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Production of Bovine Transgenic Embryos Derived from Non-transfected and Transfected Adult Cells

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외부유전자가 도입된 체세포를 이용한 소 형질전환 복제란 생산

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SUMMARY

The present study was conducted for the production of transgenic cloned cows those secrete human lactoferricin into milk by somatic cell nuclear transfer (NT). To estimate detrimental effects of gene transfection on transgenic cloned embryo production, development rates of NT embryos were compared between transfected and non-transfected cumulus and ear fibroblast cells. An expression plasmid for human lactoferricin (pbeta-LFC) was constructed by inserting a bovine beta-casein promoter, a green fluorescent protein (GFP) marker gene, and human lactoferricin target gene into a pcDNA3 plasmid. Two bovine somatic cell lines (cumulus cell and ear fibroblast) were established and transfected with the expression plasmid using a liposomal transfection reagent, Fugene6 as a carrier. Cumulus cell and ear fibroblast were transfected at the passage of 2 to 4, trypsinized and GFP-expressing cells were randomly selected and used for somatic cell NT. Developmental competences (rates of fusion, cleavage, and blastocyst formation) in bovine transgenic somatic cell NT embryos reconstructed with non-transfected cells were significantly higher than those from transfected cells in cumulus cell and ear fibroblast ($P<0.05$). This study indicated that transfection of donor cell has detrimental effect on embryo development in bovine transgenic NT.

(Key words : bovine, transgenic, somatic cell nuclear transfer, human lactoferricin, GFP)

INTRODUCTION

Transgenic animals are useful tools for the mass production of human therapeutic proteins (Houdebine, 2000; Petters and Sommer, 2000). Various gene delivery systems with different efficiency including pronuclear microinjection (Bowen et al.,

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1994; Krimpenfort et al., 1991), retroviral vector (Haskell and Bowen, 1995; Chan, 1999), sperm vector (Gandolfi et al., 1989; Schellander et al., 1995), embryonic stem cells (Stice et al., 1996), and somatic cell nuclear transfer (NT) (Cibelli et al., 1998; Schnieke et al., 1997) were employed and successfully generated the transgenic animals. Out of these methods, NT with transfected or gene -targeted cells has been proven to be a more efficient method of producing transgenic livestock than other methods (McCreath et al., 2000; Baguisi et al., 1999; Cibelli et al., 1998; Schinekes et al., 1997). However, both the low efficiency and developmental abnormalities exhibited by clones preclude widespread application of this technology (Westhusin et al., 2001; Hill et al., 2000).

To produce transgenic animals by NT, donor cells must undergo transfection and selection procedures resulting in extended culture *in vitro*. The effects of transfection, drug selection, and growth as single-cell clones on the ability of cells to support development of NT embryos were not investigated in detail, although it is urgently required to elucidate potential correlations between developmental abnormalities of cloned fetuses/offspring and extended culture or genetic manipulation of nuclear donor cells.

Consequently, this study was conducted to compare the efficiency of bovine transgenic NT between transfected and non-transfected cell including cumulus cells and ear fibroblast for determining detrimental effects of transfection.

MATERIALS AND METHODS

1. Collection of Somatic Cell and Primary Cell Culture

Ear tissues were obtained from a Holstein, washed in Ca^{2+}- and Mg^{2+}- free PBS (DPBS-; Life Technologies, Rockville, MD), and minced by surgical blade. The minced adult tissues were dissociated with DMEM (Life Technologies) supplemented with 0.1% (w/v) trypsin and 1 mM EDTA (Life Technologies) for 1 to 2 hours. Trypsinized cells were washed once by centrifugation at 300 × g for 10 minutes and subsequently seeded into 100 mm plastic culture dishes. Seeded cells were subsequently cultured for 6 to 8 days in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies), 1 mM sodium pyruvate (Sigma-Aldrich Co., St. Louis, MO), 1% (v/v) non-essential amino acids (Life Technologies) and 10 μg/mL penicillin-streptomycin solution (Sigma-Aldrich Co.) at 39℃ in a humidified atmosphere of 5% CO_2 and 95% air. After removal of unattached clumps of cell or explants, attached cells were further cultured until confluent, and then subcultured at intervals of 5 to 7 days. Cumulus cells for donor cells were collected by aspirating the ovarin antral follicles using an ultrasound-guided ovum pick-up transvaginal probe (Lee et al., 1994). Retrieved cumulus oocyte complexes (COCs) were collected into DPBS- supplemented with 50 μg/ml heparin (Sigma-Aldrich Co.). In the presence of 0.1 % (w/v) hyaluronidase (Sigma-Aldrich Co.), cumulus cells were mechanically separated by mouth -pipetting method. Seperated cumulus cells were centrifuged and washed once in culture medium before seeding onto a 100 mm culture dishes. Three types of cells were cultured and were then stored after 1~2 passages in liquid nitrogen at −196℃. Frozen media consisted of 80% (v/v) DMEM (Life Technologies), 10% (v/v) DMSO (Sigma-Aldrich Co.) and 10% (v/v) FBS.

2. Generation of Transfected Cells and Serum-starvation

Transfected cell lines were generated through lipid-mediated gene transfer. An expression plasmid for human lactoferricin (pbeta-LFC) was constructed by inserting a bovine beta-casein promoter, a green fluorescent protein (GFP) marker gene, and human lactoferricin target gene into a pcDNA3 plasmid (Life Technologies). For transfection, frozen cells

scheduled to be used as donor cells were thawed, cultured until 50% confluency at 35 mm culture dish and transfected with the plasmid using FuGene 6® (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. The transfected cells were cultured for another 2~3 days in order to have chromosomal intergration of transgene and G0 stage of cell cycle. For production of serum-starved groups, transfected cells were cultured for an additional 5 days in DMEM containing 0.5% FBS. Before injection of donor cell, starved and non-starved cells were collected by trypsinization of the monolayer and resuspended the pellets after centrifugation in PBS supplemented with 0.5% FBS. GFP-expressing cells were selected under ultraviolet light using a standard fluorescein isothiocyanate (FITC; excitation wavelength: 450~490 nm; B-mode filter, Nikon, Japan) filter set and used for microinjection.

3. Preparation of Recipient Oocytes

Ovaries were collected at a local slaughterhouse and transported to the laboratory in 0.9% (v/v) NaCl solution at 30 to 35℃. Follicular fluid and COCs from follicles 2 to 8 mm in diameter were aspirated using an 18-gauge needle attached to a 10 ml disposable syringe. COCs with evenly granulated cytoplasm and encompassing compact cumulus cells of more than three layers were selected, washed three times in HEPES-buffered tissue culture medium-199 (TCM-199; Life Technologies), and cultured for *in vitro* maturation (IVM) in bicarbonate-buffered TCM-199 supplemented with 10% (v/v) FBS, 0.005 IU/ml FSH (Antrin, Teikoku, Tokyo, Japan), 1 ug/ml estradiol (Sigma) at 39℃ in 5% CO_2 in a humidified atmosphere of 95% air. At the end of maturation culture, COCs were then transferred to Hepes-buffered calcium -free CRaa medium (Rosenkrans and First, 1991) (handling medium) containing 0.1% (w/v) hyaluronidase for 1 minute and the cumulus cells of COCs were subsequently removed by gentle pipetting.

3. Reconstruction of Embryos and Subsequent Culture

Handling medium supplemented with 10% FBS and 5 μg/ml cytochalasin B (Sigma-Aldrich Co.) was used for oocyte manipulation. The zona pellucida of oocytes were partially dissected using a fine glass needle and the first polar body and adjacent cytoplasm presumably containing the metaphase-II chromosomes were extruded by squeezing with the needle. Oocytes were then stained with 5 μg/ml bisbenzimide (Hoechst 33342, Sigma-Aldrich Co.) for 5 minutes and observed under an inverted microscope equipped with an epifluorescence apparatus. Oocytes still containing DNA material were excluded. GFP-expressing donor cells under FITC filter set from starved and non-starved group were aspirated into injection pipette and introduced into the perivitelline space of an enucleated oocyte.

Reconstructed embryos were placed in a 0.3 M mannitol solution containing 0.5 mM Hepes, and 0.1 mM $MgCl_2$ for 4 minutes and transferred to a chamber consisting of two electrodes overlaid with mannitol medium. Embryos were fused with double DC pulses of 1.75 kV/cm for 15 μsec using a BTX Electro-cell Manipulator 2001 (BTX, Inc., San Diego, CA) and transferred reconstructed oocytes to mSOF medium (Takahashi and First, 1992). At 4 h after fusion, fused oocytes were chemically activated by treatment with 5 μM ionomycin for 4 min, followed by 1.9 mM 6-dimethylaminopurine (DMAP, Sigma-Aldrich Co.) treatment for 4 h. All treated embryos were washed in handlimg medium and placed 25 μl microdrops (5~10 oocytes per drop) of mSOF supplemented with 0.8% (w/v) BSA and cultured at 39℃, 5% CO_2, 5% O_2 and 90% N_2 atmosphere. The reconstructed embryos were cultured for 7 days after fusion, and cleavage and blastocyst formation were monitored under a stereomicroscope.

4. Statistical Analysis

All values in each parameter were analyzed using a general linear model (PROC-GLM) in a SAS 8.12 program. Statistical significance was determined when the P value was less than 0.05.

RESULTS

1. Cumlus Cell as Donor Cell

The developmental competence of bovine SCNT embryos reconstructed with transfected and non-transfected cumulus cells is summarized in Table 1. NT embryos reconstructed with non-transfected cumulus cells developed to the blastocyst stage significantly better than those derived from transfected cumulus cells (43.0 vs. 28.4%; $P<0.05$). And then, there was no significant difference in fusion rates between transfected and non-transfected groups ($P<0.05$).

2. Ear Fibroblast as Donor Cell

The results of ear fibroblast showed similar tendancy with those of cumulus cells. Development rates to blastocyst were significantly higher in NT embryos reconstructed with non-transfected cells than transfected cells (40.6 vs. 10.8%), whereas there was no significant difference infusion rates ($P<0.05$).

DISCUSSION

Human lactoferricin has bacteriostatic activity

Table 1. Development rates of NT embryos derived from non-transfected and transfected cumulus cells

Transfection	No. of embryos			
	Injected	Fused (%)[a]	Cleaved (%)[b]	Develop to bl*(%)[b]
Non-transfected	259	181 (69.9)	108 (59.7)[c]	76 (43.0)[c]
Transfected	213	167 (78.4)	121 (72.5)[d]	48 (28.4)[d]

Model effect of transfection on the number of embryos fused, cleaved, and developed to the blastocysts, which was indicated as a P value, was 0.1238, 0.0001, and 0.0001, respectively.

* Blastocyst.

[a] Percentage of the number of embryos injected.

[b] Percentage of the number of embryos fused.

[cd] Within a parameter in the same treatment, values with different superscripts differed significantly, $P<0.05$.

Table 2. Effect of transfection of ear fibroblast on the development of nuclear transferred bovine embryos

Transfection	No. of embryos			
	Injected	Fused (%)[a]	Cleaved (%)[b]	Develop to bl*(%)[b]
Non-transfected	311	197 (63.3)	184 (93.4)[c]	80 (40.6)[c]
Transfected	308	213 (69.2)	134 (62.9)[d]	23 (10.8)[d]

Model effect of transfection on the number of embryos fused, cleaved, and developed to the blastocysts, which was indicated as a P value, was 0.1267, 0.0001, and 0.0001, respectively.

* Blastocyst.

[a] Percentage of the number of embryos injected.

[b] Percentage of the number of embryos fused.

[cd] Within a parameter in the same treatment, values with different superscripts differed significantly, $P<0.05$.

(Reiter and Perraudin, 1998), bactericidal activity (Arnold et al., 1977), fungicidal activity (Ellison and Giehl, 1991), and many other potential functions, such as regulation of myelopoiesis (Yamada et al., 1987) and inflammatory-immune response (Kulics and Kijlstra, 1987), production of reactive oxygen metabolites (Klebanoff and Waltersdorph, 1990). It is also found in plaques of Alzheimer disease (Kawamata et al., 1993). In order to establish the production system for human lactoferricin -secreting transgenic clone cows by somatic cell NT, the present study were conducted to investigate the detrimental effect of donor cell transfection in development of transgenic NT embryos. In the present study the efficiency of NT was higher with non-transfected than with transfected cumulus cell and ear fibroblast.

There are many reports that non-transgenic NT results in high rates of fetal losses, high birth weight, perinatal deaths, and congenital defects and malformations with more extreme effects after somatic cell NT (Hill et al., 2000; Wells et al., 1999; Cibelli et al., 1998; Wilmut et al., 1997). These abnormalities may be due to either the NT procedure itself, leading to incomplete nuclear reprogramming of donor cells orthe *in vitro* maturation and embryo culture systems, singly or in combination, may lead to in appropriate patterns of gene expression in embryos, fetuses, or in extra embryonic tissue contributing to the failure of normal development. In addition, the use of transfected cells as donors for NT may also cause some problems in the development of reconstructed embryos. It was suggested that transfection of somatic donor cells decreases cloning efficiency most likely due to the detrimental effect of cell transfection (Zakhartchenko et al., 2001). Results of the present study support these suggestions because of low efficiency of NT embryos using transfected cell as donors. Manipulation of donor cells by transfection may affect their viability resulting in the retardation of growth and extended time of proliferation, and then its phenomenon also affect reprogramming of transfected donor cell in recipient cytoplasm.

In summary, this study demonstrates that embryonic developmental potency was decreased in NT embryos derived from adult cell (cumulus cell and ear fibroblast) transfected with a gene of interest and selected *in vitro*.

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