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Epigenetic Characterization of the CpG Islands of Bovine *Leptin* and *POU5F1* Genes in Cloned Bovine Fetuses

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Abstract. Abnormal development and fetal loss during postimplantation period are concerns for production of nuclear transferred animals. Aberrant DNA methylation is one of the reasons for poor survival of cloned animals. In mammalian genome DNA, CpG islands are preferentially located at the start of transcription of housekeeping genes and are associated with tissue-specific genes. The correct and consecutive mechanisms of DNA methylation in the CpG islands are necessary for selective gene expressions that determine the properties of individual cells, tissues, and organs. In this study, we investigated the methylation status of the CpG islands of the bovine *Leptin* and *POU5F1* genes in fetal and placental tissues from fetuses produced by artificial insemination (AI) and nuclear transfer (NT) at days 48 and 59 of pregnancy. Altered DNA methylation was observed in the normal and cloned fetal, placental, and endometrial tissues using bisulfite sequencing and pyrosequencing. Different tissue-specific methylated regions in the bovine *Leptin* and *POU5F1* genes show a variable methylation status in NT fetuses compared to AI control.

Key words: Bovine, CpG islands, DNA methylation, *Leptin*, Nuclear transfer, *POU5F1*

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DNA methylation is involved in the development of mammals through various gene functions, including gene-silencing, X inactivation, and imprinting. DNA methylation occurs mainly at the cytosine in CpG dinucleotides. CpG islands are the stretches of the DNA sequence (over 200 bp) with higher CG contents (C+G

frequency; %CG>50%) and higher CpG frequencies with a ratio of observed to expected based on CpG content (CpG_{obs}/CpG_{exp}) over 0.6 [1]. Many housekeeping genes and almost half of the tissue-specific genes have CpG islands [2, 3]. CpG islands and genes with regions of higher CpG frequencies were once considered to be unmethylated in all tissues except for those related to X inactivation, genomic imprinting, and cancer [4, 5]. However, there are tissue-dependent and differentially

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methyated regions (T-DMRs) even in CpG-rich unique sequences in rat and murine normal tissues and cells [6–9]. The human genome also shows DNA methylation in the CpG-rich regions of normal tissues [10, 11].

Production rate of cloned animals, which are obtained by transferring nuclei from adult somatic cells, is quite low due to early death after implantation, although production has been successful in several species [12–15]. We previously found that there are several loci with aberrant DNA methylation in fully developed cloned mice [16]. CpG islands associated with T-DMRs are widespread in the mammalian genome [17], and their DNA methylation status illustrates the genome-wide profile unique to distinct cell types and tissues [6–8]. Since proper formation of DNA methylation profiles underlies normal differentiation and development, diagnosis based on determination of methylation profiles would be useful for evaluating the development of nuclear transferred (NT) animals.

T-DMRs in the CpG islands have been investigated in the mouse, rat, and human genome, but not in the bovine genome. Leptin is involved in regulation of interaction between maternal and fetoplacental compartments, and plays a functional role in fetal and placental growth [18]. *POU5F1* encodes octamer-binding transcription factor 4, which is involved in the development of animals. In the present study, we characterized the CpG islands of the bovine *Leptin* and *POU5F1* genes in normal bovine fetuses, and compared their DNA methylation status with those of cloned fetal and placental tissues.

Materials and Methods

NT and control animals

Bovine NT embryos were produced as described previously by using cumulus cells as the donor cells [19]. Control pregnancies were derived by artificial insemination (AI) of Japanese black cows (day 0=day of insemination). Diagnosis of pregnancy was made from days 30 of gestation by transrectal ultrasonography (7.5 MHz linear probe, SSD-1700, Aloka, Tokyo, Japan). Viability of fetuses was confirmed by detectable heartbeat 2–3 days prior to slaughter. NT and AI cows were slaughtered around days 48 and 59 of gestation.

The endometrial (caruncular and intercaruncular) and placental tissues (cotyledonary and intercotyledonary) from two control and two cloned animals were collected and separated as detailed by Nikitenko *et al.* [20]. Also, fetal brains were collected, and all tissues were immediately placed in liquid nitrogen and stored at –80 C till processing. (Fig. 1, Table 1).

Preparation of genomic DNA

Genomic DNA was extracted as described previously [6]. 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, the genomic DNA was precipitated in ethanol, pelleted, and redissolved in TE buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA).

Analysis of CpG frequency

The bovine *Leptin* and *POU5F1* gene sequences were obtained from the NCBI database (AB070368 and AF022986) (<http://www.ncbi.nlm.nih.gov>). The structures of these genes were described previously [21, 22]. The CG content and CpG frequency of the DNA sequences of these genes were analyzed by CpG View (version 1.4.6) on a Macintosh computer.

Bisulfite modification of DNA and sequencing

Genomic DNA digested with *Pst*I was denatured by adding 0.3 M NaOH and incubated for 15 min at 37 C. After incubation, sodium metabisulfite (pH 5.0) and hydroquinone were added to final concentrations of 2.0 M and 0.5 mM, respectively, and the mixture was further incubated at 55 C for 14 h in darkness. The modified DNA was purified using the Wizard DNA Clean-Up System (Promega, Madison, WI), and the bisulfite reaction was terminated by 15 min incubation at 37 C in 0.3 M NaOH. The solution was then neutralized by adding ammonium acetate (pH 7.0) to a final concentration of 3 M. The ethanol-precipitated DNA was resuspended in water and amplified by PCR using primers designed as follows: B_Lept_BS2_F, 5'-GAGGATTTTTATATTGTTT-AGTT-3'; B_Lept_BS2_R, 5'-TAAACTAAATAA-CCTACCAAAAAA-3'; POU5F1_BSPr_F, 5'-TTTTTATTTTGGTGTATGTGGGT-3'; and

Fig. 1. (A and B) Pictures of bovine maternal and fetal parts of the placenta containing a control fetus on day 59 of pregnancy (scale in centimeters). (C) Schematic view of fetal-maternal interaction in bovine uterus. In cattle, maternal (intercaruncular tissue: ICT) and fetal (ICFM: intercotyledonal fetal membrane) tissues fuse in discrete areas forming a disc-shaped placentome that consists of a fetal component (cotyledonal fetal membrane: CFM) and a maternal component (carunculolous tissue: CT). FB: fetal brain.

Table 1. Samples used for study methylation status of the bovine *Leptin* and *POU5F1* genes

			Leptin gene	POU5F1 gene	
Days			Bisulfite sequencing	Pyrosequencing	Bisulfite sequencing
Control	#1	48	ICFM, FB	ICFM, CFM, FB, CT	ICFM, FB
	#2	59			
Clone	#1	48			
	#2	59			

*POU5F1*_BSpr_R, 5'-AAAAACCCTTAAAA-ACTCATCCAAA-3'.

PCR amplification was performed with 10 ng of bisulfite-converted DNA in a total reaction volume of 50 μ l using Immolase DNA polymerase (Bioline, London, UK). The PCR conditions were as follows

: 95 C for 10 min; 40 cycles of 94 C for 30 sec, 55 C for 30 sec, and 72 C for 60 sec; and final extension 72 C for 10 min. Amplified PCR products were cloned into pGEM-T easy vector (Promega, Madison). Individual clones were sequenced using an ABI Prism 310 sequencer (Genetic Analyzer).

Table 2. Primers for pyrosequencing analysis of the bovine *POU5F1* gene

Region	PCR primers	Length	Sequencing primers	CpG #
Region I	POU5F1_BS2_F (Forward)	541	5'-GAAGTGTTAGTTATGTT-3'	1
	5'-ATGGAAGTTGGTTGGTAGTTTGTT-3'		5'-TTGATTTATAGATTGTAGTT-3'	2,3
	POU5F1_BS_R (Reverse)		5'-AGGTAAGTATATTGGAGT-3'	4
	5'-CTCCCAAACCCACATACACCAAAA-3'		5'-GGGGTTTTGAATAA-3'	6
Region II	POU5F1_BSpr_F (Forward)	474	5'-TTGGAAGTGAAGGT-3'	8
	5'-TTTTTATTTTGGTGTATGTGGGTT-3'		5'-CCAAACTCCTAAACT-3'	9
	POU5F1_BSpr_R (Reverse)		5'-AAATATTTACCTACTAATAA-3'	10
	5'-AAAAACCTTTAAAACTCATCCAAA-3'		5'-TATTAGTAGGTAAATATTTT-3'	11
			5'-ACCTTAACCTCTAAC-3'	13

Pyrosequencing

Bisulfite-treated genomic DNA was amplified by PCR with primers for the *POU5F1* gene promoter region (Table 2). One of the PCR primers was biotinylated in order to create a single-stranded DNA template. The PCR conditions were the same as for bisulfite sequencing PCR. Purification of the PCR products with streptavidin sepharose HP beads (Amersham Biosciences Ab, Uppsala, Sweden) was conducted according to the manufacturer's instructions. After completion of primer annealing, 500 ng of single-strand DNA-binding protein (Sigma, St Louis, MO) was added to the sequencing reaction mix. Sequencing was performed on a PSQ 96MA system with a SNP reagent kit (Pyrosequencing, Uppsala, Sweden) according to the manufacturer's instructions. The raw data were analyzed using the allele quantitation algorithm of the provided software.

Results

DNA methylation status of the *Leptin* gene in cloned bovine embryos

Bovine *Leptin* gene contains a CpG island with a length of 282 bp that spans from -204 bp to +78 bp and a CG frequency and CpG_{obs}/CpG_{exp} of 66% and 0.716, respectively. We investigated a 367 bp-long fragment carrying this CpG island that contained 35 CpGs (Fig. 2A) by sodium bisulfite sequencing (Fig. 2B).

In the control animals, all CpGs were almost completely unmethylated in both the intercotyledonal fetal membrane (ICFM) and fetal brain (FB). Thus, the CpG island of the *Leptin* gene

may serve as methylation free area in these bovine fetal tissues. In clones #1 and #2, the CpGs in the CpG island were also unmethylated in the ICFM, suggesting that the animal cloning process by nuclear transfer did not disturb normal DNA methylation in ICFM. Intriguingly, however, there was a different DNA methylation status in the CpG island of the FBs of the cloned animals. In clone #1, the CpGs at the 5'-end of CpG island, the 3rd CpG (-195 bp), and the 4th CpG (-186 bp) showed moderate methylation (Fig. 2B). Similarly, the 3rd and 22nd CpGs showed methylation in clone #2. At the 3'-end of the studied CpG island fragment, there was abnormal methylation of CpGs at the +44, +67, and +70 bp (33rd, 34th, and 35th CpGs, respectively) in the FBs of clones #1 and #2. Therefore, abnormal DNA methylation occurred in the cloned animals depending on the tissue.

DNA methylation of the *POU5F1* gene in cloned bovine embryos

Bovine *POU5F1* gene also has a CpG island with a length of 189 bp that spans from -44 bp to +145 bp, a CG frequency of 72%, and a CpG_{obs}/CpG_{exp} of 0.654. There are 11 CpGs arranged in the transcription start site and 3 CpGs in the upstream region covering the CpG island. The region further upstream of the bovine *POU5F1* gene is CpG poor, containing 13 CpGs within -815 bp and a SINE sequence from -578 bp to -762 bp with 4 CpGs (Fig. 3A).

We investigated 10 CpGs (1, 2, 3, 4, 6, 8, 9, 10, 11, and 13) in the 5'-upstream region of the bovine *POU5F1* gene in caruncular tissue (CT) and cotyledonal fetal membranes (CFM) by pyrosequencing analysis following the bisulfite

Fig. 2. DNA methylation status of the 5' flanking region and 1st exon of the bovine *Leptin* gene in fetal tissues of NT and AI animals. (A) The upper panel shows the GC content and CpG frequency of the bovine *Leptin* gene (-500 bp to +500 bp). The jagged line represents the average GC content, and the black bar displays the CpG frequency. White boxes represent CpG rich regions. The middle bars show the positions of the CpG and GpC sites. A schematic view of the region (-269 bp to +98 bp) analyzed by sodium bisulfite sequencing is shown in the lower panel. Open circles display the CpG positions. (B) The DNA methylation status of each PCR clone was determined by sodium bisulfite sequencing method. Black circles display the methylated status of the CpG sites.

treatment (Fig. 3B). The analysis revealed that the methylation levels of almost all of the CpGs in CT were higher than those in the ICM. As an example, most of the 8th CpG at -281 bp was methylated in

the CT (90.1%) of control #1, whereas the methylation degree was comparatively low in the CFM (57%). The degree of methylation was 50% in the 6th CpG at -506 bp in the CTs, but 0% in the

Fig. 3. DNA methylation analysis of the bovine *POU5F1* gene promoter region and first exon. (A) The upper panel shows the GC content and CpG frequency of bovine *POU5F1* gene (-1145 bp to +766 bp). The jagged line represents the average GC content, and the black bar displays CpG frequency. White boxes represent CpG rich regions. The middle bars show the positions of the CpG and GpC sites. Open circles display the CpG positions. (B) Pyrosequencing analysis data for the 1st to 10th CpG in the promoter region of the bovine *POU5F1* gene in the CT and CFM. Each methylation level for the 1st to 6th CpG in the FB and ICFM was also analyzed by pyrosequencing. The methylation level of each CpG is represented by a gray scale (right side of the figure). (C) Bisulfite sequencing of 20 CpGs (from 8th to 27th) arranged in the promoter region (9 CpGs) and 1-st exon (11 CpGs) of the bovine *POU5F1* gene. The 14th, 15th and 16th CpGs were completely unmethylated in the ICFM of clone #1 and are enclosed by a dotted line while the ICFMs of the controls and clone #2 showed partial methylation. Furthermore, the CpGs of the 1st exon have a lower methylation level in the ICFM clone #2 (enclosed by with dotted line) compared to that of the controls and clone #1.

CFMs of control #1 and #2. Thus, the 6th CpG displayed a clear difference between the CT and CFM. In addition, the 4th, 8th, 9th, and 10th CpGs displayed methylation of more than 80% in the CT, whereas they displayed about 50% methylation in the CFM (Fig. 3B). Therefore, the region containing the 1st to 11th CpGs was T-DMR.

The pyrosequencing data of the 1st to 6th CpGs in the FBs and ICFMs confirmed the conclusion above. The DNA methylation levels of most CpGs in the FBs and ICFMs were less than those in the CTs. The profiles of the DNA methylation levels in these CpGs were different among these tissues.

The 6th CpG displayed difference in methylation status between clone #1 (9.5%) and the controls (0%) in the CFM. The methylation status of the 6th CpG was 0% in the ICFMs of controls #1 and #2. In clone #1, however, this CpG showed 20.7% methylation in the ICFM. Similarly, the methylation status of the 6th CpG was slightly different in the CFM of clone #1 compared to control. These data illustrated the locus of CpG in the bovine *POU5F1* gene with an epigenetic variation between clones and controls.

Another region with epigenetic variations in cloned animals was identified by authentic bisulfite sequencing of 20 CpGs in ICFMs and FBs (Fig. 3C). In the ICFM of clone #1, the 14th, 15th, and 16th CpGs were completely unmethylated, whereas they were moderately methylated in the control. Furthermore, in the ICFM of clone #2, the 1st exon CpGs were unmethylated in contrast to the other ICFMs. No such difference was observed in the FBs.

Discussion

Many genes have T-DMRs and the DNA methylation profiles of T-DMRs are involved in tissue-specific and developmentally regulated gene expression [23]. *Leptin* is expressed in placenta and it may have physiological effects on placental growth. Abnormal placental leptin production associated with increased trophoblast proliferation [24] and the leptin concentration in cord blood are positively correlated with placental size [25]. Ravelich *et al.* [26] showed increased *Leptin* mRNA expression in the placenta of NT pregnancies. Yokomori *et al.* [27] demonstrated changes in DNA methylation in the promoter region of the *Leptin*

gene in mouse 3T3-L1 cells during differentiation, suggesting that DNA methylation is involved in *Leptin* gene expression. In bovine NT pregnancies, altered feto-placental growth and function is associated with disturbance in *Leptin* expression, which could lead to inadequate placentation [26]. The cloned fetuses (#1 and #2) in the present study were fortunate to have a normal methylation status for the T-DMR of the bovine *Leptin* gene in the ICFM. It is important to note that abnormal methylation of the ICFM has the potential to disrupt placental development and function.

In addition to its physiological effects on placental growth, leptin is also important for fetal development because it regulates the metabolic adaptation during fetal development that is required for normal pregnancy [28]. Abnormal methylation of the *Leptin* gene was detected in the FBs of the cloned fetuses, and thus may disturb further normal development of the brains of the cloned animals.

Several reports have revealed abnormal DNA methylation in preimplantation cloned embryos [29–31]. Since a large part of the genome is composed of non-genic repetitive elements [32, 33] the overall level of DNA methylation reflects the bulk methylation status of these repetitive sequences rather than unique sequences of the genes. Therefore, careful discussion of the genic and non-genic elements is required. DNA methylation of CpG islands is involved in regulation of tissue-specific gene expression [7]. Therefore, aberrant DNA methylation at CpG islands has the potential to have a deleterious effect on the phenotypes of cloned animals. The organization of bovine *POU5F1* is similar to its human and murine orthologs. Hattori *et al.* [34] showed that in mice, *Oct-4* gene expression is under the regulation of DNA methylation and chromatin structure remodeling. The bovine *POU5F1* expression pattern could be detected in early bovine embryonic development, but was not restricted to the pluripotent cells of the blastocyst [22]. There was no significant difference in DNA methylation patterns at the CpG island of the *POU5F1* gene between normal controls and cloned fetuses. Taking this together with the fact that the methylation status of *Leptin* gene in the ICFMs of the clones was similar to that of the normal control ICFMs, our data generally suggests that the extent of the abnormal DNA methylation profile is not

severe in cloned bovine fetus that are allowed to develop until 48 and 59 days.

Characterizing the gene associated with CpG islands is necessary to evaluate the development of normal and cloned cows. To our knowledge, this is

the first report on the CpG islands of bovine genes. There are T-DMRs in the bovine *POU5F1* and *Leptin* genes that show a variable methylation status in cloned bovine fetuses compared to normal controls.

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