Hypomethylation of DNA in Nuclear Transfer Embryos from Porcine Embryonic Germ Cells

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ABSTRACT

Epigenetic modification including genome-wide DNA demethylation is essential for normal embryonic development. Insufficient demethylation of somatic cell genome may cause various anomalies and prenatal loss in the development of nuclear transfer embryos. Hence, the source of nuclear donor often affects later development of nuclear transfer (NT) embryos. In this study, appropriateness of porcine embryonic germ (EG) cells as karyoplasts for NT with respect to epigenetic modification was investigated. These cells follow methylation status of primordial germ cells from which they originated, so that they may contain less methylated genome than somatic cells. This may be advantageous to the development of NT embryos commonly known to be highly methylated. The rates of blastocyst development were similar among embryos from EG cell nuclear transfer (EGCNT), somatic cell nuclear transfer (SCNT), and intracytoplasmic sperm injection (ICSI) (16/62, 25.8% vs. 56/274, 20.4% vs. 16/74, 21.6%). Genomic DNA samples from EG cells (n=3), fetal fibroblasts (n=4) and blastocysts from EGCNT (n=8), SCNT (n=14) and ICSI (n=6) were isolated and treated with sodium bisulfite. The satellite region (GenBank Z75640) that involves nine selected CpG sites was amplified by PCR, and the rates of DNA methylation in each site were measured by pyrosequencing technique. The average methylation degrees of CpG sites in EG cells, fetal fibroblasts and blastocysts from EGCNT, SCNT and ICSI were 17.9, 37.7, 4.1, 9.8 and 8.9%, respectively. The genome of porcine EG cells were less methylated than that of somatic cells ($p<0.05$), and DNA demethylation occurred in embryos from both EGCNT ($p<0.05$) and SCNT ($p<0.01$). Interestingly, the degree of DNA methylation in EGCNT embryos was approximately one half of SCNT ($p<0.01$) and ICSI ($p<0.05$) embryos, while SCNT and ICSI embryos contained demethylated genome with similar degrees. The present study demonstrates that porcine EG cell nuclear transfer resulted in hypomethylation of DNA in cloned embryos yet leading normal preimplantation development. Further studies are needed to investigate whether such modification affects long-term survival of cloned embryos.

(Key words : porcine EG cell, nuclear transfer, DNA methylation, pyrosequencing)

INTRODUCTION

Cloned animals have been produced by somatic cell nuclear transfer (NT) in several mammalian species, including sheep (Wilmut *et al*., 1999), cattle (Cibelli *et al*., 1997), goats (Baguisi *et al*., 1999), and pigs (Polejaeva *et al*., 2000). However, despite intensive efforts, efficiency of cloning by somatic cell NT has been low. Most cloned embryos die *in utero*, and a few that develop to term show a high incidence of abnormalities (Yang *et al*., 2007). In pigs, only 1~3% of cloned embryos survive to term (Kues and Niemann, 2004).

Although the reasons behind this developmental failure are not yet fully understood, one of the critical factors to produce normal cloned animals might be appropriate reprogramming of nuclear donor cells. The nuclear reprogramming refers to the erasure of the donor cell epigenetic pattern after NT and the re-establishment of embryonic epigenetic characteristics and gene expression in the cloned embryo (Yang *et al*., 2007). This includes remodeling of the chromatin structure, changes in DNA-methylation, transcriptional regulation of imprinted genes, regeneration of telomere length, and inactivation of the X-chromosome (Han *et al*., 2003; Westphal, 2005). In this regards, choice of nuclear donor cells may affect subsequent development of reconstructed embryos. The differentiation status of

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the donor cells may contribute to the success of cloning since correct epigenetic reprogramming and the resulting changes in transcriptional control are the key processes in the development of somatic cell NT embryos (Jaenisch *et al*., 2004). It has been shown that the success of nuclear reprogramming decreases as donor cells become more differentiated (Gurdon *et al*., 1962; Yang *et al*., 2007). Hence, a less differentiated cell type may support greater development of NT embryos compared with terminally differentiated cell types (Faast *et al*., 2006). The use of murine ES cells increased the efficiency from 1～3% to 15% in the production of cloned offspring per transferred embryos (Wakayama *et al*., 1999). In pigs, mesenchymal stem cells isolated from porcine bone marrow resulted in the rates of preimplantation development comparable to (Colleoni *et al*., 2005; Bosch *et al*., 2006; Faast *et al*., 2006) or superior to (Jin *et al*., 2007) somatic cell counterparts. In addition, embryos cloned from porcine fetal skin-originated sphere stem cells exhibited enhanced preimplantation development compared with fibroblast-cloned embryos, as evidenced by an increased rate of blastocyst development and a higher total cell number in blastocysts (Zhu *et al*., 2004).

Primordial germ cells (PGC) are embryonic cells that migrate from the root of the allantois to the genital ridge, where they ultimately give rise to gametes. Murine embryonic germ (EG) cells share morphological, biochemical, immunological and developmental properties with embryonic stem (ES) cells, including pluripotency and the capacity to contribute to the germ line of chimeras (Matsui *et al*., 1992; Resnick *et al*., 1992; Onishi *et al*., 1994). Undifferentiated porcine EG cell lines that can be differentiated into various cell lineages, both *in vitro* and *in vivo*, have also been reported (Shim *et al*., 1997). It has been previously demonstrated that the porcine EG cell NT increased the efficiency of cloned embryo production compared with conventional somatic cell NT using fetal fibroblast cells (Ahn *et al*., 2007). Hence, porcine EG cells may be more amenable to reprogramming after reconstruction than differentiated somatic cells. Developmental hindrance due to hypermethylation of DNA in somatic cell NT embryos was commonly observed (Han *et al*., 2003). However, the global DNA methylation of EG cells derived from migratory PGC may be lower than differentiated somatic cells, since genome-wide demethylation of DNA occurs during PGC migration similar to the phenomenon during preimplantation development of embryos. Hence, NT embryos using EG cells rather than somatic cells may be more easily reprogrammable with respect to the erasure of their epigenetic marks, resulting in embryos close to the embryos from normal fertilization in terms of DNA methylation status (Ahn *et al*., 2007).

In the present study, genome-wide methylation in somatic and EG cells was investigated. Subsequently the *in vitro* development and DNA methylation of NT embryos derived from different types of donor cells were assessed. Results from the present study may elucidate the effect of DNA methylation of nuclear donor cells on developmental competence of NT embryos.

MATERIALS AND METHODS

1. Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, USA), unless otherwise stated.

2. Culture of Porcine EG Cells

Porcine EG cells isolated from PGC of day 23 embryos were maintained, as described previously (Shim *et al*., 1997). To prepare feeder-free EG cells, a mixture of trypsinized EG and feeder cells were cultured for 15 min on 0.1 % gelatin-coated plates until the fibroblasts were attached on the dish, while the majority of EG cells were floating. Then, the cells in the supernatant were collected and washed by centrifugation at 800 × g for 5 min, and transferred onto 0.1 % gelatin-coated plates. The cells were continuously grown in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, USA) supplemented with 15% (v/v) ES-qualified fetal bovine serum (FBS; HyClone, Logan, USA), 1 mM L-glutamine (Invitrogen), 0.1 M MEM non-essential amino acids (Invitrogen), 10 μM 2-mercaptoethanol (Invitrogen), 100 units/ml of penicillin, 0.5 mg/ml of streptomycin, and 1,000 units/ml of leukemia inhibitory factor (LIF; Millipore, Billerica, USA) in a humidified atmosphere of 5% CO_2 in 95% air.

3. Culture of Porcine Fetal Fibroblast Cells

Fibroblast cells were isolated from pig fetuses on day 23 of gestation. Briefly, fetuses were washed three times with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS; Invitrogen). The heads and internal organs were removed using iris scissors and forceps. The remnants were washed twice in Dulbecco's PBS (DPBS), minced with a surgical blade on a 100-mm petridish. Cells were dissociated from the tissues in 0.25% (v/v) trypsin-EDTA (Invitrogen) for 5 min at 39℃. After centrifuging cell suspension three times at 800 × g for 10 min, pellets were sub-

sequently seeded onto 100-mm plastic culture dishes (Falcon, Franklin Lakes, USA) and cultured for 6 to 8 days in DMEM supplemented with 10% (v/v) FBS, 1 mM L-glutamine, 100 units/ml penicillin and 0.5 mg/ml streptomycin in a humidified atmosphere of 5% CO_2 in 95% air. After removal of unattached clumps of cells, attached cells were further cultured until confluent, subcultured at intervals of 5 to 7 days by trypsinization for 5 min using 0.25% trypsin-EDTA and stored after two passages in freezing medium in liquid nitrogen at −196℃. The freezing medium consisted of 80% (v/v) DMEM, 10% (v/v) dimethyl sulfoxide and 10% (v/v) FBS. After thawing, cells were cultured in DMEM supplemented with 10% FBS until approximately 80% confluency and used for NT experiment.

4. *In Vitro* Maturation of Oocytes

Porcine ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in a warm box (25～30℃) within 2 h. Follicular fluid and cumulus-oocyte complexes (COC) from follicles (5～6 mm in diameter) were aspirated using an 18-gauge needle attached to a 5-ml disposable syringe. Compact COC were selected and washed 5 times in HEPES-buffered tissue culture medium (TCM)-199 (Invitrogen). The *in vitro* maturation (IVM) medium was TCM-199 supplemented with 10 ng/ml of epidermal growth factor, 10 IU/ml equine chorionic gonadotropin (eCG; Intervet, Boxmeer, The Netherlands), 10 IU/ml human chorionic gonadotropin (hCG; Intervet), and 10% (v/v) porcine follicular fluid. A group of 50 COC was cultured in 500 μl of IVM medium at 39℃ in a humidified atmosphere of 5% CO_2 and 95% air. After culturing for 22 h, the COC were transferred to eCG- and hCG-free IVM medium and cultured for another 20 h. At the end of the maturation, oocytes were freed from cumulus cells by repeated pipeting in the IVM medium containing 0.5 mg/ml hyaluronidase for 1 min.

5. Nuclear Transfer

Forty-two h after the onset of IVM, oocytes were enucleated with a 20-μm (internal diameter) glass pipette by aspirating the first polar body and the second metaphase plate with a small volume of surrounding cytoplasm in HEPES-buffered TCM-199 supplemented with 0.4% bovine serum albumin (BSA) and 7.5 μg/ml cytochalasin B. After the enucleation, oocytes were stained with 5 μg/ml bisbenzimide (Hoechst 33342) for 5 min and observed under a Nikon TE-300 inverted microscope equipped with epifluorescence (Nikon Instrument, Tokyo, Japan). Oocytes containing DNA materials were excluded from the subsequent experiments. As nuclear donor cells, EG or NP cells were trypsinized into single cells, and transferred into the perivitelline space of enucleated oocytes. The resulting couplets were equilibrated for 1 min in 0.3 M mannitol solution containing 0.5 mM HEPES, 0.05 mM $CaCl_2$, and 0.1 mM $MgCl_2$ in a chamber containing two electrodes. Then, couplets were fused with a double DC pulse (1.5 kV/cm for 40 μsec) using a BTX Electro-Cell Manipulator 2001 (Gentronics, San Diego, USA). Following the electrical stimulation, reconstructed oocytes were cultured in NCSU23 supplemented with 4 mg/ml of fatty acid-free BSA and 7.5 μg/ml cytochalasin B for 3 h to suppress extrusion of the second polar body. Then, oocytes were cultured for 4 days in NCSU23 containing 4 mg/ml of fatty acid-free BSA and transferred to NCSU23 containing 10% FBS and cultured for another 3 days. All NT embryos were cultured at 39℃ in a humidified atmosphere containing 5% CO_2 in 95% air.

6. Intracytoplasmic Sperm Injection

Thirty to 60 min before sperm cell injection, oocyte activation was induced. Cumulus-free oocytes were stimulated by a single DC pulse of 1.3 kV/cm for 30 μs at room temperature using BTX Electro Cell Manipulator.

Sperm pellet was recovered from porcine semen and washed twice by centrifugation at 800 × g for 5 min in 0.9% NaCl (w/v) supplemented with 10 mg/ml BSA. Resulting spermatozoa were suspended in 1.5 ml of TL-HEPES. To obtain sperm heads, spermatozoa were resuspended in 5 ml of cold Tris-buffered saline (TBS), and the sonication was conducted in water bath for 1 min using 100% output from Branson 8510 ultrasonic sonicator (Branson Sonic Power Co., Danbury, USA). Then, the sonicated sperm suspension was centrifugated at 1,000 × g for 10 min in two layer percoll gradient solution to isolate sperm heads.

The sperm heads were washed twice in 1 ml of TBS by centrifugation at 800 × g for 5 min. The isolated sperm heads were centrifuged at 400 × g for 5 min and resuspended in 1:1 mixture of TBS and 7% polyvinylpyrrolidone (PVP) solution. A few microdrops of this suspension were placed on a lid of Falcon 1007 petri dish (Becton Dickinson, Franklin Lakes, NJ). The dish was placed on Nikon inverted microscope equipped with Narishige micromanipulator. After freed from cumulus cells, the oocytes with visible first polar body were selected and centrifuged for 1 to 2 min at 12,000 × g in 100 μl of TBS. The injection of isolated sperm heads into the oocytes cytoplasm

was performed by the method of Lee *et al*. (1998).

7. Pyrosequencing

Nuclear donor cells and blastocysts derived from NT and ICSI were subjected to analysis for DNA methylation by pyrosequencing as previously described (Kremenskoy *et al*., 2006). Briefly, genomic DNA from fibroblast and EG cells was prepared using a DNeasy Tissue kit (Qiagen, Valencia, USA), according to the manufacturer's protocol. In NT and ICSI embryos, blastocysts were washed twice in PBS, and individual blastocysts were separately transferred into 0.2-ml PCR tubes, each containing 5 μl of embryo lysis solution (pH 8.6) composed of 20 mM Tris-Cl, 0.9% Tween 20, 0.9% IGEPAL CA-630, and 400 μg/ml protease K.

Genomic DNA was denatured by adding 0.3 M NaOH and incubated for 15 min at 37℃. After incubation, sodium metabisulfite (pH 5.0) and hydroquinone were added to final concentrations of 2.0 M and 0.5 mM, respectively, and the mixture was further incubated at 55℃ for 14 h in darkness. The modified DNA was purified using the Wizard DNA Clean-Up System (Promega, Madison, USA), and the bisulfite reaction was terminated by 15 min incubation at 37℃ in 0.3 M NaOH. The solution was then neutralized by adding ammonium acetate (pH 7.0) to a final concentration of 3 M. The ethanol-precipitated DNA was resuspended in water, and satellite region (GenBank accession no. Z75640) that includes nine selected CpG sites was amplified by PCR using primer sets of 5'-TTTGTAGAA TGTAGTTTTTAGAAG-3' and 5'-AAAATCTAAACTACCTCT AACTC-3' as described in Kang *et al*. (2001). The amplification cycle was 45 cycles at 94℃ for 60 s, 55℃ for 60 s, 72℃ for 20 s and then finishing with one cycle of 72℃ for 10 min.

Amplified PCR products were cloned into pGEM-T easy vector (Promega), and individual clones were subjected to pyrosequencing. Pyrosequencing was performed on a PSQ 96MA system with a SNP reagent kit (Pyrosequencing, Uppsala, Sweden) according to the manufacturer's instructions. The rates of DNA methylation in each CpG site of the satellite region was analyzed using the allele quantitation algorithm of the provided software.

8. Statistical Analysis

At least three replicates were conducted for each experiment in comparison of preimplantation development of embryos as well as the rate of DNA methylation in CpG sites. All data were subjected to one-way ANOVA using SPSS (version 11.0 for Windows). Then, least significant difference (LSD) was applied for post hoc multiple comparison among groups. A value of $p<0.05$ was considered to be significant.

RESULTS

1. *In Vitro* Development of Nuclear Transfer Embryos

In vitro development of embryos after NT was shown in Table 1. Between two different donor cells used for NT in this study, no differences were observed in the rate of fusion. The rates of blastocyst development were similar among embryos from EG cell NT (EGCNT), somatic cell NT (SCNT) and intracytoplasmic sperm injection (ICSI) (16/62, 25.8% vs. 56/274, 20.4% vs. 16/74, 21.6%).

2. DNA Methylation in Nuclear Transfer Embryos

From genomic DNA samples from EG cells (n=3), fetal fibroblast cells (n=4) and blastocysts from EGCNT (n=8), SCNT (n=14) and ICSI (n=6), the average methylation degrees of CpG sites in EG cells, fetal fibroblast cells and blastocysts from EGCNT, SCNT and ICSI were 17.9, 37.7, 4.1, 9.8 and 8.9%, respectively (Fig. 1). The genome of porcine EG cells were less methylated than that of somatic cells ($p<0.05$), and active DNA demethylation was observed in embryos from both EGCNT ($p<0.05$) and SCNT ($p<0.01$). However, the degree of DNA methylation in EGCNT embryos was less than half of SCNT ($p<0.01$) and ICSI ($p<0.05$) embryos, while SCNT and ICSI embryos showed similar degrees of DNA methylation.

DISCUSSION

Although NT using various types and levels of differentiated cells has generated cloned offspring in mammals (Galli *et al*., 1999; Kato *et al*., 2000; Ogura *et al*., 2000; Hoched-

Table 1. *In vitro* development of EGCNT, SCNT and ICSI embryos

Group	No. of reconstructed oocytes	No. (%)* of fused oocytes	No. (%)** of embryos developed to blastocysts
EGCNT	117	62 (53.0)	16 (25.8)
SCNT	473	274 (57.9)	56 (20.4)
ICSI	74	-	16 (21.6)

* Calculated from the number of reconstructed oocytes.
** Calculated from the number of fused oocytes.

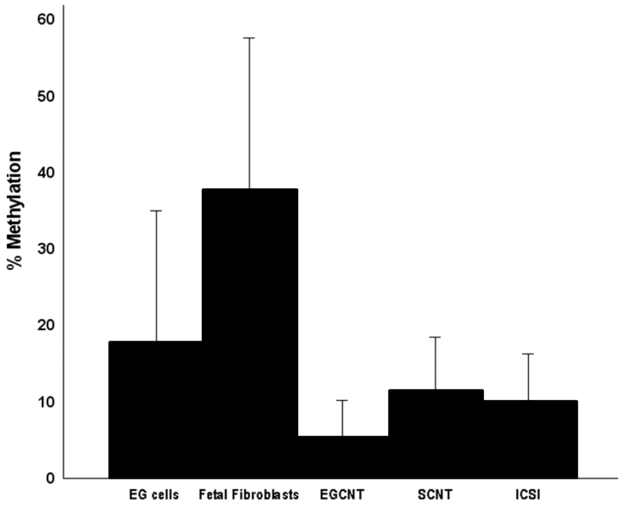

Fig. 1. Average DNA methylation degrees in nuclear donor cells and embryos.

linger and Jaenisch, 2002; Miyashita *et al*., 2002), the transfer of stem cells with low levels of epigenetic marks may be advantageous because such cells could more easily be reprogrammable and support greater development of NT embryos compared with terminally differentiated cell types (Faast *et al*., 2006). For instance, oocytes reconstructed from murine ES cells gave rise to an increase in the number of viable offspring compared with those from somatic cells (Wakayama *et al*., 1999; Rideout *et al*., 2000). It has been previously demonstrated that the porcine EG cell NT increased the efficiency of cloned embryo production compared with conventional somatic cell NT using fetal fibroblast cells (Ahn *et al*., 2007). Hence, porcine EG cells may be more amenable to reprogramming after reconstruction than differentiated somatic cells. Hampered due to hypermethylation of DNA in somatic cell NT embryos were commonly observed (Han *et al*., 2003). However, the global DNA methylation of EG cells derived from migratory PGC may be lower than differentiated somatic cells, since genome-wide demethylation of DNA occurs during PGC migration similar to the phenomenon during preimplantation development of embryos. Hence, NT embryos using EG cells rather than somatic cells may be more easily reprogrammable with respect to the erasure of their epigenetic marks, resulting in embryos close to the embryos from normal fertilization in terms of DNA methylation status (Ahn *et al*., 2007).

In the present study, developmental competence of NT embryos derived from fibroblast and EG cells was compared. Embryos from ICSI were used as normal fertilization control. The *in vitro* development of NT embryos derived from EGCNT, SCNT, and ICSI is summarized in Table 1. Between two different donor cells used for NT in this study, no differences were observed in the rate of fusion. The rates of blastocyst development were similar among embryos from all three groups. This is contrast to the previous report form Ahn *et al*., (2007) demonstrating no improvement in fusion rate but significant enhancement in the rate of blastocyst formation. It is speculated that the limited number of NT embryos used in the present study might have caused lack of statistical difference between EGCNT and SCNT. Indeed, highest proportion of embryos from EGCNT tended to develop to the blastocyst stage in the current study. Nevertheless, embryos from all three groups showed developmental competence with reasonably high rate of blastocyst development.

Further investigation on global DNA methylation in somatic and EG cells was performed in the present study. Subsequently, DNA methylation of NT embryos derived from such different types of donor cells were assessed. As demonstrated in Fig. 1, average DNA methylation degrees appeared to be reduced by NT procedure. In both EGCNT and SCNT embryos, the degrees of methylation reduced to approximately less than one half of their nuclear donor cell counterparts. Surprisingly, extensive demethylation in EGCNT embryos resulted in hypomethylation of DNA compared with ICSI control. Although this hypomethylation did not affect in vitro development of EGCNT embryos to the blastocyst stage (Table 1), long-term effect of hypomethyation during postimplantation development is needed to study further.

In conclusion, the present study demonstrates that porcine EGCNT resulted in hypomethylation of DNA in cloned embryos yet leading normal preimplantation development. However, further studies are needed to elucidate effect of DNA demethylation on epigenetic reprogramming of porcine NT embryos and to evaluate long-term effects of various degrees of DNA methylation on NT embryos to term. These future studies would lead improvement of cloning efficiency in pigs.

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