[Frontiers in Bioscience 12, 2693-2705, January 1, 2007]

Aberrant spindle assembly checkpoint in bovine somatic cell nuclear transfer oocytes

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1. ABSTRACT

Nuclear, microtubular dynamics and spindle assembly checkpoint (SAC) in bovine somatic cell nuclear transfer (SCNT) oocytes receiving G1/0 or M phase somatic cell nuclei were studied. SCNT oocytes assembled microtubules, however, the spindles were structurally abnormal, including bi-, tri-polar or elongated spindles with scattered premature chromosome condensation (PCC) in G1/0 phase nuclei, and some miniature spindles with unaligned chromosomes in M phase nuclei. In contrast, demecolcine-treated SCNT oocytes formed chromosome clusters with membrane protrusion and significantly induced maturation-promoting factor (MPF) activity elevation (up to 177%) for 3 hours, indicating that first SAC at second metaphase (MII) is established upon spindle disruption in SCNT oocytes. After parthenogenetic stimuli, unlike MII oocytes which prevent exit from MII arrest with high MPF activity upon spindle disruption by second SAC, demecolcine-treated SCNT oocytes could not prevent exit from MII arrest with inactivation of MPF activities, whereas MG132-treated SCNT oocytes could persist at MII arrest, indicating that SCNT oocytes lack the ability for second SAC establishment, however, two G1/0 phase nuclei in an ooplasm restored second SAC establishment upon spindle disruption. Furthermore, the developmental potential of demecolcine-treated SCNT oocytes receiving G1/0 phase nuclei to blastocyst stage was not significantly different than untreated SCNT oocytes (29% vs 31%). These results indicate that unlike MII oocytes, SCNT oocytes have aberrant spindle morphology and SAC at MII due to insufficient SAC signals from somatic cell nuclei, thus aberrant remodeling has started immediately after somatic cell nuclear transfer and may be responsible for chromosome instability in SCNT embryos as well as the low successful efficiency of cloning.

2. INTRODUCTION

Reasons for low successful efficiency of somatic cell nuclear transfer (SCNT) (within 5%) include problems in nuclear remodeling, genomic imprinting, cell-cycle asynchrony, manipulation procedures, mitochondria inheritance, plus cytoplasmic incompatibility between somatic cells and ooplasm. Incomplete remodeling may induce abnormal development, early abortions and death after birth with abnormal morphogenesis, however, the molecular mechanism for nuclear remodeling following SCNT has not been fully elucidated (1, 2). Nuclear remodeling starts during the first cell-cycle, as the sperm genome is modified after penetrating the oocyte by nuclear histone protein exchange (3), demethylation (4) and hyperacetylation (5) before DNA replication in the zygote. In SCNT oocytes, unlike sperm nuclei, nuclear remodeling of donor somatic cell nuclei occurred correctly (6, 7) or incorrectly (8, 9) in enucleated oocytes. The coordination of nuclear and cytoplasmic events during embryo reconstruction also influences nuclear remodeling and development (10). Previous studies by this group showed that nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC) occurred in non-activated ooplasm after transfer of bovine cumulus nuclei at all cell-cycle stages (G1/0, S, G2 and M phase), but not in pre-activated ooplasm (11). High activity of maturation-promoting factor (MPF) in ooplasm induced some changes in donor nuclei, including depolymerization of nuclear membrane lamina, PCC and microtubule reorganization into pseudo-meiotic spindle structures. However, it was reported that transferred chromosomes were misaligned to metaphase plates in ooplasm with abnormal meiotic spindle organization in several species (12-14). Abnormality in meiotic spindle organization may be one reason for failure of normal chromosome segregation and subsequent development. In oocyte maturation, a first or second meiotic (MI or MII) spindle is associated with homologous chromosomes or sister chromatids segregation, and mis-segregation is responsible for the generation of aneuploidies and pregnancy loss (15).

The spindle has an important function in cells, with the spindle assembly checkpoint (SAC) protecting genomic integrity by monitoring the attachment of sister chromatid to the spindle via a specialized structure on the chromatid, the kinetochore. If a single kinetochore is incorrectly attached to the spindle or there is a lack of tension between the kinetochore and spindle, the SAC promotes a cell-cycle delay or inhibits exit from metaphase prior to anaphase, by chromosome segregation until the defect is corrected (16, 17). If the SAC is non-functional, abnormal chromosome segregation is permitted and a following generation of aneuploidy results. In mouse oocytes, MI or MII spindle has also been shown to have functional SAC machinery, like in mitotic spindle, since microtubule-depolymerization-agent nocodazole-treated oocytes prevent homologous chromosomes segregation and extrusion of the first polar body after germinal vesicle break down at MI phase (18) or extrusion of the second polar body and pronuclear formation after fertilization or parthenogenetic stimuli at MII phase (19-21). Additionally, SAC-related component Mad2 and BubR1 in MII oocytes is localized to kinetochores, supporting the hypothesis that cytostatic factor (CSF) may act via activation of SAC, inhibiting the anaphase-promoting complex/cyclosome (APC/C) (18, 22). However, CSF-induced MII arrest is independent of the SAC where CSF arrest can be established even if the SAC component Mad2 or BubR1 is destroyed (23). In contrast, destruction of SAC component cannot prevent exit from MII arrest following second polar body excursion and pronuclear formation after parthenogenetic stimuli in the presence of nocodazole (23). These finings demonstrate that meiotic SAC is functional from MI to AI or MII to AII transition in an SAC component-dependent manner. Age dependent reduction of SAC component in human oocytes also strongly supports the incidence of aneuploidy and pregnancy loss from non-functional SAC (24).

During SCNT, extruding a pseudo-polar body should be prevented to maintain a diploid karyotype when donor somatic cell nuclei are at G1/0 phase, indicating that second meiotic division as haploidization or correct chromosome segregation is not required for normal development (11). However, ploidy errors are frequently observed in bovine SCNT embryos (25), even in live-born cloned calf (26), thus abnormalities of spindle and/or SAC may contribute to mis-segregation of correct chromosome number at mitotic division. In rhesus monkey SCNT embryos, deficiencies in the spindle association proteins (HSET, EG5, NuMA) lead to mitotic defects and prevent successful cloning due to removal of essential factors during enucleation from the recipient MII oocyte (27, 28). In SCNT oocytes from bovine (29) and other species (30, 31), the somatic centrosome is transferred from a somatic cell and contributes to mitotic spindle organization, however, mouse oocytes rely on maternal centrosomes in the ooplasm (32). These observations raise the possibility that aberrant spindle organization or SAC in the SCNT oocytes contributes to chromosome instability and the low efficiency of somatic cloning.

Thus, an understanding of first cell cycle events after SCNT may be important for improving nuclear remodeling and the following development. However, whether meiotic spindle organization and SAC in SCNT oocytes is functional remains to be elucidated. Meiotic spindles in bovine SCNT oocytes, especially spindle morphology and SAC which prevents APC/C-mediated cyclin B degradation following MPF elevation at MII phase (33) or exit from MII arrest following inactivation of MPF and MAP kinase activity after parthenogenetic stimuli upon spindle disruption (19-21), were investigated. Findings demonstrate that SCNT oocytes have aberrant spindle morphology and SAC, but that these abnormalities are not related to the developmental potential of SCNT oocytes to blastocyst stage.

3. MATERIALS AND METHODS

3.1. Culture of Immature Bovine Oocytes

Cumulus-oocyte-complexes (COCs) were aspirated from 1 to 10 mm follicles of slaughtered bovine

ovaries, preserved overnight at 10°C in saline solution, and cultured in Medium 199 supplemented with 10% FBS (Gibco, Grand Island, NY) for 20 to 24 h at 39°C (34). Matured oocytes were denuded of cumulus cells in 0.1% hyaluronidase (Sigma, St. Louis, MO) and MII oocytes with first polar body were used in the experiments.

3.2. Somatic Cell Nuclear Transfer and Embryo Culture

Somatic cell nuclear transfer was carried out according to the method reported previously (35). In brief, primary cell lines were established from COCs collected by aspirating 1 to 10 mm follicles of a bovine ovary and cultured for three to seven passages. For synchronizing to G1/0 or M phase, cumulus cells were cultured in starvation medium contained 0.5% FBS for 3-7 days or 3 μg/ml nocodazole for 24 hours. Donor nucleus was electrically fused with an enucleated oocyte by two direct current (DC) pulses of 150 V/mm for 25 μsec with a 0.1s interval in 0.3 M mannitol containing only 0.1 mM Mg^{2+} for prevention of parthenogenetic activation. Fused oocytes were cultured in culture medium with or without 0.5 μg/ml demecolcine (WAKO, Tokyo, Japan) or 50 μM MG132 (Calbiochem; San Diego, CA) for 0 to 3 hours. Some fused oocytes were parthenogenetically activated by exposure to 5 μM ionomycin (Sigma) for 5 min followed by treatment with 10 μg/ml cycloheximide (CHX; Sigma) with or without demecolcine for 6 hours and cultured in CR1-aa medium supplemented with 5% FBS in 5% CO_2, 5% O_2, and 90% N_2 at 39°C for 8 days.

3.3. Immunofluorecence Microscopy

The oocytes were fixed and permeabilized in 4% paraformaldehyde in PBS-PVA containing 0.2% Triton X-100 at room temperature for 30 min, then washed several times in PBS-PVA and incubated overnight in PBS supplemented with 3% bovine serum albumin (PBS-BSA). The oocytes were incubated with a FITC-conjugated anti-alpha-tubulin monoclonal antibody diluted 1:100 (Sigma) in PBS-BSA at room temperature for 60 min for microtubule staining. After several washes the oocytes were mounted on a glass slide with glycerol containing 1 μg/ml propidium iodide (Sigma) for DNA staining, and observed under a Nikon microscope equipped with epifluorescence.

3.4. Histone H1 and MBP Double Kinase Assay

For analysis of the MPF and MAP kinase activity, histone H1 and MBP were used as substrates for *in vitro* kinase assay. Five oocytes were washed three times with PBS-PVA and quickly frozen on dry ice in 1μl cell lysis buffer (Cell Signaling Technology Inc; Beverly, MA) and stored at –80°C. Samples were lysed by freezing and thawing for three times and then incubated at 37°C for 30 min in the presence of 2 μg Histone H1 (Sigma) or/and 0.5 μg myelin basic protein (MBP) (Sigma) and 0.1 mCi/ml [gamma-P^{32}]ATP in 10μl kinase-buffer (80 mM beta-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 1mM DTT, 1% protease inhibitor cocktail). The kinase reaction was stopped by adding 2μl of 5×Laemmli buffer and boiled at 100°C for 4 min, then analyzed by electrophoresis in 15 % SDS-PAGE followed by autoradiography. The autoradiograph was quantified by using BASS software (Fuji film, Tokyo, Japan).

3.5. Immunoblotting

Immunoblotting was carried out according to the method reported previously. In brief, 10 oocytes were collected in cell lysis buffer supplemented with 1% protease inhibitor cocktail (Nakalai, Kyoto, Japan) and stored at –80°C. Samples were added SDS Laemmli buffer and boiled at 100°C for 4 min. The proteins were separated by SDS-PAGE with 12.5% polyacrylamide gels for 1.5 h at 150 V and then electrophoretically transferred onto PVDF membrane for 30 min at 15 V. After washing in TBS containing 0.05% Tween-20 and blocking with 5% skim milk for 1 hour at room temperature, the membrane was incubated overnight at 4°C with antibodies specific for Mad2 (MBL, Nagoya Japan) diluted with 1:1000. After three times wash in TBS containing 0.05% Tween-20, the membranes were incubated for 1 hour at room temperature with anti-mouse horseradish peroxidase-conjugated secondary antibody (Amersham Bioscience Corp; Piscataway, NJ) diluted 1:3000. After three times wash, protein was detected by ECL Plus detection system (Amersham Bioscience Corp).

3.6. Statistical Analysis

Values were compared using one-way ANOVA for the kinase activity and chi-square analysis for the developmental rate. In all case, experiments were repeated three times at least and $P<0.05$ was considered to be statistically significant. Descriptive statistics were obtained by using the Sigma Stat software (Jandel Scientific Corporation, San Rafael, CA).

4. RESULTS

To investigate remodeling of microtubules and chromosomes in the donor cell nucleus, the staining pattern in 20-30 bovine SCNT oocytes was determined by immunofluorescence microscopy using monoclonal antibody against alpha-tubulin in all experiments. Control MII oocytes aligned chromosomes to the metaphase plate with a barrel-shaped bipolar spindle (Figure 1F). After nuclear transfer of G1/0 phase somatic cell nuclei into enucleated MII oocytes, all transferred cell nuclei showed PCC in the ooplasm. However, randomly arrayed or elongated microtubules including bi- or tri-polar spindles were assembled in the vicinity of scattered chromosomes in the ooplasm and observed in all cases (n=28; Figure 1A-C). Like an MII oocyte, 3 of 28 oocytes (10%) organized a barrel-shaped bipolar spindle, but the chromosomes were not aligned to the metaphase plate in the spindle (Figure 1D). None of the SCNT oocytes underwent alignment of chromosomes to the metaphase plate with a barrel-shaped bipolar spindle like control MII oocytes. During culture for 1 hour in the presence of 0.5 μg/ml demecolcine, spindles of SCNT oocytes were disrupted and induced a chromosome clusters, indicating that microtubules were completely depolymerized (Figure 1E).

Bovine MII oocytes arrest at M phase with a bipolar spindle for several hours, for up to at least 24 hours

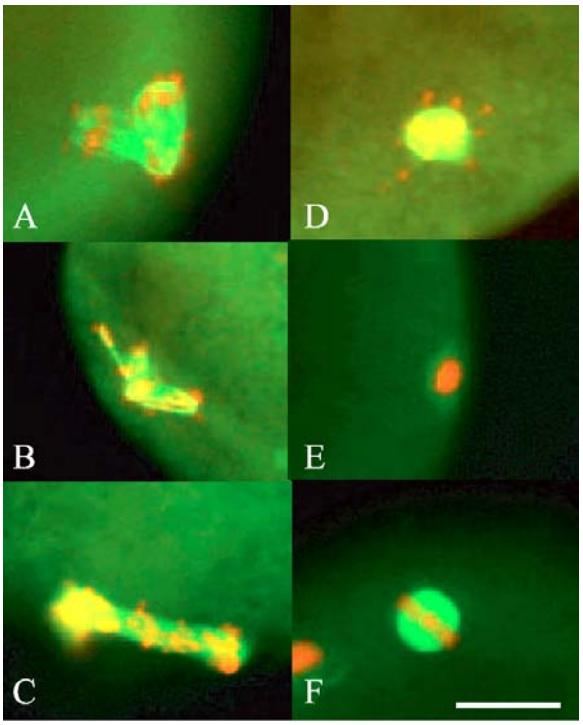

Figure 1. Abnormal microtubules of meiotic spindles in SCNT oocytes. Disarrayed microtubule organization pattern around PCC of G1/0 phase somatic cell nuclei in SCNT oocytes were seen for 1-2 hours after cell fusion (A-D), however, microtubules were not seen around chromosome clusters in the presence of demecolcine (E). Normal bipolar spindles with aligned chromosomes to the metaphase plate were seen in control MII oocyte (F). Microtubules are stained green and chromosomes are stained red. Bar is 10 μm.

with persistent high MPF and MAP kinase activity until fertilization or parthenogenetic stimuli due to CSF (36). Microtubule-disruption induced MPF elevation by the first SAC at MII phase due to inhibition of APC/C-mediated cyclin B degradation (19, 37) and membrane protrusion around a chromosome cluster (37, 38). In order to examine this feature in SCNT oocytes receiving G1/0 phase somatic cell nuclei, the MPF and MAP kinase as histone H1 kinase and MBP kinase activities in the absence or presence of demecolcine for up to 3 hours after somatic cell fusion were measured. Like control MII oocytes with persistent high MPF and MAP kinase activity, plus elevated MPF activity after demecolcine treatment, the SCNT oocytes also had persistently high MPF and MAP kinase activity in the culture medium for 3 hours (Figure 2A), with demecolcine treatment inducing significant MPF activity elevation, by up to 130% at 1 hour and 177% at 3 hours compared to control enucleated oocytes (Figure 2B). MAP kinase activity remained stable during demecolcine treatment. In addition, membrane protrusion was only induced after demecolcine treatment for 1 hour (Figure 2D). These results indicate that spindle disruption induced the first SAC and MPF activity elevation in SCNT oocytes, since SCNT oocytes have the functional SAC at MII phase upon spindle disruption being consistent with MII oocyte features.

The second SAC in MII oocytes which stabilizes MPF or MAP kinase activity and prevents exit from MII arrest following second polar body excursion and pronuclear formation after parthenogenetic stimuli upon spindle disruption was investigated (19-21). To examine the features of second SAC in SCNT oocytes receiving G1/0 phase somatic cell nuclei, the MPF and MAP kinase activity after parthenogenetic stimuli by exposure of ionomycine and CHX in the absence or presence of demecolcine for 7 hours was measured. In bovine MII oocytes, kinetics of inactivation of MPF and MAP kinase activity after parthenogenetic stimuli takes place within 1-2 hours or 6-7 hours following second polar body excursion or pronuclear formation, respectively. This is consistent with a previous report (35) and was found in the present study conditions (Figure 3A). In contrast, in the presence of demecolcine, control MII oocytes prevented inactivation of MPF or MAP kinase after parthenogenetic stimuli (Figure 3C), with prevention of second polar body excursion (30 of 30 oocytes) or pronuclear formation (30 of 30 oocytes) (data not shown), indicating that spindle disruption in MII oocytes induces the second SAC, despite the presence of CHX. However, unlike demecolcine-treated MII oocytes, SCNT oocytes failed to prevent inactivation of MPF or MAP kinase activity in the presence of demecolcine with similar kinetics to control MII oocytes (Figure 3D). Thus, in SCNT oocyte receiving G1/0 phase somatic cell nuclei, spindle disruption is not associated with stabilization of MPF or MAP kinase activity after parthenogenetic stimuli, as SCNT oocytes lack the functional second SAC for prevention of exit from MII arrest.

M phase cell nuclei synchronized by nocodazole induce functional SAC in the somatic cell cycle for prevention of chromosome segregation and arrest until release from the drug. Additionally, nocodazole is a reversible drug, not only in somatic cells but also oocytes, as M phase cell nucleus retains the ability to reassemble a bipolar spindle after removal from nocodazole treatment for 1 hour in somatic cells or MII oocytes (Figure 4D). In order to investigate whether synchronized M phase somatic cell nuclei induce the SAC in ooplasm upon spindle disruption, M phase somatic cell nuclei synchronized by nocodazole as donor cells for SCNT were used. Figure 4A-D displays the pattern in 20-30 bovine SCNT oocytes using immunofluorescence microscopy for alpha-tubulin. After nuclear transfer of M phase somatic cell nuclei into enucleated oocytes, unlike SCNT oocytes receiving G1/0 phase somatic cell nuclei, scattered chromosomes and spindle assembly patterns are apparently different, with 2 to 4 microtubules assembling some bipolar miniature spindle in the vicinity of small chromosome clusters and other smaller clusters not assembling spindle for 1-2 hours (Figure 4A, B). However, demecolcine-treated SCNT oocytes formed a chromosome clusters (Figure 4C) with a membrane protrusion (data not shown), and MPF activity was significantly elevated by up to 127% at 1 hour and by 149% at 3 hours compared to control enucleated oocytes, such as SCNT oocytes receiving G1/0 phase somatic cell

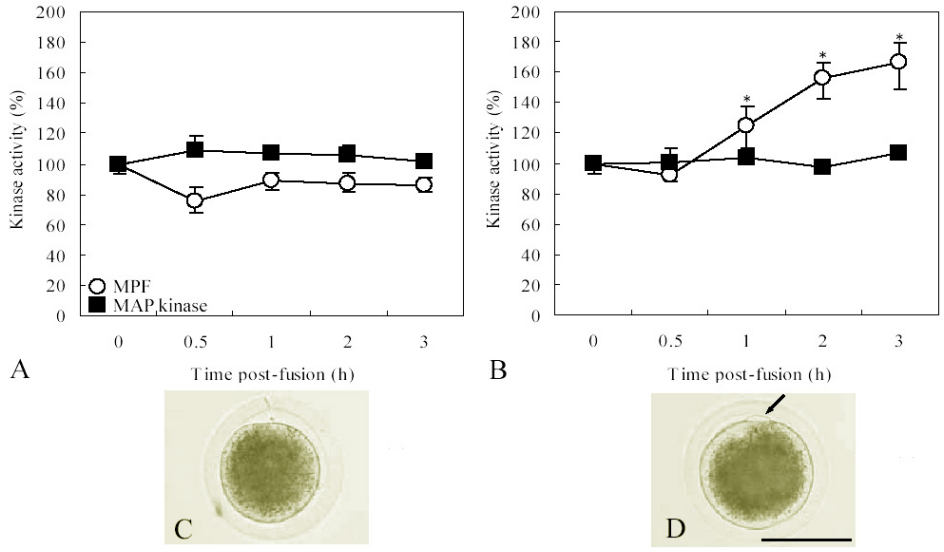

Figure 2. Demecolcine treatment induces elevation of MPF activity rather than MAP kinase activity in SCNT oocytes. MPF activity (open circle) and MAP kinase activity (black square) are shown as relative activity (mean±SEM) to that in enucleated MII oocytes. Experiments were repeated three times. SCNT oocytes receiving G1/0 phase somatic cell nuclei were cultured for 0.5-3 h (A, C) or in the presence of demecolcine (B, D). Arrow shows a membrane protrusion. Scale bar is 100 μm. *Significantly different from 0 hours for the same activity group ($p<0.05$).

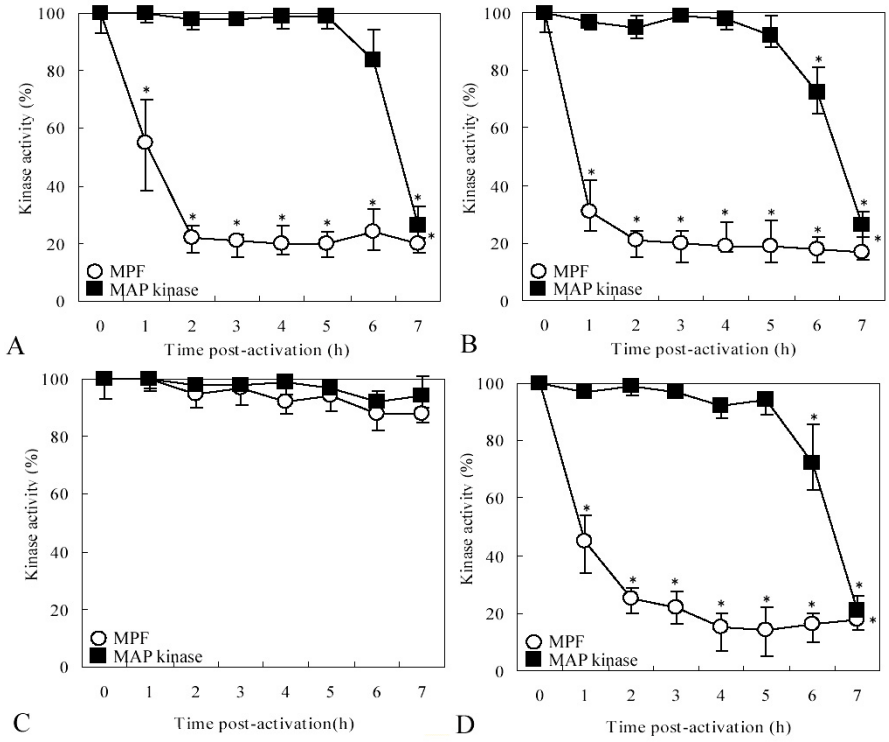

Figure 3. Inactivation of MPF and MAP kinase activities after parthenogenetic stimuli in MII or SCNT oocytes. MPF activity (open circle) and MAP kinase activity (black square) are shown as relative activity (mean±SEM) to that in intact and enucleated MII oocytes. Experiments were repeated three times. The MII and SCNT oocytes receiving G1/0 phase somatic cell nuclei were parthenogenetically activated via exposure of ionomycine and CHX (A or B) or in the presence of demecolcine (C or D) for 7 hours. *Significantly different from 0 hours for the same activity group ($p<0.05$).

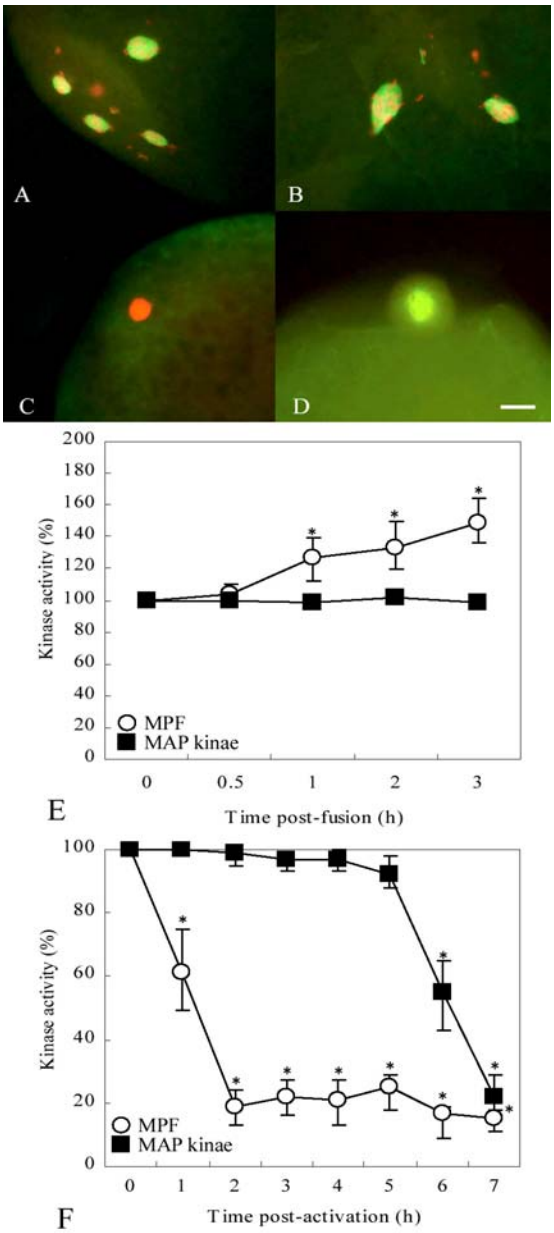

Figure 4. Functional SAC of M phase somatic cell nuclei is non-functional in SCNT oocyte. Bipolar spindle microtubule organization pattern around unaligned chromosome in SCNT oocytes receiving M phase somatic cell nuclei were seen for 1-2 hours (A, B), but microtubules were not seen around chromosome clusters in the presence of demecolcine (C). M phase somatic cell nuclei reorganized spindle after release from nocodazole (D). SCNT oocytes were cultured for 0.5-3 hours (E) and parthenogenetically activated in the presence of demecolcine (F). Microtubules are stained green and the chromosomes are stained red. Scale bar is 10 μm. MPF activity (open circle) and MAP kinase activity (black square) are shown as relative activity (mean±SEM) to that in enucleated MII oocytes. Experiments were repeated three times. *Significantly different from 0 hours for the same activity group ($p<0.05$).

nuclei (Figure 4E). MAP kinase activity remained stable during demecolcine treatment. Thus, as well as SCNT oocytes receiving G1/0 phase somatic cell nuclei, SCNT oocytes receiving M phase somatic cell nuclei could also induce the first SAC at MII phase upon spindle disruption. To examine the second SAC for prevention of exit from MII arrest in SCNT oocytes, SCNT oocytes were parthenogenetically activated in the presence of demecolcine for 7 hours. As well as SCNT receiving G1/0 phase somatic cell nuclei, SCNT oocytes failed to prevent inactivation of MPF or MAP kinase activity in the presence of demecolcine with similar kinetics to control MII oocytes (Figure 4F), indicating that the functional SAC in M phase somatic cells synchronized by nocodazole is non-functional or loses the ability to re-establish SAC in the ooplasm.

Ubiquitin-proteasome pathway contributes degradation of cyclin B and securin, which is downstream enzyme of the SAC machinery (16, 17). In addition, low level of Mad2 in ooplasm, a pivotal component of SAC, induces instability of SAC (23, 39, 40). Deficiency in the ubiquitin-proteasome pathway or low levels of Mad2 in ooplasm may be induced during enucleation or demecolcine treatment. To eliminate this possibility, firstly the effect of ubiquitin-proteasome inhibitor MG132 on MPF and MAP kinase activities in SCNT oocytes receiving G1/0 phase somatic cell nuclei was examined. When MG132-treated SCNT oocytes were cultured for 3 hours, MPF activity significantly elevated by up to 132% at 2 hours and by 151% at 3 hours compared to control enucleated oocytes (Figure 5A). MAP kinase activity remained stable during MG132 treatment, like demecolcine-treated SCNT oocytes. Unlike demecolcine-treated SCNT oocytes that inactivated MPF and MAP kinase activity after parthenogenetic stimuli with similar kinetics to MII oocytes, MG132-treated SCNT oocytes had persistently high MPF or MAP kinase activities after parthenogenetic stimuli (Figure 5B) like MG132-treated MII oocytes (data not shown). The amount of Mad2 protein was measured by Western blotting using monoclonal antibody against Mad2 during enucleation and demecolcine treatment. The amount of Mad2 protein reached a maximum from GV to MII stage and was not affected by enucleation or demecolcine treatment (Figure 5C). These results indicate that ooplasmic ubiquitin-proteasome and amount of Mad2 protein are not deficient and remain stable during SCNT procedures, as aberrant SAC machinery in SCNT oocytes is from the chromosomes of somatic cell nuclei.

To establish a functional SAC, ratio of cytoplasmic volume and nuclear density is important in Xenopus egg extract (41). Influence of ratio between nucleus and oocyte for the establishment of SAC in SCNT oocytes was investigated. Figure 6A-C displays the pattern in 20-30 bovine SCNT oocytes using immunofluorescence microscopy for alpha-tubulin. Two G1/0 phase somatic cell nuclei were transferred to an enucleated oocyte and the fused two nuclei underwent PCC, scattered with randomly arrayed or elongated microtubules in ooplasm like in single SCNT. Demecolcine treatment of SCNT oocytes induced two membrane protrusions around two chromosome

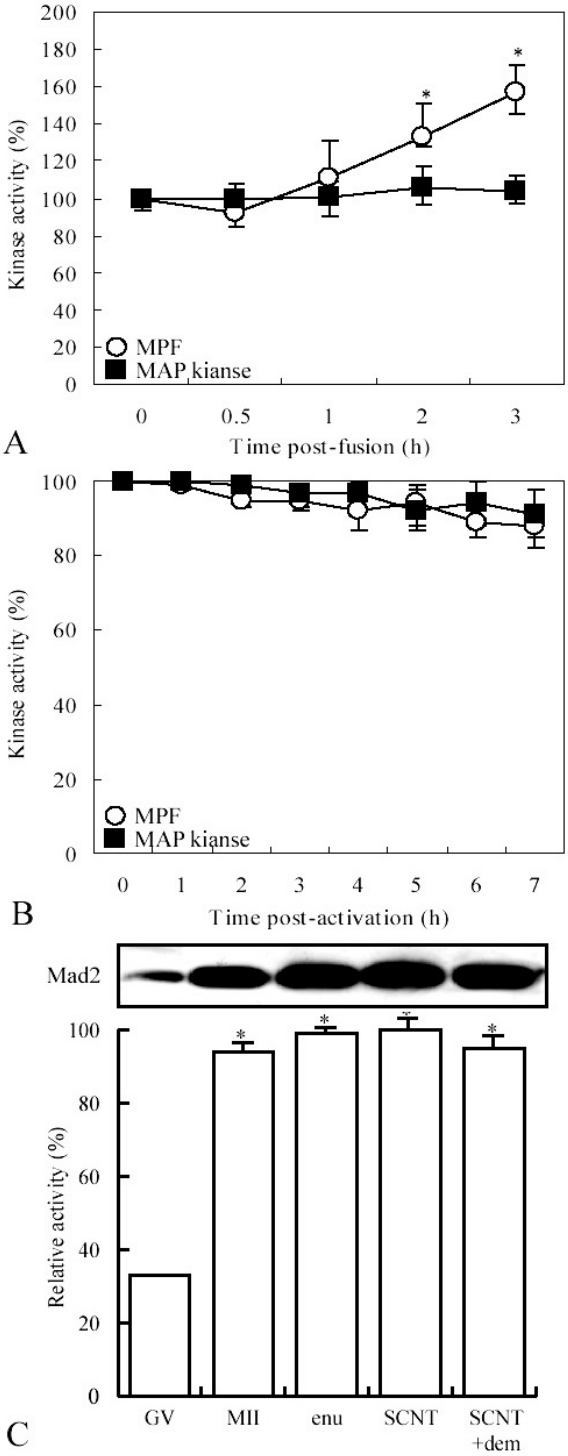

Figure 5. Effect of ubiquitin-proteasome inhibitor MG132 on MPF and MAP kinase activities and amount of Mad2 protein in SCNT oocytes. MPF activity (open circle) and MAP kinase activity (black square) are shown as relative activity (mean±SEM) to that in enucleated MII oocytes. Experiments were repeated three times. SCNT oocytes receiving G1/0 phase somatic cell nuclei were cultured for 0.5-3 h (A) and parthenogenetically activated in the presence of MG132 (B). *Significantly different from 0 hours for the same activity group ($p<0.05$). Amount of Mad2 protein is quantified by Western blotting in GV, MII, enucleated MII (enu), and SCNT oocytes receiving G1/0 phase somatic cell nuclei in the absence or presence of demecolcine. Upper panel shows Western blotting results and lower panel shows relative Mad2 density. *Significantly different from immature GV oocytes ($p<0.05$).

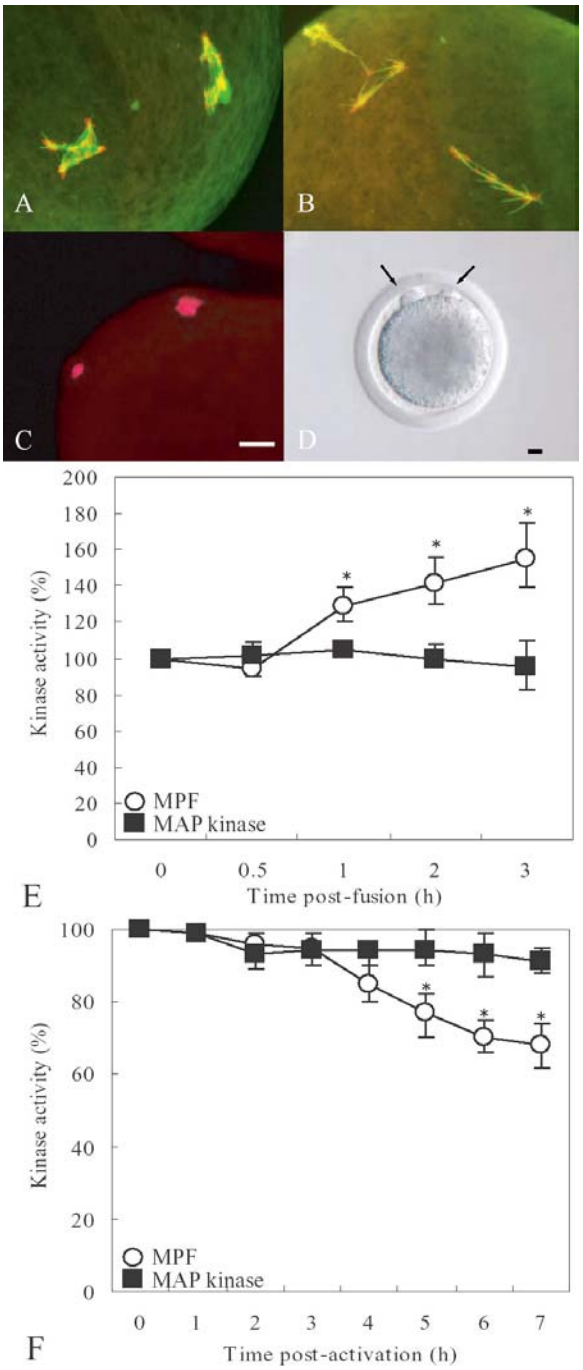

Figure 6. Two somatic nuclei restore SAC in SCNT oocytes. SCNT oocytes receiving two G1/0 phase somatic cell nuclei were cultured for 1-2 h (A, B) or in the presence of demecolcine (C, D) and SCNT oocytes were parthenogenetically activated in the presence of demecolcine (F). Arrows show two membrane protrusions. Microtubules are stained green and the chromosomes are stained red. Scale bar is 10μm. MPF activity (open circle) and MAP kinase activity (black square) are shown as relative activity (mean±SEM) to that in enucleated MII oocytes. Experiments were repeated three times. *Significantly different from 0 hours for the same activity group ($p<0.05$).

clusters (Figure 6C, D). Like single SCNT oocytes, MPF activity significantly elevated by up to 129% at 1 hour and by 155% at 3 hours compared to control enucleated oocytes (Figure 6E). MAP kinase activity remained stable during demecolcine treatment. Unlike single SCNT oocytes, demecolcine-treated SCNT oocytes prevented inactivation of MPF and MAP kinase after parthenogenetic stimuli. However, MPF activity gradually declined to 68%, significantly different compared to enucleated oocytes, as some SCNT oocytes might be activated or delay MPF inactivation. These results indicate that two somatic cell nuclei in an ooplasm restore the establishment of SAC upon spindle disruption.

Developmental potential of SCNT oocytes receiving G1/0 phase somatic cell nuclei to blastocyst stage *in vitro* in the absence or presence of demecolcine during parthenogenetic activation for 6 hours was investigated. Table 1 shows that the efficiency of development to 2 cells, 8 cells and blastocyst stage was not significantly different between the absence or presence groups (71%, 40% and 31% vs 79%, 43% and 29%). Thus, SCNT oocytes ignored the effect of demecolcine during parthenogenetic activation and supported development to blastocyst stage, indicating that SCNT oocytes apparently lost the second SAC.

5. DISCUSSION

This study found that PCC and meiotic spindle organization in SCNT oocytes are early events for remodeling in ooplasm, and that SCNT oocytes have aberrant spindle organization and SAC. To our knowledge this is the first report on meiotic SAC in SCNT oocytes. The data also reveal for the first time somatic cell nuclei number-dependent establishment of meiotic SAC in the mammalian ooplasm that may help to explain the difference in diffusible SAC signal between meiotic maternal and somatic chromosomes.

Bipolar spindle establishment requires bipolar accurate kinetochore-microtubule interaction and attachment where sister kinetochores face opposite poles and each kinetochore binds only spindle microtubules, with maintenance of mechanical tension between kinetochores and spindles in mitosis (16, 17). In MII oocytes, meiotic bipolar spindle structures are not related to centrosomes or chromosomes (42), and they can be established and persist until fertilization or parthenogenetic stimuli from CSF (36). However, in SCNT, meiotic spindle complex and chromosomes are completely removed from MII oocytes before donor cell nuclei induction, then the enucleated oocyte can reform pseudo-meiotic spindles in the vicinity of donor chromosomes under high MPF activity. All SCNT oocytes receiving G1/0 phase somatic cell nuclei failed to organize normal bipolar spindle structures. This is consistent with several reports in mouse (12), rabbit (13) and non-human primate oocytes (14). However, some reports have shown that well organized normal bipolar spindles with aligned chromosomes are observed in SCNT oocytes, like in MII oocytes (29, 43, 44). This discrepancy may be caused by differences in micromanipulations, donor cells origin or protocols for introduction of somatic cells

Table 1. Developmental potential of demecolcine-treated SCNT oocytes receiving G1/0 phase somatic cells nuclei

Demecolcine	No. oocytes	No. cultured	2cell (%)	8cell (%)	Blastocyst(%)
-	276	187	147 (79)	80 (43)	54 (29)
+	292	228	162 (71)	92 (40)	71(31)

into enucleated oocytes. Under simultaneous fusion/activation conditions for induction of donor nuclei, exposure of donor nuclei to ooplasm with high MPF activity is temporally limited to within 1 hour following inactivation of MPF activity. Bipolar meiotic spindle can persist for several hours via CSF with high MPF and MAP kinase activities until there are parthenogenetic stimuli (36). Therefore, the experimental conditions used here, in which induction of donor nuclei into recipient oocytes without parthenogenetic stimuli can persist at MII arrest-like stage with high MPF and MAP kinase activity, are likely to be more suitable for analyzing meiotic spindle morphology and SAC for comparing to MII oocyte spindle. Several modifications in donor nuclei are required for meiotic bipolar spindle organization in ooplasm, accurate chromatin condensation, kinetochore structure capture by the side of a probing spindle microtubule, maintaining attachment and tension between chromosomes and microtubules of spindles. Abnormal spindle organization with unaligned chromosomes was reported even in MII oocytes due to low cytoplasmic quality (45), aging (46) or culture conditions (47). Under the *in vitro* maturation conditions used in this study, a number of MII oocytes organized barrel shaped bipolar spindles (29 of 30 oocytes, data not shown). Enucleated MII oocytes have the ability to organize a bipolar spindle. Bipolar spindle organization is a flexible type of machinery in ooplasm, even in artificial DNA beads without kinetochores in Xenopus egg extract (48) and single chromatid with a single kinetochore in mouse MIII oocytes (49) or enucleated cytoplasm without chromosomes (42). Previous reports showed that kinetochores from G1-PCC fused with G1 and M phase Hela cells have the ability to associate with kinetochore proteins CENP-A, B, C, E and F that normally appear at the kinetochores of mitotic chromosomes as they become functional (50). However, abnormal spindle patterns and unaligned chromosomes in SCNT oocytes with G1/0 or M phase somatic cell nuclei indicate that not only the correct capturing of microtubules onto kinetochores, but also that aligned chromosomes with tension cannot establish in ooplasm even in M phase somatic cell nuclei. Recently a gradient of Ran-GTP generated by mitotic chromosomes in Xenopus egg extract and localized predominantly to chromosomes was found to contribute to mitotic spindle assembly (51), therefore scattered chromosomes in PCC of G1/0 phase somatic cell nuclei may not establish gradient Ran-GTP in ooplasm or an established Ran-GTP gradient in M phase somatic cell nuclei may be disrupted in ooplasm.

Meiotic spindle in MII arrested oocytes is responsible for equilibration of MPF activity stability due to continuous cyclin B synthesis and degradation by APC/C activity, an E3 ubiquitin ligase whose activity is required for cyclin B and securin degradation (33). If meiotic chromosomes with spindle are removed from MII oocytes, enucleated oocytes can stabilize the MPF activity due to CSF machinery, which may be defined by targeting the APC/C inhibitor as Emi2/XErp1 that is degraded by calcium (52). Additionally, spindle disruption in MII oocytes prevents APC/C activation by first SAC following elevation of MPF activity due to inhibition of APC/C-mediated cyclin B1 degradation (19, 21, 33, 37). In the present study, untreated SCNT oocytes receiving G1/0 or M phase somatic cell nuclei persisted in MII arrest even with abnormal spindles or unaligned chromosomes by CSF. Demecolcine-treated SCNT oocytes induced elevation of MPF activity by first SAC, indicating that SAC signals from chromosomes of G1/0 or M phase somatic cell nuclei upon spindle disruption are sufficient for inhibiting APC/C activity in MII arrested oocytes.

Functional SAC monitors chromosome alignment and spindle integrity, prevents progression from metaphase to anaphase when chromosomes are not aligned to the metaphase plate or are unattached to sister kinetochores on the opposite spindle (16, 17). However, meiotic SAC is not functional for checking an aligned metaphase plate during oocyte maturation, as shown in XO mice with misalignment of X chromosome (53), but treatment of microtubule-disrupting agent prevents exit from MI (18) or MII following first or second polar body extrusion and pronuclear formation after fertilization or parthenogenetic stimuli (19-21). Thus, meiotic SAC can monitor the lack of attachment of sister kinetochores on chromosomes even on unaligned chromosomes. SAC related-protein Mad2, Bub1, BubR1 and cdc20 are localized to the kinetochores of unaligned chromosomes, generating a diffusible anaphase-inhibitory-signal that inhibits APC/C activator cdc20 by formation of Mad2-cdc20 complex. Inactivated APC/C cannot ubiquitinate securin and cyclin B1 for inactivation of separase and MPF, as exit from metaphase is prevented (16, 17). This suggests that Mad2-dependent SAC is functional in MI or MII mouse oocytes, with over expression of Mad2 inducing MI arrest (18) or destruction of Mad2 failing to induce MI arrest (54) upon spindle disruption, indicating Mad2 is pivotal component for establishment of meiotic SAC. In the present study, demecolcine-treated MII oocytes had functional second SAC that prevented inactivation of MPF and MAP kinase after parthenogenetic stimuli, however, demecolcine-treated SCNT receiving G1/0 or M phase somatic cell nuclei induced inactivation of MPF and MAP kinase at 2 and 7 hours after parthenogenetic stimuli, consistent with similar kinetics for untreated MII oocytes. This indicates that even M phase somatic cell nuclei with functional SAC cannot be functional in ooplasm, as somatic cell nuclei may be incompatible and non-functional in ooplasm. In a recent study, oocyte maturation by replacement of mouse GV with nuclei from various types of cells, produced the maintenance of high MPF activity by the GVs from other oocytes or pronuclei from fertilized eggs, but not in the presence of cumulus nuclei (55). These data suggest that meiosis-specific chromosome structure as the cohesion

complex (SMC1-beta, REC8 and STAG3) (56) or other factors may contribute to control of meiosis division with MPF activity. Therefore, non-functional SAC in ooplasm at MI phase may be induced by differences in chromosome structure.

During establishment of the SAC, unknown diffusible SAC-signal, an anaphase-inhibitory-signal, from unattached kinetochores on chromosomes prevents APC/C activity following cytoplasmic ubiquitin-proteasome enzyme activity, downstream of SAC (16, 17). This suggests that SAC establishes not only attachment of kinetochores and spindle or aligned chromosomes but also cytoplasmic enzyme activity or component. In SCNT oocytes, cytoplasmic ubiquitin-proteasome is functional because ubiquitin-proteasome inhibitor MG132 induces elevation of MPF activity at MII phase and prevents exit from MII arrest with high MPF and MAP kinase activity after parthenogenetic stimuli. In addition, destruction of SAC-related protein Mad2 or BubR1 revealed non-functional SAC for exit from MII arrest in mouse oocytes after parthenogenetic stimuli in the presence of nocodazole (23). However, the amount of Mad2 protein remained stable during SCNT, although the Mad2 localization to unattached kinetochores in SCNT oocytes was not examined by immunostaining, and consequently there is a possibility of aberrant localization of Mad2 to kinetochores on the chromosomes of somatic nuclei. Taken together these findings suggest the reason for non-functional second SAC is from somatic cell nuclei, indicating that interaction between nucleus and ooplasm is incompatible or insufficient for functional second SAC establishment.

Mitotic somatic chromosomes can release enough diffusible SAC-signals to inhibit APC/C activity upon spindle disruption in a somatic cell cytoplasm (16, 17). In Xenopus eggs, SAC is non-functional during oocyte maturation, as the ratio of nuclei and cytoplasm volume is 1000 to 10000 fold less compared to mammalian oocytes or somatic cells (57). In Xenopus egg extract, sperm nuclei density and cytoplasmic volume are important for establishment of SAC (41). This shows that for SAC establishment, diffusible SAC-signals from kinetochores on chromosomes are dependent on the ratio between nuclei and cytoplasmic volume. It is intriguing that functional SAC in SCNT oocytes could be restored by at least two G1/0 phase somatic cell nuclei. Kinetochores on somatic chromosomes from G1/0 or M phase nuclei in SCNT oocytes have less diffusible SAC-signals for inhibiting APC/C activation, even if M phase somatic cell nuclei cannot restore functional SAC, indicating that establishment of SAC by somatic cell nuclei in ooplasm is dependent on somatic cell number rather than kinetochore or chromosome number. These results raise the possibility that diffusible SAC-signals have apparently different concentrations between meiotic maternal and mitotic somatic chromosomes. This difference may contribute to the understanding of control of progression through meiotic maturation by GV, pronucleus or cumulus cell replacement (55).

Developmental potential to blastocyst stage of demecolcine-treated SCNT oocytes was not significantly different to untreated SCNT oocytes and mouse SCNT oocytes even at full term development (58). Therefore, SCNT oocytes completely lack the meiotic SAC during second meiosis and support the developmental potential to blastocyst stage. However, it is not yet known whether aberrant second SAC at MII phase contributes to mitotic SAC and/or chromosome instability during mitotic division following aneuploidy/polyploidy throughout development.

Nuclear reprogramming is characterized by functional modification of the transferred nucleus to direct normal development. For correct nuclear reprogramming, somatic nucleus is required for normal nuclear remodeling in ooplasm. Aberrant meiotic spindle organization and non-functional SAC in SCNT oocytes do not contribute to the developmental potential to blastocyst stage. However, whether the relationship between aberrant meiotic spindle organization or non-functional SAC and chromosome instability in SCNT embryos plays a role in the low cloning efficiency of SCNT embryos and development that are commonly observed in cloned animals remains to be elucidated. Further studies are needed to determine these fragile regulatory mechanisms and to evaluate whether mis-organization of meiotic spindle and non-functional SAC after SCNT produce aneuploidy/polyploidy SCNT embryos and development. Restoring the ability to organize a normal bipolar spindle or establishment of functional second SAC could provide one avenue for improving cloning efficiency, together with other strategies to address other deficiencies in nuclear remodeling or reprogramming. Additionally, such studies could provide new light on the differences in chromosome origin, meiotic chromosomes or somatic chromosomes, for establishment of the SAC in ooplasm or identify SAC-signals from kinetochores on chromosomes.

6. ACKNOWLEDGEMENT

This work was supported by grants form the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), and supported by a grant from the Ministry of Education, Culture, Sports, Science, and Technology (16200030).

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Key Words: Spindle Assembly Checkpoint, Microtubule, Nuclear Transfer, MPF, MAP kinase, Nuclear Remodeling, Nuclear Reprogramming, Bovine Oocyte

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