Influence of oocyte nuclei on demethylation of donor genome in cloned bovine embryos

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Abstract We recently demonstrated that satellite regions exhibit an aberrant DNA methylation in cloned bovine embryos. Here, we examined, using bisulfite-sequencing technology, whether the inefficient demethylation of cloned donor genomes could be rescued by the presence of oocytic nuclei. Both *Aci*I digestion and sequencing analyses showed that satellite sequence was demethylated more efficiently in cloned tetraploid blastocysts than in diploid clones. When methyl-CpG density (the number of methyl-CpG sites per string) was scored, a significant decrease was observed in tetraploids ($P < 0.001$). These results suggest that unknown mechanisms provided by oocytic nuclei could assist the demethylation of satellite sequences in tetraploid clones.

Key words: Epigenetic; Nuclear transfer; Bisulfite; Reprogramming

1. Introduction

Regardless of the recent successes in cloning various animal species, the use of somatic cells as the source of donor nuclei has raised a number of practical and significant issues such as increased abortion rates, high birth weight, various disease phenotypes and perinatal death [1–5]. These anomalies may be related with incomplete epigenetic reprogramming of donor DNA that occurs in cloned embryos [6]. In mice, a remarkable change in DNA methylation, a so-called genome-wide demethylation, occurs during early development, erasing gamete-specific methylation patterns inherited from the parents [7–11]. This process may be a prerequisite for the formation of pluripotent stem cells that are important for later development [12].

We recently provided evidence supporting the existence of the demethylation process in early bovine embryos developed from in vitro fertilization (IVF). All the genomic regions examined showed considerably undermethylated states in IVF blastocysts, which may be assumed to be the result of a genome-wide demethylation process [6]. In the *Bov-B* LINE sequences of early IVF embryos, we observed a gradual pattern of endogenous demethylation. However, this appears not to be the case with cloned bovine embryos. Various genomic repeats of cloned bovine embryos were observed to carry just the donor-type methylation patterns such that they were heavily methylated in levels similar to the donor genome. This observation is sufficient to raise the possibility that an unknown activity, or activities, exists that hinders demethylation of the donor genome in cloned embryos, and that this function may be carried out by donor cells. Such a factor could be a protein complex that can actively maintain the donor-type methylation patterns in the absence of oocytic genomic material and its associated elements. Alternatively, it could be the chromatin structure itself of the donor genome that interferes with the passive demethylation process that is regularly taking place in embryonic environment. In combination with the presence of donor cell-specific factors, the absence of oocytic nuclei may be the reason for the incomplete demethylation observed in cloned embryos. Thus, an intriguing result could be expected if such a donor cell-specific factor(s) is forced to compete with the oocytic nuclei-associated factors by placing them in the same space. To do this, here, we made tetraploid clones that carry both donor genome and oocytic nuclei, and tested whether any epigenetic changes occur in tetraploid clones.

2. Materials and methods

2.1. Cell culture and embryo manipulations

We isolated fetal fibroblasts from the carcass of a 40-day-old male fetus and cells after three passages were used in nuclear transfer as donor cells [13].

For nuclear transfer, we removed both the first polar body and metaphase plate from the oocytes matured in vitro [14]. The resulting oocytes were individually fused with fibroblasts using one electrical pulse of 1.6 kV/cm for 30 μs (BTX; model ECM2001), activated and cultured in vitro as described previously [13]. We obtained embryos derived from IVF procedure, as described before [15]. Blastocysts were collected at 168 h after activation (cloned one) or fertilization (IVF). The mean numbers of blastomeres were about 130 and 100 for IVF and cloned blastocysts, respectively. By the procedure of in vitro maturation, we collected 200–250 oocytes at the presumptive metaphase II stage. Pronase (0.5%, Sigma) was used to remove zones from embryos and mature oocytes to exclude the possibility of genomic contamination, such as by residual sperm or attached cumulus cells. Parthenogenesis was induced with in vitro matured oocytes by chemical activation [13]. The overall procedure for production of cloned embryos carrying the oocytic genome was the same as the nuclear transfer procedure except the enucleation step was omitted. In vitro matured oocytes were cleared of the first polar body, fused with donor cells, subsequently activated, and then cultured in vitro for 7 days. The number of blastocysts analyzed was seven for each group. Differ-

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ences in methylation rates among experimental groups were analyzed by two independent population *t*-tests.

2.2. Bisulfite treatment, PCR and AciI digestion

Genomic DNAs isolated from blastocysts were digested with *Bam*HI (New England Biolabs), denatured with 0.3 N NaOH, and treated with sodium bisulfite (pH 5, Sigma) [16]. Following desalting and desulfonating steps, DNA was precipitated and resuspended in 20 μl dry weight. For the amplification of satellite DNA region, 4 μl of bisulfite-treated embryonic DNA was applied to PCR and triplicated. PCR products were pooled before cloning or labeling. The number of blastomeres used as template in PCR was approximately 500–600 per sample. PCR was done with 30 (cells) or 40 (embryos) cycles of 94°C for 60 s, 46°C for 60 s and 72°C for 20 s. The primer set used was 5′-AATACCTCTAATTTCAAACT-3′ and 5′-TTTGTGAATGTAGTTAATA-3′. PCRs were finished with one cycle of 72°C for 10 min. We cloned and sequenced PCR products using ABI PRISM-377. For labeling PCR products, we reamplified PCR products with 25 cycles in the presence of 2 μCi of [^{32}P]dCTP. 100 ng of purified PCR products was incubated with ten units of *Aci*I (New England Biolab) at 37°C for 16 h. Since the strands of genomic DNA were no longer complementary to each other after bisulfite treatment, the *Aci*I enzyme would work only on DNA fragments amplified from one of the two genomic strands. The reaction products were resolved on 5% non-denaturing polyacrylamide gel electrophoresis (PAGE), dried, and exposed to autoradiographic film. We duplicated or triplicated the enzyme digestion assays in each experiment. Band intensity was calculated using an image analyzer, Tina20.

3. Results

We tested whether the inefficient demethylation of cloned genomes could be rescued by the presence of oocytic nuclei. Non-enucleated oocytes carrying intact metaphase II plates were fused with fibroblast cells and then chemically activated in a condition that suppresses extrusion of the second polar body. Tetraploidy of the resulting embryos was confirmed by karyotype analysis (data not shown). The satellite I DNA region [6] was chosen as a target for methylation analysis. Genomic DNAs of blastocyst-stage embryos derived from IVF procedure, parthenogenetic activation (PG), reconstituted diploid (2n) or tetraploid clones (4n) were all treated with bisulfite [16]. We also included donor fetal fibroblasts for a reference. Bisulfite causes deamination of unmethylated cytosines to uridine, thereby allowing discrimination between unmethylated and methylated cytosine residues through restriction enzyme analysis or sequencing.

A 211 bp segment of the satellite I DNA region that has 12 CpG sites and two *Aci*I recognition sequences (5′-GCGG-3′) was amplified from bisulfite-treated genome by PCR, and digested with *Aci*I enzyme (Fig. 1, upper panel). Complete digestion of two *Aci*I sites should yield 35-, 86-, and 90-bp fragments; however, because of their excessive signal intensities and low resolution on a 5% polyacrylamide gel, individual DNA bands were not clearly resolved. As can be seen in Fig. 1, lower panel, both IVF (10%) and PG (3%) blastocysts have considerably undermethylated satellite sequences. Interestingly, the satellite I region was found to be 14% methylated in reconstituted tetraploid blastocysts, a value that is similar to that of IVF blastocysts but is considerably lower than that of reconstituted diploid counterparts (67%) carrying somatic nuclei only. Since the chemically activated parthenogenetic blastocysts showed only 3% of methylation in the satellite region, a simple calculation would yield an average of $(67+3\%) \div 2 = 35\%$ methylation rate to be expected in the cloned tetraploid blastocysts (if the potential effects of oocytic nuclei on the methylation status of the donor genome coexisting in the same cell are precluded). Thus, the methylation rate of 14% shown in tetraploid clones suggests that the donor genome is more efficiently demethylated in the presence of the oocytic nucleus than by the donor nucleus alone.

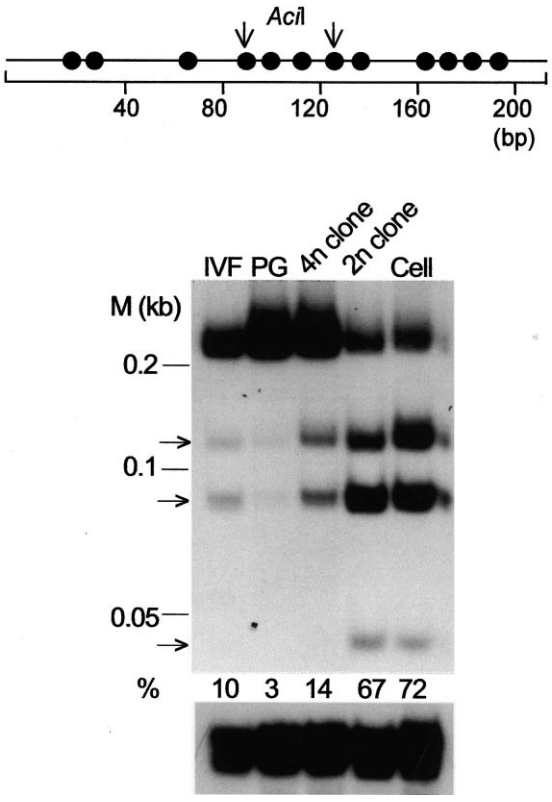

Fig. 1. *Aci*I digestion pattern of satellite I region amplified from bisulfite-treated genomic DNA isolated from cloned diploid (2n clone) and tetraploid (4n clone) blastocysts. The relative locations of 12 CpG sites (closed circle) and two *Aci*I recognition sites (standing arrows) are schematically represented. For comparison, the patterns of in vitro fertilized (IVF) blastocysts, parthenogenetic blastocysts (PG) and donor cell (Cell) are also included. Intact, undigested PCR bands are shown below the digestion panel. Arrows indicate the positions of *Aci*I-digested bands. Numbers indicate percent digestion (%), calculated from summed band intensity of digested fragments relative to that of the whole fragments.

In order to evaluate methylation profiles of 12 conserved CpG sites at the DNA sequence level, PCR products amplified from bisulfite-treated genomic satellite sequences were individually cloned and sequenced. The tetraploid genome was shown to carry 24% of methylated CpG sites (methyl-CpGs) in the satellite I sequence (Fig. 2); of 236 CpG dinucleotides, 56 CpG sites were methylated. PG chromosomes were only negligibly methylated (3%, or 3 of 95), and thus it appears that most of the methylated strings (PCR clones) in the methylation profile of tetraploid clones are derived from somatic chromosomes. The proportion of methyl-CpGs among the somatically derived satellite I region sequences in tetraploids would be $(24-3\%) \times 2 = 42\%$ in theory. This value is, when compared with the result of our accompanying report [6], higher than the one seen in IVF embryos (23%, 152 of 670) but much lower than the one seen in diploid clones (70%, 499 of 709) or donor cells (79%, 110 of 140), suggesting that a methylation modification occurs in donor genome-derived satellite sequences of cloned 4n embryos. This result further con-

firmed the *Aci*I restriction enzyme analysis results shown in Fig. 1.

For a more detailed analysis of the methylation changes occurring in satellite sequences of tetraploids, the methyl-CpG density (the number of methylated CpGs per string) was scored (Fig. 3). It should be noted that only the strings carrying at least one or more methylated CpG sites were included for calculation. For comparison, methyl-CpG densities of both diploid clones and donor cells were also scored from the results of our accompanying paper [6]. When the methyl-CpG density was examined, a significant difference ($P < 0.001$) was observed between tetraploids and diploid clones. The diploid clones had methylated strings mostly carrying seven to 12 methyl-CpGs (centered on 11) in the satellite sequences, much like the donor cells. In contrast, the tetraploids were shown to have mainly four to nine methyl-CpG containing strings (centered on five). The average methylation rate per string was $77 \pm 22\%$ (ranging from 8 to 100%) and $42 \pm 22\%$ (8–75%) in diploid and tetraploid clones, respectively. These results indicate that the satellite I region of the somatically originated chromosomes becomes demethylated more extensively in the tetraploid clones than in the diploid versions, presumably assisted by a mechanism provided by the oocytic nuclei.

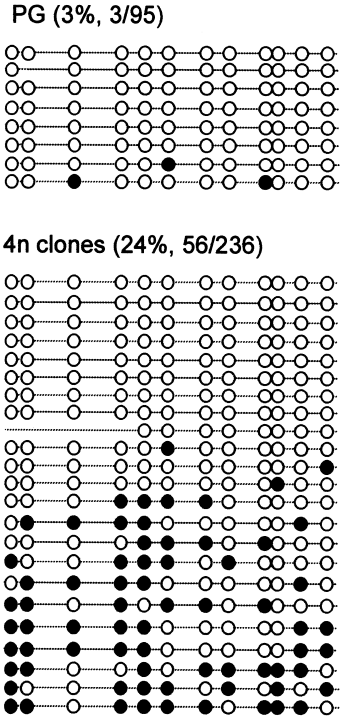

Fig. 2. Methylation profiles of 12 conserved CpG sites in the satellite sequences of parthenogenones and cloned tetraploid blastocysts. The methylation status of each CpG site was scored by sequencing the PCR clones derived from bisulfite-treated genomic DNAs. Open and closed circles indicate unmethylated and methylated CpGs, respectively. Some CpG sites are absent from the satellite sequence in some clones due to mutations in the particular copies of the satellite sequence. Numbers in the parentheses indicate the proportion of methylated CpG sites relative to the whole CpG sites examined.

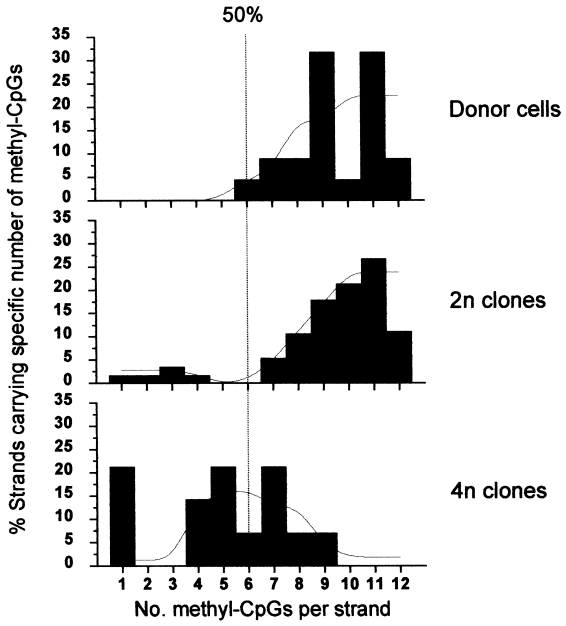

Fig. 3. Diagrams showing the proportions of variously methylated strands counted from the methylation profiles of donor cells (Donor cells), cloned diploid blastocysts (2n clones) and cloned tetraploid blastocysts (4n clones). Only the strands carrying at least one or more methyl-CpGs were scored. The position of 50% methylation is indicated by a dotted vertical line. A clear distinction in the methyl-CpG density is seen between the diploid and tetraploid clone groups. Curves in each panel represent regressed plots of the primary data, obtained by using the Savitzky–Golay filter (Micrococal Origin version 6.0).

4. Discussion

Our observations suggest the existence of an oocyte-specific demethylating activity that could modify the epigenetic status of genomic DNA in early bovine embryos, like the one demonstrated in the mouse [10,11,17,18]. In fact, it appears that the demethylation process at the preimplantation stage is not limited to the mouse. Our findings with the *Bov-B* bovine LINE sequences [6] showed that the preimplantation-stage bovine embryo clearly undergoes a demethylation process. The promoter regions of tissue-specific genes, which are known to be considerably methylated in the mouse oocyte and sperm DNA [19], are initially highly methylated in bovine embryos and then dramatically demethylated at later cleavage stages (unpublished data). Thus, it seems likely that, similar to mouse embryos, normal bovine counterparts undergo a genome-wide demethylation that carries out epigenetic modification of early embryonic DNA.

Our results suggest that the maintenance of abnormal methylation in satellite I sequences of cloned embryos is, directly or indirectly, overcome by the presence of oocyte-specific factors associated with oocyte nuclei. However, it is currently completely unknown what elements are involved in the demethylation of satellite sequences in tetraploid embryos. However, there are some possible explanations for this. Tetraploid clones are different from diploid ones in that they retain entire genomic (genomic DNA and associated oocyte-specific factors) and cytoplasmic materials (such as proteins and mRNA stocks). The integrity of ooplasm in tetraploid clones may be the reason. It is believed that oocytes carry a constant amount of cytoplasmic components to support their early

development. From the removal of oocytic nuclei (enucleation), the cytoplasmic contents are generally reduced to 60–70%, which may cause some essential embryonic processes, including demethylation, to become disrupted. There is yet another possible explanation. In mice, oocyte-specific DNA methyltransferase is actively retained in the cytoplasm of oocyte and embryo as a mechanism of early embryonic demethylation [17,18]. Such a functional organization of oocytic constituents may be entangled and interrupted by manipulations such as the enucleation and electrical fusion in cloning procedure. It seems also plausible that an unknown factor(s) that participates in the regulation of endogenous demethylation in early embryos may be also expressed from the genome of tetraploid clones. This unknown activity may compete for epigenetic modification of the satellite sequences with some donor cell-specific factors with an activity of maintenance methylation [20], resulting in demethylation. Our observation could be a composite result of all these possibilities. Currently, however, it appears premature to draw conclusions about our observations since much remains unknown regarding the mechanism of demethylation in normally fertilized bovine embryos as well as in cloned ones. Therefore, much more should be learnt about the molecular events that control the competition between the oocyte-specific and donor-specific factors in the presence or absence of oocytic nuclei.

In this study, the methylation status of the satellite DNA region was characterized through PCR amplification using a small quantity of embryonic DNA as templates. PCR was performed three times using genomic DNA of about 500 to 600 cell quantity in total, each with approximately 150–200 diploid genomes. Thus, the use of such a small template quantity in a methylation study might evoke skepticism for the reliability and suitability of our experiments. However, if it is taken into account that the satellite DNA region consists of highly repeated units, this template quantity could be recognized as sufficient for PCR analysis without significant experimental errors.

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