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Development of Nuclear Transfer Embryos using Somatic Cell Nuclei from Korean Native Cattle (Hanwoo) with High Genetic Value

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고능력 한우 종모우 체세포를 이용한 핵이식 배아의 발달에 관한 연구

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ABSTRCT

This study was conducted to determine developmental ability of reconstructed embryos by nuclear transfer using somatic cell of Korean bull with high genetic value. Fibroblast cells obtained from ear biopsy of the bull were cultured in Dulbecco's Modified Eagle's medium (DMEM) at 37℃ in air containing 5% CO_2. The cummulus-oocyte complexes were collected from slaughterhouse and were matured *in vitro* for 20 h in TCM 199 culture medium and the oocytes were then enucleated in modified phosphate buffered saline with cytochalasin B. Matured bovine oocytes were enucleated by aspirating the first polar body and metaphase chromatin using a beveled pipette in modified phosphate buffered saline. The ear fibroblast cells were fused into enucleated oocyte by electrical stimulation. The reconstructed oocytes were activated with ionomycine and 6-dimethylaminopurine, and then cultured in CR1aa medium for 7.5 days. Out of 524 bovine eggs reconstructed by nuclear transfer 65.6%(277/422) embryos were cleaved, and 30.7% (85/277) cleaved embryos were developed to the morula to blastocysts. There was no difference of developmental ability *in vitro* of reconstructed embryos regardless of donor cell passages. In order to determine fate of foreign mitochondria of donor nucleus, the Mito Tracker stained cells were fused into enucleated oocytes. The donor mitochondria were detected early stage of embryos, but disappeared rapidly. The developmental ability of reconstructed embryos was not impaired by Mito Tracker treatments. The results indicate that viable reconstructed embryos can be producted by nuclear transfer using somatic cell of Korean bulls.

Ⅰ. INTRODUCTION

Nuclear transfer (NT) is a technique to produce cloned animals by transferring the complete genetic material in a nucleus from one cell into an unfertilized egg whose own nucleus has been removed. Nuclear transfer methods developed in amphibians (Briggs and King 1954; McKinne 1962) and later in mammals (Solter et al., 1983; Willadsen et al., 1986) offer the opportunity to produce cloned animals as well as to address fundamental questions

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about cell differentiation and its reversibility. After being introduced into an enucleated recipient oocyte, nuclei from various differentiation states have been shown to be reprogrammed and were able to initiate another round of embryonic development. Offsprings have been obtained from embryos created by the NT of embryonic blastomeres (Prather et al., 1987), inner cell mass cell (Keefer et al., 1994), cultured embryonic cells (Sims et al., 1994; Campbell et al., 1994), embryonic stem cell-derived epithelial cells (Wells et al., 1997), primordial germ cells (Robertson et al., 1997), fetal fibroblasts (Schnieke et al., 1997; Wilmut et al., 1997; Cibelli et al., 1998), and adult somatic cells (Wilmut et al., 1998; Wakayama et al., 1998).

During nuclear transfer, the interactive roles of foreign mitochondria in the enucleated cytoplasm are poorly understood. In cytoplasmic environment, mitochondria play an important role in supply ATP for all energy-requiring cellular activities. These organlles are self-replicating, materially inherited organelles (Smith and Alcivar., 1993). During normal fertilization, after egg-sprem fusion, the sperm's mitochondria are destroyed and the oocyte-derived mitochondria are assumed to be transmitted to the offspring. However, it is not known of fate of foreign mitochondria following nuclear transfer.

Embryonic development is enhanced when donor nuclei are in the G0/G1 phase of the cell cycle (Campbell et al., 1996; Wilmut et al., 1997). Dolly, the sheep was developed from an enucleated oocyte electrofused with a mammary derived cell presumed to be in G0 following culture in serum deficient medium for 5 days. In contrast, Cibelli (2000) reported successful full term development. They chose fibroblasts from fetuses because they can grow rapidly in culture and have an inherently long G1 phase and have very distinctive morphologies, making these easy to identify with confidence.

The objective of this study is to investigate embryonic development by introducing nuclei from non-cultured fibroblast cells taken from Korean Bull into enucleated Hanwoo (Korean Cattle) oocytes. Developmental potential and fate of foreign mitochondria during the preimplantation development were also determined after nuclear transfer in cattle.

Ⅱ. MATERIALS AND METHODS

1. Cell Line

The cell line was derived from a surgical excisional biopsy performed on a Korean Bull (KPN 279 : EPD Carcass Weight : 12.764 Rel : 72 Musculi Longissimus Dorsi Area(cm^2) : 12.764 Rel : 70 Marbling : 0.538 Rel : 70) in December 1999. Thin sections of the tissue were sliced into 1-to 3-mm pieces using a blade, and explants were transferred into 25-mm^2 flasks containing Dulbecco's modified eagle's medium (DMEM; Gibco Laboratories Inc.) + 15% fetal bovine serum (Summit, Fort Collins, CO) + 1% peniciline/streptomycine (Gibco Laboratories Inc.) and then cultured at 37℃ in air containing 5% CO_2. When confluence was achieved at 14 days, cells were trypsinized for 3 min, and total cell count was determined using a Coulter cell counter. The recovered cells were centrifuged, and the pellet was resuspended at a concentration of 1 million cells per ml. Aliquots were either frozen in DMEM contain 10% dimethyl-sulfoxide (DMSO) before storage at −80℃, or 250,000 cells were transferred into a new 25-mm^2 flask. As confluence was approached, the cells were passaged by trypsinization and again counted.

2. *In Vitro* Maturation of Oocytes

Slaughterhouse ovaries were collected from matured Korean cattle, placed in saline (32℃), and transported within 2 h to the laboratory. Cumulus -oocyte complexes (COCs) were recovered by aspiration of 3∼6 mm follicles using an 18-gauge

needle. COCs were collected into HEPES-buffered tissue culture medium 199 (TCM-HEPES) supplemented with 50 mg/ml Gentamycin stock (Sigma), 10 μl/ml Pyruvate stock (2.2 mg/ml), 10% FBS. They were washed twice in TCM-HEPES before being washed once in maturation media (TCM199 supplemented with 10% FBS, 0.5 μl/ml Gentamycine stock (50 mg/ml), 100 μl pyruvate stock (2.2 mg/ml), 10 IU FSH and 1 mg/ml β-Estadiol stock). About 50 COCs were transferred in 20 μl of this medium and placed into 0.5 ml maturation media in 4-well dish overlaid with dimethyl polysiloxane (DMPS). COCs were cultured at 39℃ in a humidified 5% CO_2 in air for 18~19 h. After the maturation, the cumulus cells were completely removed by pipetting COCs in 0.1 % hyaluronidase (from bovine testis; Sigma) in TCM-HEPES for 3 min; this was followed by three washes in TCM-HEPES.

3. Enucleation

Before enucleation, oocytes were incubated in modified phosphate buffered saline (DPBS) containing 10% FBS and 7.5 μg/ml cytochalasin B (CCB; Sigma) for 15 min. Oocytes matured for 18~19 h were enucleated with a 20-μm (internal diameter) glass pipette, by aspirating the first polar body and the M Ⅱ plate in a small volume of surrounding cytoplasm. The oocytes were previously stained in DPBS (modified phosphate buffered saline) containing 10% FBS, 5 μg/ml Hoechst 33342, and 7.5 μ g/ml CCB (cyto chalasin B) for 15 min. Enucleation was confirmed by visualizing the karyoplast, while still inside the pipette, under ultraviolet. After enucleation, the resulting cytoplasts were washed extensively in DPBS containing 10% FBS and were held in this medium until injection of donor cells.

4. Preparation of Cells

Fibroblasts were prepared by trypsinization of early-passage cells at 70~100% confluence. Mito

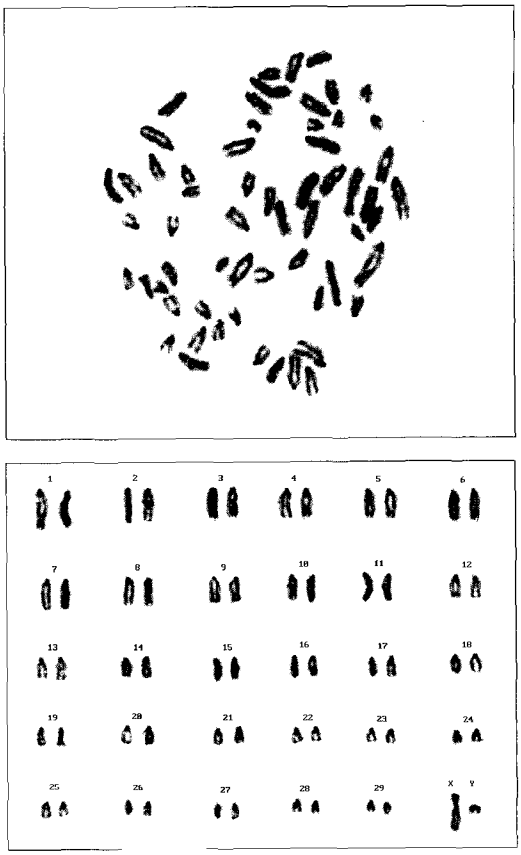

Fig. 1. Donor cell (Korea Bull) karyotype.

Tracker Green FM (Molecular Probes, Eugene, OR) was prepared as a stock 1 M solution in dimethyl sulphoxide (DMSO) and stored at −20℃. Fibroblast cells were stained at a final concentration of 400 nM (Sutovsky et al., 1996) for 10 min before use for microinjction. Chromosome karyotyping was conducted to check normality of cell's chromosome(Fig. 1).

5. Microinjection

A 20-μm pipette (internal diameter) containing a donor cell was introduced through the same slit in the zona pellucida as made during enucleation, and the cell was wedged between the zona pellucida and the cytoplast membrane to facilitate close mem-

brane contact for subsequent fusion. After the injection, the reconstructed embryos were remained in CR_{1aa} medium until fusion.

6. Cell Fusion

Reconstructed embryos were electrically fused at 24 h post maturation (hpm) in fusion buffer comprising 0.26 M mannitol (Sigma), 0.1 mM calcium, 0.1 mM magnesium and 0.01% poly vinyl alcohol (PVA, Sigma). Fusion was performed at room temperature, in a chamber with two stainless steel electrodes 3.2 mm apart filled with fusion buffer. The reconstructed embryos were manually aligned with a fine glass needle, so that the contact surface between the cytoplast and the donor cell was parallel to the electrodes. Cell fusion was induced with two DC pulses of 1.8 kV/cm for 30 μs each, by a BTX Electrocell Manipulator 2001 (BTX). After the electrical stimulus, the reconstructed embryos were washed in DPBS + 10 % FBS. They were then checked for fusion by microscopic examination.

7. Activation

The fused embryos were cultured for 4 h in CR_{1aa} medium (Rosenkrans and First, 1991) before chamical activation. The activation was induced by an incubation in CR1aa with 5 μM ionomycine (Sigma) for 4 min at 37℃. Embryos were then extensively washed in CR1aa for 5 times before culture in 1.9 mM 6-dimethy-aminopurine (6-DMAP; Sigma) for 4 h.

8. *In Vitro* Culture

The reconstructed eggs were cultured in a 4-well dish of CR_{1aa} medium containing 0.3% (w:v) BSA under monolayer of pregnant mouse oviductal epithelial cells. Embryos were cultured in a humidified incubator at 39℃ in a 5% CO_2 in air.

On Day 5 post-fusion, embryos were trasferred into CR_{1aa} + 10% FBS. On Day 7.5 post-fusion, the development of embryos to the blastocysts were recorded.

9. Statistical Analysis

Effect of cell passage and embryo development experiments were repeated at least three times. Effect of cell passage of group was analyzed by χ^2 test and embryo development of group was analyzed by ANOVA test.

Ⅲ. RESULTS

1. *In Vitro* Development of Reconstructed Embryos

Developmental ability *in vitro* of reconstructed embryos by nuclear transfer using ear fibroblast cell was summarized in Table 1. The fusion rate of reconstructed oocytes was 80.5% (422/524). Within 24 h of culture, 65.5% (277/422) of reconstructed oocytes were cleave, and 30.7% (85/277) of the cleaved embryos developed to the morula or blastocyst stage (Table 1). Following nuclear transfer, developmental stage was recorded every 24 h. Out of 40 reconstructed embryos 34 embryos were cleaved, and 14 embryos developed to the morura or blastocysts between 144 h and 168 h of culture

Table 1. *In vitro* development of reconstructed embryos by nuclear transferred using fibroblast cells

Donor cells	NT embryos produced (A)	Fusion embryos (B) (B/A)	Cleavage (C) (C/B)	Morula/Blastocyst (D)(D/C)
Korean bull ear cells	524	422 (80.5%)	277 (65.5%)	85 (30.7%)

Table 2. Time sequence of developmental stage of reconstructed embryos following after somatic cell nuclear transfer

Culture time	No.(%) of embryos developed to				
	2-cell	4-cell	8~16 cell	Molura	Blastocyst
24 h	34 (85)	–	–	–	–
48 h	10 (25)	20 (50)	4 (10)	–	–
72 h	10 (25)	5 (12.5)	19 (47.5)	–	–
96 h	10 (25)	5 (12.5)	18 (45)	1 (2.5)	–
120 h	10 (25)	5 (12.5)	7 (17.5)	12 (30)	–
144 h	10 (25)	5 (12.5)	5 (12.5)	10 (25)	4 (10)
168 h	10 (25)	5 (12.5)	5 (12.5)	4 (10)	10 (25)

(Table 2).

2. Effect of Ccell Passage on the Development of Reconstructed Embryos

To test the cloning competence of adult somatic cell after prolonged culture, skin fibroblast cells from a Korean Bull were cultured to 11 passages. Cleavage and development to morula / blastocysts rates were examined between early (A, 6~7) and late (B, 10~11) passage following nuclear transfer. The developmental abilities *in vitro* were not different between early passage and late passage.

3. Embryo Transfer of NT Embrtyos

Day 6 or 7 embryos (late morula and blastocysts) were transferred to synchronized recipient cows. A total 15 pregnancies were achieved from NT embryos. All pregnancies were maintained up to 40 days of embryo transfer.

4. Effect of Labelling with Mito Tracker Green FM on Development of Embryos after Somatic Nuclear Transfer

In order to trace foreign derived mitochondria following nuclear transfer, we injected Mito Tracker labelled fibroblast cells. Table 5 showed incidence

Table 3. Effect of donor cell passages on developmental ability of NT embryos

Donor	Passage group (Passage No.)	Total embryos	Cleavage (%)	Morula/ blastocyst(%)
Korean bull	A (6~ 7)	75	54 (72)	20 (26.7)
	B (10~11)	40	30 (75)	10 (25)
Total		115	84 (73)	30 (26)

* No significant differences ($P > 0.05$)

Table 4. Pregnancy rate after embryo transfer of nuclear transfer embryos

Donor	No. of recipients	No. of embryos transferred	No. of pregnancy(%)	No. of abortion (up to 40 days)	No. of maintained (over 40 days)
Ear fibroblast cell	*60*	*128*	15	11	5

Table 5. Persistence of foreign mitochondria following nuclear transfer

Developmental stage of embryos	No. of oocytes	
	Examined	Detected (%)
1-cell	40	40 (100)
2-cell	36	30 (83)
4-16 cell	24	13 (54)
Morula	14	0 (0)
Blastocyst	12	0 (0)

Table 6. Effect of labelling with Mito Tracker Green FM on development of embryos after somatic cell nuclear transfer

Donor cell treatment	No. of embryos injected	No.(%) of embryos developed to			
		2-cell	4-16 cell	Morula	Blastocyst
Fibroblast cell	40	10 (25)	10 (25)	4 (10)	10 (25)*
Mito-tracker labelled fibroblast cell	40	12 (30)	10 (25)	2 (5)	12 (30)*

* No significant differences ($P > 0.05$)

of pronuclear formation in reconstructed oocytes using Mito Tracker treated fibroblast cells. The foreign mitochondria were rapidly disappeared following nuclear transfer (Table 5). The donor cells were easily confirmed in reconstructed embryos after nuclear transfer (Fig. 2A & B) There were no difference of developmental abilities of reconstructed embryos using control and Mito Tracker treated fibroblast cells (Table 6).

Ⅳ. DISCUSSION

The present study examined developmental ability of nuclear transferred embryos using fibroblast cells of Korean bull. The data have shown that ear fibroblast cells obtained from a living Korean Bull can be reprogrammed by nuclear transfer and resulted in the production of cloned Korean Bull embryos. The results also showed establishment of pregnancies from transfer of blastocysts derived from fibroblast cell taken from adult native bull into enucleated recipient oocytes in cattle. About 30.7% of the oocytes cleaved to morula cells and more on day 6.

In this study the adult fibroblast cells were used for a donor nucleus.

The karyotypies showed normal numbers and characteristics of Bull's chromosome. The adult cells would be close to senescence, which may preclude development, although fetal fibroblasts close to senescence have been cloned successfully (Cibelli et al., 1998). The adult cell cultures were approaching senescence, as indicated by the slow population - doubling time. However, it is unlikely that adult cells selected for NT were senescent as only those cells of median diameter were chosen, while the large cells, which are more likely to be senescent, were discarded (Goldsetein et al., 1990). The use of regenerated cloned cells has been proposed to allow a second round of reprogramming with a goal of avoiding the high incidence of gestational losses and neonatal abnormalities reported

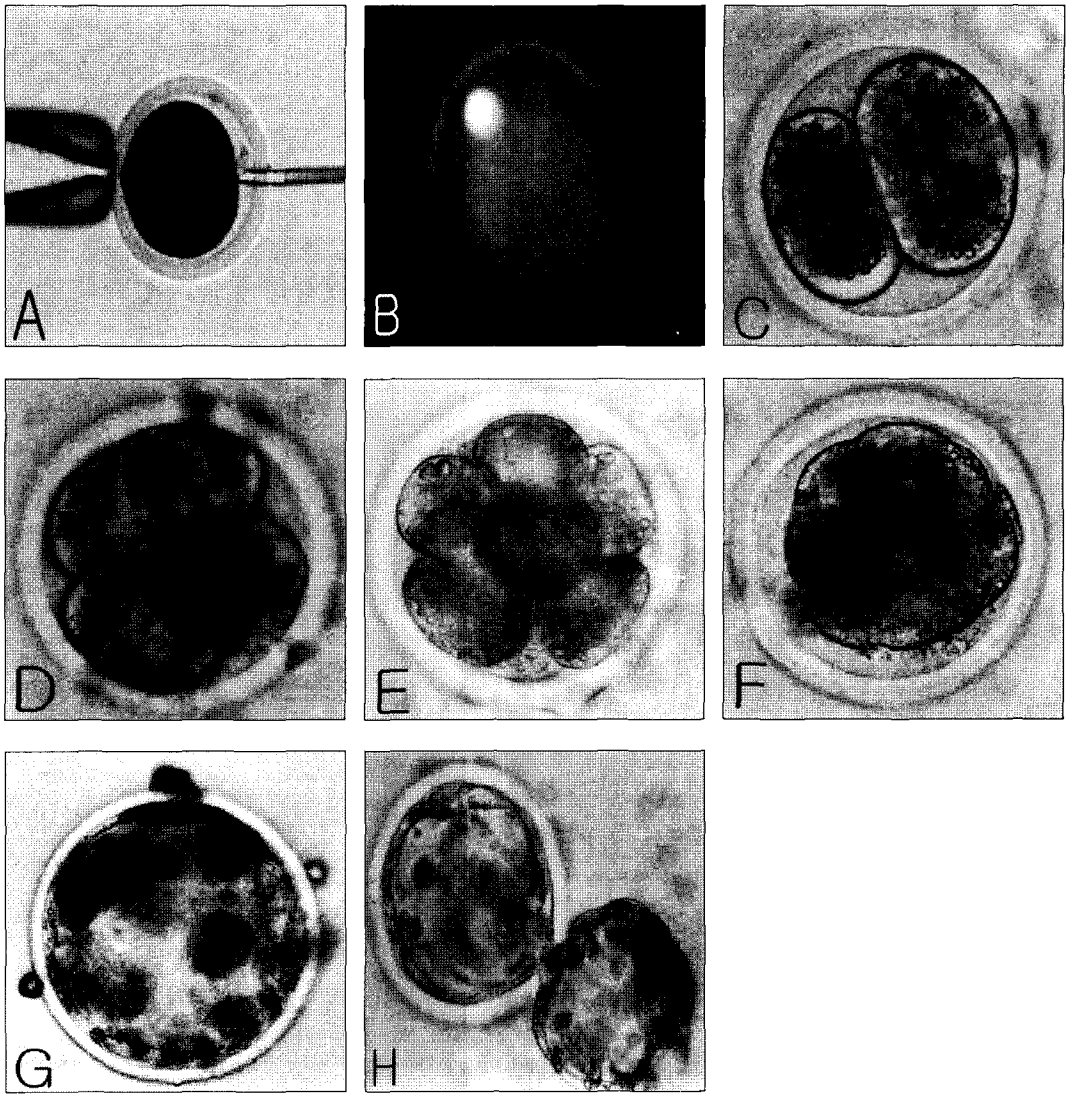

Fig. 2. *In vitro* development of reconstructed embryos by nuclear transfer.

A. Reconstructed embryo B. 1-cell embryo
C. 2-cell D. 4-cell
E. 8~16 cell F. Compaction morula
G. Blastocyst H. Hatched blastocyst

for cloned animals (Robertson et al., 1997).

With the introduction of the donor nucleus before activation, it is vital to control the ploidy of the reconstructed embryo after the activating stimulus is applied, in order for normal development to proceed. The relatively high incidence of embryo development has been in part due to the presence of 6-DMAP in the medium following exposure to ionomycine. This protein kinase inhibitor inhibited phosphorylations necessary for the spindle apparatus

(Susko-Parrish et al., 1994) and therefore prevented micronuclei formation known to occur when fusion precedes activation (Czolowska et al., 1984). Other researchers have used cytochalasin B as a microfilament inhibitor to control ploidy, but in their studies in sheep, with a quiescent embryonic cell line (Wilmut et al., 1997), there was no apparent benefit, either in terms of embryos development or embryo survival.

The high rate of fetal loss following early pregnancy diagnosis has been observed in cloned pregnancies in cattle, sheep, and mice (Wilmut et al., 1997; Cibelli et al., 1998; Wakayama et al., 1998; Wells et al., 1999; Schnieke et al., 1997). The high abortion rate appears to be related to abnormalities of placentation (Wakayama et al., 1998; Stice et al., 1998). The exception to this was the Kato et al. (1998) who used granulosa and oviductal cells as NT donor cells and recorded an 80% pregnancy rate with all of these pregnancies progressing to term, although 50% of the calves died soon after birth. It is also possible that *in vitro* cell culture condition influence pregnancy rate (Sinclair et al., 1999). We used a serum-containing medium and co-culture system previously shown to result in NT embryo pregnancy rate of 20~30% (Cibelli et al., 1998). Others have used similar serum-based co-culture system (Cibelli et al., 1998; Kato et al., 1998), *in vivo* sheep oviduct culture (Wilmut et al., 1997; Campbell et al., 1996), or serum-free defined media (Wells et al., 1999).

Although much work is yet to be done comparing development from cells derived from different animals, the result suggest that it will be possible to produce cloned Korean Bulls. If cloned korean bulls will be produced by nuclear transfer that will be exciting in terms of animal agriculture -in the quality of Hanwoo produce natually and cloned Korean bulls can artificially produce to cure some of the most serious disease by gene targeting.

Regenerated fetuses will be of great use for enabling gene targeting using homologous recombination. This process will require many cell divisions to allow for selection of transgenic cells, and thus regeneration of the cell by successive round of NT may assist in increasing the number of cell divisions (Cibelli et al., 1998; Kato et al., 1998).

In order to examine fate of foreign mitochondria during the preimplantation development after nuclear transfer in cattle, the isolated fibroblast cells were used as donor cells in nuclear transfer. This study showed demonstrated that donor somatic cell-derived mitochondria were rapidly eliminated from the cytoplasm of nuclear transferred bovine embryos at the 8-cell to 16-cell stage. It is possible that DNA from donor cell-derived mitochondria may remain in the cytoplasm (Gyllensten et al., 1991) and transported to endogenous mitochondial DNA (King and Attardi, 1988). Thorsness and Weber (1996) also suggested that mitochondrial DNA could move between the mitochondria. There have been several reports on the inheritance of mitochondria following nuclear transfer. Cloned sheep have inherited their mitochondria entirely from the oocyte and not from donor cell (Evans et al., 1999). Steinborn et al. (1998) reported that mtDNA originating from donor blastomeres was detected in cloned cattle using the allele-specific TaqMan PCR. In the other hand, Takeda et al. (1999) reported that the amount of mtDNA derived from 30-cell stage blastomere in nuclear transfer embryos decreased at the four-to eight-cell stage and was almost absent at the blastocyst stage.

Cattle derived by the transfer of blastomere nuclei showed ambiguous from both the donor cell and the oocyte (Hiendleder et al., 1999). 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 the early

embryonic development has not yet been examined in nuclear transfer using adult somatic cell.

The present study described the developmental potential and the fate of somatic cell-derived mitochondria after nuclear transfer in cattle. During the pronuclear formation, the donor mitochondria dispersed to the cytoplasm and became distributed between blastomeres and finally eliminated from the cytoplasm at the 4-cell 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 fertilization.

In conclusion, the results showed that the reconstructed embryos by nuclear transfer using Korean bull's fibroblast cell can be reprogramed. Further studies are required to understand mechanism related to interaction of foreign mitochondria and cytoplasm, and to developmental capacities of reconstructed embryos to term development.

V. 요 약

본 연구에서는 한우 수소에서 유래된 체세포로 핵 이식한 복제 난자의 발달능력을 조사하였다. 종모우의 귀세포를 배양하면서 공여핵으로 사용하였고, 수핵란은 도축장에서 얻은 난소에서 난구-난자 복합체를 채취한 후, TCM 199 배양액에서 20시간 정도 체외 배양하여 사용하였다. 성숙된 난자는 Cytochalasin B가 있는 dPBS에서 핵과 극체를 제거하였고, 이어 이 난자에 귀의 섬유 아세포의 핵을 삽입시키고 전기 자극법에 의해서 융합하였다. 재조합된 난자들은 Ionomycine과 6-dimethylaminopurine으로 활성화 한 후, 7.5일 동안 CR_{1aa} 배양액에 배양하였다. 총 524개의 난자가 핵 이식되었고, 융합된 난자중 65.6% (277/422)의 난자가 난할이 되었으며, 그 중 30.7% (85/277)의 난자가 상실배에서 배반포까지 발달하였다. 계대배양에 따른 재조합된 난자의 체외 발달능력은 차이가 없었다. 공여 세포에 의한 외래 미토콘드리아의 분포 및 생존 여부를 조사하기 위하여 Mito Tracker로 공여 세포의 미토콘드리아를 염색하여 핵이 제거된 난자와 융합하였다. 외래 미토콘드리아는 초기 배아 발달 단계에서는 발견이 되었지만, 급격히 사라졌다. Mito Tracker 염색은 재조합된 난자의 발달율에는 지장을 주지는 않았다. 이러한 연구 결과는 한우 수소의 체세포를 이용한 핵 이식에 의해 이식 가능한 난자를 생산 할 수 있음을 보여 주는 것이다.

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