Methylation Pattern of *H19* Gene at Various Preimplantation Development Stages of *In Vitro* Fertilized and Cloned Porcine Embryos

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ABSTRACT

Insulin-like growth factor II (IGF2) and *H19* genes are mutually imprinted genes which may be responsible for abnormalities in the cloned fetuses and offspring. This study was performed to identify putative differentially methylated regions (DMRs) of porcine *H19* locus and to explore its genomic imprinting in *in vitro* fertilized (IVF) and somatic cell nuclear transferred (SCNT) embryos. Based on mice genomic data, we identified DMRs on *H19* and found porcine *H19* DMRs that included three CTCF binding sites. Methylation patterns in IVF and SCNT embryos at the 2-, 4-, 8~16-cells and blastocyst stages were analyzed by BS (Bisulfite Sequencing)-PCR. The CpGs in CTCF1 was significantly unmethylated in the 2-cell stage IVF embryos. However, the 4- (29.1%) and 8~16-cell (68.2%) and blastocyst (48.2%) stages showed higher methylation levels ($p<0.01$). On the other hand, SCNT embryos were unmethylayted (0~2%) at all stages of development. The CpGs in CTCF2 showed almost unmethylation levels at the 2-, 4- and 8~16-cell and blastocyst stages of development in both IVF (0~14.1%) and SCNT (0~6.4%) embryos. At all stages of development, CTCF3 was unmethylated in IVF (0~17.3%) and SCNT (0~1.2%) embryos except at the blastocyst stage (54.5%) of IVF embryos. In conclusion, porcine SCNT embryos showed an aberrant methylation pattern comprised to IVF embryos. Therefore, we suggest that the aberrant methylation pattern of *H19* loci may be a reason for increased abnormal fetus after embryo transfer of porcine SCNT embryos.

(Key words : DNA methylation, DMR, *H19* gene, SCNT, Porcine)

INTRODUCTION

Somatic cell nuclear transfer (SCNT) technique is a powerful tool for the production of genetically identical animals and therapeutic stem cells. The cloned offspring have been produced by this technique in various species including sheep, cattle, mice and pigs (Wilmut *et al.*, 1997; Kato *et al.*, 1998; Wakayama *et al.*, 1998; Onishi *et al.*, 2000). However, its efficiency is still extremely low and cloned animals often exhibit abnormal phenotype such as an overgrowth of fetus and placenta. Although exact causes of these malformations are still not known, they are largely believed to be associated with incomplete epigenetic reprogramming (Dean *et al.*, 2001; Kang *et al.*, 2001a,b,c; Rideout *et al.*, 2001; Boiani *et al.*, 2002).

Genomic imprinting is a mechanism whereby only one of the two parental alleles is expressed while transcription from the other parental allele is suppressed (Li *et al.*, 1999; Reik and Walter, 2001; Ferguson-Smith and Surani, 2001). Insulin-like growth factor II (*IGF2*) and *H19* are the most studied imprinted genes that are reciprocally imprinted in mouse and human. Differential methylation is observed in differentially methylated region (DMR) of *H19* which contains CC-CTC-binding factor (CTCF) protein binding sites in an imprinting control region (ICR). The zinc finger protein, CTCF, binds to the unmethylated maternal allele and activates the boundary function of this element which prevents a common set of enhancers from interacting with the *IGF2* promoters (Bell and Felsenfeld, 2000; Hark *et al.*, 2000; Kanduri *et al.*, 2000). On the other hand, methylation prevents binding of CTCF to the ICR allowing *IGF2* expression whereas the *H19* gene is silenced on the paternal allele (Thorvaldsen *et al.*, 1998; Szabo *et al.*, 2000).

Many researchers have focused on the epigenetic reprogramming of cloned individuals and approached through diverse studies including the gene expression, histone modification, nuclear remodeling and cytosine methylation. DNA methylation is a major epigenetic

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modification of genome that regulates crucial aspects of gene function (Kang *et al.*, 2001b) and is essential for normal embryonic development (Li *et al.*, 1993). In the preimplantation period, a genome-wide demethylation occurs both actively and passively after fertilization, followed by *de novo* methylation at the later stages, which is dependent on the species (Howlett and Teik, 1991; Oswald *et al.*, 2000). Aberrant genome-wide demethylation followed by immediate remethylation resulting in maintenance of donor type hypermethylation during preimplantation development of cloned bovine embryo has been reported as a plausible cause of abnormal phenotype observed in cloned offspring (Kang *et al.*, 2001a; Han *et al.*, 2003b). However, the epigenetic reprogramming of repetitive DNA sequence in cloned porcine embryos appears to be concordant with that of normal mouse embryos which exhibited a gradual demethylation pattern similar to the endogenous demethylation process (Kang *et al.*, 2001c). These results suggest the species specific differences for methylation state in cloned embryo. However, DNA methylation changes of gene specific sequences and in particular, imprinted genes have not been studied in porcine cloned embryos. Therefore, this study was performed to identify putative DMRs of porcine *H19* locus and to explore its genomic imprinting in embryos produced by IVF and SCNT.

MATERIALS AND METHODS

Oocyte Retrieval and *In Vitro* Maturation

Prepubertal porcine ovaries were collected from a local abattoir and transported to the laboratory in saline maintained at 30~37℃. Cumulus oocyte complexes (COCs) were aspirated from follicles (2~6 mm diameter) using 10 ml syringe fitted with an 18 G needle. The COCs were washed thrice in TL-HEPES media containing 1 mg/ml BSA (low carbonate TALP) and matured in groups of 50 in 500 μl of Tissue Culture Medium 199 with Earle's salts (TCM-199; Gibco BRL, Grand Island, NY, USA) supplemented with 25 mM $NaHCO_3$, 10% (v/v) porcine follicular fluid, 0.57 mM cysteine, 0.22 μg/ml sodium pyruvate, 25 μg/ml gentamicin sulfate, 0.5 μl/ml p-FSH (Folltropin V; Vetrepharm, Canada), 1 μg/ml estradiol-17β, and 10 ng/ml epidermal growth factor (EGF; E-4127, Sigma, St. Louis, MO, USA) under mineral oil at 39°C in a humidified atmosphere of 5% CO2 in air for 40~42 hr (for nuclear transfer) or 42~44 hr (for IVF) as described earlier (Gupta *et al.*, 2007).

In Vitro Fertilization (IVF) of Oocytes

IVF of oocytes was carried out as described earlier (Park *et al.*, 2005). Briefly, oocytes were washed three times with fertilization medium (modified Tris-buffered medium) containing 1 mM caffeine sodium benzoate and 0.1% BSA and were placed into groups of 10~15 oocytes per 50 μl fertilization droplets of the fertilization medium. Porcine testis were collected from a local slaughterhouse and transported to the laboratory at 30~37℃ in 0.9% (w/v) saline supplemented with 75 μg/ml penicillin G and 50 μg/ml streptomycin sulfate. Sperm were retrieved from cauda epididymis in TL-HEPES, and pelleted by centrifugation at 800 × g for 5 min. The soft pellet was then subjected to swim-up in Sp-TALP medium for 10 min. The supernatant was collected and washed twice by centrifugation at 800 × g for 5 min. Finally, the sperm pellet was resuspended in the fertilization medium and added to the fertilization droplets to obtain a final sperm concentration of 5×10^5 sperm/ml. Sperm and oocytes were co-incubated at 39℃ in a humidified atmosphere of 5% CO_2 in air for 6 hr.

Preparation of Donor Cells for Nuclear Transfer

Primary porcine fetal fibroblasts (PFFs) were isolated from fetuses retrieved from a pregnant sow from abattoir essentially as described by Uhm *et al.* (2000). The PFFs were cultured in 60 mm tissue culture dishes (Falcon, Franklin Lakes, NJ, USA) in Dulbecco's Modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% FBS. After attainment of confluence, PFFs were trypsinized, washed three times in Ca^{++} / Mg^{++} free PBS (Gibco BRL), pelleted by centrifugation at 800 × for 5 min and resuspended in DPBS with 10% FBS. The PFFs were routinely maintained in 50 ml tissue culture flasks (Falcon) for 2~7 passages before use as donor cells in the NT.

Somatic Cell Nuclear Transfer (SCNT) of Oocytes

Nuclear transfer was performed as described earlier (Uhm *et al.*, 2000) with partial modifications. Briefly, *in vitro* matured oocytes were stripped of cumulus cells in TL-HEPES supplemented with 0.1% hyaluronidase and washed three times in TL-HEPES containing 0.1% BSA. Denuded oocytes were incubated for 20 min in m-NCSU23-BSA medium supplemented with 7.5 μg/ml cytochalasin B and 5 μg/ml Hoechst 33342. Enucleation was performed by the aspirating the first polar body and adjacent cytoplasm (approximately 30% of ooplasm) using a beveled pipette (25 μm in diameter) in HEPES buffered mNCSU23-BSA medium. Enucleation was confirmed by UV assisted visualization of fluorescent metaphasic plate in the aspirated cytoplasm contained within the enucleation pipette. Enucleated oocytes were subsequently reconstructed by inserting a small sized (~15 μm in diameter), smooth bordered fibroblast cell into the perivitelline space of each enucleated oocyte using the same pipette used for enucleation. Reconstructed oocytes were placed in m-

NCSU-BSA medium for 30 min to allow recovery. Fusion was achieved at room temperature in a chamber with two platinum electrodes at 1 mm apart overlaid with fusion solution that was composed of 0.3 M mannitol, 1.0 mM $CaCl_2$ and 0.1 mM $MgSO_4$. The reconstructed oocytes were aligned manually with a fine pasteur pipette, so that the contact surface between the donor cell and oocyte was parallel to the electrodes. Membrane fusion was induced with single DC pulse of 2.1 kV/cm for 30 μsec delivered by a BTX Electro Cell Manipulator 2001 (BTX, San Diego, CA, USA). This pulse was also utilized to simultaneously induce activation of the reconstructed embryos. Following activation, activated oocytes were cultured in NCSU23 medium supplemented with 7.5 μg/ml cytochalasin B for 4 hr before transferring to IVC medium for further culture (Park *et al.*, 2005).

Comparative Sequencing

Porcine *H19* sequences were analyzed using variety of software programs. Sequence comparisons of *H19* between mouse and porcine species were done with BLAST at URL http://www.ncbi.nlm.nih.gov and pair wise alignment algorithms at URL http://www.ebi.ac.uk. In order to detect CpG islands in porcine sequence, Methprimer program was employed (http://www.urogene.org/methprimer/). By default, a CpG island was defined as a DNA stretch of at least 200 bp with a GC content of more than 50% and an observed expected ratio of CpG dinucleotides more than 0.6 (Gardiner-Garden and Frommer, 1987).

Genomic DNA Isolation

The 2-, 4- and 8~16 cells and blastocyst stage embryos for genomic DNA isolation were collected from porcine IVF and SCNT embryos on Day 2, 3, 4 and 7 of *in vitro* culture, respectively. Each sample was solubilized in 300 μl of lysis buffer containing 200 μg/ ml of proteinase K (Roche, Molecular Biochemicals, Mannheim, Germany), and incubated at 55℃ for overnight. The DNA was then purified by extraction with phenol : chloroform : isoamyl alcohol, precipitated with absolute ethanol in the presence of 5 μg of glycogen as carrier and resuspended in 20 μl of distilled water. Genomic DNAs extracted from individual samples were subjected to analysis of DNA methylation at all regions of *IGF2* and *H19* genes under study.

Bisulfite (BS) Treatment and Polymerase Chain Reaction (PCR)

Genomic DNAs were treated with sodium bisulfite to convert all unmethylated cytosines to uracil using One Day MSP kit (In2Gen, Korea) according to the manufacturer's protocol. Briefly, purified genomic DNAs (0.5~1 μg) were denatured with 3N NaOH at 37℃ for 10 min. After alkaline denaturation, sodium bisulfite (5 M) was added and incubated at 50℃ for 16 hr in dark. Modified DNAs were then diluted with 20 μl of DW after desulfonation, neutralization and desalting. Aliquots of 1~2 μl DNA were used for subsequent BS-PCR amplification. For amplification of porcine *H19* genes, PCR was performed two times for each embryo sample with 3~4 μl of the bisulfite converted genomic DNA as template. Primer sets used are shown in Table 1. For nested PCR, 3 μl of the 1st PCR product was added to 2nd PCR reaction. The amplified products we-

Table 1. PCR primer sets for DMR three CTCFs

	Primer set	Sequence (5′ → 3′)	Annealing temp. (℃)	Product size (bp)
CTCF1 1st	Sense	5′ -ATGGGGAGGATTGGTTTTGGTGT-3′	50	252
	Antisense	5′ -CCTTACCTAATCCCTAATCCCAAAG-3′		
2nd	Sense	5′ -GGAGTTTAAGGTGGTTGGAGAGAG-3′	55	
	Antisense	5′ -CTAACTTCCACAAATCCACTACAC-3′		
CTCF2 1st	Sense	5′ -GTAGTTTAGTTTGGGGTGATT-3′	50	198
	Antisense	5′ -CTAACTACACCCCAAACAAC-3′		
2nd	Sense	5′ -GGGATGATTTGGAGGGGTTTA-3′	55	
	Antisense	5′ -CTAACTACACCCCAAACAAC-3′		
CTCF3 1st	Sense	5′ -GTAGGGTTTTTGGTGGTATAGGGTTTT-3′	50	205
	Antisense	5′ -ACACACTAAACACCCAACCTTTAACAC-3′		
2nd	Sense	5′ -GGTTGTGGGTGTGGAGGTAGAAG-3′	55	
	Antisense	5′ -CACTAAACACCCAACCTTTAACAC-3′		

re verified by electrophoresis on 1.0% agarose gel.

Cloning and Sequencing

Successful PCR products were cloned into pGEM-T easy vector (Promega, USA) according to the manufacturer's protocol. Plasmid DNAs were purified with GENE ALL™ Plasmid Quick (General biosystem, Korea) and then individual clones were sequenced (Macrogen, Korea). Clones were only accepted with ≥ 95% cytosine conversion. The non-converted cytosine residues were used to ensure each accepted clone originated from a different template DNA. At least two separate bisulfite treatments were conducted for the samples to verify the results.

Experimental Designs

Experiment 1: Sequence homology. This set of experiments evaluated the identification of putative CTCF binding sites within porcine *H19* DMR.

Experiment 2: Bisulfite sequencing analysis of putative DMRs. This set of experiments evaluated the methylation status of putative DMRs of the porcine genes between mature porcine sperm and oocyte by bisulfite sequencing PCR analysis.

Experiment 3: Bisulfite sequencing analysis of pocine IVF and SCNT embryos. This set of experiments evaluated the DNA methylation pattern at DMRs of *H19* gene at the 2-, 4- and 8~16-cell and blastocyst stages of porcine IVF and SCNT embryos by bisulfite sequencing PCR analysis.

Statistical Analysis

The methylation percentages were obtained for individual clone within each sample. Statistical analysis was carried out using SAS software (Statistical Analysis System Inc., Cary, NC, USA). These were then used to calculate the overall methylation level, standard deviation and standard error of the mean of each sample. The samples are considered significantly different when $p<0.01$.

RESULTS

Sequence Homology

The putative DMRs of the porcine *IGF2* and *H19* genes were deduced by aligning porcine sequences with those of mouse sequence which revealed that all exons were very well conserved with several conserved CpG islands existing throughout the porcine sequences. Putative DMR of *H19* was located 2 kb upstream of promoter region. To find CTCF binding sites which play a key role in imprinting of mouse and human, we aligned 14 bp conserved element (CCGCNNGGNGG

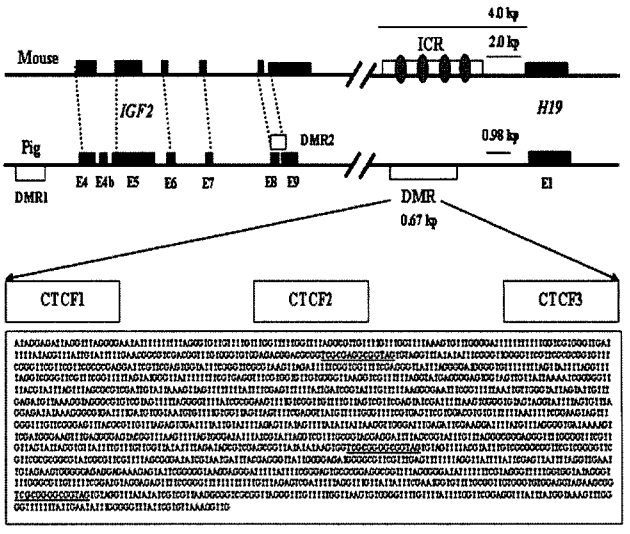

Fig. 1. Map of the conserved elements of putative *H19* DMR. 14 bp conserved sequence and its composition. Identification of putative CTCF binding sites within *H19* DMR. Closed boxes represent exons of *IGF2* and *H19* genes and open boxes indicate DMRs. The 14 bp consensus sequence for CTCF was aligned with porcine counterpart. Nucleotides identical to the CTCF consensus are underlined and boldfaced.

CAG) with the porcine *H19* and then we found three CTCF binding sites within putative *H19* DMR (Fig. 1).

Bisulfite Sequencing Analysis of Putative DMRs

To confirm the uncharacterized DMRs of the porcine *IGF2* and *H19*, bisulfite sequencing PCR analysis was performed to investigate the methylation status of putative DMRs of the porcine genes between mature sperm and oocyte. Previously, the region containing CTCF binding sites, which is located within 2 kb upstream of the transcription site of *H19*, was reported to show allele specific methylation in mouse and human. For this reason, three different types of primers were designed to detect all CTCF binding sites which were found within putative DMR region of *H19* by homology search result showed that all putative DMRs including CTCF binding sites were differentially methylated (Fig. 2).

Bisulfite Sequencing Analysis of Pocine IVF and SCNT Embryos

To determine the relationship between the methylation of these DMRs and abnormal expression of imprinted genes, in IVF and SCNT embryos at the 2-, 4- and 8~16-cell and blastocyst stages were analyzed for DNA methylation pattern at DMRs of *H19* gene. The CpGs in CTCF1 (1st CTCF binding site) was significantly unmethylated in 2-cell stage IVF embryos. However, 4- (29.1%) and 8~16-cell (68.2%) and blastocyst (48.2%) stages showed higher methylation levels ($p<$

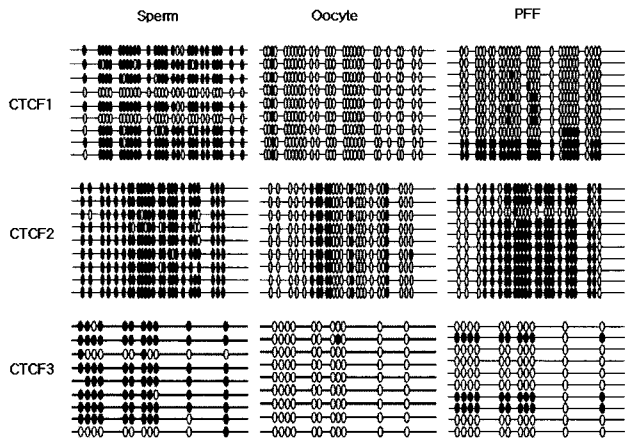

Fig. 2. Differential methylation pattern of putative DMR of the three CTCFs in sperm, oocytes, and PFFs.

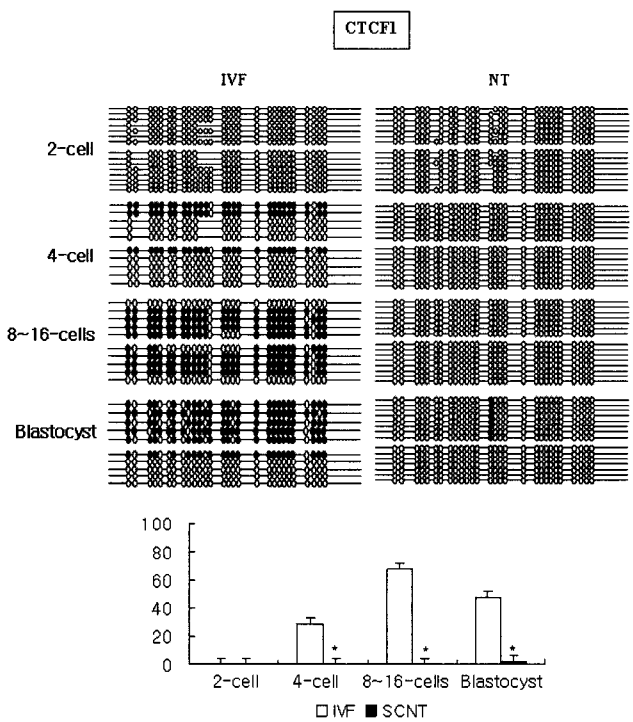

Fig. 3. Differential methylation pattern of putative DMR of CTCF1 in IVF and SCNT embryos. Value with asterisk upper the different color bar differ significantly ($p<0.05$).

0.01). On the contrary, SCNT embryos were unme- thylayted (0~2%) at all stages of development (Fig. 3). The CpGs in CTCF2 (2nd CTCF binding site) showed almost unmethylation levels at the 2-, 4- and 8~16-cell and blastocyst stages of development in both IVF (0~14.1%) and SCNT (0~6.4%) embryos (Fig. 4). Similarly, CTCF3 (3rd CTCF binding site) was unmethylated at all stages of development in IVF (0~17.3%) and SCNT (0~1.2%) embryos except at the blastocyst stage (54.5 %) of IVF embryos (Fig. 5).

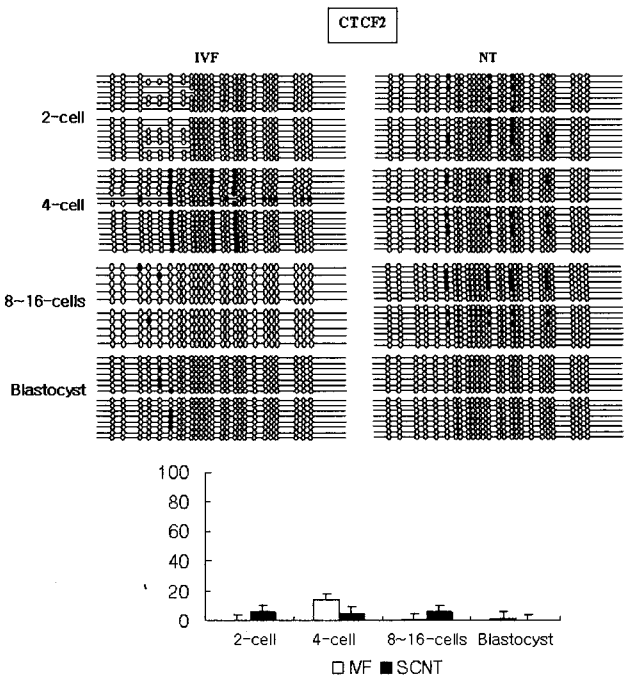

Fig. 4. Differential methylation pattern of putative DMR of CTCF2 in IVF and SCNT embryos.

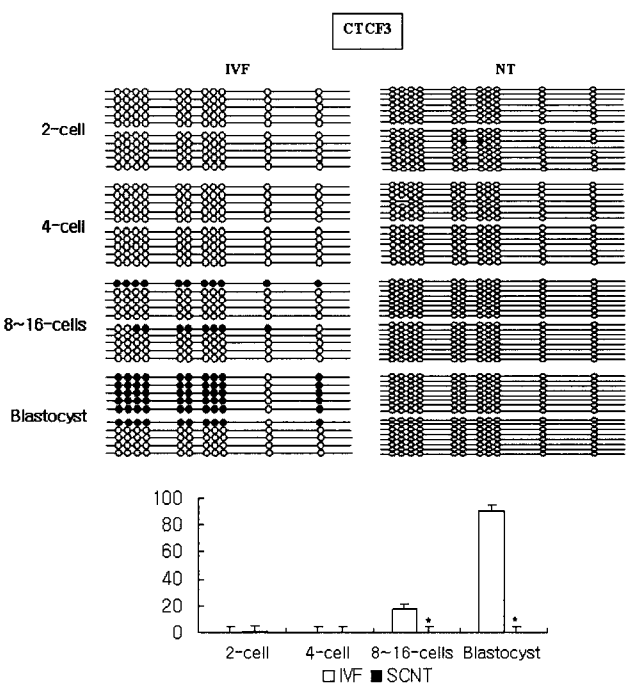

Fig. 5. Differential methylation pattern of putative DMR of CTCF3 in IVF and SCNT embryos. Value with asterisk upper the different color bar differ significantly ($p<0.05$).

DISCUSSION

Imprinting status of farm animal especially porcine

is largely unknown. Therefore, to investigate the imprinting status of porcine, this study compared the methylation pattern of putative DMRs of porcine *H19* gene between IVF and SCNT derived porcine embryos. Earlier studies on analysis of cytosine methylation utilized methylation-sensitive restriction enzymes (Kang *et al.*, 2003). However, such techniques are not sufficient to investigate large range of genomic regions owing to limited restriction sites (Warnecke *et al.*, 1998). Thus, in order to overcome these disadvantages associated with enzyme based methods, we utilized BS-PCR that could detect the methylation status of all cytosines in a sequence of interest. Putative DMRs of porcine *H19* were deduced by alignment with the known mouse DMRs. This result suggests that the genomic imprinting of porcine is highly conserved in porcine, similar to mouse.

The *IGF2* and *H19* genes are closely linked imprinted genes which are expressed only from the paternal and the maternal allele, respectively. Interestingly, the reciprocal imprinting of the two genes is dependent upon the methylation sensitive CTCF binding sites within the DMR located upstream region of *H19*. There are four specific binding sites for CTCF binding protein in mouse (Thorvaldsen *et al.*, 1998). In human, the DMR upstream of *H19* contains seven CTCF binding sites (Hark and Sasaki, 2000) and only the sixth of seven CTCF binding sites has been demonstrated to have allele-specific differential methylation (Takai *et al.*, 2001). To identify these sites in porcine, a 14 bp sequence for the core region (CCGCNNGGNGGCAG), which is known to be conserved in mouse and human (Ishihara *et al.*, 2002), was aligned with porcine *H19* upstream sequences. As a result, this experiment identified three candidate CTCF binding sites within *H19* DMR. These CTCF binding sites showed completely differential methylation in the porcine mature sperm and oocytes (see Fig. 2).

Abnormal methylation and expression of developmentally important genes, especially imprinted genes have been reported by several research groups (Dean *et al.*, 1998; Humpherys *et al.*, 2001; Kang *et al.*, 2002; Han *et al.*, 2003a; Mann *et al.*, 2003). In cloned fetus and preimplantation embryos, the methylation of cytosines is an important criterion to investigate the reprogramming status (Kang *et al.*, 2003). Contrary to IVF embryos, cloned bovine embryos did not show normal methylational changes resulting in the maintenance of donor cell type hypermethylation throughout preimplantation development. Interestingly, the gradual demethylation pattern similar to the endogenous demethylation process was observed in cloned porcine embryo (Kang *et al.*, 2001c). These results suggest that these could be the differences of epigenetic reprogramming among species. Therefore, it is essential to explore the correlation between abnormal expression and aberrant methylation of imprinted genes in cloned embryo and offspring. In order to determine the relationship between the methylation of these DMRs and abnormal expression of imprinted genes, we analyzed the methylation status of IVF and SCNT embryos. In case of *H19* DMR, this experiment analyzed three CTCF binding sites which were confirmed in our preliminary experiment. The CTCF1 showed highly menthylated in IVF embryos than SCNT embryos (see Fig. 3). On the other hand, the CTCF2 was almost unmethylated on both embryos (see Fig. 4). Furthermore, the CTCF3 showed differential methylation pattern as hypomethylated in the 2-, 4- and 8~16-cell stages, however, the blastocyst stage indicated more highly methylation in IVF embryos than SCNT embryos (see Fig. 5). Therefore, these results suggest that the methylation of all CTCF is likely to be related with allele specific methylation pattern of epigenetic process.

In conclusion, our data suggest that genomic imprinting gene at *H19* loci in porcine SCNT embryos showes the aberrant mathylation pattern compared to IVF embryos. Therefore, we suggest that the aberrant methylation pattern of *H19* loci may be a reason for higher rate of abnormal fetus after embryo transfer of porcine SCNT embryos.

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