—Full Paper—

DNA Methylation Profiles of Donor Nuclei Cells and Tissues of Cloned Bovine Fetuses

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Abstract. Methylation of DNA in CpG islands plays an important role during fetal development and differentiation because CpG islands are preferentially located in upstream regions of mammalian genomic DNA, including the transcription start site of housekeeping genes and are also associated with tissue-specific genes. Somatic nuclear transfer (NT) technology has been used to generate live clones in numerous mammalian species, but only a low percentage of nuclear transferred animals develop to term. Abnormal epigenetic changes in the CpG islands of donor nuclei after nuclear transfer could contribute to a high rate of abortion during early gestation and increase perinatal death. These changes have yet to be explored. Thus, we investigated the genome-wide DNA methylation profiles of CpG islands in nuclei donor cells and NT animals. Using Restriction Landmark Genomic Scanning (RLGS), we showed, for the first time, the epigenetic profile formation of tissues from NT bovine fetuses produced from cumulus cells. From approximately 2600 unmethylated *NotI* sites visualized on the RLGS profile, at least 35 *NotI* sites showed different methylation statuses. Moreover, we proved that fetal and placental tissues from artificially inseminated and cloned cattle have tissue-specific differences in the genome-wide methylation profiles of the CpG islands. We also found that possible abnormalities occurred in the fetal brain and placental tissues of cloned animals.

Key words: Bovine, CpG islands, DNA methylation, Nuclear transfer, RLGS

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Production of cloned mammals by nuclear transfer (NT) of adult cells into enucleated oocytes has been successful. However, only a small proportion of reconstructed embryos develop to

term. The highest success rate of all mammalian cloning attempts was achieved in Japanese black cattle [1]. Placentomegaly is a common disorder observed in phenotypically abnormal cloned embryos [2, 3]. In addition, fully grown cloned animals showed many abnormal symptoms, such as pulmonary hypertension, pneumonia, kidney defects, liver defects, and obesity [3].

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In the mammalian genome, DNA methylation occurs at cytosines of CpG dinucleotides and is involved in various gene functions, including X-chromosome inactivation and gene imprinting [4]. Tissue-dependent differentially methylated regions (T-DMRs) have been identified and revealed to be involved in the regulation of specific gene expression in various tissues and cell types [5–7]. The T-DMRs were identified as widespread in the euchromatin regions of the genome [8], and every tissue and cell had a unique DNA methylation profile consisting of T-DMRs [9–12]. Moreover, the expression of developmental master genes is controlled by methylation of their T-DMRs [13, 14]. It has been shown that the methylation status of T-DMRs changes dynamically with developmental stage [15].

Aberrant DNA methylation in the T-DMRs of fully grown cloned mice has been detected by genome-wide analysis of T-DMRs [16, 17]. Data suggests that complete re-establishment of the DNA methylation profiles was quite difficult to achieve in full-term cloned fetus [18]. Genome-wide DNA methylation analysis in T-DMRs is desired to clarify which DNA methylation marks affect the reorganization of correct DNA methylation profiles in nuclear transferred fetuses. In this study, we report genome-wide DNA methylation analysis of donor cells and NT bovine fetuses by Restriction Landmark Genomic Scanning (RLGS). Our results suggest that DNA methylation profiling will be useful for evaluating the success rate of cloned animals.

Materials and Methods

Cell culture

Ovaries were obtained from a local abattoir and transported at 25–30 °C to the laboratory. Cumulus-oocyte complexes (COCs) were aspirated from follicles 2–8 mm in diameter and placed into sterile plastic tubes. Cumulus cells were harvested from the COCs and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA USA) containing 10% fetal bovine serum (FBS, JRH, Lenexa, USA), 1 mM L-glutamine (Invitrogen), 100 U/ml of penicillin, and 100 µg/ml of streptomycin (Invitrogen). Cultured cells were grown at 37 °C in 5% CO₂ at high humidity and were routinely passaged 6–8 times until confluent for genomic

DNA extraction.

NT and control animals

Bovine NT embryos were produced from cumulus cells as described previously [19]. Control pregnancies were derived by artificial insemination (AI) of Japanese black cows (day 0 = day of insemination). Diagnosis of pregnancy was made on day 30 of gestation by transrectal ultrasonography (7.5 MHz linear probe, SSD-1700, Aloka, Tokyo, Japan). Fetal viability was confirmed by heartbeat detection 2–3 days prior to slaughter. Fetal and placental tissues of two NT and two AI cows were collected after the cows were slaughtered on days 48 and 59 of gestation. Sex determination was carried out with Y specific primers (5'-AAGCGCCCCATGAATGCATTTATGGT-3' and 5'-ACACTTTAGCCCTCCGATGAGGCTGA-3') and revealed that all animals studied were female. Endometrial tissue (caruncular), placental tissues (cotyledonary and intercotyledonary) and fetal brains from control and cloned animals were collected and separated as detailed by Nikitenko *et al.* [20] and Regnault *et al.* [21]. All tissues were immediately placed in liquid nitrogen and stored at –80 °C until processing.

Preparation of genomic DNA

Genomic DNA was extracted as described previously [12]. Briefly, cells and tissues were suspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM EDTA, 1% SDS) with proteinase K (Merck, Darmstadt, Germany). The mixture was incubated at 55 °C for 20 min. Following two phenol/chloroform/isoamyl alcohol (50:49:1) extractions, genomic DNA was precipitated in ethanol, pelleted, and redissolved in TE buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA).

Restriction landmark genomic scanning (RLGS)

The RLGS was performed as described previously [9]. Briefly, 3.5 µg of genomic DNA was treated with 10 units of Klenow fragment (TaKaRa, Kyoto, Japan) in the presence of 0.4 µM dGTPαS, 0.2 µM dCTPαS (Amersham Pharmacia, Buckinghamshire, UK), 0.4 µM ddATP and 0.4 µM ddTTP (TaKaRa). The DNA was first digested with 20 units of *Not*I as a landmark enzyme (Nippon Gene, Toyama, Japan) and the cohesive ends were isotopically labeled with 1.3 units of Sequenase Ver 2.0 (USB Corporation, NE) in the presence of 0.33

Table 1. Distribution of *NotI* sites in the bovine and mouse genomes

	Total number	In non-repetitive sequence	In repetitive sequence	Size of database (bp)	RLGS profile
Bovine	8240	6529 79.2 %	1645 20.0 %	2.41×10^9	2600 31.6%
Mouse	6087	3808 62.5 %	2227 36.6 %	2.68×10^9	1500 24.6%

The genomic sequence data was downloaded from the UCSC genome browser site (<http://genome.ucsc.edu/>) and analyzed by Perl script as described previously [23]. Mouse genome: build 33 assemblies (mm5, May 2004). Bovine genome: btau_1.0 assembly (bosTau1, Sep 2004). Note that in spite of similar genome size, the number of *NotI* sites is higher in the bovine genome compared to those of the mouse. Moreover, in the bovine genome, 79.2 % of all *NotI* sites are localized in non-repetitive sequences.

Fig. 1. Typical RLGS profile showing genome-wide DNA methylation status in various CpG islands of the bovine DNA. Approximately 2600 *NotI* sites appeared as spots on a single RLGS profile. From them, 35 spots (open red circles with numbers) are methylated differently among the DNA of the cumulus cells, ICFM, and fetal brains of the AI and NT animals. These *NotI* sites were named tissue-dependent differentially methylated regions or T-DMRs.

μM [α - ^{32}P]dCTP and $0.33 \mu\text{M}$ [α - ^{32}P]dGTP (Amersham). The labeled DNA was then treated with 20 units of *PvuII* (Nippon Gene) and subjected to first dimension electrophoresis in a 0.9 % agarose disc gel for approximately 23 hrs at 230 V. The DNA fragments in the gel were treated with 1.000 units of *PstI* (Nippon Gene). The second

electrophoresis was carried out in 5% polyacrylamide gel for 20 hrs at 150 V. The resulting gel was dried and exposed to X-ray film (Kodak, XAR 5, Eastman Kodak, Rochester, NY) for 2–3 weeks at -80°C . The spot profile was reproduced at least two times.

Fig. 2. Methylation profiles of T-DMRs among donor nuclei cells, and control and cloned fetal tissues. Open and closed circles represent the unmethylated and methylated status of *NotI* sites, respectively. T-DMRs have been combined into several groups as follows: tissue-specifically methylated in cumulus cells (A, B), ICFM (C), and brain (D, E). Arbitrarily methylated loci were also detected (F). Aberrant methylated T-DMRs in cloned fetuses are represented in groups I, II, III, and IV.

Results

Analysis of the DNA methylation profiles of the bovine genome

Profiles of DNA methylation of T-DMRs using RLGS with *NotI* methyl-sensitive restriction enzyme as a landmark enzyme have been produced for the mouse, rat, and human genomes

[9, 11, 22]. These RLGS profiles represent the DNA methylation patterns of thousands of *NotI* sites that are mainly located in genic regions [23]. Bioinformatic analysis of bovine draft genome sequence data indicated that 79% of *NotI* sites are distributed in non-repetitive sequences (Table 1), suggesting that RLGS profiles of the bovine genome show DNA methylation patterns of *NotI* sites corresponding to unique sequences widespread in the entire genome.

We used RLGS to analyze the DNA methylation profiles of fetuses resulting from NT, AI, and donor nuclei cells. Approximately 2600 *NotI* sites were visualized as spots on a single bovine RLGS profile (Fig. 1). The numbers of *NotI* sites estimated from the draft sequence data from mouse and bovine genomes were compared in Table 1. In contrast to the 1500 RLGS-detectable mouse *NotI* sites (24.6%), the efficiency of bovine RLGS analysis is higher, revealing 2600 *NotI* sites or 31.6% of all genomic *NotI* sites.

Bovine fetus-specific T-DMRs

We previously showed that aberrant DNA methylation mainly occurred in T-DMRs [8]. To investigate the establishment of methylation profiles in cloned bovine fetuses, we compared their epigenetic profiles with those of donor cumulus cells and tissues from fetuses obtained by artificial insemination. Intercotyledonal fetal membranes (ICFM) were used as placental tissue and fetal brain as fetal tissue for this study. Although almost all spots in the RLGS profiles matched each other, there were fifteen cumulus-specific T-DMRs: four hypo-methylated (Fig. 2-A) and eleven hyper-methylated regions (Fig. 2-B). We found six *NotI* sites specifically hypo-methylated in the ICFM (Fig. 2-C), and four specifically hyper-methylated (Fig. 2-D) and two hypo-methylated T-DMRs (Fig. 2-E) in the brain tissue. Six arbitrarily methylated loci were observed with methylation profiles that were not conserved among individuals (Fig. 2-F).

DNA methylation status of cloned animals

We then analyzed the RLGS profiles of the ICFM and brains of cloned fetuses to examine how adequately the DNA methylation profiles of the cloned fetuses could be established from those of the donor nuclei genome. The spots on the RLGS profiles of both types of tissue from the cloned

Fig. 3. Location and appearance of spots corresponding to *NotI* sites on RLGS profiles. A, B, C, D, and IV correspond to the groups displayed in Fig. 2. Arrowheads indicate detectable RLGS spots. (ICFM, intercotyledonal fetal membrane; FB, fetal brain)

bovine fetuses were nearly identical with those of the AI animals; however, aberrantly methylated loci were found in the cloned animals as shown in Fig. 3. Hyper- and hypo-methylation specific to cloned animals (Fig. 2-IV) were observed in two loci. Some arbitrarily methylated loci in the control animals also appeared in the cloned animals (Fig. 2-F).

In cumulus-specific T-DMRs, the methylation profiles of the cloned animals were different from those found in the T-DMRs of ICFM and fetal brains. The methylation profiles of eight of nine T-DMRs were established in the brains and ICFM of the cloned animals (Fig. 2-III). However, twelve of fifteen aberrantly methylated loci were found to be methylated in the same manner as the cumulus cells (Fig. 2-I, II).

Discussion

In this study, we compared the DNA methylation patterns of donor cells with those of fetal tissues and found genome-wide alterations in DNA methylation patterns after nuclear transfer. Also, we discovered some aberrantly methylated loci in the cloned fetal tissue. This data supports previous observations in the cloned mouse [16]. As DNA methylation is involved in regulation of gene function [4, 24], failure to reestablish previous DNA methylation patterns appears to be a major cause of cloning rate inefficiency [18].

Incorrect DNA methylation during early developmental stages has been reported previously [25–27]. Moreover, delayed and insufficient DNA demethylation was observed during early developmental stage in bovine cloned embryos [28, 29]. The mammalian genome mainly consists of

non-genic repetitive sequences [30–32], and these repetitive sequences have been shown to be hyper-methylated. Therefore, global analysis of genome methylation should reflect the methylation level of these repetitive sequences [33]. In contrast, RLGS displays the methylation profiles of *NotI* sites, which are located in non-repetitive sequences and are often in CpG islands (Table 1). Over half of the CpG islands are localized in promoter regions [34, 35], and their methylation statuses have been shown to be involved in the regulation of tissue-specific gene expression [36]. Thus, it is possible that the aberrant methylation in the T-DMRs found in the cloned animals by RLGS could cause deleterious effects on development of these animals.

In the cloned bovine fetuses, we found that aberrant methylation occurred in the T-DMRs of donor cumulus cells. Insufficient and slow demethylation has been reported in early cloned bovine embryos [28, 29, 37]. In addition, another study reported that the hyper-methylation observed in donor cells was retained in cloned blastocysts [38]. In this study, incorrect inheritance of methylation status was observed in both hypo-

and hyper methylated T-DMRs (Fig. 2-II, III). Insufficient global demethylation may not be the sole mechanism for aberrant DNA methylation profiles. Furthermore, these profiles could not be explained by loss of global methylation that has been observed in some NT fetuses undergoing normal pregnancies [39].

Skewed X chromosome inactivation [40] and loss of DNA methylation information in imprinted genes [41] have occurred in cloned mice. In this study, we observed incorrect DNA methylation statuses in cloned tissues that seemed to be inherited from donor nuclei, especially in the T-DMRs of donor cells, since all cell types have distinct tissue-specific DNA profiles [11]. Analysis of locus specific reestablishment of DNA methylation status will help us to understand how proper DNA methylation and demethylation occurs during mammalian fetal development.

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