Analysis of TIMP-2 and Vimentin Protein Expression and Epigenetic Reprogramming in Cloned Bovine Placentae

Hong-Rye Kim[1], Rong-Xun Han[1], Hye-Ran Lee[1], Jong-Taek Yoon[2], Hee-Tae Cheong[3], Chang-Sik Park[1] and Dong-Il Jin[1,†]

[1]*Research Center for Transgenic Cloned Pigs, Chungnam National University, Daejeon 305-764, Korea*
[2]*Genetic Engineering Institute, Hankyong National University, Anseong 456-749, Korea*
[3]*School of Veterinary Medicine, Kangwon National University, Chuncheon 200-701, Korea*

ABSTRACT

The objective of this study was to analyzed pattern of proteins expression abnormally in cloned bovine placenta. TIMP-2 protein whose function is related to extracellular matrix degradation and tissue remodeling processes was one of differentially up-regulated proteins in SCNT placenta. And one of down-regulated protein in SCNT placenta was identified as vimentin protein that is presumed to stabilize the architecture of the cytoplasm. The expression patterns of these proteins were validated by Western blotting. To evaluate how regulatory loci of TIMP-2 and vimentin genes was programmed reprogramming in cloned placenta, the status of DNA methylation in the promoter region of TIMP-2 and vimentin genes was analyzed by sodium Bisulfite mapping. The DNA methylation results showed that there was not difference in methylation pattern of TIMP-2 and vimentin loci between cloned and normal placenta. Histone H3 acetylation state of the nucleosome was analyzed in the cloned placental and normal placenta by Western blotting. A small portion of the protein lysates were subjected to Western blotting with the antibodies against anti acetyl-Histone H3. Overall histone H3 acetylation state of SCNT placenta was significantly higher than those of normal placenta cells. It is postulated that cloned placenta at the end of gestation seems to be unusual in function and morphology of placenta via improper expression of TIMP-2 and vimentin by abnormal acetylation states of cloned genome.

(Key words : Bovine cloned placenta, TIMP-2, Vimentin, Methylation, Acetylation)

INTRODUCTION

Cloning animals by somatic cell nuclear transfer (SCNT) has been hampered by severe problems including abortion before still birth and growth abnormalities of cloned offspring (Hill *et al.*, 2000; Wakayama *et al.*, 2001). Currently, the cause of difficulties for the developmental failure of cloned animals has been suggested to be related with the inappropriate reprogramming of somatic nucleus (Han *et al.*, 2003; Reik *et al.*, 2003). Epigenetic reprogramming is a necessary feature of normal development and is associated with the epigenetic modifications (Oswald *et al.*, 2000). A major epigenetic modification in mammals is the addition of a methyl group to the 5' position in the symmetrical CpG dinucleotide and histone acetylation in nucleosome. Alteration of the 5-methylcytosine content of specific regions of the genome is thought to be important in controlling gene expression that must undergo radical changes in both normally fertilized embryos and those reconstructed by SCNT. Evidence for abnormal gene expression patterns by insufficient imprinting or methylation of genes in reconstituted embryos with SCNT were reported (Young *et al.*, 1998). Also, the abnormal gene regulation of SCNT embryos was an association of unsuitable remodeling of chromatin on developmentally specific genes by histone acetylation (Cezar *et al.*, 2003; McGraw *et al.*, 2003). Appropriate genetic reprogramming of donor nuclear materials is prerequisite for successful cloning by somatic cell nuclear transfer. However, reprogramming errors on the nucleus of donor cell during SCNT process have occurred with most case studies. These errors lead to incomplete gene expression and could arise at any of the levels at which regulation of gene expression occurs, including the organization of the nucleus, chromatin structure and the accessibility of regulatory molecules. For this reason,

* This work was supported by a grant (No R11-2002-100-03001-0) the ERC program of the Korean Science & Engineering Foundation and by a grant (No 20070101034010) from BioGreen 21 Program, Rural Development Administration, Korea.

† Corresponding author : Phone: +82-42-821-5876; E-mail: dijin@cnu.ac.kr

recently abnormal expression levels of genes derived by epigenetic errors were reported at the mRNA levels by cDNA microarrays (Humpherys *et al.*, 2002; Suemizu *et al.*, 2003). The differential protein profile of bovine cloned placenta derived from SCNT has been analyzed and compared with normal placenta in our laboratory (Kim *et al.*, 2005). Even though various investigations about reprogramming error in cloned animals have been reported, clear diagnosis for complete reprogramming has not been reported yet. Even though it is not achieved reprogramming process has to be understood to solve the abnormality of cloned animals.

The objective of this study is to confirm whether discrepancy of epigenetic programming in nuclear transfer causes abnormal gene expression. The status of DNA methylation modifications of TIMP-2 and vimentin gene locus is investigated. In addition, histone H3 acetylation state of the nucleosome is analyzed in the SCNT placenta and normal placenta cells.

MATERIALS AND METHODS

Placental Samples

Protein patterns of 2 placentae derived from SCNT of Korean Native calves died suddenly at two months after birth and 2 normal placentae obtained from afterbirth of AI-derived fetuses were analyzed. The two SCNT-derived samples were collected in March 2004 in Hankyong National University, and normal placentae were obtained from Hankyong National University in June 2004. Placental samples were stored in liquid nitrogen just after collection until use.

Extraction of Solubilized Proteins from Bovine Placentae

Soluble proteins were extracted as previously described, with some modifications (Kim *et al.*, 2005). Briefly, lysis buffer A containing 1% SDS, 1 mM PMSF, protease inhibitor cocktail (Roche Diagnostics, Germany), and 100 mM Tris-HCl, pH 7.0 was applied to placenta samples at an equal volume to tissue weight (e.g. 100 μl buffer / 100 mg wet weight), and samples were sonicated for 15 sec and then chilled on ice. Lysis buffer B containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.1 M DTT, 1 mM PMSF, protease inhibitor, and 40 mM Tris-HCl, pH 7.0, was applied to the sonicated samples with the same volume of lysis buffer A. The samples were shaken gently for 1 hr at room temperature, and the insoluble material was removed by centrifugation (15,000 ×g, 20 min, 4℃). The resulting supernatant was treated with 100 units/ml endonuclease (Benzonase; Sigma, catalog No. E8263, USA) for 1 hr at 30℃. The solubilized protein extracts were quantified by Bradford protein assay (Bio-Rad, CA, USA) and the protein samples were stored at −70℃ until use.

2-D Gel Electrophoresis, MALDI-TOF MS and Protein Identification

Placental proteins were separated by 2-D gel electrophoresis (2-DE) as previously described (Kim *et al.*, 2005), and the resulting gels were stained using colloidal coomassie brilliant blue G-250. The stained gels were scanned at an optical resolution of 63.5 μm/pixel using a GS-710 calibrated densitometer (Bio-Rad, USA), and the Melanie III software (GE Healthcare Bio-Sciences, Sweden) was used to search for protein spots having differential intensities. Spots were manually excised, digested with trypsin and analyzed using a Voyager-DE STR MALDI-TOF-MS apparatus (Applied Biosystems, USA). Peak data were searched manually using the DataExplorerTM (Applied Biosystems, USA) and spectra were saved as peak table files and used to search against web-based non-redundant protein sequence databases (SWISS-PROT and/or NCBInr (2006/ 11/ 03) Data Bank).

Genomic DNA Isolation and Bisulfite Treatment.

Placental samples were incubated overnight at 60℃ in 500 μl of extraction buffer (1 M Tris-HCl, pH 8.0, 5 M NaCl, 0.5 M EDTA), 12.5 μl 20% SDS (sodium dodecylsulfate) and 5 μl Proteinase K (Qiagen GmbH, Germany). The samples were extracted twice with phenol : chloroform (1:1) and precipitated with one volume of ethanol. Following a wash with two volumes of 70% ethanol, the DNA was resuspended in 500 μl of diluted water. Purified genomic DNA (1 μg) was treated with sodium bisulfate solution by using a one day MSP kit (In2Gen Co., Ltd. Seoul, Korea). After treatment, DNA was purified by using a one day MSP kit, precipitated with ethanol, and resuspended in 20 μl of diluted water.

PCR Amplification and Sequencing of the Bisulfite-Treated Genomic DNA

The 5′ UTR region of TIMP-2 was amplified by PCR with specific primers 5′-TTT AGA GTT AAG AAA GTT TGT GTG G-3′and 5′-CCC AAC AAC AAA AAA CAA AAC-3′ during which cytosine was converted to uracil. Also, for amplification of the promoter region of vimentin gene, a set of primers, 5′-GGA AAG TTT TTG AAA GTT TTA GTT TAG TT-3′and 5′-AAA TAA AAA AAT TCC CTC CCA CTA C-3′, were used. DNA fragments were amplified using ExTaq Hot start version (Takara, Japan) for 35 cycle under the following condition: 94℃ for 30 sec, 57℃ for 45 sec, 72℃ for 1 min. The PCR product was ligated into T-plasmid by using the pGEMP®P-T easy vector systems (Promaga, USA). Twenty subclones were pocked and sequenced

using automatic sequencer (Applied Biosystems, PRISM 377, USA) by Macrogen.

Western Blot Analysis

Briefly, lysates containing 30 μg of protein were resolved using 12% SDS-PAGE gels. After SDS-PAGE, gels were transferred electrophoretically onto PVDF membranes (Bio-Rad lab, USA) and blocked for 1 hr at room temperature with TBS containing 5% skim-milk. Subsequently, the membranes were incubated for 1 hr with anti-acetyl-Histone H3 rabbit polyclonal antibody (Upstate, Catalog No. 06-599, USA), mouse monoclonal anti-TIMP-2 (Abcam; Catalog No. ab1828, UK), mouse monoclonal anti-vimentin (Santa Cruz Biotechnology; Catalog No sc-6260, USA), or anti-beta-actin rabbit polyclonal (Abcam; Catalog No. ab8227, UK). The blot was than washed six times for 10 min with TBS-T buffer and incubated with 1:5,000 or 1:10,000 dilution of HRP-conjugated anti-mouse antibody (for anti-TIMP-2 and anti-vimentin) or HRP-conjugated anti-rabbit antibody (for anti-actin, anti-acetyl-Histone H3). The detection procedure was carried out in a dark room by using an ECL kit (GE Healthcare Bio-Sciences, Sweden). The detection reagent was poured onto the membrane and incubated for 1 min. The detection reagent was drained off and the membrane was exposed with a sheet of diagnostic film in the film cassette.

Statistical Analysis

All experiments were repeated at least three times. Data are presented as means ± SEM. A p value of less than or equal to 0.05 was considered statistically significant. The statistical significance of the results was assessed by ANOVA followed by t-test, using Microsoft Excel.

RESULTS

TIMP-2 Expression in SCNT Placentae

We previously used 2-DE and mass spectrometry to identify proteins showing differential expression between SCNT cloned and normal bovine placentae (Kim *et al.*, 2005). A total of 33 spots that displayed to be up-regulated in SCNT against normal placentae were detected. One of these spots was putatively identified as matching to TIMP-2, which has been previously associated with extracellular matrix (ECM) degradation and remodeling (Fig. 1A). The TIMP-2 was detected as a protein band of approximately 24 kDa in size (Fig. 1B). Fig. 1B confirmed that TIMP-2 protein expression in SCNT placenta increased compared with that in normal placenta (Fig. 1). To examine whether abnormal TIMP-2 gene expression in cloned placenta is related to the status of DNA methylation in the promoter region (214 bp) of TIMP-2 gene including 26 CpG sites were investigated by Bisulfite mapping (Fig. 2A). Bisulfite treatment causes deamination of unmethylated cytosines to uridine, thereby allowing discrimination between unmethylated and methylated cytosine residues through sequencing analysis. The Fig. 2 shows the methylation profiles of TIMP-2 promoter locus in the SCNT and normal placentae. Most CpG sites of TIMP-2 locus were hypomethylated in cloned placenta and normal placenta. Any significant difference in methylation status was not detected between normal and SCNT placentae.

Vimentin in SCNT Placentae

We identified a total of 27 spots were identified to

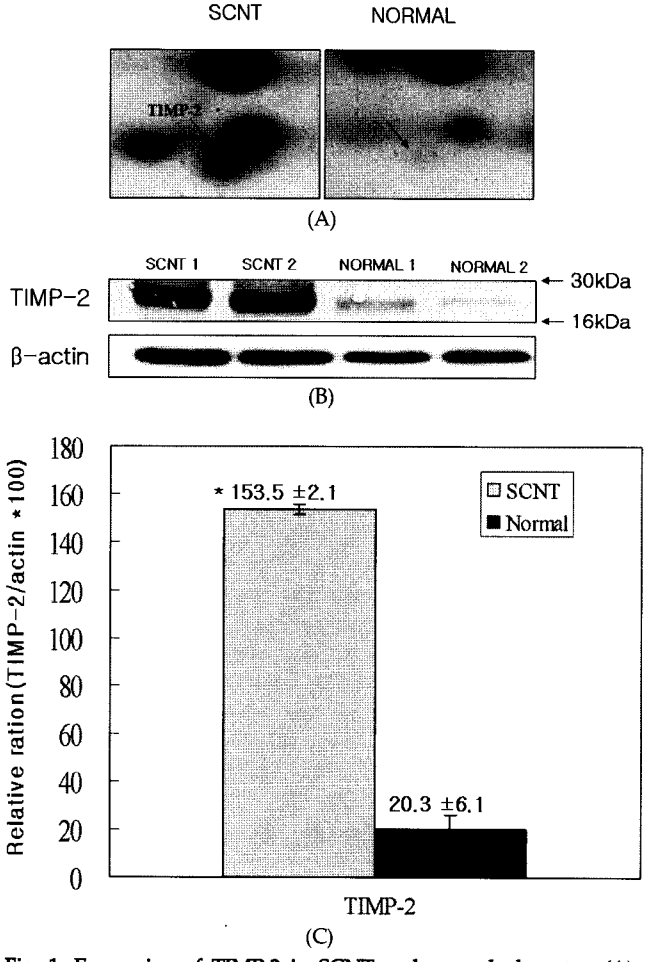

Fig. 1. Expression of TIMP-2 in SCNT and normal placentae. (A) Up-regulation of TIMP-2 in SCNT versus normal placentae, as shown on 2-DE gels. (B) Western blot analysis showing high-level expression of TIMP-2 in SCNT and normal bovine placenta, with β -actin detected as a loading control. (C) Densitometric quantification of protein levels in normal and SCNT placenta, normalized against the levels of β-actin protein using the NIH Image software. TIMP-2 protein levels are presented as the ratio of band intensity in SCNT versus normal placenta samples (*$p < 0.05$).

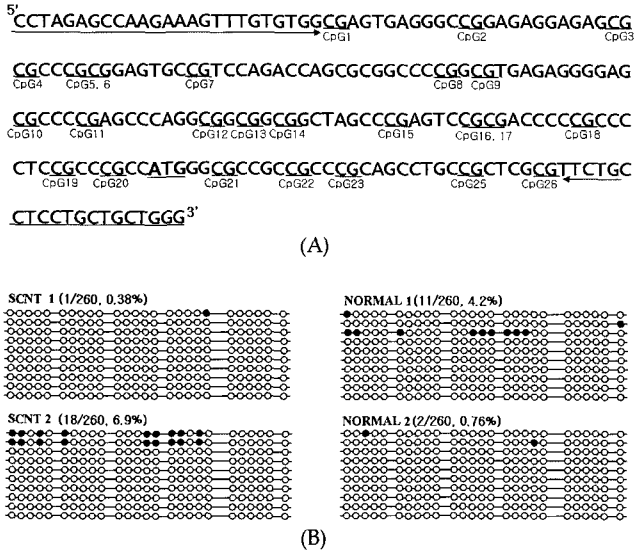

Fig. 2. Methylation patterns at the TIMP-2 promoter region. (A) Positions of the CG dinucleotides up to the 214 bp upstream region of the TIMP-2 gene are indicated (Arrow is primer region). (B) A total of 26 are present in the upstream region from the transcription initiation site. The methylation status of 10 clones obtained Normal placenta and NT placenta DNA is shown. Open circle, unmethylated; closed circle, methylated. Percentage in parentheses indicated the proportion of methylated CpG sites relative to the whole CpG island examined.

be down-regulated in SCNT versus normal placentae in the previous report (Kim *et al.*, 2005). One of the down-regulated proteins in SCNT placenta was vimentin protein that is presumed to stabilize the architecture of the cytoplasm (Morishima *et al.*, 1999). The 2DE-gel image of vimentin protein in the normal and SCNT placentae were shown in Fig. 3A. Western blot analysis revealed a significant decrease in vimentin protein level in SCNT placenta compared with normal (Fig. 3B). Vimentin protein was detected as a band of approximately 57 kDa in size in the blot. A short 5' UTR region of vimentin gene about 390 base-pair region upstream of the first exon was cloned in order to examine methylation pattern in placental samples. The surrounding sequence showed a characteristic typical of CpG island in vimentin promoter locus which is contains a total 33 CpG dinucleotides. For methylation analysis, genomic DNA was isolated from the placenta, and treated with bisulfite followed by amplication of the target sequence by PCR. A 180-bp fragment (including a set of primers) of PCR product contained 10 CpG sites (Fig. 4A). The most CpG sites were hypomethylated in cloned and normal placenta (Fig. 3B).

Overall Histone H3 Acetylation State of SCNT Placenta

Based on the above results, to evaluate whether histone modification is associated with aberrant gene expression in cloned placenta, histone-H3-acetylation state of the nucleosome in the SCNT placenta and normal

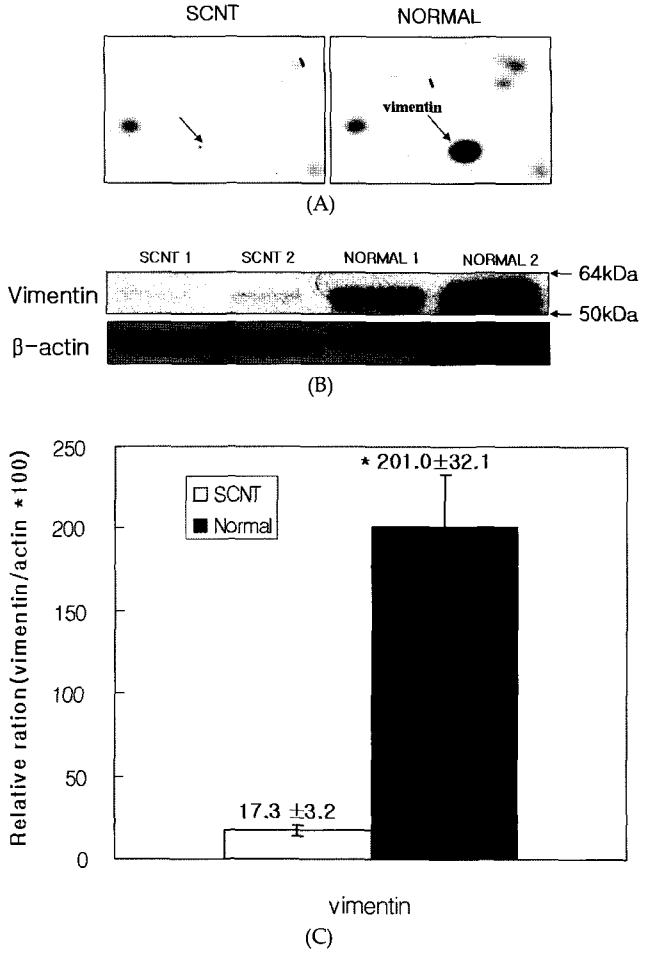

Fig. 3. Expression of vimentin in SCNT and normal placentae. (A) Down-regulation of vimentin in SCNT versus normal placentae, as shown on 2-DE gels. (B) Western blot analysis showing down-regulation of vimentin in SCNT and normal bovine placentae, with β-actin detected as the loading control. (C) Densitometric quantification of protein levels in normal and SCNT placenta, normalized against the levels of β-actin protein using the NIH Image software. Vimentin protein levels are presented as the ratio of band intensity in SCNT versus normal placental samples ($*p < 0.05$).

placenta was analyzed. Thirty microgram of the placental protein lysates were subjected to Western blotting with the antibodies against anti acetyl-histone H3. The acetyl-histone H3 was detected as a protein band of roughly 17 kDa (Fig. 5A). The overall histone H3 acetylation of SCNT placenta cells was significantly higher than in those of normal placenta cells (Fig. 5B). These results showed that differential gene expression between SCNT and normal placentae is closely correlated with overall histone H3 acetylation state.

DISCUSSION

In this study, two proteins that differentially expre-

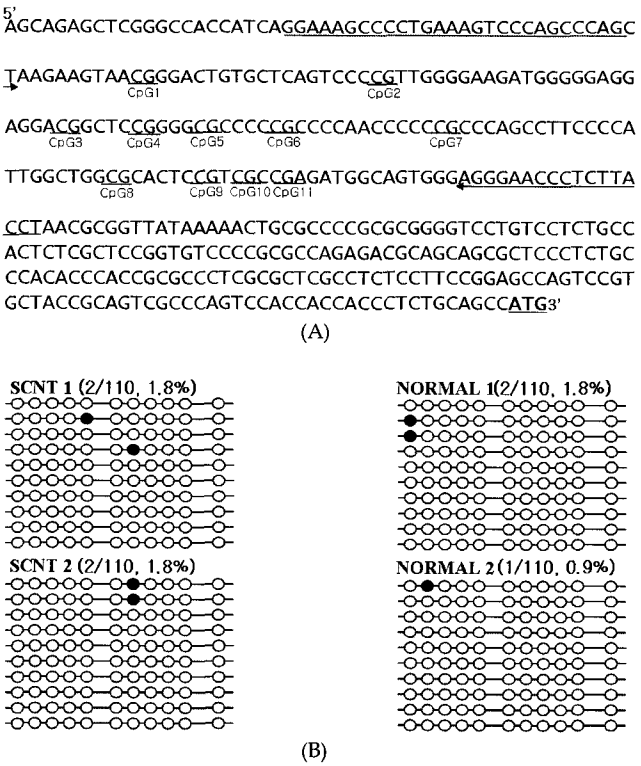

Fig. 4 Methylation patterns at the vimentin promoter region. (A) Positions of the CG dinucleotides up to the 180 bp upstream region of the vimentin gene are indicated (Arrow is primer region). (B) A total of 11 are present in the upstream region from the transcription initiation site. The methylation status of 10 clones obtained from normal placenta and NT placenta DNA is shown. Open circle, unmethylated; closed circle, methylated. Percentage in parentheses indicated the proportion of methylated CpG sites relative to the whole CpG island examined.

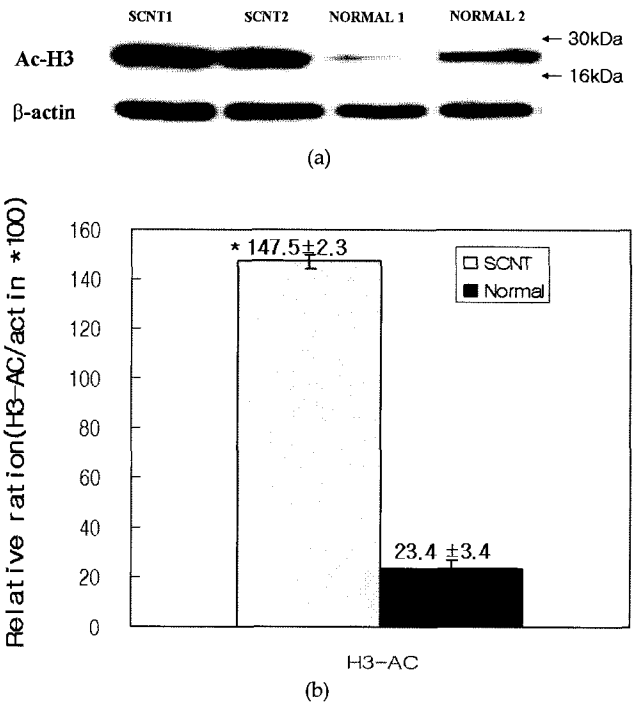

Fig. 5. Overall histone H3 acetylation in SCNT placentae. (A) Western analysis of protein extracts from SCNT and normal placental tissues using Acetyl-Histone H3 (Ac-H3) and β-actin antibodies. (B) Relative histone acetylation level by a ratio of band intensity in placental samples. Acetyl Histone H3 protein levels are presented as the ratio of band intensity in SCNT versus normal placenta samples ($*p < 0.05$).

ssed in SCNT placenta including TIMP-2 and vimentin were confirmed with Western blotting. Level of TIMP-2 protein in SCNT placenta was increased compared with that in normal placenta, while level of vimentin protein was decreased in SCNT placenta. Previous research reported that the TIMP expression level was decreased at the end of pregnancy when the number of binucleate cell was lowered (Walter *et al.*, 2001). And the loss of binucleate giant cells is necessary for the release of fetal membranes and discharge of placenta at the preparation period of parturition that is dependent on enzymatic extracellular matrix degradation (Williams *et al.*, 1987). The reduction in TIMP-2 release may be one of the several changes necessary for placental detachment and successful labor. For this view point, abnormal expression of TIMP-2 in the SCNT placenta may be the cause of placental abnormality and dysfunctional placenta.

Vimentin, a major component of intermediate filament, is degraded in response to apoptotic inducers (Van Engeland *et al.*, 1997; Hashimoto *et al.*, 1998; Prasad *et al.*, 1998). The cleavage of vimentin by caspases probably results in disruption of its filamentous structure, which may facilitate nuclear condensation and subsequent fragmentation (Morishima, 1999). It has been demonstrated that proteolysis of vimentin promotes apoptosis by dismantling intermediate filaments and amplifying the cell death signal via, pro-apoptotic cleavage products (Byun *et al.*, 2001). Reduced level of vimentin is known to be accompanied with remarkable reorganization of the cellular matrix followed by cellular disintegration and morphological changes (Morishima *et al.*, 1999). Our findings suggested that the reduced level of vimentin in the cloned placenta might be involved in the morphological alterations.

During development of embryo, appropriate gene expressions were achieved epigenetic reprogramming of genome by histone modifications and DNA methylation (Jenuwein *et al.*, 2001; Jones *et al.*, 2001). DNA methylation is one of the best studied epigenetic reprogramming of DNA (Kang *et al.*, 2002). To elucidate whether the abnormal expression of TIMP-2 and vimentin proteins in cloned placenta is related with epigenetic reprogramming, DNA methylation of TIMP-2 and vimentin loci and histone acetylation pattern were analyzed. In this experiment, there was no difference in methylation status between normal and SCNT placentae, suggesting that methylation status did not critically

affect aberrant TIMP-2 and vimentin gene expression at the SCNT placenta, and methylation pattern of TIMP-2 and vimentin locus might be correctly reprogrammed in SCNT bovine placenta. In general, acetylation of histone H3 is correlated with gene activation, while deacetylation is related with gene silencing (Fry *et al.*, 2001). In this experiment, histone acetylation difference between cloned and normal placenta may result in differential gene expression involved TIMP-2 and vimentin between SCNT and normal placenta. However, to elucidate the role of histone modification in differential gene expressions of TIMP-2 and vimentin, histone acetylation state of each differential gene in the SCNT placenta has to be analyzed. In conclusion, failure of reprogramming in SCNT could result in abnormal gene expression of placental genes and aberrant placental phenotype.

REFERENCES

1. Byun Y, Chen F, Chang R, Trivedi M, Green KJ, Cryns VL (2001): Caspase cleavage of vimentin disrupts intermediate filaments and promotes apoptosis. Cell Death Differ 8:443-450.
2. Cezar GG, Bartolomei MS, Forsberg EJ, First NL, Bishop MD, Eilertsen KJ (2003): Genome-wide epigenetic alterations in cloned bovine fetuses. Biol Reprod 68:1009-1014.
3. Fry CJ, Peterson CL (2001): Chromatin remodeling enzymes: who's on first? Curr Biol 11:185-197.
4. Han YM, Kang YK, Koo DB, Lee KK (2003): Nuclear reprogramming of cloned embryos produced *in vitro*. Theriogenology 59:33-44.
5. Hashimoto M, Inoue S, Ogawa S, Conrad C, Muramatsu M, Shackelford D, Masliah E (1998): Rapid fragmentation of vimentin in human skin fibroblasts exposed to tamoxifen: a possible involvement of caspase-3. Biochem Biophys Res Commu 247:401-406.
6. Hill JR, Burghardt RC, Jones K, Long CR, Looney CR, Shin T, Spencer TE, Thompson JA, Winger QA, Westhusin ME (2000): Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. Biol Reprod 63:1787-1794.
7. 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.
8. Jenuwein T, Allis CD (2001): Translating the histone code. Science 293:1074-1080.
9. Jones PA, Takai D (2001): The role of DNA methylation in mammalian epigenetics. Science 293: 1068-1070.
10. Kang YK, Park JS, Koo DB, Choi YH, Kim SU, Lee KK, Han YM (2002): Limited demethylation leaves mosaic-type methylation states in cloned bovine preimplantation embryos. EMBO 21:1092-1100.
11. Kim HR, Kang JK, Yoon JT, Seong HH, Jung JK, Lee HM, Sik Park C, Jin DI (2005): Protein profiles of bovine placenta derived from somatic cell nuclear transfer. Proteomics 5:4264-4273.
12. McGraw S, Robert C, Massicotte L, Sirard MA (2003): Quantification of histone acetyltransferase and histone deacetylase transcripts during early bovine embryo development. Biol Reprod 68:383-389.
13. Morishima N (1999): Changes in nuclear morphology during apoptosis correlate with vimentin cleavage by different caspases located either upstream or downstream of Bcl-2 action. Genes Cells 4:401-414.
14. Oswald J, Engemann S, Lane N, Mayer W, Olek A, Fundele R, Dean W, Reik W, Walter J (2000): Active demethylation of the paternal genome in the mouse zygote. Curr Biol 10:475-478.
15. Prasad SC, Thraves PJ, Kuettel MR, Srinivasarao GY, Dritschilo A, Soldatenkov VA (1998): Apoptosis-associated proteolysis of vimentin in human prostate epithelial tumor cells. Biochem Biophys Res Commun 249:332-338.
16. Reik W, Santos F, Dean W (2003): Mammalian epigenomics: reprogramming the genome for development and therapy. Theriogenology 59:21-32.
17. Suemizu H, Aiba K, Yoshikawa T, Sharov AA, Shimozawa N, Tamaoki N, Ko MS (2003): Expression profiling of placentomegaly associated with nuclear transplantation of mouse ES cells. Dev Biol 253:36-53.
18. Van Engeland M, Kuijpers HJ, Ramaekers FC, Reutelingsperger CP, Schutte B (1997): Plasma membrane alterations and cytoskeletal changes in apoptosis. Exp Cell Res 235:421-430.
19. Wakayama T, Yanagimachi R (2001): Mouse cloning with nuclear donor cells of different age and type. Mol. Reprod 58:376-383.
20. Walter I, Boos A (2001): Matrix metalloproteinases (MMP-2 and MMP-9) and tissue inhibitor-2 of matrix metalloproteinases (TIMP-2) in the placenta and interplacental uterine wall in normal cows and in cattle with retention of fetal membranes. Placenta 22:473-483.
21. Williams WF, Margolis MJ, Manspeaker J, Douglass LW, Davidson JP (1987): Peripartum changes in the bovine placenta related to fetal membrane retention. Theriogenology 28:213-223.
22. Young LE, Sinclair KD, Wilmut I (1998): Large offspring syndrome in cattle and sheep. Rev Reprod 3:155-163.

(Received: 11 May 2007 / Accepted: 8 June 2007)