Premature Chromosome Condensation Is Not Essential for Nuclear Reprogramming in Bovine Somatic Cell Nuclear Transfer

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Running Title: Unnecessary PCC in bovine nuclear reprogramming

Summary Sentence: A study with laser scanning confocal microscopy revealed that the occurrence of premature chromosome condensation is not a necessary prerequisite for reprogramming of a differentiated somatic genome in cattle. An improved preimplantational development in cloned embryos was achieved via an immediate activation after the fusion of donor cells with cytoplasts of matured oocytes.
Key Word: Nuclear transfer, Reprogramming, Premature chromosome condensation,

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**ABSTRACT**

Premature chromosome condensation (PCC) was believed to promote nuclear reprogramming and to facilitate cloning by somatic cell nuclear transfer (NT) in mammalian species. However, it is still uncertain whether PCC is necessary for successful reprogramming of an introduced donor nucleus in cattle. In our study, fused NT embryos were subjected to immediate activation (IA, simultaneous fusion and activation), delayed activation (DA, activation applied 4 h post fusion) and immediate activation with aged oocytes (IAA, activation at the same oocyte age with Group DA). The morphological changes, such as nuclear swelling, the occurrence of PCC, and microtubule/aster formation were analyzed in detail by laser scanning confocal microscopy. When embryos were subjected to immediate activation in both IA and IAA groups, the introduced nucleus gradually became swollen, and a pronuclear-like structure formed within the oocyte, but PCC was not observed. In contrast, delaying embryo activation resulted in 46.5-91.2% of NT embryos exhibiting PCC. This PCC was observed beginning at 4 h post cell fusion shown as one, two, or multiple chromosomal complexes. Subsequently, a diversity of pronuclear-like structures existed in NT embryos, characterized as single, double and multiple nuclei. In the oocytes exhibiting PCC, the assembled spindle structure was observed to be an interactive mass, closely associated with condensed chromosomes, but no aster had formed. Regardless of whether they were subjected to IA, IAA or DA treatments, if the oocytes contained pronuclear-like structures, either one or two asters, were observed in proximity to the nuclei. A significantly higher rate of development to blastocysts was achieved in embryos that were immediately activated (IA, 59.1%; IAA, 40.7%), compared to those in
which activation was delayed (14.2%). The development rate was higher in Group IA to
Group IAA, but it was not significant (P=0.089). Following embryo transfer, there was
no statistically significant difference in the pregnancy rates (d 70) between two groups
(Group IA, 11.7 %, n=94 vs. Group DA, 12.3 % n=130; P>0.05), as well as live term
development (Group IA, 4.3 % vs. Group DA, 4.6 %, P>0.05). Our study has
demonstrated that immediate activation of bovine NT embryos results in embryos with
increased competence for preimplantational development. Moreover, PCC was shown to
be unnecessary for the reprogramming of a transplanted somatic genome in a cattle
oocyte.
INTRODUCTION

Somatic cell nuclear transfer (NT) has successfully produced live clones in several mammalian species. In most NT studies, a highly differentiated somatic nucleus is transferred into a recipient oocyte at metaphase II, where nuclear modification and reprogramming take place[1-4]. During the several hours of exposure to the MII cytoplasm, prior to parthenogenetic activation, the introduced somatic nucleus (at the G0 and/or G1 phase) usually undergoes nuclear envelope breakdown (NEBD) and subsequent premature chromosome condensation (PCC)[5], likely due to the high concentrations of maturation promoting factor (MPF) present in the oocyte[6, 7].

The degree of PCC varies, depending upon the MPF activity and the duration that a transplanted nucleus is exposed to the MII cytoplasm[2, 5]. In most cloning studies in mice [8, 9] and cattle [10-12], parthenogenetic activation was delayed for 1 to 4 h after nuclear transplantation. This was believed to allow extensive nuclear-oocyte interaction, under the hypothesis that a longer exposure of the somatic donor nucleus to the oocyte cytoplasm would induce PCC and facilitate nuclear reprogramming. The mechanism for the molecular basis of nuclear reprogramming is still unknown, and appears to be highly complicated[13]. In mice[8] and pig NT[14], a higher embryo development rate was achieved by inducing PCC, suggesting that PCC might promote effective nuclear reprogramming of the donor nucleus and promote developmentally competent gene expression in cloned embryos [7]. However, it is not known whether PCC is essential for efficient reprogramming of a somatic cell in cattle NT. We found that a 30-40% blastocyst development rate was achieved when reconstructed oocytes were activated immediately after cell fusion [15, 16]. Meanwhile, we demonstrated that the use of an
MII oocyte was required to reprogram a somatic nucleus[15], and, furthermore, the
reprogramming factors present in the MII oocyte became inactive within hours of oocyte
activation [2, 3, 15]. Choi et al (2004)[17] reported that a higher rate of development was
achieved in restructured oocytes activated within 2 h after fusion, compared to those with
a prolonged exposure (3 to 5 h) to MII oocytes. Therefore, we believe it is important to
explore a series of cellular events taking place in the oocytes, and the morphology of an
introduced somatic nucleus, when being subjected to a brief exposure to MPF. We further
wish to evaluate whether PCC is required to promote cloning efficiency.

In the present study, we determined the effect of exposing a bovine donor nucleus
to MII oocyte cytoplasm by examining the morphological progression of nuclear
structures. The study, in particular, focused on the occurrence of PCC and the changes in
the intracellular microtubule cytoskeleton; subsequently, the developmental potential of
cloned embryos was determined in vitro and in vivo.

MATERIALS AND METHODS

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical
Co. (St. Louis, MO). The basic cell culture medium was Dulbecco’s Minimum Eagle’s
medium (DMEM; Gibco, Grand Island, NY, 31600); the basic oocyte culture medium
was Medium 199 (M199) with Earle’s salts, L-glutamine, 2.2 g/L sodium bicarbonate,
and 25 mM HEPES (Gibco, Grand Island, NY, 12340-014). Dulbecco’s phosphate
buffered saline (D-PBS; Gibco, Grand Island, NY, 15240-013) containing 20% fetal
bovine serum (Hyclone, Logan, UT, SH0070.03) was used as the standard manipulation
medium. Oocyte and embryo cultures were maintained at 39 °C in 5% CO₂ and humidified air, unless otherwise specified.

All animal care related procedures described within were reviewed and approved by the University of Connecticut Institutional Animal Care and Use Committee, and Livestock Research Institute, Council of Agriculture of Taiwan according to The Guide for the Care and Use of Agricultural Animals in Agricultural Research. The procedures were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

**Donor Cell Collection, Culture, and Cell Cycle Analysis**

For *in vitro* study, cultured bovine cumulus cells were used as the source of nuclear donors in this study. Bovine cumulus oocyte complexes (COCs) were collected from a 4-year-old Holstein dairy cow of high merit, from the University of Connecticut's Kellogg Dairy Center (KDC), by standard oocyte ultrasound-guided retrieval (ovum pickup, OPU). Briefly, COCs were recovered using an Aloka 5005 scanner fitted with a human vaginal probe (5MHz), and sterile hypodermic needle. With the aid of vacuum pressure, follicular fluid was aspirated along with the COCs. Cumulus oocyte complexes, in groups of 3 to 5, were collected and cultured in DMEM containing 20% FBS and antibiotics in Falcon 35x10 mm culture dishes (Becton Dickinson, Franklin Lakes, NJ, 3001). Cumulus cells were expanded to different passages by a brief washing with D-PBS, and subsequently subjected to a three-minute digestion by 0.05% trypsin (ICN, Aurora, OH, 103140) and 0.5 mM EDTA (Baker, Phillipsburg, NJ, 8991) at 37 °C.

Cumulus donor cells at passages 5 to 10 were used for nuclear transfer. The cell cycle stage of quiescent cumulus cells was determined by flow cytometry [16], which revealed
95 ± 2% of the cultured cumulus cells at the G0/G1 stage, as previously published by our laboratory[18].

For embryo transfer to test in vivo viability of cloned embryos, both cumulus cells collected from the above cow, and skin fibroblasts from several cow donors in the USA and Taiwan were used for nuclear transfer. Skin tissues were collected by ear notching. Skin explants were subsequently cultured in Falcon 35x10 mm culture dishes (Becton Dickinson, 3001) with 10% FBS DMEM at 37 °C in 5% CO2 humidified air. Fibroblast monolayers formed around the tissue explants in about two weeks. The explants were then removed and placed into new culture dishes. For passaging, cells were washed with 1 mL of Dulbecco’s PBS, then gently digested by a three-minute incubation in 250 L 0.05% trypsin (ICN, 103140) and 0.5 mM EDTA (Baker, 8991) at 37 °C. The reaction was terminated by adding 5% FBS in DMEM. Subsequently, the collected cells were then resuspended and divided into three new dishes and maintained for 6-7 days. For cell storage, cells cultured to various numbers of passages were collected and frozen in 7% dimethylsulfoxide (DMSO, D-5879) and 7% glycerol (G–2025) at −80 °C for 1 d, then stored in liquid nitrogen.

Nuclear donor cells were disassociated by 2 to 3 min of trypsinization at 37 °C, and resuspended in 1 mL 5% FBS in DMEM. Prior to NT, cell suspensions were allowed to recover for approximately 30 min at 37 °C.

**Oocyte Maturation, Nuclear Transfer, Parthenogenetic Activation and Embryo**

**Culture**

Bovine COCs from slaughterhouse ovaries with at least 4 intact, tight layers of cumulus cells, were selected, washed three times in D-PBS containing 0.1% polyvinyl
alcohol (PVA; P-8136) (D-PBS+PVA), placed in CO₂ gas balanced maturation medium
in 1.0 mL vials, and shipped overnight to the laboratory in a portable incubator at 39 °C.
Maturation medium was M199 containing 7.5% (v/v) FBS and supplemented with 0.5
g/mL ovine FSH (NIDDK), 5.0  g/mL ovine LH (NIDDK), 1.0  g/mL estradiol (E-
8875) and antibiotics. After 20-22 h post maturation (hpm) (average 21 hpm) matured
oocytes with well-expanded cumulus layers were selected. The cumulus cells were then
denuded according to the method of Du et al. 2006[19]; the COCs were placed into 0.1%
hyaluronidase in PBS followed by vortexing for 3 min and repeated pipetting until
cumulus cells were removed completely. Oocytes with a polar body were selected for
enucleation and NT.

All micromanipulations were carried out using our standard procedure [16, 19].
Enucleation was performed by making a slit in the zona pellucida using a glass needle
and applying pressure until the polar body, along with the surrounding cytoplasm
(estimated at approximately 1/8 total cytoplasm) was extruded. Successful enucleation
was confirmed by fluorescent microscopy after staining with 10  g/mL Hoechst 33342.
A donor cell with a diameter of around 20  m was selected and transferred into the
perivitelline space of an enucleated oocyte. Donor cell-cytoplasm pairs were fused by
applying two direct current pulses of 1.67 kV/cm for a duration of 10  s /each by BTX
200 Electro Cell Manipulator (Biotechnologies & Experimental Research Inc., San Diego,
CA)[19]. Following the completion of electric fusion, reconstructed oocytes were
incubated at room temperature for 15 min to facilitate cell fusion. According to our
observations, most cell fusion was completed within 15 min following the electrical
pulses. All fused oocytes were randomly assigned to three groups, according to the
experimental design. The parthenogenetic activation of cloned embryos was
accomplished by a 1 h incubation in M199 +7.5% FBS (M199-FBS) containing 10
g/mL cycloheximide (CHX, C-6255) and 2.5 g/mL cytochalasin D (CD, C-8273),
followed by culture in M199-FBS containing 10 g/mL CHX for an additional 4h.
Following activation, reconstructed oocytes were cultured for 44 h in CR1aa
containing 6 mg/mL BSA in a mixed gas environment of 5% CO₂, 5% O₂, and 90% N₂ at
39 °C. Cleavage rates were recorded, and 4-8 celled embryos were cultured further in
CR1aa containing 10% FBS on a cumulus cell monolayer for an additional 5 days.

**Immunohistochemistry and Laser-Scanning Confocal Microscopy**

The reconstructed oocytes were collected at different time periods, according to
the experimental design, and fixed in a microtubule stabilizing buffer containing 2%
formaldehyde (Fisher Scientific, New Jersey, F79-500), 0.5% Triton X-100 (T-8717), 1
mM taxol (T-7402), 10 U/mL aprotinin (A-6279), and 50% deuterium oxide (D-4501) at
37 °C for at least 30 min[20]. Fixed oocytes were washed in washing buffer (PBS
containing 3 mM NaN₃ (S-8032), 0.01% Triton X-100, 0.2% nonfat dry milk, 2% normal
goat serum, 0.1 M glycine(G-7126), and 2% BSA (A-3311) three times, and subsequently
maintained in the same washing buffer overnight at 4 °C for blocking and
permeabilization. Cloned embryos were double stained to visualize microtubules and
DNA [18]. Samples were incubated overnight at 4 °C with the first antibody--mouse
anti-tubulin (T-5168, 1:200 dilution). After rinsing three times in the washing buffer,
reconstructed oocytes were kept at 4 °C overnight in a second antibody--fluorescein
isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (F-0257, 1:200
dilution). The reconstructed oocytes were mounted in PBS containing 10 M propidium
iodide (P-4170) to stain for DNA, and observed under laser-scanning confocal microscopy (Leica TCS SP2; Mannheim, Germany).

Sampling of Reconstructed Bovine Embryos and Morphological Evaluation

This study consisted of three experimental treatments that were defined as immediate activation (IA), also called simultaneous fusion and activation; delayed activation (DA); or immediate activation with aged oocytes (IAA). Nucleation (removal of the MII plate) began at 21 h post oocyte maturation. In the IA and DA groups, cell fusion was performed at 25 h post maturation. The time of completion of cell fusion (25 hpm was designated as 0 h, and marked as the onset for sample collection). In Group IA, immediately after cell fusion (activation at 25 hpm), restructured oocytes were allocated to the activation regime of 5 h incubation in combined CD/CHX and CHX medium, as described above. With this activation procedure, MPF activity in the oocyte decreases to a basal level within 1 h post activation, and the oocyte is dramatically driven away from MII phase into, presumably, the S-phase [21]. In Group DA, fused oocytes were incubated in M199-FBS for 4 h after cell fusion prior to parthenogenetic activation (activation at 29 hpm); subsequently, they were allocated to the same activation regime as Group IA. This incubation period allowed for extended exposure of the introduced G0/G1 nucleus in the oocyte’s cytoplasm that contained a high level of MPF to induce PCC. In Group IAA, oocytes were enucleated at 21 hpm. Enucleated cytoplasts were subsequently cultured for 4 h before being subjected to donor cell insertion, cell fusion and a simultaneous activation. Therefore, Groups DA and IAA were treated with a regime that eliminates the age difference between two groups at the time of activation (Groups DA and IAA, 29 hpm). In Groups IA and IAA, the activation treatments were
the same except for oocyte age difference at activation (Group IA, 25 hpm vs. Group IAA, 29 hpm). In all three groups, reconstructed oocytes were collected and fixed at 0, 1, 2, 4, 6, 12, 18, or 24 h post fusion (hpf). The samples were also collected from three groups at 44 hpf to determine the events of mitosis of NT embryos. The progressive changes over time of the donor nucleus in the oocyte were categorized as: enlarged nucleus or swelling, PCC, and the arrangement of microtubules/aster; these parameters were examined by confocal microscopy. To determine nuclear change, the nuclear area of each reconstructed embryo was analyzed by the Leica Confocal Software program (Leica TCS SP2; Mannheim, Germany). The degree of nuclear swelling was determined by comparison to the average area value of cumulus nuclei from donor cells prior to NT. The evaluation of nuclear swelling was subjectively defined. Those nuclei with areas below (<100%) or similar (100-120%) to that average value determined for cumulus nuclei were categorized as unswollen; while those with areas 120 % or greater than the average value were designated as swollen.

**In Vivo Developmental Potentials of NT Embryos Derived From IA and DA Protocols**

The cloned embryos, derived from either the IA or DA groups, were transferred into recipient cows to test their in vivo viability. The NT embryos were either freshly transferred or cryopreserved by liquid nitrogen surface vitrification [19] prior to ET. Recipient cattle were synchronized by a regime of 2 injections of prostaglandin F2 (Lutalyse, Upjohn Co., Kalamazoo, MI; 25 mg/injection, i.m.) at an interval of 11 d. The onset of standing heat estrus of recipients was monitored closely and recorded as estrus d 0. On d 7 post estrus, recipients were selected by palpation per rectum to verify the presence and the size of the corpus luteum (CL). Either fresh or thawed blastocysts (one
or two per straw) were loaded into 0.25 mL French straws containing ViGro Holding
Plus (AB Technology, Pullman, WA). One or two embryos were deposited non-
surgically into the uterine hom ipsilateral to the ovary with the CL. Pregnancy was
determined by palpation per rectum or ultrasound monitoring on d 70 after transfer. All
pregnancies were allowed to carry on to term.

Statistical Analysis

The data on nuclear swelling (nuclear area) were subjected to an arc sine
transformation. The transformed data were then analyzed by ANOVA (General Linear
Model, SPSS 11.0, Chicago, IL) [22] For the analysis of in vitro and in vivo development
of cloned embryos and the proportions of embryos that reached cleavage, developed to
the 8-cell stage, and to the blastocyst, as well as the conception rates on d 70 and calving,
were transformed by an arcsin transformation, and analyzed by a Student’s t-test. A P
value of less than 0.05 is considered to be statistically significant.

RESULTS

Nuclear Progression and PCC in Reconstructed Bovine Embryos

Cultured cumulus cells, presumably at G0/G1[18], were designated for use in our
nuclear transfer experiments. The nuclear areas of introduced donor cells, in IA, DA and
IAA groups, regardless of their activation regime, were found to increase progressively
and directly with the time interacted within a recipient oocyte (FIG.1). As a comparative
control, the average areas of cumulus nuclei, from donor cells prior to NT, were
measured as 61 ± 11  m² (n=29, FIG. 2A). There was no significant nuclear swelling in
oocytes from Groups IA, DA, and IAA at 0 h (FIG. 2B), 1-2 hpf (FIG. 1B, FIG. 2C),
when the area was compared with the average cumulus nuclear value; however, a
dramatic increase in the size of the nucleus (P<0.05) was observed from 4 to 24 hpf (FIG.
1B, FIG. 2D-F) in the three groups. The range of nuclear area of donor nuclei in Group
DA varied from 61 ± 11 (unswollen) to as high as 680 ± 24 μm² (hugely swollen) (FIG.
1B). Pair-wise comparisons between Groups IA and IAA embryos did not show any
difference in nuclear swelling (FIG. 1B) at all collecting periods. With the same pair-
wise comparison model between either Groups IA and DA, or Groups IAA and DA
embryos (FIG. 1B), the degree of swelling remained similar from 0 to 6 hpf; however, the
oocytes in Group DA showed significantly greater nuclear swelling than those in Groups
IA or IAA at 12-24 hpf, (FIG. 1B). The size of the nuclear area peaked at 18 hpf in
Groups IA (FIG. 2F), IAA and DA (FIG. 3D), and then declined, in the three groups, by
24 hpf (FIG. 1B).

The onset of mitosis in the three groups of NT embryos, began to be manifest at
12-18 hpf (FIG. 1A). This was evidenced by the varied appearance of prophase (FIG.
2G), a typical metaphase (FIG. 2H), a telophase (FIG. 2I) at the first cell cycle, and a
metaphase at the second cell cycle (FIG. 2J) during the period from 18-24 hpf (FIG. 1A).
The continuation of nuclear development in these NT embryos was evident by the 4-(FIG.
2K), and 8-cell (FIG. 2L) stage embryos observed at 44 h after cell fusion.

As indicated in FIG. 1A, PCC was not observed in Groups IA and IAA at any of
the time points examined (0 –24 hpf). In Group DA (FIG.1), PCC was not found at 0, 1
and 2 hpf, respectively, however, it appeared, by 4 hpf in 72.5 % of embryos, and
increased at 6 hpf to the peak of 91.2 % of embryos. After that, the proportion of oocytes
showing PCC subsequently decreased to 54.8% (12 hpf), 68.6 % (18 hpf), and 46.5% (24
hpf), respectively (FIG. 1). In addition, the morphology of PCC was discerned as one
FIG. 3A), two (FIG. 3B) and multiple (FIG. 3C) scattered chromosomal clusters;
however, the number of chromosomes was difficult to examine in those PCC
constructions. The percentage of chromosome condensation into a single cluster was
observed to decrease in collected embryos from 75.8% (n=29) at 4 hpf to 15.0% (n=20)
at 24 hpf. Instead, many of these embryos in Group DA displayed various numbers of
scattered and condensed chromosome formations between 4 and 24 hpf (FIG. 3B, 3C).
Among those NT embryos having pronuclear appearances in Group IA, all
possessed one nucleus until 12 hpf. Embryos began to show double nuclear structure at
18 hpf; by 24 hpf, as many as 28.0% (n=25) had two nuclei (FIG. 4), presumably
indicating that these embryos had entered either telophase or the post-telophase stage of
the first cell cycle (FIG. 2I). In Group IAA, all embryos with nuclear appearances
showed one pronuclear-like structure from 0 to 24 hpf. In contrast, a varied range of
embryos in Group DA (17.6-40.0%) contained either two (FIG. 3E) or multiple (FIG. 3F)
nuclear structures during 12-24 hpf (FIG. 4). Overall, irregular chromosomal clusters
and the appearance of multiple nuclei were predominant features of embryos from
delayed activation (Group DA) (FIG. 3). The emission of the second polar body was not
observed in all groups (IA, IAA and DA) probably to the treatment of cytochalasin
during the activation.

**Microtubule Organization in Cloned Cattle Embryos**

The microtubule organization in cloned embryos was examined under laser-scanning confocal microscopy. In Groups IA and IAA, highly stained microtubule
structures were evident in the donor cell prior to fusion (FIG. 2A). However, an aster or
a microtubule organization center (MTOC) could not be seen in most reconstructed
oocytes shortly after fusion (FIG.2B, 2C). With time and the progression of swelling of
the introduced nucleus, one (FIG. 2D) or two (FIG. 2E) tufts of microtubules developed
in the proximity of the donor nucleus 1 hpf (FIG. 4), and these structures continued to
develop into characteristic asters (two asters, FIG. 2F,G) in the embryos collected starting
at 2 hpf (FIG. 4). At 24 hpf, all NT embryos with nuclei in Group IA showed two asters
(FIG. 4). Likewise, of NT embryos in Group IAA, 84.2 % (n=36) showed two asters at 6
hpf, and a similar percentage of appearance of double asters was maintained until 24 hpf
(90.0%, n=10) (FIG. 4). In Group DA, the aster was observed beginning at 1 hpf (FIG.
4), and it continued to develop in those embryos with one aster (FIG. 3D) and two asters
(FIG. 3E, F) around the swollen nucleus/nuclei (FIG. 4). The formation of asters seemed
to be associated only with the presence of a nucleus/nuclei (FIG. 3D, E, F). When PCC
clusters were induced in Group DA, microtubules were found in the region of the
chromosomal clusters; however, the typical aster structure was not observed in any
oocytes displaying PCC (FIG. 3A, 3B and 3C). All NT embryos in Group DA that
contained a pronuclear structure showed an aster after 4 h of incubation in M199 prior to
activation (FIG. 4), indicating that aster formation was independent of parthenogenetic
activation.

*Developmental Potential of Cloned Embryos Derived From Immediate or Delayed
Activation*

The results of nuclear transfer (Table 1) indicated that a higher fusion rate was
observed in aged oocytes (Group IAA, fusion at 29 hpm) compared to young oocytes
(Groups IA and DA, fusion at 25 hpm). A significant improvement of cleavage (2-8 cells)
rates, and subsequent development to blastocyst (50.0 % vs. 11.6 %) were achieved by immediate activation (Group IA) when compared to delayed activation (Group DA). The efficiency of NT in Group IA, as judged by the rate of blastocyst development based upon the number of oocytes fused, reached as high as 59.1 %. Overall development of cloned embryos in Group IAA was also significantly higher than that from Group DA (40.7 % vs. 14.2 %) although the oocyte age of both treatments was same at the time of parthenogenetic activation (29 hpm). Thus, immediate activation proved much more effective than delayed activation; the latter resulted in a blastocyst development rate of only 14.2% (P<0.05).

The cleavage between Groups IA and IAA was similar, and subsequent preimplantational development to blastocysts, as well as overall blastocyst (BL) percentage (BL/fused oocytes) was higher in Group IA compared to Group IAA, but it was not significant (59.1% vs. 40.7%, P=0.089).

Based on the similar nuclear remodeling and progression results, and comparable in vitro developmental potential between Groups IA and IAA, cloned embryos derived from either IA or DA were subjected to embryo transfer (ET) to examine their viability to term development. Table 2 indicates the pregnancy and calving data with either fresh NT embryos or vitrified NT embryos, derived from fibroblasts and cumulus cells as nuclear donors. In DA group, all NT embryos (cumulus and fibroblast origin) were freshly transferred into recipients. In IA group, NT embryos derived from cumulus donors were vitrified prior to ET. In contrast, a total of 113 fibroblast derived NT embryos in Group IA were either freshly produced (n=49) or vitrified (n=64), subsequently transferred into recipients (fresh ET, n=44, vitrified ET, n=32; total ET, n=76, Table 2). Established
pregnancies, on d 70 of gestation, indicated no statistically significant difference in
established pregnancies between transfers of blastocysts from Group IA (cumulus, 22.2
\%, n=18; fibroblast, 9.2 \%, n=76; overall, 11.7 \%), and Group DA (cumulus, 11.1 \%,
n=18; fibroblast, 12.5 \%, n=112; overall, 12.3 \%)(P>0.05). There was no difference of
pregnancy on d 70 between fresh and vitrified embryos in Group IA when fibroblasts
were used as donor for NT (Table 2). There were 2 live calves born in each of Groups IA
(11.1 \%) and DA (11.1\%) in which cumulus cells were used as donor cells. When
fibroblast cells were used for NT, a high fetal loss was observed after d 70 of embryo
transfer in both Groups IA (4 abortions) and DA (9 abortions) (Table 2). One stillborn
clone from each activation treatment was observed; 2 (2.6 \%) and 4 (3.6 \%) live clones
were delivered from Groups IA and DA, respectively. The overall term development to
live clones was 4.3 \% for Group IA (4 live clones, n=94), and 4.6 \% for Group DA (6
live clones, n=130), respectively.

**DISCUSSION**

Our nuclear transfer study in cattle clearly demonstrated that an occurrence of PCC,
presumably induced by MPF, is not essential for the effective reprogramming/remodeling
of a somatic nucleus introduced into the cytoplasm of an MII oocyte. The direct
exposure of a somatic genome to an MII oocyte rich in MPF, was proven to be sufficient
for the successful reprogramming of a differentiated somatic nucleus [3, 7, 15]. Nuclear
envelope breakdown (NEBD) and PCC have been shown to occur within a short duration
(usually 2-4 h) after a donor nucleus was transferred into an enucleated metaphase II
(non-activated) mammalian oocyte [2, 5, 8, 12, 23]. Nevertheless, the mechanism(s)
involved during the interaction of a donor nucleus with an oocyte’s cytoplasmic
environment, containing high levels of MPF, and the extent of chromosomal remodeling
due to PCC, remain obscure and quite controversial. Several previous studies reported
that a prolonged exposure of a donor nucleus, particular a G0/G1 nucleus, to a non-
activated oocyte, in order to induce PCC, was beneficial for nuclear reprogramming [14,
17, 23]. In mice, Wakayama et al. (1998) showed a high proportion of enucleated
oocytes developed to morulae/blastocysts when they were activated following a
prolonged exposure of the adult somatic nucleus to the oocyte’s cytoplasm [8]. The
inclusion of a prolonged interval between nuclear injection and oocyte activation was
believed to be beneficial for both pre- and post-implantational development [24].
Somatic pig clones could be produced by the combined approaches of simultaneous NT
fusion/activation and followed with serial nuclear exchange technology [25]; however,
pigs [26, 27] shared a similarity with mice [24] in that the induction of PCC was in
association with beneficial nuclear reprogramming. The proportion of reconstructed
porcine oocytes developing to the blastocyst stage was lower when activation was
immediate [14]. Wakayama et al. (1998) believed one of the key steps for successful
cloning of mice was to induce PCC and the subsequent pronuclear-like vesicle formation
in the injected nuclei [8]. It was believed that in other species, such as rats, the failure to
produce live clones was attributed to insufficient PCC induction [28].

Nevertheless, our results show that PCC is not a necessary process for nuclear
reprogramming and subsequent embryo development in cattle. In both Groups IA and
IAA, when donor nuclei were exposed to the presumably MPF-rich cytoplasm for only a
short time prior to chemical activation, PCC was not observed. We did observe, however,
rapid nuclear swelling that might be capable of inducing nuclear de-differentiation into a
pronuclear-like stage in both groups. Our results are in agreement with those of Fulka et al. (1996) [29], who reported that exposure of the donor nucleus to the non-activated oocyte, even for a very short time, has beneficial effects on nuclear remodeling. Our results indicate that the molecular remodeling of an introduced nucleus still occurs within the cytoplasm of an activated oocyte, along with the progressively increasing nuclear swelling; however, inducing dramatic chromosomal structural reformation, such as PCC, can, and likely should, be avoided [3]. Our present results demonstrate that direct nuclear and cytoplasmic interactions are sufficient for reprogramming and subsequent embryo development, regardless of the presence of PCC.

It has been known that the formation of PCC may lead to dramatic chromosomal changes, possibly causing a range of DNA damage (fragmented chromatin, joined chromatin, chromosomal breakage), or loss of chromosomes [29, 30], especially when the donor cell cycle is not compatible with that of the recipient oocyte [5]. Our results showed that a prolonged exposure (up to 4 h) of a donor nucleus to the presumptively high-MPF levels of the pre-activation oocyte was not beneficial for the preimplantational blastocyst development of cloned embryos. The in vitro developmental potential was significantly lower in Group DA (14.2%), compared to Groups IA (59.1%) and IAA (40.7%) (Table 1). The development of NT embryos, derived from cultured skin fibroblasts, was also higher with immediate activation than in those with delayed activation (Du and Yang, data not shown). The effects of immediate or delayed activation in previous reports of bovine somatic cloning are controversial. Wells et al. (1999) achieved a rate of 27.5% blastocysts when cultured adult mural granulosa cells were exposed to a cytoplast for a prolonged period of 4-6 h prior to activation; however,
a direct comparison between immediate activation and a 4-6 h incubation prior to
activation (delayed activation) was not performed in their study. The discrepancies in the
results from various studies might also be explained by differences in selecting somatic
donor cells (bovine ES-like cells vs. somatic cumulus cells) [31], age of oocyte recipients
[32], the experimental conditions [23] and protocols used [32-34]. Akagi et al. (2003)
found that the DA method improved in vitro development potential of NT embryos. In
Akagi et al.’s (2003) study, relative aged oocytes (24 hpm) were subjected to immediate
activation; in our experiment, young oocytes at 21 hpm were arranged for IA treatment.
In our IAA treatment, oocytes at a similar age (25 hpm) were used for NT and subsequent
activation. In this case, the age difference between immediate and delayed activation
groups was eliminated at the time of activation (IAA and DA, activation at 29 hpm). As
shown in our results, Group IAA demonstrated significantly improved preimplantational
development in comparison to that in Group DA. While the pattern of nuclear
progression and remodeling in Group IAA was similar to what occurred in Group IA with
relatively young oocytes, blastocyst development was higher in Group IA (P=0.089). We
believe that young oocytes had more competent capability to reprogram an oocyte if
better oocyte activation and pre-implantational culture were used [15, 21]. On the other
hand, recently, more reports demonstrated that the proportion of embryos with normal
chromosomal ploidy decreased as the incubation time prior to activation was prolonged.
Decreased blastocyst development (0-8.6%) was reported when the exposure period was
longer than 3 h [17]. A plausible explanation for inferior in vitro development of NT
embryos in cattle may be abnormal chromatin structure and/or anomalous pronuclear
formation [17] resulting in numerical chromosome errors, such as polyploidy and
mixoploidy [35]. Our embryo transfer results demonstrated that vitrified NT bovine
embryos derived from immediate activation had similar in vivo survivability as embryos
derived from a delayed activation treatment. This suggests that reprogramming events, in
the absence of PCC, did not have a detrimental effect on in vivo viability of cloned cattle
embryos. In fact, our immediate activation procedure had resulted in fully developed and
healthy newborns in the past [16, 19, 36]. In the present study, 4 and 6 live clones were
produced from either immediate or delayed activation, respectively, indicating the further
implantational development potential was equivalent in the resultant cloned blastocysts
regardless of the manner of activation. Nevertheless, the efficiency of generating cloned
blastocysts was significantly increased with immediate activation (40.7-59.1% vs. 14.2%).

Nuclear reprogramming is a complicated process, involving not only cellular
nuclear-cytoplasm interaction [5], but also epigenetic modification and molecular
differentiation [13]. It is believed that the processes of nuclear swelling, remodeling of
the somatic nucleus, and chromosomal modification were required for successful
reprogramming [3]. NT oocytes in Group DA had significantly larger nuclear sizes than
those in Groups IA and IAA during 12-24 hpf. We assumed that during the process of
reformation of a pronuclear-like structure from the PCC chromatin phase, swelling
factors existing in cytoplasm are likely to more readily participate in nuclear
reconstruction. As a result, dramatically enlarged nucleus/nuclei could be formed in the
DA group. Because only 91.2% of the NT oocytes were observed to show PCC in Group
DA, we cannot also exclude the possibility that some of oocytes in Group DA actually
did not involve PCC-dependent nuclear reformation. A small proportion of oocytes in
Group DA might directly develop nuclear swelling similar to that which occurred in
Group IA; however, this proportion was believed to be relatively minimal. The molecular reprogramming factors, or at least the reprogramming initiation molecules, certainly reside in the cytoplasm of the matured oocyte, and their function may vanish post-parthenogenetic activation [15]. MPF activity in an oocyte was reported to be at a basal level 1 h post activation [3, 21]. MPF or mitogen-activated protein kinase activity was shown not to be a direct regulatory factor for reprogramming in cattle [3]. However, our results cannot rule out the possibility that MPF is acting as an initiator of reprogramming because a somatic nucleus was introduced into a cytoplasm containing high concentrations of MPF [21, 37]. In the cases of our Groups IA and IAA, the nuclear envelope was observed to be intact and the entire area of the nucleus had expanded during activation. We hypothesize that the reprogramming factors can be incorporated into the nucleus during nuclear swelling without disrupting the nuclear envelope membrane; thus, reprogramming of the nucleus occurs without dramatic chromosomal restructuring, such as PCC.

Cattle may represent a unique and excellent species as a model to extrapolate about humans, while they appear to be distant from mice and pigs with respect to immediate and delayed activation. At the very least, improving reprogramming in the context of somatic cell NT requires erasure of cellular memory inherent in the donor cell and re-establishment of patterns of gene expression and their regulation, such as epigenetic methylation and acetylation, for competent embryogenesis and differentiation [38]. One report demonstrated that DNA methylation supported intrinsic epigenetic memory in mammalian cells [39]. They found that DNA methylation is not required for the establishment of the maintenance of silent chromatin status; however, it conferred to
the chromatin structure a long-term, intrinsic epigenetic memory that prevents gene
reactivation. Recently, a typical egg protein nucleoplasmin was reported to induce
massive chromatin decondensation that resulted in nuclear swelling, and to significantly
influence epigenetic modification, such as histone phosphorylation and acetylation [40].
Although it is possible that molecules, such as nucleoplasmin, may serve to catalyze the
exchange of somatic and embryonic histones and de-repress gene expression, the long-
term benefit has not been determined. A comprehensive study with molecular/cellular
mechanistic approaches in combination with a cattle cellular reconstruction system via
somatic cell nuclear transfer will help address interesting questions related to
reprogramming events. Among these questions are whether immediate activation
improves the process of demethylation and histone acetylation, or whether induced PCC
affects a delayed demethylation, alters exchange of DNA proteins, and suppresses de-
repression of genes [41].

According to our data, it was evident that the aster was associated with the
introduced nucleus, suggesting that the centrosome, or microtubule organization center
(MTOC), was introduced into the oocyte with the donor cell via membrane fusion. We
observed the duplication and splitting of the aster following NT in all Groups--IA, IAA
and DA. The aster initially formed at the nuclear poles as a fusiform structure during the
first mitotic phase. This phenomenon is similar to that described by Navara et al. [42].
Due to the experimental design, we were able to observe that the formation and
distribution of asters was independent of parthenogenetic activation but was closely
associated with pronuclear-like structures (FIG. 2 and FIG. 3). We found it most
interesting that no aster formation was observed in reconstructed oocytes that were
induced to PCC by delaying activation. The mechanism(s) inherent in PCC that inhibits or prevents aster formation is unknown and warrants further investigation.

In conclusion, our study has demonstrated that premature chromosome condensation (PCC) is not an indispensable prerequisite for the competent reprogramming of a differentiated somatic genome in cattle. The direct exposure of the donor nucleus to the MII cytoplasm, presumably containing high levels of MPF, for a relatively short period of time, is sufficient to trigger a cascade of nuclear reprogramming and developmental events. Higher efficiency of blastocyst development was obtained by immediate activation. Similar in vivo developmental potentials of NT embryos derived from substantially varied protocols (IA vs. DA), and the birth of live clones from both treatments, suggest that cattle may represent a unique species with a greater plasticity available for mechanical, biochemical, and physiological manipulation.

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FIGURE LEGENDS

FIG. 1. The profile of nuclear dynamics during the first 24 h after NT in cattle. Panel A represents the different nuclear morphologies observed at time points dictated by the experimental design. The morphological appearance of the nucleus was classified as unswollen, swollen, PCC, or having mitotic chromosomal structures. Panel B represents the progressive change in nuclear area of the reconstructed oocytes, comparing immediate activation (IA) and with aged oocytes (IAA), to delayed activation (DA). Star (*) indicates a statistically significant nuclear enlargement in oocytes from Group DA, as compared to those of Groups IA and IAA (P<0.05).

FIG. 2. Laser-scanning confocal images of bovine cumulus cells and NT embryos subjected to immediate activation (IA). (A) A cumulus cell was inserted into the perivitelline space of the oocyte prior to membrane fusion, (B) following fusion with an enucleated oocyte; the introduced nucleus underwent a progressive swelling (C, D, E and F), but without any evidence of PCC. Post-electrical fusion, one aster was observed in proximity of the nucleus (D); subsequently, two asters positioned at opposite poles of an enlarged nucleus (E and F). Microtubules developed in association with the aster(s) (D), and were gradually distributed throughout the cytoplasm (E and F). At 18 h post fusion (hpf) (see FIG. 1), mitotic prophase (G) was established; subsequently, (H) the metaphase spindle, shown to be a fusiform structure, was formed, but apparently lacking the interphase microtubules in the cytoplasm. Metaphase chromosomes are regularly aligned along the spindle equator (H). A prominent astral microtubule structure developed at telophase (I). A metaphase embryo (J) and telophase embryo (K) were evident at the
second cell cycle, and an 8-celled NT embryo (L) was found at 44 hpf. Red color, green
color, and arrow(s) indicate DNA, microtubules, and the position of presumptive aster(s),
respectively. Bars represent 50 μm.

FIG. 3. Laser-scanning confocal microscopy of microtubules and nuclear dynamics in NT
oocytes following delayed activation (DA). Premature chromosomal condensation (PCC)
was indicated by one cluster of chromosomes (A), two scattered (B), and multiple scattered
(C) PCC structures. Microtubules (arrowhead) were observed associated with PCC;
however, no asters or aster-like structures were found in oocytes that had displayed PCC.
A pronuclear structure with one aster (arrow) (D), two nuclei with two asters (arrows) (E),
and multiple nuclear structures with two asters (F) were visualized in NT oocytes. Red
and green color indicated DNA and microtubules, respectively. Bars represent 50 μm.

FIG. 4. The dynamics of aster formation and its association with nuclear structures in
reconstructed oocytes during the first 24 h after nuclear transfer. The aster began to form
at 1 h post fusion (hpf) in embryos from immediate (IA, IAA) and delayed (DA) activation
treatments, and continued to develop in all oocytes observed, with a nucleus appearing at
4-6 phf. The duplication of the aster occurred gradually and was complete at 24 hpf, in
both groups. An aster was observed only in NT oocytes containing nuclei, and never in
oocytes that had displayed PCC.
Table 1. Effects of activation regimen on the development of cloned bovine embryos 
*in vitro.*

| Treatment | Total No. * | No. of reps | % Oocytes Fused | % Embryos developed to 2-8 cell | % Embryos developed to Morula | % Embryos developed to D7 BLs | % BL/fused oocytes§ |
|-----------|------------|-------------|-----------------|---------------------------------|-------------------------------|-------------------------------|-------------------|
| IA        | 145        | 4           | 81.0 ± 1.9<sup>a</sup> | 73.1 ± 1.5<sup>a</sup> | 64.8 ± 8.0<sup>a</sup> | 50.0 ± 6.3<sup>a</sup> | 59.1 ± 9.1<sup>a</sup> |
| DA        | 233        | 4           | 80.9 ± 1.6<sup>a</sup> | 26.6 ± 3.9<sup>b</sup> | 15.0 ± 3.0<sup>b</sup> | 11.6 ± 0.1<sup>b</sup> | 14.2 ± 2.6<sup>b</sup> |
| IAA       | 240        | 4           | 88.9 ± 1.0<sup>b</sup> | 70.9 ± 4.1<sup>a</sup> | 41.2 ± 5.7<sup>a</sup> | 36.2 ± 5.7<sup>a</sup> | 40.7 ± 6.2<sup>a</sup> |

<sup>a,b</sup>Values with different superscripts within columns differ, P<0.05. 2-8 cell, 2 to 8 cell; BL, blastocyst; D7, day 7; DA, delayed activation; IA, immediate activation; IAA, immediate activation aged. NT embryo development to cleavage (2 to 8 cell stage), morula and blastocyst stage evaluated on d2, d4, and d7, respectively, according to the standard of the International Embryo Transfer Society. *Fused oocytes were randomly allocated to treatment groups following membrane fusion. The percentage of embryonic development was calculated based upon the total number of oocytes in each treatment. §P value between Groups IA and IAA was 0.089.
Table 2. Pregnancy and newborn outcomes of fresh and vitrified bovine NT embryos derived from different activation regimens (IA vs. DA)

| Activation | Treatment | Nuclear donor | Embryo type | No. Embryos Transferred | No. Recipients | Day 70 Pregnancy (%) | Development to term (%) |
|------------|-----------|---------------|-------------|-------------------------|----------------|----------------------|-------------------------|
| IA         | Cumulus   | Vitrified     | 36          | 18                      | 4 (22.2)       | 0                    | 2 (11.1)                |
|            | Fibroblast| Fresh         | 49          | 44                      | 3 (6.8)        | 1 (2.3)              | 0                       |
|            |           | Vitrified     | 64          | 32                      | 4 (12.5)       | 0                    | 2 (6.25)                |
| DA         | Cumulus   | Fresh         | 23          | 18                      | 2 (11.1)       | 0                    | 2 (11.1)                |
|            | Fibroblast| Fresh         | 209         | 112                     | 14 (12.5)      | 1 (0.9)              | 4 (3.6)                 |

IA, immediate activation; DA, delayed activation. One to two NT embryos were transferred into each recipient. The percentage in each group was calculated based on the number of recipients transferred in ET trials. NT bovine cloned embryos were cultured for 7 days, and subsequently either transferred into recipients directly, or vitrified by liquid nitrogen surface vitrification on day 7 of culture, then thawed and transferred into recipients. There was no significant difference among the treatments.
FIG. 1
FIG. 2
FIG. 4