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Fuliang Du

Li-Ying Sung

X Cindy Tian

Xiangzhong Yang

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Fuliang Du, Li-Ying Sung, X. Cindy Tian and Xiangzhong Yang*

Department of Animal Science and Connecticut Center for Regenerative Biology, University of Connecticut, Storrs, CT 06269, USA

Running Title: Development of cloned embryos from embryonic vs. somatic cells

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*Correspondence addressed to: Xiangzhong (Jerry) Yang, PhD, Connecticut Center for Regenerative Biology/Department of Animal Science U-4163, University of Connecticut, 1390 Storrs Road, Storrs, CT 06269-4163.

Tel: 860-486-2406, Fax: 860-486-0534, E-mail: jyang@canr.uconn.edu

ABSTRACT Effective activation of a recipient oocyte and its compatibility with the nuclear donor are critical to the successful nuclear reprogramming during nuclear transfer. We designed a series of experiments using various activation methods to determine the optimum activation efficiency of bovine oocytes. We then performed nuclear transfer (NT) of embryonic and somatic cells into cytoplasts presumably at G1/S phase (with prior activation) or at metaphase II (M II, without prior activation). Oocytes at 24 h of maturation *in vitro* were activated with various combinations of calcium ionophore A23187 (A187) (5 μM, 5 min), electric pulse (EP), ethanol (7%, 7 min), cycloheximide (CHX) (10 μg/ml, 6 h), and then cultured in cytochalasin D (CD) for a total of 18 h. Through a series of experiments (Expt 1-4), an improved activation protocol (A187/EP/CHX/CD) was identified and used for comparison of NT efficiency of embryonic vs. somatic donor cells (Expt 5). When embryonic cells from morula and blastocysts were used as nuclear donors, a significantly higher rate of blastocyst development from cloned embryos was obtained with G1/S phase cytoplasts than with M II-phase cytoplasts (36% vs. 11%, $P<0.05$). In contrast, when skin fibroblasts were used as donor cells, the use of an M II cytoplast (vs. G1/S phase) was imperative for blastocyst development (30% vs. 6%, $P<0.05$). Differential staining showed that parthenogenetic, embryonic, and somatic cloned blastocysts contained 26%, 29% and 33% presumptive inner cell mass (ICM) cells, respectively, which is similar to that of frozen-thawed *in vivo* embryos at a comparable developmental stage (23%). These data indicate that embryonic and somatic nuclei require different recipient cytoplast environment for remodeling/reprogramming, and this is likely due to the different cell cycle stage and profiles of molecular differentiation of the transferred donor nuclei.

INTRODUCTION

Nuclear remodeling/reprogramming represents re-establishment of the totipotency of an introduced nucleus with a progressive pattern of gene expression similar to that occurring during the development of a fertilized embryo. The mechanisms involved in reactivation of the genome from either embryonic or differentiated somatic nucleus during reprogramming remain unclear (Kikyo and Wolffe, 2000; Kühholzer and Prather, 2000; Reik et al., 2001; Rideout III et al., 2001). In early nuclear transfer studies conducted in sheep (Willadsen, 1986), cattle (Prather et al., 1987), rabbits (Stice and Robl, 1988), and pigs (Prather et al., 1989), the genome of an embryonic nucleus, was introduced into a metaphase II cytoplasm. Later, studies in sheep demonstrated that the use of recipient cytoplasm at a presumable G1/S phase by pre-activation of the oocyte led to an improved developmental competence of the resultant embryos (Campbell et al., 1994, 1996). This finding was confirmed by several other studies (Stice et al., 1994; Du et al., 1995; Loi et al., 1998; Piotrowaska et al., 2000). More recently, the innovation of somatic cell nuclear transfer has produced live clones in sheep (Wilmut et al., 1997), cattle (Cibelli et al., 1998; Kato et al., 1998; Wells et al., 1999; Hill et al., 2000; Kubota et al., 2000), mice (Wakayama et al., 1998), goats (Baguisi et al., 1999, Zou et al., 2001, Keefer et al., 2002), pigs (Betthauser et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000), cat (Shin et al., 2002) and rabbits (Chesne et al., 2002). In most of these cases, the nucleus from highly differentiated G_o cell or active dividing cell (G1) was transferred into a metaphase II oocyte. No efforts have been made, however, to directly compare the developmental competence of embryos cloned from different donor cells, embryonic vs. adult somatic, and recipient cytoplasts of different activation status, G1/S (pre-activated)

or MII phase. Furthermore, efficient and reliable activation of the recipient oocyte is crucial for competent interaction between a donor nucleus and the recipient cytoplast, and is thought to greatly enhance the efficiency of successful NT (Kono et al., 1994; Stice et al., 1994; Campbell et al., 1996; Wilmut et al, 1997; Wells et al., 1999).

In this study we tested a series of combined activation procedures and selected the best protocol for subsequent nuclear transfer using embryonic and somatic cells as nuclear donors. We report here that while pre-activated cytoplasts are beneficial for embryonic nuclear transfer, M II cytoplasts are essential for somatic cell nuclear transfer.

MATERIALS AND METHODS

Media and Chemicals

Basic culture was in Medium 199 (M199) with Earle's salts, L-glutamine, 2.2 g/l sodium bicarbonate, and 25 mM HEPES (Gibco, 12340-014) containing 7.5% (v/v) fetal calf serum (Gibco, 26140-012) (M199+FCS). Maturation medium consisted of M199+FCS supplemented with 0.5 μg/ml ovine FSH, 5.0 μg/ml ovine LH (NIDDK) and 1.0 μg/ml estradiol (Sigma, E-8875). The media utilized for washing ovaries and oocytes consisted of Dulbecco's phosphate buffered saline (D-PBS; Gibco, 15240-013) containing 0.1% polyvinyl alcohol (PVA; Sigma, P-8136) (D-PBS+PVA). Calcium free D-PBS+PVA was used for preparing 0.2% hyaluronidase (Sigma, H-3506) solution in addition to some activation solutions. Activation solutions were as follows: 5 μM calcium ionophore A23187 (A187) (Sigma, C-7522), 7% ethanol in calcium free D-PBS-PVA (ETOH), and 10 μg/ml cycloheximide (CHX) (Sigma, C-6255) in M199+FCS. Electric-pulse (EP) treatment medium consisted of 0.3 M mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$ and 0.5 mg/ml bovine serum albumin (BSA) (Fraction V, Sigma, A-9647).

Cytochalasin B (CB) (Sigma, C-6762) at concentrations of 2.5, 5, 7.5 μg/ml, and cytochalasin D (CD) (Sigma, C-8273) at 2.5 μg/ml were dissolved in M199+FCS depending on the experimental design. Frozen embryos were thawed by a stepwise procedure in: a) D-PBS with 0.4% BSA, 6% glycerol and 0.3 M sucrose, b) D-PBS with 0.4% BSA, 3% glycerol and 0.3 M sucrose, c) D-PBS with 0.4% BSA and 0.3 M sucrose, and d) 0.4% BSA in D-PBS for 5 min each. The zona pellucida of the donor embryos were removed by acidic D-PBS (pH 2.3) and 0.5% pronase (Sigma, P-6911) in M199. The solution to desegregate embryonic blastomeres was 0.25% trypsin (Sigma, T-0646) in Hank's balanced salt solution (HBSS; Gibco, 450-1250EB). Micromanipulation medium for enucleation and donor cell transfer was M199+FCS containing 7.5 μg/ml CB. Skin fibroblast cells were cultured in Dulbecco's Minimum Eagle's medium (DMEM; Gibco, 31600) supplemented with 10% FBS (Hyclone, SH0070.03) and antibiotics (Gibco, 15240-013) at 37^{o}C in 5% CO_2 humidified air. Medium M2, containing 4 mg/ml BSA was the basic solution for differential staining. Before staining, the following chemicals and solutions were prepared: 10 mM 2, 4, 6-Trinitrobenzenesulfonic acid (TNBS) (Sigma, P-2297), 0.1 mg/ml anti-DNP-BSA (ICN, 610006-1), specified concentration of guinea pig complement (Sigma, S-1639), 2.5 mg/ml propidium iodide (PI; Sigma, P-4170), and 5 μg/ml Hoechst 33258 (Sigma, B-2883).

Oocyte Maturation *In Vitro*, Selection and Activation

Oocytes used in this study were aspirated from antral follicles of slaughterhouse ovaries as described previously (Yang et al., 1993). Oocytes with at least 4 layers of cumulus cells were selected, washed three times in D-PBS+PVA, one time in maturation

medium, and then cultured for 20-22 h in 5% CO_2 and 95% humidified air at 39 ^{o}C. Cumulus cells were then stripped from the oocytes by 5 min of incubation in 0.2% hyaluronidase and 1.5 min vigorous vortexing. Denuded oocytes with a polar body were selected and randomly allocated to various activation or enucleation treatments.

In a series of activation studies different repetitive and combined activation protocols were compared according to the experimental design detailed below. Briefly, oocytes were activated by various activation procedures beginning at 24 h post maturation (hpm). Activation stimuli included A23187, 5 μM for 5 min; EP, 1.2 kV/cm, for 30 μsec unless indicated otherwise; CHX 10 μg/ml in M199-FCS for 6 h (24-30 hpm), and subsequently 2.5 or 5 μg/ml CB or 2.5 μg/ml CD in M199-FCS for 18 h (24-42 hpm).

Donor embryos and embryonic cell isolation for nuclear transfer

Embryonic donor cells for NT were derived from frozen embryos produced *in vivo* at late morulae and early blastocyst stage. Embryos were thawed at 21-27°C in air for 10 sec, then in a 27°C water bath for less than 10 sec. Glycerol was removed in four steps of 5 min each by systematically pre-washing with the thawing solutions described above with decreasing concentrations of glycerol. The thawed embryos were then transferred into M199-FCS and cultured for 30-45 min.

After removal of the zona pellucida, embryos were incubated for 2-3 min in cell disaggregating solution and gently pipetted with a fine, fire-polished capillary pipette in M199+FCS until individual cells were disaggregated. Isolated cells had diverse morphology, and only the small, round-shaped and healthy looking cells were selected for NT.

Adult fibroblast cell culture and donor cell preparation

Skin explants taken from the ear of Aspen, a 13-year-old dairy cow with a high milk yield from the University of Connecticut's herd, were cultured in Falcon 35x10 mm culture dishes (Becton Dickinson, 3001) with 10% FBS DMEM at 37°C in 5% CO_2 humidified air. Fibroblast monolayers formed around the tissue explants in about two weeks. The explants were then removed and placed into new culture dishes. Cultures of the fibroblasts were continued until confluency was reached. For passaging, cells were washed with 1 ml of Dulbecco's PBS, then gently digested by a three-minute incubation in 250 µl 0.05% trypsin (ICN, 103140) and 0.5 mM EDTA (Baker, 8991) at 37 °C. The reaction was terminated by adding 10% FBS in DMEM. Subsequently, the cell suspension was centrifuged at 1000 rpm for 5 min, and cells were then resuspended and divided into three new dishes and maintained for 6-7 days. Cells cultured to different passages were collected and frozen in 10% dimethylsulfoxide DMSO (Sigma, D5879) at –80 °C and stored in liquid nitrogen.

In this study fibroblast cells at passage 5 or 6 were used for nuclear transfer. Briefly, after reaching confluency, donor cells were serum starved in 0.5% FBS DMEM for 4-5 days. Cells were then disassociated by 2-3 min of trypsinization at 37°C, and resuspended in 0.5 % FBS in DMEM. Finally, cell suspensions were allowed to recover for about 45 min at 37°C before nuclear transfer.

Culture and evaluation of parthenogenetic and cloned embryos *in vitro*

Activated oocytes and nuclear transferred embryos were cultured in M199 medium supplemented with 7.5% FBS for 8 days (initiation of activation = day 0) on buffalo rat liver cell (BRLC) monolayers. Cleavage and blastocyst development rates

were recorded on Day 3 and Day 8, respectively. The cell number of the blastocysts was evaluated either by fluorescent microscopy following staining with 10μg/ml Hoechst 33342, or by differential staining described below.

Experiment 1. Comparison of activation protocols

Five activation treatments were included. Electric pulse field strength was 1.2 kV/cm in this experiment except for Treatment D (1.0 kV/cm, 90 μsec). In Treatment A, oocytes were sequentially activated with A187 at 24 hpm, EP at 25 hpm, then cultured in CHX for 6 h, followed by two electric pulses (EP) 30 min apart at 31 hpm (A187/EP/CHX/EPx2). In Treatment B oocytes were stimulated as for Treatment A except that EP stimulation was given at 24 hpm instead of A187 (EP/EP/CHX/EPx2). In Treatment C, oocytes were activated with EP at 24 hpm, ETOH at 25 hpm, followed by the same procedures as for treatments A and B (EP/ETOH/CHX/EPx2). In Treatment D, oocytes were treated with A23187 at 24 hpm, EP, 1.0 kV/cm, 90 μsec at 25 hpm, CHX for 6 h and CD for 18 h (A187/EP90/CHX/CD). Treatment E was a non-stimulation control (Table 1).

Experiment 2. Comparison of electric pulses

Five treatments were designed to examine the effect of the intensity and duration of electric pulses on embryonic development of activated oocytes. Oocytes in Treatment D (A187/EP90/CHX/CD) were handled as in Treatment D in Exp. 1, while oocytes in treatments A, B, and C were activated as in Treatment D except for the EP stimulus being varied to 1.2 kV/cm 30, 45, and 60 μsec for treatments: A (A187/EP/CHX/CD), B (A187/EP45/CHX/CD) and C (A187/EP60/CHX/CD), respectively. Treatment E was conducted as for Treatment A but without CD incubation (A187/EP/CHX) (Table 2).

Experiment 3. Effect of cytochalasins on parthenogenetic development

Treatments A, B, and C were the same as in Treatment A of Exp. 1. However, the oocytes were incubated for 18 h in 2.5 μg/ml cytochalasin B (CB) (A, A187/EP/CHX/CB2.5/EPx2), 5 μg/ml CB (B, A187/EP/CHX/CB5/EPx2) and 2.5 μg/ml cytochalasin D (CD) (C, A187/EP/CHX/CD/EPx2), respectively. Treatment D was the same as for Treatment D in Exp.1 (A187/EP90/CHX/CD) (Table 3).

Experiment 4. Effect of fusion pulses on parthenogenetic development

In previous studies, we had found that two EPs (30 min apart) at 31 hpm had enhanced the membrane fusion and development of NT embryos (Du et al., 1995). We, therefore, directly compared whether there was a difference between *in vitro* development of activated oocytes from Treatment C in Exp. 3 (A187/EP/CHX/CD/EPx2), and Treatment A in Exp. 2 (A187/EP/CHX/CD).

Experiment 5. NT with metaphase II (MII) and pre-activated (G1/S) cytoplasts

Recipient oocyte enucleation was conducted in M199+FCS containing 7.5 μg/ml CB at 22 hpm by aspiration of the first polar body and its surrounding cytoplasm, ~1/8 total oocyte volume. Successful enucleation was confirmed by fluorescent microscopy after staining with 10μg/ml Hoechst 33342. For activation prior to NT, enucleated oocytes were activated with the optimal procedure as determined from previous experiments (A187/EP/CHX) from 24 to 30 hpm that induced pre-activated G1/S cytoplasts. Embryonic donor cell insertion was completed during 30-31 hpm and membrane fusion was induced at 31 hpm with two EPs (1.2 kV/cm, 30 μsec) 30 min. apart. In the case of NT into MII cytoplasts, donor cells were transferred at 24 hpm, and electric fusion was completed by about 25 hpm. After the second electric pulse, oocyte-

donor cell complexes were incubated for 15-30 min in 20% FBS in PBS at room temperature before being subjected to further activation procedures (A187/EP/CHX) between 25 and 31 hpm. Fusion rates were determined 90 min after the first fusion pulse. Following activation the fused embryos were cultured in M199+FCS on BRLC monolayers (Rehman et al., 1994).

In NT with embryonic cells, Treatment A was NT into pre-activated cytoplasts (A187/EP/CHX/NT) while Treatment B was NT into a cytoplast without prior activation (MII/NT/A187/EP/CHX) (Table 5).

When somatic nuclei from skin fibroblasts were used as the donors, NT with pre-activated (Treatment C) and metaphase II (Treatment D) cytoplasts was completed as described above (Table 5). Small donor cells with an approximate diameter of 12-15 μm were allocated for transfer into the perivitelline space of enucleated oocytes (Vignon et al., 1998). Somatic donor cell-cytoplasm pairs were fused by applying two direct current pulses at 2.0 kV/cm for a duration of 10 μsec/each pulse. Following the completion of electric fusion, there was also an 15 min incubation at room temperature before activation with the optimal regime of A187/EP/CHX was applied.

Differential staining

Embryos were allowed to develop to Day 8. Early blastocysts (BL), regular BL, expanded BL and hatched BL were harvested from parthenogenetic, cloned and *in vivo* produced embryos. Expanded and hatched BLs were subjected to differential staining. After removal of the zona pellucida, embryos were treated with 10 mM TNBS for 10 min at 4°C, washed 3 times in M_2-BSA and incubated in 0.1 mg/ml anti-DNP-BSA for 10 min at 39°C, washed in M_2-BSA again to remove surplus antibody, then treated by a 12

min incubation with guinea pig complement solution and 0.25 mg/ml propidium iodide. The embryos were then stained with 0.5 mg/ml Hoechst 33258 in ethanol for at least 1 h to distinguish presumptive inner cell mass (ICM) whose nuclei stained blue. Presumptive trophectoderm (TE) cells were stained by both propidium and Hoechst 33258, and differentially indicated by a pink stain. Embryos were mounted and gently squashed under a cover slip for counting of nuclei under fluorescent microscopy.

Statistical Analyses

Proportions of embryos reaching cleavage and developing to the blastocyst stage from various treatments within each experiment were analyzed by Chi-square (Snedecor and Cochran, 1980) or student's t-test. The mean number of nuclei for each embryo was compared by one-way ANOVA. The P values less than 0.05 are considered as significant between the treatments.

RESULTS

Experiment 1

After *in vitro* maturation of oocytes for 20 h, cumulus cells had expanded as shown in Fig 1.A. Following parthenogenetic activation, oocytes usually cleaved to the 4-8 cell stage at 44-48 hr of *in vitro* culture, and further developed to compacted morula (Fig 1.B) on Day 4, and to expanding blastocysts (Fig 1.C) on Day 8 in accordance to the developmental pace expected for *in vitro* fertilized embryos. The proportion of degenerated oocytes following activation in Treatments B (27%), and D (38%) were significantly higher than in Treatments A (4%), C (4%) and control E (0%) ($P<0.05$). Oocyte degeneration frequently took place before two electric pulses were applied at 31 hpm. Among the survived oocytes in Treatments A, B, C, and D, the oocytes with good

quality were selected for continuous *in vitro* culture. There was no difference among Treatments A, B, or C in terms of cleavage and blastocyst development after culture *in vitro*, and no difference among Treatment A, B, C, and D for the overall rate of blastocyst development (Table 1). Despite the high percentage of oocyte lysis in Treatment D when compared to Treatments A, B and C, a significant higher rate of cleavage to 2-8 cells (74% vs. 42-53%, $P<0.05$) and subsequent embryonic development to blastocyst stage (31% vs. 8-14%, $P<0.05$) (Table 1) were observed. In the control group (Treatment E), 11% of oocytes underwent spontaneous activation and cleavage to the 2-8 cell stage, but no further development was observed. Thereafter, experiments were specifically designed to determine whether the higher degree of oocyte lysis was due to the duration of the EP and cytochalasin incubation (Exp.2), or if the development was improved by the cytochalasin treatment (Exp. 3).

Experiment 2

This experiment was designed to test if a longer duration of electrical pulse resulted in a significantly higher incidence of oocyte lysis. As shown in Table 2, when 1.2 kV/cm was applied and the duration was increased from 30 μsec in Treatment A to 45 μsec in Treatment B, 11% and 17%, respectively, of the oocytes were lysed, while 28% in Treatment C were degenerated after subjected to a pulse of 1.2 kV/cm for 60 μsec, significantly higher than Treatments A and B ($P<0.05$). When the duration of the pulse (1.0 kV/cm) was increased to 90 μsec in Treatment D, up to 42% of the oocytes were completely lysed, significantly higher than Treatments A, B and C ($P<0.05$). In contrast to other groups, Treatment E, in which oocytes were activated as for Treatment A but without cytochalasin D incubation, represented the lowest lysis (2%), significantly lower

than Treatments A, B, C and D ($P<0.05$). The overall blastocyst development varied from 22 to 29% in Treatments A and B, which was significantly higher than Treatments D and E. The highest rate of development (29%) was achieved in Treatment A that was significantly higher than Treatments C, D and E (Table 2). We concluded, therefore, that oocyte lysis was caused by both prolonged exposure to electric pulse and culture with cytochalasin D. Interestingly, the cleavage and blastocyst development of oocytes in Treatments A, B, C and D were not different from each other. These values, however, were significantly higher than those in Treatment E ($P<0.05$), indicating the beneficial effect of cytochalasin D on the development of parthenogenetic oocytes.

Experiment 3

From the results of Exp. 2, Treatment A (least oocyte lysis) was selected to determine the effect of cytochalasins on embryo development (Table 3). There was no difference in the extent of cell lysis between Treatments A to C with various concentrations of cytochalasin D or cytochalasin B (A: 15%; B: 14%; C: 12%; $P>0.05$). A significantly higher rate of oocyte lysis was found in Treatment D (53%, $P<0.05$). However, live parthenogenetic oocytes showed similar cleavage rates and blastocyst development among Treatments A and B ($P>0.05$). In contrast, oocytes in Treatment C gave rise to 38% blastocyst development, better than any cytochalasin B treatments although there was no difference between the cytochalasin D groups. The overall efficiency of blastocyst development in Treatment C was 26% when all oocytes used were taken into consideration and this is the highest among all treatments (Table 3). As a result, Treatment C in Exp.3 was selected as the optimal regime to parthenogenetically activate bovine oocytes in the next series of experiments.

Experiment 4

To further determine the effect of repetitive electrical pulses in initiating membrane fusion (30 min apart, applied at 31 hpm) following NT, Treatment A in Exp. 2 and Treatment C in Exp. 3 were compared. Data showed neither cleavage (78%, n=228 vs. 81%, n=245) nor subsequent development to blastocyst (35%, n=228 vs. 40%, n=245) was influenced by these treatments ($P>0.05$), a consistently high 40% blastocyst development rate was achieved.

Experiment 5

With the optimized activation protocol selected (A187/EP/CHX), we conducted a series of NTs with a 2x2 factorial combination of MII vs. pre-activated cytoplasts and embryonic vs. somatic nuclei. The fusion rate was higher in embryonic NT group (A, 75% and B, 79%) than that in the somatic NT group (C, 43% and D, 49%) ($P<0.05$). When the donor nuclei were embryonic cells (Fig 1, E to G), pre-activation of recipient cytoplasts (Treatment A, Fig 1, F) significantly improved the cloned embryo's ability to undergo cleavage (77% vs. 50%, $P<0.05$) and blastocyst (Fig 1, G) development (36% vs. 11%, $P<0.01$) as compared to NT into M II cytoplasm (Table 4). Interestingly, enucleated oocytes without transfer of donor nuclei can also undergo parthenogenetic development. Some cytoplasts could finish several cell divisions developing to the 8-cell stage before degenerating (data not shown). In contrast, when NT was conducted with cultured somatic cells as donors (Fig 1, I), metaphase phase (MII) recipient cytoplasm (Fig 1, J) greatly enhanced the extent to which early embryos cleaved (Fig 1, K) (76% vs. 58%, $P<0.05$) or developed to blastocysts (30% vs. 6%, $P<0.05$).

Analysis of Cell Allocations to TE or ICM

As shown in Table 5, following embryo immunosurgery and differential staining, the total number of nuclei in expanded or hatching blastocysts between parthenogenetic (Fig 1, C, D) and NT (Fig 1, G, H) groups was not significantly different (Table 5). The proportion of ICM in different types of blastocysts was not significantly different for embryonic NT, somatic NT, parthenogenetic and frozen *in vivo* derived embryos, shown as 29%, 33%, 26% and 23%, respectively. Cloned blastocysts developed from an activated cytoplast and an embryonic nucleus (Fig 1, G, H), or from an MII cytoplast and a somatic nucleus (Fig 1, K, L) showed a similar total cell number and ICM/TE ratio. There was also no difference between NT and parthenogenetic hatched embryos with respect to the percentage of pycnotic cells. However, some parthenogenetic embryos were observed with a sporadic and dislocated distribution of ICM cells (58%, n=38) and an apparent variation in nuclear volume ranging from less to more than the average of the group (45%, n=38).

DISCUSSION

In the present study, we compared various activation protocols and showed that the development of cloned embryos reconstructed from either embryonic or somatic nuclei require cytoplasts in different activation status for optimal development. In our study with embryonic donor nuclei, nuclear transfer into pre-activated oocytes resulted in a high percentage, 36%, of blastocysts, while only 11% of embryos without prior activation developed blastocysts. During early development, the embryonic cells divide very rapidly and mitosis is relatively short. The interphase of cell cycle in most embryonic cells in pre-implantation embryos of mice, sheep and cattle is notably occupied by S phase (Collas et al., 1993, Campbell et al., 1994). After fusion between an

S phase cell and an metaphase cell (Johnson and Rao, 1970), active maturation promoting factor (MPF) in the metaphase cell or oocyte initiates nuclear envelope breakdown (NEBD) and premature chromosome condensed (PCC), resulting in a pulverized chromatin appearance (Sperling and Rao, 1974; Szöllösi et al., 1988; Barnes et al., 1993). Pulverized PCC may cause the breakage of chromatin and the damage to DNA duplexes in donor nuclei. Therefore, it is essential to synchronize the recipient oocyte and the S phase nucleus during nuclear transfer. Activation of cytoplasts prior to nuclear transfer makes the recipient oocyte transit from MII to G1/S phase, resulting in a universal cytoplasm for the donor nucleus, and allowing continuous DNA synthesis to occur in the S phase donor nucleus (Campbell et al., 1994, 1996). Although transplanting blastomere nuclei into M II cytoplasts has produced full term development in sheep (Willadsen, 1986), rabbits (Stice and Robl, 1988), cattle (Prather et al., 1987), and pigs (Prather et al., 1989), nuclear-cytoplasm synchronization in embryonic cell nuclear transfer increases the developmental efficiency of the reconstructed embryos (Collas et al., 1993; Campbell et al., 1994; Kono et al., 1994). Recently, prior activation of recipient oocytes resulted in successful nuclear transfer of embryonic cells in cattle (Kubota et al., 1998), Rhesus monkeys (Meng et al., 1997) and rabbits (Piotrowaska et al., 2000).

Alternatively, nuclear transfer with differentiated skin cells and M II cytoplasts yielded significantly higher early development when compared to those transferred into pre-activated recipient cytoplasts. In our study, cultured skin fibroblasts were serum starved for 4-5 days, and were thus synchronized at G_o/G1 phase (Kubota et al., 2000). An M phase cell induces the G1 nucleus into a pattern of PCC with intact single

chromatids (Sperling and Rao, 1974). During somatic nuclear transfer, this chromatin modification due to NEBD and PCC by the MII cytoplast may facilitate the course of reprogramming of differentiated nucleus such as an epithelial cell (Wilmut et al., 1997), cumulus cell (Kato et al., 1998) adult mural granulosa cell (Wells et al., 1999), and skin fibroblast (Hill et al., 2000; Kubota et al., 2000). It is possible that certain degrees of PCC can induce chromatin rearrangement in the donor nucleus that facilitates the process of demethylation of the highly methylated genome. It is unclear, however, that the minimum time somatic nucleus should be exposed to a high level of MPF in the cytoplast for complete nuclear reprogramming (Wilmut et al., 1997; Wakayama et al., 1998; 1999). Wells et al. (1999) demonstrated that exposure of a quiescent nucleus to enucleated MII cytoplast for 4-6 h before activation resulted in an increased proportion (up to 27.5 %) of fused embryos developing into blastocysts. Similarly, nuclei introduced either by electric fusion in cattle (Cibelli et al., 1998; Wells et al., 1998) or direct nuclear injection in pigs (Onishi et al., 2000) were subjected to a 2-6 hour exposure to MII cytoplast before activation. In our study, however, a 30% blastocyst development was obtained when oocyte-donor cell complexes were activated no longer than 15 min after cell fusion. When cumulus cells from the same donor animal were used with the same timing of activation, as high as 50% blastocysts development was achieved (data not shown). Recently, short exposure of somatic nuclei from a genetically modified fetal cell line in MII cytoplasts resulted in cloned blastocysts that produced pregnancies (Du and Yang, 2002, unpublished data). Further experiments will be of interest to determine the minimum period of exposure necessary for the complete reprogramming of a differentiated nucleus.

The factors affecting nuclear reprogramming are mysterious and unclear (Fulka et al., 1998), nevertheless, it is unambiguous that these remodeling factors are uniquely present in the oocyte cytoplasm, and this oocyte reprogramming ability for differentiated somatic nucleus vanishes after pre-activation. Our finding with skin fibroblasts (from a 13-year-old cow) as nuclear donors is in accordance with the results of Tani et al. (2001) using cumulus cells. We believe that unknown somatic remodeling factors have a critical impact on the reprogramming of a differentiated nucleus, and on the developmental potential of the fused embryos, however, they appear to be unstable and lose their function after parthenogenetic activation. In contrast, it seems likely that this influence of remodeling factors in the oocyte on the embryonic nucleus is nominal. Donor cells from compacted morula and blastocysts, as those used in our study, are in a state of undifferentiated development, and possess a low degree of methylated genomic DNA (Kühholzer and Prather, 2000; Ridout III et al., 2001). Due to reduced DNA methylation, cloned embryos derived from embryonic nuclei will share similar processes with ES cell-derived clones that may need little or no reprogramming of genes for early development (Ridout III et al., 2001). Therefore, we assume that the differentiation state of a transplanted donor karyoplast may have some influence over the extent of its reprogramming. In other words, remodeling of a donor genome by a recipient oocyte is dependent upon the molecular differentiation existed in this nucleus, such as methylation, one of the major differentiation events and epigenetic modifications of the genome during mammalian development (Reik et al; 2001).

The efficiency of combined activation could still be improved to further increase nuclear transfer efficiency. In the present study, we have shown that *in vitro* matured

bovine oocytes can be effectively activated and as many as 40% of them can undergo further parthenogenetic development to blastocysts (Expt 1-4). The optimal parthenogenetic activation procedure is a combined treatment of 5 μM A23187 for 5 min at 24 hpm, 1.2 kV/cm of EP for 30 μsec 1 hr later, 10 μg/ml cycloheximide for 6 h (24-42 hpm) in addition to culture in 2.5 μg/ml cytochalasin D (A187/EP/CHX/CD). The synergistic effects of repetitive and combined activation treatments cause destruction of existing MPF and prevents further synthesis of new MPF in the oocytes. The commonly used activation reagents/stimuli are broad-spectrum modulators of calcium concentration (Cuthberston, 1981; Ware et al., 1992; Stice et al., 1994), inhibitors of protein synthesis (Presicce and Yang, 1994; Piotrowska et al., 2000) and phosphorylation (Susko-Parrish et al., 1994; Loi et al., 1998). In addition, the detrimental effects of high intensity electric shock is possibly attributed to damage to the oocyte's membrane and cytoplasmic components (Zimmerman and Vienken, 1982). Cytochalasins are microfilament inhibitors and serve to suppress the extrusion of the second polar body, which sustain the diploid state of the activated oocytes. Our study confirms the observation that both cytochalasins B (Kono et al., 1989, Fukui et al., 1992) and D (Minamihashi et al., 1993) improve parthenogenetic development. It makes no doubt that the development of reagents specific for modulations of proteins involved in oocyte activation will greatly improve the nuclear transfer efficiency.

In the present study, we found that parthenogenetically activated and NT blastocysts have similar TE and ICM cells to those in frozen *in vivo* embryos, but both parthenogenetic and cloned embryos had reduced total cell numbers, as well as ICM numbers, when compared to those of *in vivo* produced embryos (Du and Yang,

unpublished data). It is unknown whether the lower number of cells in the cloned embryos was a result of nuclear reprogramming or was due to the developmental potential of the parthenogenetically activated recipient cytoplasts.

In conclusion, more effective activation and parthenogenetic development in cattle was achieved with a combination treatment consisting of calcium ionophore, electric pulse and cycloheximide. Higher *in vitro* development was achieved when embryonic and somatic donor cells were transferred into pre-activated and MII cytoplasts, respectively.

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Table 1. Development of bovine oocytes following different activation procedures

Treatment	No. rep	Total No. oocytes	No. (%) oocytes lysed	No. of oocytes cultured*	No. (%) cleaved	No. (%) BL	% (overall BL rate)
A. A187/EP/CHX/EPx2	4	134	5 (4)[a]	90	48 (53)[a]	13 (14)[a]	10[a]
B. EP/EP/CHX/EPx2	4	156	42 (27)[b]	90	45 (50)[a]	7(8)[a]	4[a,b]
C. EP/ETOH/CHX/EPx2	4	135	5 (4)[a]	90	38 (42)[a]	11 (12)[a]	8[a]
D. A187/EP90/CHX/CD	4	211	80 (38)[b]	90	67 (74)[b]	28 (31)[b]	13[a]
E. Control	4	102	0 (0)[a]	102	11 (11)[c]	0[c]	0[b]

[abc]Values with different superscripts within columns differ, $P<0.05$. A187, calcium ionophore A23187; CD, cytochalasin D; CHX, cycloheximide; EP, electrical pulse; EPx2, two electrical pulses applied; EP90, electrical pulse at 90 μsec; ETOH, ethanol. *The oocytes from each group are selected for further culture experiment, and leftover oocytes are fixed and subjected to morphological evaluation (data not shown). The overall blastocyst rates were calculated using total number of oocytes in each treatment.

Table 2. Effect of intensity of electric pulse on parthenogenetic development

Treatment	No. rep	Total No. oocytes	No. (%) oocytes lysed	No. of oocytes cultured	No. (%) cleaved	No. (%) BL	% (overall BL rate)
A. A187/EP/CHX/CD	3	122	13 (11)[a]	109	86 (79)[a]	36 (33)[a]	29[a]
B. A187/EP45/CHX/CD	3	122	21 (17)[a]	101	74 (73)[a]	27 (27)[a]	22[a,b]
C. A187/EP60/CHX/CD	3	122	34 (28)[b]	88	62 (70)[a]	24 (27)[a]	20[b,c]
D. A187/EP90/CHX/CD	3	122	51 (42)[c]	71	52 (73)[a]	15 (21)[a]	12[c,d]
E. A187/EP/CHX	3	122	2 (2)[d]	120	55 (46)[b]	11 (9)[b]	9[d]

[abcd]Values within columns with different superscripts differ, $P<0.05$. A187, calcium ionophore A23187; CD, cytochalasin D; CHX, cycloheximide; EP, electrical pulse; EP 45, EP60, and EP90 represent electrical pulse at 45, 60 and 90 μsec, respectively. The overall blastocyst rates were calculated using total number of oocytes in each treatment.

Table 3. Effect of cytochalasins on parthenogenetic development

Treatment	No. rep	Total No. oocytes	No. (%) oocytes lysed	No. of oocytes cultured*	No. (%) cleaved	No. (%) BL	% (overall BL rate)
A. A187/EP/CHX/CB5/EPx2	5	206	31 (15)[a]	139	97 (70)[a]	29 (21)[a]	14[a]
B.A187/EP/CHX/CB2.5/EPx2	5	210	29 (14)[a]	142	96 (68)[a]	37 (26)[a]	17[a]
C. A187/EP/CHX/CD/EPx2	5	205	25 (12)[a]	141	104 (74)[a]	54 (38)[b]	26[b]
D. A187/EP90/CHX/CD	5	264	141 (53)[b]	113	84 (74)[a]	35 (31)[ab]	13[a]

[ab]Values within columns with different superscripts differ, P<0.05. A187, calcium ionophore A23187;CB2.5, cytochalasin B at 2.5 μg/ml; CB5, cytochalasin B at 5.0 μg/ml; CD, cytochalasin D; CHX, cycloheximide; EP, electrical pulse; EP90, electrical pulse at 90 μsec. *The oocytes from each group are selected for further culture experiment, and leftover oocytes are fixed and subjected to morphological evaluation (data not shown). The overall blastocyst rates were calculated using total number of oocytes in each treatment.

Table 4. Development of NT embryos with embryonic and somatic donor nuclei

Treatment	No. rep	No. of donor oocyte pairs	No.(%) of fused	No. (%) cleaved*	No. (%) BL*
Embryonic donor NT					
A. A187/EP/CHX/NT	5	233	184 (79)[a]	142 (77)[a]	66 (36)[a]
B. MII/NT/A187/EP/CHX	5	97	73 (75)[a]	37 (50)[b]	8 (11)[b]
Somatic donor NT					
C. A187/EP/CHX/NT	5	249	107 (43)[b]	62 (58)[b]	6 (6)[b]
D. MII/NT/A187/EP/CHX	5	253	124 (49)[b]	94 (76)[a]	37 (30)[a]

[a,b]Values within columns with different superscripts differ, $P<0.05$. A187, calcium ionophore A23187; CHX, cycloheximide; EP, electrical pulse; MII, metaphase II; NT, nuclear transfer. *The rates of development to cleavage and blastocyst in NT embryos were calculated from the number of fused embryos.

Table 5. Analysis of TE/ICM in different types of parthenogenetic and NT blastocysts

Blastocyst type (Day 8)	No. of embryos	Cells/ BL	No. (%) ICM cells	No. (%) pycnotic cells
Parthenogenetic	12	133±14[a]	36±6 (26)[a]	9±1 (7)[a]
Embryonic NT	14	142±18[a]	44±9 (29)[a]	5±2 (3)[a]
Somatic NT	12	139±10[a]	47±8 (33)[a]	4±2 (3)[a]
Frozen *in vivo*	7	145±17[a]	32±6 (23)[a]	9±3 (6)[a]

[a]Values within columns with the same superscript do not differ, P>0.05.

Parthenogenetic, blastocysts developed from parthenogenetically activated oocytes;

Frozen *in vivo*, blastocysts thawed from cryo-preserved *in vivo* fertilized embryos;

NT, nuclear transfer.

Fig. 1. Parthenogenetic activation and nuclear transfer in cattle. (**A**) Bovine oocytes after maturation for 20-22 h *in vitro* showed the expansion of cumulus cells. After activation oocytes have undergone development *in vitro* to compacted morulae (**B**) at Day 4.5 and expanded blastocysts (**C**) at Day 8. (**D**)The inner cell mass (ICM) and trophectoderm (TE) cells were stained blue and pink, respectively, when a parthenogenetic blastocyst was treated with differential staining (arrows indicating pycnotic inner nuclei shown as blue fragments). (**E-H**) Nuclear transfer of embryonic donor nuclei into pre-activated cytoplasts. Oocytes are enucleated by aspirating the first polar body (arrow) and surrounding cytoplasm containing the metaphase plate (**E**), then subjected to an activation protocol, followed by insertion of embryonic cells (**F**) and cell fusion as shown by the arrow in the insert of (**F**). Fused embryos developed to hatched blastocysts (**G**) at Day 8 with a proportional allocation of ICM (blue)/TE (pink) cells (**H**). (**I-L**) Nuclear transfer of somatic cells into M II cytoplasts. Fibroblasts (**I**) at passage 5-6 were transferred into the perivitelline space of oocytes (**J**), and after optimal activation fused embryos developed to hatched blastocysts (**K**) *in vitro* at 8 days of culture. The resultant blastocysts possessed ICM (blue) and TE (pink) cells (**L**). Bar=100 μm.