Effect of the time interval between fusion and activation on nuclear state and development *in vitro* and *in vivo* of bovine somatic cell nuclear transfer embryos

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Abstract

This study indicated that prolonged exposure of donor cell nuclei to oocyte cytoplasm before activation results in abnormal chromatin morphology, and reduced development to compacted morula/blastocyst stage *in vitro*. However, after transfer of embryos to recipients, there was no difference in pregnancy rates throughout gestation. Chromatin morphology was evaluated for embryos held 2, 3, 4 and 5 h between fusion and activation. In embryos held 2 h, 15/17 (88.2%) embryos contained condensed chromosomes, while only 12/24 (50.0%) embryos held 3 h exhibited this characteristic. The proportion of embryos with elongated or fragmented chromosomes tended to increase with increased hold time. While 15/19 (78.9%) of embryos held 2 h developed a single pronucleus 6 h after activation, only 8/22 (36.4%) had one pronucleus after a 4-h hold. Embryos held 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 h cleaved at rates of 207/281 (73.7%), 142/166 (85.5%), 655/912 (71.8%), 212/368 (57.6%), 406/667 (60.9%), 362/644 (56.2%) and 120/228 (52.6%) respectively. Further development to compacted morula/blastocyst stage occurred at rates of 78/281 (27.8%), 42/166 (25.3%), 264/912 (28.9%), 79/368 (21.5%), 99/667 (14.8%), 94/644 (14.6%) and 27/228 (11.8%) respectively. Embryos held less than 2.5 h between fusion and activation established pregnancies in 18/66 (27.3%) of recipients, while embryos held over 2.5 h established pregnancies at a rate of 17/57 (29.8%). This study indicates that holding bovine nuclear transfer embryos less than 2.5 h between fusion and activation results in improved nuclear morphology and increased development to compacted morula/blastocyst stage, and results in pregnancy rates equivalent to embryos held over 2.5 h.

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Introduction

Successful somatic cell nuclear transfer (NT) has been achieved in domestic animals and rodents, as reported by the birth of offspring. The overall efficiency of this technique, however, remains low, generally less than 2% (Hill 2002). A high frequency of early postimplantation developmental arrest and abortion occurs, especially in cattle. The exact mechanism(s) contributing to losses are still unclear. Epigenetic alterations (Wrenzycki *et al.* 2001, Cezar *et al.* 2003) and chromosomal abnormalities (Burgoyne *et al.* 1991, Li *et al.* 2004a, 2004b) probably contribute to developmental failure.

After the transfer of a differentiated nucleus into an enucleated MII oocyte, the nucleus is disassembled, an event involved in reprogramming the differentiated donor nucleus to a totipotent embryonic state. This disassembly involves nuclear envelope breakdown (NEBD) and

premature chromosome condensation (PCC), mediated by high levels of mitosis/meiosis/maturation-promoting factor (MPF) in the oocyte cytoplasm (Czolowska *et al.* 1984, Barnes *et al.* 1993, Campbell *et al.* 1996). These events are followed by erasure of epigenetic modification of DNA, including changes in histone acetylation (Nakao 2001) and DNA methylation (Kang *et al.* 2003, Shi *et al.* 2003). Since maternal transcripts are responsible for the events of early embryonic development (Telford *et al.* 1990), nuclear reprogramming is presumably mediated by factors in the oocyte cytoplasm.

Several groups have shown that the duration of exposure of the donor nucleus to oocyte cytoplasm after NT affects *in vitro* development; however, the conclusions are mixed. Some reports have indicated that prolonged exposure to the oocyte cytoplasm before activation may be beneficial in promoting embryo development for bovine (Wells *et al.* 1998, 1999) and murine NT

(Wakayama *et al.* 1998). Another study reported that exposure of transferred nuclei to cytoplasm for less than 30 min before activation yielded significantly lower blastocyst development than a 2 h exposure (Liu *et al.* 2001). Conversely, other research indicates that excessive exposure of the donor DNA to oocyte cytoplasm results in lower rates of *in vitro* development in cloned embryos (Akagi *et al.* 2001). Most recently, Choi *et al.* (2004) demonstrated that *in vitro* development of bovine NT embryos to blastocyst decreased as time in hold increased from 1 to 5 h.

Given the conflicting data on the subject, we have evaluated the effect of timing between fusion and activation on NT development. The present study was designed to examine the effect of different time intervals between fusion and activation on structure of the transferred nucleus and embryonic development *in vitro* and *in vivo*.

Materials and Methods

Unless otherwise noted, all reagents used were obtained from MP Biochemicals, Irvine, CA, USA.

Donor cell culture

Primary bovine fibroblast cultures were established from either lung tissue or ear biopsy. Previous data have demonstrated no difference in *in vitro* development between lung- and ear-derived donor cells (Kato *et al.* 2000). Tissues were washed thoroughly and minced, suspended in DMEM/Ham's F12 (1:1) (Hyclone Laboratories, Logan, UT, USA) supplemented with 15% fetal bovine serum (FBS) (Hyclone Laboratories) and 100 U/ml penicillin/100 µg/ml streptomycin (Hyclone Laboratories), seeded in 25 cm² tissue culture flasks, and cultured at 39°C in a humidified atmosphere of 5% CO₂ in air for several days. Cells were then harvested in tissue culture medium containing 10% DMSO and stored in liquid N₂ until use in NT. Frozen/thawed cells were grown to 80–100% confluence, and passages 2–16 were used as nuclear donors.

Oocyte maturation

Maturation of bovine oocytes was performed as described previously (Li *et al.* 2004a, 2004b). Briefly, cumulus oocyte complexes (COC) were aspirated from 3–8 mm follicles with an 18 gauge needle from ovaries collected from a local abattoir. Only those with uniform cytoplasm and intact layers of cumulus cells were selected and matured in TCM 199 containing 10% FBS, 0.5 µg/ml follicle-stimulating hormone (FSH) (Sioux Biochemicals, Sioux City, IA, USA), 5 µg/ml luteinizing hormone (LH) (Sioux Biochemicals), and 100 U/ml penicillin/100 µg/ml streptomycin for 18–22 h.

Nuclear transfer (NT)

After maturation, cumulus cells were removed by vortexing COC in PB1 (calcium and magnesium containing PBS (Hyclone Laboratories), 0.32 mM sodium pyruvate, 5.55 mM glucose and 3 mg/ml BSA) medium containing 10 mg/ml hyaluronidase. Oocytes with a first polar body were used as recipient cytoplasts. Enucleation was employed to remove the first polar body and metaphase plate, and single cells were subsequently transferred to the perivitelline space of recipient cytoplasts. Fusions of NT couplets were performed in mannitol fusion medium (Wells *et al.* 1999) by two electric DC pulses of 2.2 kV/cm for 25 µs. After fusion, embryos were held in CR2 medium supplemented with 3% FBS for 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 h before activation. Fused embryos were activated at 23–25 h after the onset of maturation by exposure to 5 µM ionomycin for 5 min followed by 5 h incubation in 10 µg/ml cycloheximide.

Nuclear and microtubule assessment by immunofluorescent staining

Reconstructed embryos were fixed 2, 3, 4 and 5 h after fusion. Some embryos activated 2–4 h after fusion were fixed 6 h after initial activation to evaluate pronuclear morphology. Immunofluorescent staining was performed as reported (Zhu *et al.* 2003) with some modifications. Briefly, embryos were fixed with 3.7% (w/v) paraformaldehyde in PBS overnight at 4°C. Fixed embryos were extracted in PBS containing 1% (w/v) Triton X-100 and 0.3% BSA for 1 h at 37°C. After two washes with PBS containing 0.01% Triton X-100, embryos were blocked in PBS containing 150 mM glycine and 1% Triton X-100 for 1 h at 37°C. The embryos were then incubated for 1 h at 37°C in a mouse monoclonal antibody against α -tubulin (T-5168; Sigma) diluted 1:100 in PBS. They were then washed with PBS and incubated in fluorescein isothiocyanate-labeled goat antimouse IgG (Southern Biotechnology Associates, Birmingham, AL, USA; Cat no. 1030-02) diluted 1:100 in PBS for 1 h at 37°C. Chromatin was stained with 10 µg/ml propidium iodide. Finally, embryos were mounted on slides with a solution of glycerol and PBS (1:1). The samples were examined under a Zeiss epifluorescent microscope (Carl Zeiss Optical, Chester, VA, USA). Images were captured by digital camera with the PIXERA Viewfinder Program (Pixera Corporation, Los Gatos, CA, USA).

Embryo culture

After activation, embryos were cultured under mineral oil in 50 µl droplets of CR2 with 3% FBS on a monolayer of bovine cumulus cells at 39°C in an atmosphere of 5% CO₂ in air for 6–7 days. Medium was changed every 48 h. Cleavage and compacted morula/blastocyst rates

were recorded 48 h and 6–7 days after activation respectively.

Embryo transfer

On day 6 or 7, compacted morulae and blastocysts were shipped overnight in equilibrated CR2 at 38.5°C to the site of transfer. One to two embryos were transferred non-surgically to cows synchronized ± 1 day to the stage of embryonic development. Pregnancy was detected by transrectal ultrasound at embryonic days 25–30.

Statistical analyses

Data were pooled from at least four replicates per group for the *in vitro* development studies. Chi-square analysis was used to determine differences in cleavage and development to the compacted morula stage between hold times. Differences in remodeling and nuclear morphology between groups were analyzed by Student's *t*-test. Unless otherwise noted, a probability of $P < 0.05$ was considered statistically significant.

Results

Nuclear morphology

When the preactivation-reconstructed embryos were examined 2 h after fusion, the majority of the embryos (88%, 15/17) possessed condensed chromosomes (Fig. 1a) or a chromosome array resembling the maternal metaphase plate (metaphase-like chromosomes, Fig. 1b) that was significantly higher ($P < 0.05$) than for embryos examined 3 h after fusion (50%, 12/24). The proportion of

the embryos possessing elongated or scattered chromosomes tended to increase with increasing time between fusion and activation (Table 1). Eighty-two percent of the embryos held 4–5 h between fusion and activation possessed elongated (Fig. 1c and d) or scattered (Fig. 1e) chromosomes.

Of embryos observed 6 h after activation, 78.9% of the embryos activated 2 h after fusion developed a single pronuclear structure (PN) that was significantly higher than those activated 4 h after fusion (36.4%) (Table 2). Of the embryos activated 4 h after fusion, 63.6% possessed two or more PN (Fig. 1f–i).

Embryo development in vitro and in vivo

Cleavage of embryos derived from groups activated 1–2 h after fusion (71.8–85.5%) was significantly higher ($P < 0.01$) than that of embryos held 2.5–4.0 h between fusion and activation (52.6–60.9%) (Fig. 2 and Table 3). The compacted morula/blastocyst development of embryos activated 1.0–2.5 h after fusion (21.5–28.9%) was higher ($P < 0.01$) than that of embryos activated 3–4 h after fusion (11.8–14.8%) (Fig. 2 and Table 3). After transfer of the cloned, compacted morulae/blastocysts to recipients, no differences were observed in day-30 pregnancy rates between embryos held under 2.5 h (18 pregnancies/66 transfers (27.3%)) and embryos held over 2.5 h (17 pregnancies/57 transfers (29.8%)). Pregnancy retention was evaluated around days 60, 90 and 180, and term, and no differences in pregnancy retention were observed at any stage of gestation, three calves being born in the < 2.5 h group and one calf in the > 2.5 h group.

Figure 1 Nuclear remodeling and morphology of bovine nuclear transferred embryos. (a–e) Remodeling of nuclei after fusion. (a) Condensed chromosomes and small microtubule aster among chromatin; (b) metaphase-like chromosomes with strongly stained microtubules; (c and d) elongated chromosomes and microtubules connecting the chromosomes; (e) scattered chromosomes; (f–i) the representatives of 1 PN, 2 PN, 3 PN and 7 PN respectively after activation of fused nuclear transferred embryos. Bars represent 10 μ M.

Table 1 Remodeling of transferred bovine somatic cells at various times after fusion.

Time after fusion (h)	No. embryos examined	Nuclear remodelling types (%)				
		Condensed chr.	Metaphase-like	Elongated chr.	Scattered chr.	PN
2	17	9 (52.9) ^a	6 (35.3) ^b	2 (11.8) ^c		
3	24	2 (8.3) ^c	10 (41.7) ^a	9 (37.5) ^b	3 (12.5) ^a	
4	25		2 (8) ^c	12 (48) ^a	9 (36) ^a	2 (8)
5	25		2 (8) ^c	9 (36) ^b	11 (44) ^a	3 (12)

^{a–c} Values with different superscripts in the same column differ from each other at $P < 0.05$. Chr.: Chromosomes.

Discussion

For NT to be successful, the donor nucleus must be properly reprogrammed. During nuclear reprogramming, epigenetic marks are erased from the donor nucleus genome, resulting in an erasure of tissue-specific gene expression patterns, and effectively resetting the cell to a totipotent state (Santos & Dean 2004). Our studies indicate that the timing between fusion and activation probably has a critical impact on reprogramming.

Studies evaluating DNA methylation patterns in developing NT embryos indicate that demethylation and remethylation events are not always faithfully recapitulated in the mouse (Shi & Haaf 2002, Chung *et al.* 2003, Mann *et al.* 2003) and the cow (Bourc’his *et al.* 2001, Dean *et al.* 2001, Kang *et al.* 2002). It is also clear that histone acetylation is sometimes aberrant in bovine NT embryos (Santos *et al.* 2003). This incomplete epigenetic reprogramming is the predominant explanation of the frequent aberrant gene expression in NT embryos and the subsequent failures in development (Santos *et al.* 2003).

The mechanisms responsible for DNA demethylation may follow a pattern of activity similar to MPF with high activity before activation and diminished activity after activation. Further research is required to determine those dynamics, but the study by Dean *et al.* (2001) suggests a critical window of time in which active demethylation can occur after fusion. The idea of this critical window between fusion and activation is supported by Bourc’his *et al.* (2001), who did not observe active demethylation when activation was performed at the time of fusion.

Nuclear remodeling is an important element in the process of reprogramming that must occur in NT embryos. Our research, as well as that of others, has demonstrated that chromatin remodeling and blastomere ploidy frequently deviate from normal after NT in various species,

including cattle (Booth *et al.* 2003, Bureau *et al.* 2003, Li *et al.* 2004a, 2004b), rabbits (Shi *et al.* 2004) and pigs (Kim *et al.* 2005). Several significant morphologic changes occur in the donor nucleus after NT into cytoplasts with high MPF activity. These changes include nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC) (Czolowska *et al.* 1984, Campbell *et al.* 1996). After these two events, the nuclear envelope is reformed, and DNA synthesis commences (Campbell *et al.* 1993). Aberrations or deficiencies in these events might result in some of the problems associated with early development in NT embryos.

Figure 1C and D show elongated chromosome sets. It is clear how these cells might end up with 2–3 PN (as in Fig. 1G and H) after activation, as areas where microtubules are thinner are probably more prone to depolymerization and fragmentation. This is supported by the fact that more elongated chromosome sets were observed with increased time after fusion, and more embryos displayed multiple PN when held 4 h between fusion and activation than embryos activated 2 h after fusion. Likewise, a scattered chromosome arrangement, as observed in Fig. 1E, would result in multiple PN after activation. It is also probable that embryos with more than one PN after activation result in nuclear fragmentation and unbalanced chromosome constitutions.

The amount of time the donor nucleus is exposed to oocyte cytoplasm before activation is critical in subsequent development of NT embryos. Our data suggest that prolonged exposure to arrested MII oocyte cytoplasm results in more frequent structural abnormality in nuclear material, manifesting itself as elongated chromatin before activation and the development of multiple pronuclei after activation.

While it is important to note that the number of morphologically ‘normal’ embryos before activation (embryos

Table 2 Nuclear morphology of bovine NT embryos 6 h after activation.

Activation time after fusion (h)	No. embryos examined	Pronucleus (PN) status (%)				
		1 PN	2 PN	3 PN	4–8 PN	Scattered Chr.
2	19	15 (78.9) ^a	3 (15.8)	1 (5.3)	0	0
4	22	8 (36.4) ^b	4 (18.2)	2 (9.0)	6 (27.3)	2 (9.0)

^{a,b}Values with different superscripts in the same column differ from each other at $P < 0.05$. Chr.: Chromosomes.

Figure 2 *In vitro* development of bovine NT embryos based on time between fusion and activation. (a–c) Values with different superscripts in the same group differ from each other at $P < 0.01$.

with compacted or metaphase-like chromosomes) and after activation (single PN) declines when embryos are held longer than 3 h before activation, it is also noteworthy that some embryos held longer do appear normal and develop to compacted morula/blastocyst stage. Our data also indicate that embryos that develop to compacted morula stage have an equal probability of establishing and maintaining pregnancy regardless of hold time, indicating that most embryos negatively affected by prolonged hold time will stop developing before reaching compacted morula stage.

This study evaluated the effect of the duration of exposure of the donor nucleus to MII oocyte cytoplasm before activation on nuclear structure, *in vitro* development and pregnancy rates after transfer. The data indicate that prolonged exposure to oocyte cytoplasm results in more embryos with elongated or scattered chromosomes before activation, as well as fewer embryos developing a single PN 6 h after activation. We found that a hold of

1–2 h results in higher *in vitro* development and lower rates of nuclear fragmentation. While *in vitro* development declines and fragmentation increases with increased hold time, embryos that develop to compacted morula or blastocyst stage are equally likely to establish pregnancy after transfer. According to these data, embryos that are chromosomally compromised probably cease development before reaching compacted morula stage. The data further indicate that 1 h between fusion and activation provides the donor nucleus with sufficient exposure to MII cytoplasm to initiate critical reprogramming events, and that longer than 2 h results in reduced viability of embryos *in vitro*.

The process of nuclear reprogramming during NT is extremely complex and not well understood. There are undoubtedly numerous proteins involved in the process of dedifferentiation that occurs in NT. Even under conditions where proper chromosomal composition is maintained, NT efficiency is still quite low. This indicates that while

Table 3 *In vitro* development of bovine NT embryos with different hold times.

Time in hold (h)	No. cultured	No. cleaved (%)	No. compacted morulae/blastocysts (%)
1.0	281	207 (73.7) ^b	78 (27.7) ^{ab}
1.5	166	142 (85.5) ^a	42 (25.3) ^{ab}
2.0	912	655 (71.8) ^b	264 (28.9) ^a
2.5	368	212 (57.6) ^a	79 (21.5) ^b
3.0	667	406 (60.9) ^c	99 (14.8) ^c
3.5	644	362 (56.2) ^c	94 (14.6) ^c
4.0	228	120 (52.6) ^c	27 (11.8) ^c

^{a–c}Values with different superscripts in the same column differ from each other at $P < 0.01$.

compromised chromosomal composition is a factor that reduces NT efficiency, improper epigenetic reprogramming of the donor nucleus probably has a greater impact on NT efficiency. Further research evaluating the molecular machinery involved in nuclear reprogramming before and after activation will pave the way to a better understanding of the mechanisms of nuclear reprogramming and the development of new strategies to improve the efficiency of the process.

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