Molecular Characterization of Porcine DNA Methyltransferase I

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ABSTRACT

During normal early embryonic development in mammals, the global pattern of genomic DNA methylation undergoes marked changes. The level of methylation is high in male and female gametes. Thus, we cloned the cDNA of the porcine DNA methyltransferase 1 (*Dnmt*1) gene to promote the efficiency of the generation of porcine clones. In this study, porcine *Dnmt*1 cDNA was sequenced, and *Dnmt*1 mRNA expression was detected by reverse transcription-polymerase reaction (RT-PCR) in porcine tissues during embryonic development. The porcine *Dnmt*1 cDNA sequence showed more homology with that of bovine than human, mouse, and rat. The complete sequence of porcine *Dnmt*1 cDNA was 4,774-bp long and consisted of an open reading frame encoding a protein of 1611 amino acids. The amino acid sequence of porcine *DNMT*1 showed significant homology with those of bovine (91%), human (88%), rat (76%), and mouse (75%) *Dnmt*1. The expression of porcine *Dnmt*1 mRNA was detected during porcine embryogenesis. The mRNA was detected at stages of porcine preimplantation development (1-cell, 2-cell, 4-cell, 8-cell, morula, and blastocyst stages). It was also abundantly expressed in tissues (lung, ovary, kidney and somatic cells). Further investigations are necessary to understand the complex links between methyltransferase 1 and the transcriptional activity in cloned porcine tissues.

(Key words : Porcine *Dnmt*1, Cloning, Characterization, Expression)

INTRODUCTION

Embryos produced by nuclear transfer consistently display a myriad of developmental abnormalities caused by improper patterns of gene expression, likely due to incomplete epigenetic reprogramming (Daniels *et al.*, 2001; Wrenzycki *et al.*, 2001). Eukaryotic genomes are not methylated uniformly, but they contain methylated regions interspersed with unmethylated domains (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).

Three enzymes with DNA (cytosine-5)-methyltransferase (*Dnmt*) activity have been identified in mammals (Bestor, 2001). *Dnmt*1, a large protein with a molecular mass of 190 kDa, predominantly catalyzes maintenance methylation via binding to proliferating cell nuclear antigen in replication foci during the S phase (Bestor *et al.*, 1998). Epigenetic modification such as DNA methylation is defined as heritable modification to the DNA with the potential to alter gene expression while conserving the primary DNA sequence. DNA methylation occurs primarily in the context of 5'-CpG-3' dinucleotides (Jabbari and Bernardi, 2004). 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 first eukaryotic DNA-MTase gene cloned from mouse (Bestor *et al.*, 1988) is now referred to as *Dnmt*1. "*De novo* methylation" induces the initial methylation of DNA, this methylated DNA replicates and is then again subjected to methylation, which is called "maintenance methylation" (Singal and Ginder, 1999).

DNA methylation is involved in a number of important biological processes, e.g., control of gene expression, imprinting, development, X-chromosome inactivation, genomic integrity, and protection of the genome against selfish DNA elements (Fuks *et al.*, 2003; Li, 2002; Reik *et al.*, 2001). Eckardt and McLaughlin (2004) discussed the use of tetraploid embryos as a model to evaluate reprogramming using gene expression and showed that somatic cell nuclei can be reprogrammed by blastomeres to re-express embryo specific genes but not to contribute to post-implantation development.

Dnmtases have been identified in mammals: *Dnmt*1, *Dnmt*2, and *Dnmt*3 (Bestor, 2000) (Fig. 1). All *Dnmt*1s contain a domain of 500 amino acid residues, which is

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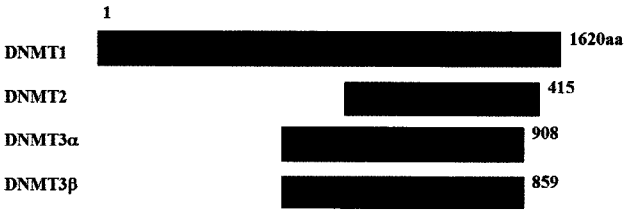

Fig. 1. Structure of *Dnmt*1, 2, 3α and 3β. Comparison of *Dnmt*-1, *Dnmt*2, *Dnmt*3α and *Dnmt*3β was shown. *Dnmt*1 was consisted of about 1620aa. It is the most longer molecules in the *Dnmt* family. *Dnmt*3a and *Dnmt*3b are encoding proteins of 908 and 859 amino acids, respectively.

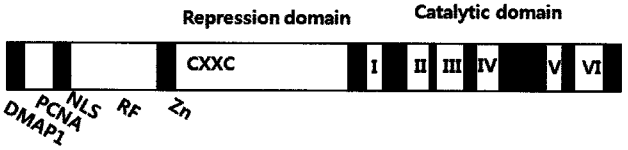

Fig. 2. Schematic diagram of *DNMT*1. *Dnmt*1 shows sequence similarity with the cysteine-rich region (CXXC motif) and the basic motif of HRX. The C-terminal domain is related to the bacterial DNA methyltransferase. The large N-terminal domain has accreted domains that serve functions required by eukaryotes. These include nuclear localization, co-ordinatiion of methylation and replication via a domain that targets *Dnmt*1 to replication foci at S phase, and the partial suppression of *de novo* methylation (Bestor, 2000). DMAP1: *DNMT*1-associated protein binding region; PCNA: proliferative cell nuclear antigen binding domain; NLS: nuclear localization signal; RF: replication fork targeting peptide sequences; Zn: zinc-binding region; HDAC: histone deacetylase binding region.

characterized by the presence of 10 conserved amino acid motifs, shared between prokaryotic and eukaryotic methyltransferases (Jeltsch, 2002) (Fig. 2). Cloning of porcine *Dnmt* plays an important role for pattern of DNA methylation and is considered as a significant factor to understand problems in cloning scientifically and basically (Golding and Westhusin, 2003; Liu *et al.*, 2008; Kurihara *et al.*, 2008).

Therefore, we conducted this study to understand the function and roles of porcine *Dnmt*1 during embryonic development and in tissues, including somatic cells.

MATERIALS AND METHODS

Cloning of Porcine *Dnmt*1 cDNA

Porcine tissues of lungs and ovaries were homogenized with Trizol. Total RNA was extracted from homogenized porcine tissues, and RNA pellets obtained from these tissues were dissolved in 50 ul of DEPC-treated water.

cDNA synthesized using superscript first-strand cDNA synthesis kit subjected for RT-PCR and PCR. Total RNA was mixed with 1 μl of oligo (dT) 12~18, 10 mM dNTP, and added a reaction buffer (10× RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, RNase inhibitor). Then the reaction was incubated at 42℃ for 2 min, after which Superscript II (1 μl) was added. The mixture was incubated at 42℃ for 50 min, 70℃ for 15 min, and 37 ℃ for 20 min, and then, subjected to PCR. A partial region of porcine *Dnmt*1 cDNA was cloned using PCR with mixed primers and sequenced. Then, rapid amplification of cDNA ends (RACE) techniques were employed to sequence the 5' and 3'-region.

5'- and 3'-RACE System

The gene-specific GeneRacer 5'-primer and GeneRacer 5'-nested primer for 5'-RACEs were designed according to the nucleotide sequence of the PCR results. The 3'- and 5'-RACEs were performed using the GeneRacer Kit (Invitrogen, USA). The 3'- and 5'- RACE experiments were conducted according to the manufacturer's instructions.

In Vivo Derived Porcine Embryos

We examined all *in vivo*-derived porcine preimplantation stages from 1-cell stage to the blastocyst-stage. Pig embryos were collected from superovulated female pigs as described previously (Park *et al.*, 2006). Gilts were administered 1,500 IU of pregnant mare serum gonadotrophin (PMSG) by subcutaneous injection, 16 days after standing estrus, and then administered 750 IU of human chorionic gonadotropin (hCG) intramuscularly, 72 h after PMSG injection. Next, gilts were bred by natural mating 24 h after hCG injection. Embryos were recovered surgically at 30~32 h after insemination. Embryos were collected from the oviducts by flushing with 20 ml of sterile Dulbecco's phosphate buffered saline. The embryos were then cultured until the indicated stage.

RT-PCR of Tissues and Embryos

RT-PCR was performed with the AccuPower RT-PCR Kit (RT/PCR PreMix). Total RNA (1.0 ug) extracted from the lung, ovary, liver, kidney, and somatic cells was mixed with the reverse primer (5'-GAT GTT GCT GAC AAA CTT CT-3'), incubated at 70℃ for 5 min, and placed on ice. The forward primer (5'-CCT GGA GAA CGT CAG GAA TT-3') was added, and the reaction volume was adjusted up to 20 ul with DEPC-distilled water. The cDNA synthesis reaction was performed using the following parameters: 42℃ for 60 min and, 94℃ for 5 min. PCR was performed using the following parameters: 94℃ for 1 min followed by 30 cycles of 94℃ for 1 min, 58℃ for 1 min, and 72℃ for 1 min, and a final extension step at 72℃ for 8 min. The amplified products were analyzed a 2% agarose gel. A single embryo and the same method were used to detect mRNA expression.

Real Time PCR

Real-time PCR was carried out using the one-step SYBR RT-PCR kit. The real-time PCR amplification mixture consisted of the following reagents: 5 μg of RNA, 1x of 2x one-step SYBR RT-PCR buffer, 5 pmol of each primer, 2.5 U of Takara Ex-Taq HS (Takara, Japan), 50 U of Moloney murine leukemia virus (MMLV) RTase (RNase H free), and 20 U of RNase inhibitor. This mixture was transferred to PCR tubes, and the reaction volume (25 μl) was adjusted with RNase-free distilled water. The thermocycler profile was as follows: 10 min at 95℃; 30 cycles of 10 s at 95℃, 15 s at 58℃, and 20 s at 72℃ followed by 45 s at 72℃.

RESULTS

Cloning of Procine *Dnmt*1 cDNA

Porcine *Dnmt*1 cDNA was cloned using step-by-step PCR method and first-strand cDNA synthesized from porcine adult ovary (GenBank: DQ060156). It consisted of 1611 amino acids. Porcine *Dnmt*1 cDNA displays high homology with those reported for bovine (88%), human (86%), rat (78%), and mouse (77%) (Fig. 3 and Table 1). The amino acid sequence showed homology to those of bovine (91%), human (88%), rat (76 %), and mouse (75%). Phylogenetic tree analysis of the porcine *Dnmt*1 amino acid sequence across various vertebrate species showed that porcine *Dnmt*1 clusters with high bootstrap in the lineage of mouse and rat, thereby sharing the highest homology with bovine *Dnmt*1 (Fig. 4).

*Dnmt*1 mRNA Expression in Preimplantation Porcine Embryos and Tissues

During porcine preimplantation development, we detected mRNA expression at each developmental. We did not observe any difference in the mRNA expression during embryogenesis (Fig. 5). In addition, we found that porcine *Dnmt*1 mRNA was expressed in all tissues analyzed (Fig. 6). The real-time PCR result showed that there was a little difference in the expression. However, there is no significant difference.

Table 1. Homology of porcine *Dnmt*1 with other mammals

Homology of Amino acid sequences

Homology of cDNA sequences

	Porcine	Bovine	Human	Rat	Mouse
Porcine		91%	88%	76%	75%
Bovine	88%		89%	76%	76%
Human	86%	86%		77%	77%
Rat	78%	78%	79%		89%
Mouse	77%	77%	79%	89%	

The similarity of amino acid and nucleotide sequences of *Dnmt*1 is shown. Percentage identities were calculated on sequences arranged for maximum alignment using the DNASIS program.

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pDnmt1 MPARTAPARVAALASRAFSLPDDVRRRLKDLERHSLTEKECVKEKLNLLHEFLQTEIKNQ 60
bDnmt1 MPARTAPARVPALASRAFSLPDDVRRRLKDLERDSLTEKECVKEKLNLLHEFLRTEIRNQ
rDnmt1 MPARTAPARVPALASPAGSLPDHVRRRLKDLERDGLTEKECVKEKLNLLHEFLQTEIKSQ
mDnmt1 MPARTAPARVPALASPAGSLPDHVRRRLKDLERDGLTEKECVREKLNLLHEFLQTEIKSQ
hDnmt1 MPARTAPARVPTLAVPAISLPDDVRRRLKDLERDSLTEKECVKEKLNLLHEFLQTEIKNQ

       LCDLETKLHKEELSEEGYLAKVKSLLNKDLSLENGAHAFSREVNGYLENGSQTSGEDRRV 120
       LCDLETKLHKEELSEEGYLAKVKSLLNKDLSLENGAHAFSREANGCLENGSQTSGEDCRV
       LCDLETKLHKEELSEEGYLAKVKTLLNKDLCLENGTLSLTQKANGCPANGSRP----WKA
       LCDLETKLHKEELSEEGYLAKVKSLLNKDLSLENGTHTLTQKANGCPANGSRP----WRA
       LCDLETKLRKEELSEEGYLAKVKSLLNKDLSLENGAHAYNREVNGRLENGNQARSEARRV

       EMAEENKSPKPVSRLGTPRRSKSDGEAKSAEVSSSPRITRQTTRQTTITSHFTRGPGKRK 180
       VMAEKGKPPKPVSRLYTPRRSKSDGETK--EVSSSPRITRKTTRQTTITSHFPRGPAKRK
       EMADSNRSPRSRPKPRGPRRSKSDSET--IEASSSSVATRRTTRQTTITSHF--GPAKRK
       EMADSNRSPRSRPKPRGPRRSKSDSDTLSVETSPSSVATRRTTRQTTITAHFTKGPTKRK
       GMADANSPPKPLSKPRTPRRSKSDGEAK--EPSPSPRITRKSTRQTTITSHFAKGPAKRK

       PEEDTAKAKPDSPVEEEEKDQEEKRRKVTSRDSV--RT--PTEEPERVRPGTHME-E--- 232
       PEEEPEKVKSDDSVDEE-KDQEEKRRRVTSRERV--WA--PAEEPGRVRPGTHME-EEGR
       PKEDSEKGNANESAAEE-RDQD-KKRRVAGTESRASRAGESVEKPERVRPGTQLCQEEQG
       PKEESEEGNSAESAAEE-RDQD-KKRRVVDTES---GAAAAVEKLEEVTAGTQLGPEEPC
       PQEESERAKSDESIKEEDKDQDEKRRRVTSRERV--XT-PAEEPERAKSGTRTEKEEER

VGKACDPAARQFNTLIPWCLPHTGNRHNHWAGLYGRLEWDGFFSTTVTNPEPMGKQGRVL 1534
--KPCEPAARQFNTLIPWCLPHTGNRHNHWAGLYGRLEWDGFFSTTVTNPEPMGKQGRVL
--KTCDPASRQFNTLIPWCLPHTGNRHNHWAGLYGRLEWDGFFSTTVTNPEPMGKQGRVL
--KACDPESRQFSTLIPWCLPHTGNRHNHWAGLYGRLEWDGFFSTTVTNPEPMGKQGRVL
AGKACDPAARQFNTLIPWCLPHTGNRHNHWAGLYGRLEWDGFFSTTVTNPEPMGKQGRVL

       HPEQHRVVSVRECARSQGFPDTYRLFGNILDKHRQVGNAVPPPLAKAIGLEIKRCMLAKA 1594
       HPEQHRVVSVRECARSQGFPDTYRLFGNILDKHRQVGNAVPPPLAKAIGLEIKRCMLAKA
       HPEQHRVVSVRECARSQGFPDTYRLFGNILDRHRQVGNAVPPPLAKAIGLEIKLCLLASA
       HPEQHRVVSVRECARSQGFPDSYRFFGNILDRHRQVGNAVPPPLAKAIGLEIKLCLLSSA
       HPEQHRVVSVRECARSQGFPDTYRLFGNILDKHRQVGNAVPPPLAKAIGLEIKLCMLAKA

       RESASV--KVKEEETTKDX 1611
       RESASA--KIKEE--AKDX
       QESASAAVKGKEETTTEDX
       RESASAAVKAKEEAATKDX
       RESASA--KIKEEEAAKDX
```

Fig. 3. Comparision of deduced amino acid sequence of *Dnmt*1. Amino acid residues are numbered from the first amino acid of the *Dnmt*1 protein as 1. Amino acids corresponding to deleted sequences are shown by hyphens. And it was deleted amino acids from 232aa to 1474aa.

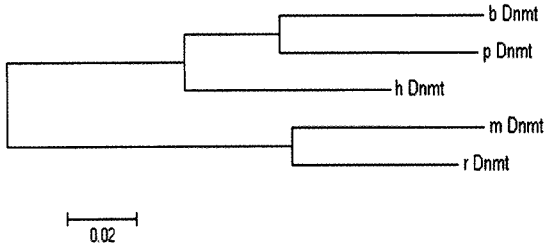

Fig. 4. Comparison of amino acid sequence of *Dnmt*1 with related *Dnmt*1s by dendorgram analysis. The other *Dnmt*1 amino acid sequences were obtained from GeneBank, and the phylogenetic tree was constructed using the unrooted analysis PHYLYP & View.

DISCUSSION

The sequencing of porcine *Dnmt*1, which plays an important role for pattern of DNA methylation and the

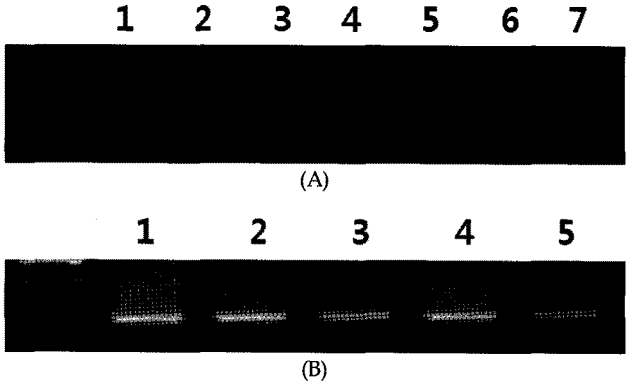

Fig. 5. *Dnmt*1 mRNA expression during developmental embryo and tissues by RT-PCR. One embryo was used in the RT-PCR. Each data was obtained by triplicated experiment. Each tissues were randomly collected from slaughterhouses. Total RNA was extracted and then subjected to RT-PCR. PCR products were subjected to electrophoresis. (a): 1: 1 cell; 2: 2 cell; 3: 4 cell; 4: 8 cell; 5: blastocyst; 6: morula; 7: negative control. (b): 1: lung; 2: ovary; 3: liver; 4: kidney; 5: somatic cell.

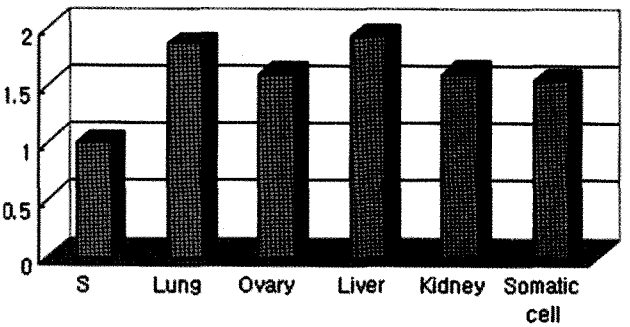

Fig. 6. Real-time PCR analysis for *Dnmt*1 expression. *Dnmt*1 transcripts were quantified in the samples of total RNA isolated from lung, ovary, liver, kidney and somatic cell. The amplified products of *Dnmt*1 were separated on agarose gel and stained with ethidium bromide. S: standard.

specific localization of *Dnmt*1 in cloned embryos, has been considered as a significant factor to solve problems in cloning.

*Dnmt*1 has already been cloned from mouse (Bestor *et al.*, 1988), rat (Ohsawa *et al.*, 1996), human (Bestor *et al*, 1988), and bovine (Chang *et al.*, 2009) tissues in our lab. It has been known to play an important role in preimplantation embryos. The predominant form of *Dnmt* in mammals is *Dnmt*1, a protein with a relative molecular weight of 190,000 Da composed of a large amino-terminal regulatory domain and a small carboxyl-terminal catalytic domain, which is closely related to the bacterial C_5-specific restriction methyltransferase (Ratnam *et al.*, 2002; Chang *et al.*, 2009).

Kanai *et al.* (2003) reported that mutation of the *Dnmt*1 gene in human colorectal cancer was detected in appriximately 7 % of coding exons of the *Dnmt*1 gene. No mutation was detected in stomach cancer and in the 5'-flanking region of the *Dnmt*1 gene. These data suggest mutational inactivation of the *Dnmt*1 gene that potentially causes a genome-wide alteration of the methylation status, which may be a rare event during carcinogenesis. *Dnmt* 3B depletion reactivated methylation-silenced gene expression but did not induce global or juxtacentromeric satellite demethylation as did specific depletion of *Dnmt*1 (Beaulieu *et al.*, 2002).

Golding and Westhusin (2003) reported that *Dnmt* mRNA expression is present at each stage in preimplantation bovine embryos. In addition, significant differences in *Dnmt* mRNA expression levels were found among different tissue types as well as between fetal and adult development stages. Chang *et al.* (2009) reported that bovine *Dnmt*1 mRNA expression was different in the expression sites during development. Kang *et al.* (2001) reported that a genomic demethylation process occurs in pig preimplantation embryos produced by either normal fertilization or somatic cell nuclear transfer. They also insisted that species-specific differences exist in modifying the epigenetic status of cloned donor genomes. In addition, the developmental anomalies of cloned bovine embryos could be due to incomplete epigenetic reprogramming of donor genomic DNA (Kang *et al.*, 2001).

Although *Dnmt*1 northern blots of clones and controls of each tissue have not been examined, many researchers have reported that the *Dnmt*1 gene in both fetal tissues and placenta, mediates fetal and maternal, was hypermethylated. Suimizu *et al.* (2003) reported that abnormalities occur frequently in embryo and placenta in NT and it is not clear yet what causes these abnormalities. However, it was indicated that placentomegaly is associated with dysregulation of methylation by determining the expression of 5 imprinting genes. Cardoso and Leonhardt (1999) described that the DNA methylation level decreases from the zygote to the blastocyst stage and that the enzyme is localized in the cytoplasm of early embryos.

According to Dean *et al.* (2001) who had used 5-MeC antibodies reported there was further reduction in methylation and passive demethylation during DNA replication from the 2-cell to the 8-cell stage in mice. Considerable *de novo* methylation was noted in the bovine embryos from the 8-cell to the 16-cell stage. In addition, an initial loss of methylation has been reported in the male pronucleus. Thereafter, the remaining decline in the methylation signal occurs at the morula stage. Bovine zygote also showed loss of methylation from the pronucleus to the 8-cell stage. *De novo* methylation at the 16-cell stage results in highly and moderately methylated nuclei at the blastocyst stage in which the inner cell mass (ICM) and trophectoderm contain highly and moderately methylated nuclei, respectively. In mice. the ICM has become remethylated, but in bovine nuclei both ICM and trophectoderm are methylated. After the 2-cell stage, however, cloned embryos did not ap-

pear to undergo further demethylation.

According to other studies, *Dnmt*1 accumulates in the nuclei of early growing oocytes, but it is sequestered in the cytoplasm of mature oocytes. In 2-cell and 4-cell embryos, *Dnmt*1 is cytoplasmic, but at the 8-cell stage, it is present only in the nucleus. At the blastocyst stage, oocyte-specific *Dnmt*1 (*Dnmt*1o) is again found only in the cytoplasm. During the 8-cell stage, *Dnmt*1o enters the nucleus just for that 1 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 *Dnmt*1o in preimplantation embryos is limited to the 8-cell stages. After implantation, *Dnmt*1 is localized in the nucleus in mice (Howell *et al.*, 2001).

However, we did not use a porcine-specific antibody for *Dnmt*1 when we investigated. This reason may be due to the different antibodies used in confocal microscopy. Thus, it has to be solved the differences between the other results. In addition, we did not detect any porcine *Dnmt*1o existence in the porcine ovarian tissues. This difference may be due to absence of oocyte- specific *Dnmt*1. Further genetic manipulations of *Dnmt* in the germ line are 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 *Dnmt*1s and *Dnmt*1o expression and cytoplasmic-nuclear trafficking may prevent clones from completing essential early developmental stages. Furthermore, aberrant *Dnmt*1 localization and expression may contribute to the defects in DNA methylation and the developmental abnormalities seen in clones.

Lie *et al.* (2008a) reported that the methylation status was different in methylated regions of 4 imprinted genes in 4 spontaneously aborted somatic nuclear transfer-cloned fetuses. Abnormal methylation imprints were observed in each individual to different extents. The paternally expressed gene 3 (Peg3) and monoamine oxidase A (MAOA) were either extensively demethylated or showed aberrant methylation patterns, but the X-inactive-specific transcript (Xist) gene and Peg10 exhibited a relatively better maintained methylation status in aborted cloned fetuses. Using the bisulfite sequencing method, Lie *et al.* (2008b) analyzed methylation patterns of the 5'-regions of 4 *Dnmt* genes, including *Dnmt*3a, *Dnmt*3b, *Dnmt*1, and *Dnmt*2 in 4 aborted bovine clones. They found that 3 out of 4 aborted bovine clones showed either hypermethylation or hypomethylation in the 5'-regions of *Dnmt*3b and *Dnmt*3a. Thus, the *Dnmt*3a and *Dnmt*3b genes are not properly reprogrammed and are probably associated with the high abortion rate of bovine clones. Kurihara *et al.* (2008) suggested that the somatic form of *Dnmt*1 (*Dnmt*1s) is present in association with chromatin in MII-stage oocytes as well as in the nucleus throughout preimplantation development. At the early 1-cell stage, *Dnmt*1 is asymmetrically localized in maternal pronuclei. Thereafter, *Dnmt*1s is recruited to the paternal genome during pronuclear maturation.

Little is known on the functions of *Dnmt*1 during preimplantation developmental embryos. Our findings may contribute to the better understanding of methylation pattern by observing several epitopes of antibodies, obtained by sequencing.

REFERENCES

1. Antequera F, Bird A (1993): Number of CpG islands and genes in human and mouse. Proc Natl Acad Sci USA 90:11995-11999.
2. Beaulieu N, Morin S, Chute IC, Robert MF, Nguyen H, MacLeod AR (2002): An essential role for DNA methyltransferase DNMT3B in cancer cell survival. J Biol Chem 277:28176-28181.
3. Bestro TH (2000): The DNA methyltransferases of mammals. Hum Mol Genet 9:2395-2402.
4. Bestor TH, Verdine GL (1994): DNA methyltransferase. Curr Opin Cell Biol 6:380-389.
5. Bestor TH, Laudano A, Mattaliano R, Ingram V (1988): Cloning and sequencing of cDNA encoding DNA methyltransferase of muse cell. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. J Mol Biol 203:971-983.
6. Bird AP (1986): CpG-rich islands and the function of DNA methylation. Nature 321:209-213.
7. Bird AP (1995): Gene number, noise reduction and biological complexity. Trends Genet 11:94-100.
8. Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt Biochem 72:248-254.
9. Cardoso MC, Leonhardt H (1999): DNA methyltransferase is actively retained in the cytoplasm during early development. J Cell Biol 147:25-32.
10. Chung YG, Ratnam SJ, Chaillet R, Latham KE (2003): Abnormal regulation of DNA methyltransferase expression in cloned mouse embryos. Biol Reprod 69: 146-153.
11. Daniels R, Hall V, French A, Korfiatis N, Trounson A (2001): Comparison of gene transcription in cloned bovine embryos produced by different nuclear transfer technique. Mol Reprod Dev 60:281-288.
12. Dean W, Santos F, Stojkovia M, Zakhartchenko V, Walter J, Wolf E, Reik W (2001): Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. Proc Natl Acad Sci USA 98:13734-13738.
13. Eckardt S, McLaughlin KJ (2004): Interpretation of

reprogramming to predict the success of somatic cell cloning. Anim Reprod Sci 82:97-108.
14. Fuks F, Hurd PJ, Wolf D, Nan X, Bird AP, Kouzarides T (2003): The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. J Biol Chem 278:4035-4040.
15. Golding MC, Westhusin ME (2003): Analysis of DNA (cytosine 5) methyltransferase mRNA sequence and expression in bovine preimplantation embryos, fetal and adult tissues. Gene Expression Patterns 3:551-558.
16. Hogan B, Beddington R, Constantini F, Lacy E (1986): Manipulating the Mouse Embryos (2nd Edition). Cold Spring Harbor Laboratory, pp 390-410.
17. Howell CY, Bestor TH, Ding F, Latham KE, Mertineit C, Trasler JM, Chaillet JR (2001): Genomic imprinting disrupted by a maternal effect mutation in the *Dnmt*1 gene. Cell 104:829-838.
18. Humpherys D, Eggan K, Akutsu H, Friedman A, Hochedlinger K, Yanagimachi R, Lander ES, Golub TR, Jaenisch R (2002): Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei. Proc Natl Acad Sci USA 99: 12889-12894.
19. Jabbari K, Bernardi G (2004): Cytosine methylation and CpG, TpG (CpA) and TpA frequencies. Gene 333: 143-149.
20. Jeltsch A (2002): Beyond Watson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases. Chembiochem 3:274-293.
21. Kanai Y, Ushijima S, Nakanishi Y, Sakamoto M, Hirohashi S (2003): Mutation of the DNA methyltransferase (DNMT 1) 1 gene in human colorectal cancers. Cancer Lett 192:75-82.
22. Kang YK, Koo DB, Park JS, Choi YH, Kim HN, Chang WK, Lee KK, Han YM (2001): Typical demethylation events in cloned pig embryos. J Biol Chem 276:39980-39984.
23. Kang YK, Koo DB, Park JS, Choi YH, Kim HN, Chung AS, Lee KK, Han YM (2001): Aberrant methylation of donor genome in cloned bovine embryos. Nature Genetics 28:173-177.
24. Kurihara Y, Kawamura Y, Uchijima Y, Amamo T, Kobayashi H, Asano T, Kurihara H (2008): Matenance of genomic methylation patterns during preimplantaion development requires the somatic form of DNA methyltransferase I. Developmental Biology 313: 335-346.
25. Lee RSF, Peterson AJ, Donnison MJ, Ravelich S, Ledgard AM, Li N, Oliver JE, Miller AL, Tucker FC, Breier B, Wells DN (2004): Cloned cattle fetuses with the same nuclear genetics are more variable than contemporary half-siblings resulting from artificial insemination and exhibit fetal and placental growth deregulation even in the first trimester. Biol Reprod 70:1-11.
26. Li E (2002): Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 3:662-673.
27. Liu JH, Liang X, Zhu J, Wei L, Hou Y, Chen DY, Sun QY (2008b): Aberrant DNA methylation in 5'-regions of DNA methyltransferase genes in aborted bovine clones. J Genet Genomics 35:559-568.
28. Liu JH, Yin S, Xiong B, Hou Y, Chen DY, Sun QY (2008a): Aberrant DNA methylation imprints in aborted bovine clones. Mol Reprod Dev 75:598-607.
29. Ohsawa K, Imai Y, Ito D, Kohsaka S (1996): Molecular cloning and characterization of annexin V-binding proteins with highly hydrophilic peptides structure. J Neurochem 67:89-97.
30. Park HJ, Ko YG, Hwang S, Yang BC, Seong HH, Oh SD, Hwang SY, Min KS, Yoon JT (2009): Analysis of differentially expressed genes in cloned bovine placenta. Reprod Dev Biol 33:41-48.
31. Ratnam S, Carmen M, Ding F, Howells CY, Clarke HJ, Bestor TH, Chaillet JR, Trasler M (2002): Dynamics of *Dnmt*1 methyltransferase expression and intracellular localization during oogenesis and preimplantation development. Dev Biol 245:304-314.
32. Reik W, Dean W, Walter J (2001): Epigenetic reprogramming in mammalian development. Science 293: 1089-1093.
33. Singal R, Ginder GD (1999): DNA methylation. Blood 93:4059-4070.
34. Tanaka S, Oda M, Toyoshima Y, Wakayama T, Tanaka M, Yoshida N, Hattori N, Ohgane J, Yanagimachi R, Shiota K (2001): Placentomegaly in cloned mouse concepti caused by expression of the spongiotrophoblst layer. Biol Reprod 65:1813-1821.
35. Trasler JM, Trasler DG, Bestor TH, Li E, Ghibu F (1996): DNA methyltransferase in normal and $Dnmt^N$ /$Dnmt^N$ mouse embryos. Dev Dyn 206:239-247.
36. Wakayama T, Yanagimachi R (1999): Cloning of male mice from adult tail-tip cells. Nat Genet 22:127-128.
37. Yang BC, Im GS, Kim DH, Min KS, Yoon DH, Park HS, Kim SW, Hwang IS, Seo JS, Seong HH, Yang BS (2006): Development of transgenic NT embryos using bovine fetal fibroblast transfected with hFSH gene. Korean J Emb Trans 21:13-20.
38. Wrenzycki C, Wells D, Herrmann D, Miller A, Oliver J, Tervit R, Niemann H (2001): Neclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. Biol Reprod 65:309-317.

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