Control of MPF Activity of Recipient Oocytes and Subsequent Development and DNA Methylation of Somatic Cell Nuclear Transfer Bovine Embryos

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ABSTRACT

We attempted to control the maturation promoting factor (MPF) activity and investigated the subsequent reprogramming of bovine somatic cell nuclear transfer (SCNT) embryos. Serum-starved adult skin fibroblasts were fused to enucleated oocytes treated with 2.5 mM caffeine or 150 μM roscovitine. The MPF activity, nuclear remodeling patterns, chromosome constitutions and development of SCNT embryos were evaluated. Methylated DNA of embryos was detected at various developmental stages. The MPF activity was increased by caffeine treatment or reduced by roscovitine treatment ($p<0.05$). Blastocyst development was higher in the caffeine-treated groups (27.6%) than that of the roscovitine-treated group (8.3%, $p<0.05$). There was no difference in the apoptotic cell index among the three groups. However, the mean cell number of blastocysts was increased in the caffeine-treated group ($p<0.05$). Higher methylation levels were observed in the Day 3 embryos of the roscovitine-treated group (50.8%), whereas lower methylation levels were noted at Day 5 in the caffeine-treated group (12.5%, $p<0.05$). These results reveal that the increase in MPF activity via a caffeine-treatment creates a more suitable condition for nuclear reprogramming after SCNT.

(Key words : Somatic cell nuclear transfer, Reprogramming, Maturation promoting factor, Methylation status)

INTRODUCTION

Despite the great number of somatic cell nuclear transfer (SCNT) research, nucleo-cytoplasmic interaction remains to be not fully understood. The oocyte cytoplasm can modify a differentiated donor nucleus to the same state as a zygote nucleus-this process is generally referred to as reprogramming. The reprogramming of donor cells can be influenced by a number of factors; among these factors, the remodeling type of a transferred nucleus has been suggested to affect early embryonic development (Cheong *et al*., 1993; Alberio *et al*., 2000; Lai *et al*., 2001; Tani *et al*., 2001).

The remodeling type of the transferred nucleus is affected by recipient cell cycle stage, which is associated with the activity of the maturation promoting factor (MPF) (Collas and Robl, 1991; Cheong *et al*., 1993). MPF is a serine/threonine protein kinase composed of $p34^{cdc2}$ kinase and cyclin B (Hashimoto and Kishimoto, 1988), which regulates the cell cycle stage of cells. MPF activity is highest at metaphase II (MII) and is reduced by fertilization or activation owing to the inactivation of cyclin B (Nurse, 1990; Campbell *et al*., 1993). The activity of $p34^{cdc2}$ kinase promotes the meiotic or mitotic division of cells, and induces nuclear envelope break down (NEBD), chromosome condensation, and cytoskeletal reconstruction (Matsui and Markert, 1971). Therefore, a donor nucleus transferred to a recipient cytoplasm with a high level of MPF activity undergoes NEBD and premature chromosome condensation (PCC), whereas a donor nucleus transferred to a recipient cytoplasm with a low level of MPF activity does not undergo NEBD, and forms a pronucleus (PN)-like structure (Szollosi *et al*., 1998; Tani *et al*., 2001). However, the mechanism of nuclear reprogramming following nuclear remodeling type remains uncertain.

MPF activity is regulated by the phosphorylation of $p34^{cdc2}$ at the tyrosine-15 (Y15) and threonine-14 (T14) residues (Norbury and Nurse, 1992; Kikuchi *et al*., 2000). The cdc25 promotes MPF activity via the promotion of dephosphorylation at T14 and Y15, whereas Myt1/Wee-1 inhibits MPF activity by promoting phosphorylation at T14 and Y15. Caffeine induces an increase in MPF activity via the inhibition of Myt1/Wee1 activity, whereas vanadate (Kikuchi *et al*., 2000, Kwon *et al*., 2008)

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and roscovitine (Ito *et al.*, 2005) reduce MPF activity by suppressing cdc25 phosphatase activity.

In this study, we attempted to control the MPF activity by caffeine and roscovitine treatments, and investigated the subsequent *in vitro* development and DNA methylation patterns of SCNT embryos in bovine.

MATERIALS AND METHODS

Preparation of Somatic Cells

Skin cells were collected via ear biopsy of an adult female of Korean native cattle, followed by enzymatic digestion with 0.05% trypsin-EDTA in PBS. The supernatants containing the disaggregated cells were centrifuged for 5 min at 500 × g. The cell pellets were then diluted with 3 ml of Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% FBS (Gibco-BRL), 0.2 mM Na-pyruvate (Sigma, St. Louis, MO, USA), and 50 μg/ml of gentamicin, then cultured in a 25 ml culture flask at 37℃, 5% CO_2 in air. The cells were passaged four times prior to being frozen in DMEM with 10% FBS and 10% dimethylsulfoxide (DMSO), and then stored in liquid nitrogen. Prior to the nuclear transfer, the thawed cells were cultured in 4-well dish for 2~3 days, and serum-starved by replacing culture medium with DMEM supplemented with 0.5% FBS for 5 days (Wilmut *et al.*, 1997).

In Vitro Maturation of Oocytes

Bovine cumulus-oocyte complexes (COCs) were aspirated from the antral follicles (2~7 mm diameter) of ovaries with an 18 G needle and a 10 ml syringe. COCs with uniform ooplasm and compact cumulus cells were selected in Tyrode's lactate (TL)-Hepes containing 0.1% (w/v) polyvinyl alcohol (PVA; Sigma, St. Louis, MO, USA). Approximately 10 COCs were transferred into 50 μl of tissue culture medium 199 (TCM199; Gibco-BRL) supplemented with fetal bovine serum (FBS; Gibco-BRL), 0.02 U/ml of FSH (Sigma), and 1 μg/ml of estradiol (Sigma), then cultured for 20~22 h at 39℃ in 5% CO_2 in air for *in vitro* maturation (IVM).

Enucleation of Oocytes and Treatment with Caffeine and Roscovitine

After *in vitro* maturation, the cumulus cells of COCs were removed via vortexing in PBS supplemented with 0.1% hyaluronidase (Sigma) and 0.1% PVA (Sigma). Prior to enucleation, the MII chromosome masses of the oocytes were extruded by culture in TCM199 containing 0.4 μg/ml of demecolcine for 40 min (Yin *et al.*, 2002). Enucleation was conducted via the removal of the extruded chromosome mass and 1st polar body in Hepes-buffered TCM199 (Gibco-BRL) supplemented with 3 mg/ml of BSA and 5 μg/ml of cytochalasin B (CB; Sigma). More than 95% of oocytes were successively enucleated by this method (Baek *et al.*, 2005). The enucleated oocytes were cultured in TCM199 containing 3 mg/ml of bovine serum albumin (BSA; Sigma) and 5 mM caffeine (Sigma) or 150 μM roscovitine (Sigma) for 6 h or 1.5 h, respectively, prior to nuclear transfer.

MPF Kinase Assay

The MPF kinase assay was conducted by measuring the activity of its catalytic subunit, cdc2 kinase, with a MESACUP cdc2 kinase assay kit (MBL, Nagoya, Japan) as previously described by Anas *et al.* (2000) and Kwon *et al.* (2008). In brief, MII and enucleated oocytes with or without (control) caffeine or roscovitine treatment were washed twice in cdc2 kinase sample buffer and then stored frozen at −80℃. For the assay, the oocytes were lysed by freezing and thawing in liquid nitrogen and water, respectively. Five μl of oocyte extracts were mixed with 45 μl of kinase assay buffer and incubated for 30 min at 30℃. The phosphorylation reaction was terminated using 200 μl of stop reagent, and the solution was centrifuged for 15 sec at 13,000 × g. The cdc2 kinase activity was detected via ELISA analysis. Each of the reaction mixtures (100 μl) was transferred to a microwell strip coated with monoclonal antibody (MBL) recognizing the phosphorylated form of the biotinylated MV peptide. The microwells were incubated for 60 min at 25℃, and then washed five times in washing solution. Horseradish peroxidase (POD)-conjugated streptavidin solution (100 μl) was added to each well and incubated for 30 min at 25℃. After washing, 100 μl of POD substrate solution was added and incubated for an additional 5 min. Finally, 100 μl stop solution was added to each well, and the optical density was read at 492 nm using a microplate reader.

Nuclear Transfer, Electrofusion, and Activation

Nuclear transfer (NT) was conducted in Hepes-buffered TCM199 (Gibco-BRL) supplemented with 3 mg/ml of BSA and 5 μg/ml of CB (Sigma) at 27 h of IVM, regardless of treatments. Donor cells were trypsinized and centrifuged in TCM199 supplemented with 3 mg/ml of BSA. A donor cell was transferred into the perivitelline space of enucleated recipient oocytes. The reconstituted oocytes were fused electrically. They were placed between the wire electrodes of a fusion chamber (1 mm apart) overlaid with 0.3 M mannitol solution supplemented with 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$ and 0.1% BSA. A single DC pulse of 1.3 kV/cm was applied for 30 μsec using a BTX Electro Cell

Manipulator 200 (BTX, San Diego, CA, USA). The fused oocytes were activated 1 h after fusion treatment by 10 μM Ca-ionophore (A23187; Sigma) for 5 min, followed by 4 h of treatment with 2 mM 6-dimethlyaminopurine (6-DMAP; Sigma) prior to *in vitro* culture. Parthenogenetic embryos were made from MII oocytes treated with the same volumes of caffeine and roscovitine that were used for enucleated oocytes via the same activation condition of reconstituted oocytes in order to evaluate the effects of chemicals on embryonic development.

In Vitro Culture of Embryos

SCNT and parthenogenetic embryos were cultured in 50-μl droplets of CR1aa supplemented with 3 mg/ml of BSA for 4 days under an atmosphere of 5% CO_2 in air at 39℃. Afterward, the embryos were transferred to 50-μl droplets of CR1aa supplemented with 10% FBS (Gibco-BRL) and cultured for an additional 3 days under identical conditions.

Apoptotic Cell Analysis

A TUNEL assay was used to determine the number of apoptotic cells in blastocysts from NT embryos. Blastocysts were fixed for 40 min in PBS (Gibco-BRL) containing 3.7% (w/v) paraformaldehyde (Sigma) and permeabilized for 30 min in PBS containing 0.5% Triton X-100 and 3% (w/v) BSA at 37℃. The fixed blastocysts were incubated for 1 h in TUNEL reaction medium (*in situ* cell death detection kit, TMR red; Roche, Indianapolis, IN, USA) in darkness at 39℃. The broken DNA ends of the blastomere were labelled with TdT and fluorescein-dUTP. The blastocysts were stained for 30 min with 1 μg/ml of Hoechst 33342 (Sigma) at 39℃ and mounted on a glass slide in Vecta-Shield anti-fade (Vector Laboratories, Burlingame, CA, USA) under a cover slip. The numbers of total and apoptotic nuclei were determined via fluorescence microscopy.

Immunodetection of DNA Methylation

SCNT embryos at Day 3 (4 to 16-cell stage), 5 (16 to 32-cell stage) and 7 (morula to blastocyst) were washed with 0.05% Tween 20 in PBS, fixed for 15 min in 4% paraformaldehyde in PBS, and permeabilized for 15 min with 0.2% Triton X-100 in PBS at room temperature. To detect 5-methylcytosine (5-MeC), the embryos were treated for 30 min with 2 M HCl at room temperature and then neutralized for 10 min with 100 mM Tris/HCl buffer (pH 8.5). After 30 min of washing with PBS containing 0.05% Tween 20, the embryos were stored overnight at 4℃ in a blocking solution of PBS containing 1% BSA and 0.05% Tween 20. The embryos were incubated sequentially with 1 μg/ml of mouse monoclonal antibody against 5-MeC (Calbiochem, San Diego, CA, USA) at room temperature for 1 h and labelled with Alaxa-488 goat anti-mouse IgG (1:200 dilution; Molecular Probes, Eugene, OR, USA) for 30 min at 37℃. DNA was stained for 20 min with 3 μg/ml 4,6-diamidino-2-phenylindole (DAPI; Sigma) and the embryos were mounted on glass slides in Vecta-Shield anti-fade (Vector Laboratories). Z-stack images were obtained via the sequential scanning of samples at 1-μm intervals using an LSM 510 Meta NLO microscope (Zeiss, Jena, Germany) and merged with the Zeiss LSM image browser (ver. 3.2.0.70). Each nucleus in each embryo was manually outlined, and the fluorescence intensity emitted by each nucleus was measured using ImageJ 1.37v (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Data of MPF activity, embryo development, apoptotic cells, and methylation pattern were analyzed using Duncan's multiple range tests, using the general linear model procedure in SAS (SAS Institute, Inc., Cary, NC). Cell numbers were analyzed via *t*-test.

RESULTS

$p34^{cdc2}$ Activity of Recipient Oocytes

The cdc2 kinase activity in the MII oocytes and enucleated bovine oocytes treated with caffeine or roscovitine or left untreated (control) is shown in Fig. 1. Caffeine treatment resulted in a significantly higher cdc2 kinase activity than was observed with the MII and enucleated control oocytes ($p<0.05$). By way of contrast, roscovitine treatment resulted in a lower cdc2 ki-

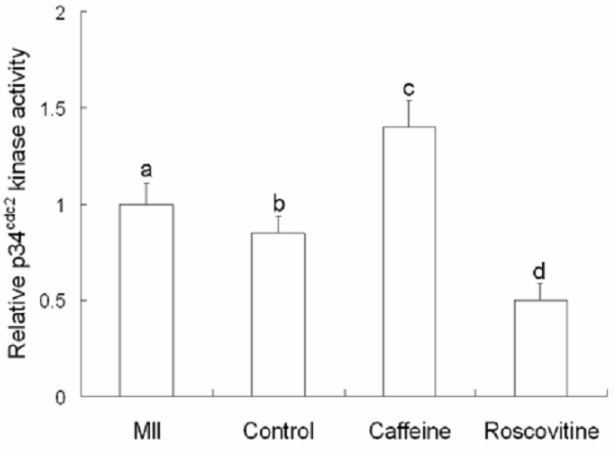

Fig. 1. Effects of caffeine and roscovitine on the activity of $p34^{cdc2}$ kinase in enucleated bovine oocytes. The $p34^{cdc2}$ kinase activity in the MII and enucleated oocytes of each treatment group were analyzed (n=20×3 trials for each group). The activity level in the MII oocytes was defined as 1. Error bars indicate± S.E.M. $^{a\sim c}$ Different letters indicate significant differences ($p<0.05$).

Table 1. Effects of caffeine and roscovitine treatments on the development of parthenogenetic bovine embryos

Treatments	Number of embryos cultured	Number (%) of embryos developed to				Cell number in blastocysts (mean±S.E.)
		2-Cell	8-Cell	Morula	Blastocyst	
Control	114	98(86.0)	46(40.4)	38(33.3)	22(19.3)	61.7± 7.9
Caffeine	116	104(90.0)	38(32.8)	26(22.4)	22(19.0)	62.2±13.8
Roscovitine	114	88(77.2)	48(42.1)	30(26.3)	20(17.5)	54.6± 8.1

nase activity compared to those in the MII, enucleated control, and especially, the caffeine-treated oocytes ($p<0.05$).

Effect of Chemical on *In Vitro* Development of Parthenogenetic Embryos

The effects of caffeine and roscovitine on embryonic development were evaluated using parthenogenetic embryos. Caffeine and roscovitine did not affect the development of bovine parthenogenetic embryos. We noted no significant differences in the proportion of blastocyst formation (17.4～19.3%) and cell number in the blastocysts (54.6±8.1 to 62.2±13.8) among the three groups (Table 1).

In Vitro Development and Apoptotic Cells of NT Embryos

The developmental ability of embryos did not differ between the control and caffeine-treated groups (Table 2). However, the developmental ability of embryos in the roscovitine-treated group was significantly lower than those of the embryos in the control and caffeine-treated groups ($p<0.01$). The proportion of embryos developed to the blastocyst stage in the roscovitine-treated group was only 8.3% (15/180), whereas the blastocyst development rate in the control and caffeine-treated groups were 25.9% (42/162) and 27.6% (42/162), respectively. The cell number of blastocysts in the caffeine-treated group (125.6±10.5) was significantly ($p<0.05$) higher than those of the control (81.6±5.0) and roscovitine-treated group (72.5±4.0). There was no difference in the number of apoptotic cells among the three groups (Fig. 2).

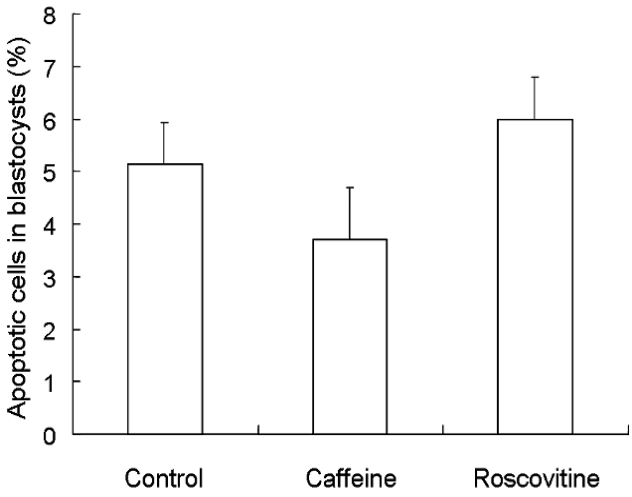

Fig. 2. Apoptotic cell index in blastocysts derived from somatic cell nuclear transfer embryos following caffeine and roscovitine treatments. The apoptotic cell index (TUNEL-positive cells/total cells in blastocysts) is presented for control (144/2,774, n=34), caffeine-treated (130/3,516, n=28) and roscovitine-treated (48/797, n=11) groups. Error bars indicate±S.E.M.

DNA Methylation Status of NT Embryos

Higher methylation levels (Fig. 3) were observed in the Day 3 embryos (4 to 16-cell stage) in the roscovitine-treated group (50.8%), whereas lower methylation levels (Fig. 3) were noted at Day 5 (16 to 32-cell stage) in the caffeine-treated group (12.5%) as compared to the other two groups ($p<0.05$). We noted no difference in methylation status (24.8～26.9%) at Day 7 (morula to blastocyst stage) among the three groups (Fig. 3).

Table 2. Effects of caffeine and roscovitine treatments on the development of bovine somatic cell nuclear transfer embryos

Treatments	Number of embryos cultured	Number (%) of embryos developed to				Cell number in blastocysts (mean±S.E.)
		2-Cell	8-Cell	Morula	Blastocyst	
Control	162	117(72.2)^{a}	94(58.0)^{a}	45(27.8)^{a}	42(25.9)^{a}	81.6± 5.0^{a}
Caffeine	152	103(67.8)^{ab}	89(58.6)^{a}	44(28.9)^{a}	42(27.6)^{a}	125.6±10.5^{b}
Roscovitine	180	105(58.3)^{b}	72(40.0)^{b}	17(9.4)^{b}	15(8.3)^{b}	72.5± 4.0^{a}

^{a,b} Different letters indicate significant differences within columns ($p<0.05$).

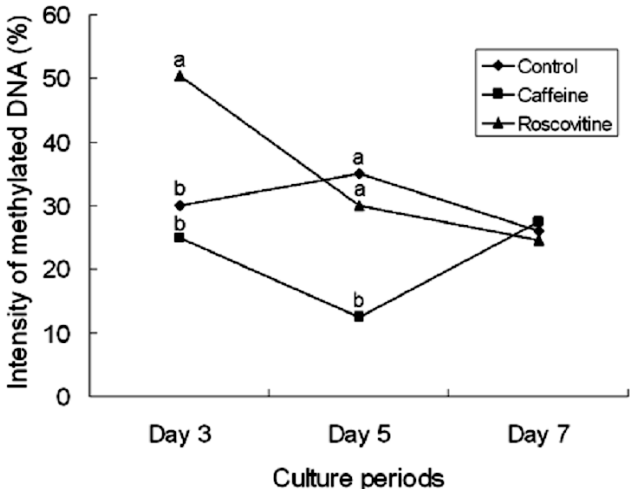

Fig. 3. Dynamic of methylation patterns in bovine somatic cell nuclear transfer (SCNT) embryos. The methylation intensity in SCNT embryos at various developmental stages are presented as methylated DNA/total DNA intensity. Day 3; 4 to 16-cell stage, Day 5; 16 to 32-cell stage, Day 7; morula to blastocyst stage. The numbers of embryos corresponding to each developmental stage are 32, 23 and 12 in control, 35, 25 and 14 in caffeine-treated group, and 33, 18 and 11 in roscovitine groups, respectively.

DISCUSSION

In this study, we confirmed that the MPF activity of recipient cytoplasm of bovine SCNT embryos could be regulated via caffeine and roscovitine treatments. Furthermore, we also suggested that the increase in the MPF activity of recipient cytoplasm creates a more suitable condition for nuclear reprogramming after SCNT than the NPCC state, as has also been reported with porcine SCNT (Kwon *et al*., 2008).

Tani *et al*. (2001) previously suggested that the exposure of donor chromosomes to nonactivated oocyte cytoplasm is crucial to the reprogramming of donor nuclei. However, Sung *et al*. (2007) reported that the PCC state is not an essential factor in the reprogramming of bovine SCNT embryos. It was recently reported that the PCC state, rather than the NPCC state, is more suitable for the development of SCNT porcine embryos (Kwon *et al*., 2008). The findings of the present study are similar to the previous results reported by Kwon *et al*. (2008). Although blastocyst development in the caffeine-treated group did not improve as compared to the control, it was significantly high as compared to the roscovitine-treated group. Mean cell number in blastocysts were also increased significantly in the caffeine-treated group as compared to the other two groups, which is different from the result that achieved in porcine cells (Kwon *et al*., 2008). This increase in development and cell number may be attributable to the induction of PCC, but not caffeine treatment itself, because the *in vitro* development and cell number in parthenogenetic embryos was neither increased nor reduced by caffeine or roscovitine treatments.

DNA demethylation in embryos relative to their developmental stage is critical to ensuring normal embryonic development (Shi and Haaf, 2002; Beaujean *et al*., 2004). Aberrant methylation status or demethylation patterns of embryos during the early developmental stages can lead to developmental failure (Shi and Haaf, 2002; Beaujean *et al*., 2004). Therefore, it is suggested that the embryos that developed to the blastocyst stage might undergo sufficient epigenetic modification for early embryonic development. The methylation patterns of cloned embryos as an epigenetic reprogramming marker have been reported previously in cattle (Kang *et al*., 2001a), pigs (Kang *et al*., 2001b), mice (Dean *et al*., 2001), and sheep (Beaujean *et al*., 2004). In bovine, the global methylation level is reduced up to the eight-cell stage, after which de novo methylation is observed (Dean *et al*., 2003). In this study, the methylation level of SCNT embryos in roscovitine-treated group was significantly high at Days 3 (4 to 16-cell stage) and 5 (16 to 32-cell stage), and reduced up to Day 7 (morula to blastocyst stage), which may be associated with low development to the blastocyst stage and cell numbers. Whereas the methylation level of SCNT embryos in the caffeine-treated group was significantly low at Day 3 and was reduced up to Day 5 (16 to 32-cell stage) prior to an increase at Day 7, which looks closer to a normal methylation pattern (Dean *et al*., 2003). We cannot determine, at this point, whether the relationship between the global demethylation level and the expression of specific sequences at genomic loci is associated with further embryonic development, largely because we used an immunostaining method in this study. Overall, it is suggested that SCNT embryos that have undergone PCC can be characterized by efficient demethylation.

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