Methylation Status of *H19* Gene in Embryos Produced by Nuclear Transfer of Spermatogonial Stem Cells in Pig

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ABSTRACT

The faulty regulation of imprinting gene lead to the abnormal development of reconstructed embryo after nuclear transfer. However, the correlation between the imprinting status of donor cell and preimplantation stage of embryo development is not yet clear. In this study, to determine this correlation, we used the porcine spermatogonial stem cell (pSSC) and fetal fibroblast (pFF) as donor cells. As the results, the isolated cells with laminin matrix selection strongly expressed the *GFR α-1* and *PLZF* genes of SSCs specific markers. The pSSCs were maintained to 12 passages and positive for the pluripotent marker including OCT4, SSEA1 and NANOG. The methylation analysis of *H19* DMR of pSSCs revealed that the zinc finger protein binding sites CTCF3 of *H19* DMRs displayed an androgenic imprinting pattern (92.7%). Also, to investigate the reprogramming potential of pSSCs as donor cell, we compared the development rate and methylation status of *H19* gene between the reconstructed embryos from pFF and pSSC. This result showed no significant differences of the development rate between the pFFs (11.2±0.8%) and SSCs (13.3±1.1%). However, interestingly, while the CTCF3 methylation status of pFF-NT blastocyst was decreased (36.3%), and the CTCF3 methylation status of pSSC-NT blastocyst was maintained. Therefore, this result suggested that the genomic imprinting status of pSSCs is more effective than that of normal somatic cells for the normal development because the maintenance of imprinting pattern is very important in early embryo stage.

(Key words : Porcine SSCs, bFGF, Laminin, Reprogramming, SCNT, Imprinting gene)

INTRODUCTION

Since the first cloned animal 'Dolly' was generated in 1997 (Wilmut *et al.*, 1997), a number of mammalian species have been successfully generated as cloned animal through the somatic cell nuclear transfer (SCNT). However, the efficiency of this procedure for the livestock species is extremely low (Jaenisch, 2002) and cloned animals often showed abnormal phenotypes, such as overgrowth of the fetus and placenta. The main reason for these problems was thought to be due to faulty epigenetic reprogramming of the donor cell (Rideout *et al.*, 2001). The epigenetic reprogramming was involved in genomic methylation, the modification of histone, and the regulation of imprinting gene (Latham, 1999). Due to this mechanism of reprogramming, many different donor cell types have been examined how to improve the reprogramming of reconstructed embryo after nuclear transfer. Previously, many reports suggest that the differentiated level of donor cells was considerably important for the reprogramming after nuclear transfer. Generally, the less differentiated donor cells have higher efficiency of the reprogramming potential than the terminally differentiated cells after SCNT. Furthermore, despite the fact that several reports have addressed the correlation between epigenetic reprogramming and genomic imprinting in the mouse and human genomes, the correlation has remained largely elusive in pig.

Imprinting genes are epigenetically regulated so that only one allele is expressed from the maternally or the paternally inherited chromosome (Carr *et al.*, 2007). Although they represent only a small subset of the mammalian total genome, imprinting genes were indispensable for normal development. The methylation status of differentially methylated region (DMR) in imprinting genes regulated paternal- or maternal-allele specific gene expression (Lucifero *et al.*, 2002) and regulation of imprinting gene expression is important for the normal development of early embryo, placental function, and post-natal phenotype. Both gain and loss of imprinting are associated with various diseases (Reik *et al.*, 2001). In the previous study, the putative DMRs of the *H19* genes were identified in order to investigate their im-

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printing status in produced piglets by SCNT (Han *et al.*, 2008). There are three of the identified CTCF binding sites conserved in the mouse and human genes. The CTCF1 and 2 sites of control piglets, natural birth, did not exhibit a differential methylation pattern compared with cloned piglets. These results suggest that the epigenetic modification of CTCF1 and 2 of *H19* were normal, at least in the cloned piglets. However, the CTCF3 site of cloned piglets exhibited a differential methylation pattern compared with that of control piglets. Thus, the methylation status of the CTCF3 site of *H19* gene was very important for normal development of cloned animals in pig.

In the mammalian testis, gonocytes migrate to the basement membrane of seminiferous tubule and converted into the spermatogonial stem cells in order to spermatogenesis in the mature testis throughout life of the male. Although SSCs represent an extremely small subset (0.02~0.03%) of the cells of the testis (Tegelenbosch and de Rooij, 1993). The isolated SSCs from the adult mouse can also acquire a pluripotent state when cultured under standard embryonic stem (ES) cell culture conditions (Zovoilis *et al.*, 2008; Guan *et al.*, 2006). The cellular and molecular characterizations of these cells revealed many similarities among the mouse, rat, bovine and human. Moreover, the SSCs exhibited similarly the androgenic methylation pattern of DMR site of imprinting genes. However, very little is known about the culture condition and methylation pattern of SSCs in pig.

Therefore, this study was designed to optimize the culture condition of porcine spermatogonial stem cell (pSSC). Furthermore, to investigate a correlation of between the imprinting status of donor cells and development of early embryos, the methylation status of *H19* gene was analyzed in cloned embryos after nuclear transfer of the PFF and pSSC.

MATERIALS AND METHODS

All chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise specifically indicated. Each experiment consisted of at least three replicates and in each of the replication, oocytes were from the same collection of abattoir-derived ovaries collected on the same day.

Isolation and Culture of Porcine SSCs

Testes were collected from 3 week old crossbred piglets (Landrace×Large White Yorkshire×Duroc). The obtained porcine testes tissues were mechanically disrupted and 2 step enzymatically dissociated with the 1st step 0.5 g/ml collagenase type VI: 0.5 g/ml hyaluronidase, 1:1 mixture in DPBS buffer with Ca^{2+} and Mg^{2+} (Gibco) for 20 min at 37℃ and 2nd step 0.25% Trypsin-EDTA solution. Then the digest was pelleted at 800 g for 5 min, washed twice with DMEM culture medium (Gibco) with 15% ES cell qualified FBS and 1% L-glutamine and filtered through a 40 μm-pore size mesh to obtain a single cell suspension. To remove more than 95% of somatic testis cells from the germ cell population, negative selection on plastic dish, cells were plated into 10 cm^2 culture dishes at 2×10^6 cells per cm^2 containing medium with 10 ng/ml GDNF (Invitrogen) and incubated at 39℃, 5% CO_2 for 4 h. After 1st negative selection (Plastic non-bounded cells; $Pl\text{-}_{NB}$), non-bounded cells were harvested from adherent somatic cells by repeated pipetting with 4 ml of DMEM. The pooled suspension was pelleted at 800 g and suspended in 10 ml DMEM. For further purification the $Pl\text{-}_{NB}$ cells were suspended in basic medium and plated at $0.5\sim1\times10^6$ cells per ml per well in 6-well plates pre-coated with laminin (4.4 μl/cm). The plated $Pl\text{-}_{NB}$ cells were incubated for 45 min at 39℃ and laminin non-bounded cells ($Pl\text{-}_{NB}$/$Lam\text{-}_{NB}$ cells) were removed from laminin bounded cells ($Lam\text{-}_{B}$ cells) by pipetting and discarded. After washing one time with 1 ml DPBS, the $Lam\text{-}_{B}$ cells were continuously cultivated in basic pSSCs medium, DMEM, 5% ES cell qualified FBS with 10 ng/ml GDNF and 10 ng/ml b-FGF (invitrogen) on laminin coated dish for 1~2 weeks. Under these conditions, the spermatogonial cells proliferated first and aggregated, and then pSSCs clusters were formed.

Generally the colonies were subcultured mechanically every 10~14 days and manually isolated and cut into pieces and plated on laminin coated dish. Medium was replaced by fresh medium supplemented with the same combination of growth factors at every 2~3 days.

Immunohistochemistry

Testicular samples were fixed in Bouin fixative, dehydrated, embedded in paraffin, and sectioned (8 μm thickness). Dilutions of lectin DBA (*dolichos biflorus agglutinin*) and the primary and secondary antibodies were performed in PBS with 1% (v/w) BSA. Sections were dewaxed, rehydrated, and stained with DBA, as described previously (Izadyar *et al.*, 2002). In briefly, after removal of paraffin wax (for tissue sections only) and rehydration, sections were treated with 3% (v/v) H_2O_2 (Merck, Darmstadt) for 10 min to inhibit endogenous peroxidase and were subsequently rinsed in PBS. Incubation in PBS containing 50 mg BSA/ml for 15 min before lectin incubation was advantageous to block non-specific adhesion sites. The sections were then incubated in DBA conjugated with horseradish peroxidase (DBA-HRP; E. Y. Laboratories, San Mateo, CA) at 1:100 in PBS and 1% (w/v) BSA for 1 h at 37℃ in a moist chamber. After incubation with lectin, the sections were

rinsed three times in PBS. Staining of the DBA-HRP was performed by treating the sections for 5~15 min with PBS containing 25 mg 3,3'-diaminobenzidine tetrahydrochloride (DAB), 1 ml nickel ammonium sulphate solution (10 mg/ml), 1.25 ml cobalt chloride solution (10 mg/ml) and 17 ml of 35% (v/v) H_2O_2 per 50 ml. The slides were rinsed thoroughly in distilled water and, if necessary, counterstained with haematoxylin. The sections were dehydrated in graded alcohol, cleared in xylol and mounted with Pertex (Cell Path; Compulink, Bedford) and observed under an Olympus IX 71 microscope. Negative control sections were incubated in 1 % BSA in PBS without lectin.

Immunocytochemistry

The pSSCs were characterized by their morphology, alkaline phosphatase (AP) activity and immunocytochemistry according to the protocol previously described (Ju *et al.*, 2008). AP activity was assessed using an AP staining kit following the manufacturer's protocol. For analyzing the expression of OCT-4, SSEA-1, NANOG and PGP 9.5, pSSCs colonies were fixed in 4% paraformaldehyde/absolute methanol and were incubated with appropriate dilutions of mouse monoclonal antibodies against OCT-4, SSEA-1 (1:50, ES-cell marker sample kit chemicon international), NANOG (1:300, Abcam) and PGP9.5 (1:100, BIOMOL International). The colonies were then incubated in 1:500 normal goat serum (Vector Laboratories, Burlingame, CA) for 1 h to block nonspecific binding and bound primary antibodies were localized with FITC-conjugated IgG second antibody. The colonies were then washed, mounted on clean glass slides and stained Hoechst 33342 with 12.5 μl/ml. The colonies were observed under an Olympus IX 71 microscope (Olympus, Tokyo, Japan).

Oocyte Retrieval and *In Vitro* Maturation

Retrieval and IVM of pig oocytes, from abattoir-derived ovaries, were performed essentially as described earlier (Gupta *et al.*, 2008). Briefly, cumulus-oophorus-complexes were aspirated from medium-sized follicles (3~6 mm diameter) 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) supplemented with 25 mM $NaHCO_3$, 10% (v/v) pig follicular fluid, 0.57 mM cysteine, 0.22 μg/ml sodium pyruvate, 25 μg/ml gentamicin sulfate, 0.5 μg/ml FSH (Folltropin V; Vetrepharm, Canada), 1 μg/ml estradiol-17β, and 10 ng/ml epidermal growth factor under mineral oil at 39℃ in a humidified atmosphere of 5% CO_2 in air for 40~42 h.

Somatic Cell Nuclear Transfer

Nuclear transfer was performed essentially as described earlier (Uhm *et al.*, 2009). Briefly, *in vitro* matured oocytes were denuded of cumulus cells in TL-HEPES medium supplemented with 0.1% hyaluronidase and enucleated by aspirating the first polar body and adjacent cytoplasm (approximately 30% of ooplasm) using a beveled borosilicate pipette (25 μm internal diameter). Successful enucleation was confirmed by UV assisted visualization of fluorescent metaphase plate in the aspirated cytoplasm contained within the enucleation pipette. Enucleated oocytes were subsequently reconstructed by inserting a small sized (15 μm in diameter), pFF or pSSC into the perivitelline space of each enucleated oocyte using the same pipette used for enucleation. pFF and pSSCs for SCNT were prepared essentially the same as we described earlier (Gupta *et al.*, 2007). Membrane fusion of donor cell with cytoplast was induced with single DC pulse of 2.1 kV/cm for 30 μs delivered by a BTX Electro Cell Manipulator 2001 (BTX, San Diego, CA). The same pulse was also utilized to simultaneously induce activation of reconstructed embryos.

Bisulfate Treatment

Genomic DNA was treated with sodium bisulfite to convert all unmethylated cytosine to uracil residues using One Day MSP kit (In2Gen) according to the manufacturer's protocol. Briefly, purified genomic DNA (0.5~1.0 μl) was denatured with 3N sodium hydroxide at 37℃ for 10 min. After alkaline denaturation, sodium bisulfate (5 M) was added and the sample was incubated in the dark at 5℃ for 16 h. Modified DNA was then desulfonated, neutralized, and desalted, and was finally diluted with 20 μl of distilled water. Subsequently, bisulfite PCR (BS-PCR) amplification was carried out using aliquots of 1~2 μl of modified DNA per PCR run.

PCR Amplification and Sequencing of Bisulfite-treated Genomic DNA

Based on previous study of *H19* DMR site (Han *et al.*, 2008), one genomic locus were selected for amplification, CTCF3 region in the *H19* gene. The region was amplified in each sample using a nested primer approach. Primer pairs used were specific for bisulfite-treated DNA, and are shown in Table 1. PCR amplification was performed using AccuPower PCR premixR (Bioneer) in 20 μl reaction volume with a thermoprofile of 94℃ for 5 min, 94℃ for 30 sec, 72℃ for 30 sec, for 40 cycles, followed by 72℃ for 10 min. The annealing temperature used for primer pair is shown in Table 1. Three microliters of products from the first amplification reaction were used in the second PCR reaction. The amplified products were verified by electrophoresis on a 1.5% agarose gel, and were subsequently cloned into the PCR 2.1-TOPO vector (Invitrogen, California) according to the manufacturer's pro-

Table 1. Primer sequences and cycling conditions used in RT-PCR and BS-PCR analysis

	Gene	Sequence (5'-3')	GenBank accession number	Annealing temp. (℃)	Product size (bp)
RT-PCR	*GFR α-1*	AGCTGTCTAAAGGAAAACTACG CAGAAATTTCAAGCACTCCT	XM_001925122	55	162
	PLZF	TGAAGACGTACGGGTGTGAG TATGGGTCTGCCTGTGTGTC	LOC100157336	57	165
	PGP9.5	GAGATGCTGAACAAAGTGCTG CATGGTTCACCGGAAAAGG	AY459531	58	526
	SOX9	CCTAATCTCGATATGTTTGATGAC GTCTTCGATTTGTTAAGGTTCTTG	NM_213843	56	383
	GATA4	TCTCGATATGTTTGATGACTTCTC GTCTTCGATTTGTTAAGGTTCTTG	AY11549531	56	378
	H2A	TGTCGTTGTCATGTCTGGTC CAACAGCTTGTTCAGCTCCT	BF703857.1	56	304
BS-PCR	*H19-CTCF3* Outer	GTAGGGTTTTTGGTGGTATAGGG CCAAACCTAACCACACCTTAAAC	AY044828	50	205
	H19-CTCF3 Inner	GGTTGTGGGTGTGGAGGTAGAAG CACTAAACACCCAACCTTTAACAC	AY044828	55	

tocol. Plasmid DNA was purified using the QIAprep Spin Miniprep kit (Qiagen, Valencia) and individual clones were sequenced (Macrogen), and only clones with ≥95% cytosine conversion were selected. At least 3 replicates were performed for the selected DMR locus in PFFs, pSSCs, IVF-blastocysts, PFF-NT blastocysts and SSC-NT blastocystsat.

Reverse Transcriptase-polymerase Chain Reaction

RT-PCR was performed essentially as described earlier (Oh *et al.*, 2009). Briefly, total RNA was extracted using RNeasy extraction kit (Qiagen, Germany) with on-column DNase digestion according to the manufacturer's recommendations. RNA (500 ng) was then reversetranscribed to cDNA using AccuPowerR RT-Premix (Bioneer) in 20 μl of reaction mixture for 60 min at 42℃. Amplification of cDNA was carried out in 20 μl of reaction mixture using AccuPowerR PCR-Premix (Bioneer) according to the manufacture's recommendations. The primer sets used for cDNA amplification are listed in Table 1. Housekeeping gene, *H2A*, was used as an internal standard in all experiments.

Statistical Analyses

Statistical analyses were performed using SAS software (Statistical Analysis System Inc., Cary, NC, USA) for Chi-square test as appropriate. The percentage data were subjected to arc sine transformation before statistical analyses. Data are presented as Mean±SEM and differences at $p \leq 0.05$ were considered significant.

RESULTS

The Histological Analysis of Porcine Testis

In the mammalian testis, gonocytes migrate to the basement membrane and undergo a conversion to SSCs, which establish and maintain spermatogenesis in the mature testis. In case of mice, migration of gonocytes occurred from Day 0 to 5 postnatally. Therefore, to investigate the migration and conversion time of the SSCs in pig, the localization of gonocytes was examined by immunohistochemistry. For the analysis the gonocytes, this study used the DBA that is specific affinity for α-D-N-acetyl-galactosamine at testis. Section of testis showed that there were significantly more DBA-positive cells in seminiferous tubules from 5-day-old testis than those from 3-day-old testis (Fig. 1). In addition, while the DBA positive cells from day 5 testis localized within center of the seminiferous tubule (Fig. 1A and C), the positive cells from 3 week testis are rarely found on the basement membrane of seminiferous tubule (Fig. 1B and D). These findings suggest that gonocytes migrated at the basement membrane and give rise to SSCs from Day 0 to Day 21 postnatally in the porcine testis. In other words, the pSSC may be contained much more pSSCs in 3 week old testes than in 5 day testes. Thus, the 3 week old testes were used to obtain a high yield of SSCs in the rest of this study.

The Isolation and Characterization of pSSCs

After the pSSCs were isolated through the laminin

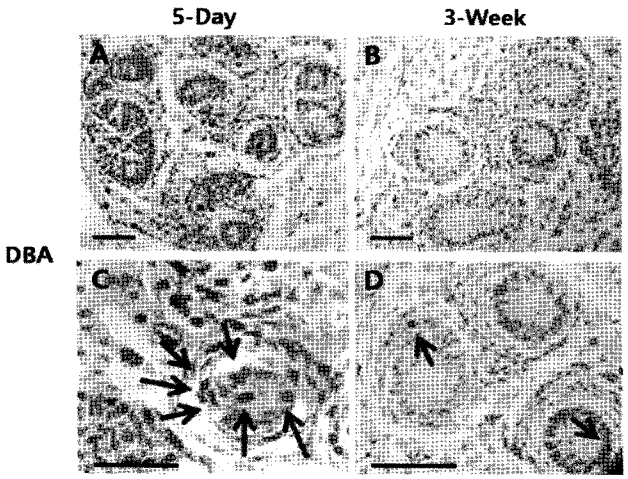

Fig. 1. Histological analysis of porcine testes from males at 5 days and 3 weeks old. Testis sections from 5-day-old pigs (A, C) and 3-week-old (B, D) were stained with DBA. Arrows indicate DBA-positive germ cell. Note that DBA-positive germ cells were significantly fewer in the 3-wk old section than in 5-day old section, and that no somatic cells were stained (Bar=50 μm).

matrix selection (Fig. 2A), the lam-$_B$ cells were continuously cultivated onto laminin coated dish in low-serum (5%) culture medium with GDNF. After 7~14 days, colonies appeared and showed the typical clusters of grape-like morphology (Fig. 2C), and then ES like colonies were found after 3 passage and were strongly positive for alkaline phosphatase (Fig. 2D).

To examine the expression of several genes, these cells were analyzed with RT-PCR. The results showed that the *PGP9.5* of pSSCs maker was strongly expressed in Lam-$_B$ cells compared with Lam-$_{NB}$. Furthermore, the *PLZF* and GFR α-1, undifferentiated germ cell marker, were expressed much higher level in Lam-$_B$ group than in Lam-$_{NB}$ group. However, the expression level of *GATA-4*, somatic cell marker in testis, was much higher in the Lam-$_{NB}$ cells than Lam-$_B$ cells (Fig. 2E). Moreover, we determined the cell contamination of pre-sertoli cells because pre-sertoli cells can form the colonies. Thus, the exclusion of these cells was necessary to prevent the contamination of pre-sertoli cells in primary culture. To identify the pre-sertoli cells, we analyzed the

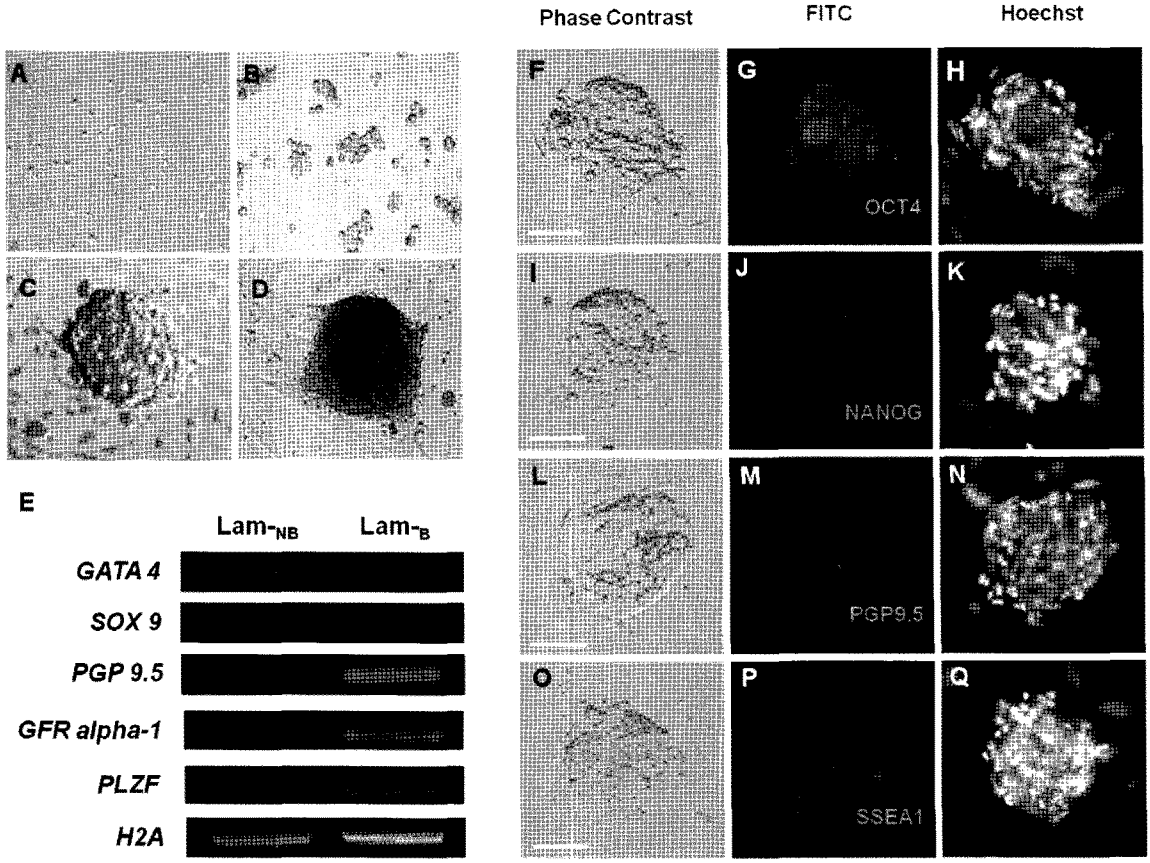

Fig. 2. The isolation and characterization of pSSCs. A: the bounded cells on laminin coated dish (Lam-B cells). B: the proliferated pSSC clusters. C: the pSSCs colony (Passage 3). D: that the pSSC colony was positive for alkaline phosphatase activity. E: RT-PCR analysis for *GFR alpha-1, PLZF, PGP9.5, GATA-4* and *SOX-9*. F~Q: Analysis of immunocytochemistry for pSSCs. The colonies of pSSCs expressed OCT4, NANOG, PGP9.5 and SSEA1 proteins. Columns from left to right, left: bright field, middle: staining of each markers and right: staining of nucleus (Bar=50 μm).

Table 2. Development rate of pFF and pSSC nuclear transfer embryos

Type of donor cell	No. of oocytes	Rate (n) of				
		Fusion	2~4 cell	8~16 cell	Morula	Blastocyst
pFF	169	68.6±1.2 (116)	66.3±1.4 (77)[a]	37.9±2.0 (44)[a]	14.6±0.8 (17)[a]	11.2±0.8 (13)[a]
pSSC	125	72.0±2.1 (90)	72.2±1.2 (65)[b]	40.0±1.0 (36)[a]	16.6±1.5 (15)[a]	13.3±1.1 (12)[a]

[a,b] Values within column with different superscripts differ significantly ($p<0.05$).

expression level of *SOX9* gene. Results showed that Lam-$_B$ group contained significantly less number of the pre-sertoli cells compared with Lam-$_{NB}$ group (Fig. 2E). These cells were maintained 12 passages for 3 month. The generated pSSC colonies were positive for PGP 9.5 of pSSCs marker and pluripotent marker including Oct-4, Nanog and SSEA-1 (Fig. 2F-Q). Thus, these results suggest that our pSSCs exhibited not only undifferentiated germ cell but also pluripotent cell characteristics.

Analysis of the Methylation Status in pSSCs and pSSC-NT Embryo

To investigate the appropriation of pSSC as donor cells at the somatic cell nuclear transfer, this study evaluated not only the rate of blastocyst development but also compare to methylation pattern between somatic cell transfer and pSSCs transfer. First, to compare the efficiency of pSSCs with that of pFF after nuclear transfer into an enucleated recipient oocyte, the development rate was investigated from 2~4 cell cleavage to blastocyst. However, the examination found no significant differences in the developments of cloned embryos between the two donor cells (Table 2). Furthermore, the analysis of mathylation patterns demonstrated that the pSSCs showed androgenic mathylation pattern as like as sperm, in contrast, pFF showed somatic cell pattern (Fig. 3). Thus, the methylation pattern of *H19* gene was investigated after nuclear transfer of pSSC and pFF at blastocyst stage of embryonic preimplantation. This study showed that cloned embryos exhibited loss of methylation during development to the blastocyst stage at the *H19* DMR-CTCF3 region. However, when the pSSC was transferred into the enucleated oocyte, the methylation pattern of this region was maintained (Fig. 4). In other words, pSSCs can improve the epigenetic modification of reconstructed embryo after nuclear transfer compared with pFF.

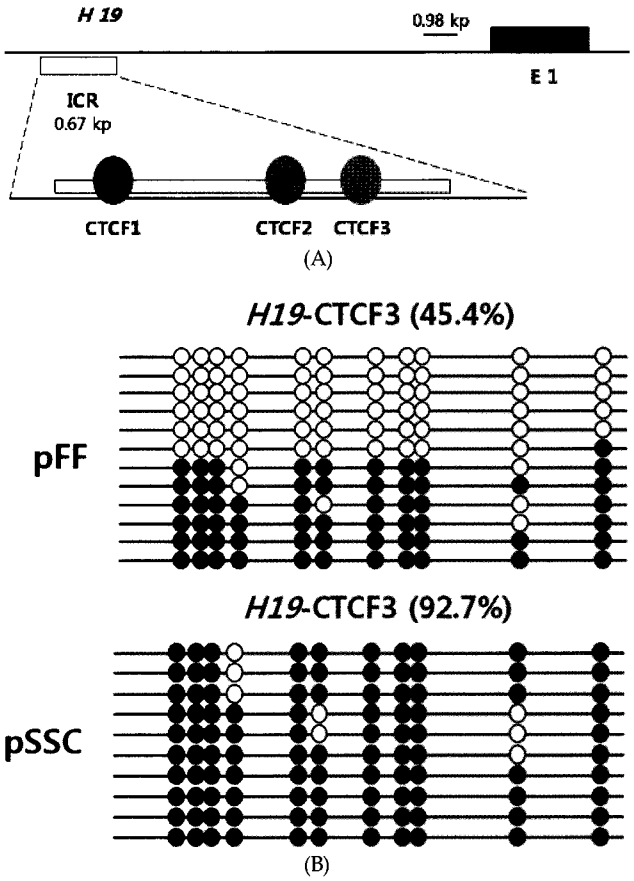

Fig. 3. Analysis of methylation status of *H19* CTCF3 site in pFFs and pSSCs. A: The three CTCF binding site of DMR of porcine *H19* gene. B: The CTCF3 site was analyzed in pFFs and pSSCs. Open and filled circles indicate unmethylated and methylated CpGs, respectively.

DISCUSSION

Previously, several research groups claimed that less differentiated cells can increase the efficiency of somatic cell nuclear transfer compared with terminally differentiated cell (Eggan *et al.*, 2002; Jaenisch, 2002; Rideout *et al.*, 2001; Wakayama and Yanagimachi, 1999). For this reason, many different donor cell types have been investigated to understand genomic reprogramming and how to improve the efficiency of cloning by nuclear transfer. However, these studies were limited in scope, inasmuch as the examined donor cells almost exhibit the imprinting status of somatic cell. The imprinting status of somatic cells lead to abnormal epigenetic modification in reconstructed embryos (Cattanach

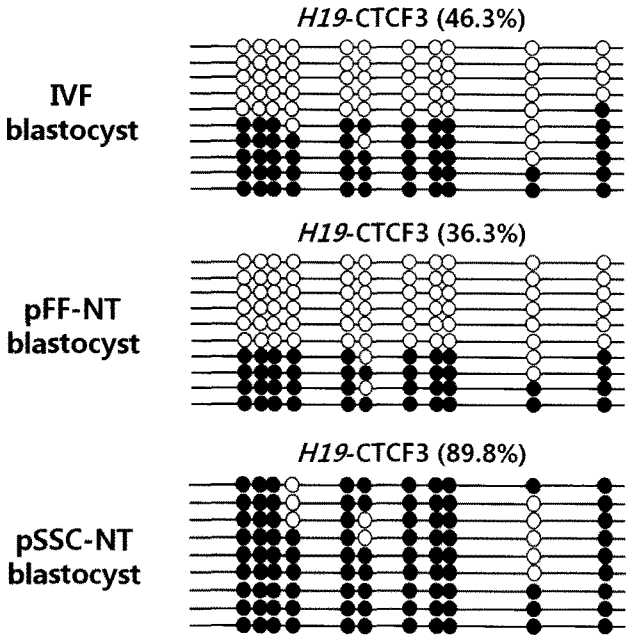

Fig. 4. Analysis of methylation status of *H19* CTCF3 site in IVF blastocyst, pFF-NT blastocyst and pSSC-NT blastocyst.

and Beechey, 1990; Surani *et al.*, 1990). In the present study, the pSSCs were examined as donor cells, whether it can improve the development rate or not and this study investigated the methylation status of reconstructed embryos after pSSC nuclear transfer.

In the mammalian testis, gonocytes migrate to the basement membrane and undergo a conversion to SSCs after postnatal (Brinster, 2002). In the mouse, this migration and conversion occurred from 0 to 5 days (Shinohara *et al.*, 2002). To find the optimal age to obtain SSCs in pig, this study demonstrated the time of gonocytes migration and conversion to SSCs via DBA location, because DBA has specific affinity for gonocytes in porcine testis (Takagi *et al.*, 1997; Goel *et al.*, 2007). The data indicated that the migration and conversion to SSCs have occurred from 5 days to 3 weeks in porcine testis. In other words, the testes of 2~3 weeks old contained much more pSSCs than neonatal testes. Therefore, the pSSCs were isolated from 2~3 weeks old testis in this study, while other previous studies used the samples of neonatal testis (Luo *et al.*, 2006; Dirami *et al.*, 1999; Kuijk *et al.*, 2009). The pSSCs can be isolated with laminin positive selection because the SSCs have α-6 and β-1 integrin surface receptor (Shinohara *et al.*, 1999). In the lack of highly selective antibodies for pSSCs, matrix selection like as laminin is an important tool in order to obtain a pure pSSCs population. This study showed that the isolated pSSCs, laminin bounded cells (Lam-$_B$), strongly expressed the undifferentiated germ cell marker, including *PLZF* and *GFR α-1*. This observation is consistent with those of previous reports (Meng *et al.*, 2000; Costoya *et al.*, 2004). Generally it was known that *GFR α-1* mediates GDNF signaling and thereby supports SSCs self-renewal (Meng *et al.*, 2000). Furthermore, the Lam-$_B$ cells expressed the *PGP9.5*. The *PGP9.5* was identified not only the marker of porcine undifferentiated germ cells (Luo *et al.*, 2006), but also the marker of SSCs in other several species recently. However, the differentiated germ cell marker and sertoli cell marker were weekly expressed in Lam-$_B$. Especially, since pre-sertoli cell can form the colonies in primary culture (Mizukami *et al.*, 2008), the exclusion of these cells was a crucial process. These Lam-$_B$ cells appeared to form typical clusters, grape-like morphology, within 7~14 days in culture. And ES like morphology was found after 3 passages. The colonies were strongly positive for the alkaline phosphatase activity. Thus, these findings were consistent with characteristics of many other species, including mouse, rat, bovine and human. To further determine the undifferentiated state of the pSSCs, immunocytochemistry was performed in this study. The pSSCs were positive for the marker of ES-stem cell, including OCT-4, NANOG and SSEA-1, even, positive for PGP9.5. Therefore, these results suggested that the pSSCs possessed not only undifferentiated germ cell characteristic and pluripotent ability. Moreover, these results proposed that pSSCs may be able to obtain the properties of stem cell in optimal culture condition *in vitro* culture.

The pSSCs exhibited characteristics of multipotent germ-line derived stem cells with respect to gene and protein expression. However, until recently, the methylation patterns of pSSCs have not been analyzed. In the previous study, the putative DMRs of the *H19* genes were identified in order to investigate their imprinting status in cloned piglets (Han *et al.*, 2008). There are three the identified CTCF binding sites conserved in the mouse and human genes. The CTCF1 and 2 sites of control piglets, natural birth, did not show a differential methylation pattern compared with cloned piglets. This result suggested that the methylation patterns of CTCF1 and 2 were normal, at least in the produced piglets through nuclear transfer. However, the CTCF3 site of cloned piglets exhibited a differential methylation pattern compared with that of control piglets. Thus, the methylation status of the CTCF3 site of *H19* gene can be considered an epigenetic criterion to analyze the reprogramming status of produced embryo through nuclear transfer. The data of present study showed that CTCF3 site of *H19* gene of pFFs exhibited a somatic imprinting pattern, while that of pSSCs exhibited an androgenic pattern (Fig. 3). Recently, report showed that the treated somatic cell of testicular extract changed the characteristic into male germ like cells and improve the efficiency of the nuclear transfer in porcine embryos (Roh *et al.*, 2009). Furthermore, several research groups claimed that less differentiated cells can increase the efficiency of somatic cell nuclear

transfer compared with terminally differentiated cell (Rideout *et al.*, 2001; Eggan *et al.*, 2002; Jaenisch, 2002; Rideout *et al.*, 2000; Wakayama and Yanagimachi, 1999). Therefore, the efficiencies of pSSCs were investigated as donors after nuclear transfer into recipient oocyte because the pSSCs have characteristics both undifferentiated status and androgenetic pattern. Contrary to the expectation, this study found no significant differences of development rate between the pFFs and pSSCs. Interestingly, the CTCF3 methylation of *H19* of pFF cells was decreased compared with that of pFF cell after nuclear transfer. However, the methylation pattern of pSSC was maintained after nuclear transfer. Generally, most of the genome is demethylated by the morula stage (Mayer *et al.*, 2000). Despite these global changes, imprinting genes must maintain the methylation pattern of DNA in order to be properly expressed later in postimplantation stage development (Stoger *et al.*, 1993; Tremblay *et al.*, 1995). In other words, the maintenance of imprinting pattern in preimplantation stage was important for accurate epigenetic reprogramming of post-implantation. Thus, these results suggest that pSSCs may improve the normal epigenetic modification of reconstructed embryos after nuclear transfer compared with embryos produced by pFF nuclear transfer, while the development rates of blastocyst showed no difference.

In this study, we demonstrates that pSSCs obtained from 3 weeks old testis can be propagated and maintain the stem cell properties under relatively simple culture condition. The pSSCs exhibit the androgenetic status of DMRs site of *H19* gene. Furthermore, this study demonstrates that the pSSCs are definitely appropriative as donor cells in nuclear transfer, while the development rate was no difference between pFF and pSSCs. Therefore, this study suggest that the imprinting status of donor cells is crucial as well as differentiated state of donor cell in reprogramming of reconstructed embryos. In the future, more research will need to be conducted on culture conditions with different combinations of growth factors, the need for feeder plate and the function of protein interactions. Moreover, further investigations will be needed to analyze whether other ES-like cells are correct donor cell and will provide important information for epigenetic reprogramming of embryo development.

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