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Bovine Ooplasm Partially Remodels Primate Somatic Nuclei following Somatic Cell Nuclear Transfer

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Abstract

Interspecies somatic cell nuclear transfer (iSCNT) has the potential to become a useful tool to address basic questions about the nucleus–cytoplasm interactions between species. It has also been proposed as an alternative for the preservation of endangered species and to derive autologous embryonic stem cells. Using chimpanzee/bovine iSCNT as our experimental model we studied the early epigenetic events that take place soon after cell fusion until embryonic genome activation (EGA). Our analysis suggested partial EGA in iSCNT embryos at the eight-cell stage, as indicated by Br-UTP incorporation and expression of chimpanzee embryonic genes. Oct4, Stella, Crabp1, CCNE2, CXCL6, PTGER4, H2AFZ, c-MYC, KLF4, and GAPDH transcripts were expressed, while Nanog, Glut1, DSC2, USF2, Adrbk1, and Lin28 failed to be activated. Although development of iSCNT embryos did not progress beyond the 8- to 16-cell stage, chromatin remodeling events, monitored by H3K27 methylation, H4K5 acetylation, and global DNA methylation, were similar in both intra- and interspecies SCNT embryos. However, bisulfite sequencing indicated incomplete demethylation of Oct4 and Nanog promoters in eight-cell iSCNT embryos. ATP production levels were significantly higher in bovine SCNT embryos than in iSCNT embryos, TUNEL assays did not reveal any difference in the apoptotic status of the nuclei from both types of embryos. Collectively, our results suggest that bovine ooplasm can partially remodel chimpanzee somatic nuclei, and provides insight into some of the current barriers iSCNT must overcome if further embryonic development is to be expected.

Introduction

Interspecies somatic cell nuclear transfer (iSCNT), defined as the procedure by which the oocyte's cytosol of one species is fused with the nucleus of a different species, has the potential to become a useful tool to address basic questions regarding nucleus–cytoplasm interactions between species. It may also have practical applications for the preservation of endangered species (Dominko et al., 1999; Thongphakdee et al., 2006; Williams et al., 2006). More importantly, iSCNT has been proposed as a technique to generate human embryonic stem cells, potentially solving the ethical and logistical problems associated with the procurement of human oocytes (Chen et al., 2003; Fulka and Mrazek, 2004; Minger, 2007; St John et al., 2008). Countries like the UK have recently legalized this procedure, allowing fusions between animal enucleated oocytes and human somatic cells.

A variety of oocyte–somatic cell combinations have been tested, and more than 30 studies have been published reporting different degrees of success (Beyhan et al., 2007). Live offspring have been obtained when using closely related species—such as cow/gaur, cow/buffalo, domestic cat/wild cat, and pig/bovine (Mastromonaco et al., 2007; Uhm et al., 2007)—however, the efficiency of the procedure (measured by the number of reconstructed embryos/animals born) is very low. Preimplantation development seems easy to obtain, but further development of these embryos has either failed or not been reported (Beyhan et al., 2007).

From the onset of cell division, the SCNT embryo must maintain the stability of its genome and correct separation of sister chromatids is a prerequisite for maintaining proper ploidy (Hanada et al., 2005; Tani et al., 2007). A series of reports have indicated failure of the mitotic spindle assembly in the context of SCNT. Monkey SCNT embryos showed unequal segregation of chromosomes due to the deficiency of a spindle protein, NuMA (Simerly et al., 2003), and mouse and bovine SCNT embryos have shown chromosome instability and misconstruction of the spindle (Balbach et al., 2007;

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Hanada et al., 2005). Aneuploidy in blastomeres has the added detrimental effect of triggering cell death by apoptosis, a phenomenon commonly seen in bovine SCNT embryos (Hanada et al., 2005). It is possible that in the context of iSCNT, the donor nuclei and cytoplasmic control elements are incompatible, leading to an even more pronounced instability of chromosomes.

During SCNT, the genome of the somatic cell must undergo massive reprogramming. This process is still poorly understood. It has been shown that the epigenetic remodeling of the chromatin leads, in ideal circumstances, to silencing of somatic-cell-related genes and reactivation of developmentally regulated embryonic genes (Hattori et al., 2007; Yamazaki et al., 2006). Direct modifications of DNA and histones play an essential role in chromatin structure and transcriptional regulation. Epigenetic changes in histones—such as acetylation, methylation, phosphorylation, and ubiquitinylation—will, in turn, lead to reactivation or silencing of specific genes (Turner, 2002). Hyperacetylation of histones facilitates the access of transcription factors to nucleosomes and induces a more transcriptionally permissive state (Kikyo et al., 2000). DNA methylation is a well-characterized epigenetic change required for cell differentiation and embryonic development. Experiments in *Xenopus* SCNT and bovine SCNT embryos have shown that complete demethylation in Oct4 gene and in whole genome, respectively, are necessary for cloned animals to develop normally (Bourc'his et al., 2001; Simonsson and Gurdon, 2004).

During early stages of embryonic development, embryos rely on maternal RNA transcripts to regulate development. Embryonic genome activation (EGA) in bovine embryos occurs at the 8- to 16-cell stage (Memili and First, 1998), and is necessary to sustain further embryonic development of fertilized embryos. The same requirement applies to SCNT embryos, but has the added challenge that the somatic cell nuclei must undergo rapid reprogramming, that is, silencing of somatic-related genes, during the first three cell cycles, and then go through EGA successfully to establish an embryonic profile compatible with development. It is not surprising that aberrant gene expression is frequently observed in SCNT embryos, leading to abnormal development and early fetal losses (Boiani et al., 2002; Daniels et al., 2000; Rideout et al., 2001). Whether the genome of the somatic cell, in the context of iSCNT, can reactivate its embryonic genes has not yet been categorically answered.

Another important requirement for preimplantation embryos is the availability of functional mitochondria and their related enzymatic activity (Reynier et al., 2001; Stojkovic et al., 2001). SCNT, just like IVF embryos and *in vivo* produced embryos, need to maintain a minimum production of adenosine triphosphate (ATP) (Reynier et al., 2001; Stojkovic et al., 2001). It is then possible that the failure of iSCNT embryos to progress in development correlates directly with the evolutionary distance between the nucleus and the oocyte cytoplasm. This idea has been supported by cybrid experiments where mitochondria were incapable of maintaining proper respiration levels when the species providing the nuclei and mitochondria were evolutionarily more than 16 million years apart from each other (Kenyon and Moraes, 1997). Further cybrid experiments have indicated that there might be subtle disparities in metabolic functions even among those closely related species (Barrientos et al., 1998).

The chimpanzee (*Pan troglodytes*) is considered the primate most closely related to humans (Culotta, 2005), and offers a valuable experimental model for studying interspecies nuclear transfer without having to create cloned human embryos. We hypothesized that enucleated bovine oocytes would be capable of taking epigenetic control of the chimpanzee somatic cell nuclei and reprogramming them into embryonic nuclei. Reconstructed interspecies SCNT embryos were compared with same-species reconstructed embryos (bovine/bovine, B/B). We characterized iSCNT embryos' genetic stability, gene expression pattern, and epigenetic state, as well as their rate of cell death. While Chimpanzee/Bovine (C/B) iSCNT embryos did not progress beyond the 8- to 16-cell stage, we observed significant remodeling activity in the chimpanzee nucleus, as measured by histone H3K27 methylation, H4K5 acetylation, and BrUTP incorporation. Our study provides insight into some of the current barriers iSCNT must overcome if further embryonic development is to be expected. The developmental limitations we observed in iSCNT embryos could be the same as those limiting the efficiency of same-species SCNT embryos, only being more pronounced.

Materials and Methods

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise stated.

Maturation and preparation of recipient oocytes

Recipient bovine oocytes were matured according to procedures previously shown to produce developmentally competent oocytes (Fischer-Brown et al., 2005). Bovine oocytes were obtained by aspiration of small antral follicles (from 2 to 5 mm) on slaughterhouse-derived ovaries. Immature cumulus–oocyte complexes were cultured in Tissue Culture Medium 199 (TCM-199) supplemented with 10% fetal calf serum (FCS), 0.2 mM pyruvate, 25 μL/mL gentamicin, 0.5 μg/mL Luteinizing Hormone (LH; Sioux Biochemical, Sioux Center, IA) and 1 μg/mL estradiol-17β for 16 to 18 h at 38.5°C with 5% CO_2 in the air. Eighteen hours after the start of maturation, cumulus cells were removed from the oocytes by vortexing in a 14-mL tube for 4 min in the presence of 2 mg/mL of hyaluronidase, and oocytes with extruded first polar bodies were selected for enucleation. The oocytes were labeled with 0.5 μg/mL of DNA fluorochrome (Hoechst 33342) for 20 min at 38.5°C in KSOM medium. All manipulations were performed in HEPES-buffered HECM media (HH) (supplemented with 7.5 μg/mL of cytochalasin B) on a Nikon TE2000-U microscope equipped with Hoffman optics and Narishige micromanipulators. The MII plate was removed by aspiration, using an enucleation pipette with a 20-μm inner diameter. To ensure that oocyte chromatin was removed, the aspirated cytoplasm was exposed to UV light and examined for the presence of the removed polar body and metaphase plate.

Adult fibroblast culture

Chimpanzee cells were purchased from American Type Culture Collection (ATCC) Donor cells were cultured in Dulbecco's Modified Minimum Essential Medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 10% FCS

(Hyclone, Logan, UT) and antibiotic–antimycotic (Gibco BRL) at 37°C under a gas phase of 5% CO_2 in air at high humidity until they reached approximately 70% confluence. Prior to nuclear transfer, cells were disaggregated by pronase treatment (100 μg/mL).

Somatic cell nuclear transfer, activation, and fusion

A single donor cell was deposited into the perivitelline space of each enucleated oocyte using a micropipette. Then, nuclear transfer couplets were fused in sorbitol fusion medium by applying a single electric pulse (20-μsec pulse, 2.4 kV/cm). One hour after fusion, the fused embryos were selected. SCNT embryos were activated using 5 μM of ionomycin (Calbiochem, La Jolla, CA) in HH medium supplemented with 1 mg/mL of bovine serum albumin for four minutes, 24 to 25 h after the start of maturation. This was followed by incubation of SCNT units in KSOM supplemented with 5 μg/mL 6-DMAP for 4 h. At the end of incubation, SCNT units were washed in KSOM medium and placed into culture medium KSOM drops at 38.5°C.

Embryo culture

Activated SCNT embryos were cultured (50 embryos/100 μL drop) in KSOM embryo culture medium for the first 72 h, followed by incubation with the addition of 5% FCS until 7.5 days postactivation.

Embryo collection and RNA isolation

Groups of 10 embryos were selected after checking nuclei by labeling with 0.5 μg/mL of Hoechst 33342 for 20 min at 38.5°C in KSOM medium, rinsed in sterile PBS, and lysed in 20 μL of extraction buffer (XB; Arcturus, Mountain View, CA) in an RNase-/DNase-/pyrogen-free 0.5-mL microcentrifuge tube 65 h after activation for eight-cell stage embryos. Each sample was incubated for 30 min at 42°C, centrifuged at 3000 $\times$ g for 2 min, and stored at −80°C until use.

Total RNA was isolated by using the PicoPure RNA Isolation Kit (Arcturus) following the manufacturer's instructions, except the elution volume was 6.5 μL. All RNA samples within the purification column were treated with RNase-Free DNase (Qiagen, Valencia, CA) and eluted with RNase-free elution buffer. Extracted RNA was stored at −80°C until use.

RT, cDNA amplification, and PCR

First-strand cDNA synthesis was performed based on our previously published protocol (Kocabas et al., 2006). Briefly, the following reagents were added to each 0.5 mL of RNase-free tube: 5 μL total RNA and 300 ng of anchored T7-Oligo(dT)$_{24}$V Promoter Primer (Ambion, Austin, TX). The reaction tubes were incubated in a preheated PCR machine at 70°C for 2 min and transferred to ice. After denaturation, the following reagents were added to each reaction tube: 1.4 μL of SMART II A oligonucleotide (5′AAGCAGTGGTATCAACGCAGAGTACGCGrGrGr-3′) (Clontech, Mountain View, CA), 4 μL of 5$\times$ first-strand buffer, 2 μL of 20 mM DTT, 0.6 μL of 5 μg/μL T4 Gene 32 Protein (Roche, Indianapolis, IN), 2 μL of 10 mM dNTPs, 20 U of RNase Inhibitor (Ambion) and 1 μL of PowerScript Reverse Transcriptase (Clontech). Total reaction volume was 20 μL. After gentle mixing, reaction tubes were incubated at 42°C for 60 min in a hot-lid thermal cycler. The reaction was terminated by heating at 70°C for 15 min and purified by a NucleoSpin Extraction Kit (Clontech) following the manufacturer's instructions.

PCR Advantage 2 Mix (9 μL) was prepared as follows: 5 μL of 10X PCR Advantage Buffer (Clontech), 1 μL of 10 mM dNTPs, 100 ng of 5′SMART upper primer (5′-AAGCAGTGGTATCAACGCAGAGTA-3′), 100 ng of 3′SMART lower primer (5′-CGGTAATACGACTCACTATAGGGAGAA-3′), and 1 μL of Advantage 2 Polymerase Mix (Clontech). This mix was added to 41 μL of the first-strand cDNA synthesis reaction product, and thermal cycling was carried out in the following conditions: 95°C for 1 min followed by 19 cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec, and extension at 68°C for 10 min. The cDNA was purified using a NucleoSpin Extraction Kit (Clontech) following the manufacturer's instructions.

We diluted cDNA into 10 ng/μL for PCR detection; 10 ng cDNA was used. The reaction was performed in a final volume of 20 μL of 10 μL 2$\times$ SYBR master (Applied Biosystems, Foster City, CA), 1 μL 10 μM primers, 1 μL cDNA and 8 μL water. The amplification conditions were as follows: preincubation at 50°C for 2 min and DNA polymerase activation at 95°C for 10 min, followed by 40 amplification cycles at 95°C for 15 sec, 55°C for 30 sec, and 72°C for 45 sec. The primers used in this research are listed in Table 4. PCR products were visualized under ultraviolet light on 2% agarose gels (Gibco).

*BrUTP incorporation (*in vitro *transcriptional activity assay)*

The *in vitro* transcriptional activity assay was conducted as described by Aoki et al. (1997). The protocol was slightly modified for bovine embryo specificity. In the plasma membrane penetration step, we used 0.1% Triton X-100 in physiological buffer (PB). Briefly, the following protocol was fol-

Table 1. Preimplantation Development of C/B and B/B SCNT Embryos

				Developmental stages (%)		
Embryos	N	n	*Fused*	*Two-cell*	*8–16-cell*	*Blastocyst*
C/B SCNT	10	1224	776 (63.4)	494 (63.6)	258 (52.2)[a]	0 (0.0)[a]
B/B SCNT	10	465	306 (65.8)	205 (67.0)	153 (74.6)[b]	55 (26.8)[b]

C/B SCNT: chimpanzee/bovine somatic cell nuclear transfer, B/B SCNT: bovine/bovine somatic cell nuclear transfer. *N*: number of replications, *n*: total number of constructed SCNT couplets. Frequency of 8–16-cell and Blastocyst stages were calculated based on the number of cleaved embryos. Different superscripts in the same column indicate statistical significance at $p < 0.05$.

Table 2. Chromosome Composition of Four-Cell Stage B/B and C/B SCNT Embryos

	No. of embryos analyzed	No. of metaphase cells (%)		% Abnormal
		2n	4n	
B/B SCNT	19	18 (95)	1 (5)	5%
C/B SCNT	18	17 (95)	1 (5)	5%

C/B SCNT: chimpanzee/bovine somatic cell nuclear transfer, B/B SCNT: bovine/bovine somatic cell nuclear transfer.

lowed. All treatments were performed at room temperature unless otherwise specified. Embryos were washed in a drop of PB that consisted of 100 mM potassium acetate, 30 mM KCl, 1 mM $MgCl_2$, 10 mM Na_2HPO_4, 1 mM ATP supplemented with 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 50 units/mL of RNase Inhibitor (Promega, Madison, WI), and the plasma membrane was permeabilized by treating embryos for 1 min with 0.1% Triton X-100 in PB. Following this treatment, the embryos were briefly washed three times with PB and then transferred to 100 mM potassium acetate; 1 mM $MnCl_2$; 50 mM $(NH_4)_2SO_4$; 30 mM KCl, 10 mM Na_2HPO_4 containing 2 mM ATP; 0.4 mM each GTP, CTP, and BrUTP; and 1 mM $MgCl_2$. After 30 min of incubation at 33°C, the embryos were washed briefly three times with PB, and the nuclear membrane was permeabilized by a 3-min treatment in PB containing 0.2% Triton X-100. The embryos were then washed in PB three times and fixed for 1 h with 3.7% paraformaldehyde in PB. The incorporated BrUTP was detected by indirect immunostaining with anti-BrdU antibodies. The embryos were washed five times in 15-μL drops of PBS containing 10% normal donkey serum (PBS/NDS) (Jackson ImmunoResearch, West Grove, PA) over a period of 15 min and then incubated for 45 min with PBS containing 2 μg/mL anti-BrdU monoclonal antibody (Boehringer Mannheim, Indianapolis, IN). The embryos were then washed four times with PBS/NDS over the course of 15 min and subsequently incubated in PBS containing 0.5 μg/mL antimouse IgG antibody conjugated with Texas Red (Jackson ImmunoResearch, West Grove, PA) for 1 h. The samples were then washed with PBS/NDS and mounted on glass slides in antibleaching solution (ProLong with DAPI, Invitrogen, CA).

Karyotyping and tunnel detection

Embryos were incubated in demecolcine 400 μg/mL for 12 h before karyotyping. Chromosome preparations were made of four-cell stage embryos using an air-drying method, and were stained with DAPI. Embryos at the four- and eight-cell stages were collected at 34 and 64 h postactivation, respectively; fixed in 4% PF for 15 min and stored at 4°C in PBS-PVA (1 mg/mL) until TUNEL analysis was performed using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche), following the manufacturer's recommendations. Briefly, embryos were permeabilized in 0.1% Triton X-100 and 0.1% sodium citrate for 15 min. Then, positive and negative control embryos were treated with DNase I (50 IU/mL; Promega) at 37°C for 1 h. Sample embryos and positive controls were incubated in microdrops containing the enzyme terminal deoxynucleotidyl transferase and fluorescein-conjugated dUTP at 37°C for 1 h. Negative controls were incubated in the same buffer, but omitting the enzyme. After washing in PBS-PVA, embryos were mounted with an antifade solution containing DAPI (ProLong with DAPI, Invitrogen) and evaluated by epifluorescence microscopy at 400×.

Immunofluoresence of histone H3K27me3, H4K5ac, and 5-methylcytosine

Fixed embryos were washed in Dulbecco's phosphate-buffered saline (DPBS) containing 1 mg/mL of PVA, fixed with 4% paraformaldehyde for 15 min in DPBS (Gibco) and stored at 4°C in DPBS containing 1 mg/mL of PVA. Embryos were washed in DPBS containing 1 mg/mL of PVA, fixed with 4% paraformaldehyde for 15 min in DPBS. Then, embryos were permeabilized in 1% Triton X-100 for 30 minutes at room temperature, incubated with Image-iT FX signal enhancer (Invitrogen) for 30 min, and blocked with 10% normal goat serum for 2 h. After that, the embryos were incubated overnight with the following antibodies: Antihistone H3 trimethyl K27 (Abcam, ab6002) and antiacetyl-histone H4 lys5 (Upstate, 07-327). After 6 h of washing in DPBS containing 0.1% Triton X-100, embryos were incubated with a secondary antibody conjugated with Alexa 594 (Invitrogen) or Alexa 488 (Invitrogen) for 1 h at room temperature. DNA was visualized by Hoechst 33342 staining. For 5-methyl cytosine staining, embryos were treated with 4 N HCl for 1 h at 37°C and then incubated with anti-5-MeCyt antibody (Eurogentec BI-MECY-0100 and Abcam ab1884) for 2 h at room

Table 3. Apoptosis in C/B and B/B SCNT 4- and 8–16-Cell Stage Embryos

	Four-cell			8–16 cell		
Embryo	No. of embryos	No. of TUNEL-positive embryos (%)	No. of embryos with mitotic figures (%)	No. of embryos	No. of TUNEL-positive embryos (%)	No. of embryos with mitotic figures (%)
B/B SCNT	10	0	1 (10)	17	1 (5.9)	7 (41.2)
C/B SCNT	10	0	0	21	1 (4.7)	10 (47.6)

C/B SCNT: chimpanzee/bovine somatic cell nuclear transfer, B/B SCNT: bovine/bovine somatic cell nuclear transfer.

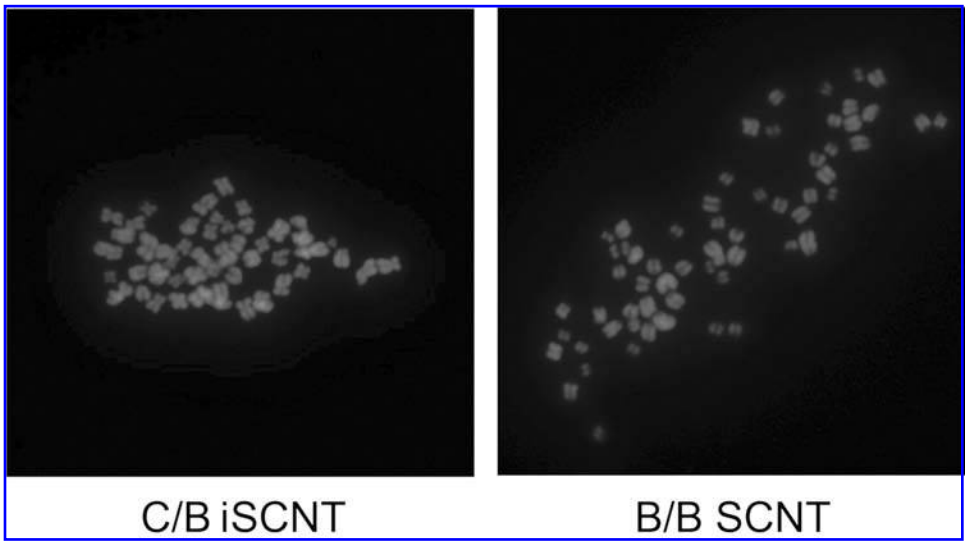

FIG. 1. Representative pictures of karyotype analyses on C/B and B/B SCNT embryos. Left panel: karyotype of C/B iSCNT four-cell embryo; right panel: karyotype of B/B SCNT four-cell embryos. C/B SCNT: chimpanzee/bovine somatic cell nuclear transfer, B/B SCNT: bovine/bovine somatic cell nuclear transfer.

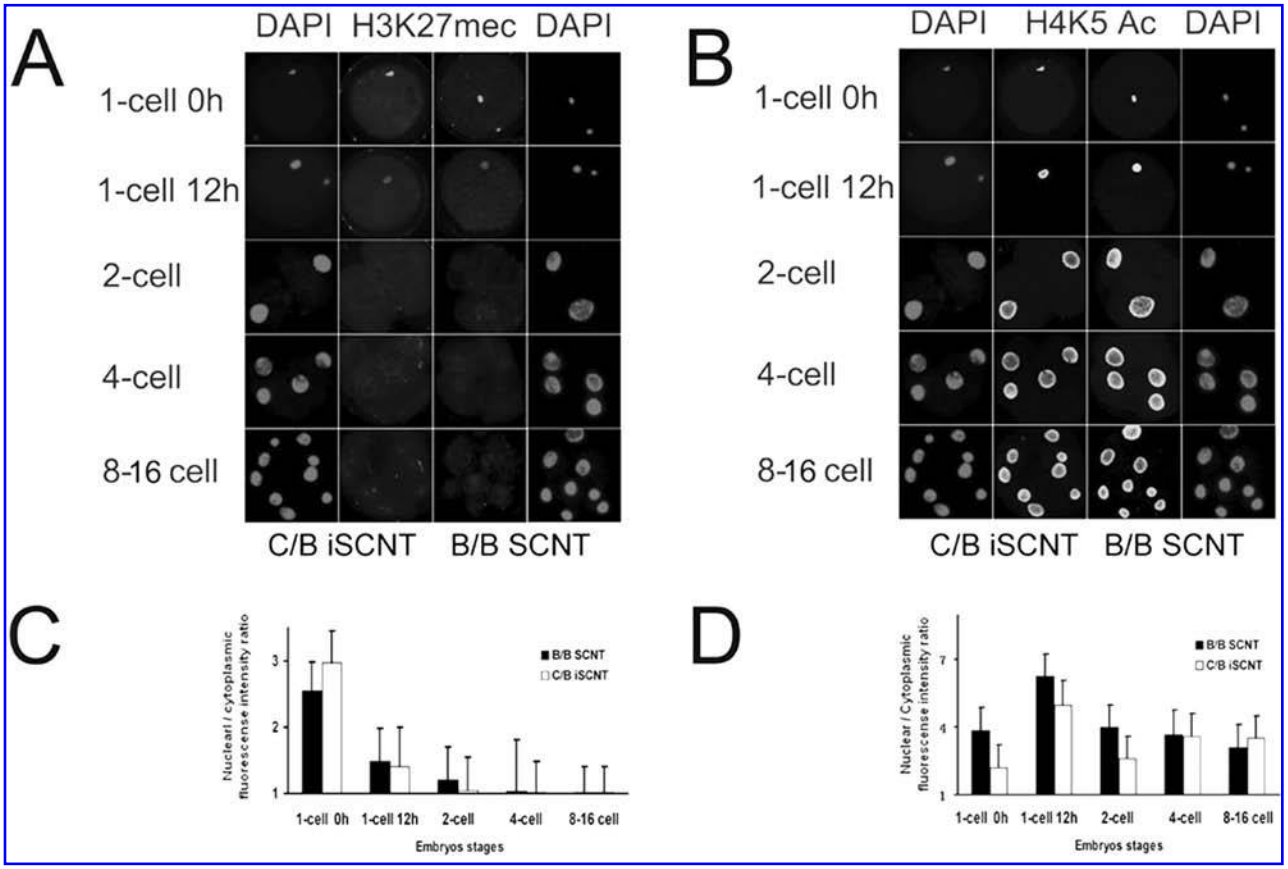

FIG. 2. Histone acetylation and methylation in C/B and B/B SCNT embryos. (**A**) H3K27me3 immunostaining in B/B and C/B SCNT embryos from 1-cell to 8–16-cell stage. Left two columns are images of C/B iSCNT embryos, and the right two columns are B/B SCNT embryos. The red signal is H3K27me3 and the blue signal is DAPI DNA counterstaining. (**B**) H4K5ac immunostaining in B/B and C/B SCNT embryos from 1-cell to 8–16 cell stage. The left two columns are images of C/B SCNT embryos, and the right two columns are B/B SCNT embryos. The green signal is H4K5ac, and the blue signal is DAPI DNA counterstaining. (**C**) Relative levels of H3K27me3 per nucleus (mean ± SEM) in B/B SCNT (black bars) and C/B iSCNT (white bars). (**D**) Relative levels of histone acetylation of H4K5 per nucleus (mean ± SEM) in B/B SCNT (black bars) and C/B iSCNT (white bars). For the analysis of H4K5 and H3K27 16, 12, 10, 5, and 20 embryos were analyzed at 1-cell 0 h, 1-cell 12 h, 2-cell, 4-cell, and 8–16-cell stage for B/B SCNT groups respectively, and 13, 7, 9, 14, and 20 embryos for C/B iSCNT groups. C/B SCNT: chimpanzee/bovine somatic cell nuclear transfer, B/B SCNT: bovine/bovine somatic cell nuclear transfer.

temperature. After washing for 6 h, the embryos were incubated with a secondary antibody conjugated with Alexa 488. For DNA staining, the embryos were incubated in PI (25 μg/mL) for 15 min at 37°C. For imaging, the embryos were mounted in 12 μL of antifade solution and compressed with a coverslip.

Confocal microscopy

The embryos were visualized using a spinning disk confocal system mounted on a Nikon TE-2000 microscope. The optical sections were acquired every micrometer, and images were analyzed using Metamorph software. The nuclei were delineated on maximum projections of all sections to calculate the average pixel intensity. Two cytoplasmic regions per embryo were used as background fluorescence. The average pixel intensity of each nucleus was divided by the average of two cytoplasmic regions.

Statistical analysis

Data analyses for differences in embryonic development and for DNA methylation and histone methylation acetylation, as assessed by the relative signal ratio between the nuclei and cytoplasm area of arbitrary Alexa 594 and Alexa 488 emissions, respectively, were carried out by ANOVA using the GLM procedure of SAS (version 9.0, SAS Institute Inc., Cary, NC). The main effects were embryo type (B/B SCNT or C/B iSCNT), stage of embryo development. The interaction of embryo type and stage also was included in the model. We tested the normality and homogeneity of variance assumptions of all data sets before ANOVA. A p value of <0.05 was considered significant.

DNA methylation analysis by bisulfite sequencing

The embryos or the cells were freshly collected, then kept at −80°C to until detection. Two hundred cells were used perform this assay, and for the eight-cell stage, 15 to 20 embryos were used in each assay, two replicates are performed. The extraction of genomic DNA and bisulfite mutagenesis sequencing analysis were conducted using the ReadyAmp Genomic Kit (Promega, Madison, WI) and EZ DNA Methylation Kit (Zymo Research, Orange, CA), respectively (Yamazaki et al., 2006) according to the manufacturers' instructions. After mutation, DNA was eluted in 15 μL elution buffer, and used for two successive PCR rounds of PCR with nested primer (outer and inner) pairs. The PCR products were verified by running on a 2% agarose gel. Then, the PCR products were ligated into the pTopo vector system (Invitrogen) for sequencing.

Measurement of net cytoplasmic ATP content

Two commercial assay kits (ATP Detection Kit, Roche and somatic cell assay kit, Sigma) based on the luciferin–luciferase reaction were used, according to the manufacturer's instructions and a protocol previously described for bovine oocyte ATP detection (Rieger, 1997).

For ATP Detection Kit from Roche, a single embryo was added to 50 μL dilution buffer. Next, 50 μL lysis buffer was added and a sample was kept at room temperature for five minutes. After mixing 100 μL lysate with 100 μL luciferin–luciferase, 10 sec measurements were performed in Berthold Lumat LB 9501 Luminometer (Berthold Technologies, Bad Wildbad, Germany) (Van Blerkom et al., 1995). A standard curve, containing 11 ATP concentrations from 10 fmol to 10 pmol, was generated for data analysis. The ATP content was calculated using the formula derived from the linear regression of the standard curve.

For the ATP Detection Kit from Sigma, a single embryo was added to a 50-μL dilution buffer. Samples were frozen in liquid nitrogen and stored at −80°C. When all samples were collected, they were thawed all at once and 50 μL of dilution buffer was added to each one of them along with 100 μL ATP releasing buffer. Samples were kept on ice for 5 min. Subsequently, 100 μL of the original lysate was mixed with 100 μL luciferin–luciferase samples were left in room temperature for 5 min. Measurements were performed as described above.

Sigma detection kit was used in one-cell, two-cell, and four-cell stage embryos. Roche kit was used in 8–16-cell stage embryos. Our intention was to use only one source throughout the experiment however Roche discontinued its product before we finished our tests. For this reason, our statistical analysis was performed within groups (B/B vs. C/B) and not between groups.

Results

Development of iSCNT embryos and SCNT embryos

Enucleated bovine oocytes were fused either with chimpanzee or bovine fibroblasts. Out of 306 fused bovine–bovine SCNT embryos, 205 were cleaved (67.0%). Of these successfully cleaved embryos, 153 developed to the 8–16-cell stage (74.6%) and 55 to blastocyst stage (26.8%). A total of 1224 C/B SCNT embryos were reconstructed (10 replicates), of which 494 cleaved (63.6) and 258 developed to the 8–16-cell stage (52.2%). No iSCNT embryos developed to the blastocyst stage (Table 1). Fusion and cleavage rates prior to the 8–16-cell stage were similar between C/B and B/B SCNT embryos.

Genetic stability of donor nuclei in iSCNT embryos and SCNT embryos

In an effort to ascertain possible reasons for the developmental block at the 8–16-cell stage in C/B SCNT embryos, we first looked at the genome stability of interspecies donor nuclei. This was evaluated by karyotype analyses and apoptosis assays because it is possible that chromosomal segregation cannot occur as expected in same-species SCNT embryos, impairing development and triggering programmed cell death. Ninety-five percent (percent of total metaphase nuclei counted) of chimpanzee nuclei had a normal chromosome number in iSCNT embryos ($2n$; $n = 46$), and this was not different from bovine SCNT embryos. The incidence of embryos with apoptotic cells, measured by TUNEL assay, was 5% (percent of total nuclei), in line with the results obtained from same species SCNT embryos. When iSCNT embryos were collected at 64 h postactivation for analysis, we observed 41% of nuclei in metaphase, indicating that, at least at this time point, blastomeres within iSCNT embryos had no sign of apoptosis and were still undergoing cell division (Tables 2 and 3, and Fig. 1).

Epigenetic reprogramming in iSCNT embryos and SCNT embryos Histone acetylation and methylation

While hyperacetylation of histones could facilitate the access of transcription factors to the nucleosomes and facilitate RNA transcription, methylation of specific histone residues such as H3K27 trimethylation (H3K27me3) triggers the opposite effect (Lee et al., 1993). We measured the levels of histone acetylation and methylation in SCNT embryos immediately after fusion of the somatic cell and oocyte, and at the 1-, 2-, 4-, and 8–16-cell stages. Overall, we observed similar patterns and levels of two histone modifications in the nuclei of C/B and B/B SCNT embryos.

Histone H4 is acetylated at lysine 16, lysine 8 or 12, and lysine 5 in a sequential fashion (O'Neill and Turner, 1995). Therefore, it has been suggested that acetylation of H4K5 (H4K5ac) corresponds to a hyperacetylated state, and represents a transcriptionally permissive chromatin conformation (Turner, 2000). The profile of H4K5 acetylation was compared between iSCNT and SCNT embryos at different stages starting from 1 h postfusion to 8–16-cell stage. Immunofluorescent signals for H4K5ac appeared in all embryonic stages, and a similar pattern of expression was observed in iSCNT and SCNT embryos. For the average intensity of H4K5ac signal in nuclei, the signal intensity increase at 1-cell stage (12 h) and later decrease at the 2-cell stage, and remained constant through the 4- and 8–16 cell stages.

Embryonic and stem cell related genes are silenced in differentiated cells by H3K27me3 (Lee et al., 2006). Thus, H3K27me3 represents a transcriptionally inhibitory chromatin state. We sought to determine the levels of H3K27me3 in iSCNT and SCNT embryos via immunostaining. In both iSCNT and SCNT groups, the level of H3K27me3 was greatest in newly fused embryos and declined dramatically at 12 h postfusion. In 2-cell embryos the signal was barely detectable, only to be completely lost in blastomere nuclei by the 4-cell through 8–16-cell stages (Fig. 2).

DNA methylation

C/B and B/B SCNT embryos were immunostained with antibodies against 5-methylcytosine (Fig. 3). In both types of SCNT embryos, the relative levels of DNA methylation per nuclei decreased from the 1-cell stage to the 8–16-cell stage.

Oct4 and Nanog demethylation in iSCNT and SCNT embryos

We analyzed the DNA methylation pattern at specific promoter regions of Oct4 (also named POU5F1) and Nanog using a bisulfite mutagenesis sequencing method. First, we determined differentially methylated regions (DMR) of the Oct4 and Nanog promoters in chimpanzee. Figure 4A shows a comparison of the DMRs in chimpanzee and human promoters of Oct4 and Nanog (Freberg et al., 2007). Coincidentally, the Nanog gene DMR sequence in chimps and humans is the same, having four CpG islands suitable for analysis. Humans have eight CpG islands in the Oct4 DMR while chimps have only six. For the bovine Oct4 gene, we validated the DMR in Intron 1, which was previously described by others (Kremenskoy et al., 2006). For bovine NANOG, we determined the proximal promoter region of the gene and designed bisulfite primers (Table 4) with the help of Methprimer software (http://www.urogene.org/methprimer).

For this study, DNA from both chimpanzee and bovine fibroblasts (~200 cells), as well as 8–16-cell stage SCNT embryos (15–20) was collected. The pattern of DNA methylation in the promoter regions of donor fibroblasts was compared with those in B/B SCNT embryos. Likewise, promoter DNA methylation in chimpanzee fibroblasts was compared with that in C/B 8–16-cell embryos. In bovine donor cells, Oct4 and Nanog DMR methylation was found to be 34 and 30%, respectively. In the resulting reconstructed SCNT embryos, methylation decreased from 34 to 1% for Oct4, and 30 to 21% for Nanog. In chimpanzee donor cells, Oct4 and Nanog DMRs showed 60 and 46% methylation, respectively. After iSCNT, Oct4 and Nanog methylation decreased to 42 and 32% in 8–16-cell stage, respectively. While the similar pattern of demethylation of the Nanog DMR happened in iSCNT (46 to 32%) and SCNT (30 to 21%), Oct4 demethyla-

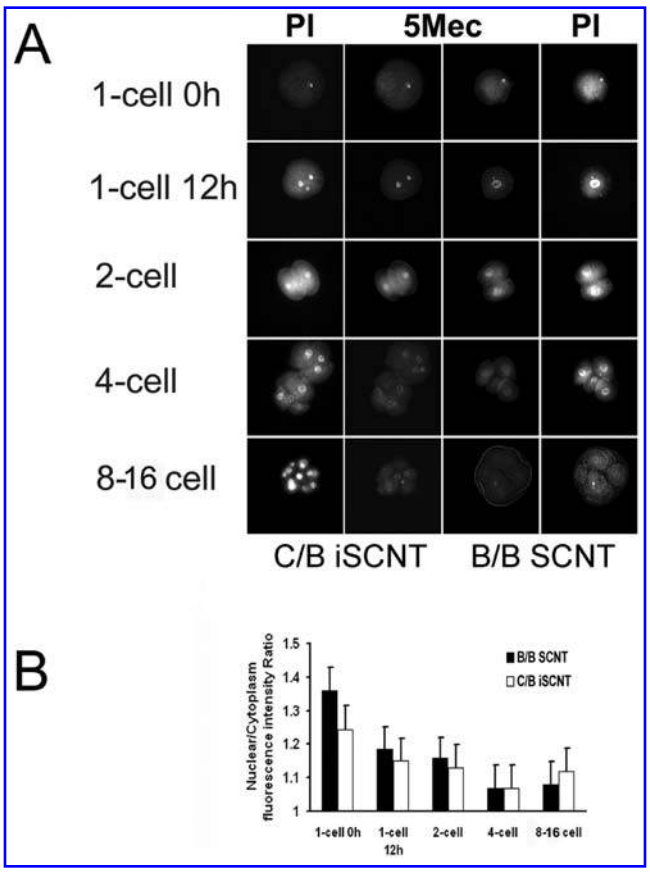

FIG. 3. Global DNA demethylation in C/B and B/B SCNT embryos. (**A**) Representative images of C/B and B/B SCNT embryos from the 1- to 8–16 cell stage. The right two columns are B/B SCNT embryos, and the left two columns are C/B iSCNT embryos. 5-Methylcytosine (5MeC) was detected by immunofluorescence (green) and counterstained with PI (white). (**B**) Relative DNA methylation level per nucleus (mean ± SEM) in B/B SCNT (black bars) and C/B iSCNT (white bars) embryos. Twelve, 14, 12, 12, and 12 embryos were analyzed at 1-cell 0 h, 1-cell 12 h, 2-cell, 4-cell, and 8–16 cell stage for B/B SCNT groups, respectively, and 10, 15, 11, 10, and 12 embryos for C/B iSCNT groups. C/B SCNT: chimpanzee/bovine somatic cell nuclear transfer, B/B SCNT: bovine/bovine somatic cell nuclear transfer.

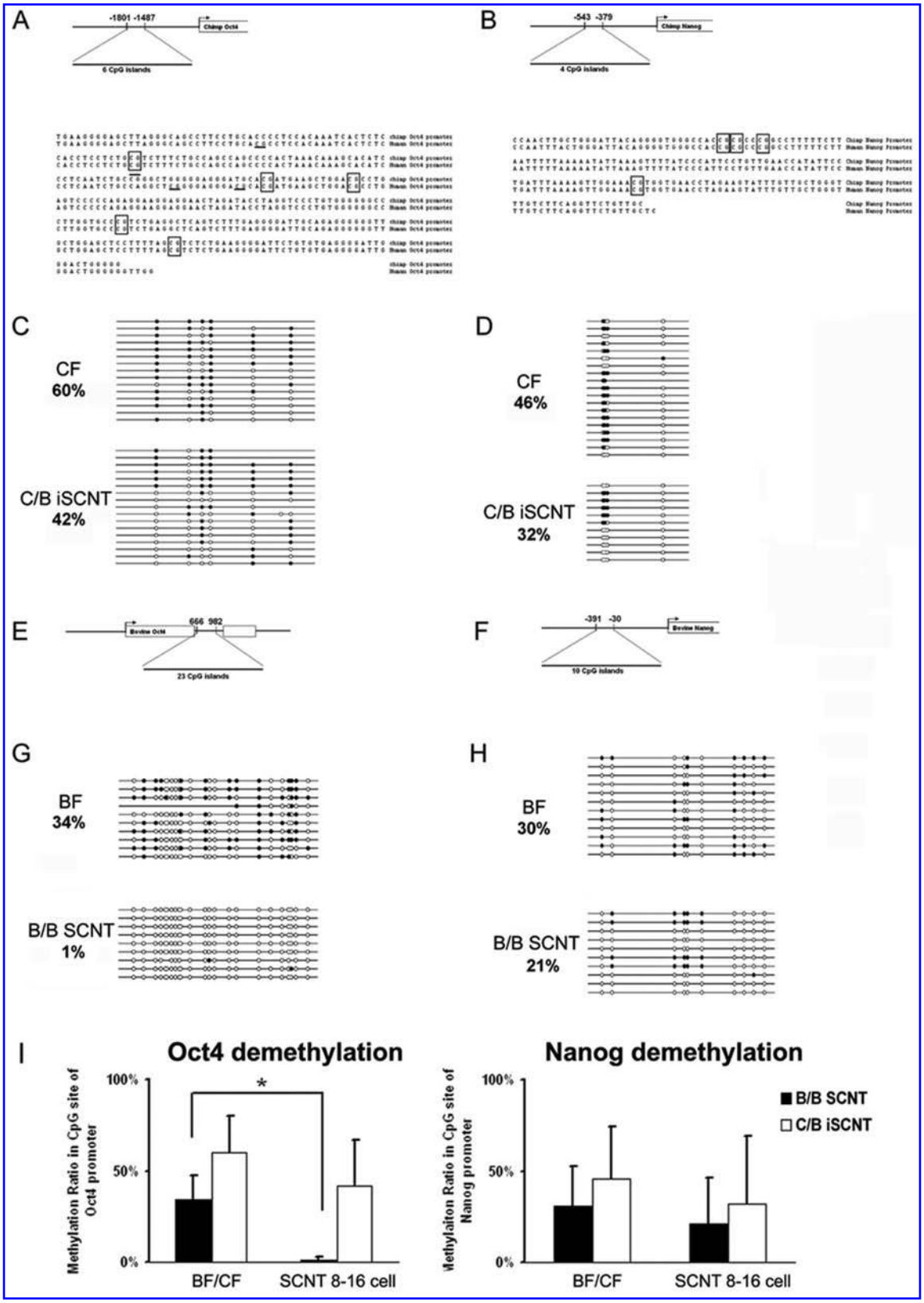

FIG. 4. Demethylation of Oct4 and Nanog promoter DMRs in C/B and B/B SCNT 8–16-cell embryos. (**A, B**) CpG site location in chimpanzee Oct4 and Nanog gene and the comparison between human and chimpanzee CpG sites in this region. (**C, D**) Analysis of DNA methylation of chimpanzee Oct4 and Nanog in donor cell (chimpanzee fibroblast, CF) and 8–16 cell iSCNT embryos by bisulfite sequence. Each line represents an independent clone, and open and closed circles represent nonmethylated and methylated CpGs, respectively. (**E, F**) CpG site location in bovine Oct4 and Nanog gene. (**G, H**) Analysis of DNA methylation of bovine Oct4 and Nanog in donor cell (bovine fibroblast, BF) and 8–16-cell SCNT embryos. (**I**) Total methylation levels in B/B SCNT (black bars) and C/B iSCNT (white bars) embryos are shown (mean ± SEM). Denotes $p = 0.00006$. C/B SCNT: chimpanzee/bovine somatic cell nuclear transfer, B/B SCNT: bovine/bovine somatic cell nuclear transfer.

Table 4. Primers for RT-PCR

Gene	Primer sequences	Accession number	Amplification region	Length of PCR product (bp)
Bovine OCT4	GGCAAACGATCAAGCAGTG TAATCCCAAAGGCCTGGTAC	NM_174580	1153–1470	317
Bovine NANOG	GTGTTTGGTGAACTCTCCTG GGGAATTGAAATACTTGACAG	NM_001025344	715–1022	307
Bovine GLUT 1	TCCGGCAGGGAGGAGCAAGT TGCTGAGATCTATCAGTTTGAG	NM_174602	1570–1747	177
Bovine GAPDH	CACTCACTCTTCTACCTTCGAT TGTGGCGGAGATGGGGCAGG	BC102589	932–1157	225
Bovine DSC2	TGTTGCAGCGAACGACAAG CCGCAAGTGTCCTAAATTTGG	XM_615164	2572–2647	75
Bovine STELLA	ATGGATTCATCAGAAGATAACCCAACCTG CTCACTAGGTAACTGCCCAGTC	EU143548	(−65)–155	220
Bovine USF2	GCCCGGTTTGCCTATTTCCC AATTGTCCTCTGCGTTCCTGTCTG	NM_001001162	493–642	150
Bovine PTGER4	TGGTCATCTTACTCATCGCC AGGATGTATATCCAGGGGTC	NM_174589	832–1015	173
Bovine CRABP1	ATGCGCAGCAGCGAGAATTTC CACCGTGGTGGACGTCTTGATG	NM_18102	315–464	149
Bovine CCNE2	CTGCCTTATGCCACTTTACC TGTGTCTGTATGTTGTGTCTGTCT	NM_001015665	941–1124	183
Bovine CXCL5	TGTGAGAGAGCTGCGTTGTGTGTG TCTGGGTCCAGACAGACTTC	NM_174300	175–330	155
Bovine ADRBK1	ATGTCAACCGGAGGCTAGGCTG AAAGGCGTCGGCTGCATTCAC	NM_174710	1379–1533	154
Bovine H2AFZ	TCAAGGCTACAATTGCTGGTGGTGG CACTGGAATCACCAACACTG	NM_174809	346–504	158
Bovine Myc	CGAGCTACTAGGAGGCGACATGGTG TGGTAAGAGGCCAGCTTCTCCGAG	NM_001046074	721–870	150
Bovine KLF4	GGAAAGCACTACAATCATGG CCTTGGCGTTTTGTAAGTC	NM_001105385	1531–1720	189
Bovine Lin28	AAGGGAAGAATATGCAGAAAC TCAGTGGGCACGAAGCTAC	XM_866673	362–517	155
Human Oct4	TTTCTCAGGGGGACCAGTG TTTTCTTTCCCTAGCTCCTC	NM_002701	966–1195	229
Human Nanog	GCCTTAATGTAATACAGCAG TTGACTGGATAGGCATCATG	NM_024865	1034–1271	237
Human Stella	ATGGACCCATCACAGTTTAATCCAACC TTATGGCTGAAGTGGCTTGGTGTCTTGATTCCC	NM_199286	79–558	479
Human Glut1	CAGCAAGAAGCTGACGGGT ACAGAAAAGATGGCCACTGAG	NM_006516	191–400	209
Human GAPDH	TGGCCTCCAAGGAGTAAGAC CTCTTCAAGGGGTCTACATGG	NM_002046	1094–1248	154
Human DSC2	TCTGCGAATGGCCTTCACAAC ATCCCACGGTGCCACAAG	NM_004949	2697–2763	66
Human USF2	CCTTAGCACAGAGAGGGACAC TGAGACCACTAGAAGTGCCG	NM_003367	1213–1420	207
Human PTGER4	ATCTAAGCAGCTTATTGTTTCTC TTAAGTGCCCCAATTCTGC	NM_000958	3009–3201	190
Human PTGFR	AAAATTCTGTTGAGAGCAGGT TCTCAATTATTATTGGAAAATTTG	NM_000959	2348–2525	167
Human CRABP1	GACCGCCCTTTTCCCCTAC GTCTAACCAGTTTAATGACTTCG	NM_004378	566–725	159
Human CCNE2	AAAAAAAGACATGAAAACTTGC AATGTATAGTTTCTTCAAAGGG	NM_057749	2371–2541	170
Human CXCL5	TATTTGTTTTTCCAGTAGTTAGC ACACTATAGTCAATTGCCAAAAC	NM_002994	575–755	180
Human ADRBK1	CTCCCAGTGTCTTCCTGTG AAAATTCAAGTCGGATCCC	NM_001619	2555–2736	181
Human H2AFZ	GATGAGCAATCCGAGTTCC GAAAAGGCTAATCGGACCCAC	NM_002106	9–149	140
Human C-Myc	ATGTAAACTGCCTCAAATTGG ATTCTGTGTAACTGCTATAAACG	NM_002467	2021–2213	192
KLF4	TCCAAAAGACAAAAATCAAAGA	XM_520171.2	2194–2356	162 bp
Lin28	CCCTCCCCCAACTCACGGATA CCAGCAGGGCCCTAGTGCAC CTATTCCCACTTTCTCCACTC	XM_513232.2	578–750	180 bp

Table 5. Primers for Bisulfite-PCR

Gene		Primer sequences	Accession number	Amplification region	Length of PCR product (bp)
Bovine OCT4	Out F	GGGTGGAGAGTAATTTT(C)GAGGG	AF022986	3322–3638	316
	In F	GAAGTTGGATAAGGAGAAGTTGGAG			
	In R	AATAAAAAAACCTACTTAACAAAAACC			
	Out R	TAATACTAACTAATAATAAATAACC			
Bovine NANOG	Out F	AGAGTGAATTAAAGAGGAAAATGG	NC_007303.2	(−391)–(−30)	361
	In F	GTAGTTTTTGTTATAAATTAGTTTGA			
	In R	AAATAAAACTCAACCATACTTAACC			
	Out R	TATAAAAATAAAAACCATCCAATCCA			
Human OCT4	Out F	GTAGTATGAGTTTTAGGATATTTAGG	AJ297527	1403–1717	314
	In F	TGAAGGGGAGTTTAGGGTAGT			
	In R	CCAACCCCCCAATCCCAATCC			
	Out R	CTTAAATAAACCTAAATAACTCC			
Human Nanog	Out F	GTTTTAATTTTTGATTTTAGGTGA	ENSG00000111704	(−543)–(−379)	164
	In F	TTAATTTATTGGGATTATAGGGGTG			
	In R	AACAACAAAACCTAAAAACAAACCCA			
	Out R	AAAATAACTACAAAATAACCCAAAC			

tion in iSCNT was incomplete and different from that of bovine–bovine SCNT (42 vs. 1%, respectively) (Fig. 4).

Gene expression in C/B and B/B SCNT embryos

The expression of developmentally relevant genes was investigated at the 8–16-cell stage SCNT embryos. Transcript abundance of 16 genes was monitored using RT-PCR (primers are listed in Table 4). B/B SCNT 8–16-cell stage embryos showed robust expression of all 16 genes (Glut1, Stella, Nanog, Oct4, DSC2 , USF2, Crabp1, Adrbk1, CCNE2, CXCL6, PTGER4, H2AFZ, c-MYC, KLF4, Lin28, and GAPDH). However, C/B 8–16-cell stage iSCNT embryos showed expression of only Stella, Oct4, Crabp1, CCNE2, CXCL6, PTGER, H2AFZ, c-MYC, KLF4, and GAPDH (Fig. 5).

BrUTP incorporation

In this study, we used BrUTP incorporation to quantify transcription levels in C/B SCNT and B/B SCNT eight-cell embryos. Figure 6 shows a representative image of BrUTP incorporation in a SCNT embryo. Relative quantification revealed that C/B SCNT eight-cell embryos have only 70% of the transcriptional activity of B/B SCNT counterparts ($p = 0.016$).

ATP production in SCNT embryos

Mitochondria are a major source of ATP synthesis in embryos, and most enzymes related to energy production come from the ooplasm in SCNT embryos (Stojkovic et al., 2001). Thus, a functional crosstalk between the donor nuclei and recipient ooplasm must be established to guarantee normal metabolism following interspecies SCNT. ATP levels in the one-cell, two-cell, and four-cell stage of SCNT and iSCNT embryos ranged between 2.8 and 2.5 pMol/embryo. In the four-cell stage, a significant drop of ATP level was observed in both iSCNT and SCNT embryos, 2.1 and 2.2 pMol/embryo, respectively. At the 8–16-cell stage, B/B SCNT embryos had significantly higher ATP levels (20%) than C/B iSCNT embryos. ATP levels in bovine–bovine and C/B SCNT embryos were 1.22 and 1.0 pMol/embryo, respectively ($p = 0.008$) (Fig. 7).

Discussion

Can the nucleus of one species completely reprogram its gene expression profile when introduced into the oocyte of a different species? If so, to what extent this is possible? In theory, oocytes should have highly conserved capacity to perform such tasks; however, in practice, it has been found that only iSCNT embryos from closely related species can develop to term (Uhm et al., 2007). When evolutionary distant species are used, only preimplantation development can be achieved (Beyhan et al., 2007). The only reported interspecies nuclear transfer ES study used a rabbit/human model (Chen et al., 2003), It is thought that the closer evolutionary relationship between human and rabbit in terms of multiple gene loci (Wildman et al., 2007) might have contributed to fortunate outcome of the study. The recent announcement by the UK parliament and the Human Fertilization Embryology Authority allowing the use of nonhuman oocytes to attempt reprogramming of human nuclei, had renewed the interest on performing these experiments. Two groups have announced their plans to conduct these studies; one of them will use bovine oocytes http://www.hfea.gov.uk/en/1581.html. Our study thoroughly explore the extent to what a nucleus from a primate somatic cell can be reprogrammed by a bovine oocyte. We have found that although iSCNT embryos do not progress beyond the 8–16-cell stage, some aspects of early embryonic development are recapitulated, including the expression of some embryonic genes.

To our knowledge, bovine is one of the most utilized animal models to study SCNT. The availability of oocytes and the breadth of knowledge in preimplantation development

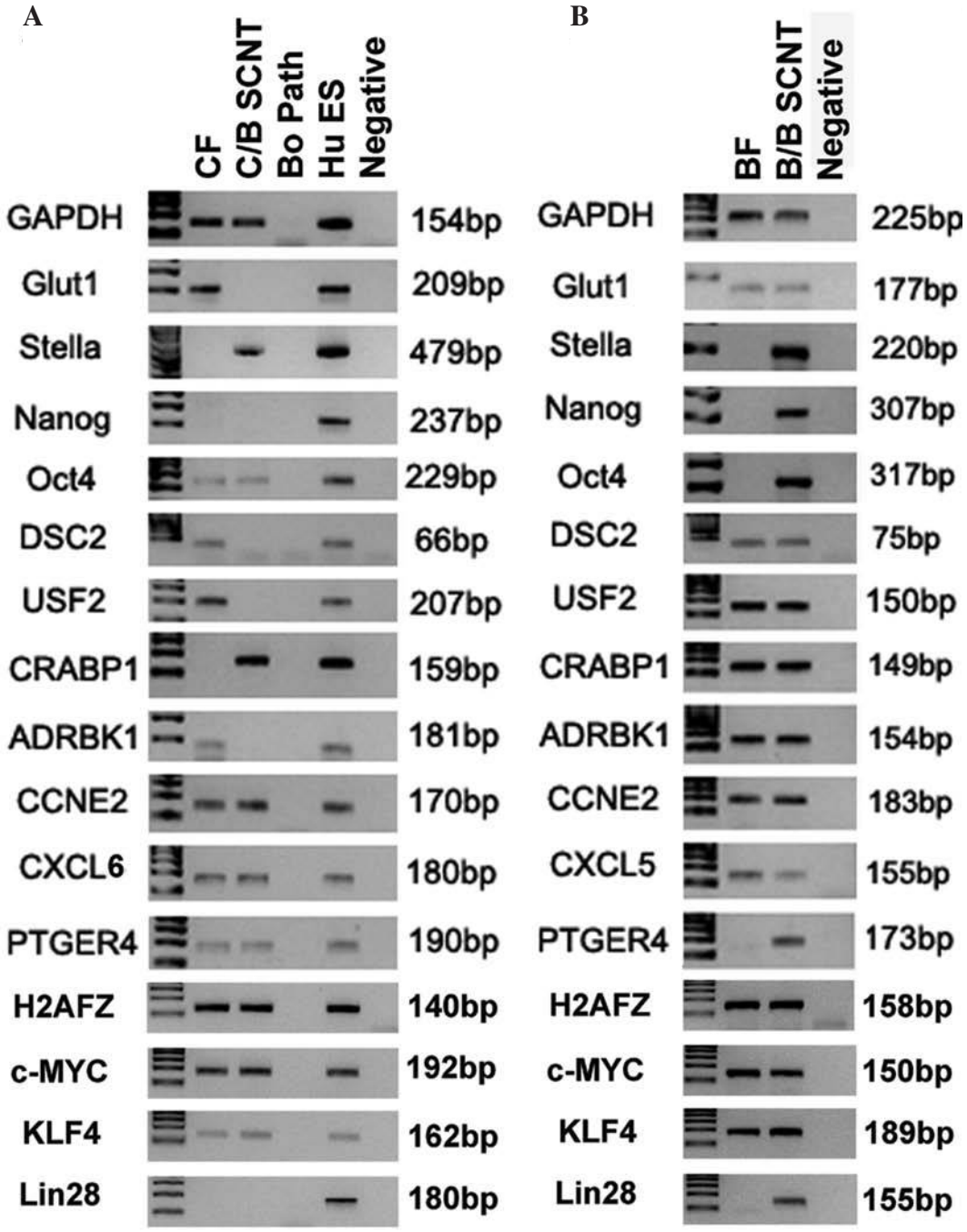

FIG. 5. mRNA expression of developmentally important genes in C/B and B/B SCNT 8-16 cell embryos, and bovine and chimpanzee donor cells. (**A**) mRNA expression in chimpanzee donor fibroblast (CF) and C/B iSCNT 8–16-cell stage embryos. The loading order of the gel is 100-bp DNA marker, CF, C/B iSCNT 8–16-cell embryos, bovine parthenogenetic 8–16-cell stage embryos (Bo Path), human ES cell (H9) (Hu ES), and negative control. (**B**) mRNA expression in bovine donor fibroblast (BF) and B/B SCNT 8–16 cell stage embryo. The loading order of the gel is 100-bp DNA marker, BF, B/B SCNT 8–16-cell stage embryos, and negative control. C/B SCNT: chimpanzee/bovine somatic cell nuclear transfer, B/B SCNT: bovine/bovine somatic cell nuclear transfer.

made this species an obvious choice for interspecies SCNT experiments. Considering that chimps are the closest species to humans (Culotta, 2005), we performed C/B iSCNT and used B/B SCNT as control. We hypothesized that enucleated bovine oocytes would be capable of taking epigenetic control of the chimpanzee somatic cell nuclei and reprogramming them into embryonic nuclei. Based on the parameters assessed, we found that C/B iSCNT embryos develop similar to B/B SCNT embryos from the one-cell to the eight-cell stage. However, C/B iSCNT embryos arrest at the 8- to 16-cell stages. We have evaluated a number of experimental parameters such as nuclear transfer procedure, oocyte activation, and embryo culture conditions to overcome developmental arrest, but none improved the development of C/B

SCNT embryos (unpublished data). Our results were consistent with previous mouse/bovine iSCNT studies that also showed mouse/bovine iSCNT developing to the eight-cell stage (Park et al., 2004).

In an attempt to identify the cause(s) of C/B iSCNT embryo developmental failure, our experimental approach included looking for evidence in the processes of epigenetic reprogramming, EGA defects, and/or energy supply deficiency.

The genetic stability of the genome is a prerequisite for proper development of nuclear transfer embryos. When the nucleus of a donor cell is injected into an enucleated oocyte, it must integrate into the ooplasm structure and faithfully recapitulate the complex and dynamic process of cytokinesis in a way that resembles that of the gametes, this include among other events, normal chromosome alignment and segregation during the first cell cycles. This is process is prone to errors. In 2003, following monkey SCNT, a group reported that the donor nuclei could not form a functional spindle, and the lack of development beyond the eight-cell stage was attributed to aberrant chromosome segregation (Simerly et al., 2003). Our study found comparable ratios of normal karyotype (95%) in cells of B/B SCNT and C/B iSCNT embryos. These results are similar to previously published data in bovine (Hanada et al., 2005) and suggest that chimpanzee donor nuclei can form functional spindles and separate properly when introduced to bovine ooplasm.

Genomic instability is known to trigger apoptosis, which contributes to embryo loss or developmental arrest. It has been reported that more apoptosis occurs in SCNT embryos than in IVF embryos (Hao et al., 2003). Our study compared 4- and 8–16-cell stage B/B and C/B SCNT embryos. Their apoptosis rates were comparable, being 4.7 and 5.9% of the cells counted, respectively. This by no means rules out the possibility that subtle mutations in the genome have taken place; however, it deemphasize the role of apoptosis in the developmental block of iSCNT embryos.

Bovine ooplasm partially reprograms epigenetic marks in chimpanzee donor nuclei in a global and gene-specific manner

To trace dynamic changes in histone modifications in iSCNT embryos, we chose to monitor histone acetylation and methylation by H4K5ac and H3K27me3 immunochemistry. Our study found that, from the 1-cell stage to the 8–16-cell stage, H4K5ac levels remained stable in both types of SCNT embryos. The observation that chimpanzee histone 4 at the lysine 5 residue remained acetylated up to 8–16-cell stage, could be attributed to the presence of maternal histone acetyl transferases. Alternatively the chimpanzee nuclei may have remained transcriptionally active and the enzyme with all its subunits was actively transcribed and translated into functional proteins in the bovine oocyte cytosol. Regarding H3K27me3, we observed that, at the one-cell stage, histone methylation is relatively high and decreases dramatically to undetectable levels at the four- and eight-cell stages. Both SCNT groups were similar as to this trend. These results suggest that changes in H3K27me3 may share a close relationship with EGA events in bovine embryos. Taken altogether, our findings show that the bovine ooplasm possesses the ability to dynamically regulate posttranslational histone modifications in bovine and chimpanzee donor nuclei.

DNA methylation, another epigenetic modification, is essential for mammalian development (Reik, 2007). It is well documented that SCNT embryos undergo DNA demethylation, a process that has been described as a prerequisite for cloned embryos to develop successfully into healthy animals (Kikyo et al., 2000). In this study, we used immunostaining to determine the global DNA methylation (5-MeC) profile of one- to the eight-cell stage SCNT embryos. In addition, we employed bi-sulfite sequencing to evaluate the methylation status of DMRs on embryonic gene promoters in similar embryos.

5-MeC immunostaining results revealed that, in both SCNT groups, DNA methylation was greatest in the newly fused nuclei of SCNT embryo. Then, from the pronuclear through the two- and four-cell stages, the signal decreased and remained low until the eight-cell stage (Fig. 3). Our results are consistent with data reported by others, which claimed that 5-MeC levels decreased to a nadir from the one-cell to the eight-cell stages, and then increased in the morula and blastocyst stage bovine SCNT embryos (Suteevun et al., 2006). These results indicate a common trend in SCNT and iSCNT embryos from the one- to eight-cell stages (Fig. 8), and suggest that global demethylation depends on the ooplasm but not on the species of the donor nucleus. These findings are similar to that of Chen et al. (2006) compared rabbit/pig and pig/rabbit SCNT embryos and found that the DNA demethylation patterns depended on the species of the oocyte.

Global demethylation likely aids the somatic genome in acquiring an embryonic state. Specific demethylation of promoters of embryonic genes is also essential for its reactivation of such genes; among them, Oct4 and Nanog are two genes required for proper embryonic development and for regaining pluripotency (Aoi et al., 2008; Lowry et al., 2008; Meissner et al., 2007; Nakagawa et al., 2008; Okita et al., 2007; Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007). However, in the bovine SCNT system, there is no data available regarding Oct4 and Nanog promoter methylation control in early embryonic development. In the mouse SCNT model, Yamazaki et al. (2006) reported that the Oct4 promoter is gradually demethylated during the first cell cycles and failure to do so is associated with impaired development of the cloned embryos. We observed that, in B/B SCNT 8–16-cell stage embryos, the Nanog and Oct4 gene promoter DMRs are partially and completely demethylated, respectively (relative to that in bovine donor fibroblasts). On the other hand, in eight-cell C/B iSCNT embryos, the Nanog and Oct4 promoters DMRs have undergone partial demethylation in both genes, compared with that in chimpanzee donor cells (Fig. 4). Although the trend of global demethylation seems to be similar in both species genomes, only in bovine–bovine SCNT a complete demethylation of the Oct4 promoter was observed. Taken together, our results indicate that bovine ooplasm cannot recapitulate the DNA demethylation events observed in the same species SCNT. In turn, this could potentially have a significant effect on transcription of chimpanzee specific transcripts in the iSCNT embryos.

This evidence shows that although the bovine ooplasm is capable of globally modifying epigenetic marks in chimpanzee donor nuclei, such modifications fall short from mimicking those observed in the same species SCNT embryos.

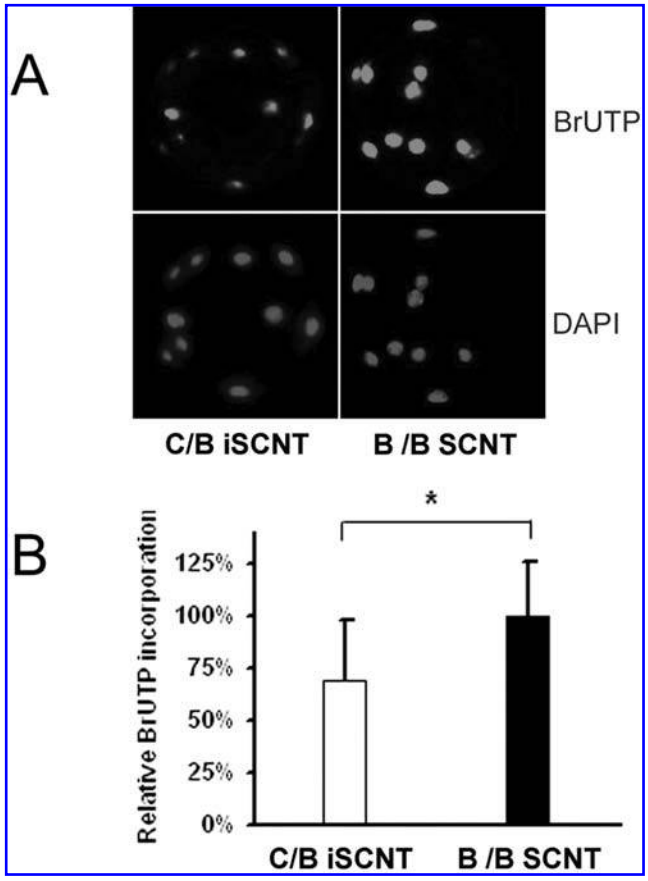

FIG. 6. BrUTP incorporation and immunostaining in C/B and B/B SCNT 8–16-cell embryos. (**A**) Representative images of C/B and B/B SCNT 8–16-cell embryos. First row, red signal indicates incorporated BrUTP, and second row, blue signal is DAPI counterstaining. (**B**) Relative BrUTP incorporation level per nucleus (mean ± SEM) in B/B SCNT (black bars) and C/B SCNT (white bars) embryos. The signal in B/B SCNT 8–16-cell embryos is set as 100%. The number of embryos evaluated per group ranged from 15 to 18. *Denotes $p = 0.016$. C/B SCNT: chimpanzee/bovine somatic cell nuclear transfer, B/B SCNT: bovine/bovine somatic cell nuclear transfer.

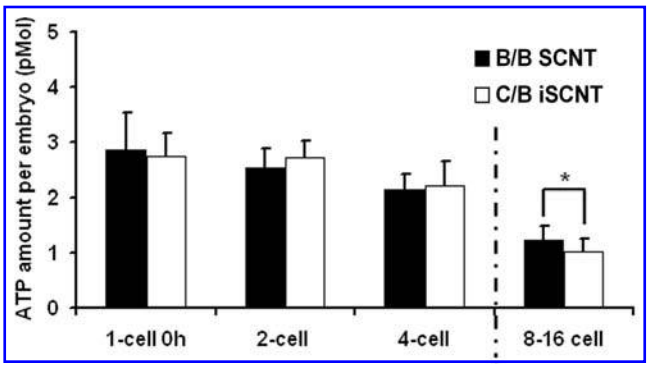

FIG. 7. Relative ATP levels in C/B and B/B SCNT embryos by the luciferin–luciferase reaction-based ATP bioluminescent kit. ATP amount per embryos (mean ± SEM) in B/B SCNT (black bars) and C/B iSCNT (white bars) embryos is showed. The number of embryos per group ranged from 16 to 33. At one-cell, two-cell, and four-cell stage, ATP in embryos was measured using ATP bioluminescent somatic cell assay kit (FL-ASC, Sigma). At the 8–16-cell stage, ATP in embryos was measured by ATP bioluminescent kit (Roche). C/B SCNT: chimpanzee/bovine somatic cell nuclear transfer, B/B SCNT: bovine/bovine somatic cell nuclear transfer. *Denotes $p = 0.008$.

EGA and gene expression in iSCNT embryos

EGA is a critical event for the onset of transcription and the transfer of developmental control from the maternal RNA and protein stored in the cytosol to the nuclei of the embryo. During this period of time, the embryonic nuclei start transcribing a series of specific transcripts that are essential for pluripotency, energy metabolism, for cell differentiation—ICM and trophectoderm differentiation—and an array of ubiquitous physiological events, that is, cell cycle progression and protein metabolism. EGA in the bovine occurs at the eight-cell stage, while in humans it takes place between the four- to eight-cell stage (unfortunately, no data in chimpanzee is available) (Memili and First, 1998). We hypothesized that iSCNT embryos fail to progress beyond the 8–16-cell stage due to a failure of reactivation of the embry-

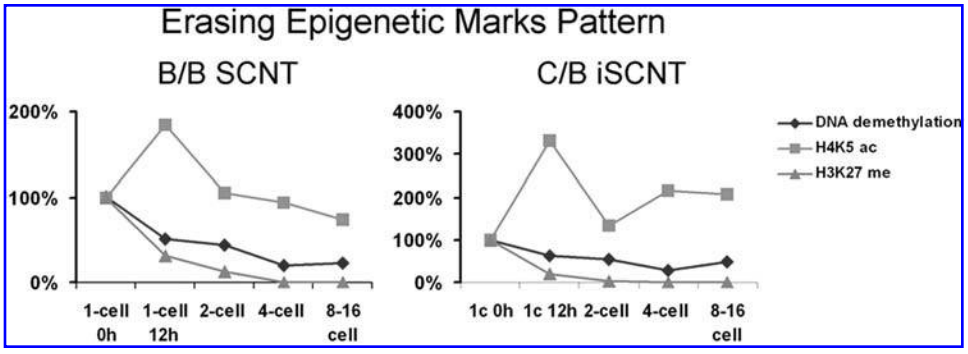

FIG. 8. Summary of global epigenetic changes from the time of cell fusion with the enucleated oocyte until the 8–16-cell stage SCNT embryo. Expressed in relative values setting the signal intensity of the one cell embryo as 100%. Following SCNT, the bovine oocyte cytosol is capable of remodeling the chromatin of the cell from the time of fusion. Twelve hours after fusion, there is an increase in the level of histone acetylation a transcriptionally permissive mark. At the two-cell stage histone acetylation decreases to a level relatively similar to that of the original somatic cell and remains so until the 8–16-cell stage embryo. Overall DNA methylation and H3K27me3—both transcriptionally repressive marks—decrease rapidly during the first 12 h after fusion and then gradually thereafter.

onic genome. Measured by BrUTP incorporation and contrary to our expectations, C/B iSCNT embryos indeed showed transcriptional activity albeit it was 70% of transcription level recorded in B/B SCNT counterparts (Fig. 6). These results are encouraging; they indicate that primate somatic cells can transcribe RNA in the environment of a bovine cytosol; however, it was not clear whether there was a complete silencing of somatic-related genes and reactivation of embryonic ones or that transcription of the primate nucleus was never shut down in the first place, and genes transcribed were merely somatic genes. We decided to analyze the expression levels of 16 developmentally important genes, Stella, Nanog, Oct4, DSC2, USF2, Crabp1, Adrbk1, CCNE2, CXCL6, PTGER4, Glut1, H2AFZ, c-MYC, KLF4, Lin28, and GAPDH, at the 8–16-cell stage, when BrUTP incorporation was measured.

Of significance in the iSCNT embryos was the presence of chimpanzee-specific Stella, Oct4, Crabp1 CCNE2, CXCL6, PTGER4, H2AFZ, c-MYC, and KLF4, showing that transcription of these genes, also shown to be present in same-species preimplantation embryos, allowed to occur in the cytosol of an evolutionary distant species; however, we also found that although robustly expressed in SCNT embryos, Glut1, Nanog, DSC2 , USF2, Adrbk1, and Lin28 were absent in iSCNT counterparts. The absence of NANOG is significant because it was shown that in mouse, NANOG null embryos fail to form a discernible epiblast and are incapable of give rise to ES cells (Mitsui et al., 2003), and lack of Lin28 suggests defect in pluripotency in iSCNT. Lack of DSC2 in iSCNT indicate a potential defect in the formation of trophectoderm (Lorimer et al., 1994) and lack of USF2 imply the lower global transcription level in iSCNT. Lack of Adrbk1 implies the defect of signal translation in signal pathway in iSCNT. The absence of Glut1 in iSCNT embryos may indicate a reduced glucose metabolism, be detrimental to embryo development, and manifested by low ATP levels in such embryos. Taken together, this data demonstrate that although bovine cytosol can activate transcription of some genes in the chimpanzee genome, the profile of transcription is different when compared to the same species counterpart and seems to reflect the stochastic nature of the phenomenon.

Energy supply and ATP levels in iSCNT embryos

The coexistence of foreign mitochondria with maternal mitochondria was previously addressed in an interspecies SCNT trial (Yang et al., 2004). These experiments clearly demonstrated that mitochondria derived from the donor cell are gradually excluded from the functional pool to an undetectable level after two rounds of cell division. These finding raise the concern as to what extent the mitochondria from bovine would be able function under the control of a primate genome. A direct and functional way to compare the compatibility between donor nuclei and ooplasm, at least in terms of energy supply, is to measure the level of ATP produced by the iSCNT embryos and compare it to that of same-species SCNT embryos. In our study, the dynamics of ATP levels in both type of SCNT embryos from the 1-cell to the 8–16-cell stage were similar between them, that is, there was a decrease in the level of ATP production as the embryos progressed in development; however, significantly low ATP level was observed in iSCNT embryos at the 8–16-cell stage, where iSCNT embryos had only 80% of the ATP level of that in SCNT embryos. It remains to be determined whether the lack of Glut1 expression is one of the causes of such decrease in ATP production.

In conclusion, C/B iSCNT embryos have only limited developmental ability compared with B/B SCNT counterparts. When compared interspecies and same-species SCNT embryos as to their genetics, epigenetics, global transcription levels, specific gene expression, and ATP levels the data strongly suggest that chimpanzee nuclei are stable in cell division and that bovine ooplasm can, at least partially, globally reprogram chimpanzee nuclei similar to bovine SCNT embryos; however, gene promoter demethylation and gene expression for the genes analyzed is incomplete. This, together with the low ATP levels we observed in C/B SCNT embryos, may be the reason for arrested development. It is interesting to notice that although global epigenetic changes can be exerted in the chimpanzee genome, to levels undistinguishable from those of the same species SCNT experiments, these changes are not sufficient to drive a similar gene expression pattern. It reinforces the notion that there are species-specific regulatory transcriptional and/or posttranscriptional regulatory molecules responsible for proper gene expression and embryonic development. Finding such regulators could shed light in the SCNT process itself and underscore the importance of interspecies models to study the mechanism of cellular reprogramming. These data should be taken into consideration when devising experiments that involve fusion between human somatic cells and enucleated oocytes from animal sources.

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Author Disclosure Statement

The authors declare that no competing financial interests exist.

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