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Developmental Potentials of Clone Embryos Derived from Bovine Fetal Fibroblast Cells

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소 태아섬유아세포 유래 복제란의 발육능에 관한 연구

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ABSTRACT

This study was conducted to investigate the developmental potential of cloned embryos derived from bovine fetal fibroblast cells, and the effect of quiescent treatment, passage number and origin of donor cells on in vitro development of cloned embryos. Fetal skin and liver-derived fibroblast cells were transferred to enucleated oocytes after serum starvation or nontreatment (cycling). After electrofusion, reconstituted embryos were activated with Ca^{++}-ionophore and cycloheximide, and cocultured for 7~9 days with BRL cells. Some blastocysts were transferred to recipient cows 7~8 days post estrus. The development rate to the blastocyst stage of serum starved cell-derived embryos was higher (25.3%) than that of actively dividing cells-derived embryos (15.9%), The rates of blastocyst formation were 23.1~25.0% after transfer of cell passaged 4 to 6 times, and 23.8 and 25.2% after transfer of fetal skin and liver cells, respectively. After embryo transfer, 34.4% and 15.6% of recipient cows were pregnant on Day 60 and 120, respectively, and one male calf was produced from skin-derived vitrified blastocyst. The result of this study showed that the development of cloned embryos·was enhanced by quiescent treatment, but did not different among the cells passaged 4 to 6 times, and between skin and liver cells. This result also confirms that offspring can be obtained from the vitrified clone embryo derived from fetal skin cell.

(Key words: Nuclear transfer, Fetal fibroblast, Serum starvation, Clone bull)

I. INTRODUCTION

Since the first report of a lamb born after nuclear transfer of an adult somatic cell nucleus into an enucleated oocyte (Wilmut et al., 1997), the birth of clone mice (Wakayama et al., 1998, 1999), goat (Baguisi et al., 1999) and cattle

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(Kato et al., 1998; Shiga et al., 1999; Vignon et al., 1999; Wells et al., 1999, Zakhartchenko et al., 1999) were obtained after transfer of somatic cell nuclei. Furthermore, transgenic clone lambs and cattles were born from the nuclear transfer of transfected somatic cells (Schnike et al., 1997; Cibelli et al., 1998).

It has been considered that cloning by nuclear transfer depends upon various factors. One important factor is the cell-cycle-stage of a donor cell (Cheong et al., 1993; Campbell et al., 1996; Wilmut et al., 1997). Cell cycle synchronized nuclear transfer was previously reported in embryonic cell nuclear transfer (Collas et al., 1992; Cheong et al., 1993). Transfer of a G1 nucleus into an enucleated metaphase Ⅱ (MII) oocyte facilitated the nuclear reprogramming and lead to the high development of nuclear transfer embryos (Cheong et al., 1993). Normal development of somatic cell-cloned embryos may also be affected by the quiescent status of donor cells. The productions of live offspring from adult and fetal somatic cells were reported to be possible when quiescent G0 cells were transferred into the enucleated oocytes (Wilmut et al., 1997; Kato et al., 1998; Wells et al., 1999).

The quiescent G0 state may be induced in somatic cells by serum starvation (Campbell et al., 1996; Wilmut et al., 1997) or by using the naturally arrested cells in this state directly from the animal, such as Sertoli and neuronal cells in the mouse (Wakayama et al., 1998). The G0 phase of the cell cycle is implicated in the reduction of transcriptional activity and chromatin modification (Whitfield et al., 1985), which may facilitate the reprogramming of the donor nucleus in the oocytes cytoplasm (Campbell et al., 1996; Wilmut et al., 1997; Kato et al., 1998; Wells et al., 1999). Some recent studies, however, suggested the use of G0 cells might not be a critical factor for successful somatic cell cloning. Nonquiescent proliferating cells could support development to term after nuclear transfer (Cibelli et al., 1998; Zakhartchenko et al., 1999). Furthermore, full term development was also obtained from cumulus cells of mouse, in which about 90% of cells were in G0/G1 (Wakayama et al., 1998).

On the other hand, the donor cell types may affect the development of clone embryos. In the sheep, *in vitro* development of clone embryos derived from adult mammary gland cells was lower than that of fetal fibroblast cell-derived clone embryos (Wilmut et al., 1997). In the bovine, higher percentage of cloned blastocysts was obtained from the use of cumulus cells compared to oviductal cells as donor nuclei (Kato et al., 1998), which suggested that the development potential of clone embryos was affected by the origin of the donor cell, even though obtained from the same animal.

This study was conducted to examine the developmental potential of cloned embryos derived from bovine fetal fibroblast cells, and the effect of quiescent treatment, passage number and origin of donor cells on *in vitro* development of clone embryos.

Ⅱ. MATERIALS AND METHODS

1. *In Vitro* Maturation of Oocytes

Bovine follicular oocytes were obtained by aspiration of 2~7mm follicles. About 10 cumulus-oocytes complexes (COCs) were placed into a 50μl droplet of maturation medium previously prepared in a ψ35mm culture dish overlaid with mineral oil (Sigma, St Louis, MO, USA), and cultured for 18~20 h at 39℃, 5% CO_2 in air. The maturation medium comprised TCM-199 supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, NY, USA), 0.2mM Na-pyruvate, 50μg/ml gentamycin (Sigma), 0.02U/ml FSH (Sig-

ma) and 1μg/ml estradiol-17 (Sigma). After maturation, cumulus cells were removed by vortexing the COCs in 200IU/ml hyaluronidase (Sigma), and oocytes with extruded first polar body were selected for recipient cells.

2. Isolation, Culture and Preservation of Donor Cells

A male fetus was obtained from a pregnant (Day 100) cow. After through washing in sterilized physiological saline solution, the liver and some skin tissues were isolated separately and then chopped into small pieces. The tissues were enzymatically digested with 0.05% trypsin-EDTA in PBS for 30 min at 37℃ with occasional stirring. The digestion procedure was repeated two or three times, and the digested tissues were allowed to settle for 5 min and the supernatants containing disaggregated cells were transferred into a 15ml conical tube. Cells were collected by centrifugation at 500×g for 5 min and washed once in culture medium. The cell pellets were diluted with Dulbeccos modified Eagles medium (DMEM)/F12 (Gibco-BRL) supplemented with 10% FBS and 50μg/ml gentamycin, and cultured at 37℃, 5% CO_2 in air. At about 90% confluency, the cells were passaged with a 1:2 division, and subsequently regrown cells were passaged 2~4 times more before being frozen in DMEM/F12 with 15% FBS and 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen. Thawed cells from each tissue were cultured to form a monolayer. After 2~3 days of culture, cells were induced to enter quiescence by serum starvation (Campbell et al., 1996) before the nuclear transfer. Culture medium was replaced with DMEM/F12 supplemented with 0.5% FBS and further cultured for 5 days. Nonquiescent proliferating cells at 2~3 days after culture (grown to 60~70% confluency) were used as controls.

3. Nuclear Transfer

Micromanipulation procedures were performed in Dulbecco's PBS supplemented with 5μg/ml cytochalasin B (Sigma) and 3mg/ml BSA. Oocytes were enucleated by aspirating the first polar body (PB) and the MⅡ plate with a small volume of surrounding cytoplasm. The oocytes were stained with 1μg/ml Hoechst 33342 (Sigma) for 15~20 min (Westhusin et al., 1992) in TCM-199 containing 3mg/ml BSA and examined under fluorescence to confirm enucleation. After the enucleation, the cytoplasts were held in TCM-199 containing 3mg/ml BSA until injection of donor cells.

Donor cells used for nuclear transfer were between passages 4 to 6 of culture. Cells were harvested by trypsinization and resuspended in TCM-199 containing 3mg/ml BSA, and remained in this medium until injection. A single fibroblast cell was injected into the perivitelline space of an enucleated oocyte through the same slit in the zona.

4. Electrofusion and Activation

Reconstituted embryos were placed in TCM-199 supplemented with 3mg/ml BSA for 10~30 min prior to fusion. Then they were transferred into a fusion chamber consisting of two wires, 0.5mm apart, overlaid with 0.3M mannitol containing 0.1mM $MgSO_4$, 0.05mM $CaCl_2$ and 0.05mg/ml BSA. The reconstituted embryos were manually aligned with a pipette, after which two direct current (DC) pulses of 1.25kV/cm for 70μsec were applied to the chamber using a BTX Electrocell Manipulator 200 (BTX, San Diego, CA, USA). After fusion treatment, the reconstituted embryos were placed in TCM-199 supplemented with 3mg/ml BSA, and checked for fusion. The above fusion pulses did not activate the oocyte cytoplasm (data not shown).

Fused embryos were activated 1~1.5 h after fusion, by exposing to 10μM Ca^{2+}-ionophore (A23-187;Sigma) for 5 min at 37℃, followed by incubation in 10μg/ml cycloheximide (Sigma) in TCM-199 containing 3mg/ml BSA for 6h at 39℃, 5% CO_2 in air.

5. Embyro Culture and Transfer

After activation, the nuclear transferred embryos were checked for the existence of a PB-like structure, and then cultured in drops of TCM-199 supplemented with 3mg/ml BSA overlaid with mineral oil. Cleaved embryos were transferred into 50μl drops of CRlaa containing with 10% FBS after 2 days, and further cocultured with Buffalo rat liver (BRL) cells for 5~7 days. Cloned blastocysts derived from the fetal skin and liver cells were transferred to synchronized recipient cows 7~8 days post estrus. Some embryos were vitrified by GESD solution (Saito et al., 1994) and thawed before transfer. Pregnancy was determined by ultrasound and rectal palpation at Days 60 and 120.

6. Statistical Analysis

Data were analyzed by chi-square test.

Ⅲ. RESULTS

1. Effect of Quiescent Treatment

When the nonquiescent cells were transferred, significantly high cleavage rate was obtained (93.7%, P<0.05), however, developmental rate to the blastocyst stage (15.9%, 20/126) was significantly lower (P<0.05) than that of the serum starved cells (25.3%, 40/158) (Table 1). Some embryos extruded a PB-like structure after activation. The proportion of embryos that extruded a PB-like structure was slightly lower when serum starved cells were transferred (22.8%), compared to the nonquiescent proliferating cell (30.2%).

2. Effect of Passage Number

The development of nuclear transferred embryos derived from skin cells passaged 4 to 6 times were assessed. Proportions of fused embryos (69.6~73.1%) and developed to the blastocyst stage (23.1~25.0%) were not different among the different passages. Cleavage rate at passage 5 was significantly higher (92.1%, P<0.05) than the other groups, but the same trend was not observed in the advanced stages (Table 2). Some embryos extruded a PB-like structure after activation (18.4~24.4%).

3. Effect of Origin of Cell

The developmental potential of nuclear transferred embryos derived from different tissues were examined. Proportions of embryos that developed to the two-cell (81.9~88.7%) and blastocyst stage (23.8~25.2%) were not different when fetal skin or liver cells were used as donor cells (Table 3). The proportions of embryos

Table 1. Effect of quiescent treatment on the development of cloned bovine embryos derived from fetal fibroblast cells*

Treatments	No. (%) of eggs fused/manipulated	No. (%) of embryos developed to		
		2-Cell	Morula	Blastocyst
SS	158/233 (67.8)	130 (82.3)	45 (28.5)[a]	40 (25.3)[a]
NQ	126/190 (66.3)	118 (93.7)	21 (16.7)[b]	20 (15.9)[b]

* The cells were derived from fetal skin. SS: serum starvation, NQ: nonquiescent

[a,b] Values with different superscripts differ (P<0.05).

Table 2. Effect of the number of cell passage on the development of cloned bovine embryos derived from fetal fibroblast cells*

No. of passage	No. (%) of eggs fused/manipulated	No. (%) of embryos developed to		
		2-Cell	Morula	Blastocyst
P4	78/112(69.6)	66(84.6)[a]	20(25.6)	18(23.1)
P5	76/104(73.1)	70(92.1)[b]	20(26.3)	19(25.0)
P6	82/113(72.6)	65(79.3)[a]	23(28.0)	20(24.4)

* The donor cells were derived from serum starved fetal skin.

[a,b] Values with different superscripts differ($P<0.05$).

Table 3. Effect of the origin of donor cell on the development of cloned bovine embryos derived from fetal fibroblast cells*

Origin of cells	No. (%) of eggs fused/manipulated	No. (%) of embryos developed to		
		2-Cell	Morula	Blastocyst
Skin	160/225(71.1)	131(81.9)	43(26.9)	38(23.8)
Liver	159/247(64.4)	141(88.7)	45(28.3)	40(25.2)

* Cells were serum starved for 5 days.

Table 4. Development *in vivo* of nuclear transfer bovine embryos derived from fetal fibroblast cells

Origin of cells	Type of embryos	No. of transferred embryos	No. of recipients	No. (%) of pregnant on day*		No. (%) of offspring**
				60	120	
Skin	Fresh	13	11	4(36.4)	2(18.2)	0
	Vitrified	7	6	2(33.3)	1(16.7)	1(16.7)
Liver	Fresh	10	10	3(30.0)	1(10.0)	0
	Vitrified	6	5	2(40.0)	1(20.0)	0
Total		36	32	11(34.4)	5(15.6)	1(2.8)***

* Based on the number of recipients.

** Based on the number of transferred embryos.

*** Born on Day 255, but died 40 min after birth(male, birth weight: 18kg).

extruded a PB-like structure were also similar between two groups (about 24%).

4. Embryo Survival after Embryo Transfer

A total of 36 cloned blastocysts were transferred in pairs or single to 32 recipient cows, of which 11 (34.4%) and 5 (15.6%) animals were pregnant on Days 60 and 120, respectively (Table 4). There were no differences in pregnancy rates between the embryos derived from fetal skin and liver cells, and likewise from fresh and vitrified. A male calf (birth weight 18kg) was born from a recipient that received a vitrified blastocyst derived from a fetal skin cell on Day 255 (spontaneous dilivery), but died 40 min later probably due to premature birth and weakness.

Ⅳ. DISCUSSION

The result of the present study showed that

the development of bovine fetal cell-derived clone embryos was affected by the quiescent treatment, however, the number of cell passage (4 to 6 times) and the origin of the cells (skin and liver) did not influence the development. This result also confirms that offspring can be obtained from the vitrified clone embryo derived from a fetal skin cell.

It has been considered that cell cycle stage of the donor cells is an important factor for successful nuclear transfer in mammals. It was reported that G1 (Collas et al., 1992; Cheong et al., 1993) and G0 (Wilmut et al., 1997) cells are essential for nuclear reprogramming. In somatic cell nuclear transfer, a quiescent G0 cell produced by serum starvation was transferred into an enucleated M II oocyte to induce the nuclear reprogramming (Wilmut et al., 1997; Kato et al., 1998; Wells et al., 1999). On the other hand, mouse cumulus cells arrested in G0/G1 by 90% (Wakayama et al., 1998), or actively dividing bovine fetal fibroblast cells were also reprogrammed successfully (Cibelli et al., 1998; Zakhartchenko et al., 1999). In this study, serum starvation of the cells enhanced the development of the nuclear transfer embryos likewise in previous studies (Galat et al., 1999; Hill et al., 1999; Zakhartchenko et al., 1999), but it was not essential for the development to the blastocyst stage. About 16% of the nuclear transfer embryos derived from proliferating cells without serum starvation also developed to the blastocyst stage. This result is coincided with the results of Cibelli et al. (1998) and Zakhartchenko et al. (1999), and suggests that some nuclear transfer embryos injected with a proliferating cell in G1 may developed to the blastocyst stage.

Little research has been conducted on the effect of cell passage number on the development of cloned bovine embryos using somatic cells as donor. In the sheep, development of cloned embryos from cultured embryonic epithelial cells was not affected by the cell passage number from 6 to 13 (Campbell et al., 1996). Likewise the development of cloned rabbit embryos using fetal cells was not affected by the cell passage number from 2 to 8 (Galat et al., 1999). In the previous studies, cells passaged 3 to 6 times in sheep (Wilmut et al., 1997), and cells passaged 3 to 8 times (Wells et al., 1999), 2 to 12 times (Vignon et al., 1999), and 4 or 6 times (Kato et al., 1998) in bovine were used as nuclear donor, but the influence of the cell passage number on the development of the nuclear transfer embryos was not reported in these studies.

Kato et al. (1998) reported that the *in vitro* development of bovine embryos cloned from cumulus cells was significantly higher than that of the oviductal cell-derived embryos. Zakhartchenko et al. (1999) also reported that the blastocyst development of ear cell-derived embryos was higher than that of the mammary gland cell-derived embryos in bovine. In bovine nuclear transfer using the fetal fibroblast cells, skin-derived fibroblast cells had higher developmental potential than muscle-derived fibroblast cells which had many heterogeneous cells in culture and might be more differentiated (Vignon et al., 1998). In this study, however, no differences were observed in the development and the morphology in culture between the fetal skin- and liver-derived fibroblast cells.

In mouse embryonic cell nuclear transfer, nuclear remodeling types depend on the cell cycle stage of the donor cells. A donor nucleus undergoes premature chromosome condensation (PCC), and is modified in various types after activation depending on the cell cycle stage (Cheong et al., 1993). A PB-like structure was extruded from the embryos that received a donor nucleus which cell cycle stage was not in G1 (Cheong et al., 1993). In other animals, ex-

trusion of a PB-like structure was not reported. In this study, however, some nuclear transfer bovine embryos that received a fetal fibroblast cell extruded a polar body-like structure after activation. Similar phenomenon was shown in bovine embryonic cell nuclear transfer (Cheong et al., 1999). The extrusion of a PB-like structure might be resulted from the transfer of a cell in S or G2 phase to the enucleated MⅡ oocyte. This was suggested by the fact that the proportion of embryos extruded a PB-like structure was high in the embryos that received a nonquiescent proliferating cell compared to embryos that received a serum starved cell.

In this study, an offspring was obtained from the clone embryo derived from fetal skin cell, but a high proportion of embryonic death was observed before midterm of gestation, especially beyond Day 120. It was reported that mortality of cloned embryos was high after implantation and birth when somatic cells were used as donor nuclei (Cibelli et al., 1998; Wells et al., 1999), with an exception from the report of Kato et al. (1998). The reason of high embryonic death was not known in this study.

Ⅴ. 요 약

본 연구는 소 태아섬유아세포를 이용하여 핵이식 후 세포의 휴면처리, 세포의 passage 수 및 세포의 기원이 복제란의 발육에 미치는 영향을 검토하였다. 3.5개월령 한우 수컷 태아의 피부 및 간 조직에서 세포를 채취하여 체외에서 4~6회 계대배양 후 동결하였다가 핵이식에 사용하였다. 세포는 핵이식 전에 혈청기아처리를 하였으며, 대조구로는 활발히 분열 중의 무처리 세포를 사용하였다. Donor 세포는 미수정란의 탈핵세포질에 이식 후 전기융합 및 활성화를 실시하였고, 재구축배는 7~9일간 체외배양하여 발육능을 검토하였다. 배반포로 발육된 일부 복제란은 발정 7~8일째의 수란우에 이식하였다. 복제란의 배반포 발육율은 혈청기아처리구(25.3%)가 무처리구(15.9%)에 비하여 유의적으로 높았으나(P<0.05), 세포의 passage수(4~6회)에 관계없이 23.1~25.0%로 나타났고, 피부 및 간 유래 복제란의 배반포 발육율도 23.8~25.2%로 두 조직세포 간에 차이가 없었다. 복제란의 이식 후 60일 및 120일에 수란우의 34.4% 및 15.6%가 각각 임신이 확인되었으며, 초자화동결된 태아 피부세포 복제란으로부터 1두의 수컷 송아지가 생산되었다. 본 연구의 결과는 복제란의 체외발육능이 세포의 휴면처리에 의하여 향상되었으나, 세포의 passage수(4~6회) 및 세포의 두 기원(피부 및 간)에 의해서는 영향을 받지 않으며, 태아 피부세포 유래 복제란으로부터 산자가 생산될 수 있음을 확증한다.

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