, we found *IGF2*

mRNA levels were also elevated abnormally in the placentas compared to the placentas of normally reproduced calves and live clones (Su et al., 2010). In humans, upregulation of *IGF2* is thought to be important in the pathogenesis of Beckwith-Wiedemann Syndrome (BWS) (Joyce et al., 1997; Reik et al., 1996; Sun et al., 1997), which is characterized by somatic overgrowth similar to LOS in ruminants. These studies indicate that upregulation of *IGF2* would have promoted overall fetal growth and therefore could be related to LOS in the somatic cloning. The present study agreed with the results from Han et al. (2003) in that *IGF2* mRNA was significantly higher in cloned bovine embryos compared to IVF controls. However, the abundance of *IGF2* mRNA in T-NT cloned blastocysts was reduced to the levels of IVF blastocysts. The 5-aza-dC and TSA treatment might also have a "correcting" effect on *IGF2* expression.

In conclusion, we found that treatment of both donor cells and early cloned embryos with combination of 5-aza-dC and TSA affected the expression of DNA methylation-, chromatin structure-, and development-related genes at the blastocyst stage. It selectively reduced *DNMT1*, *DNMT3b*, *HDAC2*, and *IGF2*, whereas it increased *OCT4* and *SOX2* expression in NT bovine blastocysts. The treatment also reduced the methylation levels of the *satellite 1* sequence of NT blastocysts. Although the mechanisms underlying the effects of 5-aza-dC and TSA treatment on gene expression and DNA methylation are not clear, it is likely that the developmental potential (*in vitro* and full term) of treated NT embryos could be enhanced by such gene modifications and changes in epigenetic status such as DNA methylation.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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