

The DNA methylation events in normal and cloned rabbit embryos

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Abstract To study the DNA methylation events in normal and cloned rabbit embryos, we investigated the methylation status of a satellite sequence and the promoter region of a single-copy gene using bisulfite-sequencing technology. During normal rabbit embryo development, both sequences maintained hypermethylation status until the 8- to 16-cell stage when progressive demethylation took place. In cloned embryos, the single-copy gene promoter sequence was rapidly demethylated and precociously de novo methylated, while the satellite sequence maintained the donor-type methylation status in all examined stages. Our results indicate that unique sequences as well as satellite sequences may have aberrant methylation patterns in cloned embryos.

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1. Introduction

DNA methylation represents a major epigenetic modification of the genome. During early mouse embryo development, a dramatic wave of DNA methylation occurs which is essential for normal mouse development [1]. The paternal pronucleus undergoes an active demethylation process while passive demethylation occurs in the maternal genome, resulting in the lowest DNA methylation level at the morula stage. Subsequent genome-wide de novo methylation occurs preferentially in the inner cell mass of mouse blastocysts [2–4]. Similar DNA methylation events are also observed in cattle and pig [5–7], but no active demethylation occurs in rabbit and sheep [8,9]. The species-specific DNA methylation reprogramming may have an impact on later development.

Nuclear transfer (NT) has been successfully performed in various species, but the low cloning efficiency and the frequent anomalies inherent to cloned animals indicate that the technique is still in its infancy stage [10]. One possible cause for the low rate in cloning success is inaccurate epigenetic reprogramming of donor cell nuclei [10,11]. Recent findings revealed

that aberrant DNA methylation occurs in cloned bovine and sheep embryos [6,12,13]. In cloned pig embryos typical demethylation takes place in two examined repeated sequences [7], but the possibility of abnormal methylation in other sequences cannot be ruled out.

Rabbits are widely used experimental animals. Rabbit oocytes are relatively easy to manipulate and rabbit embryos have a high capability to develop to blastocyst stages in vitro culture systems [14,15]. However, rabbit cloning is difficult and so far there has only been one successful report [16]. Here, by analyzing *Rsat IIE*, a centromeric satellite DNA, and the promoter region of surfactant protein A (*SP-A*), a single-copy gene, we investigated DNA methylation of normal and NT rabbit embryos to examine whether the low cloning efficiency in rabbits is related to incorrect reprogramming of DNA methylation.

2. Materials and methods

2.1. Production of rabbit embryos

For recovery of in vivo-derived embryos, mature female Japanese Big Eared white rabbits were superovulated by administering 150 IU pregnant mare serum gonadotropin (PMSG) followed by 100 IU human chorionic gonadotropin (hCG) 4 days later. Female rabbits were mated immediately after hCG injection. Mature oocytes and embryos were surgically collected at various stages of development. For NT, we isolated skin fibroblasts from a fetus at embryonic day 17 and cells at passage 3–10 were used as donors. NT was performed as previously described [14]. Embryos at each of the specific developmental stages were pooled into a quantity of ~150–600 diploid genomes, representing about 300 matured oocytes, 150 two-cell embryos, 60 four-cell embryos, 30 eight- to sixteen-cell embryos, 10 morulae and 6 blastocysts. We used 0.5% pronase (Sigma) to remove zonae from all embryos and mature oocytes to exclude the possibility of genomic contamination. Differences in methylation rates among experimental groups were analyzed by two independent population *t* tests.

2.2. Bisulfite treatment

We used a procedure based on methods by Olek et al. [17] with some modifications. Briefly, cells were washed with PBS and incubated in lysis buffer containing 1 mM SDS and 280 g/mL proteinase K at 37 °C for 1–1.5 h. Following boiling for 15 min, the sample DNA was denatured with 0.3 N NaOH at 50 °C for 15 min, then mixed with low melting point agarose to form beads, which were treated with freshly made 5 M bisulfite solution (2.5 M sodium metabisulfite, Merck; 125 mM hydroquinone, Sigma; pH 5) at 50 °C for 8–10 h 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 H₂O and stored at –20 °C until use. For lung tissues, genomic DNA was isolated and digested with *EcoRI*. After denaturing in 0.3 M NaOH, samples were mixed with agarose to form beads which were treated as described

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Abbreviations: NT, nuclear transfer; ZGA, zygotic gene activation

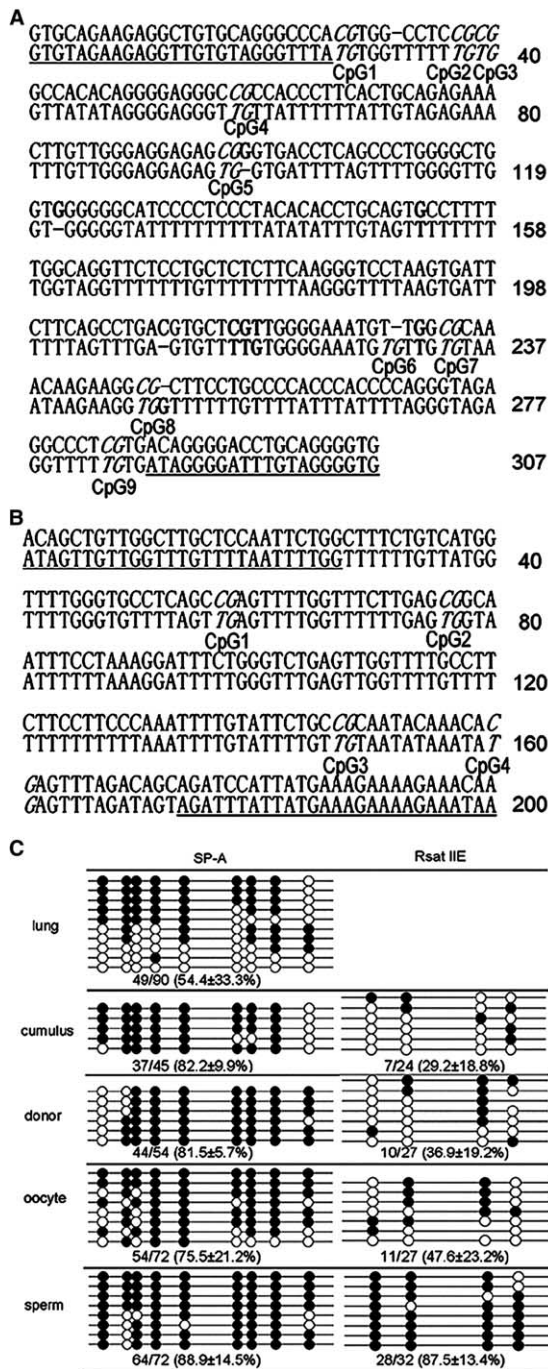


Fig. 1. DNA methylation pattern in rabbit lung tissue, cumulus, donor fibroblasts, spermatozoa and oocytes. (A) Nucleotide sequence of *SP-A* promoter region fragment (upper strands) and the sequence of bisulfite-PCR (lower strands). Note that two CpG sites were lost and one CpG site was created in PCR amplicon compared to the sequence derived from GenBank due to sequence variety between individual animals (shown in bold). (B) Nucleotide sequence of satellite DNA *Rsat IIE* (upper strands) and the corresponding bisulfite-converted sequence (lower strands). CpG dinucleotides are numbered and marked in italic. Primer sequences are underlined. (C) Methylation profiles of CpG dinucleotides of *SP-A* promoter and satellite sequence *Rsat IIE* in rabbit lung tissue (lung), cumulus cells (cumulus), fetal fibroblasts used as donors in NT (donor), oocyte and spermatozoa (sperm). 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.

above. Using this method, we never found any indication of incomplete bisulfite reactivity.

2.3. PCR amplification, cloning and sequencing

Because of the limited quantity of preimplantation embryos, we amplified both sequences by hemi-nested PCR. For amplification of *SP-A* promoter region (GenBank Accession No. L19387), the primary PCR consisted of 30 cycles of 94 °C for 40 s, 52 °C for 1.5 min and 72 °C for 1.5 min using primers 5'-GTG TAG AAG AGG TTG TGT AGG GTT TA-3' and 5'-CCT ACA CAC AAA ACC TCC AAA CTA TA-3', and then another 30 cycles of 94 °C for 40 s, 55 °C for 50 s and 72 °C for 50 s using the primer set 5'-GTG TAG AAG AGG TTG TGT AGG GTT TA-3' and 5'-ACA CCC CTA CAA ATC CCC TAT-3'. For amplification of satellite sequence *Rsat IIE* (GenBank Accession No. AY114136), the primary PCR was accomplished with 30 cycles of 94 °C for 40 s, 48 °C for 1 min and 72 °C for 1 min with the primer set 5'-TTT AAT TAG GGT ATA GTT GGT TTG T-3' and 5'-GGT TAT TTC TTT TCT TTC ATA ATA AAT CTA-3'; this was followed by 30 cycles of 94 °C for 40 s, 52 °C for 50 s and 72 °C for 50 s, with primers 5'-ATA GTT GGT TTG TTT TAA TTT TGG T-3' and 5'-GGT TAT TTC TTT TCT TTC ATA ATA AAT CTA-3'. The PCR products pooled from three independent amplifications were cloned into TA-cloning vector (TaKaRa). Individual clones were sequenced using an automatic sequencer (ABI PRISM 337).

3. Results

3.1. DNA methylation of rabbit somatic cells, spermatozoa and oocytes

The promoter region of *SP-A*, a single-copy gene, and *Rsat IIE*, a satellite DNA, were chosen as targets for methylation analysis (Fig. 1A and B). We first examined the methylation of both sequences in fetal skin fibroblast cells (used as donors in NT) and cumulus cells. As shown in Fig. 1C, both sequences had similar methylation patterns in the two types of cells with the *SP-A* promoter sequence over-methylated (82.2 ± 9.9% in cumulus cells and 81.5 ± 5.7% in donor cells, mean ± S.D.) and the satellite DNA moderately methylated (29.2 ± 18.8% in cumulus cells and 36.9 ± 19.2% in donor cells). We also examined the methylation status of *SP-A* promoter sequence in lung where the *SP-A* gene was particularly expressed [18], and found a significantly lower methylation value (54.4 ± 33.3%, $P < 0.05$) compared to those of cumulus cells and primary cultured cells.

We subsequently examined the DNA methylation status of the two sequences in rabbit spermatozoa and oocytes. As shown in Fig. 1C, the *SP-A* promoter region was hypermethylated in both spermatozoa (88.9 ± 14.5%, mean ± S.D.) and oocytes (75.5 ± 21.2%), but for satellite sequence *Rsat IIE* there was a distinct difference between the methylation pattern of the two kinds of germ cells, with spermatozoa being hypermethylated (87.5 ± 13.4%) and oocytes moderately methylated (47.6 ± 23.2%).

3.2. DNA methylation of in vivo-derived embryos

As shown in Fig. 2A, the promoter region of the *SP-A* gene was over-methylated in two-cell embryos (84.3 ± 20.9%, mean ± S.D.) and four-cell embryos (84.1 ± 21.1%). This hypermethylation pattern was gradually lost as the embryos developed to 8- to 16-cell embryos (63.2 ± 40.7%), morulae (34.4 ± 25.9%) and blastocysts (17.7 ± 15.4%). Satellite sequence *Rsat IIE* displayed a similar methylation pattern, except that no demethylation occurred between morula and

blastocyst stages. Considering that the composite methylation values of the two parental genomes in theory were ~83% for *SP-A* promoter region and ~68% for satellite sequence *Rsat IIE* (calculated from $(89 + 76)\%/2$ and $(88 + 48)\%/2$, respectively, see Fig. 1C), it appears that no demethylation occurred in both sequences during the first two cleavages. Fig. 2B shows that the methylation pattern of the satellite sequence closely resembles that of *SP-A* promoter region.

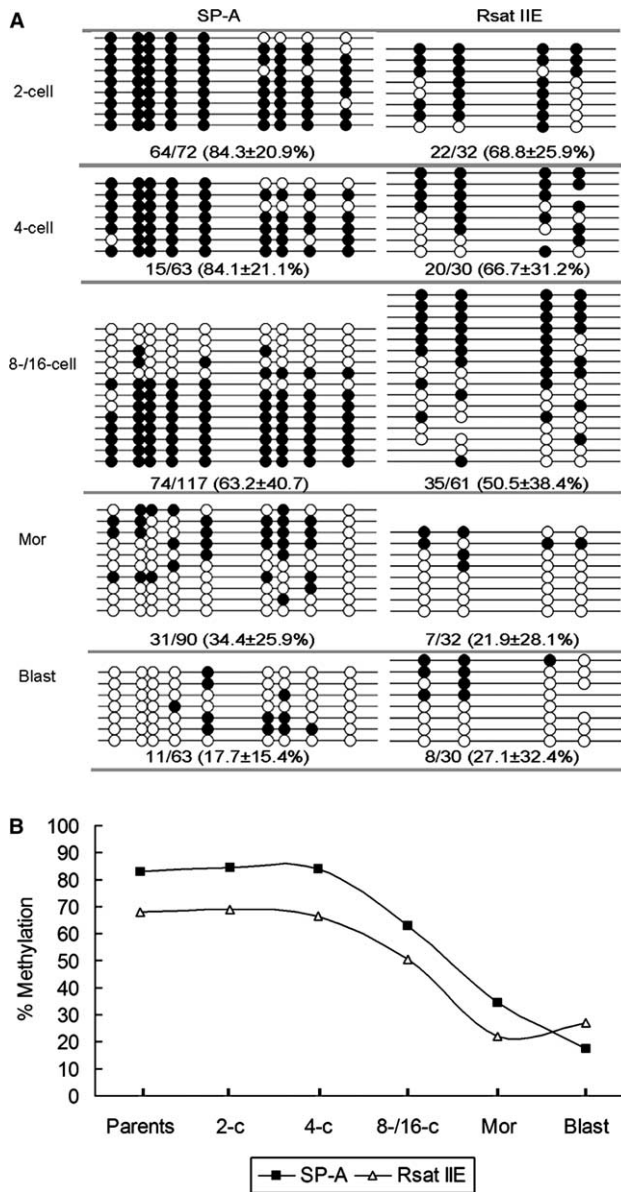


Fig. 2. Methylation profiles in normal rabbit embryos. (A) Methylation pattern of *SP-A* promoter region and satellite sequence *Rsat IIE* under various developmental stages. Open and closed circles indicate unmethylated and methylated CpG sites, respectively. Percent methylation is shown in parentheses. (B) Methylation changes of *SP-A* promoter sequence (closed square) and satellite sequence *Rsat IIE* (open triangle) in accordance with developmental stages. For a reference, the composite methylation value of the two parental genomes in theory (Parents) is included. 2-c, two cell embryos; 4-c, four cell embryos; 8-/16-c, eight- to sixteen-cell embryos; Mor, morulae; Blast, blastocysts.

3.3. DNA methylation of cloned rabbit embryos

Our data in Fig. 3A show a dramatic change of DNA methylation in the promoter region of the *SP-A* gene. An intermediate level ($59.3 \pm 46.0\%$) of methylation was seen at the two-cell stage, which further dropped to 6.7% in 8- to 16-cell embryos. After this stage, de novo methylation took place and the methylation status was recovered to 61.9% in morulae and to 80.2% in blastocysts. However, the satellite sequence showed remarkable differences in NT embryos. During NT embryo development, the methylation level of *Rsat IIE* in donor nuclei was maintained without significant changes ($P > 0.05$). The aberrant methylation patterns of the two sequences are shown in Fig. 3B.

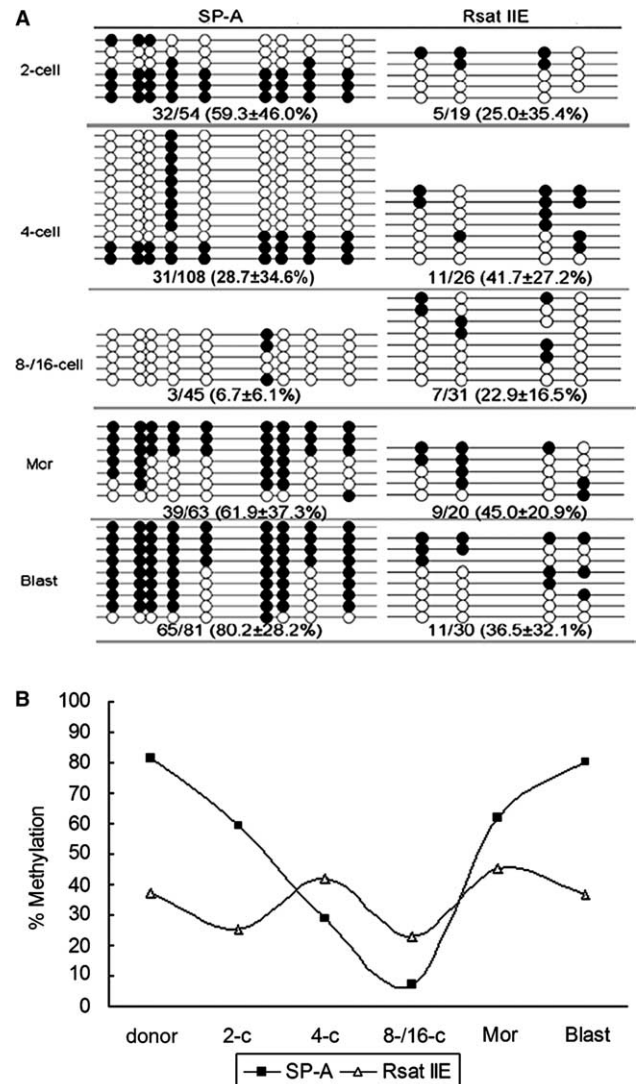


Fig. 3. Methylation profiles in cloned rabbit embryos. (A) Methylation pattern of *SP-A* promoter region and satellite sequence *Rsat IIE* of various developmental stages. Open and closed circles indicate unmethylated and methylated CpG sites, respectively. Percent methylation is shown in parentheses. (B) Methylation changes of *SP-A* promoter sequence (closed square) and satellite sequence *Rsat IIE* (open triangle) in accordance with developmental stages. For a reference, the methylation status of donor fibroblasts (Donor) is included. 2-c, two cell embryos; 4-c, four cell embryos; 8-/16-c, eight- to sixteen-cell embryos; Mor, morulae; Blast, blastocysts.

4. Discussion

In this study, the promoter region of *SP-A*, a single-copy gene, and *Rsat IIE*, a satellite DNA, was chosen for the analysis of methylation patterns in normal and cloned rabbit embryos. Our findings revealed that the *SP-A* promoter sequence was hypermethylated in both spermatozoa and oocytes, whereas the satellite sequence was only hypermethylated in spermatozoa. The methylation pattern of the repeated sequence is similar to the gamete-specific methylation pattern observed in pig, but different from that observed in mouse and cow [6,7,19]. We also found that the *SP-A* promoter sequence displayed a significantly lower methylation level in lung than in cumulus and fetal skin fibroblast cells, suggesting that the tissue-specific expression of *SP-A* may be regulated by DNA methylation.

In normal rabbit embryos, both sequences maintained the hypermethylation status during the first two cleavages. Progressive demethylation began at the 8- to 16-cell stage. Whether other genomic sequences display similar patterns is not known at the present time. However, a previous report using restriction endonuclease digestion also showed that the rabbit embryo genome had uniform hypermethylation at least to the eight-cell stage [20], and it had been reported that no active demethylation was observed in rabbit embryos [8,9]. This delayed demethylation pattern, which is quite different from that observed in mouse [2] and cattle [5,6], may represent a general reprogramming mode in rabbit embryos. It is interesting to note that in rabbit embryos, zygotic gene activation (ZGA) also occurs at the 8- to 16-cell stage [21], whereas in mouse [22] and most likely in cattle [23] ZGA is already seen at the two-cell stage. It appears that there is an interrelationship between the delayed demethylation pattern and the delayed activation of the embryonic genome [9].

In rabbit NT embryos, both sequences showed abnormal DNA methylation patterns. The promoter region of the single-copy gene showed fast demethylation and early de novo methylation, while the satellite sequence maintained the methylation status of donor nuclei through all examined stages. Recently, a study using immunofluorescence techniques showed that cloned rabbit preimplantation embryos displayed no detectable DNA methylation changes [9]. Since the immunofluorescence technique used in their study mainly reflects the methylation status of genomic repeats [24], the observation is consistent with our results of the stable methylation level of the satellite sequence in NT embryos. We also do not find any evident methylation change in rabbit short interspersed C repeat sequence in NT embryos (our unpublished data). In cloned bovine embryos, an absence of demethylation in repeated sequences was also seen [6], but the promoter region of several tissue-specific genes had similar demethylation patterns as those of in vitro fertilized bovine embryos [12]. However, our results indicate that inaccurate reprogramming can also take place on single-copy genes, at least in cloned rabbit embryos. The reprogramming differences of single-copy genes in cloned cattle and rabbit embryos may be due to different genes selected, but it is also possible that the methylation patterns of unique sequences in rabbit are more vulnerable to NT than in cattle. Support for this notion comes from the fact that the

production of cloned offspring in cattle is now routine, while rabbit cloning is difficult and success has only been documented in one report [16]. We have observed that major loss of rabbit cloned embryos occurred at the 8- to 16-cell stages (our unpublished data), when ZGA and demethylation take place during normal embryo development. We suggest that the aberrant methylation pattern in cloned rabbit embryos perturbs ZGA and leads to subsequent developmental failures and embryo death.

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