The mRNA Expression and Methylation Pattern of Apoptosis-related and Imprinted Genes in Day 35 of Cloned Pig Fetuses

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ABSTRACT

This study was conducted to examine the mRNA expression of apoptosis-related and imprinted genes and methylation pattern of the differentially methylated region (DMR) of H19 gene in day 35 of SCNT pig fetuses. The day 35 of natural mating (control) or cloned (clone) pig fetuses were recovered from uterus. Endometrium from dam and liver from fetus were obtained, respectively. mRNA expression was evaluated by real-time PCR and methylation pattern was analyzed by bisulfite sequencing method. The Bcl-2 mRNA expression in clone was significantly lower than that of control ($p<0.05$). The mRNA expression of H19 gene in both endometrium and liver was significantly higher in clone than that of control, respectively ($p<0.05$). The level of IGF-2 mRNA in liver of clone was significantly lower than that of control ($p<0.05$), whereas the mRNA expression of IGF2-R gene in liver of clone was significantly higher than that of control ($p<0.05$). The DMR of H19 was lower methylation pattern in clone than that of control. These results suggest that the aberrant mRNA expression of apoptosis-related and imprinted genes and the lower DMR methylation pattern of imprinted gene may be closely related to the inadequate fetal development of cloned fetus.

(Key words : Cloned pig fetus, Apoptosis-related and imprinted genes, Methylation, Fetal growth)

INTRODUCTION

Although cloned animals have been successfully produced, the success rate of obtaining viable offspring from cloning remains less than 5% (Tamada and Kikyo, 2004). The low efficiency of animal production by SCNT is considered to be the result of an incomplete reprogramming of donor cell nucleus, which leads to abnormal expression of developmentally important genes. The expression patterns of some important genes, such as apoptosis-related and imprinted genes, expressed during preimplantation and in early implantation stages were abnormal (Daniels *et al.*, 2000; Wrenzycki *et al.*, 2001). Moreover, even viable cloned animals have varied methylation patterns (Eggan *et al.*, 2001; Ohgane *et al.*, 2001), which might be due to insufficient reprogramming of somatic cell nuclei. Recently Jiang *et al.* (2007) reported imprinted genes in cloned piglet died at or shortly birth, however, little is known about the expression of the apoptosis-related and imprinted genes and methylation pattern in the cloned fetuses.

This study was conducted to examine the mRNA expression of apoptosis-related and imprinted genes and to analyze methylation pattern of the differentially methylated region (DMR) of H19 gene in Day 35 of normal and SCNT pig fetuses.

MATERIALS AND METHODS

Ovaries were obtained from prepubertal crossbred gilts at a local slaughterhouse. Fibroblast cells were obtained from 30 days of landrace pig fetuses. Nuclear transferred eggs were surgically transferred into the oviducts of synchronized recipients. Approximately 100~200 SCNT embryos (2~4 cell stage) were transferred into oviduct of each synchronized recipients. The pregnancy status of recipients between Day 30 and Day 35 was determined by ultrasound scanning.

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Recovery of Control or Cloned Fetuses

The day 35 of natural mating (control) or SCNT clone (clone) pig fetuses were recovered from uterus. The fetuses were counted and weighed the body weight. The day 0 was indicated the day of natural mating or cloned embryo transfer. Samples of endometrium from dam and liver from fetus were isolated, frozen in liquid nitrogen, and stored at −80℃ until use.

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from the endometrium and liver tissues using TRIzol reagent (15596, Invitrogen, Carsbad, USA). The RNA was treated with RNas-free DNase I to remove possible contaminating DNA and stored at −80℃.

First-strand cDNA was synthesized by cDNA synthesis kit (Invitrogen). The RNA/primer mixtures (2 μg of total RNA, 1 μl of 50 μM oligo (dT), 1 μl of 10 mM dNTP, and 6 μl of DEPC-treated water) were mixed and incubated at 65℃ for 5 min, and then placed on ice for at least 1 min. The reaction mixture was prepared by adding each component (2 μl of 10× RT buffer, 4 μl of 25 mM $MgCl_2$, 2 μl of 0.1 M DTT, and 1 μl of RNaseOUT™ Recombinant RNase Inhibitor). The reaction mixture (9 μl) was added to 10 μl of the RNA/primer mixture, mixed gently, and collected by brief centrifugation. After incubation at 42℃ for 2 min, the mixture was added 1 μl of SuperScript™ II reverse transcriptase (50 U/μl: Invitrogen) and incubated at 42℃ for 50 min. The reaction were terminated at 79℃ for 50 min and then chilled on ice. All cDNA samples were stored at −20℃ until amplification.

Quantitative Real-Time PCR

Real-Time PCR reactions were performed according to the manufacturer's instruction using LightCycler (Roche, Germany). The sequences of target genes and β-actin were shown in Table 1. β-actin mRNA was used as an internal standard. The FastStart DNA SYBR Green I contains Taq DNA polymerase enzyme, 10× buffer, SYBR Green, optimized PCR buffer, 5 mM $MgCl_2$, and dNTP mix that includes dUTP. The PCR protocol was as follows: a preliminary denaturation program (95℃ for 10 min), 45 cycles of amplification and quantification (95℃ for 10 sec, 56~64℃ for 30 sec, 72℃ for 30 sec with a single fluorescence measurement), and a melting curve program (65~95℃ with a heating rate of 0.2℃ per second and continuous fluorescence measurement). The products were then cooled to 48℃.

For the mathematical model, it is necessary to determine the crossing points (CP) for each transcripts, CP is defined as the point at which the fluorescence rises appreciably above the background fluorescence. The relative quantification of gene expression was analyzed by the 2-ddCt method (Livak and Schmittgen, 2001). The sizes of PCR products were confirmed by gel electrophoresis on a standard 2% agarose gel stained with ethidium bromide and visualized by exposure to ultraviolet light.

Preparation of Genomic DNA

Genomic DNA was isolated from the liver and endometrium tissues using 500 μl of lysis buffer consisting of 100 mM Tris-HCl (pH 8.0), 150 mM EDTA, 1% SDS, and 5 μl of proteinase (20 mg/ml). The mixture was incubated at 55℃ for 60 min. After that the mixture was added 1 μl of RNase and incubated for 60 min at 37℃. An equal volume of phenol chloroform isoamyl alcohol was added, mixed gently, and centrifuge at 13,000 rpm for 15 min at 4℃. The supernatant was transferred into a new tube, added 1/10 vo-

Table 1. Primer sequences and real-time PCR conditions for apoptosis-related and imprinted genes

Gene		Primer sequence (5'-3')	Size (bp)	PCR condition (cycles)
	β-actin	F-ACGTGGACATCAGGAAGGAC R-ACATCTGCTGGAAGGTGGAC	210	58℃, 5s (55)
Apoptosis-related	Bcl-2	F-GGACAGAAAAGACAGGCTTCA R-GGCTAGGTCTTCCGTTTTCA	170	64℃, 5s (45)
	Bax	F-AAGCGCATTGGAGATGAACT R-AGTTGCCGTCAGCAAACATT	130	64℃, 5s (45)
Imprinted	H19	F-AAAGAGCATCTCAAGCGAGTCT R-GCTCCTGTACCTGCTACTAAATGAA	180	56℃, 5s (45)
	IGF-2	F-CTCGTGCTGCTATGCTGCTTAC R-CAGGTGTCATAGCGGAAGAACT	300	62℃, 12s (45)
	IGF2-R	F-ATAAACACCAATATAACACTCATCTGC R-GCACACGTTAATATAAAACTCGTACTT	216	62℃, 12s (45)

lume of 3 M sodium acetate and 2 volumes of absolute ethanol, shaken gently, and stayed for 2 hr at −20℃. The DNA pellet was washed with 70% ethanol followed by air-drying. The DNA pellet was dissolved and stored at −20℃ until use.

Sodium Bisulfite Genomic Sequencing

Bisulfite genomic sequencing was carried out as previously described (Herman *et al.*, 1996) with slight modification. Briefly, HindIII-digested genomic DNAs (0.5~2.0 μ g) were denatured in 0.33 M NaOH and incubated for 15 min at 37℃, and then added sodium metabisulfite solution (2.5 M) and hydroquinone (0.5 mM), respectively. After incubation in the dark at 55℃ for 12 hr, the DNAs were purified with Wizard DNA purification resin (Promega, Madison, WI). The reaction was stopped by treatment with NaOH at a final concentration of 3 M at 37℃ for 15 min followed by ethanol precipitation. The purified DNAs were suspended in 20 μ l of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA and used for two successive rounds of PCR with nested primer (outer and inner) pairs, which are specific to the top strand of mutagenized DNA (Table 2). All PCR reactions were preformed using Immolase™ DNA polymerase Mix (Bioline).

The condition of the first PCR was as follows: The thermocycling program was 43 cycles of 94℃ for 30 sec, 55℃ for 30 sec, and 72℃ for 1 min, preceded, and followed by 10 min of incubation at 94℃ and 72℃, respectively. The first PCR product were eluted in 100 μ l of water and used for the second round of PCR reactions. The entire PCR products were run on a 2% agarose gel to verify the amplification of specific bands, which were then excised from the gel for purification with the affinity column. The PCR fragments were cloned into TOPO TA cloning kits (Invitrogen, USA) and the cloned samples were sequenced.

Statistical Analysis

Data were analyzed by *t*-test statistic for number of fetuses recovered and body weight and by Duncan's multiple range test for mRNA expression assayed by quantitative real-time PCR. *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

Recovery Data of Fetuses from Uterus

The number of cloned fetuses per dam (Table 3) and the mean body weight of cloned fetuses (Fig. 1) were lower tendency compared to control, although the difference was not statistically significant.

mRNA Expression of Apoptosis-Related and Imprinted Genes

The mRNA level of Bax gene in both tissues was

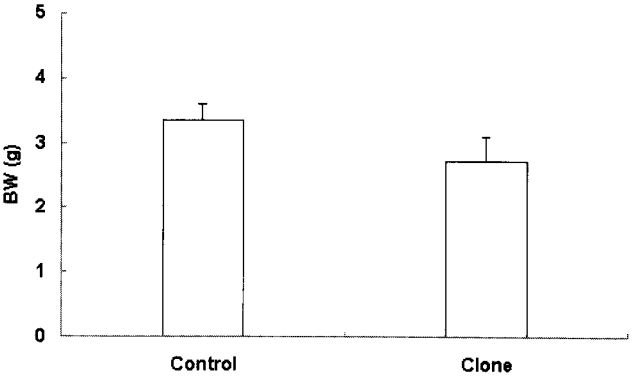

Fig. 1. Body weight of control and cloned fetuses at day 35 of pregnancy. Control, natural mating; Clone, SCNT clone. Data were expressed as mean±SD.

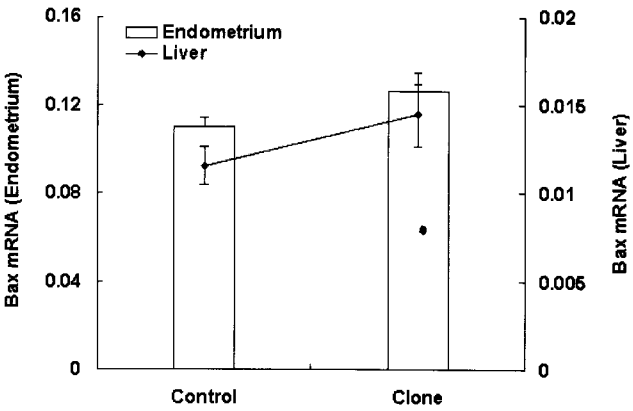

Fig. 2. Relative mRNA expression levels of Bax in both endometrium and liver of control and clones. β-actin was used as an internal control. Data were expressed as mean±SE.

Table 2. Primer sequence of H19 for bisulfite PCR

Gene	Primer sequence (5'-3')	Size (bp)
H19 - Outer	F:GTTGTTTAGAGTGGATTTTA R:ACCCAAAACCCTATACCACC	600
H19 - Inner	F:AGGATTTTAGCGGTATTTGT R:CCGAAAATAAAAATCCCTCCC	356

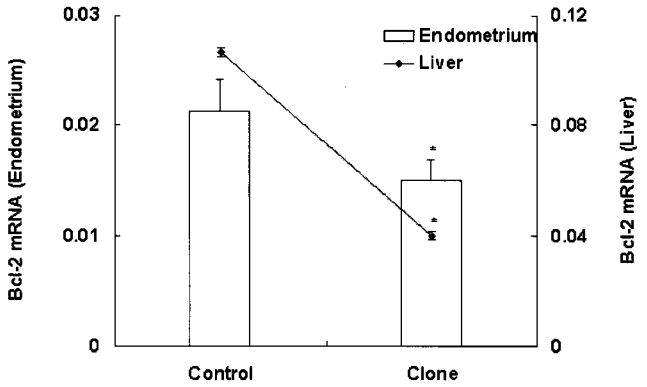

Fig. 3. Relative mRNA expression levels of Bcl-2 in both endometrium and liver of control and clones. β-actin was used as an internal standard. Data were expressed as mean±SE. Asterisk indicates significant difference ($p<0.05$) with control.

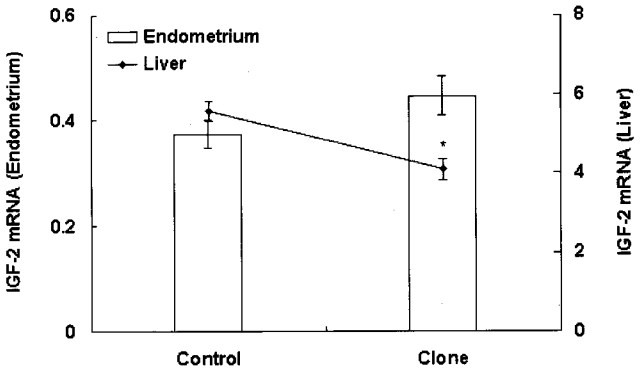

Fig. 5. Relative mRNA expression levels of IGF-2 in both endometrium and liver of control and clones. β-actin was used as an internal standard. Data were expressed as mean±SE. Asterisk indicates significant difference ($p<0.05$) with control.

not different between control and cloned groups (Fig. 2). The mRNA expression of Bcl-2 in cloned group was significantly lower than that of control ($p<0.05$) in both tissues, respectively (Fig. 3). The relative abundance of Bax/Bcl-2 was significantly higher in cloned fetus than in control (data not shown).

The mRNA expression of H19 gene in both tissues was significantly higher in cloned group than that of control ($p<0.05$), respectively (Fig. 4). The level of IGF-2 mRNA in liver was significantly lower in cloned group than that of control ($p<0.05$), the difference in endometrium, however, was not different in both groups (Fig. 5). The level of IGF2-R mRNA in cloned group was significantly over-expressed in liver compared to that of control ($p<0.05$), but in endometrium the mRNA expression of IGF2-R was similar to that of control (Fig. 6).

DMR Methylation Pattern in H19 Gene

We examined the methylation status of DMR of H19 gene in day 35 of fetus or dam somatic tissues. The

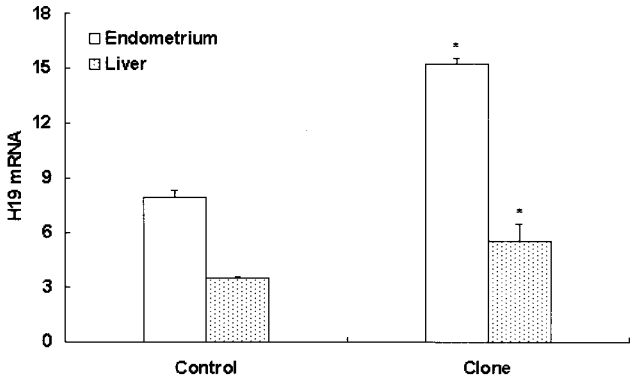

Fig. 4. Relative mRNA expression levels of H19 in both endometrium and liver of control and clones. β-actin was used as an internal standard. Data were expressed as mean±SE. Asterisk indicates significant difference ($p<0.05$) with control.

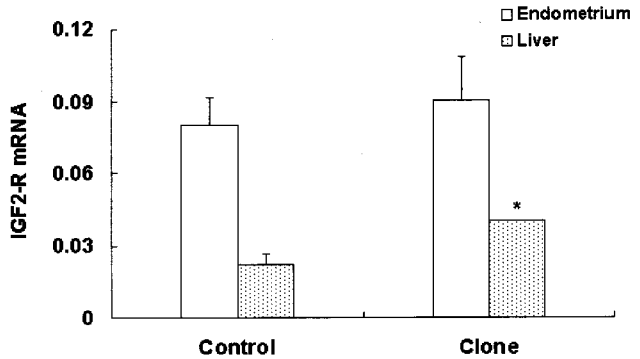

Fig. 6. Relative mRNA expression levels of IGF2-R in both endometrium and liver of control and clones. β-actin was used as an internal standard. Data were expressed as mean±SE. Asterisk indicates significant difference ($p<0.05$) with control.

methylation pattern (A) and its numerical value (B) of H19 gene in both endometrium and liver tissues were shown in Fig 7. The both tissues were moderately methylated (43% in endometrium and 44% in liver) in control, clones, however, showed lower methylation status in both tissues (33% in endometrium and 33% in liver).

DISCUSSION

In this study, we evaluated the mRNA expression of apoptosis-related and imprinted genes and determined the methylation pattern of H19 gene in day 35 of normal or SCNT cloned pig fetuses and endometrium from dam.

Pregnancy rate after transfer to recipients are relatively high (Tsunoda and Kato, 2002), somatic cell nuclear transfer, however, has shown a high rate of abortion during early gestation (Han *et al.*, 2003). Like previous report, we obtained small number of cloned

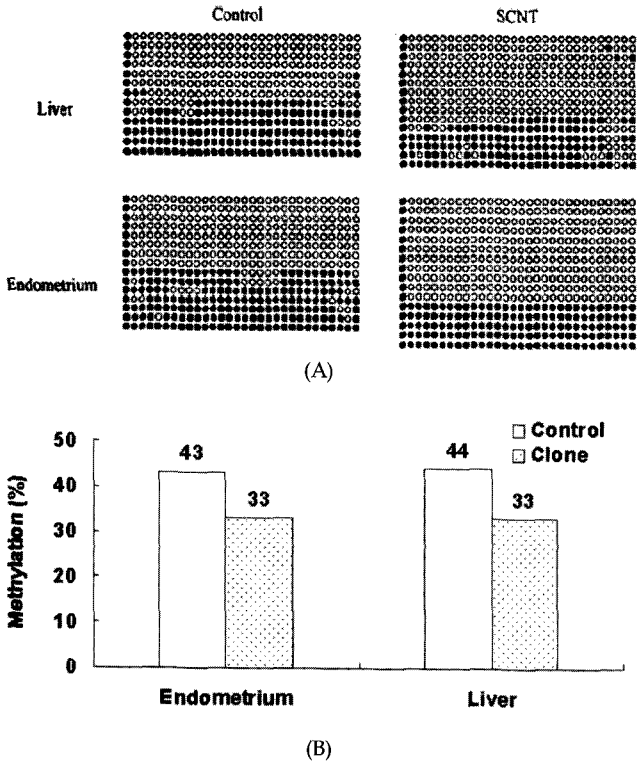

Fig. 7. Methylation analysis of differentially methylated region (DMR) of H19 gene in day 35 of cloned fetus and dam somatic tissues. (A) Filled and open circle correspond to methylated CpG and unmethylated CpG sites, respectively. (B) Numeral value of DMR of H19 in both endometrium and liver.

fetuses and their body weight was lower than control, although the difference was not statistically significant because of a wide range of variation. The number of cloned fetuses was varied from 1 to 9 per dam. And the number of mummy fetuses was higher in cloned group. It can be postulated that the cloned fetuses are not developed normally at early pregnancy.

Chae *et al.* (2006) reported that cloned porcine embryos were identified abnormal apoptosis in the extra-embryonic tissue during early pregnancy. And apoptosis occurs during the preimplantation stage in both *in vivo*- and *in vitro*-produced embryos, and it may contribute to embryonic loss. In rat granulosa cells, Bax and Bcl2 mRNA level was closely correlated with apoptosis (Choi *et al.*, 2004). However little is known about the regulation of apoptotic gene expression in day 35 of cloned fetuses. Here we revealed that the relative abundance of Bax/Bcl-2 was significantly higher in cloned fetus than in the control, because lower expression of anti-apoptotic gene (Bcl-2) in both liver and endometrium.

Imprinted genes may regulate some crucial processes associated with reproduction, placentation, energy homeostasis, lactation, and behavior. To date, more than 50 imprinted genes have been identified in the mouse and/or human, and many of them are involved in regulation of fetal growth (Dean *et al.*, 2003).

H19 gene has been found in the fetus and placenta and in the liver of clones produced from ES cells (Humpherys *et al.*, 2001; Ogawa *et al.*, 2003). Although the function of the H19 transcript is unclear, it is closely linked to the IGF-2 gene in both the mouse and human and regulates imprinting of the IGF-2 gene by sharing an imprinting control element (Bartolomei *et al.*, 1991).

There are some controversies about the mRNA expression of H19 in fetus and maternal. Inoue *et al.* (2002) reported that the expression of H19 mRNA in the placenta of normal was not different from that of somatic cell cloned mice. However, Khatib and Schutzkus (2006) reported that significant higher H19 mRNA levels were found for liver, lung, heart, and spleen in fetus compared to those of maternal. In the present study, the expression of H19 mRNA in both liver and endometrium was significantly higher in cloned fetuses and dams compared to control. These results imply that further studies are needed to evaluate the functions of this gene in fetal development.

The insulin-like growth factor 2 (IGF-2) encodes a growth factor that plays a crucial role in tissue differentiation, fetal growth, and placental development (Constancia *et al.*, 2002). The insulin-like growth factor 2 receptor (IGF2-R) expressed from the maternal allele, has been shown to be an important regulator of fetal growth and embryonic development. Mice that inherit a maternal allele with a targeted mutation at IGF2-R die at birth, show an increase in body weight of 20.25 %, have higher circulating levels of IGF-2, and feature a slightly kinked tail and other abnormalities (Lau *et al.*, 1994). The evidence of similar fetal overgrowth reported as a result of livestock cloning and *in vitro* embryo culture suggests that alterations in imprinted genes such as IGF2-R may be involved in 'large offspring syndrome' (Young *et al.*, 2001). In the present study, the mRNA expressions of IGF-2 and IGF2-R in endometrium were not different, but the IGF-2 mRNA level was significantly lower and IGF2-R mRNA expression was significantly over-expressed in liver of cloned fetuses, respectively. Although it is difficult to conclude because of limitation of organ tissues used, the aberrant mRNA expression of IGF-2 and IGF2-R may affect to fetal growth of clones.

Although the mechanism for aberrant expression of some imprinted genes in the cloned fetus is unclear, several factors including histone acetylation and DNA methylation may be involved. Recent reports revealed that cloned embryos exhibit abnormal patterns of DNA methylation and gene expression, leading to high rates of embryonic, foetal, perinatal and neonatal deaths, as well as offspring with various abnormalities (Kang *et al.*, 2001a, b; Kelly, 2005). Regulation of imprinted ge-

nes correlates well with DNA methylation (Reik and Water, 2001). Im *et al.* (2007) reported that SCNT embryos were demethylayted (0~2%) at all stages of development. The aberrant methylation pattern of H19 loci may be a reason for increased abnormal fetus after embryo transfer of porcine SCNT embryos. In this study, we analyzed DNA methylation of the H19 DMR. The status of H19 DMR in cloned group was lower methylation pattern in both endometrium and liver than that of control. This unmethylated status may be the result of over-expression of H19 mRNA and affect to abnormal development of cloned fetus. Unlike our result, low H19 mRNA expression in bovine was observed for adult spleen, caruncle, and endometrium (Khatib and Schutzkus, 2006). One possibility is that the endometrium used might be contaminated with extraembryonic tissue, because it was difficult to separate perfectly.

These results suggest that the aberrant mRNA expression of apoptosis-related and imprinted genes and the lower DMR methylation pattern of imprinted gene are closely related to the inadequate fetal development of cloned fetus.

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