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In vitro development of reconstructed bovine embryos and fate of donor mitochondria following nuclear injection of cumulus cells

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Summary

In this study we examined the developmental potential of reconstructed embryos and the fate of donor mitochondria during preimplantation development after nuclear transfer in cattle. Isolated cumulus cells were used as donor cells in nuclear transfer. Cumulus cells labelled with MitoTracker Green FM fluorochrome were injected into enucleated bovine MII oocytes and cultured *in vitro*. MitoTracker labelling on donor cells did not have a detrimental effect on blastocyst formation following nuclear transfer. Cleavage rate was about 69% (56/81) and blastocyst formation rate was 6.2% (5/81) at 7 days after nuclear transfer. The labelled mitochondria dispersed to the cytoplasm and became distributed between blastomeres and could be identified up to the 8- to 15-cell stage. Small patches of mitochondria were detected in some 8- to 15-cell stage embryos (5/20). However, donor mitochondria were not detected in embryos at the 16-cell stage and subsequent developmental stages. In the control group, mitochondria could be identified in arrested 1-cell embryos up to 7 days after nuclear transfer. These results suggest that disappearance of the labelled donor mitochondria in nuclear transfer bovine embryos is not due to fading of the fluorochrome marker, but is rather an as yet undefined cytoplasmic event.

Keywords: Bovine embryos, Cumulus cell, Mitochondria, Nuclear transfer

Introduction

Nuclear transfer has the potential to produce a number of identical progeny and would greatly benefit current research efforts. To date, cloned offspring from adult somatic cells have been achieved in sheep (Wilmut *et al*., 1997), mice (Wakayama *et al*., 1998; Wakayama & Yanagimachi, 1999) and cattle (Kato *et al*., 1998; Wells *et al*., 1999). In cloning using adult somatic cells, the introduced differentiated somatic cells must be programmed to the zygotic state by exposing them to the oocyte that includes cytoplasmic organelles and other cytoplasmic factors. Therefore, an appropriate cytoplasmic environment is important for cell–cell communication involved in the reprogramming of the introduced nucleus. The cytoplasmic environment actively changes according to the cell cycle and developmental stage. Reprogramming is, therefore, the procedure that obtains compatibility between a cytoplast and karyoplast that consist of different cytoplasmic environments. During this process, some elements of the donor cell may be destroyed, as shown in sperm mitochondria in *in vitro* fertilisation.

In the cytoplasmic environment, mitochondria play an important role in supplying ATP for all energy-requiring cellular activities. Mitochondria are self-replicating, maternally inherited organelles (Smith & Alcivar, 1993). During normal fertilisation, after sperm–egg fusion, the sperm's mitochondria are destroyed and the oocyte-derived mitochondria are

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assumed to be transmitted to the offspring. However, the fate of foreign mitochondria following nuclear transfer is not known.

There have been several reports on the inheritance of mitochondria following nuclear transfer. Cloned sheep produced by somatic cell nuclear transfer have inherited their mitochondria entirely from the oocyte and not from the donor cell (Evans *et al*., 1999). In addition, Takeda *et al*. (1999) reported that the amount of mitochondrial DNA (mtDNA) derived from the 30-cell stage blastomere in nuclear transfer embryos decreased at the 4- to 8-cell stage and was almost absent at the blastocyst stage when analysed with the PCR-SSCP method. On the other hand, Steinborn *et al.* (1998) reported that mtDNA originating from donor blastomeres was detected in cloned cattle using the allele-specific TaqMan PCR. Furthermore, cattle derived by the transfer of blastomere nuclei showed ambiguous contributions from both the donor cell and the oocyte (Hiendleder *et al*., 1999). Therefore, data concerning the transmission of parental mitochondria and mtDNA after manipulation of mammalian embryos are still controversial. Moreover, to our knowledge, the fate of mitochondria during early embryonic development has not been examined in nuclear transfer using adult somatic cells. The objective of this study was to monitor the fate of donor mitochondria in reconstructed embryos produced by injection of cumulus cells into enucleated bovine oocytes.

Materials and methods

Cumulus–oocyte complex collection and *in vitro* maturation

Bovine ovaries were obtained from an abattoir and brought to the laboratory at a temperature of 37 °C. Follicles (2–10 mm in diameter) were aspirated and the cumulus–oocyte complexes (COCs) with compacted cumulus cells and evenly pigmented cytoplasm were collected and provided for this study.

Collected oocytes were washed three times in Tyrode-Hepes medium (TL Hepes; Parrish *et al*., 1985) and cultured in tissue culture medium (TCM-199; 400–1100; Gibco BRL, USA) supplemented with 2.2 g/l sodium bicarbonate ($NaHCO_3$), 10% (v/v) heat-treated fetal bovine serum (FBS; 200–614, Gibco), 0.22 µg/ml sodium pyruvate, 25 µg/ml gentamycin sulphate, 1 µg/ml FSH-p (Schering, UK) and 1 µg ml oestradiol-17β (BCE-8875, Sigma, Missouri, MO). The oocytes were cultured in 50 µl drops of TCM-199 under mineral oil (Sigma) for 20–22 h at 39 °C, 5% CO_2 in a humidified atmosphere.

Preparation of cumulus cells as donor cells

Twenty hours after the onset of maturation, cumulus cells were isolated by pipetting in phosphate-buffered saline (PBS) supplemented with 2.5 mg/ml hyaluro-

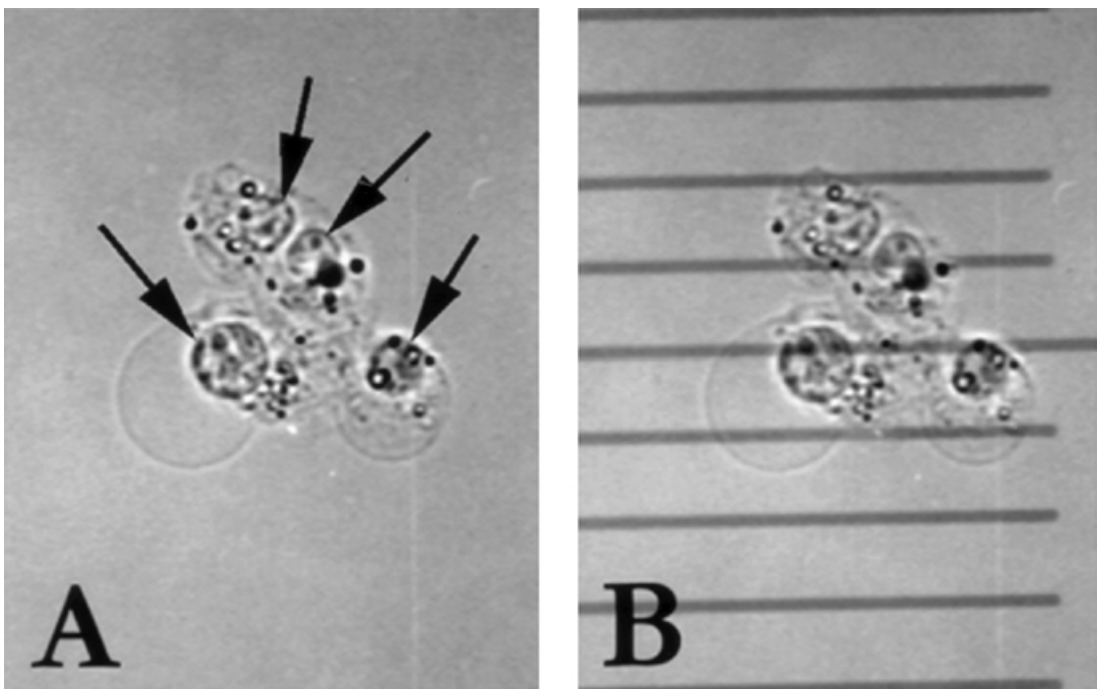

Figure 1 Cumulus cells isolated from *in vitro* cultured cumulus-oocyte complexes (COCs). (*A*) Isolated cumulus cells and their nuclei (arrows). (*B*) When applied to a 10 µm scale, cumulus cells were about 12–18 µm in diameter.

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nidase (Sigma) and transferred to TCM-199 for use as donor cells. The cumulus cells were about 12–18 µm in diameter (Fig. 1). MitoTracker Green FM (Molecular Probes, Eugene, OR; catalogue no. M7514) was prepared as a stock 1 M solution in dimethyl sulphoxide (DMSO) and stored at –20 °C. Cumulus cells were stained at a final concentration of 400 nM (Sutovsky *et al*., 1996) for 10 min before microinjection into the enucleated oocytes.

Oocyte enucleation and donor cell microinjection

The enucleation of recipient oocytes and the injection of cumulus cells were performed as previously described (Collas & Barnes, 1994) with some modification. Denuded oocytes were enucleated through a slit previously cut in the zona pellucida by transpiercing it at two adjacent points with a finely drawn glass microneedle and opening it by rubbing the area between the two puncture sites against the holding pipette. Oocytes were enucleated with a 25 µm glass pipette, by aspirating the first polar body and MII plate in a small volume of surrounding cytoplasm. Successful enucleation was confirmed by Hoechst 33342 (Sigma) fluorescent staining of the pushed-out karyoplasts. Stained karyoplasts with observable chromatin were considered enucleated.

At 1 h post-enucleation, prepared cumulus cells were placed in a 2 µl drop of PBS under mineral oil. Enucleated oocytes were placed in an adjacent drop of PBS containing 10% FBS (v/v) and individual cumulus cells were picked up with the injection pipette and the pipette moved to the injection drop. A 6µm pipette (inner diameter) containing the cumulus cell was introduced through the slit in the zona pellucida made during enucleation, and injected into the cytoplast. A small amount of cytoplasm was then drawn into the micropipette and the cytoplasm together with the cumulus cell and a small amount of medium expelled into the enucleated oocyte. Immediately after injection, the injecting micropipette was quickly withdrawn, and the oocytes were released from the holding pipette to reduce the intracytoplasmic pressure exerted on the oocyte.

Activation and *in vitro* culture of nuclear transfer embryos

After the injection, oocytes were transferred to a drop of CR1aa supplemented with 5 µm ionomycin (Sigma) for 4 min at 37 °C for activation. Then the same volume (50 µl) of PBS containing bovine serum albumin (BSA; 6 mg/ml) was added to stop the activation process (Keefer *et al*., 1990). Embryos were then extensively washed in PBS containing 30 mg/ml fatty-acid-free (FAF) BSA for 5 min before culturing for 3 h in 2 mM 6-dimethylaminopurine (6-DMAP; Sigma) in CR1aa containing 10% FCS. Activated oocytes were transferred to drops of embryo culture medium: CR1aa (Rosenkrans & First, 1991) supplemented with 3 mg/ml FAF BSA, 30 µl/ml MEM essential amino acids, 10 µl/ml MEM non-essential amino acids, 0.44 µg/ml; sodium pyruvate, 1.45 µg/ml glutamine and 25 µg/ml gentamycin.

Fluorescence microscopy

The nuclear transferred oocytes were fixed with 1% formalin solution for 10 min at room temperature, then cultured with propidium iodide (5 mg/ml) in PBS for 1 h to detect the DNA. Stained oocytes were washed with PBS containing 0.5% Triton-X 100 and 0.5% BSA, and mounted under a coverslip with antifade mounting medium (Universal Mount; Fisher Scientific, Huntsville, AL) to retard photobleaching. Slides were examined using a fluorescence microscope (BX60; Olympus, Tokyo, Japan).

Statistics

Data from experiments were analysed with a chi-square test. A value of $p < 0.05$ was considered to be statistically significant.

Results

Embryo development following nuclear transfer

We used the activation protocol of calcium ionomycin followed by 6-DMAP to induce full activation. After activation by ionomycin/DMAP, 69% of embryos (56/81) were cleaved (Table 1). This result indicated that the activation procedure applied in this experiment worked efficiently. In bovine nuclear transfer, oocyte activation is of significance for effective cloning by nuclear transfer because successful cloning requires full activation of the recipient cytoplast, but the nuclear transfer process itself does not induce adequate activation in bovine oocytes (Liu *et al*., 1998). Embryo developmental potential after microinjection of MitoTracker-labelled cumulus cells into enucleated oocytes is shown in Table 1. There seemed to be no detrimental effect on subsequent embryo development of labelling donor cells with MitoTracker (Table 2). Two-cell stage embryos were first observed after 16 h (12/81) of culture. The embryos reached the 4- and 8-cell stage by 24 h and 38 h after activation of reconstructed embryos, respectively. By 72 h of post-activation 16% (13/81) of embryos reached the 9- to 16-cell stage. However, of the total number of reconstructed embryos, 4.9% (4/81) reached the morula stage and relatively fewer (5/81) developed to the blastocyst stage after 168 h of culture.

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Table 1 Development of embryos from a time-lapse recording after somatic cell nuclear transfer by microinjection

Culture time (*h*)[a]	No. (%) of embryos developed to:						
	1-cell	2-cell	4-cell	8-cell	16-cell	Morula	Blastocyst
16	69	12	–	–	–	–	–
24	43	30	8	–	–	–	–
38	27	13	25	16	–	–	–
48	25	11	23	22	–	–	–
72	25	11	15	17	13	–	–
96	25	11	14	15	11	5	–
120	25	11	14	14	10	7	–
144	25	11	14	14	8	9	–
168	25 (30.9)	11 (13.5)	14 (17.3)	14 (17.3)	8 (9.9)	4 (4.9)	5 (6.2)

[a]Elapsed time after activation by ionomycin.

Table 2 Effect of labelling with MitoTracker Green FM on the development of embryos after somatic cell nuclear transfer by microinjection[a]

Donor cell treatment	No. of embryos injected	No. (%) of embryos developed to:			
		2-cell	16-cell	Morula	Blastocyst
Intact CC	45	15 (33.3)	26 (57.8)	2 (4.4)	2 (4.4)
MitoTracker labelled CC	45	14 (31.1)	26 (57.8)	3 (6.7)	2 (4.4)

CC, cumulus cell.
[a]Three replicates were performed for each experiment.
There is no significant difference between experimental groups ($p<0.05$).

Fate of donor mitochondria following nuclear transfer by microinjection of cumulus cells into enucleated oocytes

We identified the fate of mitochondria derived from donor cells by using MitoTracker Green FM. At a final concentration of 400 nM, MitoTracker Green FM seemed to have no detrimental effect on subsequent embryo development (Table 2). This probe appears to preferentially accumulate in mitochondria regardless of the mitochondrial membrane potential, making it an important tool for determining mitochondrial mass (Molecular Probes' Product Information).

The labelled mitochondria from donor cells could be easily identified up to 7-cell stage embryos (19/25; 76%) following nuclear transfer, but by the time embryos reached the 8- to 15-cell stage, only 25% of embryos (5/20) had detectable mitochondria in their cytoplasm. Moreover, at the 16-cell to blastocyst stage, no labelled mitochondria could be detected in any of the 26 embryos examined (Table 3).

Immediately after injection of cumulus cells into enucleated embryos, the mitochondria remained closely associated with the nucleus (Fig. 2*B*, *C*). At the time of pronucleus formation, the mitochondria dispersed to the cytoplasm (Fig. 2*D*) and became distributed between blastomeres in 2-cell stage embryos (Fig. 2*E*). Stained mitochondria could also be identified in 4-cell (Fig. 2*F*) and 8-cell embryos (Fig. 2*G*), but considering the disappearing fluorescent mitochondria, mitochondria originated from donor cumulus cells were presumably disintegrating. The foreign mitochondria were not identified at the 16-cell stage and subsequent development stages (Fig. 2*H*). In 1-cell stage arrested embryos after 7 days of culture, donor mitochondria were identified (Fig. 2*I*).

Table 3 Persistence of labelled mitochondria following nuclear transfer

Developmental stage of embryos	No. (%) of oocyte mitochondria detectable/total
1-cell	44/45 (98)
2- to 3-cell	28/33 (85)
4- to 7-cell	19/25 (76)
8- to 15-cell	5/20 (25)
16-cell	0/14
Morula	0/6
Blastocyst	0/6

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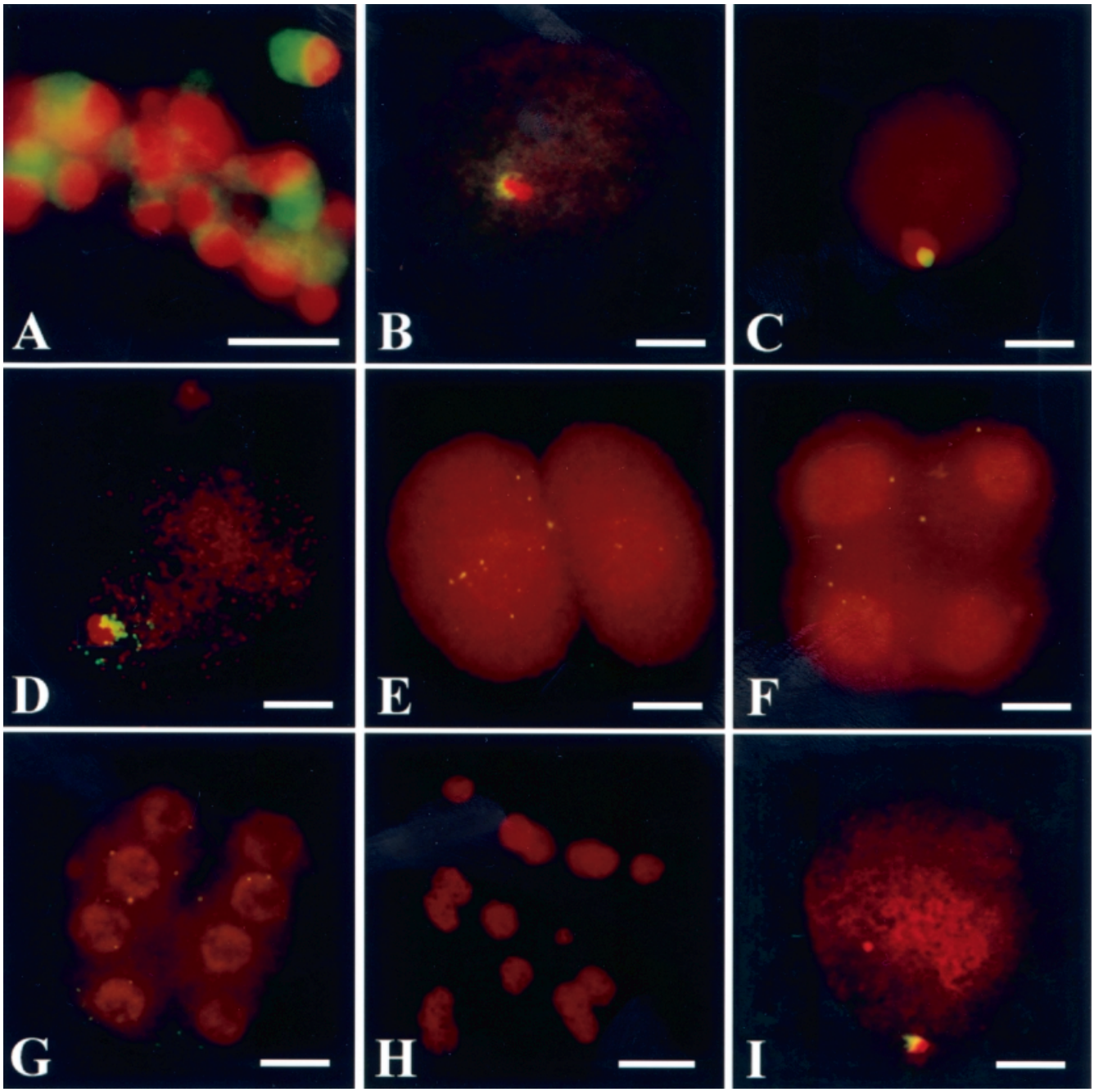

Figure 2 Fluorescence microscopic images of mitochondria and chromatin in nuclear transfer bovine embryos. Green, mitochondria; red, chromatin; yellow, overlapping image of mitochondria and chromatin. Scale bars represent 25 μm. (*A*) Cumulus cell mass labelled with MitoTracker Green FM and propidium iodide. (*B, C*). Injected cumulus cell was enlarged and its mitochondria remained associated closely with the nucleus. (*D*) Donor mitochondria were dispersed to the cytoplasm and randomly scattered. (*E*) Donor mitochondria distributed between blastomeres in a 2-cell stage embryo. (*F, G*) By the 4-cell to 8-cell stage, donor mitochondria were detectable but had largely disappeared. (*H*) By the 16-cell stage, donor mitochondria could not be identified. (*I*) Embryos arrested at the 1-cell stage maintained MitoTracker labelling of cumulus cell for 7 days after nuclear transfer.

Discussion

We have demonstrated that donor somatic cell-derived mitochondria were eliminated from the cytoplasm of nuclear transferred bovine embryos at the 8-cell to 16-cell stage. This disappearance of donor-cell-derived mitochondria is not due to lack of sensitivity of our technique, which can detect mitochondria in embryos arrested at the 1-cell stage at 7 days after nuclear transfer (Fig. 2*I*). However, it is possible that DNA from

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donor-cell-derived mitochondria may remain in the cytoplasm (Gyllensten *et al*., 1991) and be transported to endogenous mitochondrial DNA (King & Attardi, 1988, 1989). Thorsness & Weber (1996) also suggested that mitochondrial DNA could move between the mitochondria.

In this study cumulus cells were used as donor cells because they have been shown to serve as ideal somatic cells for cloning. In an earlier study, bovine cumulus granulosa cells produced blastocysts after nuclear transfer (Collas & Barnes, 1994) and live offspring were produced using cumulus or granulosa cells as donor cells in mice (Wakayama *et al*., 1998) and cattle (Kato *et al*., 1998; Wells *et al*., 1999). The cumulus cytoplasm may be compatible with the oocyte cytoplasm because the precursor cells of cumulus cells can exchange cytoplasmic factors through cytoplasmic bridges. The initial development of clones to the blastocyst stage may be dependent on the compatibility between cell cycles of the donor nucleus and the oocyte (Campbell *et al*., 1996). Also the aging effects were fewer than with other cell types, because cumulus cells in human (Dorland *et al*., 1998) and granulosa stem cells in cow (Lavranos *et al*., 1999) have telomerase activity. Their small size and predominant G0/G1 stages give cumulus cells an advantage over other cell types. Collas & Barnes (1994) reported the development to term and to blastocysts by microinjection of inner cell mass and granulosa cell into enucleated oocytes, respectively. Recently, Trounson *et al.* (1998) reported that more reconstituted embryos developed to blastocysts after isolated nuclear injection than after electrofusion. These reports indicate that the microinjection method as well as electrofusion technology could be an efficient method for somatic cell nuclear transfer.

There has been intense interest in the question of mitochondrial inheritance. Gresson (1941) thought that the mouse sperm mitochondria became dispersed from the midpiece and distributed evenly between cells at the first cell cycle. More recent studies using genetic markers (Kaneda *et al*., 1995), antibodies to fibrous sheath and mitochondrial membrane (Shalgi *et al*., 1994) and electron microscopy (Szollosi, 1965; Hiraoka & Hirao, 1988) all independently indicated that the mitochondria were destroyed in early embryonic development. These reports reinforced the findings that the mitochondrial genome was maternally inherited (Hutchinson *et al*., 1974; Giles *et al*., 1980). Several theories have been advanced to explain the maternal inheritance of mammalian mitochondria or mtDNA.

To date, the most reliable explanation seems to be the selective destruction theory. Sutovsky *et al.* (1996) suggested that ubiquitin of paternal mitochondria was the proteolytic target for the destruction machinery of the cytoplasm. They observed that in bovine embryos fertilised *in vitro* the sperm mitochondria became undetectable at the late 4-cell stage (Sutovsky *et al*., 1996). They also observed that both the ubiquitin and the MitoTracker signals were lost by the 8-cell stage in most embryos, and the sperm mitochondria were ubiquitinated during spermatogenesis (Sutovsky *et al*., 2000). It is wondered, however, whether mitochondria of somatic cells for nuclear transfer are ubiquitinated and identified by the cytoplasmic machinery as in spermatozoa. It has also been reported that specific elimination of sperm mtDNA from the fertilised egg occurred at the pronuclear stage in intraspecific mouse hybrids (Kaneda *et al*., 1995). These authors suggested that degradation of paternal mitochondria during fertilisation was signalled by loss of their membrane potential in the egg cytoplasm of early stage embryos. These reports indicate that the mitochondrial destruction mechanism is triggered not by mtDNA but paternal mitochondrial membrane.

Second, mitochondrial morphology varies during oogenesis and throughout the early cleavage stages. Somatic morphology and normal replication patterns are regained at the blastocyst stage. During bovine embryonic development, mitochondria show extensive morphological transformation (Plante & King, 1994). In bovine embryos, mitochondrial maturation begins at the 8-cell stage and is completed by the blastocyst stage. So, it was speculated that mature mitochondria transferred from donor cell or spermatozoa might be broken down during morphological transformation of mitochondria in early development (Takeda *et al*., 1999). This hypothesis may provide further support for our initial observation that donor mitochondria disappear from cytoplasm at the mitochondrial maturation stage following nuclear transfer.

Third, there is the dilution of paternal mDNA by the overwhelming 1000:1 copy number ratio of oocyte to sperm mtDNA (Smith & Alcivar, 1993). Considering the amount of mitochondria in most somatic cells, an average of 200 copies, a 1:500 dilution effect was created immediately after somatic cell nuclear transfer (Nass, 1969). But, donor cell-derived mtDNA would not be diluted by selective replication in the early embryo, because the mtDNA content remains constant from the 1-cell to the blastocyst stage (Piko & Matsumoto, 1976).

Fourth, oxidative-damage theory (Allen, 1996) suggests that cumulative damage of sperm mitochondria from free radicals generated by the electron transport chain causes degradation of sperm mitochondria by the time the sperm enters the oocyte. Natural selection would therefore select the mechanisms that permit the zygote to start life with perfect bioenergetic mechanisms (Cummins *et al*., 1997).

Fifth, selective inactivation or destruction of the

paternal mtDNA by differential methylation has been suggested, but there is no evidence to support this (Hecht *et al*., 1984). However, as an epigenetic DNA modification mechanism, DNA methylation has been proposed as a likely candidate for controlling reprogramming after nuclear transplantation.

Since reliable nuclear transfer procedures were established, there have been many studies attempting to produce identical domestic and laboratory animals. Nevertheless, how donor nuclei transferred into ooplasts are reprogrammed so that totipotency is restored remains unknown. In this study, we examined the observation that mitochondria disappear following nuclear transplantation. In most reports, it is suggested that the mitochondria in nuclear transfer calves are not derived from donor cells, but rather from the recipient oocytes. The finding may be important for breeders who want to use cloning to create transgenic animals, because mitochondria, although encoding only 37 genes, may be important for certain traits desired in superior livestock. Many mtDNA polymorphisms identified in cattle were associated with milk production (Schutz *et al*., 1994). Thus the mitochondrial genetic effect can be an important factor for establishing a transgenic line producing a pharmaceutical protein in milk.

In summary, the present study has described the developmental potential and fate of somatic-cell-derived mitochondria after nuclear transfer in cattle. During pronucleus formation, the donor mitochondria dispersed to the cytoplasm and became distributed between blastomeres and finally eliminated from the cytoplasm at the 8- to 16-cell stage. Further studies are required to determine the mechanism of mitochondrial destruction and transmission of mtDNA after somatic cell nuclear transfer as well as in normal fertilisation.

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