Nuclear Modeling and Developmental Potential of Bovine Somatic Nuclear Transfer Embryos Cloned by Two Different Activation Methods

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ABSTRACT

The present study investigated the nuclear remodeling, development potential with telomerase activity and transcription level of X-linked genes (ANT3, HPRT, MeCP2, RPS4X, XIAP, XIST and ZFX) in the bovine somatic cell nuclear transfer (SCNT) embryos using two different fusion and activation methods. Female adult fibroblasts were injected into perivitelline space of *in vitro* matured oocytes. The oocyte-nucleus complexes were fused and followed by immediately either activated (Group 1), or activated at 1 h post-fusion (hpf) (Group 2), respectively. The incidence of normal premature chromosome condensation (PCC) at 1 hpf was slightly increased in the Group 2, compared to those of Group 1, but there was no significant ($p<0.05$) difference. The incidence of normal pronucleus (PN) and chromosome spread at 5 and 18 hpf were significantly ($p<0.05$) higher in the Group 2 than those of Group 1. The cleavage rate to 2-cell stage, developmental rate to blastocyst stage, and the mean number of total and ICM cell numbers were significantly ($p<0.05$) higher in the Group 2, compared to those of Group 1. Level of telomerase activity was significantly ($p<0.05$) higher in the SCNT blastocysts of Group 2, compared to those of Group 1. Transcript levels of HPRT, MeCP2 and XIST were not significantly ($p<0.05$) different between blastocysts of Group 1 and 2. However, transcript level of ANT3, RPS4X, XIAP and ZFX were significantly ($p<0.05$) up-regulated in the SCNT blastocysts of Group 2, compared to those of Group 1. Taken together, it is concluded that oocyte activation at 1 hpf induces the enhanced developmental potential by efficient nuclear remodeling and subsequent facilitation of the nuclear reprogramming of bovine SCNT embryos.

(Key words : Somatic cell nuclear transfer, Oocyte activation, Nuclear remodeling, Telomerase activity, X-linked gene expression)

INTRODUCTION

Although the production rate of live offspring by somatic cell nuclear transfer (SCNT) technology is still low by the limited reprogramming process of donor nucleus, SCNT has been broadly applied for the production of the genetically unified and endangered animals, and fundamental research of genetic and epigenetic contributions to development events (reviewed by Keefer, 2008; Niemann *et al.*, 2008).

In SCNT procedure, donor cells were generally introduced into the matured and enucleated oocytes by electro-fusion methods. The injected donor nucleus undergoes the dynamic changes called as nuclear remodeling, including nuclear envelope breakdown (NEBD), premature chromosome condensation (PCC) and pronucleus (PN) formation by re-arrangement of microtubules and chromosomes (Collas and Robl, 1991; Kawahara *et al.*, 2005; Shin *et al.*, 2002). PCC of the injected donor nucleus was attributed to a high level of maturation promoting factor (MPF) activity in the non-activated and matured oocyte cytoplasm (Kawahara *et al.*, 2005; Whitacker and Patel, 1990). It has been reported that the rarely and abnormal PCC formations are induced in the donor nucleus injected into the activated oocytes with low level MPF activity, and the developmental potential of their SCNT embryos are noticeably reduced (Shin *et al.*, 2002). Further, the degradation of MPF induced by artificial stimulation of oocytes was subsequently developed to the chromosome structure of swollen PN (Collas and Robl, 1991; Kawahara *et al.*, 2005; Shin *et al.*, 2002). However, the embryo development was also decreased with the increased abnormal pattern of PN formation in the sperm nucleus fertilized or donor nucleus injected into oocytes with low level of MPF activity (Kawahara *et al.*, 2005; Kikuchi *et al.*, 1995). Thus, it has been suggested that PCC formation of the injected donor nucleus by high MPF level in the non-activated oocytes and subsequent PN formation with fully swollen status in the activated oocytes is an important role for subsequent development of SCNT embryos

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and nuclear reprogramming (Mitalipov *et al.*, 2007; Whitworth and Prather, 2010).

Cell fusion for injection of donor cell and artificial oocyte activation for degradation of MPF level is an essential step in the procedure of SCNT, and most efficient fusion and activation methods have been attempted for nuclear remodeling of the injected donor nucleus in connection with MPF activity. However, there have been still inconclusive in the fusion and activation methods such as simultaneous fusion/activation and delayed activation after fusion.

In the present study, we investigated the nuclear remodeling and developmental potential in the bovine SCNT embryos derived from simultaneous fusion/activation or delayed activation after fusion methods. In addition, up-regulated telomerase activity is required for telomere extension of injected donor nucleus with short telomere length in the SCNT embryos. Dynamic changes for X-chromosome re-activation and in-activation also occur in the reprogramming processes of the female SCNT embryos. The level of telomerase activity and transcript level of genes linked to X-chromosome have been used as useful indexes for reprogramming of bovine SCNT embryos (Jeon *et al.*, 2008). Further, reprogramming potential in their SCNT blastocysts were compared by the level of telomerase activity and the transcript levels of X-linked genes.

MATERIALS AND METHODS

Media and Chemicals

Chemicals were purchased from the Sigma Chemical Company (USA) and media from Gibco BRL (USA), unless otherwise described. The medium used for *in vitro* maturation (IVM) was TCM-199 containing 2.5 mM Na-pyruvate, 1 mM L-glutamine, 1% (v/v) penicillin-streptomycin (10,000 IU and 10,000 μg/ml, respectively, Pen-Strep, Gibco), 10% (v/v) fetal bovine serum (FBS), 1 μg /ml estradiol-17 β, 0.5 μg/ml FSH, and 1 μg/ml LH. Embryo culture medium was modified synthetic oviduct fluid (mSOF) supplemented with 2.9 μg/ml EDTA and 8 mg/ml BSA (essentially fatty acid free). The medium used for culture of donor cells was Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS). Tyrode's albumin lactate pyruvate medium containing 2 mg/ml bovine serum albumin (BSA, essentially fatty acid free) and 10 mM HEPES (HEPES-TALP) was used for oocyte manipulation. For all the media, the pH was adjusted to 7.4 and osmolality to 280 mOsm/kg.

Preparation of Donor Cells

Donor cells were established from skin fibroblasts of ear tissues. Briefly, the ear tissue pieces (1~2 mm^2) were incubated in 0.5% collagenase at 38.5℃ for 3~6 h with agitation, followed by dispersal in DMEM and cultured at 38.5℃ for 5 days in a humidified atmosphere of 5% CO_2 in air until sufficient outgrowth of fibroblasts. Attached fibroblasts were dissociated with 0.05% Trypsin-EDTA. After washing in DMEM by centrifugation at 300 ×g for 10 min, fibroblasts at a final concentration of 2×10^5 cells/ml were subsequently seeded onto 35 mm plastic culture dishes (Nunc, Denmark) and cultured to confluence for 6~8 days. Confluent fibroblasts at 4~6 passages were trypsinized immediately before nuclear transfer.

Preparation of Recipient Cytoplasms

The SCNT procedure was performed according to the previously described protocol (Jeon *et al.*, 2008). Briefly, ovaries were obtained from a local abattoir and cumulus-oocyte complexes (COCs) were aspirated from antral follicles of 2~7 mm in diameter using an 18-gauge needle using vacuum pressure and collected into Ham's F10. Sets of 10 COCs with ≥4 layers of cumulus cells and a finely granulated homogeneous cytoplasm were matured in 50 μl droplets of IVM medium covered with mineral oil at 38.5℃ in a humidified atmosphere of 5% CO_2 in air. After 18 h of maturation, the oocytes were denuded their cumulus cells by vortexing for 2 min in 3% (v/v) sodium citrate solution and removed the nucleus with the first polar body (PB) in HEPES-TALP under a micromanipulator. Enucleation of oocytes was confirmed by staining with 5 μg/ml bisbenzimide (Hoechst 33342) under a fluorescence inverted microscope equipped with UV light.

Nuclear Transfer and Oocyte Activation

Nuclear transfer and cytoplast activation were conducted as previously described (Jeon *et al.*, 2008). Briefly, fibroblasts at passage 4~6 with intact membranes were selected and transferred into the perivitelline space of each enucleated oocyte. Oocyte-nucleus complexes were divided to two groups. In the Group 1, oocyte-nucleus complexes were fused with two electric pulses at 1.0 kV/cm for 15 μsec in 0.26 M mannitol solution supplemented with 100 μM $CaCl_2$ and 100 μM $MgCl_2$ using an ECM 2001 BTX cell manipulator (VWR Laboratories, Canada) equipped with electro-needles, and immediately activated. In the Group 2, oocyte-nucleus complexes were fused and activated at 1 h post-fusion (hpf). Oocyte activation was composed of 5 μM ionomycin in HEPES-TALP for 5 min, followed by exposure to 30 mg/ml BSA for 4 min, and further treated with 10 μg/ml cycloheximide in mSOF for 5 h at 38.5 ℃ and 5% CO_2 in air.

In Vitro Culture of Embryos

After cycloheximide treatment, fused eggs were selected, and a set of 30 eggs without polar body-like extrusion were cultured in 30 μl droplets of mSOF for 3 days, and further cultured in mSOF supplemented with MEM amino acids for 6 additional days at 38.5℃ in a humidified atmosphere of 5% CO_2, 5% O_2, and 90 % N_2. Cleavage and blastocyst rates were assessed on day 2 and 9, respectively. Total cell numbers and inner cell mass (ICM) cell numbers of Day-9 blastocysts were counted with differential staining method as previously described by Thouas *et al.* (2001).

Immunofluorescent Staining of Embryos

Fused eggs were fixed at 1, 5 and 18 hpf with 3.7% paraformaldehyde in phosphate buffered saline (PBS) for 30 min at room temperature (RT), and washed with PBS supplemented with 1% BSA (PBS+BSA) for 30 min. The eggs were permeabilized in PBS+BSA containing 0.1% Triton X-100 at RT. After several washing steps with PBS+BSA, the eggs were blocked in PBS+BSA for 30 min. Microtubule configuration was conducted with a fluorescein isothiocyanate conjugated mouse monoclonal α-tubulin antibody in PBS+BSA overnight at 4℃ on slides and washed with PBS + BSA for 30 min. Finally, stained egg samples were mounted with Vectashield mounting medium (Vector Laboratory, USA) containing 10 μg/ml propidium iodide (PI) on glass slides for staining DNA and covered with cover slips. The samples were observed with a laser-scanning confocal microscope (Olympus, Japan). The elongated and scattered nuclei with disarranged microtubules (Fig. 1. D, E, and F) were considered as abnormal remodeling patterns.

Absolutely Quantitative Analysis of Transcripts by Real Time RT-PCR

Total RNA was extracted using the Qiagen RNeasy Micro Kit with carrier RNA (Qiagen, USA) from Day-9 SCNT blastocysts. Homogenization, isolation, precipitation and purification of RNA were performed according to the manufacturer's procedure with an extra step of DNase I treatment for removal of DNA contamination. The concentration of extracted total RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer. A total of 1 μg RNA was synthesized for the first-strand cDNA with Omniscript RT Kit (Qiagen, USA). Each of cDNA samples contained 2 μl of 10 μM Oligi-dT12-18 primer (Invitrogen, Canada), 1 μl of 10 U/μl RNase Inhibitor (Invitrogen, Canada), 2 μl RT buffer, 2 μl dNTP, and 1 μl Omniscript (Qiagen, USA), was adjusted to a total volume of 20 μl using H_2O. The cDNA samples were then incubated in a thermal cycler (Effendorf, Germany) at 42℃ for 1 h, followed by 5 min at 95℃ to inactivate the enzyme. A total of three reverse transcription reactions was used for each RNA sample. The real time RT-PCR was carried out using the LightCycler 4.0 and the LightCycler Faststart DNA Master SYBR Green I (Roche, Germany) according to the manufacturer's protocols. Each reaction mix contained 2 μl of the cDNA reaction, 2 μl of the FastStart DNA Master SYBR Green I reaction mix, 3 mM $MgCl_2$, and 2 μl of each the forward and reverse primer (0.1 μg/μl), adjusted to a total volume of 20 μl using H_2O. The amplification protocol consists of an initial denaturation step at 95℃ for 10 min followed by 40 cycles of denaturation for 15 sec at 95℃, annealing of 6 sec at 57~65℃ and extension of 16 sec at 72℃. X-linked genes (ADP/ATP translocase 3; ANT3, growth factor receptor bound protein 2-associated binder 3; HPRT, methyl CpG binding protein 2; MeCP2, 40S ribosomal protein S4; RPS4X, X-linked inhibitor of apoptosis protein; XIAP, X-inactive specific transcript; XIST and Zinc finger X-chromosomal protein; ZFX) and reference gene (β-actin; ACT-B) were analyzed for the pattern of the transcripts by quantitative real time RT-PCR. Primer sequences, the size of amplified products, and the annealing and acquisition temperatures of primer were used as described previously by Jeon *et al.* (2008). In each of the cDNA samples, at least five replicates of PCRs were carried out. All the samples were quantified using the LightCycler Quantification Software's (Roche, USA) second derivative method of crossing point (Cp) determination, and absolutely quantitative analysis of transcripts were calculated with standard curve of the each gene.

Relative-quantitative Telomerase Repeat Amplification Protocol (RQ-TRAP)

Relative telomerase activity (RTA) was analyzed by RQ-TRAP assay modified from a conventional TRAP assay for use on the LightCycler 4.0 (Roche, Canada) as previously described by Jeon *et al.* (2008). A pool of 5 Day-9 SCNT blastocysts in three replicates from each groups was immediately frozen at −80℃ for future analysis or lysed in 20 μl 0.5% (v/v) 1·3-[(3-cholamidopropyl) dimethylam-monio] propanesulfonic acid (CHAPS) lysis buffer (pH 7.5) supplemented with 10 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM benzamidine, 5 mM 2-mercaptoethanol and 10% glycerol for 30 min on ice. Following lysis, samples were centrifuged for 20 min at 12,000 ×g at 4℃ to remove cell debris. Eighty percent (by volume) of the lysis was then transferred to a fresh microcentrifuge tube and analyzed by RQ-TRAP. Each run included measurements of 1000, 100, 10, and 1 293T (Chemicon, USA) telomerase-positive control cell(s) for generation of a standard curve. As a negative control, a portion of the 293T sample was heat-inactivated by incubation for 10 min at 85℃. The RQ-TRAP was optimized according to the manufacturer's protocol using the PCR reagent LightCycler FastStart DNA Master SYBR Green 1 (Roche, Canada), con-

taining 2.5 mM $MgCl_2$, 0.02 μg of primer TS (5′-AAT CCG TCG GAG CAG AGT T-3′), 0.04 μg of primer ACX (5′-GCG CGG CTT ACC CTT ACC CTT ACC CTA ACC-3′), and 2 μl of sample was analyzed. The assay-run included 20 min incubation at 25℃, followed by 10 min incubation at 94℃, and 40 cycles of PCR at 94℃ for 30 s and 60℃ for 90 s. All samples were quantified utilizing the LightCycler Quantification Software's (Roche, Canada) second derivative method of crossing point (Cp) determination, and RTA of Day-9 SCNT blastocysts in the Group 2 was calculated to ratio based on the level of telomerase activity in the SCNT blastocysts of Group 1.

Statistical Analysis

Differences among treatments were analyzed by using one-way analysis of variance (ANOVA). Differences in the telomerase activity, transcripts level of X-linked genes and the total cell number of blastocyst were analyzed using a Student's *t*-test. Differences in the percentage of nuclear remodeling and *in vitro* developmental rate to blastocyst stage were analyzed using a Chi-square test. A five percent probability ($p<0.05$) was used as the level of significance.

RESULTS

Nuclear Remodeling of SCNT Embryos

The incidence of nuclear remodeling was examined by confocal microscope in the SCNT embryos of Group 1 and Group 2, as shown in Fig. 1. The percentage of embryos with normal PCC, PN and chromosome spread are summarized in Table 1. The incidence of normal premature chromosome condensation (PCC) at 1 hpf was slightly increased in the Group 2 (80.0%), compared to those of Group 1 (71.8%), but there was no significant ($p<0.05$) difference. However, the incidence of normal PN and chromosome spread were significantly ($p<0.05$) higher in the Group 1 than those of Group 2 (73.9 and 70.5% vs. 57.1 and 51.5%, respectively).

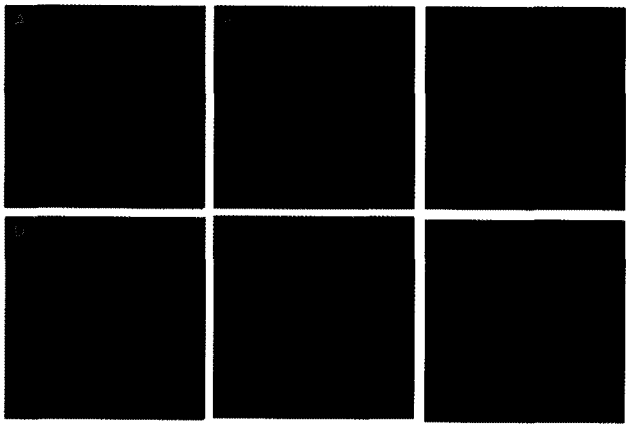

Fig. 1. Confocal microscopic images of the donor nucleus transferred to enucleated oocytes. Nucleus (red) and spindle (green) were stained with propidium iodide and α-tubulin antibody, respectively. A, normal PCC 1 h post-fusion; B, normal PN at 5 h post-fusion; C, normal chromosome spreads at 18 h post-fusion; D, abnormal PCC with elongated spindle at 1 h post-fusion; E, abnormal PN with scattered nucleus at 5 h post-fusion; F, abnormal chromosome and spindle structure observed at 18 h post-fusion.

Table 1. Incidence of nuclear remodeling at 1, 5 and 18 h post-fusion in SCNT embryos derived from two different activation methods

Groups	No of oocytes with normal (%)		
	PCC	PN	Chromosome spread
Group 1	23/32 (71.8)	20/35 (57.1)[a]	17/33 (51.5)[a]
Group 2	36/45 (80.0)	34/46 (73.9)[b]	31/44 (70.5)[b]

Group 1; Oocytes were immediately activated after fusion, Group 2; Oocytes were activated at 1 h post-fusion.
PCC, premature chromosome condensation; PN, pronucleus.
4 replicates.
[a,b] Different superscripts within columns indicate significant ($p<0.05$) differences.

Developmental Potential of SCNT Embryos

The rates of cleavage to 2-cell stage and development to blastocyst stage in the SCNT embryos of Group 1 and Group 2 are summarized in Table 2. The rate of cleavage was significantly ($p<0.05$) increased in the Group 2, compared to Group 1 (87.9 vs. 74.8%, respectively). The percentages of embryos that reached to hatching and hatched blastocyst stages on Day 9 were 28.8 and 14.1% in the Group 1, and 39.8 and 26.9% in Group 2, respectively. The rate of development to hatching and hatched blastocyst stages was significantly ($p<0.05$) increased in the Group 2, compared to Group 1. The mean numbers of total and ICM cells in Group 1 were significantly ($p<0.05$) higher (143±15.3 and 34.6± 8.7 vs. 183±16.2 and 44.5±9.2, respectively).

Relative Telomerase Activity (RTA) of SCNT Embryos

The telomerase activity by RQ-TRAP was investigated in the SCNT blastocysts of Group 1 and Group 2, as shown in Fig. 2. The RTA in the SCNT blastocysts of Group 1 was considered as 100% for comparison with the SCNT blastocysts of Group 2. The RTA in the SCNT blastocysts of Group 2 was 131.21% when compared with the SCNT blastocysts of Group 1, and it was significantly ($p<0.05$) higher in the SCNT blastocysts of Group 2 than those of Group 1.

Transcript Levels of X-linked Genes in SCNT Embryos

Table 2. *In vitro* developmental capacity and mean number of total and ICM cells in SCNT embryos derived from two different activation methods

Groups	No. (%) of oocyte fused	No.(%) of embryos developed to			
		2-cell	Blastocyst	H-blastocyst	Total cell no.* (ICM cell no.)
Group 1	138/145 (95.1)	101/135 (74.8)[a]	39/135 (28.8)[a]	19/135 (14.1)[a]	143±15.3[a] (34.6±8.7)
Group 2	268/289 (92.7)	225/256 (87.9)[b]	102/256 (39.8)[b]	69/256 (26.9)[b]	183±16.2[b] (44.5±9.2)

Group 1; Oocytes were immediately activated after fusion, Group 2; Oocytes were activated at 1 h post-fusion.
H-blastocyst, hatching and hatched blastocysts.
5 replicates.
[a,b] Different superscripts within columns indicate significant ($p<0.05$) differences.

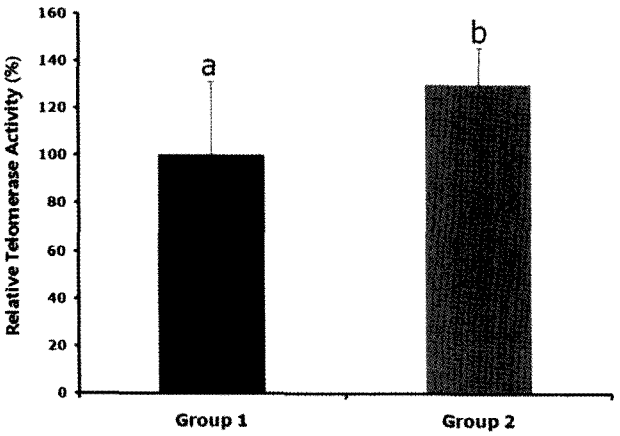

Fig. 2. Relative telomerase activity of SCNT blastocysts. Group 1; Oocytes were immediately activated after fusion, Group 2; Oocytes were activated at 1 h post-fusion. Telomerase activity of SCNT blastocysts in the SCNT embryos of Group 1 was considered as a 100% for comparison with the SCNT embryos of Group 2. [a,b] Different superscripts indicate significant ($p<0.05$) differences.

Transcript levels of X-linked genes were investigated by quantitative real time RT-PCR in the SCNT blastocysts of Group 1 and Group 2, as shown in Fig. 3. Transcript levels of ACT-B, HPRT, MeCP2, and XIST did not differ between the Group 1 and Group 2. However, transcript levels of ANT3 and RPS4X, XIAP and ZFX were significantly ($p<0.05$) higher in the SCNT blastocysts of Group 2 than those in the SCNT blastocysts of Group 1.

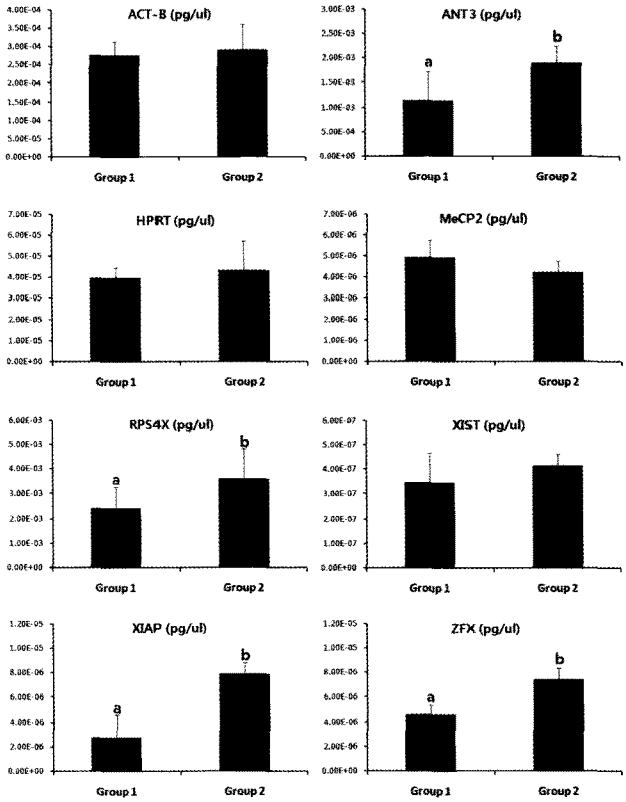

Fig. 3. Transcript levels (Mean±SEM) of X-linked genes in SCNT blastocysts. Group 1; Oocytes were immediately activated after fusion, Group 2; Oocytes were activated at 1 h post-fusion. [a,b] Different superscripts indicate significant ($p<0.05$) differences.

DISCUSSION

Cell fusion of the oocyte-nucleus complexes is a critically important step in the SCNT procedure, and the subsequently reconstructed eggs are required to oocyte activation for initiating development of SCNT embryos. However, the most efficient methods for the timing of fusion and oocyte activation between simultaneous fusion/activation and delayed activation after fusion have been still inconclusive. Here, we have definitely proven that delayed oocyte activation after fusion induces the increased incidence of normal nuclear remodeling, including formation of PCC, PN and chromosome spread, thus increasing subsequent developmental potential of the SCNT embryos. Moreover, delayed activation me-

thod induces more reprogrammed SCNT embryos by consequence of increased telomerase activity and several genes related with X-chromosome, when compared with simultaneous fusion/activation. We have considered that use of delayed activation method is advantageous in the fusion/activation step for production of SCNT embryos.

The production of SCNT embryos and offspring by simultaneous fusion/activation methods has been successfully reported in several species, including porcine and bovine (Akagi *et al.*, 2003; Kurome *et al.*, 2003; Park *et al.*, 2001). It has been also reported that the developmental potential to blastocyst stage is similar in the simultaneous fusion/activation and delayed activation after fusion in porcine SCNT embryos (Kurome *et al.*, 2003). On the contrary to these reports, it has been reported that the increased developmental potential of SCNT embryos and successful production of SCNT offspring are observed in the delayed activation after fusion method when compared with simultaneous fusion/activation method in porcine and bovine, as shown in our results (Akagi *et al.*, 2003; De Sousa *et al.*, 2003). Furthermore, the high fusion rate was observed in the SCNT embryos using needle fusion method in present study, thus we have considered that needle fusion method is highly increased by more exact fusion stimulations than wire chamber method in the oocyte-cell complexes.

Ii is well known that MPF activity is induced to the considerable changes of donor nucleus as called nuclear remodeling, including NEBD, PCC and PN formation (Collas and Robl, 1991; Kawahara *et al.*, 2005; Shin *et al.*, 2002). Oocytes with low level of MPF activity exhibit a limited nuclear remodeling capacity and embryos developmental potential of the introduced donor nucleus or sperm nucleus. Further, it has been proven that the increased nuclear remodeling and reprogramming is induced by extended exposure of donor nucleus to recipient cytoplasm with high level of MPF activity, suggesting that high level of MPF activity is closely responsible for nuclear remodeling (Wakayama *et al.*, 1998). The level of MPF activity of reconstructed eggs was gradually degraded by the increased intracellular level of calcium into oocytes by electro-fusion or chemicals. It has been reported that the MPF activity at 1 h after oocyte activation is reached to ~70% level, when compared to those of non-activated oocytes in bovine (Akagi *et al.*, 2003; Tian *et al.*, 2002). However, it has been reported that NEBD and PCC formation by high level of MPF activity is not immediately induced in the donor nucleus introduced into oocytes, rather than induced is fully formed at 1 or 1.5 h at fusion of donor nucleus in porcine and bovine (Akagi *et al.*, 2003; Kawahara *et al.*, 2005; Kurome *et al.*, 2003). Even though the donor nucleus is introduced into oocytes by simultaneous fusion/activation method, NEBD and PCC formation is occurred during the degradation of MPF activity of the oocytes. Therefore, it has assumed that NEBD and PCC formation is not fully achieved in the donor nucleus introduced into oocytes by simultaneous fusion/activation method. From this point of view, we have considered that delayed activation after fusion method may be induced to full NEBD and PCC formation of the donor nucleus without degradation of MPF level till oocytes are activated at 1 hpf. Interestingly, our results have shown that the incidence of normal PCC formation with normal spindles formation is slightly increased in Group 2 embryos using delayed activation after fusion method, compared to those of Group 1 using simultaneous fusion/activation. Furthermore, the incidence of normal PN formation with fully swollen status and chromosome spreads in Group 2 embryos was higher than Group 1 embryos. After NEBD and PCC formation, nuclear envelope of the donor nucleus is subsequently reformed in the activated recipient cytoplasm with low level of MPF activity. The size of PN was obviously increased in sperm-fertilized or donor nucleus-injected oocytes when compared to nuclei of most somatic cell (Prather *et al.*, 1990). The donor nucleus with PN status was efficiently reprogrammed by exchange of proteins and/or reprogramming factors, and it has been reported that the fully swollen PN is an important aspect of the remodeling and reprogramming process of the SCNT embryos (Prather *et al.*, 2004). Further, DNA replication of the donor nucleus is initiated in the SCNT embryos with PN status. As shown in confocal images of Fig. 1. E, F and G, we have considered that the scattered and non-swollen nuclei with nuclear fragmentation may be induced from the elongated PCC by disarrangements of microtubule. Thus, reprogramming and DNA replication of the donor nucleus was induced to aberrant patterns, resulting in decreasing developmental potential of SCNT embryos.

Meanwhile, it has been reported that the prolonged time exposure of donor nucleus in the non-activated oocytes over 2 h is increased in the incidence of fragmented chromosome and nuclear aneuploidy, and decreased in the developmental potential of bovine and porcine SCNT embryos (Aston *et al.*, 2006; You *et al.*, 2010). Thus, we have considered that prolonged exposure interval to non-activated recipient cytoplasm after fusion may have deleterious effects on the nuclear remodeling and reprogramming processes.

Further, somatic cells used for donor cells used is generally exhibited to down-regulated telomerase activity with a limited proliferation capacity, except for embryos, embryonic stem cells and cancer cells, and up-regulated telomerase activity is required for the extension of telomere length in the SCNT embryos (Betts *et al.*, 2001). Even though telomerase is composed telomerase reverse transcriptase (TERT) catalytic subunit, telomerase RNA component (TERC) and some proteins, the ex-

pression level of telomerase activity is tightly regulated by TERT transcribed from DNA, and TERT expression is regulated by epigenetic patterns, including methylation and histone modification and expression of TERT is repressed by methylation and histone modification in most somatic cells (Guilleret *et al.*, 2003; Hoare *et al.*, 2001). Thus, the up-regulated telomerase activity could imply to re-initiate TERT expression in the SCNT embryos (Betts *et al.*, 2001; Jeon *et al.*, 2008). It is well known that epigenetics modification of the fused donor nucleus is a most important index of cellular reprogramming to early embryonic totipotency in the SCNT embryos (Niemann *et al.*, 2008). Further, it has been proven that the noticeably up-regulated telomerase activity is exhibited in the bovine SCNT embryos derived from fibroblasts treated with de-methylation when compared with non-treated counterparts (Jeon *et al.*, 2008). The elongated proliferative and self-renewal capacity of undifferentiated embryonic stem cells was also maintained by up-regulated telomerase activity (Yang *et al.*, 2008). As shown in our result, it has implied that the up-regulated telomerase activity is probably due to more efficiently reprogrammed blastocysts with extended proliferative and self-renewal capacity in the blastocysts of Group 2.

Inactivated one of the two X-chromosome of the donor cells fused into recipient cytoplasm was re-activated and subsequently re-inactivated during SCNT development. Inactivation of X-chromosome (XCI) is a silencing process of genes for dosage compensation which equally expressed in the male and the female. Even though silencing genes was firstly induced and continually maintained by coating of XIST RNA on one of the two X-chromosome which will be inactivate in female embryos, maintains of the inactivated X-chromosome are synergistically archived through histone modification and DNA methylation (Chang *et al.*, 2006; Heard, 2004). Further, it has been reported that re-activation of the inactivated X-chromosome with changed transcript levels of X-linked genes was observed in the demethylated bovine fibroblasts and these fibroblasts-derived SCNT embryos (Jeon *et al.*, 2008). Therefore, transcript levels of X-linked genes by dynamic changes of the X-chromosome related to DNA methylation and histone modification have been used for an index of reprogramming in the female SCNT embryos (Jeon *et al.*, 2008).

Over-expressed ANT3, integral protein of the inner mitochondrial membrane, is induced to cellular apoptosis in cultured cells (Zamora *et al.*, 2004). On the contrary to ANT3, XIAP is a member of the inhibitor of apoptosis family, and reduced XIAP expression is induced to cellular apoptosis (Harada and Grant, 2003). As shown in our results, transcript levels of both ANT3 and XIAP were interestingly increased in the SCNT blastocysts of Group 2, compared to those of Group 1. Further, RPS4X is responsible for a part of cytoplasmic ribosome 40S, and ZFX is a zinc finger protein expressed from X-chromosome. It has been reported that both RPS4X and ZFX is transcribed from both the active and inactive X-chromosome that have functional Y-homologs (Boggs and Chinault, 1994; Brown and Chow, 2003). Transcript level of both RPS4X and ZFX was also increased in the SCNT blastocysts of Group 2, compared to those of Group 1. As shown in our results, transcript level of XIST, a fundamental modulator of XCI process, was similar in the SCNT blastocysts of Group 1 and Group 2, suggesting that XCI process is probably induced to similar level in the SCNT blastocysts of Group 1 and Group 2. The exact cause for the varied transcript levels of X-linked genes have not fully understood in the SCNT embryos. However, it has been demonstrated that aberrant XCI patterns and differential methylated regions (DMR) of XIST is exhibited in the SCNT embryos and aborted calf clones, suggesting that process of XIC is incompletely reprogrammed in the SCNT embryos and offspring (Liu *et al.*, 2008; Nolen *et al.*, 2005; Xue *et al.*, 2002). We have also considered that up-regulated X-linked genes may be due to more efficient reprogramming in the SCNT blastocysts of Group 2, on the other hand, varied transcript levels of X-linked genes may be also due to incomplete reprogramming of SCNT embryos.

In conclusion, oocyte activation at 1 h post-fusion could enhanced the normal chromosome remodeling incidences of the injected donor nucleus, developmental potential of the SCNT embryos, telomerase activity and X-linked genes of SCNT blastocysts. Further, this method was a useful activation method which induces more efficient nuclear remodeling and reprogramming of the donor nucleus in the bovine SCNT embryos. However, incomplete reprogramming of the donor nucleus was still displayed in the SCNT embryos.

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