Variation of Transcribed X-linked Genes in Bovine Embryos Cloned with Fibroblasts at Different Age and Cell Cycle

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ABSTRACT

The present study compared the developmental potential, telomerase activity and transcript levels of X-linked genes (ANT3, HPRT, MeCP2, RPS4X, XIAP, XIST and ZFX) in the bovine somatic cell nuclear transfer (SCNT) embryos derived from different age and cell cycle of female donor nucleus. In experiment 1, the fusion rate, cleavage rate to 2-cell stage, developmental rate to blastocyst stage, and the mean number of total and ICM cells was slightly increased in embryos cloned with fetal fibroblasts compared to those with adult fibroblasts, but there was no significantly ($p<0.05$) differences. Telomerase activity was also similar in blastocysts cloned with fetal and adult fibroblasts. Up-regulated RPS4X and down-regulated MeCP2, XIAP, and XIST transcript level were observed in blastocysts cloned with adult fibroblasts, compared to those with fetal fibroblasts. In experiment 2, the fusion rate, cleavage rate to 2-cell stage, developmental rate to blastocyst stage, and the mean number of total and ICM cells was significantly ($p<0.05$) increased in embryos cloned with fetal fibroblasts at early G1 phase of the cell cycle, compared to those of fetal fibroblasts at late G1 phase. DNMT1 transcript was observed to significantly ($p<0.05$) increased in the fetal fibroblasts at 3 hrs after trypsin treatment of confluent culture. Further, level of telomerase activity and transcribed X-linked genes was also significantly ($p<0.05$) higher in the early G1 SCNT blastocysts than those of late G1. The results imply that fetal fibroblasts at early G1 phase induces the enhanced developmental potential and up-regulated telomerase activity and X-linked gene, but aberrant transcript pattern of X-linked genes may be displayed in the SCNT embryos.

(Key words : Somatic cell nuclear transfer, Donor cell stage, Donor cell age, Telomerase activity, Transcript of X-linked genes)

INTRODUCTION

Somatic cell nuclear transfer (SCNT) technique has many benefits for production of the genetically unified and endangered animals, and cloned animal by SCNT have been successfully produced in the diverse animal species, including bovine. The numerous significant advances have been reported in the procedure of SCNT, but low efficiency in the production of cloned animals and embryos by SCNT is still a major obstacle for application of this technique (reviewed by Keefer, 2008). The low efficiency of SCNT embryos might be related with a consequence of incomplete epigenetic reprogramming of the donor cell from its differentiated status into the totipotent state of the early embryo (Niemamm *et al.*, 2008).

It has been reported that successful production of SCNT embryos is influenced by many factors, including manipulation methods, cell stage and source recipient oocytes and donor cells, interval time of activation and fusion, and donor cell stage (reviewed by Keefer, 2008). However, a most important factor is probably sources of the donor cells for the success of SCNT embryos. Even though same type of fully differentiated donor fibroblasts isolated either from fetal or adult ear skin are influenced developmental potential of bovine SCNT (Kasinathan *et al.*, 2001). Another factor affecting SCNT efficiency is the cell cycle stage of donor cells (Kasinathan *et al.*, 2001; Miyamoto *et al.*, 2007). It has been reported that the donor nucleus at the quiescent G0 or G1 phase of cell cycle is suitable for successful production of SCNT embryos by achievement of normal DNA replication (Miyamoto *et al.*, 2007). More importantly, it has been suggested that the intrinsic factor for success of SCNT embryos and offspring is nuclear reprogramming of the injected donor cells into the totipotent state of the early embryo (Oback *et al.*, 2008).

Telomerase activity is responsible for extension of telomere repeats which protect the end of the DNA strand from erosion. Up-regulation of telomerase activity in the SCNT embryos is certainly necessary for telomere extension of the injected donor nucleus with short telomere length (Betts *et al.*, 2001). Further, one of the two X-chromosomes is inactivated in most differentiated somatic cell for dosage compensation, and the injected female donor cells is also dy-

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namic modifications of re-activation of the inactivated X-chromosome. One of the two X-chromosomes is subsequently re-activated (Chang *et al*., 2006; Heard, 2004). Up-regulation of telomerase activity is controlled through epigenetic mechanisms, including DNA methylation and histone modification, and diverse X-linked genes are also transcribed by epigenetic mechanism during embryo development (Nino-soto *et al*., 2007). Therefore, both levels of telomerase activity and transcribed X-linked genes have been used as an index of cellular reprogramming of SCNT embryos (Jeon *et al*., 2008).

The present study therefore examined the developmental potential in the bovine SCNT embryos using donor fibroblasts derived from fetal and adult, and cell cycle of the donor fibroblasts at early and late G1, respectively. In addition, the level of telomerase and transcribed X-linked gene were also evaluated for comparison of reprogramming incidence in the SCNT embryos.

MATERIALS AND METHODS

Media and Chemicals

Chemicals were purchased from the Sigma Chemical Company (UAS) and media from Gibco BRL (USA), unless otherwise described. The medium used for in vitro maturation (IVM) of cumulus-oocytes complexes (COCs) 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. The medium used for *in vitro* culture of embryos was modified synthetic oviduct fluid (mSOF) supplemented with 2.9 μg/ml EDTA and 8 mg/ml bovine serum albumin (BSA, essentially fatty acid free). The culture medium of donor cells was Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) 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

Primary culture of skin fibroblasts was established from ear tissues both of a 60-days female fetus from a slaughter house and of a 4-year-old female bovine, respectively. 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% (w/v) Trypsin-EDTA solution. After being washed 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. Further, transcript levels of DNMT1 were analyzed in the fetal fibroblasts at 1, 2, 3, 4, 6, 7, 8 and 10 hrs after trypsin treatment (Fig. 3). The level of fetal fibroblasts at 1 and 3 hrs were considered as a presumptive early and late G1 stage of the cell cycle, respectively, as previously described by Kasinathan *et al*. (2001) and Kimura *et al*. (2003).

Nuclear Transfer and Oocyte Activation

The SCNT procedure was performed according to the previously described protocol (Jeon *et al*., 2008). Briefly, ovaries were obtained from a slaughter house and 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 cytoplast activation was 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. The 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 50 μM $MgCl_2$ using an ECM 2001 BTX cell manipulator (VWR Laboratories, USA) equipped with electrode needles. Fused eggs were subsequently activated with 5 μM ionomycin in HEPES-TALP for 5 min, followed by exposure to 30 mg/ml BSA for 4 min, and further incubated in modified synthetic oviduct fluid (mSOF) containing 10 μg/ml cycloheximide for 5 hrs at 38.5℃ and 5% CO_2 in air.

In Vitro Culture of Embryos

After cycloheximide treatment, 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 additional 6 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).

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 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 (Mecasys, Korea). 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-$dT_{12\text{-}18}$ primer (Invitrogen, USA), 1 μl of 10 U/μl RNase inhibitor (Invitrogen, USA), 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 were 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, USA) 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; ANT-3, 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 (beta-actin; ACT-B) were analyzed for the pattern of the transcripts by quantitative real time RT-PCR. Primer sequences, the size of amplified products, annealing and acquisition temperatures are shown in Table 1. 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 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, USA) as previously described by Jeon *et al.* (2008). A pool of 5 Day-9 blastocysts in three replicates from each groups immediately frozen at −80℃ for future analysis or lysed in 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 at a concentration of 5 blastocysts/20 μl 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 using the PCR reagent LightCycler Fast-Start DNA Master SYBR Green 1 (Roche, USA) according to the manufacturer's protocol, containing 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 to be 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, USA) second derivative method of crossing point (Cp) determination, and at least five replicates of RQ-TRAP were carried out in each sample.

Experimental Design

The present study comprised of two experiments. In Experiment 1, the developmental potential and transcript level of X-linked genes with level of telomerase activity were compared between the SCNT embryos derived from the fetal and adult fibroblasts. In Experiment 2, *in vitro* developmental potential and the transcript levels of X-linked genes with level of telomerase activity were compared between SCNT embryos derived from fetal fibroblasts at presumptive early and late G1 stage, respectively.

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 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

Developmental Potential of SCNT Embryos

In Experiment 1, the rates of fusion, cleavage to 2-cell sta-

Table 1. Sequence-specific primers used for RT-PCR

Gene	Primer sequences (5'-3')	Band size of PCR product (bp)	Annealing temperature (℃)	Acquisition temperature (℃)
ACT-B	F-CGTGACATTAAGGAGAAGCTGTGC R-CTCAGGAGGAGCAATGATCTTGAT	374	63	86
ANT3	F-TTCCCTGTGCTTCGTCTACC R-TGCCCTTGTACATGATGTCG	383	60	89
HPRT	F-CGAGATGTGATGAAGGAGATGG R-TTTCAAATCCAACAAAGTCTGG	397	61	81
MeCP2	F-GGGACCCATGTATGATGACC R-ATGTGTCGCCTACCTTTTCG	173	60	81
RPS4X	F-ATTAAGATCGATGGCAAAGTCC R-AAAAGAACCTGGATGTCTCTCC	408	59	80
XIAP	F-GGCGACACTTTCCTAATTGC R-AAGCATAAAATCCAGCTCTTGC	213	58	78
XIST	F-CCTTGTCATGTGGATATCATGG R-AATGTCCTTGGAAAGACTTTGG	224	59	84
ZFX	F-TCTATCCTTGCATGATTTGTGG R-AGAGTCTGCGGACCTATATTCG	494	57	83
DNMT1b	F-CAGTTCACATATCAAAGTACCAGCA R-TCATTTCAGCCTGAGCTCCT	152	60	81

ge and development to blastocyst stage between the SCNT embryos derived from fetal and adult fibroblasts are summarized in Table 1. No significant ($p<0.05$) differences in the fusion and cleavage rate were observed between the SCNT embryos derived from fetal and adult fibroblasts (91.3 vs. 92.0% and 86.0 vs. 91.4%, respectively). The percentages of embryos that reached to blastocysts and hatching and hatched blastocyst stages on day 9 were slightly increased in the SCNT embryos derived from fetal fibroblasts, compared to those of the SCNT embryos derived from adult fibroblasts, but there was no significant ($p<0.05$) difference (34.3 vs. 43.0% and 26.4 vs. 31.8%, respectively). The mean numbers of total and ICM cells in the SCNT embryos derived from fetal and adult fibroblasts were 161±15.7 and 36.3±7.9 cells, and 186±17.3 and 46.6±10.1 cells, respectively, and there were no significant ($p<0.05$) differences in the mean numbers of total and ICM cells of blastocyst stages.

In Experiment 2, the rates of fusion, cleavage to 2-cell stage and development to blastocyst stage between the SCNT embryos derived from fibroblasts at presumptively early and late G1 stage are summarized in Table 2. The rates of fusion and cleavage to 2-cell stage were similar between the SCNT embryos derived from presumptively early and late G1 stage fibroblasts (90.8 vs. 91.6% and 92.1 vs. 98.2%, respectively). The percentages of embryos developed to blastocysts stage were slightly increased in the SCNT embryos derived from fibroblasts at presumptively early G1 stage compared to those of late G1, but there was no significant ($p<0.05$) difference (46.5 vs. 35.5%, respectively). However, the percentages of embryos that reached to hatching and hatched blastocyst stages on day 9 were significantly ($p<0.05$) higher in the SCNT embryos derived from fibroblasts at presumptive early G1 fibroblasts than those of late G1 (31.5 vs. 13.8 %, respectively), as shown in Fig. 1. The mean numbers of

Table 2. *In vitro* developmental potential and mean number of total and ICM cells in embryos cloned with fetal and adult fibroblasts

Donor cells	Fused eggs	No. (%) of embryos developed to			No. (%) of total cell* (mean±SEM)
		2-cell	Blastocyst	H-blastocyst	
Adult	355/389 (91.3)	283/329 (86.0)	113/329 (34.3)	87/329 (26.4)	161±15.7 (36.3±7.9)[a]
Fetal	161/175 (92.0)	138/151 (91.4)	65/151 (43.0)	48/151 (31.8)	186±17.3 (46.6±10.1)[b]

H-blastocyst, hatching and hatched blastocysts.
5 replicates.
[a,b] Different superscripts within columns indicate significant ($p<0.05$) differences.

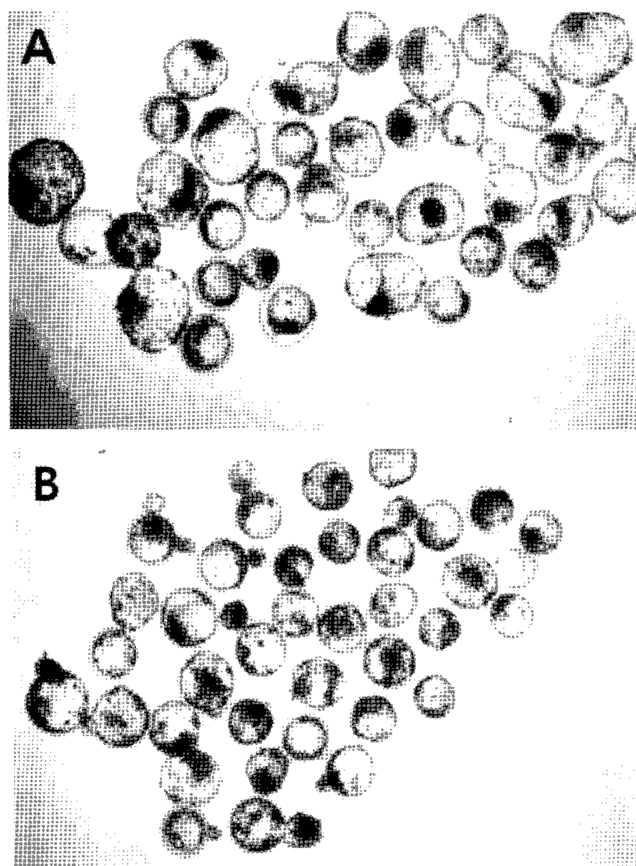


Fig. 1. Day-9 bovine blastocysts cloned with fetal fibroblasts at 1 (A) and 3 (B) hrs after trypsin treatment (×50).

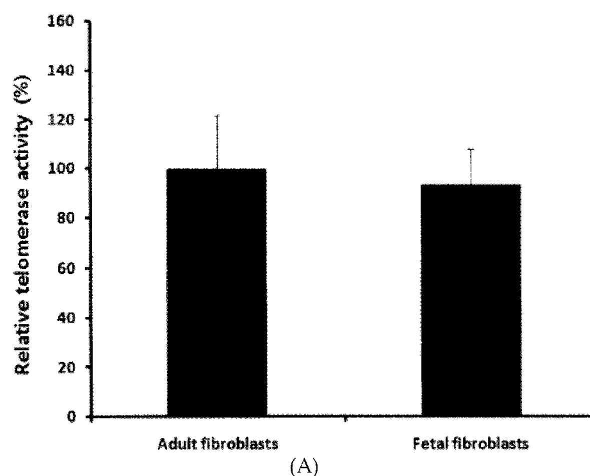
total and ICM cells in the SCNT embryos derived from fibroblasts at presumptively early G1 stage were significantly ($p<0.05$) higher than those of late G1 stage (179±19.1 and 43.4±9.8 cells compare with 121±6.2 and 29.3±8.8 cells, respectively).

Relative Telomerase Activity (RTA) of SCNT Embryos

Telomeres activity by RQ-TRAP was investigated in the SCNT blastocysts, as shown in Fig. 2. In Experiment 1, RTA in the SCNT blastocysts derived from adult fibroblasts was considered as 100% for comparison with SCNT blastocysts derived from fetal fibroblasts. RTA in the SCNT blastocysts derived from fetal fibroblasts was 94.2% compared with the SCNT blastocysts derived from adult fibroblasts. RTA was no significant ($p<0.05$) difference between the SCNT blastocysts derived from fetal and adult fibroblasts.

In Experiment 2, RTA in the SCNT blastocysts derived from fibroblasts at presumptively early G1 stage was considered as 100% for comparison with SCNT blastocysts derived from fibroblasts at late G1 stage. RTA was significantly ($p<0.05$) increased in the SCNT blastocysts derived from fibroblasts at presumptively early G1 stage than those of late G1 stage (100 vs. 65.2%, respectively).

Transcript Levels of DNMT1 in the Fibroblasts



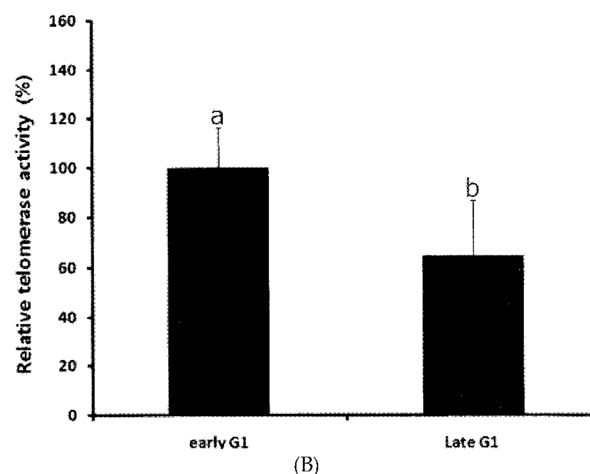


Fig. 2. Relative telomerase activity (Mean±SEM) of bovine blastocysts cloned with adult and fetal fibroblasts (A), and fibroblasts at early G1 and late G1 stage (B). Telomerase activity of blastocysts cloned with adult fibroblasts and fibroblasts at early G1 stage of the cell cycle was considered as a 100% for comparison to those with fetal fibroblasts and fibroblasts at late G1 stage, respectively (A). [a,b] Bars with different letters indicate significant ($p<0.05$) differences.

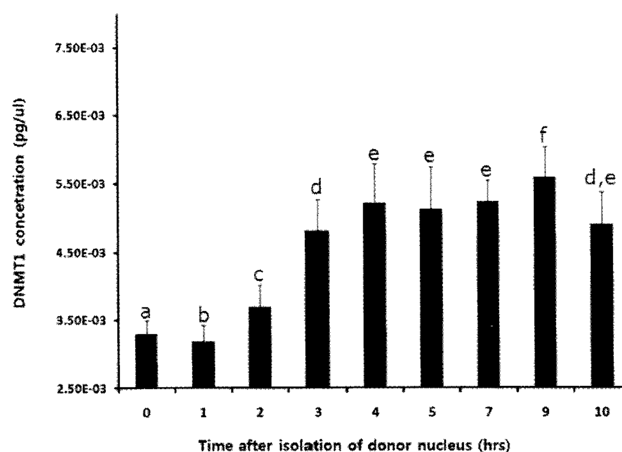


Fig. 3. Transcript levels (Mean±SEM) of DNMT1 in fetal fibroblasts at 1, 2, 3, 4, 5, 7, 9 and 10 hrs after trypsin treatment. [a~e] Bars with different letters indicate significant ($p<0.05$) differences.

Table 3. ***In vitro*** **developmental potential and mean number of total and ICM cells in embryos cloned with fibroblasts at early G1 and late G1 stage of the cell cycle**

Fusion time after trypsination	Fused eggs	No. (%) of embryos developed to			No. (%) of total cell* (mean±SEM)
		2-cell	Blastocyst	H-blastocyst	
Within 1 hr (early G1)	138/152 (90.8)	117/127 (92.1)	59/127 (46.5)	40/127 (31.5)^{a}	179±19.1 (43.4±9.8)^{b}
At 3 hrs (late G1)	99/108 (91.6)	83/93 (89.2)	33/93 (35.5)	13/93 (13.8)^{b}	121±6.2 (29.3±8.8)^{b}

H-blastocyst, hatching and hatched blastocysts.
3 replicates.
^{a,b} Different superscripts within columns indicate significant (p<0.05) differences.

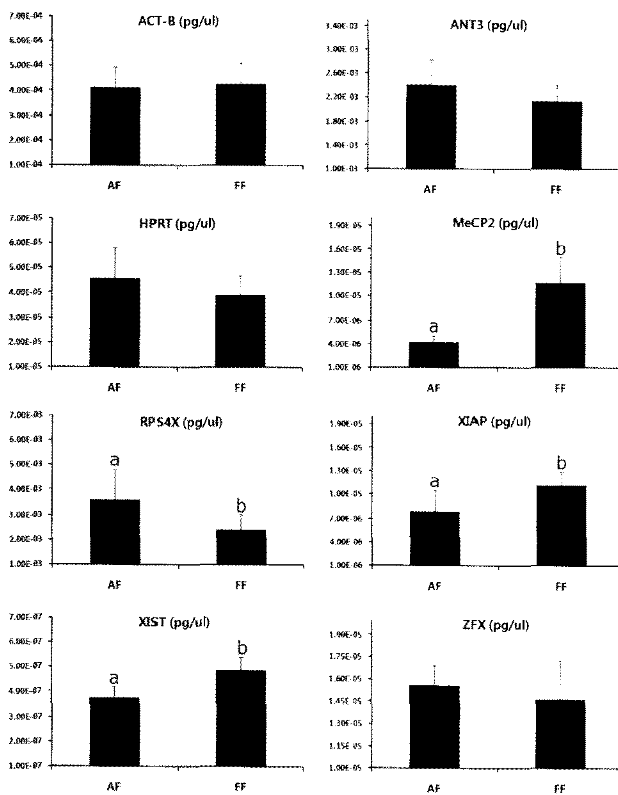


Fig. 4. Transcript levels (Mean±SEM) of X-linked genes in blastocysts cloned with adult (AF) and fetal (FF) fibroblasts. ^{a,b} Bars with different letters indicate significant (p<0.05) differences.

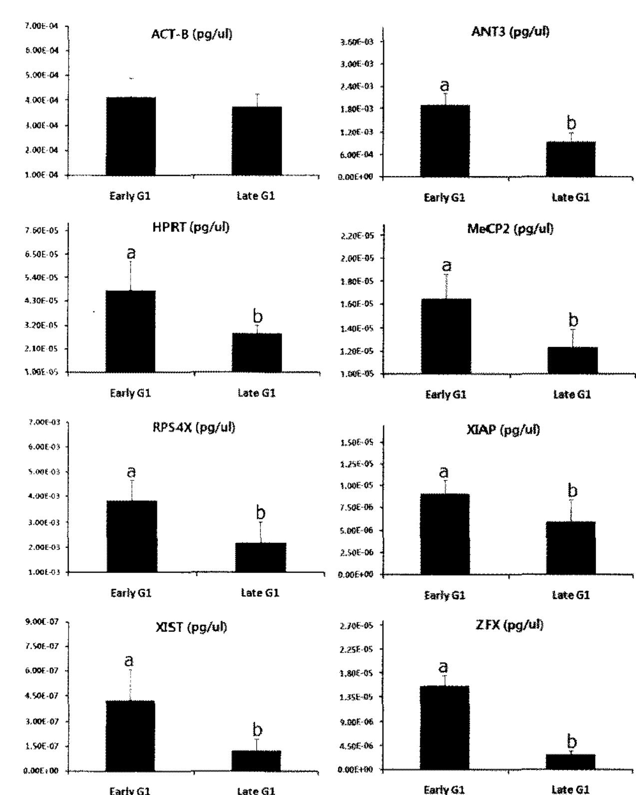


Fig. 5. Transcript levels (Mean±SEM) of X-linked genes in blastocysts cloned with fibroblasts at early G1 and late G1 stage of the cell cycle. ^{a,b} Bars with different letters indicate significant (p<0.05) differences.

Transcript level of DNMT1 was analyzed by quantitative real time RT-PCR in the fetal fibroblasts at 1, 2, 3, 4, 5, 7, 9 and 10 hrs after trypsin treatment as shown in Fig. 3. The level of DNMT1 in the fibroblasts tended to be increased in process of time after trypsin treatment. Further, transcript level of DNMT1 in the fibroblasts at 3 hrs was significantly(p<0.05) higher than those of 0, 1 and 2 hrs after trypsin treatment.

Transcript Levels of X-linked Genes of SCNT Embryos

Transcript levels of X-linked genes were evaluated by quantitative real time RT-PCR in the SCNT blastocysts. In Experiment 1 (Fig. 4), transcript levels of ACT-B, ANT3 and ZFX did not differ significantly (p<0.05) between the SCNT blastocysts derived from fetal and adult fibroblasts. However, the transcript levels of MeCP2, XIAP and XIST were significantly (p<0.05) higher in the SCNT blastocysts derived from fetal fibroblasts than those of adult fibroblasts. Meanwhile transcript level of RPS4X was significantly (p< 0.05) higher in the SCNT blastocysts derived from adult fibroblasts than those of fetal fibroblasts.

In experiment 2 (Fig. 5), transcript level of ACT-B did not differ significantly ($p<0.05$) between the SCNT blastocysts derived from fibroblasts at presumptive early and late G1 stage. However, transcript levels of ANT3, HPRT, MeCP2, RPS4X, XIAP, XIST and ZFX were significantly ($p<0.05$) higher in the SCNT blastocysts derived from fibroblasts at presumptive early G1 stage than those of late G1 stage.

DISCUSSION

Age and cell cycle stage of the donor cells is critically important factor for the successful development of SCNT embryos. In present study, our results have proven that even though fusion and cleavage rate is similar in the SCNT embryos using different age and cell cycle stage of the donor cells, developmental potential of the SCNT embryos to the blastocysts is partly influenced by the age and cell cycle stage of the donor cells. Further, telomerase activity and transcript level of X-linked genes were increased in the SCNT blastocysts derived from early G1 phase of fetal fibroblasts, but transcript level of X-linked genes was generally displayed aberrant pattern in the SCNT embryos produced using donor cells of different age and cell cycle stage.

In previous reports, it has been also demonstrated that the developmental potential to blastocyst stage is also similar in bovine SCNT embryos derived from fibroblasts of a 45-days-old fetus and, 4- and 15-years-old, as shown in our results (Kasinathan *et al.*, 2001). More importantly, the developmental potential to blastocysts has been shown to decrease in the SCNT embryos derived from late S phase of donor cells, suggesting that phase of cell cycle of the donor cells is highly influenced to developmental potential of SCNT embryos (Campbell *et al.*, 1994; Collas *et al.*, 1992). Successful animal cloning have been reported using enucleated and activated oocytes with an interphase state as a recipient cytoplasms, but enucleated and non-activated oocytes with a metaphase state have been primary used for s better reprogramming of the SCNT embryos (Keefer, 2008). The injected donor nucleus into the non-activated oocytes was induced to dynamic changes, nuclear remodeling, including nuclear envelope breakdown (NEBD), premature chromosome condensation (PCC) and pronucleus (PN) formation by high level of MPF activity (Collas and Robl, 1991; Kawahara *et al.*, 2005; Shin *et al.*, 2002). It has been reported that the dynamic changes of the injected donor nucleus is closely responsible for more reprogrammed SNCT embryos (Keefer, 2008). Further, if the injected donor nucleus is G1 phase of the cell cycle, DNA replication is normally completed at PN stage of SCNT eggs after treatment of oocytes activation. However, if the injected donor nucleus is S phase with replicated DNA, the SCNT eggs are displayed to aberrant DNA content by DNA re-replication at PN stage (Collas *et al.*, 1992). Therefore, donor nucleus at G1 phase has been often preferred for production of SCNT embryos. Interestingly, our results have shown that the developmental potential to hatching and hatched blastocysts cloned with early G1 phase of donor nucleus is remarkably increased than those with late G1 phase. In addition, number of total and ICM cells was also increased in blastocysts cloned with donor nucleus at early G1 phase. It has been suggested that efficient reprogramming ability of SCNT embryos facilitates the enhanced developmental potential with increased total cell number and ICM ratio (Jeon *et al.*, 2008; Santos *et al.*, 2008). We have also considered that high developmental potential in embryos cloned with fibroblasts at early G1 stage is due to more reprogrammed donor nucleus.

The injected donor nucleus needs to primitively reprogram to early embryonic status in the transcriptional profile by epigenetic modification, including DNA methylation and histone modification. Incomplete reprogramming of the donor nucleus in the DNA methylation and histone modification is thought to cause poor outcome of SCNT embryos and offspring (Niemann *et al.*, 2008). The methylation pattern in the somatic cells is induced with DNA methyltransferase (DNMT) family which catalyze the transfer of a methyl group to DNA. DNMT1 is especially important for maintenance of DNA methylation during DNA replication (Lin *et al.*, 2000). It has been reported that the expression of DNMT1 is increased in the mouse oncogenic transformed cells at late G1 phase of cell cycle (Kimura *et al.*, 2003). As shown in the Fig. 3, we also observed that the increased transcript level of DNMT1 is remarkably increased at 3 hrs after trypsin treatment from confluent status, and the fibroblasts at 3 hrs may be late G1 or early S phase of cell cycle. Thus, we have considered that the increased expression level of DNMT1 is influenced to methylation pattern of the injected donor nucleus, thus, donor nucleus at early G1 stage is better than late G1 stage by effective reprogramming for the production of SCNT embryos.

Further, somatic cells is generally displayed to down-regulation of telomerase activity with a limited proliferation capacity by the decreased expression of telomerase reverse transcriptase (TERT) catalytic subunit which compose telomerase, and TERT expression is also controlled by epigenetic modification of DNA (Guilleret *et al.*, 2003; Hoare *et al.*, 2001). As shown in our results, the down-regulated telomerase activity in blastocysts cloned with fibroblasts at late G1 phase of the cell cycle was certainly implied that reprogramming level of the embryos is decreased by incomplete DNA modification, compare to those at early G1. Moreover, up-regulation of telomerase activity was induced to the elongated proliferative and self-renewal capacity of undifferentiated embryonic stem cells (Yang *et al.*, 2008). The SCNT blastocysts with up-regulated telomerase activity should be exhibited to extended proliferative and self-renewal capacity.

X chromosome inactivation (XCI) in the female cells is a process for balance of X-linked genes expressed between the

male and female. The female donor nucleus injected into recipient cytoplasms was also required to re-activation of the inactivated X-chromosome and subsequent inactivation of one copy of the X chromosomes during embryo development. Initial coating by XIST RNA expressed on the future inactivated X-chromosome, subsequent histone modification and DNA methylation of the inactivated X-chromosome was induced to silencing of X-linked genes (Heard, 2004; Chang *et al.*, 2006). As shown in our result, all of transcript level of X-linked genes was down-regulated in embryos cloned with fibroblasts at late G1, compared to those with early G1. We have also considered that the decreased transcript level of X-linked genes in embryos cloned with fibroblasts at late G1 may be due to less complete reprogramming capacity than those at early G1. Further, varied transcript level of X-linked genes was displayed in embryos cloned with fetal and adult fibroblasts. Transcript level of XIST was up-regulated in embryos cloned with fetal fibroblasts, compared to those with adult fibroblasts, suggesting that process of XCI may be induced to more effective in the fetal SCNT embryos. However, transcript level of ANT3, HPRT and ZFX was similar between embryos cloned with fetal and adult fibroblasts, as shown in our results. ANT3 is integral protein of the inner mitochondrial membrane, and its over-expression is generally induced to cellular apoptosis in in vitro cultured HeLa cells (Zamora *et al.*, 2004). On the contrary to ANT3, XIAP is responsible for the inhibition of apoptosis, and decreased transcription level of XIAP is induced to cellular apoptosis (Harada and Grant, 2003). As shown in our results, the slightly increased transcript level ANT3 and the decreased transcript level of XIAP was observed in embryos cloned with adult fibroblasts, compared to those with fetal fibroblasts, suggesting that tolerance of apoptosis may be better in embryos cloned with fetal adult fibroblasts than those with adult fibroblasts. MeCP2 is a transcriptional regulator which binds specifically to methylated DNA (Bogdanović and Veenstra, 2009). However, transcription level of MeCP2 was increased in embryos cloned with fetal fibroblasts, compared to those with adult fibroblasts, implying that the incidence of methylated DNA is probably increased in embryos cloned with fetal fibroblasts.

Further, RPS4X is a 40S ribosomal protein S4 expressed from X-chromosome for a part of cytoplasmic ribosomes, and ZFX is a zinc finger protein for transcriptional activator expressed from X-chromosome. It has been reported that both RPS4X and ZFX is escape genes from XCI status which transcribed from both the active and inactive X-chromosome that have functional Y-homologs (Boggs and Chinault, 1994; Brown and Chow, 2003). In the present results, transcription level of ZFX was similar between embryos cloned with fetal and adult fibroblasts, but transcription level of RPS4X was higher in embryos cloned with adult fibroblasts than those with fetal fibroblasts. Our results on the transcription level of X-linked genes have implied to variously express in embryos cloned with fetal and adult fibroblasts. The varied expressions of X-linked genes have not been fully understood in the SCNT embryos. Further, earlier report has showed the successful XCI in some cells and the failure of XCI in other cells within the same bovine SCNT embryos (Nolen *et al.*, 2005). Differential methylated regions (DMR) of XIST were also observed in the SCNT embryos and aborted calf clones (Liu *et al.*, 2008), suggesting that the process of XCI is probably displayed an aberrant pattern in the SCNT embryos. We have considered that variation of X-linked genes is thought to be a consequence of aberrant process of XCI by incomplete reprogramming of the donor cell.

In conclusion, developmental potential and telomerase activity of SCNT embryos was influenced by type of donor cells, but varied transcription level of X-linked genes was also observed in the SCNT embryos, suggesting that incomplete reprogramming of the donor nucleus injected into recipient cytoplasms is still displayed in the SCNT embryos.

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