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Interspecies Nuclear Transfer Reveals that Demethylation of Specific Repetitive Sequences Is Determined by Recipient Ooplasm but not by Donor Intrinsic Property in Cloned Embryos

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ABSTRACT DNA methylation/demethylation of donor genomes in recipient ooplasm after nuclear transfer occurs in a species-specific way. In cloned rabbit and bovine embryos, repetitive sequences maintain the donor-type methylation status, but typical demethylation of repetitive sequences takes place in cloned porcine embryos. To clarify whether the demethylation is controlled by donor nucleus intrinsic property or by recipient ooplasm, we used interspecies somatic cell nuclear transfer (iSCNT) model to examine the methylation status of repetitive sequences in pig-to-rabbit and rabbit-to-pig interspecies embryos. We found that no demethylation of pig repetitive sequences was observed in pig-to-rabbit iSCNT embryos, while the examined rabbit repetitive sequence *Rsat IIE* was demethylated in rabbit-to-pig iSCNT embryos. These results indicate that demethylation of donor repetitive sequences is determined by ooplasm but not by donor intrinsic property and that ooplasm from different species have different capabilities to demethylate genes. *Mol. Reprod. Dev. 73: 313–317, 2006.* © 2005 Wiley-Liss, Inc.

Key Words: DNA methylation; embryo; iSCNT; rabbit; pig

INTRODUCTION

DNA methylation, one of the epigenetic modifications, is essential for mammalian development. The DNA methylation pattern is stable in somatic cells from different resources, but dynamic methylation waves occur in early embryos (Reik et al., 2001). In the mouse, the male pronucleus is actively demethylated shortly after fertilization, while the female genome undergoes passive demethylation correlated with embryo development. Then, de novo methylation takes place at the blastocyst stage especially in the inner cell mass (ICM) (Reik et al., 2001). This methylation wave eliminates parental gamete methylation differences and may be important for the formation of pluripotent stem cells that are crucial for later development (Han et al., 2003; Shi et al., 2003).

Nuclear transfer (NT) of cultured somatic cells has produced offspring in many species, but the increased abortion rate, large offspring syndrome (LOS), and perinatal death indicate that there are still many problems to be solved before practical application of this technique (Shi et al., 2003). In animal cloning, the highly differentiated donor nucleus must be properly dedifferentiated, cease its own program of gene expression, and express genes required for early embryo development. However, aberrant methylation changes of the donor genome, especially in highly repetitive sequences, are observed frequently in cloned embryos (Dean et al., 2001; Han et al., 2003). In cloned bovine embryos, euchromatin and the promoter region of single copy genes are demethylated, but centromeric heterochromatin and repetitive sequences maintain the hypermethylation status of donor cells (Bourc'his et al., 2001; Kang et al., 2001a). Our recent work also revealed the lack of demethylation in repetitive sequence *Rsat IIE* in cloned rabbit embryos (Chen et al., 2004). In contrast, two examined repetitive sequences were typically demethylated in cloned pig embryos (Kang et al., 2001b).

Is the demethylation of repetitive sequences in cloned embryos dependent on the donor nucleus or on recipient cytoplasm? Interspecies NT is a good model for studying nuclear and cytoplasmic interactions, and the different

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methylation fates of repetitive sequences in cloned pig and rabbit embryos provide a unique approach to investigate the regulatory mechanisms of DNA demethylation by using this technology. In the present study, we constructed pig-to-rabbit and rabbit-to-pig interspecies somatic cell nuclear transfer (iSCNT) embryos, and examined the methylation changes of donor repetitive sequences in recipient ooplasm of another species to address and answer this question.

MATERIALS AND METHODS

All animal experiments were approved and performed under the guidelines of the ethical committee of the State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences. All chemicals used in this study were purchased from Sigma (Sigma Chemical Company, St. Louis, MO) unless otherwise noted.

Preparation of Donor Cells

Porcine cumulus cells were obtained by vortexing matured cumulus oocyte complexes (COCs) for 1 min in TL-HEPES supplemented with 0.1% hyaluronidase. Approximately 1×10^7 isolated cells were plated in a culture bottle containing Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) and 1% antibiotic-antimycotic. The cumulus cells were cultured at 37°C to reach confluence, and then passaged with routine methods. We obtained rabbit skin fibroblasts from a fetus at day 17 of gestation as described before (Han et al., 2001; Chen et al., 2004). Both porcine and rabbit cells at passages 3–10 were used as donors. For cell synchronization, the serum concentration was decreased to 0.5% to starve the donor cells for 3–5 days before NT.

Preparation of Recipient Cytoplast

For preparation of rabbit recipient cytoplast, matured female Japanese Big Eared white rabbits were superovulated by administering 150 IU pregnant mare serum gonadotropin (PMSG, Institute of Zoology, Chinese Academy of Sciences) and 100 IU human chorionic gonadotropin (hCG, Institute of Zoology, Chinese Academy of Sciences) 4 days after the PMSG injection. Rabbits were killed 14 hr after the hCG injection. Cumulus masses were collected and treated with 300 IU/ml of hyaluronidase. The cumulus-free oocytes were transferred to M2 medium containing 7.5 μg/ml cytochalasin B, 7.5μg/ml Hoechst 33342, and 10% FBS for 10 min. A small amount of cytoplasm containing the meiotic spindle beneath the first polar body was aspirated using a 25–30 μm glass pipette, and then the aspirated karyoplast was exposed to ultraviolet light to confirm the presence of chromosomes. Only the oocytes from which the chromosomes were entirely removed were used for NT.

For preparation of porcine recipient oocytes, ovaries were collected from gilts at a local slaughterhouse and transported to the laboratory within 1 hr. Oocytes were aspirated from antral follicles (2–6 mm in diameter) with an 18-gauge needle attached to a 20-ml disposable syringe. A group of 25 oocytes with compact cumulus and evenly granulated ooplasm was cultured in a 100-ml drop of NCSU-23 medium supplemented with 75 mg/ml potassium penicillin G, 50 mg/ml streptomycin sulphate, 0.57 mM cysteine, 0.5 mg/ml FSH, 96 0.5 mg/ml LH, 10 ng/ml epidermal growth factor, and 10% (v/v) porcine follicular fluid (pFF). The pFF was aspirated from 5–7 mm follicles of prepubertal gilt ovaries. The oocytes were cultured for 44 hr at 39°C in an atmosphere of 5% CO_2 and saturated humidity. After maturation, expanded cumulus cells were removed by repeated pipetting in the presence of 300 IU/ml hyaluronidase. The cumulus-free pig oocytes were enucleated as above except that the basic medium was changed to NCSU-23 medium.

Nuclear Transfer, Activation, and Embryo Culture

For pig-to-rabbit iSCNT, a single pig donor cell was placed in the perivitelline space of an enucleated rabbit oocyte. The couplets were transferred to a fusion chamber consisting of two wires, 1 mm apart and overlaid with 100 μl fusion medium (0.25 M sorbitol, 0.5 mM magnesium acetate, 0.1 mM calcium acetate, 0.5 mM HEPES, and 100 mg/100 ml BSA). Fusion was induced with two direct current (DC) pulses of 1.4 kv/cm for 80 μs delivered by an ECM2001 Electrocell Manipulator (BTX, Inc., San Diego, CA). Fused couplets were activated by double DC pulses of 1.4 kv/cm for 40 μs, and then cultured in TCM199 medium supplemented with 10% FBS at 37°C in a humidified air containing 5% CO_2. For rabbit-to-pig iSCNT, fusion was induced with two direct current pulses of 150 V/mm for 50 μsec in fusion medium (0.28 mol/L mannitol supplemented with 0.1 mM $MgSO_4$ and 0.01% polyvinyl alcohol). This pulse was also utilized to simultaneously induce oocyte activation. The reconstructed oocytes were cultured for 4 days in NCSU-23 containing 0.4% BSA and then transferred to NCSU-23 containing 10% FBS at 38°C in humidified air containing 5% CO_2. Embryos at each specific developmental stage were collected and pooled into a quantity of ~100–500 diploid genomes. Both kinds of iSCNT embryos are difficult to develop to blastocysts. We only obtained two pig-to-rabbit blastocysts and one rabbit-to-pig blastocyst; the latter was too little for methylation analysis. To exclude the possibility of genomic contamination, we used 0.5% pronase to remove the zona pellucida from all embryos. Differences in methylation rates among different stages were analyzed by two independent population Student's *t*-tests.

Bisulphite Treatment

The procedure has been described previously (Chen et al., 2004). Briefly, cells were washed with PBS and incubated in lysis buffer (1 mM SDS; 280 g/ml proteinase K) at 37°C for 1–1.5 hr. After boiling for 15 min, the sample DNA was denatured with 0.3N NaOH and mixed with low melting point agarose to form beads, which were treated with freshly made 5 M bisulphite solution

(2.5 M sodium metabisulphite; 125 mM hydroquinone; pH 5) at 50°C for 8–10 hr in the dark. The reaction was stopped by equilibration against TE. Following desulfonation in 0.2 N NaOH, the beads were washed with TE and H_2O, and stored at −20°C.

PCR Amplification, Cloning, and Sequencing

Amplification of the pig *PRE-1* sequence and the rabbit *Rsat IIE* sequence was as described previously (Kang et al., 2001b; Chen et al., 2004). Amplification of the pig satellite sequence (GenBank accession no. Z75640) was performed with 25 cycles of 94°C for 40 sec, 48°C for 1.5 min, and 72°C for 1.5 min using primers 5′-TTT GTA GAA TGT AGT TTT TAG AAG-3′ and 5′-AAA ATC TAA ACT ACC TCT AAC TC-3′, then another 30 cycles of 94°C for 40 sec, 48°C for 1.5 min, and 72°C for 1.5 min using primers 5′-TTT GTA GAA TGT AGT TTT TAG AAG-3′ and 5′-CRT AAA CAC TAC TAC TTA CCT AAT A-3′. For amplification of the rabbit short interspersed C repeat sequence (GenBank accession no. X02216), the primary PCR consisted of 25 cycles of 94°C for 40 sec, 48°C for 1.5 min, and 72°C for 1.5 min using primers 5′-TTG TTG TAA ATA GGA AAG TGT-3′ and 5′-ACA TCA CAA CAG AAA CTA ATA C-3′, and then another 30 cycles of 94°C for 40 sec, 50°C for 50 sec, and 72°C for 50 sec using the primer set 5′-AGG TGA TAG GTA GAG TTA AAT AG-3′ and 5′-ACA TCA CAA CAG AAA CTA ATA C-3′. The PCR products pooled from three independent amplifications were cloned into TA-cloning vector (TaKaRa, Tokyo, Japan). Individual clones were sequenced using an automatic sequencer (ABI PRISM 337). The complete conversions of base Cs to Ts in the sequencing result confirmed the efficiency of the bisulfite treatment.

Restriction Analysis of PCR Products

For *Taq*I restriction analysis of the pig *PRE-1* sequence, pooled PCR products from three independent amplifications were purified and concentrated using a Wizard DNA Clean-Up System (Promega, Madison, WI). About 100 ng of purified PCR products were digested with 20 U of *Taq*I restriction enzyme (New England Biolabs, Beverly, MA) overnight at 37°C, resolved on 6% non-denaturing polyacrylamide gel, which was silver stained as previously reported (Budowle et al., 1991). To confirm the consistency, the enzyme digestion assays were repeated three times.

RESULTS

DNA Methylation Profiles of Donor Porcine Repetitive Sequences in Porcine-to-Rabbit iSCNT Preimplantation Embryos

Typical demethylation of the two repetitive sequences (*satellite I* and *PRE-1*) has been observed in intraspecies cloned pig embryos (Kang et al., 2001b). Here, we examined the methylation status of the same two sequences in pig-to-rabbit iSCNT embryos to determine whether demethylation can still occur. For the porcine *satellite I* sequence, a 206-bp DNA fragment carrying about nine CpG sites were amplified (Fig. 1A). As shown in Figure 1B, the cumulus cells cultured as donor cells showed heavy methylation status ($66.3 \pm 18.6\%$), which is comparable to the methylation level of donor fetal fibroblasts ($64.6 \pm 26.4\%$, $P < 0.05$) reported by Kang et al. (2001b). After the donor porcine cells had been introduced into enucleated rabbit oocytes by NT, the methylation value rose to $83.9 \pm 11.8\%$ in one-cell embryos. During later development, this hypermethylation status was maintained at least to the blastocyst stage ($80.3 \pm 12.1\%$).

For the porcine *PRE-1* sequence, the amplified 148-bp segment has two *Taq*I recognition sites (5′-TCGA-3′) and will be cut into three fragments (a 74 bp and two 37 bp) by *Taq*I digestion if fully methylated (Fig. 2A). As shown in Figure 2B, there were three DNA bands (111 bp, 74 bp, and 34 bp) after the PCR products of donor cells was completely digested, which is very similar to the digestion pattern of donor fibroblasts reported by Kang et al. (2001b). This digestion pattern appears unchanged in the iSCNT preimplantation embryos indicating that the hypermethylation status of donor cells is maintained during the early iSCNT embryo development.

DNA Methylation Profiles of Donor Rabbit Repeat Sequence *Rsat IIE* in Rabbit-to-Porcine iSCNT Preimplantation Embryos

We next constructed rabbit-to-pig iSCNT embryos and examined the methylation profiles of rabbit *Rsat IIE* sequence (Fig. 3A), which shows no evident demethylation in rabbit intraspecies cloned embryos (Chen et al.,

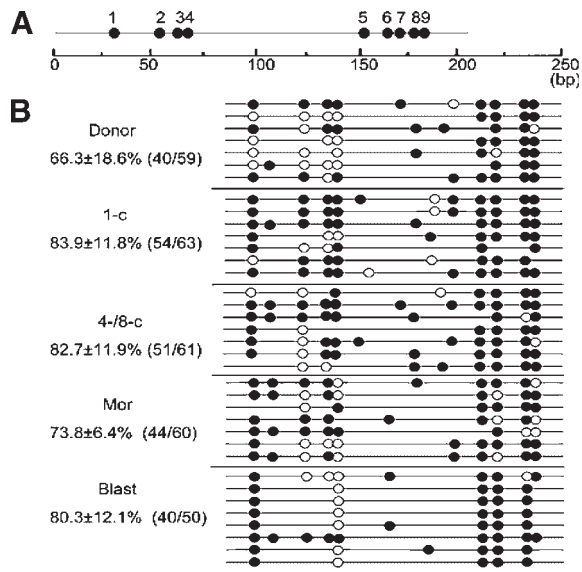

Fig. 1. Methylation profiles of pig satellite DNA in pig-to-rabbit interspecies NT embryos. **A**: The relative locations of nine CpG dinucleotides are schematically represented. **B**: Methylation profiles of CpG dinucleotides of pig satellite DNA in pig-to-rabbit interspecies cloned embryos. Open and closed circles indicate unmethylated and methylated CpG sites, respectively. Donor, donor cell; 1-c, one cell embryos; 4-/8-c, four-to-eight-cell embryos; Mor, morulae; Blast, blastocysts.

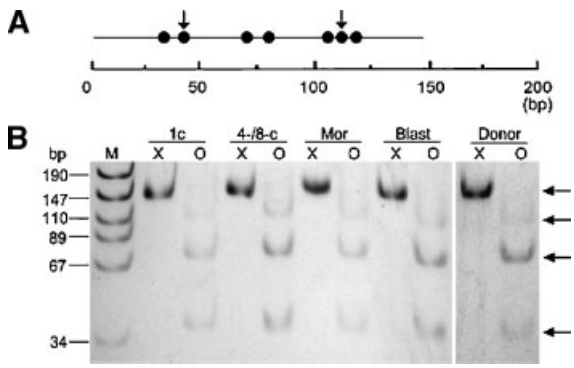

Fig. 2. Methylation patterns of pig *PRE-1* sequence in pig-to-rabbit interspecies NT embryos. **A**: Schematic diagram showing the relative locations of seven CpG dinucleotides (closed circles) and two *Taq*I recognition sites (standing arrows). **B**: The *Taq*I digestion pattern of the *PRE-1* sequence amplified from genomic DNAs of pig-to-rabbit iSCNT embryos. X, intact, undigested PCR products; O, *Taq*I-digested PCR products. Arrows indicate locations of PCR products and *Taq*I-digested PCR products. M, DNA size marker. Donor, donor cell. 1-c, one-cell embryos; 4-/8-c, four-to-eight-cell embryos; Mor, morulae; Blast, blastocysts.

2004). As shown in Figure 3B, the methylation value in donor fibroblasts was $35.7 \pm 24.3\%$, which is well consistent with the data we obtained previously ($36.9 \pm 9.2\%$). To our surprise, the methylation value in one-cell rabbit-to-porcine iSCNT embryos was only $7.3 \pm 13.7\%$, significantly lower than the donor cell methylation level ($P < 0.05$). This hypomethylation status was maintained in 2-/4-cell embryos ($9.4 \pm 18.6\%$), 8-/16-cell embryos ($16.7 \pm 19.4\%$), and morulae

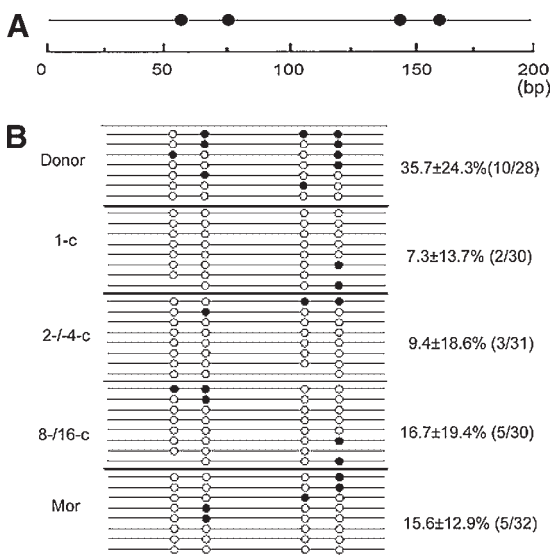

Fig. 3. Methylation profiles of rabbit satellite DNA *Rsat IIE* sequence in rabbit-to-pig interspecies NT embryos. **A**: Schematic representation of amplified DNA fragments showing the relative locations of four CpG dinucleotides. **B**: Methylation profiles of CpG dinucleotides of rabbit satellite DNA *Rsat IIE* sequence in donor fibroblasts (donor) and iSCNT embryos. Open and closed circles indicate unmethylated and methylated CpG sites, respectively. Donor, donor cell. 1-c, one-cell embryos; 2-/4-c, two- to four-cell embryos; 8-/16-c, eight- to sixteen-cell embryos; Mor, morulae.

($15.6 \pm 12.9\%$). We could not examine the methylation status of blastocysts due to the difficulty to obtain blastocyst stage embryos.

DISCUSSION

Limited demethylation has been observed in cloned bovine and rabbit embryos with repeated sequences maintaining the donor-type methylation status (Bourc'his et al., 2001; Kang et al., 2001a; Chen et al., 2004), while in cloned pig embryos, two examined repeated sequences were typically demethylated (Kang et al., 2001b). These data raise the question whether the demethylation differences are determined by recipient cytoplasts or by inherent properties of donor nuclei. Here, we constructed pig-to-rabbit and rabbit-to-pig iSCNT embryos and examined the methylation patterns of several repetitive sequences. In our results, the two donor pig repetitive sequences, which are demethylated in cloned pig embryos (Kang et al., 2001b), maintained the donor type hypermethylation status in pig-to-rabbit iSCNT embryos. On the other hand, the donor rabbit repetitive sequence *Rsat IIE*, which showed no evident demethylation in rabbit cloned embryos (Chen et al., 2004), were demethylated in rabbit-to-pig iSCNT embryos. Because we only examined the methylation status of the three sequences, whether other genomic sequences behave similar is unknown at present. Nevertheless, our data provided strong evidence showing that demethylation of donor genomes, at least some specific repetitive sequences, is controlled by the recipient cytoplasm but not by donor intrinsic properties. The different demethylation fate of donor repetitive sequences in the two kinds of iSCNT embryos may be due to variation in the demethylating activity of the different recipient cytoplasm. In this sense, porcine ooplasm may have higher demethylation activity than rabbit ooplasm. Alternatively, rabbit ooplasm may have high methylating activity, which counteracts the demethylating process of repetitive sequences.

Because we collected 1-cell iSCNT embryos at the time when the pronucleus-like structure just formed, the abrupt demethylation of donor rabbit *Rsat IIE* in rabbit-to-porcine one-cell embryos is most likely caused by active demethylation (Mayer et al., 2000). By immunostaining with anti-5-methyl-cytosine, active demethylation was observed in male pronuclei of pig, mouse, rat, and human, but not in rabbit and sheep (Dean et al., 2001; Beaujean et al., 2004a,b; Shi et al., 2004). The fact that sheep sperm DNA can be demethylated in mouse oocytes indicates that active demethylation is mainly oocyte-determined (Beaujean et al., 2004c). The enucleated pig oocyte may have enough active demethylating activity to demethylate donor rabbit genomes. Because of its hypomethylation status in one-cell embryos, the rabbit satellite sequence *Rsat IIE* did not show a passive demethylation process during cleavage of iSCNT embryos.

The feasibility of interspecies cloning has been demonstrated in previous studies (Dominko et al., 1999; Lanza et al., 2000; Loi et al., 2001). Our research

provides the first evidence showing that recipient ooplasm could demethylate donor karyoplasts from different species. An iSCNT technique using human somatic cells and animal oocytes has been suggested as an alternative therapeutic cloning approach (Chang et al., 2003). Some experiments have been conducted by using enucleated cow and rabbit oocytes, but there has not yet been a satisfactory result (Chen et al., 2002; Chang et al., 2003). It is worth to note that repetitive sequences cannot be efficiently demethylated in both bovine and rabbit-cloned embryos (Kang et al., 2001a; Chen et al., 2004). According to our experiments here, cow and rabbit ooplasm might lack the ability to demethylate repetitive sequences from other species. Considering this aspect, oocytes from other species like porcine may be a better choice for deriving human stem cells.

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