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DNA HYPOMETHYLATION OF INDIVIDUAL SEQUENCES IN ABORTED CLONED BOVINE FETUSES

Tao Chen [1,2], Yan Jiang [1,2], Yan-Ling Zhang [1,2], Jing-He Liu [1,2], Yi Hou [1], Heide Schatten [3], Da-Yuan Chen [1], and Qing-Yuan Sun [1]

[1] State Key Laboratory of Reproductive Biology, Institute of Zoology, [2]Graduate School, Chinese Academy of Sciences, Beijing 100080, China, [3]Department of Veterinary Pathobiology, University of Missouri-Columbia, Columbia, Missouri 65211

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1. ABSTRACT

Cloned bovines have a much higher abortion rate than those derived *in vivo*. Available evidence indicates that inappropriate epigenetic reprogramming of donor nuclei is the primary cause of cloning failure. To gain a better understanding of the DNA methylation changes associated with the high abortion rate of cloned bovines, we examined the DNA methylation status of a repeated sequence (satellite I) and the promoter regions of two single-copy genes (interleukin 3/cytokeratin) in aborted cloned fetuses, aborted fetuses derived from artificial insemination (AI), cloned adults and AI adults by bisulfite sequencing and restriction enzyme analysis. Two of four aborted cloned fetuses show very low methylation levels in the two single-copy gene promoter regions. One of the two fetuses also showed undermethylated status in the satellite I sequence. The other two aborted cloned fetuses have similar methylation levels to those of aborted AI fetuses. However, no difference in methylation was observed between cloned adults and AI adults. Our results demonstrate for the first time the undermethylated status of individual sequences in aborted cloned fetuses. These findings suggest that aberrant DNA methylation may contribute to the developmental failure of cloned bovine fetuses.

2. INTRODUCTION

Nuclear transfer (NT) has been successfully used in various species, but the low efficiency and the frequent anomalies inherent to cloned animals indicate that the technique is still in its infancy (1-5). The differentiated somatic nucleus introduced into the enucleated oocyte must cease its own gene expression program and restore the embryonic expression program needed for normal development. This process involves a series of complex epigenetic alterations, of which DNA methylation related to chromatin structure remodeling appears to be one of the prime targets (2, 6). In mammals, DNA methylation takes place mainly on the CpG dinucleotides. DNA methyltransferase 3a (DNMT3a) and DNMT3b are mainly involved in the *de novo* methylation, while DNMT1, with a preference for hemi-methylated substrates, acts mainly as maintenance methyltransferase (7). DNA Demethylation can occur in the absence of DNMT1 when replication takes place (passive demethylation) or independent of replication (active demethylation) (8-12).

A number of researches have reported aberrant DNA methylation patterns in cloned animals (13-20). Many of these researches use bovine as a model system since cloning technique in this species is well established and has

a relatively high efficiency. It is worthy to be note that most of the analyses have been done in preimplantation embryos and choose demethylation as main indicator for appropriate reprogramming. However, the extent of correct remethylation at late development stages may be more important for cloned animals to get proper development. Compared with *in vivo*-derived ones, cloned bovine have a much higher abortion rate. Cezar et al. examined the genomic-wide cytosine methylation levels of bovine fetuses by reverse-phase HPLC, and found great loss of methylation in aborted cloned fetuses. In the nigh examined aborted cloned fetuses, six have no detectable methylation level (21). But the global hypomethylation of DNA from aborted cloned fetuses wait sequence-level confirmations.

In order to get a better understanding of the DNA methylation changes associated with the high abortion rate of cloned bovine fetuses, in the present research we examined the methylation status of three individual sequences in aborted cloned fetuses, cloned adults, aborted AI fetuses and AI adults. Interleukin 3 is one of the cytokines that promotes the *in vitro* differentiation and proliferation of hematopoietic progenitors. It also participates in the response of the organism to various types of stress. (22). Here we choose the promoter region of this gene as target to reflect the detailed methylation profiles in various samples by bisulfite sequencing its promoter region. The methylation profiles of cytokeratin promoter region and satellite I have been well studied by restriction enzyme analysis in preimplantation bovine embryos and aging fetal cells (17, 23, 24). In this study, our investigation of the two sequences using the same restriction analysis method can not only provide additional methylation information but reveal possible links between the methylation status of preimplantation and late developmental stage.

3. MATERIALS AND METHODS

3.1 Oocyte collection and in vitro maturation

Recipient oocytes were collected from ovaries of sex mature bovine obtained from a slaughterhouse. Only oocytes with compact and homogeneous cytoplasm were selected for *in vitro* maturation. The selected cumulus oocyte complexes (COCs) were cultured in maturation medium which comprised M199 (GIBCO) supplemented with 10% FBS (GIBCO), 0.5 mg/ml FSH, 0.01 IU/ml LH and 1.0 mg/ ml estuarial at 38.5°C in the humidified 5% CO2 in air atmosphere. After culturing for 19 h, mature oocytes were subjected to NT.

3.2 Nuclear Transfer and Embryo Culture

We isolated skin fibroblasts from an adult bovine ear tissue and cells at passage 3-10 were used as donors for NT. Both the first polar body and metaphase□plate with a small amount of surrounding cytoplasm were removed from the cumulus-free oocytes inM2 medium with 7.5 mg/ ml cytochalasin B . Fusion of cell–oocyte couplets was induced by two direct current pulses (2.0 kV/cm, 10 msec each, 1 sec apart) in the electrical fusion solution which contained 0.28 M mannitol, 0.5 mM HEPES, 0.05 mM calcium, and 0.1 mM magnesium. Fused couplets were chemically activated by incubation in 5 mM A23187 for 4 min, cycloheximide (10 mg/ml) + cytochalasin D(3 mg/ml) for 1 hr , cycloheximide(10 mg/ml) for 4 hr and then cultured in CR1-aa for further development. Embryos were cultured in CR1-aa supplemented with 10% FBS at 38.5°C in the humidified 5% CO2 in air atmosphere for 48 hr. The embryos were then co-cultured with mouse fetal fibroblast cells in the same medium, which was changed by half every 48 hr.

3.3 Embryo Transfer and Artificial Insemination

On Day 7 after nuclear transfer, morphologically normal blastocysts were non-surgically transferred into synchronized recipients. All the adult clones had been confirmed to be genetically identical using microsatellite DNA analysis (25). Control fetuses and adults were generated by inseminating heifers with frozen-thawed semen by standard procedures.

3.4 Genomic DNA Isolation and Bisulfite Treatment

Skin biopsies were obtained from four *in vivo*-derived and four cloned spontaneously aborted fetuses. All of the fetuses were female with age between 60-90 days. Blood samples were obtained form four normal and four cloned adult cows, which were phenotypically normal and had no history of health or reproductive disorders. The ages of adults are between 18-24 months. Genomic DNAs were isolated from skin and blood with a Wizard Genomic DNA Purification kit (Promega, Madison, WI). Bisulfite treatment was performed as previously described by us (15). Briefly, genomic DNAs were digested with *Eco*RI restriction enzyme (TaKaRa, Japan) at 30 ^{o}C for three hours. After denaturing in 0.3 M NaOH at 50 ^{o}C for 15 min, samples were mixed with 2 volumes of 2% LMP agarose and pipetted into chilled mineral oil to form agarose beads, which were treated with freshly made bisulfite solution (2.5M sodium metabisulphite, Merck; 125mM hydroquinone, Sigma; pH5.0) at 50 ^{o}C for 8-10 hours in the dark. The reaction was stopped by equilibrations against 1ml TE for 3 times. Following desulphonation in 500μl NaOH for 30min, the beads were washed with TE and H_2O and stored at –20 ^{o}C before use.

3.5 PCR Amplification and Sequencing of the bisulfite-treated genomic DNA

For amplification of the interleukin 3 promoter region (GenBankTM U72065), the primary PCR consisted of 30 cycles of 94 ^{o}C for 40s, 52 ^{o}C for 1min and 72 ^{o}C for 1min using primers 5'-AGA AGG AGG TGT AAG ATG GAG AGA TTG T-3' and 5'-AAA TCT CTT ACC TAT CCC CAC TCT A -3', and then another 30 cycles of 94 ^{o}C for 40s, 55 ^{o}C for 50s and 72 ^{o}C for 50s using the primer set 5'- AGA AGG AGG TGT AAG ATG GAG AGA TTG T -3'and 5'- TCC AAA TTT CAC TAA TCT CAA ATA CTA A -3'. Amplification of 5' region of the bovine epidermal cytokeratin gene and satellite I DNA was as described by Kang et al (23). For the interleukin 3 promoter region, the PCR products pooled from three independent amplifications were cloned into TA-cloning vector (TaKaRa, Japan). Individual clones were sequenced using an automatic sequencer (ABI PRISM 337) and complete conversions of base Cs to Ts by bisulfite treatment were

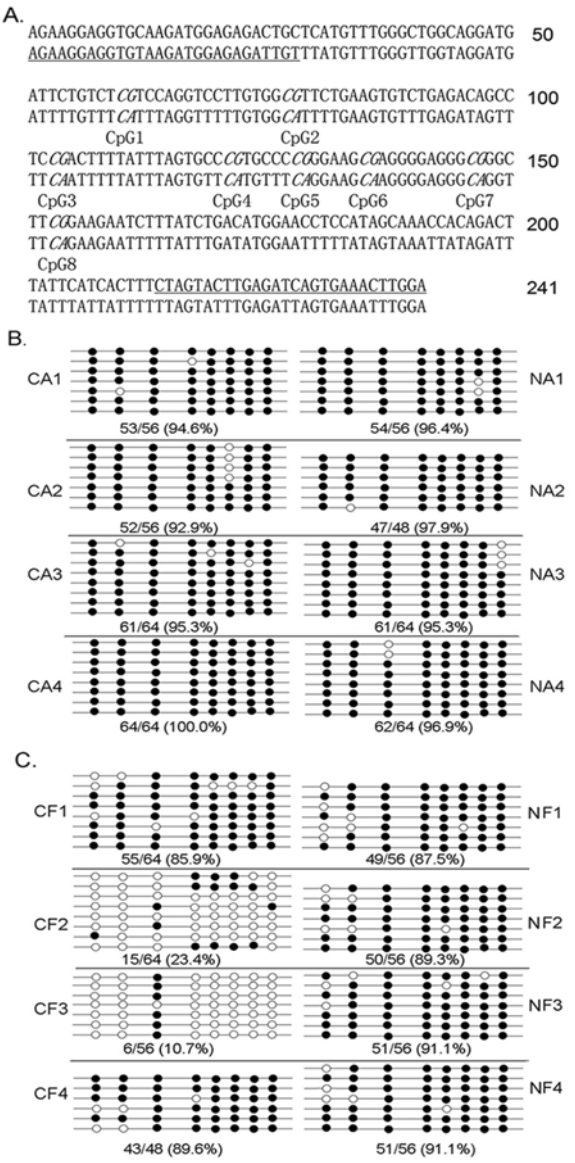

Figure 1. DNA methylation status of interleukin 3 promoter sequence. (A), Nucleotide sequence of interleukin 3 promoter region fragment (upper strands) and the sequence of bisulphite-PCR result (lower strands). CpG dinucleotides are numbered and marked in italic. Primer sequences are underlined. (B), Methylation profiles of CpG dinucleotides of interleukin 3 promoter in aborted cloned fetuses (CF1-4) and aborted AI fetuses (NF1-4). (C), Methylation profiles of CpG dinucleotides of interleukin 3 promoter in cloned adults (CA1-4) and AI adults (NA1-4). Open and closed circles indicate unmethylated and methylated CpG sites respectively. Percent methylation is shown in parentheses.

confirmed. Differences in methylation rates among experimental groups were analyzed by two independent population *t*-test.

3.6 Restriction analysis of PCR products

For *Aci*I restriction analysis of the bovine epidermal cytokeratin gene and satellite I DNA, 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 units of *Aci*I restriction enzyme (New England Biolabs, Beverly, MA) overnight at 37^{o}C, resolved on 6% non-denaturing polyacrylamide gel, which were silver stained as previously reported (26). We used duplicate or triplicate enzyme digestion assays in each experiment.

4. RESULTS

4.1 The methylation profile of the interleukin 3 promoter region

Bovine genomic DNAs were isolated from blood or skin samples for measuring the methylation status of the interleukin 3 promoter region (Figure 1A). First we examined the methylation status of the sequence in NT and AI adult cows. As shown in Figure 1B, all four examined AI adults show high methylation status, with a mean methylation level of 95.7 ± 3.0 %. The four adult cloned bovine have nearly the same high methylation level (96.6 ± 1.1 %, P>0.5). Then we examined the methylation status in aborted AI and NT fetuses (Figure 1C). The four aborted AI fetuses have high methylation status, with a mean level of 89.8 ± 1.7%. But it is significantly lower than that of AI adults (P<0.05). The four aborted cloned fetuses can be divided into two groups according to their methylation levels. One group (including CF1 and CF4, with methylation status 85.9% and 89.6% respectively) was heavily methylated. The high methylation level is similar to that of aborted AI fetus. The other group (including CF2 and CF3) has much low methylation level. The methylation status of CF2 is only 23.4%, and CF3 only 10.7%.

4.2 The methylation status of the epidermal cytokeratin promoter

For the 5' region of epidermal cytokeratin gene (Figure 2), the PCR products were nearly completely digested by *Aci*I restriction enzyme in all examined cloned adults, AI adults and aborted AI fetus, indicating the hypermethylation status of cytokeratin gene promoter region in all these samples. But in aborted cloned fetuses complete digestion of PCR products only presented in two (CF1 and CF4) out of four fetuses. A majority of PCR products from the other two fetuses (CF2 and CF3) appears free from digestion, indicating that half of aborted cloned fetuses were undermethylated in cytokeratin gene promoter region. It is worth to note that CF2 and CF3 are just the two fetuses that had low methylation status in interleukin 3 promoter region.

4.3 The methylation status of the satellite I DNA

The PCR products of satellite I DNA were digested incompletely in all the four samples of AI adult bovine (Figure 3), which is in accordance with previous reports. AI adults, cloned adults and aborted AI fetuses all have similar digestion patterns of this gene, suggesting a

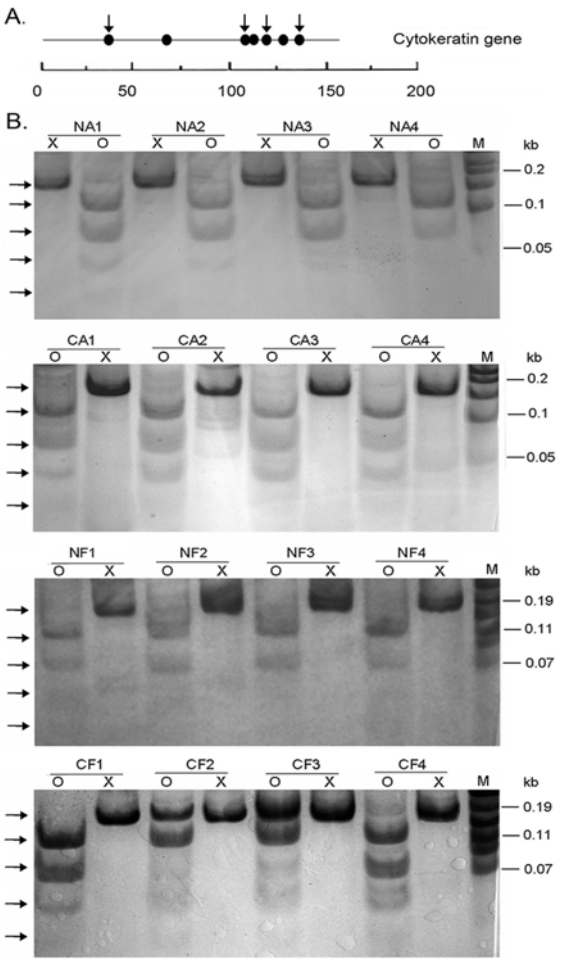

Figure 2. DNA methylation status of the cytokeratin promoter sequence. (A), Schematic diagram showing the relative locations of seven CpG dinucleotides (closed circles) and four *Aci*I recognition sites (standing arrows). (B)The *Aci* I digestion patterns of the cytokeratin promoter sequence amplified from genomic DNAs of AI adult (NA1-4), cloned adults (CA1-4), aborted AI fetuses (NF1-4) and aborted cloned fetuses (CF1-4). X, intact, undigested PCR products; O, *Aci* I-digested PCR products. *Arrows* indicate locations of PCR products and *Aci* I–digested PCR products. M, DNA size marker.

similar methylation level of satellite I in these samples. The PCR products of aborted cloned fetuses also have similar extent of digestion, except for one fetus (CF3), where a larger proportion of PCR products were shown to be resistant to enzyme digestion. Aborted cloned fetus CF2, which has hypomethylation status in both the interleukin 3 and the cytokeratin promoter regions, appears to have a normally methylation level in the satellite I sequence.

5. DISCUSSION

Animal cloning is still an inefficient technique, and inappropriate epigenetic reprogramming of donor nuclei has been reported by many researchers (13-18, 20, 27). However, most of the studies were performed in preimplantation embryos, while little is known about the methylation status at late developmental stages. Day 30-90 is a crucial stage when more than 80 percent bovine fetus abortions occur during this period (28). It has been proposed that the main cause of pregnancy failure may be due to aberrant placenta development (24, 28-30), but it cannot rule out the possibility of death caused by aberrant fetus. Cezar et al. have reported hypomethylation of DNA from cloned bovine fetuses using reverse-phase HPLC (21). Moreover, in their research six out of nigh aborted cloned fetuses had no detectable level of cytosine methylation (21). In contrast, Hiendleder et al. found significant hypermethylation in cloned bovine fetuses by capillary electrophoresis (31), but they did not examine the DNA methylation level of aborted cloned fetuses. In our results, two of four aborted cloned fetuses (CF2 and CF3) have much lower methylation levels in both examined single-copy genes than aborted AI fetuses. CF3 also has undermethylated status in satellite I sequence. The hypomethylation of individual sequences in aborted cloned fetuses is in agreement with the no detectable genomic-wide methylation level observed by Cezar et al (21). This hypomethylation status may induce aberrant gene expression, resulting in abnormal development of fetus (30).

Our bisulfite sequencing results of the interleukin 3 promoter sequence showed that two of aborted cloned fetuses had similar methylation levels to aborted AI fetuses, which were slightly, but significantly lower than the methylation level of AI or NT adults ($P<0.05$). This methylation difference cannot be simply attributed to different materials we used to extract DNA from adults and fetuses (blood and skin respectively), for the methylation status of the interleukin 3 promoter sequence in adult skin was nearly the same to that in adult blood (our unpublished data). Unfortunately, the methylation status of the interleukin 3 gene in AI fetuses recovered from ongoing normal pregnancies was not examined, so we don't know whether the hypomethylation status we observed is natural in bovine fetuses or related with abortion. Due to the limitation of the enzyme-digested technique, we did not know whether similar methylation differences existed for the cytokeratin gene promoter sequence and the satellite I DNA.

It is also interesting to note that there are two aborted cloned fetuses being undermethylated in both examined single-copy genes, while only one of them has low methylation status in satellite I sequence. Repeated sequences may be easier to be methylated than unique sequences. It has been reported that SINEs could be good elicitors of methylation spreading (32). On the other hand, Satellite I sequence may be reluctant to lose methylation. In bovine fetal fibroblasts satellite I DNA show a stable methylation level during aging while unique sequences are demethylated (23). In cloned preimplantation embryos, satellite I sequence can keep the donor type methylation status instead of being demethylated as single-copy genes (17).

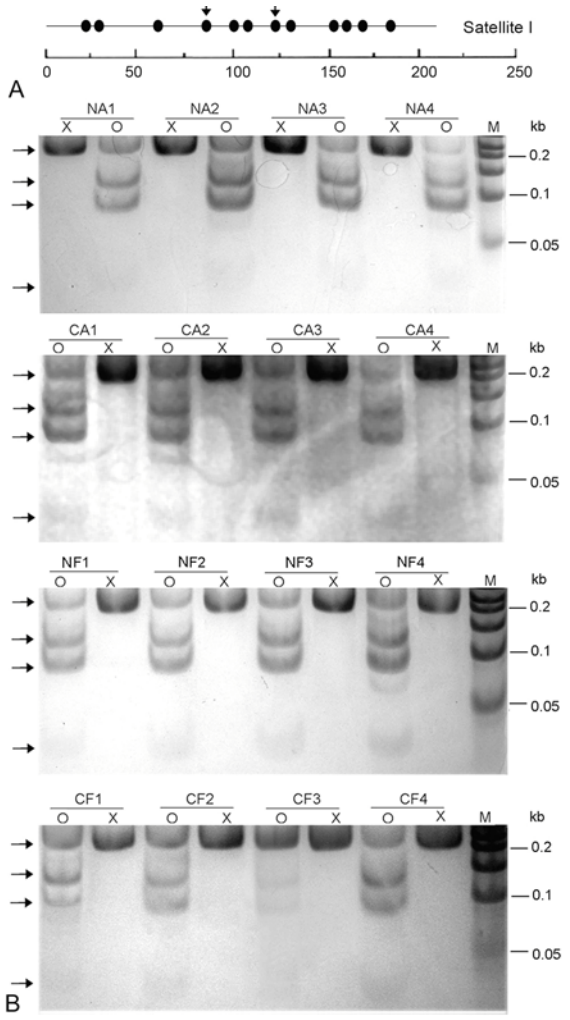

Figure 3. DNA methylation status of the satellite I sequence. (A), The relative locations of 12 CpG dinucleotides (closed circles) and two *Aci*I recognition sites (standing arrows) are schematically represented. (B), The *Aci* I digestion patterns of the satellite I sequence amplified from genomic DNAs of AI adult (NA1-4), cloned adults (CA1-4), aborted AI fetuses (NF1-4) and aborted cloned fetuses (CF1-4). X, intact and undigested PCR products; O, *Aci* I-digested PCR products. *Arrows* indicate locations of PCR products and *Aci* I–digested PCR products. M indicates DNA size marker.

De novo methylation has been reported in 8-/16-cell bovine embryos by immunofluorescence interphase cell with anti-5-MeC antibody (16), but this may be a visual artifact associated with decreasing nuclear intensity between progressive cell division (13, 33). Consistent with this is that immunostaining chromosome with the same antibody or bisulfite sequencing individual sequences showed little evidence of remethylation (14, 17, 18, 34, 35). Thus for most of bovine genomic sequences de novo methylation may take place after implantation, and till now only two sequences (the galanin gene and the satellite II sequence) have been reported to be de novo methylated before implantation (34, 35). In cloned bovine embryos, euchromatin or single copy gene sequences can be demethylated, resulting in an undermethylation pattern at morula and blastocyst stages, similar to that of normal embryos (14, 18). On the other hand, centromeric heterochromatin or repeated sequences appear to be free from demethylation during preimplantation NT embryo development and have a hypermethylation status in blastocysts (14, 17). Analysis of individual NT blastocysts also showed that the cytokeratin promoter region was undermethylated in most of them while the satellite I sequence was substantially methylated (18). But separately investigating the satellite I methylation profiles in ICM and TE indicate that the high methylation level in most of NT blastocysts mainly comes from TE, while in ICM the satellite sequence has a low methylation status (18). Thus, it appears more reasonable to assume that the hypomethylation status of aborted cloned fetuses may be due to dysfunction of remethylation rather than demethylation. Consisting with the assumption is that targeted disruption of de novo methyltransferase Dnmt3a and b can lead to global demethylation of the genome and associated perinatal or late development lethality (36).

Our result that NT adults have no significant methylation difference from AI adults indicates that cloned fetuses surviving into adulthood may undergo correct epigenetic reprogramming. It has been suggested that there is an epigenetic reprogramming threshold for proper development of cloned animals (21). Understanding of how this threshold is achieved may be important to increase cloning efficiency. Our multi-sequences examination indicates that aberrant methylation may be a crucial factor for achieving the threshold. Although in this research only limited animal samples were examined and more data from other genomic sequences need to be collected, the generality of our results was supported by HPLC examination of genomic-wide cytosine methylation level (21).

6. ACKNOWLEDGMENT

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Send correspondence to: Qing-Yuan Sun, PhD, Institute of Zoology, Chinese Academy of Science, Beishihuanxi Road 25, Beijing, 100080, P. R. China Tel: 8610-8262-7593, Fax: 8610-6256-5689, E-mail: sunqy1@yahoo.com