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Nuclear Remodeling and *In Vitro* Development of Bovine Oocytes Following Nuclear Transfer of Bovine Fetal Fibroblasts

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ABSTRACT

This study was investigated the developmental potential of bovine embryos following nuclear transfer with bovine fetal fibroblasts (BFF). BFF were isolated from a male 45-day-old-fetus. Non-starved BFF labeled with MitoTracker were transferred into perivitelline space of enucleated oocytes. BFF-oocyte units were fused by electric pulse, and then fused oocytes were activated with calcium ionophore A23187 and subsequently 6-dimethylaminopurine (6-DMAP). The resulting zygotes were placed into CR1aa bovine embryo culture medium. Transfer of the nucleus into enucleated oocyte led to premature chromosome condensation, swelling and pronucleus formation. Remodeled oocytes were developed to the mitotic and 2-cell stage at 18 to 26 h after nuclear transfer. The incidence of *in vitro* development to the blastocyst stages was 21% of fused oocytes. Mitochondria of BFF eliminated rapidly and were not detected at 8 h after fusion. These results suggest that BFF can be successfully reprogrammed in enucleated bovine oocytes, and that reconstructed embryos can develop to the blastocyst stage.

(Key words : Bovine, Development, Mitochondria, Nuclear transfer, Remodeling)

I. INTRODUCTION

Nuclear transfer procedure using embryonic cells as donors of genetic material is important for producing cloned animals as well as for research purposes. However, this approach has little value in introducing new genes or genetic modifications. Production of cloned offspring by nuclear transfer from a cell population that can be maintained in culture or under other appropriate conditions offers numerous advantages in the fields of research, agriculture and biotechnology. An established cell line provides the opportunity to modify the genomes and select the required cell populations with desired genomic modification before embryo reconstruction (Wolf et al., 1998).

Recently, after Dolly was produced by the transplantation of a sheep mammary-grand cells

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into an enucleated sheep oocyte, the development of somatic cell nuclear transfer has become steadily more successful in generating cloned young (Schnieke et al., 1997; Cibelli et al., 1998; Kato et al., 1998; Wakayama et al., 1998). When the nucleus is transplanted into enucleated oocyte, various changes occur in the transferred nucleus. These changes are referred as the nuclear remodeling. The first morphological indication of nuclear remodeling is premature chromosome condensation (PCC) shortly after the nuclear transfer (Czolowska et al., 1984; Collas and Robl, 1991; Cheong et al., 1994; Wakayama et al., 1998). The next indicator is swelling of the transferred nucleus upon the activation of recipient oocytes (Czolowska et al., 1984; Prather et al., 1990; Collas and Robl, 1991). Nuclear remodeling has been considered to be necessary for the complete functional reprogramming of the transferred nucleus, such that the nucleus behaves as if it were a pronucleus (Prather et al., 1990; Collas and Robl, 1991). However, it is not yet known the processes of nuclear remodeling following nuclear transfer with somatic cells.

Following the nuclear transfer, oocytes were fused with nuclear donor cells, which possibly resulted in mitochondrial heteroplasm in the reconstituted embryos. In the offspring from such embryos, the nuclear genome was the same among clonal animals, but the mitochondrial DNA (mtDNA) polymorphism was found among four cloned Holstein bulls (Takeda et al., 1999). This finding suggests that the mtDNA is originated from different recipient oocytes with different mtDNA genotypes. Strict maternal inheritance of mammalian mtDNA has been assumed. However, little information is available on the fate of donor mitochondria during reprogramming of bovine oocytes with somatic cells.

In the present study, the chromatin configuration following fusion of fetal fibroblasts with enucleated bovine oocytes was investigated. In addition, *in vitro* development of reconstructed bovine oocytes from somatic cell nuclear transfer was examined.

Ⅱ. MATERIALS AND METHODS

1. *In Vitro* Maturation of Follicular Oocytes

Ovaries were collected from a slaughterhouse and brought to the laboratory in saline at 37℃. The collected oocytes were washed three times in TL-HEPES medium (Parrish et al., 1985) and washed again three times in equibrated TCM-199 (Gibco BRL, Grand Island, NY) supplemented with 2.2 g/l sodium bicarbonate, 10% heat-treated fetal bovine serum (FBS; Gibco BRL), 0.22 μg/ml sodium pyruvate, 25 μg/ml gentamycin sulfate, 1 μg/ml FSH-p (Schering Co., UK), and 1 μg/ml estradiol-17β (Sigma Chemical Co., St. Louise, MO). Then oocytes were cultured in 50 μl drop of TCM-199 under paraffin oil for 24 h at 39℃, 5% CO_2 in humidified atmosphere.

2. Preparation of Bovine Fetal Fibroblast

Primary bovine fetal fibroblasts (BFF) were isolated from male fetuses of pregnant bovine female at 40 day in gestation. Bovine fetal fibroblasts were cultured on 60 mm tissue culture dishes (Falcon, Lincoln Park, NJ) in Dulbecco's Modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% FBS. After 7 days of culture, BFF were trypsinized and washed three times with fresh changes of Ca^{2+}-, Mg^{2+}-free PBS (Gibco BRL). The cells were pelleted and resuspended in DMEM supplemented with 10% FBS. Thereafter, BFF were routinely maintained on 50-ml tissue culture flasks (Falcon) up to passage 2 to 7 and used as donor cells for nuclear transfer.

3. Labeling of Fibroblasts with MitoTracker

MitoTracker Green FM (Molecular Probes, Eugene, OR) was prepared as 1 M stock solution in anhydrous dimethyl sulphoxide (DMSO) and stored desiccated at −20℃. To label the mitochondria, BFF were incubated for 10 min with the dye at a final concentration of 5 μM. The labeled BFF were washed twice by centrifugation in TL-HEPES. Mitochondria of BFF were stained with MitoTracker and examined at 3, 5, 8 h after fusion.

4. Nuclear Transfer

Matured oocytes were freed of cumulus cells in PBS supplemented with 0.1% hyaluronidase and washed three times in PBS containing 5% FBS. The oocytes were enucleated by the aspiration of the first polar body and adjacent cytoplasm (approximately 30% of ooplasm) using a beveled pipette (30 μm in diameter) in PBS containing 5% FBS and 7.5 μg/ml Cytochalasin B (CB; Sigma). The enucleation was confirmed by staining aspirated portion of cytoplasm with 5 μg/ml Hoechst 33342 (Sigma), then the enucleated oocytes were incubated in CR1aa containing 0.3% BSA until injection of donor cells. Single bovine fetal fibroblast cell was inserted into the perivitelline space of enucleated oocyte by the enucleation pipette (30 μm in diameter). Reconstructed oocytes were washed three times in electrofusion medium composed of 0.3 M mannitol, 0.1 mM $CaCl_2$ and 0.1 mM $MgSO_4$.

At room temperature, fusion was performed in a chamber with two platinum electrodes at 1 mm apart overlaid with electrofusion medium. The reconstructed oocytes were manually aligned with a fine pasteur pipette in order that the contact surface between the bovine fibroblast and oocyte was parallel to the electrodes. Membrane fusion was induced with double D.C. pulse of 2.5 KV/cm for 30 μsec delivered by BTX Electro Cell Manipulator 2001 (Genetronics, San Diego, CA). Oocytes reconstructed with BFF were examined during the first 24 h after fusion. Reconstructed eggs were fixed and stained with Hoechst 33342 for the evaluation of normality.

5. Oocyte Activation

The reconstructed and fused oocytes were transferred to a drop of 50 μM calcium ionophore A23187 (Sigma) for 10 min and subsequently 2.0 mM 6-dimethylaminopurine (6-DMAP) for 4 h for activation. The activated oocytes were transferred to a drop of CR1aa (Rosenkrans and First, 1991) supplemented with 3 mg/ml fatty-acid-free BSA, 20 μl/ml MEM essential amino acid, 10 μl/ml MEM non-essential amino acid, 0.44 μg/ml Na pyruvate, 1.46 μg/ml glutamine and 25 μg/ml gentamycin.

6. *In Vitro* Fertilization

In vitro fertilization was carried out by the method of Sirard et al. (1988). Matured cumulus-oocyte complexes (COCs) were washed with Sp-TALP and subsequently with Fert-TALP (Rosenkrans et al., 1993). After washing, ten mature COCs were pooled in a 44 μl of Fert-TALP droplet under paraffin oil. Bull spermatozoa recovered from frozen-thawed semen were separated on a discontinuous percoll gradient. Highly motile spermatozoa were added to Fert-TALP at a final concentration of 1×10^6 sperm/ml. Then 2 μl of PHE stock solution (2 mM Phenicillamine, 20 μM hypotaurine and 1 μM epinephrine) were added to a Fert-TALP droplet to stimulate sperm motility. Sperm and COCs were coincubated in a 50 μl drops of Fert-TALP under paraffin oil for 24 h at 39℃, 5% CO_2 in humidified atmosphere.

7. *In Vitro* Culture of Reconstructed Embryos

Both reconstructed embryos and *in vitro* fertilized embryos were cultured in 50 μl of CR1aa containing 0.3% BSA for 3 days, and then transferred to 50 μl of CR1aa containing 10% FBS and cultured for 5 days. All embryos were cultured at 39℃ in an atmosphere of 5% CO_2 in air. Hatching embryo was stained with Hoechst 33342, and the cell number was counted.

8. Statistical Analysis

The data from at least four replications were pooled. Differences in the percentages of oocytes developed to particular stages were determined by Chi-square analysis.

Ⅲ. RESULTS

Chromatin configurations of bovine oocytes following nuclear transfer were different with incubation times as shown in Table 1. Sixty-nine percent of examined oocytes at 3 h after nuclear transfer showed swelled nucleus. Most of these oocytes formed pseudo-pronucleus at 8 h after the injection and fusion. Development of these oocytes at 24 h after nuclear transfer represented pseudo-pronucleus of 53%, mitosis of 20% and 2-cell of 27%. Successfully fused oocytes contained somatic cell nucleus at the periphery of the oocyte cytoplasm, and PCC was observed in some reconstructed embryos during exposure to the MⅡ cytoplasm (Fig. 1A). Swelled and decondensed chromatin was observed within a nuclear envelope (Fig. 1B, C), and no chromatin fragmentation was seen at any times examined.

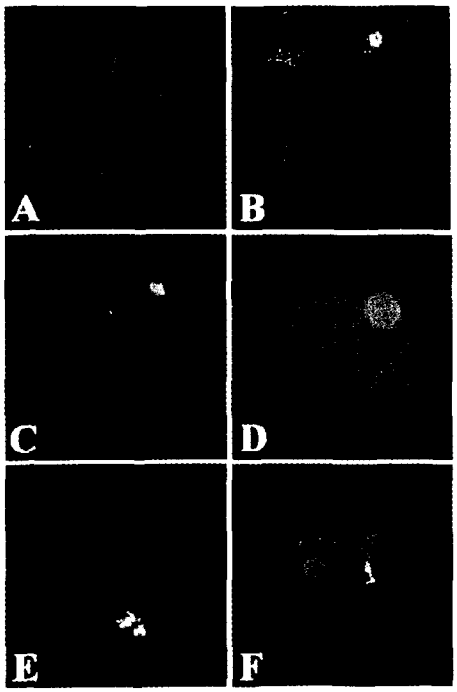

Fig. 1. Remodeling of a reconstructed bovine oocyte with nuclear transfer. Fused oocytes were stained with Hoechst 33342; A) Enucleated oocyte B) A prematurely condensed nucleus at 2 h after fusion C) A remodeled swollen nucleus at 3 h after fusion D) A pseudo-pronuclei at 12 h after fusion E) Mitotic stage at 18 h after fusion and F) Twocell stage embryo at 24 h after fusion.

Table 1. Chromatin configurations of bovine oocytes following nuclear transfer

Hours after nuclear transfer	No. of oocytes	No. (%) of oocytes with					
		Condensed chromatin	Disarrayed chromatin	Swelled nucleus	Pseudo-pronucleus	Mitosis	2-cell
3 h	42	5(11.9)	8(19.0)	29(69.0)	–	–	–
8 h	38	–	4(10.5)	3(7.9)	31(81.6)	–	–
24 h	60	–	–	–	32(53.3)	12(20.0)	16(26.7)

Table 2. Development of nuclear transferred bovine embryos

	No. of oocytes	Fused oocytes (%)	2-Cell stage (% of fused)	Morula stage (% of 2-cell)	Blastocyst stage (% of 2-cell)
Control	192	–	157(81.8)	73(46.5)	59(37.6)[a]
NT	136	102(75.0)	68(66.7)	26(38.2)	14(20.6)[b]

Control: *in vitro* fertilization, NT: nuclear transfer

[a,b]Different superscripts denote significant differences ($P<0.05$)

The cleavage occurred between 18 and 26 h after activation (Fig. 1D).

Table 2 shows *in vitro* development of reconstructed embryos after nuclear transfer. The fusion and cleavage rates of these embryos were 75 and 68%, respectively. Among cleaved embryos, 38% developed to the morula stage and 21% to the blastocyst stage. The percentage of embryos developed to the blastocyst stage was significantly higher when embryos were fertilized *in vitro* (35%). Fig. 2 demonstrates the development of reconstructed bovine embryos *in vitro*. Fig. 2A~C present enucleated eggs, cleaved eggs and blastocyst stage, respectively. Fig. 2D represents the number of cells in a hatching blastocyst developed after the nuclear transfer, and the counted number of cells was approximately 140.

Intensity of green fluorescence in reconstructed embryos declined with the time, and the mitochondria remained associated closely with the nucleus. While embryos at 3 h after nuclear transfer showed bright fluorescence. However, the intensity of fluorescence was declined at 5 h, and the fluorescence was not detectable at 8 h following fusion (Fig. 3). By contrast, in control embryos that oocytes and fibroblasts were not fused, the mitochondria remained bright.

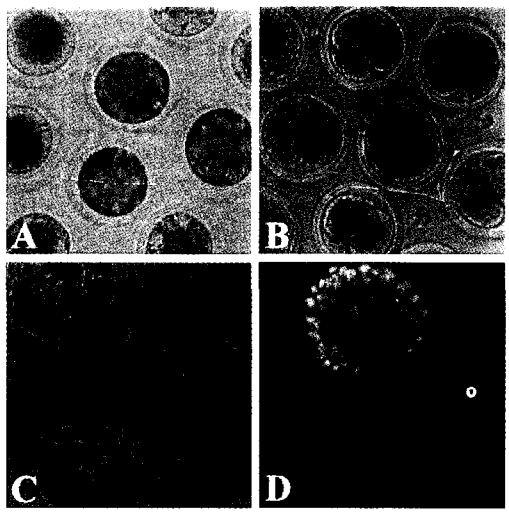

Fig. 2. *In vitro* development of nuclear transferred bovine embryos; A) Enucleated oocytes B) Cleaved embryos at 36 h after fusion C) Blastocyst stage embryos on 7 days after fusion and D) Nucleus of a hatching embryo stained with Hoechst 33342 on 8 days after fusion.

Ⅳ. DISCUSSION

The present study demonstrated nuclear remodeling of reconstructed embryos after fusion of BFF with enucleated oocytes. The remodeling of nuclei transferred to enucleated oocytes is characterized by PCC and nuclear swelling (Tarkowski and Balakier, 1980; Czolowska et al., 1984; Collas and Robl, 1991). The PCC is induced in interphase nucleus fused with M-phase cell as a result of the maturation promoting factor (MPF) activity derived from the M phase recipient oocyte (Johnson and Rao, 1970). MPF in non-activated oocyte cytoplasm is present in

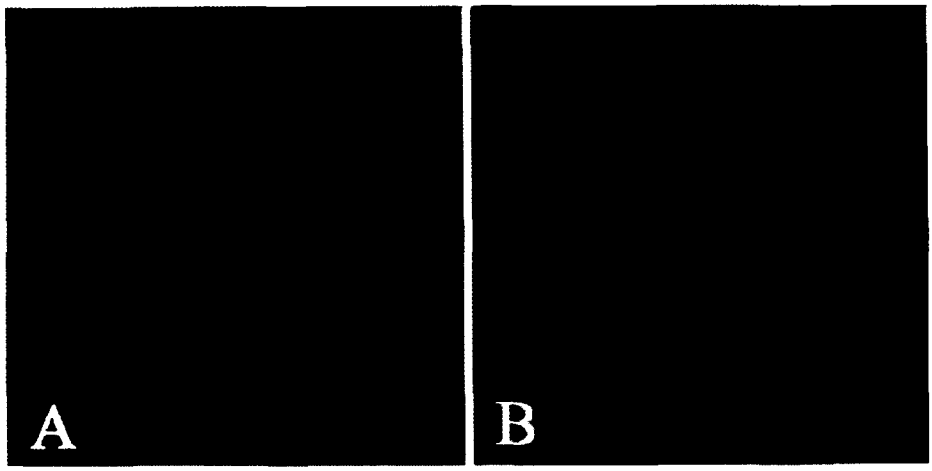

Fig. 3. Laser scanning confocal microscopic images of mitochondria in a fibroblast cell fused with enucleated bovine oocyte. Green, mitochondria; red, chromatin. A) at 3 h after fusion and B) at 5 h after fusion.

high levels. All nuclei transferred at the time of activation, when MPF activity in high, undergo nuclear envelope breakdown (NEBD), which in turn followed by PCC. In contrast, when nuclei are transferred after the disappearance of MPF activity, NEBD and PCC are not observed (Campbell et al., 1996). Therefore, the cytoplasm of metaphase II oocytes containing high MPF activity is capable of inducing PCC.

In addition, it has been suggested that NEBD and PCC are essential for the reprogramming of gene expression (Collas et al., 1992) and are important processes for the development of nuclear transfer embryos to term (Cheong et al., 1994). Nuclear reprogramming is characterized by functional modification of the transferred nucleus to be able to direct normal early embryo development, with the potential to develop to term. Nuclear remodeling, characterized by nuclear swelling and growth of nucleoli is often associated with the initial reprogramming (Stice and Robl, 1988; Collas and Robl, 1991; Modlinski and Smorag, 1991). In cattle, Wells et al. (1998) obtained a significantly higher proportion of blastocysts by fusion of fibroblasts with recipient cytoplasm 4~8 h before activation than when reconstructed embryos were activated and fused simultaneously (52 versus 25%). These results suggest that the increase in embryo development obtained is due to the facilitation of nuclear remodeling and reprogramming. In addition, recently, Dominko et al. (1999) reported that PCC were not observed in cattle when nuclei from fibroblasts were introduced into enucleated oocyte. In this study, however, the embryos fused with somatic cells formed PCC and the remodeled embryos developed to blastocyst stage.

The low efficiency of development to the blastocyst stage in cloned embryos has been raised questions regarding *in vitro* development block. However, the high rate of development for IVF embryos indicates that poor development of nuclear transferred embryos may not be do to inadequate culture system. Rather, poor development may have been due to technical difficult-

ies in the procedures of nuclear transfer (Keefer et al., 1994). Variable quality of oocytes obtainted from slaughter house and *in vitro* matured may also have accounted for low ability of recipient cytoplasm to support development after the nuclear transfer (Keefer et al., 1993).

Takeda et al. (1999) reported that only the mtDNA genotype of recipient oocytes, not of donor cells, was detected in each of the nuclear transfer calves, indicating that the mtDNA of nuclear transfer calves originated from the recipient oocytes. Although mtDNAs from both the recipient oocyte and the donor cell were detected in the nuclear transferred-embryos collected immediately after fusion of the karyoplast and the oocyte, the donor mtDNA decreased at the 4- to 8-cell stage and was hardly detectable at the blastocyst stage (Takeda et al., 1999). These results explain why donor cell mtDNAs were not detected in most of the nuclear transfer calves and indicate that the mtDNA originated from the donor cells was nearly eliminated during early embryonic development.

There might be some unknown factors that cause a reduction in donor cell mtDNA. This observation is similar to the finding that in bovine embryos fertilized *in vitro* the sperm mitochondria become undetectable at the late 4-cell stage (Sutovsky et al., 1996). In this study, the mtDNA of fibroblast in nuclear transferred oocytes was rapidly disappeared than the mtDNA of spermatozoa in fertilization. In addition, it appears that the mitochondria from somatic cells were eliminated in recipient cytoplasm faster than those from blastomeres as reported by Takeda et al. (1999). Apparently, nuclear transferred embryos are quickly exposed to recipient cytoplasm through nuclear membrane fusion, and this may be due to that the fibroblast cell have a little cytoplasm than blastomere.

In summary, this study demonstrated that reconstructed oocytes using fetal fibroblasts were remodeled through PCC and swelling and developed to the morula and blastocyst stage. In addition, mitochondrial DNA from donor cells was rapidly eliminated in recipient cytoplasm.

V. 요 약

본 연구는 핵이 제거된 소난자 내에 소의 태아 섬유아세포를 이식한 후 난자의 발달능력을 조사하였다. 소 태아 섬유아세포는 45일된 웅성 태아로부터 분리한 후 MitoTracker로 염색하고 세포 주기를 동기화시키지 않은 세포를 핵이 제거된 소난자의 위란강 내에 이식하였다. 섬유아세포와 소난자의 복합체는 전기 자극을 주어 융합시키는 방법을 이용하였으며, 융합된 난자는 Calcium ionophore와 6-DMAP를 이용하여 난활성을 유도시킨 다음 CR1aa에서 배양하였다. 핵치환된 난자의 핵은 핵질 응축과정, 팽대과정, 전핵 형성과정이 일어났으며, 이러한 과정에서 재구성된 난자는 분열 과정을 거쳐 18~26시간 사이에는 2-세포기로 발달하였다. 섬유아세포의 미토콘드리아는 핵치환시 난자 내로 이전되었는데 이것들은 난자 내에서 빠르게 사라지는 것으로 관찰되었으며, 핵융합 후 8시간째에는 섬유아세포의 미토콘드리아가 전혀 관찰되지 않았다. 2-세포기로 분열된 난자의 21%가 이식 가능한 단계인 배반포 단계까지 발달하였다. 이러한 결과는 소의 태아 섬유아세포가 탈핵된 소난자 내에서 성공적으로 재분화과정을 거치며, 이렇게 재조합된 수정란은 배반포 단계까지 발달이 가능하다는 것을 보여주고 있다.

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