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## Improvement of Porcine SCNT Embryo Development Using Histone Deacetylase Inhibitors

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Somatic cell nuclear transfer (SCNT) has been used in crucial and potential fields of the animal reproduction. Despite the successful birth of cloned animals, the cloning efficiency of SCNT has been still low. Abnormal epigenetic reprogramming has been reported as the cause of the low efficiency of SCNT in cloned embryos.

Here, the study aimed to improve the developmental competence of porcine SCNT embryos using Trichostatin A (TSA) and Scriptaid which are well known as histone deacetylase inhibitors that would enhance epigenetic reprogramming in cloned embryos by suppressing the event of histone deacetylation. Moreover, this study tested whether Scriptaid would be a substitute for TSA because it has been suggested that TSA is involved in malformation of cloned embryos.

Various concentrations of Scriptaid were tested and 500 nM Scriptaid treatment resulted in a significant improvement of the cloned embryo during development regarding the blastocyst formation rates. When cloned embryos were treated with 50 nM TSA or/and 500 nM Scriptaid for 15 h or 24 h, the blastocyst rates of reconstructed embryos were increased in comparison to the untreated control group. However, there was no dose-dependent difference among groups. When donor cells were treated with 50 nM TSA or/and 500 nM Scriptaid for 4 h the blastocyst rates of reconstructed embryos were increased in comparison with the untreated control group. Moreover the expression levels of histone deacetylase 1 (HDAC1) and histone deacetylase 2 (HDAC2) were decreased with TSA and/or Scriptaid treatment.

In conclusion, TSA and/or Scriptaid treatments significantly increased the developmental competence of porcine SCNT embryos. In addition, Scriptaid improved the development of SCNT embryos regardless of the TSA treatment. Therefore, Scriptaid would be an alternative additive to improve the development competence of cloned embryos after SCNT.

**Key words:** histone deacetylase inhibitor, porcine embryo, Scriptaid, SCNT, TSA

## INTRODUCTION

The histone modification appears the dynamic global patterns (Bannister and Kouzarides, 2011) and is involved in expression and regulation of genes during development of mammalian embryos (Morgan *et al.*, 2005; Feng *et al.*, 2010). Histones are very positively charged proteins, and have an important role on DNA coiling and regulate gene expressions. Histone acetylation is the supplement of acetyl groups in the lysine residues within the N-terminal tail of histone proteins. Regulation of histone acetylation and deacetylation is modulated by interaction of histone acetyl transferases (HATs) and histone deacetylases (HDACs).

Histone acetylation and deacetylation are necessary action for embryonic genome activation (EGA) and somatic cell reprogramming of cloned embryos (Yamanaka

*et al.*, 2009). Previous studies reported that histone deacetylase inhibitor (HDACi) improved developmental efficiency of cloned embryos in porcine (Zhang *et al.*, 2007; Zhao *et al.*, 2010; Huang *et al.*, 2011). In addition, HDACi improves nuclear remodeling, expression of development-associated genes, the quality of blastocysts, and the efficiency of cloned offspring production in varied species (Maalouf *et al.*, 2009; Kishigami *et al.*, 2006; Whitworth *et al.*, 2011). For example, the treatment of Trichostatin A (TSA), which is one of HDACi, has been known to improve blastocyst formation in pigs (Zhang *et al.*, 2007) and cows (Sawai *et al.*, 2012), and the rate of full-term development in mice (Kishigami *et al.*, 2006). However, TSA treatment in high concentration or for long exposure time induces malformation of cloned embryos (Svensson *et al.*, 1998), and influences on a significant decline of embryo developmental efficiency (Van Thuan *et al.*, 2009) and cloning rate (Tsuji *et al.*, 2009).

Scriptaid, which is one of HDACi with low toxicity, has been studied to increase cloning efficiency and proper gene expressions in porcine (Zhao *et al.*, 2009; Whitworth *et al.*, 2011). The objective of this study was to investigate, optimize, and compare the effects of TSA and Scriptaid regarding the reprogramming of somatic nuclei following SCNT. The effect of a combination of TSA and Scriptaid on *in vitro* developmental competence of cloned porcine embryos was also assessed.

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## MATERIALS AND METHODS

### Chemicals and reagents

Unless specified otherwise, all chemicals for embryo culture and manipulation were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Fetal fibroblast cell preparation

Porcine fetal fibroblast cells (PFFs) were isolated from a 35-day-old fetus. Simply, the fetus was recovered and rinsed 4–7 times with phosphate buffered saline (PBS). After removal of the head, internal organs and limbs, the remaining tissues were finely chopped into pieces. Next chopped tissues dispersed in high glucose enriched Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The dispersed cells were centrifuged, resuspended, and cultured in high glucose enriched DMEM containing 10% FBS and 1% penicillin-streptomycin at 38.5°C in 5% CO<sub>2</sub> atmosphere and saturated humidity. Cells were trypsinized, centrifuged and resuspended in FBS containing 10% dimethyl sulfoxide (DMSO), and then stored in liquid nitrogen until use. Prior to SCNT, PFFs were thawed, cultured and subsequently used in 5–7 passages.

### Collection of oocytes and *in vitro* Maturation

Porcine ovaries were collected from local slaughter house and transported to laboratory in physiological saline containing 1% penicillin/streptomycin (approximately 25°C) within 3 h. The ovaries were washed in saline three times. Antral follicles of 3–8 mm in diameter were aspirated using 18-gauge needle attached to a 10 mL disposable syringe. The aspirated follicular fluid was precipitated in 37°C water bath. The sediments were washed with saline two times. Compact cumulus-oocyte complexes (COCs) surrounded by at least three layers of cumulus cells and uniform cytoplasm were selected. The selected COCs were washed with tissue culture medium 199 supplemented with 2.5 mM fructose, 0.4 mM L-cysteine, 1 mM sodium pyruvate, 0.13 mM kanamycin, 10% (v/v) porcine follicular fluid, 10 ng/mL epidermal growth factor and 500 IU/mL gonadotropin hormone. After washing, selected COCs were cultured in maturation medium for 22 h at 39°C in 5% CO<sub>2</sub> in air. COCs were then transferred to the IVM medium without hormone, and cultured for additional 22 h at same condition. After 44 h of IVM, cumulus cells were removed by repeated gentle pipetting in 0.1% hyaluronidase. After removing cumulus cells, oocytes with good and uniformed cytoplasm and a polar body were selected and placed in porcine zygote medium-3 (PZM-3).

### Somatic Cell Nuclear Transfer

Oocyte enucleation was performed in NCSU-W medium containing with Hoechst 33342 and cytochalasin B. *in vitro* matured oocytes were enucleated by aspirating the first polar body and metaphase II chromosome under epifluorescent microscope (TE2000; Nikon, Tokyo, Japan). After enucleation, a single donor cell with a

smooth cell surface was inserted into the perivitelline space of enucleated oocyte. The oocyte-cell fusion was performed using of electrodes equipped with micromanipulator in 280 mM mannitol medium. Membrane fusion was induced by applying an alternating a direct-current (DC) pulse of 32 V for 30  $\mu$ s was applied for single pulse, using a cell fusion generator (LF101; NepaGene, Chiba, Japan). After fusion, oocytes were washed three times in PZM-3, and then cultured in PZM-3 containing with 1.9 mM N-6 Dimethylaminopurine (6-DMAP) for 3 h. After being treated with 6-DMAP in PZM-3, the oocytes were examined for fusion under stereomicroscope. The reconstructed embryos were washed three times in PZM-3. Fifteen to twenty embryos were cultured in 40  $\mu$ L PZM-3 medium droplets covered by mineral oil in dish at 39°C with 5% CO<sub>2</sub>.

### Treatment of TSA and/or Scriptaid to SCNT embryos or donor cells

Different concentrations of Scriptaid was evaluated with SCNT embryos after activation and treatment with 6-DMAP for 3 h. SCNT embryos were cultured in medium supplemented with 0, 100, 200, 500, 700, or 1000 nM Scriptaid for 15 h, and then transferred to medium without Scriptaid. SCNT embryos were also cultured in medium supplemented with TSA and/or Scriptaid (TSA 50 nM, Scriptaid 500 nM) for 15 and 24 h and then transferred to medium without TSA and/or Scriptaid. In addition, before porcine nuclear transfer, cell working media supplemented with TSA and/or Scriptaid (TSA 50 nM, Scriptaid 500 nM) for 4 h in incubator and then SCNT embryos with donor cells treated with TSA and/or Scriptaid were activated and treated with 6-DMAP for 3 h.

### Quantitative real-time RT-PCR

qRT-PCR was performed by the MJ AL079721 real-time PCR system (MJ Research, Inc., Waltham, MA, USA) using QuantiTect SYBR Green PCR Kit (Qiagen). The reaction mixture of total 25  $\mu$ L volume is consisted of 12.5  $\mu$ L QuantiTect SYBR Green PCR Master Mix (2 $\times$ ), 1  $\mu$ L of forward and reverse primers (10 mol/L), 2  $\mu$ L of cDNA, and 8.5  $\mu$ L double-distilled water. The reaction parameters were as follows: initial denaturing step at 95°C for 10 min, followed by 50 denaturing cycles at 95°C for 15 sec, annealing at 60°C for 1 min, and extension at 95°C for 20 sec. After running each PCR, a melting curve analysis was performed for each sample to assure that a single specific product was generated. Melting curves were obtained by decreasing the temperature stepwise from 95 to 60°C. The primers used for amplification of target and internal reference genes are presented in table 1.

### Statistical analysis

Statistical analysis was performed using SPSS software (version 13.0 for Windows). One-way analysis of variance (ANOVA) was used to detect differences in gene expression. Data are expressed as mean  $\pm$  standard error (SE). A value of  $P < 0.05$  was considered to represent a

statistically significant difference.

## RESULTS

### The effect of different concentrations of Scriptaid treatment on *in vitro* development of cloned embryos

Developmental competency of porcine SCNT embryos was compared, and the percentages of cleaved embryos, the rate of blastocyst formation, and the total cell numbers of blastocysts are shown in Table 2. There was no significant difference among groups regarding the total

cell numbers in blastocysts. However, the rate of blastocyst formation was significantly higher with 500 nmol/L treatment ( $P < 0.05$ ).

### The effect of TSA and/or Scriptaid treatment on *in vitro* development of cloned embryos

As shown in Table 3 and 4, cloned embryos were treated with 50 nM TSA, 500 nM Scriptaid or both 50 nM TSA and 500 nM Scriptaid for 15 h and 24 h. Even though there was significant improvement regarding the rate of blastocyst formation in treated groups compared to the

**Table 1.** RT-PCR primer sequences for canine epigenetic modification related genes

| Genes          | Primer sequences(5'→3')   | Accession number |
|----------------|---------------------------|------------------|
| DNMT 1         | F- GTGAGGACATGCAGCTTTCA   | NM_001032355     |
|                | R- AACTTGTGTGCTCCGTTGG    |                  |
| DNMT 3A        | F- CTGAGAAGCCCAAGGTCAAG   | CJ026384         |
|                | R- CAGCAGATGGTGCAGTAGGA   |                  |
| HDAC 1         | F- CGCATGACTCACAATTGCT    | BC108371         |
|                | R- AGCCATCAAATACCGGACAG   |                  |
| HDAC 2         | F- TGGGAGGAGGTGGATACACAA  | EW621236.2       |
|                | R- AGCTTGAAGTCGGGTCCAAA   |                  |
| $\beta$ -Actin | F- CTCGATCATGAAGTGCACGCT  | NM_001170157     |
|                | R-GTGATCTCCTTCTGCATCCTGTC |                  |

**Table 2.** The effect of different concentrations of Scriptaid treatment on *in vitro* development of porcine SCNT embryos

| Treatment | No. of embryos | No. of Blastocysts<br>(% $\pm$ SE) | No. of Total cells<br>(% $\pm$ SE) |
|-----------|----------------|------------------------------------|------------------------------------|
| 0 nM      | 58             | 9 (15.44 $\pm$ 1.36) <sup>ab</sup> | 28.25 $\pm$ 1.96                   |
| 100 nM    | 59             | 12 (20.20 $\pm$ 2.73) <sup>b</sup> | 29.75 $\pm$ 2.64                   |
| 200 nM    | 63             | 14 (21.88 $\pm$ 3.62) <sup>b</sup> | 30.75 $\pm$ 1.72                   |
| 500 nM    | 59             | 22 (37.22 $\pm$ 2.88) <sup>c</sup> | 31.25 $\pm$ 2.51                   |
| 700 nM    | 62             | 13 (20.96 $\pm$ 0.88) <sup>b</sup> | 29.50 $\pm$ 3.00                   |
| 1000 nM   | 60             | 7 (11.70 $\pm$ 1.98) <sup>a</sup>  | 29.25 $\pm$ 3.07                   |

Four replicates were performed.

<sup>a-c)</sup> Values with different superscripts are significantly different ( $P < 0.05$ ).

**Table 3.** Effect of TSA and/or Scriptaid treatment for 15 h on *in vitro* development of porcine SCNT embryos

| Treatment                 | No. of embryos | No. of Blastocysts<br>(% $\pm$ SE) | No. of Total cells<br>(% $\pm$ SE) |
|---------------------------|----------------|------------------------------------|------------------------------------|
| 0 nM                      | 54             | 8 (14.92 $\pm$ 0.83) <sup>a</sup>  | 28.85 $\pm$ 2.78                   |
| TSA 50 nM                 | 58             | 13 (22.49 $\pm$ 1.28) <sup>b</sup> | 31.15 $\pm$ 2.57                   |
| SCP 500 nM                | 61             | 15 (24.31 $\pm$ 2.19) <sup>b</sup> | 31.31 $\pm$ 1.96                   |
| TSA+SCP<br>(50 nM+500 nM) | 58             | 15 (25.72 $\pm$ 2.95) <sup>b</sup> | 32.18 $\pm$ 1.91                   |

Four replicates were performed.

<sup>a-b)</sup> Values with different superscripts are significantly different ( $P < 0.05$ ).

**Table 4.** Effect of TSA and/or Scriptaid treatment for 24 h on *in vitro* development of porcine SCNT embryos

| Treatment              | No. of embryos cultured | No. of Blastocysts (% $\pm$ SE)    | No. of Total cells (% $\pm$ SE) |
|------------------------|-------------------------|------------------------------------|---------------------------------|
| 0 nM                   | 56                      | 8 (13.99 $\pm$ 2.27) <sup>a</sup>  | 28.86 $\pm$ 2.50                |
| TSA 50 nM              | 53                      | 14 (26.72 $\pm$ 4.40) <sup>b</sup> | 30.99 $\pm$ 2.28                |
| SCP 500 nM             | 55                      | 14 (25.41 $\pm$ 1.31) <sup>b</sup> | 30.19 $\pm$ 2.82                |
| TSA+SCP (50 nM+500 nM) | 56                      | 15 (26.50 $\pm$ 2.02) <sup>b</sup> | 32.63 $\pm$ 2.76                |

Four replicates were performed.

<sup>a-b)</sup> Values with different superscripts are significantly different ( $P < 0.05$ ).

**Table 5.** Developmental competence of porcine cloned embryos transferred of donor cells which were treated with TSA and/or Scriptaid for 4 h

| Treatment              | No. of embryos cultured | No. of Blastocysts (% $\pm$ SE)    | No. of Total cells (% $\pm$ SE) |
|------------------------|-------------------------|------------------------------------|---------------------------------|
| 0 nM                   | 62                      | 8 (12.97 $\pm$ 0.63) <sup>a</sup>  | 28.58 $\pm$ 1.44                |
| TSA 50 nM              | 55                      | 12 (21.54 $\pm$ 2.06) <sup>b</sup> | 31.06 $\pm$ 1.93                |
| SCP 500 nM             | 65                      | 16 (24.90 $\pm$ 3.52) <sup>b</sup> | 32.69 $\pm$ 2.00                |
| TSA+SCP (50 nM+500 nM) | 61                      | 14 (22.90 $\pm$ 1.21) <sup>b</sup> | 31.88 $\pm$ 2.63                |

Four replicates were performed.

<sup>a-b)</sup> Values with different superscripts are significantly different ( $P < 0.05$ ).

non-treated group there was no significant difference among treated groups. In addition, there was no significant difference of the total cell number in blastocysts.

#### The effect of the donor cells treated with TSA and/or Scriptaid on developmental competence of porcine cloned embryos

The donor cells treated with TSA and/or Scriptaid resulted in significant difference in the rate of blastocyst formation compared to non-treated group ( $P < 0.05$ ). However, there was no difference in the treating groups of TSA and/or Scriptaid regardless of the concentration. There was also no difference in the total cell numbers of blastocyst among groups.

Exposure time of TSA and/or Scriptaid from 4 h to 24 h would be sufficiently to inducing histone deacetylation in cloned porcine embryos. Consequently, when embryos and donor cells were treated with TSA and/or Scriptaid, embryo development did not changed regardless of exposure times (embryos for 15 h, 24 h and donor cell for 4 h).

#### Expression levels of HDAC1, HDAC2, DNMT1 and DNMT3A in porcine SCNT blastocysts

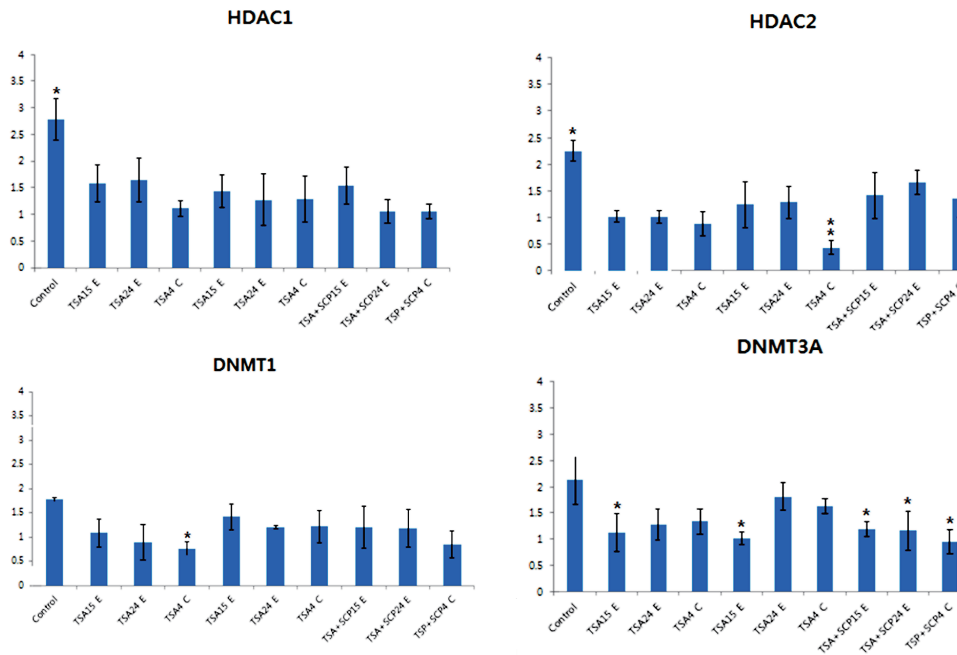
The comparative abundance of gene expressions was investigated, and is shown in Fig. 1. In blastocysts which were treated with TSA and/or Scriptaid, the expression levels of HDAC1 and HDAC2 were decreased unlike the untreated control group ( $P < 0.05$ ). The expression of

HDAC2 was especially decreased highly in embryos which were reconstructed with the donor cells treated with Scriptaid for 4 h. In addition, DNMT1 was expressed in embryos treated with TSA less than those of other groups ( $P < 0.05$ ). DNMT3A was also decreased significantly in embryos treated with TSA and/or Scriptaid for 15 h and 24 h ( $P < 0.05$ ).

## DISCUSSION

Global epigenetic modification is a crucial mechanism for successful development of SCNT embryos and complete cloning (Yang *et al.*, 2007). Histone acetylation is a process of histone modification during epigenetic reprogramming, and regarded as an important epigenetic marker in donor cells and embryos that affects on the successful SCNT embryo development (Yamanaka *et al.*, 2009). Currently, it has been known that the abnormal histone acetylation in SCNT embryos and/or donor cells would be able to be changed that redeem the developmental ability of embryos *in vivo* and *in vitro* (Wang *et al.*, 2011; Ding *et al.*, 2008; Panda *et al.*, 2012).

TSA and Scriptaid are histone deacetylase inhibitors of traditional epigenetic remodeling substances. Previous studies demonstrated that TSA treatment improves histone acetylation in SCNT embryos, and produces generalizable patterns of chromatin remodeling, histone acetylation, transcription activity, and developmental competence of SCNT embryos in several species (Maalouf



**Fig. 1.** Relative abundance of HDAC1, HDAC2, DNMT1 and DNMT3A transcripts at blastocyst stage after treating embryos or donor cells with TSA (50 nM), SCP (500 nM), or TSA+SCP for 15 h and 24 h. Control: no treatment; TSA15 E: TSA treated embryos for 15 h; TSA24 E: TSA treated embryos for 24 h; TSA4 C: TSA treated donor cells for 4 h; SCP15 E: SCP treated embryos for 15 h; SCP24 E: SCP treated embryos for 24 h; SCP4 C: SCP treated donor cells for 4 h; TSA+SCP15 E: TSA and SCP treated embryos for 15 h; TSA+SCP24 E: TSA and SCP treated embryos for 24 h; TSA+SCP4 C: TSA and SCP treated donor cells for 4 h.

*et al.*, 2009; Bui *et al.*, 2010; Fan *et al.*, 2011). In addition, Scriptaid treatment altered patterns and levels of the histone acetylation, and improved *de novo* synthesis of mRNA in the early stage of mouse cloned embryos (Van Thuan *et al.*, 2009) that is similar in cattle and pig cloned embryos compared with *in vitro* fertilized embryos (Wang *et al.*, 2011; Zhao *et al.*, 2010). Scriptaid is also able to be treated relatively in high concentrations or longer exposure because of its low toxicity to cloned embryo development. Moreover, treatment of both TSA and Scriptaid treatment reduced the DNA methylation levels of cloned embryo nuclei (Whitworth *et al.*, 2011).

In this study the treatment of TSA and Scriptaid enhanced the developmental potency of reconstructed embryos *in vitro*. This result may be cause of increased histone acetylation in the somatic nuclei of reconstructed SCNT embryos. (Chan Luo *et al.*, 2013). Treating donor cells with TSA augments the level of histone acetylation, and facilitates subsequent epigenetic reprogramming of nucleus of porcine embryo during development (Yun-Fei *et al.*, 2013). TSA can revitalize silenced genes involved with embryo development and maintain gene expression by generating histone hyper-acetylation in porcine fibroblast cells (Qingran *et al.*, 2011). In this study, TSA and/or Scriptaid treatment to donor cells before porcine nuclear transfer improved the rate of SCNT embryo development. It is possible that the combination of TSA and Scriptaid induces synergic effects on cloning efficiency of porcine embryos.

Deficiency of DNMT3A, DNMT3B or HDAC1 caused

the embryonic lethality (Okano *et al.*, 1999). HDAC1 and HDAC2 are substance of histone deacetylases, and were decreased in donor cells treated with HDACi (Wee *et al.*, 2007). In this study, the expression levels of HDAC1, HDAC2, DNMT1 and DNMT3A were examined to study the effect of TSA and Scriptaid, both individually and simultaneously. With treatment of TSA and/or Scriptaid, porcine SCNT blastocysts showed the different expression levels of HDAC1, HDAC2, DNMT1 and DNMT3A. The expression level of HDAC1 in TSA and/or Scriptaid treated embryos or donor cell was reduced, and improved the embryonic development competence. Although Scriptaid induced the lowest expression level of HDAC2 when treated to nuclear donor cells, slightly reduced expression level of HDAC2 was detected in all treated groups that was enough to improve the development competence of cloned embryos. The transcription level of DNMT1 in SCNT blastocysts was significantly decreased when TSA was treated in nuclear donor cells before SCNT. The expression of DNMT3A was significantly decreased by single TSA or Scriptaid treatment in embryos or by combination treatment of TSA and Scriptaid in embryos or the donor cells. It implied that TSA and/or Scriptaid is enough to altering abnormal epigenetic reprogramming that would enhance the developmental competence of SCNT embryos for successful production of cloned animals.

In conclusion, TSA and/or Scriptaid treatments (alone or in combination) improved blastocyst development rates, even though they differently affected on the expres-



sion of genes which are involved in epigenetic modification. Treating TSA and/or Scriptaid in nuclear donor cells before SCNT or reconstructed SNCT embryos would be an alternative method to improve the development of SCNT porcine embryos.

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