Expression and Characterization of Bovine DNA Methyltransferase I

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ABSTRACT

In this study, bovine Dnmt1 cDNA was sequenced and detected Dnmt1 mRNA level in bovine tissues by northern blot, methylation pattern of genome by southern blot, specific localization of Dnmt1 in mouse and bovine preimplantation embryos by immunocytostaining and Dnmt1 protein level in ovary and testis by western blot. Bovine Dnmt1 cDNA sequence showed more homology with that of human than mouse and rat. The RNA level of Dnmt1 was 10 times higher expression in placenta than other tissues. This indicates that placenta was hypermethylated compared to others organs. The genomic DNA could not be cut by a specific restriction enzyme (HpaII) in placenta, lung and liver of bovine. It suggests that Dnmt1 in some somatic cells was already methylated. Dnmt1, which has the antibody epitope 1316～1616, was distributed in nucleus and cytoplasm including the stage of pronuclear stage and maturation of oocyte and gradually weaken to blastocyst stage compare to negative. In addition, Dnmt1 was strongly expressed in tetraploid embryo and cloned 8-cell than IVF 8-cell. An aberrant pattern of DNA methylation in cloned embryo may be abnormal development of fetus, embryonic lethality and placenta dysfunction. The somatic specific band (190kDa) was appeared in ovary and testis, but oocyte specific band (175kDa) was not. Further investigations are necessary to understand the complex links between the methyltransferases and the transcriptional activity of genes in the cloned bovine tissues.

(Key words : Bovine Dnmt1, Characterization, Expression, Immunocytochemistry)

INTRODUCTION

Eukaryotic genomes are not methylated uniformly but contain methylated regions interspersed with unmethylated domain (Bird, 1986). During devolution, the dinucleotide CpG has been progressively eliminated from the genome of higher eukaryotes and is present at only 5% to 10% of its predicted frequency (Antequera and Bird, 1993; Bird, 1995). Approximately 70% to 80% of the remaining CpG sites contain methylated cytosines in most vertebrates, including human (Antequera and Bird, 1993; Bird, 1995).

The enzymes that transfer methyl groups to the cytosine ring, cytosine 5-methyltransferase, or DNA methyltransferases (DNA-MTase; Dnmt) have been characterized in a number of eukaryotes (Bestor and Verdine, 1994). The target site for DNA-MTase in DNA is the dinucleotide palindrome CG. The first eukaryotic DNA-MTase gene cloned from mouse (Bestor *et al*., 1988) is now referred to as Dnmt1. *De novo* methylation induces to methylate on DNA initially, this DNA that was methylated replicates and then it induced to methylation again, so it called maintenance methylation (Singal and Ginder, 1999).

Eckardt and Mclaughlin (2004) discussed the use of tetra embryos as a model to evaluate reprogramming using gene expression and demonstrate that somatic cell nuclei can be reprogramming by blastomeres to re-express embryonic specific genes but not to contribute to post-implantation development. According to pattern of methylation in preimplantion development of mammal, level of methylation was slowly decreased after fertilized and it has the lowest level of methylation on implantation. Sequencing of bovine Dnmt 1 that has an important role for pattern of DNA methylation and specific localization of Dnmt1 in clone embryo is considered as a significant factor to understand problems in clone scientifically and basically (Golding and Westhusin, 2003; Liu *et al*., 2008; Kurihara *et al*., 2008).

So, the molecular characterization of bovine Dnmt1 was studied to know the expression pattern of mRNA. A localization of bovine Dnmt1 was researched to understand the function and roles of bovine Dnmt1 during the embryo development.

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MATERIALS AND METHODS

Cloning of Bovine Dnmt 1 cDNA

Bovine tissues of lung, liver, placenta and brain were homogenized with Trizol. First-strand cDNA synthesis kit was used superscript first-strand for RT-PCR and PCR. Total RNA mixes 1 μl oligo (dT) 12~18 and 10 mM dNTP and added a reaction buffer (10× RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, RNase inhibitor) and then, reaction at 42℃ for 2 min. Super script II enzyme added 1 μl and incubated 42℃ for 50 min, 70℃ for 15 min, 37℃ for 20 min and then, operated PCR. Partial region of Dnmt1 cDNA was cloned by PCR using mixed primer and sequenced. And then, RACE techniques were employed to sequence the 5′ region.

Northern Blot Analysis

mRNA of brain, placenta, lung and liver was isolated and then cDNA probe (807bp) was labeled with DIG DNA labeling method. Total RNA was denatured, size-fractioned on 1% agarose - 2.2 M/l formaldehyde gel electrophoresis for 1.5 h at 50V in 1 × MOPS (Gibco, USA), and transferred to Hybond-N^+ blotting membranes. Hybridization was carried out at 65℃ overnight in the presence of rapid hybridization.

For signal dectection, the membrane was incubated for 5 min in 0.25 mM CDP-star, as a chemiluminescent substrate and was exposed to Kodak film for 1~20 min.

Southern Blot Analysis

Genomic DNA was extracted from tissues to analysis DNA methylation pattern in the Dnmt 1. Next, DNA was electrophoresis after treatment of restriction enzymes, HpaII (methylation insensitive) and MspI (methylation sensitive). The membrane was dried briefly and UV-crosslink. We detected with the same probe used in the northern blot.

In Vivo Derived Mouse Embryos

We examined all *in vivo*-derived mouse preimplantation stages from one-cell to blastocyst. Mouse embryos were collected from superovulated females on 1 cell-stage embryos. BCF_1 mouse embryos were derived from a cross of (C57BL/6×CBA) F_1 females. The day after mating is termed day 1. The embryos were cultured in M16 F medium *in vitro* (Hogan *et al*., 1986).

In Vitro Fertilized Bovine Embryos

Ovaries were collected from a local slaughter and brought to the laboratory in physiological saline (0.85%, w/v, NaCl). COCs were aspirated from follicles (2~7 mm in diameter) and were washed twice with D-PBS (Gibco, USA) supplemented with 5% FBS (Gibco, USA) and antibiotic antimycotic (Gibco, USA). For *in vitro* mature, the ovum were cultured in TCM 199 (Sigma, USA) supplemented with 10% FBS at 38.5℃ in 5% CO_2 in air for 22 h. For IVF generation of fertilized oocytes, the procedure followed was using frozen semen and BO.

Bovine Cloned Embryos

The somatic cells used in this experiment were derived from bovine fetal fibroblast cells collected at 45 days of pregnancy. For NT, frozen-thawed cells were cultured up to the confluent stage and were utilized after the culture was placed in a serum-deficient medium for 5 days. The methods involved in the production of nuclear embryos are described in detail in the literature (Yang *et al*., 2006, Park *et al*., 2009).

Immunocytochemistry

Oocytes and preimplatation embryos were washed in PBS, fixed for 1 h at 4℃ in 0.4% paraformaldehyde in PBS with triton X-100 and permeabilized with 1% triton X-100 in PBS for 3 min at room temperature. The embryos were blocked for 1 h at 37℃ in 3% BSA in PBS. Cells were incubated overnight at 4℃ with a monoclonal antibody and a secondary antibody coupled with fluorescein. Anti-rabbit Dnmt1 antiserum was used the reagent generated against the amino-terminal polypeptide (amino acids 1316~1616, H-300) of Dnmt1 protein that is a common region between Dnmt1o and Dnmt1s. Digital optical sections from oocytes and embryos were recorded with a confocal microscope fluoview. For each wavelength, a series of 0.2 μm slices were scanned and exported as 24-bit TIFF files. Using Image and pseudo-colored using Adobe Photoshop 6.0 later projected the images.

Western Blot Analysis of Bovine Dnmt1 Protein

Protein determinations were made following the Bradford protein protocol. Homogenized samples were denatured for polyacrylamide gels (SDS-PAGE) were prepared so that the same amount (75 μg) of protein could be loaded into each lane on the 6% gel. The transfer was carried out to PVDF membrane for overnight in blotting buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, 15% methanol, pH 8.3).

Blot was detected using an Amplified Opti-4CN detection kit. The blot was blocked for 1 h in PBST (1 × PBS, 0.1% tween-20) containing 3% blocker. Primary antibody (1:1,000) was used for blotting. The primary antibody was same for staining of embryo and detected using goat anti-rabbit IgG-HRP conjugated secondary antibody (1:10,000, sc-2004, Santa cruz, USA). The blot was washed several times with PBST after each step. The membrane was incubated with diluted BAR for 10 min and washed 2 times 20% DMSO/PBST for 5 min.

The membrane incubated in 1,000 times-diluted streptavidine-HRP (horseradish peroxidase) for 30 min and poured onto Opti-4CN diluents concentration and Opti-4CN substrate. And then, the membrane was incubated with gentle agitation in the substrate for up to 30 min. or until the desired level of sensitivity is attained.

RESULTS

Cloning of Bovine Dnmt 1 cDNA

Bovine Dnmt1 cDNA was cloned by step by step PCR method by using 1st strand cDNA synthesized from bovine adult placenta (GenBank: AY173048). It was consisted of 1612 amino acids. Bovine DNMT 1 cDNA display high homolog to those reported for mouse (76 %), rat (76%), porcine (91%; unpublished) and human (88%).

Northern and Sothern Blot Analyses

It was defined normal mRNA as shown 28S and 18S in UV light (left). Dnmt1 mRNA was expressed in all tissues and placenta was especially high than liver, lung, and brain (right).

The southern blot analysis was shown the result of Dnmt1 in bovine tissues (placenta, lung, and liver). All tissues used in southern blot were cut in MspI enzyme, but it was not cut any tissues by HapII enzyme treatment. This result insisted that Dnmt1 genome controlled by other DNA methyltransferase.

Immunocytochemistry

Fig. 3A and B show the staining of Dnmt1 of *in vitro* fertilization bovine and *in vivo* derived mouse embryos. Fig. 3C was shown specific localization of Dnmt1 in cloned 8-cell embryo. Monoclonal antibody and a secondary antibody coupled with FITC (upper). Nuclear DNA was stained PI (down). Dnmt1 was distributed nuclear and cytoplasm including pronuclear stage, the stage of mutation of oocyte and gradually weaken to blastocyst stage compare to negative. It also showed the same pattern of Fig. 3B in *in vivo* derived mouse em-bryo. In addition, tetraploid embryo and cloned 8-cell

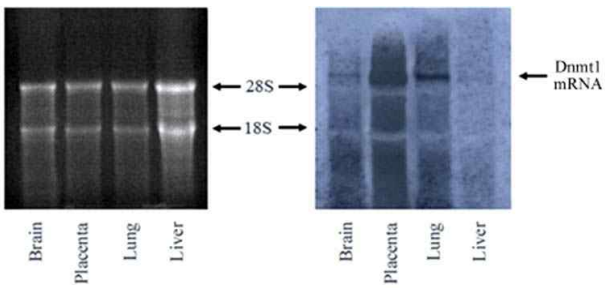

Fig. 1. Expression of Dnmt 1 mRNA in bovine tissues.

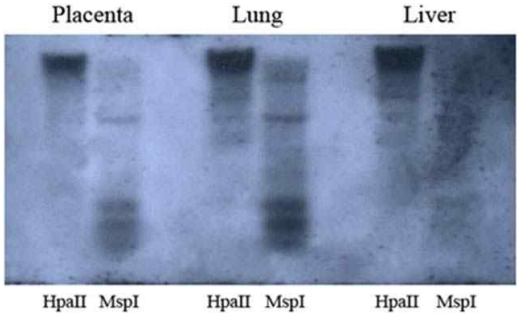

Fig. 2. Methylation patterns of Dnmt1 genome in bovine genome DNA.

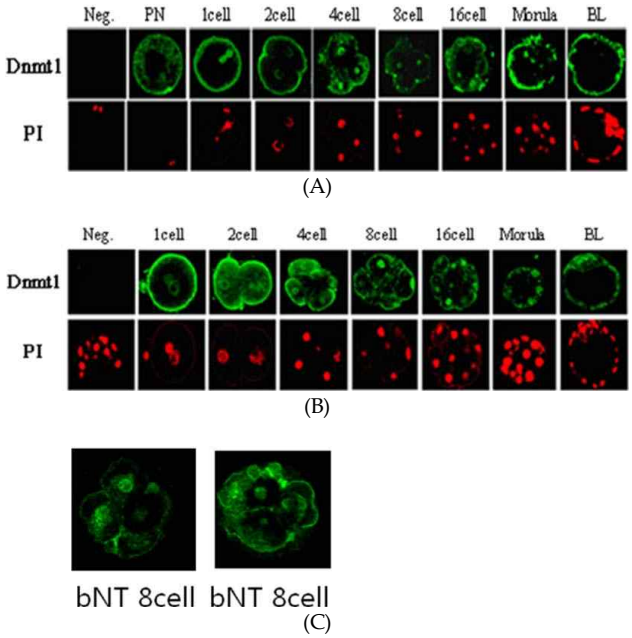

Fig. 3. Immnunostaining of *in vitro* fertilized bovine embryos (A), *in vivo* derived mouse (B) and bovine NT 8-cell (C) with the Dnmt1 antibody.

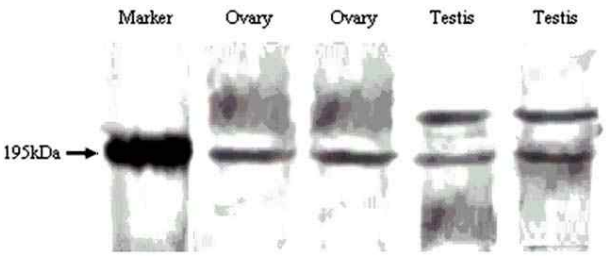

Fig. 4. Western blot of Dnmt1 in bovine ovary and testis.

embryo had stronger expression than IVF 8-cell (Fig. 3C).

Western Blot for Bovine Tissues

Fig. 4 was shown the result of western blot in bovine

ovary and testis. We used the Dnmt1 antibody, somatic specific band (190kDa) appeared in ovary and testis but not oocyte specific (175kDa) compare to rainbow maker. Interestingly, the other specific bands (about 225kDa) were found in testis.

DISCUSSION

The sequencing of bovine Dnmt1 which has an important role for pattern of DNA methylation and the specific localization of Dnmt1 in cloned embryo has been considered as a significant factor to solve problems in the cloning.

Dnmt1 has already cloned in mouse (Bestor *et al*., 1988), rat (Ohsawa *et al*., 1996) and human (Bestor *et al*, 1988), and it has known as important role in preimplatation embryo. The predominant form of Dnmt in mammals is Dnmt1, a protein of relative molecular weight 190,000 composed of a large amino-terminal regulatory domain and a smaller carboxy-terminal catalytic domain that is closely related to bacterial C_5-specific restriction methyltransferase (Ratnam *et al*., 2002).

Golding and Westhusin (2003) have been identified bovine Dnmt1 cDNA full-length (AY244709). They reported that bovine Dnmt mRNAs display strong sequence homology to those of human and mouse and similar to other species. They also suggested that repeated attempts using a variety of rapid amplification of c-DNA ends (RACE) techniques readily detected RNA coding for the ubiquitous somatic form, and yet failed to detect any Dnmt1o transcripts. But, we already enrolled bovine Dnmt 1 cDNA in the GeneBank data system (AY173048). According to the sequence of bovine Dnmt1 cDNA, it has more homology with porcine (unpublished) than mouse, rat and human.

As a northern result of Dnmt1, there is 10 times intensive expression in placenta than other tissue. It indicates that placenta may be hypermethylated than others because of high level of Dnmt1 in placenta. Therefore, Dnmt1 may have a significant role in placenta. However, it may have some problem in cloned placenta because placenta of cloned mouse is twice or three times bigger than normal`s (Tanaka *et al*., 2001; Lee *et al*., 2004). Golding and Westhusin (2003) reported that Dnmt mRNA expression in preimplantation bovine embryos is present during each stage. And then significant differences in Dnmt mRNA expression levels were found among different tissue types as well as between fetal and adult stages. Although Dnmt1 northern of clone and control to each tissue has not examined, many researches have reported that both fetal tissues and placenta, mediates fetal and maternal, were hypermethylated. Suimizu *et al*. (2003) reported that it happens frequently abnormalities in embryo and placenta in NT and in not clear yet what causes the abnormalities. However, it indicated placentomegaly is associated to large dysregulation by determining of expression of five imprinting genes

We analyzed the specific pattern of Dnmt1 in *in vitro* derived/nuclear transfer bovine and *in vivo* derived mouse embryos to monitor the epigenetic reprogramming process. Though we used the common antibody (Dnmt1 epitope: 1316~1616), immuno-localization data revealed that Dnmt1 antibody has been detected at the nucleus and cytoplasm in 1-cell to blastocyst embryos. It also showed the same pattern in *in vivo* derived mouse embryo. Cardoso and Leonhardt (1999) described that the DNA methylation level decreases from the zygote to the blastocyst stage and the enzyme is localized in the cytoplasm of early embryos. Furthermore, there was a strong Dnmt1 expression in tetraploid or cloned 8-cell embryos which is abnormal fertilization cases, and it meant there was no demethylation at 8~16 cell in bovine and Dnmt1 (maintenance methylation) was continuously appeared. So, it seems that Dnmt1 antibody (H300) has the same pattern of 5-MeC antibody not specific.

According to Dean *et al*. (2001), there was a further reduction in methylation, passive demethylation occurring during DNA replication, from the two-cell to the eight-cell stage in mouse using 5-MeC antibody. There was considerable *de novo* methylation in bovine embryos from the eight-cell to the 16-cell stage. There is an initial loss of methylation from the male pronucleus. Thereafter the remaining decline in signal occurs to the morula stage. Bovine zygote was also show the loss of methylation from one pronucleus to the 8-cell stage. *De novo* methylation by the 16-cell stages results with highly and moderately methylation nuclei at the blastocyst stage the ICM contains highly methylated nuclei and trophectoderm moderately ones. Mouse the ICM has become remethylated, but in bovine nuclei both ICM and trophectoderm are methylated. After the two-cell stage, however, cloned embryos did not appear to undergo further demethylation.

According to other studies, Dnmt1 accumulates in nuclei of early growing oocytes but is sequestered in the cytoplasm of mature oocytes. In 2-cell and 4-cell embryos, Dnmt1 is cytoplasmic, but at the 8-cell stage, it is present only in the nucleus. By the blastocyst stage, Dnmt1o is again found only in cytoplasm. During the 8-cell stage, Dnmt1o enters the nucleus just for that one cell cycle, when it is essential for maintaining imprinted gene methylation patterns (Trasler *et al*., 1996; Howell *et al*., 2001; Ratnam *et al*., 2002). Thus, nuclear localization of Dnmt1o in preimplantation embryos is limited to the 8-cell stages. After implantation, Dnmt1 is localized in the nucleus in mouse (Howell *et al*., 2001). However, we have found different patterns of Dnmt1 nucleus localization. This reason may be due

to the different of antibody used in confocal. Thus, it has to be solved the differences between the other results and patterns of Dnmt1 researched in this work. This differences may be due to absent of oocyte specific Dnmt1.

Further genetic manipulation of DNA methyltransferases in the germ line is likely to illuminate additional aspects of genomic methylation pattern dynamics in mammalian reproduction and development. Chung *et al*. (2003) found that defects in the regulation of Dnmt1s and Dnmt1o expression and cytoplasmic-nuclear trafficking may prevent clones. Furthermore, aberrant Dnmt1 localization and expression may contribute to the defects in DNA methylation and the developmental abnormalities seen in clone.

190kDa (somatic specific band) was appeared but there is no oocyte specific band, which is 175kDa in bovine ovary and testis. Using specific antibody, Mertineit *et al*. (1998) reported that a smaller (Mr 175,000) form of Dnmt1 appears in mouse oocytes and a larger (Mr 190,000) form is in adult ovary by PATH52 antibody. According to Ratnam *et al*. (2002), it was showed bands in ovary and testis tissue using UPT82, somatic specific antibody and PATH52, somatic/oocyte specific. There are two bands (175kDa, 190kDa) in ovary by PATH52 and 190 kDa in testis. RT-PCR result of Dnmt1s was founded from 4-cell to day 7.5 embryos and day 70 ovarys. In the Dnmt1o, it is expressed at 1-cell. The western result of immunoblot probed with UPT82 antibody, which it is specific for somatic cell, just only day 7.5 embryos and day 70 ovaries. It, however, did not found any band during preimplatation embryos. It indicated epitope of these antibodies was specific. However, not only 190kDa but also over 220~230kDa were shown in testis. It was thought that caused by latent or polyclonal antidoby of Dnmt1. The antibody for this study seems not oocyte specific but it is somatic specific.

Wakayama and Yanagimachi (1999) reported that placentomegaly is often associated with abnormal expression of imprinted genes, imprinted genes were initially proposed as causative aberrations. Humpherys *et al*. (2002) also assessed global gene expression by microarray analysis on RNA from the placentas and livers of neonatal cloned mice derived by NT. Direct comparison of gene expression profiles of more than 10,000 genes showed that ~4% of the expressed genes in the NT placentas differed from those in controls and that the majority of abnormally expressed genes were common.

Little is known on the functions of bovine Dnmt1o in bovine preimplantation embryos. Therefore, we thought that cloning of bovine Dnmt1 was performed prior to solve the inefficiency causes of bovine NT. It considered for understanding of methylation pattern by observing several epitope of antibody, produced by sequencing.

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