Epigenetic alteration of the donor cells does not recapitulate the reprogramming of DNA methylation in cloned embryos

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Abstract

Epigenetic reprogramming is a prerequisite process during mammalian development that is aberrant in cloned embryos. However, mechanisms that evolve abnormal epigenetic reprogramming during preimplantation development are unclear. To trace the molecular event of an epigenetic mark such as DNA methylation, bovine fibroblasts were epigenetically altered by treatment with trichostatin A (TSA) and then individually transferred into enucleated bovine oocytes. In the TSA-treated cells, expression levels of histone deacetylases and DNA methyltransferases were reduced, but the expression level of histone acetyltransferases such as Tip60 and histone acetyltransferase 1 (HAT1) did not change compared with normal cells. DNA methylation levels of non-treated (normal) and TSA-treated cells were 64.0 and 48.9% in the satellite I sequence ($P < 0.05$) respectively, and 71.6 and 61.9% in the α -satellite sequence respectively. DNA methylation levels of nuclear transfer (NT) and TSA-NT blastocysts in the satellite I sequence were 67.2 and 42.2% ($P < 0.05$) respectively, which was approximately similar to those of normal and TSA-treated cells. In the α -satellite sequence, NT and TSA-NT embryos were substantially demethylated at the blastocyst stage as IVF-derived embryos were demethylated. The *in vitro* developmental rate (46.6%) of TSA-NT embryos that were individually transferred with TSA-treated cells was higher than that (31.7%) of NT embryos with non-treated cells ($P < 0.05$). Our findings suggest that the chromatin of a donor cell is unyielding to the reprogramming of DNA methylation during preimplantation development, and that alteration of the epigenetic state of donor cells may improve *in vitro* developmental competence of cloned embryos.

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Introduction

Clone animals derived from somatic cells indicate that the chromatin of differentiated somatic cells should be reprogrammed to totipotent or pluripotent states by adapting to the ooplasmic microenvironment (Wilmut *et al.* 1997, Wakayama *et al.* 1998). However, there are several developmental anomalies including high abortion rate and death shortly after birth (Tanaka *et al.* 2001, Ogonuki *et al.* 2002, Tamashiro *et al.* 2002). For these reasons, it is persuasive that aberrant epigenetic reprogramming of the cloned embryo is responsible for the developmental failures of cloned embryos. Reprogramming of the parental genome or chromatin of somatic cells can be monitored by investigating the profile of epigenetic marks such as DNA methylation. During early embryonic development, zygotic chromatin undergoes genome-wide reprogramming of DNA methylation to acquire developmental competence. The active demethylation of the paternal genome occurs after fertilization and

before the first cell division (Mayer *et al.* 2000, Santos *et al.* 2002). The maternal genome maintains its DNA methylation status at the pronuclear stage and thereafter its genome-wide demethylation occurs progressively by a passive demethylation mechanism (Monk *et al.* 1987, Rougier *et al.* 1998). However, it is likely that the reprogramming of DNA methylation takes place independently on distinct genomic regions by different mechanisms (Kim *et al.* 2004). In normal bovine embryos, *de novo* DNA methylation begins at the 8- to 16-cell stage and higher DNA methylation status is detected in the inner cell mass of the blastocyst (Dean *et al.* 2001), whereas cloned embryos represent incomplete reprogramming of DNA methylation during preimplantation development (Bourc'his *et al.* 2001, Dean *et al.* 2001, Kang *et al.* 2001). In mouse embryos, aberrant DNA methylation patterns are related to the failure of blastocyst formation and pregnancy (Shi & Haaf 2002). This result clearly demonstrates that DNA methylation status of the early stage embryo might be essential for appropriate gene expression and normal development.

Recently, we also found that acetylation reprogramming of histone H4 at lysine 5 is abnormal in early cloned embryos, thereby leading to aberrant transcriptional expression of some imprinted genes (Wee *et al.* 2006). Further observations on epigenetic behaviors of the donor cell nuclei may contribute to our understanding of the molecular mechanism of cloned embryos. To test whether the epigenetic alteration of somatic cells influences epigenetic reprogramming of cloned embryos, donor cells treated with trichostatin A (TSA) were individually transferred into enucleated oocytes and then DNA methylation status was measured at the blastocyst stage. DNA methylation levels in the satellite I sequence were reduced in the TSA-treated cells and thereafter maintained at the same level in cloned blastocysts. Thus, the chromatin of donor cells seems to be refractory to the reprogramming of DNA methylation during early development. In addition, cloned embryos with TSA-treated nuclei showed a higher developmental competence than those with normal somatic nuclei. This result indicates that epigenetic status of the

somatic cell might influence developmental potential of cloned embryos.

Results

Treatment of donor cells with TSA

Prior to NT, we first investigated whether TSA treatment induced abnormality of bovine ear skin fibroblasts (bESFs). The cells changed morphologically at 24 h after TSA treatment. Although TSA-treated cells enlarged in size, flattened and were vague in outlines (Fig. 1A), they had normal karyotypes (Fig. 1B). The cells grew slightly by 12 h after TSA treatment and thereafter their growth was arrested, whereas normal cells continuously proliferated by 60 h (Fig. 1C). Thus, TSA treatment inhibited proliferation of bovine fibroblasts. TSA-treated cells showed reduced proportion of the S phase ($P < 0.05$) and enhanced proportion of the G1/G2/M phases compared with non-treated cells (Fig. 1D).

Figure 1 Characterization of TSA-treated bESF cells. (A) Morphology of TSA-treated cells. (B) Karyotype of TSA-treated cells. (C) Proliferation of TSA-treated cells. (D) Distribution of TSA-treated cells to the cell cycle stages. Each value is expressed as mean $\pm$ s.e.m. ^{a,b}Different superscripts denote significant differences between experimental groups ($P < 0.05$). Experiments for the cell cycle analysis were repeated thrice.

Expression of enzymes involved in epigenetic modification in TSA-treated cells

The following experiment was performed to determine whether TSA treatment affects expression of enzymes involved in epigenetic modification in bovine cells. Most TSA-treated cells had weak immunofluorescence signals for HDAC2 and Dnmt1 when compared with non-treated cells (Fig. 2A and B, $P < 0.05$). Histone acetyltransferases such as Tip60 and HAT1 were consistently expressed in TSA-treated cells, whereas expression of histone deacetylases (HDAC1 and HDAC2) and DNA methyltransferases (Dnmt1, Dnmt3a, Dnmt3b, and Dnmt3L) was decreased (Fig. 2C). Interestingly, expression of Dnmt1 was remarkably reduced after TSA treatment, indicating that expression of Dnmt1 might be more sensitive to the activity of HDACs than other Dnmts.

Reprogramming of DNA methylation in TSA-NT embryos

To monitor epigenetic behaviors of donor cell nuclei, the DNA methylation state of TSA-NT (nuclear transferred with TSA-treated cells) embryos was compared with that of NT embryos (nuclear transferred with non-treated cells). In the bovine satellite I sequence, the DNA methylation level (48.8%, 99/203 CpG sites) of TSA-treated cells was significantly lower than that (64.0%, 146/228 CpG sites) of non-treated cells (Fig. 3A, $P < 0.05$). DNA methylation levels of IVF, NT, and TSA-NT blastocysts were 28.9% (59/204 CpG sites), 67.2% (137/204 CpG sites), and 42.2% (86/204 CpG sites) respectively (Fig. 3B). Intriguingly, DNA methylation levels of NT and TSA-NT embryos were approximately similar to those of their respective donor cells. These results suggest that the satellite I region of somatic cell nuclei is unyielding to the reprogramming of DNA methylation in cloned embryos during preimplantation development. As shown in Fig. 4A, the DNA methylation level (61.9%, 78/126 CpG sites) of the α -satellite sequence in TSA-treated cells was slightly reduced compared with non-treated cells (71.4%, 75/105 CpG sites). DNA methylation levels of IVF, NT, and TSA-NT embryos in the α -satellite sequence were 32.4% (35/108 CpG sites), 33.3% (24/72 CpG sites), and 38.9% (28/72 CpG sites) respectively (Fig. 4B). Thus, CpG sites in the α -satellite sequence of somatic cell nuclei are vulnerable to be demethylated in cloned embryos.

Improved preimplantation development of TSA-NT embryos

After somatic cell NT, *in vitro* developmental competence of cloned bovine embryos with TSA-treated cells was investigated (Table 1). TSA-treated cells were individually transferred into single enucleated oocytes. Reconstructed oocytes were cultured *in vitro* for 7 days. At day 3 of *in vitro* culture, cleavage rates of IVF, NT, and

TSA-NT embryos to four- to eight-cell stages were 85.9% (146/170), 80.2% (162/202) and 79.6% (164/206) respectively. Thus, there was no difference in the cleavage rate among the experimental groups. However, *in vitro* developmental rates (46.6%, 48/170) of TSA-NT embryos to blastocysts were significantly higher ($P < 0.05$) than those of IVF and NT embryos (28.2%, 64/202 and 31.7%, 96/206 respectively).

Discussion

Differentiated somatic cell nuclei with highly methylated state appear to be incompletely reprogrammed in cloned embryos during preimplantation development. In fact, aberrant reprogramming of DNA methylation in early cloned embryos has been reported (Bourc'his *et al.* 2001, Dean *et al.* 2001, Kang *et al.* 2001, 2002). In this study, we demonstrate that the somatic cell nuclei epigenetically altered by treatment with a chemical reagent are still refractory to the epigenetic reprogramming in cloned embryos.

Epigenetic alteration of donor cells could be induced by inhibiting activities of epigenetic-modifying enzymes such as histone acetyltransferases, HDACs, and Dnmts. TSA, which inhibits the activity of HDACs by chelating of zinc atoms within their catalytic sites (Imai *et al.* 2000, Finnin *et al.* 2001), could alleviate transcriptional repression by facilitating chromatin remodeling and relieving methylated CpG sites (Jones *et al.* 1998, Nan *et al.* 1998). To modify the epigenetic state of donor cells, in this study, bovine fibroblasts were treated with TSA for 60 h. In TSA-treated cells, expression levels of HDACs and Dnmts were extremely decreased compared with non-treated cells (Fig. 2C). Interestingly, there was no significant reduction in the expression of acetyltransferases such as HAT1 and Tip60. In contrast to non-treated cells, which were acetylated in the interphase and deacetylated in the metaphase, TSA-treated cells were highly acetylated throughout the cell cycle (Wee *et al.* 2006). The results obtained in this study indicate that hyperacetylation of TSA-treated cells might be due to the decreased expression of HDACs, but not responsible for the consistent expression of histone acetyltransferases. Thus, the epigenetic status of donor cells could be altered by regulating the activity of epigenetic-modifying enzymes.

From our results that the expression of Dnmts was suppressed (Fig. 2C), we could also expect low DNA methylation levels of TSA-treated cells. In fact, TSA-treated cells showed a lower DNA methylation level than non-treated cells on two repetitive sequences (Figs 3A and 4A). Inhibitors of the HDAC resulted in global or regional DNA demethylation in various cell lines (Szyf *et al.* 1985, Selker 1998, Hu *et al.* 2000). After somatic cell NT, TSA-NT embryos showed a lower DNA methylation level in the satellite I sequence than NT embryos (Fig. 3B). However, there was no difference

Figure 2 Expression of epigenetic-modifying enzymes in TSA-treated cells. (A) Intensity of HDAC2 and Dnmt1 signals in non- and TSA-treated cells. Scale bar, 50 μ m (B) Comparison of signal intensity quantified by the ratio of HDAC2/DNA or Dnmt1/DNA signals. The quantitative value is expressed as mean $\pm$ S.E.M. ^{a,b}Different superscripts denote significant differences between experimental groups ($P < 0.05$). (C) Western blot analysis for epigenetic-modifying enzymes in TSA-treated cells.

in DNA methylation levels between donor cells and cloned embryos. The satellite I sequence did not seem to be reprogrammed in cloned embryos during early development (Kang *et al.* 2005). Intriguingly, the lower DNA methylation level of TSA-treated cells was maintained at a similar level on the satellite I sequence by the blastocyst stage after somatic cell NT. This result suggests that epigenetic alteration of somatic cell nuclei does not affect reprogramming of DNA methylation in the repetitive sequence during early development of cloned embryos. In contrast to the satellite I sequence, the α -satellite sequence was substantially demethylated even in NT and TSA-NT embryos (Fig. 4B). We previously reported that the α -satellite sequence was passively demethylated in IVF and NT bovine embryos (Kang *et al.* 2005). Our findings show that reprogramming of DNA methylation takes place in the α -satellite sequence of cloned embryos. Based on the results of two repetitive sequences examined in this study, it is conceivable that reprogramming of DNA methylation might act separately on the respective genomic regions during early development. In fact, three repetitive sequences showed differential reprogramming patterns of DNA methylation during preimplantation development of mouse embryos (Kim *et al.* 2004).

Various epigenetic events, such as active demethylation, passive demethylation, and *de novo* methylation, are crucial for normal embryo development (Reik & Dean 2001, Li 2002). In this study, we also examined whether altered epigenetic states of donor cells might influence developmental competence of cloned embryos. We found that reconstructed oocytes with TSA-treated donor cells showed a higher *in vitro* developmental rate than IVF and NT embryos (Table 1). This result is consistent with a previous report that TSA treatment of donor cells could improve *in vitro* development of cloned bovine embryos (Enright *et al.* 2003). This improved development might be explained by the fact that TSA treatment enhanced expression of embryonic genes by altering epigenetic states of donor cell nuclei. When mouse embryos were incubated in TSA, expression of the transgene and the endogenous genes was significantly increased at the preimplantation stages (Thompson *et al.* 1995, Aoki *et al.* 1997). Moreover, TSA treatment in activation medium enhanced blastocyst formation of cloned mouse embryos up to fivefold and reduced abnormality of clones (Kishigami *et al.* 2006). However, they did not show the results about reprogramming of DNA methylation in cloned embryos. From our results, it is likely that improved development of TSA-NT embryos might be responsible for epigenetic alleviation of donor nuclei such as lower DNA methylation. Thus, this study provides an insight that the developmental competence of cloned embryos could be enhanced by altering epigenetic status of somatic cell nuclei such as DNA methylation.

Figure 3 Analysis of DNA methylation status on the satellite I sequences. (A) DNA methylation levels of non-treated and TSA-treated cells. (B) DNA methylation levels of IVF, NT and TSA-NT blastocysts.

Materials and Methods

Cell culture and TSA treatment

Bovine ear skin fibroblasts (bESFs) were derived from an adult cow aged 3.5 years and cultured in Dulbecco's modified Eagle's medium (GibcoBRL) supplemented with 10% fetal bovine serum (FBS), 100 μ M non-essential amino acid (GibcoBRL) and 500 μ g/ml gentamycin sulfate (GibcoBRL) at 37 °C in 5% CO₂ in air. bESF cells were seeded at a concentration of 7×10^4 cells per well on a 24-well culture plate or 1×10^6 cells on a 100 mm culture dish. After 24 h of culture, the cells were incubated in the culture medium containing 1 μ M TSA (Sigma) for 60 h. TSA-treated cells were obtained at intervals of 12 h and subjected to subsequent experiments.

culture medium containing 0.1 μ g/ml colcemid for 60 h. After trypsinization (0.25% trypsin-EDTA), recovered cells were suspended and treated with hypotonic solution of 0.56% KCl for 30 min at room temperature. Fixative solution (methanol:acetic acid=3:1) was added to the cells and centrifuged at 700 *g* (1000 r.p.m.) for 5 min. The fixative process was repeated twice. The cell pellet was suspended in 1 ml fixative solution and was dropped onto the slide to get metaphase spreads. The slides were immersed in 0.5% barium hydroxide solution for 3 min and were incubated in $2 \times$ SSC (30 mM NaCl and 30 mM trisodium citrate) for 2 h at 50 °C. Then, the samples were stained with 0.1% Giemsa (Fluka, Gallen, Switzerland) for 30 min, washed thrice with distilled water, and dried in air. The metaphase chromosomes were observed at 400 $\times$ magnification under a microscope (Olympus, Tokyo, Japan).

Karyotype

At 60 h after TSA treatment, the karyotype of bESF cells was performed by G-banding analysis. The cells were cultured in

Analysis of cell cycle

DNA contents of the cells were determined by measuring the fluorescence in a FACScan (BD Science, San Jose, CA, USA) after

Figure 4 DNA methylation analysis on the α -satellite sequences. (A) DNA methylation levels of non- and TSA-treated cells. (B) DNA methylation levels of IVF, NT and TSA-NT blastocysts.

Table 1 *In vitro* development of cloned embryos.

Group	No. of embryos cultured	No. (%) of embryos cleaved	No. (%) of blastocysts ^a
IVF	170	146 (85.9)	48 (28.2)*
NT	202	162 (80.2)	64 (31.7)*
TSA-NT	206	164 (79.6)	96 (46.6) [†]

These experiments were contemporaneously carried out five times.

*[†]Different superscripts denote significant differences ($P < 0.05$).

^aDevelopmental rate = No. of blastocysts/No. of embryos cultured $\times 100$.

staining with propidium iodide (Sigma). Briefly, the cells were fixed in ice-cold 70% ethanol for 1 h and then placed in a solution containing 1 mg/ml RNase (Sigma) and 20 μ g/ml propidium iodide for 30 min. Approximately 10 000 cells were measured by a FACScan and proportion of the cells to G0/G1, S, and G2/M phases was determined by the manufacturer's protocol.

***In vitro* maturation, fertilization and culture of bovine oocytes**

Bovine oocytes were matured and fertilized *in vitro* as previously described (Koo *et al.* 2002). Cumulus-oocytes were incubated in maturation medium at 38.5 °C, 5% CO₂ in air for 20 h. After maturation, oocytes were fertilized with frozen-thawed sperm and were cultured in CR1aa medium supplemented with 0.3% BSA (Sigma) for 3 days. Cleaved embryos were further cultured in each drop (50 μ l) of CR1aa supplemented with 10% FBS for 4 days. At day 7 of culture, blastocyst formation was observed.

Somatic cell nuclear transfer (NT)

Somatic cell NT was carried out as previously described (Wee *et al.* 2006). Donor cells were individually inserted into the perivitelline space of single enucleated oocytes, and fused by a single direct pulse of 1.6 kV/cm for 20 μ s using Electro Cell Manipulator 2001 (BTX, San Diego, CA, USA). At 2 h after electrofusion, fused embryos were activated with 5 μ M ionomycin (Sigma) for 5 min followed by treatment with 2.5 mM 6-dimethylaminopurine (Sigma) for 4 h. Reconstructed eggs were cultured *in vitro* for 7 days in the same culture conditions as described previously.

Immunostaining

Bovine fibroblasts were fixed in 4% formaldehyde in PBS for 1 h, washed in PBS containing 0.1% Tween 20 (TPBS) for 1 h, and then permeabilized with 0.5% Triton X-100 in PBS for 2 h. The cells were blocked with 1% BSA in TPBS for 2 h. Primary antibodies were diluted with PBS (1:50) and were co-incubated with samples for 3 h at 4 °C. After washing for 3 h in TPBS, the samples were incubated for 30 min in the presence of Cy3-anti-rabbit immunoglobulin G (IgG; Molecular Probes, Eugene, OR, USA) and then washed in TPBS for 1 h. Samples were mounted on slides with a 90% glycerol-PBS containing 1 fg/ml 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes). The specific antibodies

for histone deacetylase 2 (HDAC2; cat. no. sc-7899; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and DNA methyltransferase 1 (Dnmt1; cat. no. sc-60B1220; Santa Cruz Biotechnology) were used in this experiment. Fluorescent images were observed on an epifluorescence light microscope (Olympus). Exposure times of fluorescent light were kept constant for the respective channel (Cy3 or DAPI). Quantification of images was determined by the ratio of a primary antibody signal to a DAPI DNA signal using an image analyzer system, SigmaScan-pro V5.01 (SPSS Inc., Chicago, IL, USA). The images were merged using Adobe Photoshop 6.0 software.

Western blot

Cells ($\sim 1 \times 10^7$ cells) were washed twice with PBS and lysed with 10 volume of lysis buffer on ice for 1 h. The lysis buffer consists of 25 mM HEPES (pH 7.8), 25 mM KCl, 5 mM MgCl₂, 0.05 mM EDTA, 10% glycerol, 0.1% NP40, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), and 1 mM dithiothreitol (DTT). The nuclear pellet was washed with lysis buffer to remove cytoplasmic contaminants, resuspended in nuclear extract buffer, and then incubated at 4 °C for 30 min. The nuclear extract buffer consists of 10 mM HEPES (pH 7.5), 200 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, and 1 mM PMSF. Approximately 20 μ g extracted proteins were subjected to Western blot analysis. The samples were boiled for 4 min, loaded into 10% SDS-polyacrylamide gel, resolved by electrophoresis, and then transferred to a nitrocellulose membrane (Bio-Rad). The membrane was placed in PBS containing 4% nonfat milk powder at room temperature for 1 h and then washed five times with TPBS at 4 °C. The samples were transferred to PBS containing 4% nonfat milk powder and incubated with the antibodies for HAT1 (sc-8751), Tip60 (sc-5727), HDAC1 (sc-6298), HDAC2 (sc-7899), Dnmt1 (sc-60B1220), Dnmt3a (sc-20703), Dnmt3b (sc-20704), Dnmt3L (sc-10239), and β -actin (sc-47778) at 4 °C for 6 h respectively. After washing with TPBS, the samples were treated with the secondary antibody in PBS containing 4% nonfat milk powder at 4 °C for 1 h. The membrane was washed with TPBS and then developed using the ECL system (Pierce, Rockford, IL, USA) as recommended by the manufacturer's procedure.

Bisulfite sequencing

DNA methylation states were measured as previously described (Kang *et al.* 2005). Cells or embryos were lysed in 100 μ l lysis buffer containing 200 μ g/ml proteinase K (Roche Molecular Biochemicals). Then, samples were incubated at 55 °C for 5 h. Genomic DNA was isolated from bovine embryos using ethanol precipitation method in the presence of 5 μ g *Escherichia coli* tRNA as a carrier, and then resuspended in 10 μ l distilled water. Modification of sample DNA was carried out by adding 235 μ l freshly made 5 M sodium bisulfite (pH 5; Sigma) and 13.5 μ l of 10 mM hydroquinone at 55 °C for 16 h in darkness. DNA sample was recovered by DNA purification kit (Bio-Rad) and desulfonated by adding 0.1 volume of 3 M NaOH and incubated at 37 °C for 30 min. Satellite I and α -satellite DNA sequences were amplified by PCR. Primers and PCR were the same as previously described (Kang *et al.* 2005). Amplified PCR

products were inserted into pGEM-T easy vector (Promega) and sequenced by an automatic sequencer (ABI PRISM 377).

Statistical analysis

All experimental data were analyzed by ANOVA using the SAS package (SAS Inc., Cary, NC, USA). Individual data for the signal intensity of immunostains were pooled in the respective group, and differences among groups were evaluated by least significant difference analysis of one-way ANOVA. Developmental rates of the embryos to blastocysts among experimental groups were analyzed by Duncan's analysis of one-way ANOVA. A value of $P < 0.05$ was considered statistically significant.

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