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Active Demethylation of Individual Genes in Intracytoplasmic Sperm Injection Rabbit Embryos

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ABSTRACT Intracytoplasmic sperm injection (ICSI), as an assisted reproduction technique, has been widely used in animal and human. However, its possible effect on epigenetic changes has not been well studied. To investigate whether ICSI can induce aberrant DNA methylation changes in rabbit preimplantation embryos, we examined the methylation status of the *SP-A* promoter region and the satellite sequence *Rsat IIE* by bisulfite-sequencing technology. The *SP-A* promoter region was extensively demethylated before the first round of DNA replication commences, and the unmethylated status was maintained until morula when dynamic remethylation occurred. A similar but more moderate demethylation process was observed in satellite sequence *Rsat IIE*. These results are in contrast with the previous reports of no active demethylation in normal rabbit embryos, suggesting that the active demethylation we observed may be induced by ICSI. *Mol. Reprod. Dev. 72: 530–533, 2005.*

Key Words: rabbit; DNA methylation; embryo; intracytoplasmic sperm injection (ICSI)

INTRODUCTION

DNA methylation at CpG dinucleotides is essential for normal mammalian development. Up to five methyltransferases have been found and the methylation mechanism is well known, but the demethylation process is still obscure (Hsieh, 2000; Meehan, 2003). Previous studies have indicated that DNA demethylation could be achieved when DNA replication takes place (passive demethylation) (Rougier et al., 1998) or occurred independent of replication (active demethylation) (Mayer et al., 2000; Oswald et al., 2000). Active demethylation of the paternal genome has been observed in various mammalian species except for sheep and rabbit (Dean et al., 2001; Chen et al., 2004; Beaujean et al., 2004a; Santos et al., 2005). However, sheep oocytes have been reported to be able to partially demethylate donor nuclei as well as sperm from other species, suggesting that sheep ooplasm may have residue demethylation activity (Beaujean et al., 2004b,c). It is still unknown whether the active demethylation machinery also exists in rabbit.

Since the first successful human pregnancy after intracytoplasmic sperm injection (ICSI) had been reported in 1992 (Palermo et al., 1992), numerous papers followed reporting high success with this assisted reproduction procedure. ICSI has now become a popular choice in treating male-factor infertility, but possible epigenetic factors associated with ICSI have not been well studied. There are increasing reports that associate ICSI with altered fetal development and with various diseases, leading to serious debates concerning the safety of this technique (De Rycke et al., 2002; Thompson et al., 2002; Maher et al., 2003). We have previously examined the methylation status of centromeric satellite *Rsat IIE* and surfactant protein A (*SP-A*) promoter region in normal and cloned rabbit embryos, and found that the methylation patterns of both sequences can be altered by nuclear transfer (Chen et al., 2004). Here, by investigating the methylation patterns of the same two sequences, we tested whether any epigenetic change could be induced in ICSI rabbit embryos.

MATERIALS AND METHODS

Production of Rabbit ICSI Embryos

Fresh sperm were collected from mature Japanese Big Eared White buck. Oocytes were placed in a drop of HEPES-buffered M199 medium, and single spermatozoon in 0.9% NaCl with 10% polyvinyl pyrolidone (PVP) was scored and aspirated into a glass injection pipette

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and deposited in the oocyte cytoplasm after gentle aspiration of the oolemma. After injection, oocytes without additional activation treatment were either cultured in TCM 199 medium supplemented with 10% FBS or cultured for 16 hr in the presence of the DNA replication blocker aphidicolin (10 μg/ml) at 38°C in humidified air containing 5% CO_2. We used pronase (0.5%; Sigma, St. Louis, MO) to remove zonae from all the embryos to exclude the possibility of genomic contamination from attached cumulus cells.

Bisulfite Treatment, PCR Amplification and Sequencing

Embryos at each development stage were pooled into a quantity of 150–600 diploid genomes and treated with bisulfite as previously described (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. 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 5M bisulfite solution (2.5M sodium metabisulfite, Merck, Darmstadt, Germany; 125 mM hydroquinone, Sigma; pH 5) at 50°C for 8–10 hr in the dark. After desulfonating and washing steps, the beads were stored at −20°C until use. Both sequences were amplified by heminested PCR as described previously (Chen et al., 2004). The PCR products pooled from three independent amplifications were cloned into TA-cloning vector (TaKaRa, Shiga, Japan). Individual clones were sequenced using an automatic sequencer (ABI PRISM 337).

RESULTS

DNA Methylation Status of *SP-A* Promoter Region in Rabbit ICSI Embryos

We first examined the DNA methylation status of *SP-A* promoter region from 2-cell ICSI embryos to blastocysts. As shown in Figure 1A, the *SP-A* promoter sequence was nearly completely demethylated in 2-cell embryos. This unmethylated status was maintained at least to the 8-/16-cell stages. Then dramatic de novo methylation took place, resulting in hypermethylation status in morula (88%) and blastocysts (81%).

Because passive demethylation can only result in a 50% decrease after one replication cycle, considering the hypermethylation of *SP-A* promoter sequence in sperm (88%) and oocytes (75%) (Chen et al., 2004), the abrupt drop in DNA methylation in 2-cell embryos appears to be due to active demethylation. To prove this hypothesis, we further examined the methylation status of 1-cell ICSI embryos at 8 hr after sperm injection, that is, before S phase of the first cell cycle commences (Fig. 1B). The overall methylation level was 70%, which is a little lower than the composite methylation value of the two parental genomes (82%) (Chen et al., 2004). Moreover, the methylation pattern of early 1-cell embryos was heterogeneous. In the 13 clones we examined, the majority of them (9 of 13) maintained hypermethylation

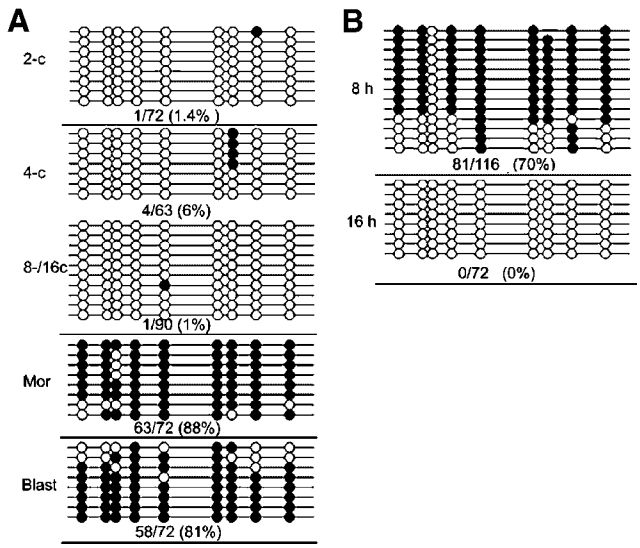

Fig. 1. DNA methylation status of *SP-A* promoter sequence in rabbit ICSI embryos. **A**: Methylation profiles of CpG dinucleotides from 2-cell embryos to blastocysts. 2-c, two cell embryos; 4-c, four cell embryos; 8-/16-c, eight- to sixteen-cell embryos; Mor, morulae; Blast, blastocysts. **B**: Methylation profiles of CpG dinucleotides in 1-cell embryos. 8 h, zygote after single sperm injection cultured for 8 hr; 16 h, zygote after single sperm injection cultured for 16 hr in the presence of aphidicolin; Open and closed circles indicate unmethylated and methylated CpG sites, respectively. Percent methylation is shown in parentheses.

status, but the other four strings were extensively demethylated, indicating that active demethylation has already taken place even at this early stage in ICSI embryos.

The active demethylation that occurred at the early 1-cell stage may grow in both scope and extent during development. To evaluate the contribution of active demethylation to the unmethylated status in 2-cell embryos, we cultured inseminated ICSI oocytes for 16 hr in the presence of DNA replication blocker aphidicolin. To our surprise, we found the *SP-A* promoter region was completely demethylated, indicating that its unmethylated status at 2-cell stages was entirely owing to active demethylation.

DNA Methylation Status of Satellite Sequence *Rsat IIE* in Rabbit ICSI Embryos

To confirm the result on *SP-A* promoter sequence, we examined the methylation patterns of satellite sequence *Rsat IIE* in ICSI embryos. As shown in Figure 2, the methylation value of the 1-cell embryos (cultured for 16 hr in the presence of aphidicolin) is 43%, which is significantly lower than the composite methylation value of the parental genome (60%) (Chen et al., 2004), indicating that active demethylation did take place in 1-cell ICSI embryos. After 1-cell cycle, the methylation level of the satellite sequence was decreased to 37% in 2-cell embryos, and further dropped to 9% in 4-cell embryos. This hypomethylation status was maintained until blastocyst stages when a slight de novo methylation occurred (25%).

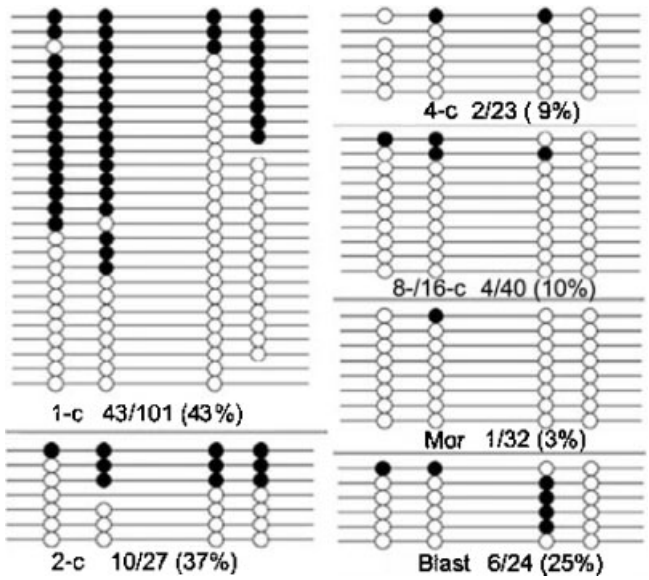

Fig. 2. DNA methylation status of satellite DNA *Rsat IIE* in rabbit ICSI embryos. Open and closed circles indicate unmethylated and methylated CpG sites, respectively. Percent methylation is shown in parentheses. Some sites are absent from the sequences in some clones due to mutations in the particular copies of the satellite sequences. 1-c, zygote after single sperm injection cultured for 16 hr in the presence of aphidicolin; 2-c, two-cell embryos; 4-c, four-cell embryos; 8-/16-c, eight- to sixteen-cell embryos; Mor, morulae; Blast, blastocysts.

DISCUSSION

In this research, we found that the DNA methylation status of the *SP-A* promoter region and the *Rsat IIE* sequence in rabbit ICSI embryos were totally different from those in rabbit in vivo-derived embryos, where both sequences were demethylated at 8-/16-cell stages and little or no remethylation occurred before implantation (Chen et al., 2004). The most interesting result is the extensive demethylation of the *SP-A* promoter sequence before the first round of DNA replication commences, indicating that active demethylation occurred in rabbit ICSI embryos. This conclusion was confirmed by the bisulfite-sequencing result of the *Rsat IIE* sequence. However, no active demethylation has been observed in in vivo-derived rabbit embryos by immunofluorescence staining with an anti-5-methylcytosine antibody (Beaujean et al., 2004a; Shi et al., 2004). We also found no signs of active demethylation of the *SP-A* promoter region and the *Rsat IIE* sequence in in vivo-derived rabbit 1-cell embryos (unpublished data).

The nature of active demethylation is still obscure. Previous work has shown that mouse oocytes can demethylate spermatozoa form ovine (Beaujean et al., 2004c), suggesting that active demethylation is maternally driven. The finding that ooplasm is not effective in demethylating paternal chromatin in germinal vesicle breakdown (GVBD) fertilized oocytes (Spinaci et al., 2004) suggests that the demethylation mechanism may need a process of activation. Thus, we may hypothesize that the rabbit oocyte has premature active demethylation machinery which may be activated by ICSI though some unknown signaling pathways (Thompson et al., 2002). Consistent with this is that sheep oocytes can partially demethylate donor nuclei as well as sperm from other species, although no active demethylation was observed in in vivo-derived sheep embryos (Beaujean et al., 2004c; Young and Beaujean, 2004).

Active demethylation has only been observed in paternal pronuclei by immunostaining with 5-mC antibody, which is confirmed by bisulfite sequencing of individual sequences. It has been proposed that the early paternal chromatin reorganization make it susceptible to active demethylation(Barton et al., 2001; Santos et al., 2002). However, our result of the totally demethylation of SP-A promoter region before the first round of DNA replication indicates that active demethylation may take place on both parental chromosomes. It seems that ICSI, by an unknown mechanism, may induce changes in the structure of maternal chromosomes, rendering them accessible to demethylation activity. The methylation statuses of additional sequences need to be investigated to examine whether the remodification is occurred in special regions or in whole chromosome.

In nuclear transfer (NT) embryos, evident demethylation of the SP-A promoter region began at the 2-cell stage and remethylation occurred at the morula stage, which is to some extent similar with the methylation pattern of ICSI embryos but is different from the in vivo-derived embryos (Chen et al., 2004). Both ICSI and NT include micromanipulation and in vitro embryo culture, so the similar methylation patterns may be due to similar processes involved in the two procedures. We also noted that the methylation changes of cloned embryos (Chen et al., 2004) were less dramatic than those of ICSI embryos. It has been suggested that factors introduced from somatic donor cells, such as Dnmt1, might interfere with the demethylation process (Dean et al., 2001; Kang et al., 2001a). In addition, during nuclear transfer part of the cytoplasmic content (20%–30%) is removed with the oocyte nucleus, which may disrupt the demethylation ability. In fact, bovine satellite sequences which maintained abnormal methylation in cloned embryos can be demethylated in tetraploid clones (Kang et al., 2001b).

During ICSI embryo development, the methylation wave occurred in the satellite sequence *Rsat IIE* is less dramatic than that occurred in *SP-A* promoter region, which may be due to the stubbornness of repeated sequences to demethylation. In cloned bovine embryos repeated sequences maintained the hypermethylation status of donor somatic cells while single-copy genes were demethylated (Kang et al., 2001a, 2002). Our previous experiments also indicated that in cloned rabbit embryos the *Rsat IIE* sequence could not be demethylated like the *SP-A* promoter regions (Chen et al., 2004).

ICSI is an assisted reproduction technique that has been well used in animal and human. Using this technique we have successfully obtained germinal vesicle transfer rabbits (Li et al., 2001). Thus, most genes must be correctly reprogrammed in ICSI embryos. ICSI has been reported to be able to recapitulate the pronuclear

methylation status observed for in vivo fertilized mouse and sheep embryos by anti-5-methylcytosine staining (Beaujean et al., 2004c). It also has no effect on the methylation status of Spalt-like gene 3 (*Sall3*) which is over methylated in the placental genome of cloned mice (Ohgane et al., 2004). However, these positive data cannot rule out the possibility of aberrant epigenetic changes on some specific genes. Until now most reports focused on imprinted genes that are thought to be prone to epigenetic changes (De Rycke et al., 2002; Thompson et al., 2002; Maher et al., 2003). Our results of the abnormal methylation pattern of the two nonimprinted genes emphasize the need to assess the potential damages of ICSI on epigenetic changes other than imprinting disorders.

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