



Optimization of Procedure for Efficient Gene Transfer into Porcine Somatic Cells with Lipofection

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ABSTRACT : The objective of this study was to establish conditions for transfection of a foreign gene into somatic cells using cationic lipid reagents and to evaluate the effects of transfection on *in vitro* development of somatic cell nuclear transfer (SCNT) embryos. Green fluorescent protein (GFP) gene was used as a foreign gene and a non-transfected somatic cell was utilized as a control karyoplast. Monolayers of porcine cells were established and subsequently transfected with a GFP-expressing gene (pEGFP-N1) using three types of transfection reagents (LipofectAMINE PLUS, FuGENE 6 or ExGen500). Donor cells used for SCNT included transfected fetal or adult fibroblasts and oviduct epithelial cells, either serum-fed or serum-starved. Oocytes matured *in vitro* for 42 h were reconstructed with either transfected or non-transfected porcine somatic cells by electric fusion and activation using a single DC pulse of 1.8 kV/cm for 30 μ s in Ca^{2+} and Mg^{2+} -containing 0.26 M mannitol solution. Reconstructed oocytes were subsequently cultured in NCSU-23 medium for 168 h and the developmental competence and cell number in blastocyst were compared. There were no significant differences ($P>0.05$) in fusion, cleavage rates or development to the blastocyst stage between non-transfected, transfected, serum-fed and serum-starved cells. However, the rates of GFP-expressing blastocysts were higher in the FuGENE 6 group (71.4%) among transfection reagents and in the fetal fibroblasts group (70.4%) for donor cells. These results indicate that fetal fibroblasts transfected with FuGENE 6 can be used as donor cells for porcine SCNT and that GFP gene can be safely used as a marker of foreign genes in porcine transgenesis. (**Key Words :** Lipofection, Porcine, Developmental Competence, Somatic Cell Nuclear Transfer (SCNT))

INTRODUCTION

Transgenic technology in livestock has enormous value in the field of biotechnology and medicine including the production of bioreactors for therapeutic proteins (Gordon et al., 1980; Wall et al., 1996; Gordon, 1989; Jaenisch, 1988). Genetic modification of the genome without conventional cross breeding of livestock is now possible by gene transfer. The objective of gene transfer is to produce an animal having stable incorporation of foreign DNA into the germ line which will be able to act as founder stock to produce many offspring carrying a desirable gene. The desired gene can be transferred into the animal by various methods including retrovirus infection (Chan et al., 2001) microinjection of foreign DNA into the pronuclei of the zygote (Onishi et al., 2000), sperm-mediated DNA

transfer (Lavitrano et al., 1989) during IVF and nuclear transfer of ES, EG and somatic cells transfected with a foreign gene (Wilmot et al., 1998; Wakayama et al., 1999; Lee et al., 2005; Hwang et al., 2006). The method of microinjecting foreign DNA into the pronuclei has been considered as a practical means for the production of transgenic livestock, although the achievement of transgenesis is less than 1% in livestock (Wall, 1996). Because the timing and site of gene integration were not stable, most of the transgenic animals showed mosaicism (Wall, 1996; Chan et al., 1999). These problems, however, could be solved by somatic cell nuclear transfer (SCNT), because it allows evaluation of the expression of the transgene in somatic cells *in vitro* before SCNT. The recent successes for animal cloning by SCNT in sheep (Schnieke et al., 1997; Wilmot et al., 1997), mice (Wakayama et al., 1998; Wakayama and Yanagimachi, 1999), cattle (Cibeli et al., 1998; Kato et al., 1998; Arat et al., 2001; Arat et al., 2002; Suzuki et al., 2004), goat (Baguish et al., 1999; Keeper et al., 2001; Park et al., 2007) and pig (Betthausen et al., 2000; Lai et al., 2002; Hyun et al., 2003) provide the possibility that transfection of donor cells may provide a

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powerful tool for incorporation of a desired gene in cloned animals. Desired genes can be transferred into the somatic cell by virus infection carrying a viral vector, electroporation and lipid (liposome) or non-lipid (polymer) reagents. Among these methods, liposomes and polymers have been thoroughly used in transfection experiments for cultured cells and *in vivo* organs (lung, liver, tumor cells) (Ledley, 1995; Scheule and Cheng, 1996) and sperm cells (Bachiller et al., 1991; Jeoung and Griswold, 2007). Benefits of cationic liposome- and polymer-mediated transfection methods include high transfection efficiency, no cytotoxicity and stable gene integration in many primary cell types (Petters and Wells, 1993).

In this study, we examined the transfection efficiency of somatic cells for SCNT among three different compositions of commercial sources of transfection reagents such as polycationic lipid (LipofectAMINE PLUS), nonliposomal proprietary lipid (FuGENE 6) and cationic polymer (ExGen500). Green fluorescent protein (GFP) gene was used as a marker gene, and porcine fetal or adult fibroblast and oviduct cells were used as donor cells.

MATERIALS AND METHODS

Preparation of donor cells

Fibroblasts were isolated from fetuses at Day 30 of gestation from a local slaughterhouse. Porcine fetus was recovered aseptically from the gravid uterus and washed three times with Ca^{2+} - and Mg^{2+} - free Dulbecco's PBS (D-PBS, Life Technologies, Rockville, MD). The head and internal organs were surgically removed and the remaining tissues were washed three times with D-PBS. After washing, the tissues were minced using a surgical blade on a 100 mm culture dish (Becton Dickinson, Lincoln Park, NJ). Adult fibroblasts were established from ear skin of a high-performance Duroc boar. The external surface of ear was shaved and cleaned aseptically. A piece of ear skin tissue about 100 mm² wide and 1-mm thick was biopsied, washed in D-PBS supplemented with 5% antibiotics and minced with a surgical blade. The minced fetal or adult tissues were dissociated with 0.25% (w/v) trypsin (Life Technologies) and 1 mM EDTA (Life Technologies.) for 1.5 h at 38°C. Trypsinized cells were washed once by centrifugation (300×g, 2 min) and subsequently seeded into 100 mm plastic culture dishes and cultured for 6 to 8 days in DMEM (Life Technologies) 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.) in a humidified atmosphere of 5% CO₂, 95% air at 38°C. After removal of unattached clumps of cells or explants, attached cells were further cultured until confluent, then subcultured

at intervals of 5 to 7 days. Oviduct epithelial cells were isolated by oviduct flushing. All types of cells were frozen and stored in liquid nitrogen at -196°C after 2 passages. The freezing medium consisted of 80% (v/v) DMEM, 10% (v/v) DMSO (Sigma-Aldrich Corp.) and 10% (v/v) FBS. Prior to SCNT, frozen cells were thawed and subsequently cultured for 3 to 4 days until confluent and serum starvation cultures were subsequently conducted in DMEM supplemented with 0.5% FBS for 3 to 5 days. Individual cells were retrieved from the monolayer by trypsinization for 30 sec and subsequently used for SCNT.

Transfection of GFP gene

The plasmid, pEGFP-N1 that encodes a red-shifted variant of wild-type GFP was purchased from Clontech Laboratories, Inc (Palo Alto, CA). The pEGFP-N1 gene was transfected into fetal fibroblast cells with three different transfection reagents (LipofectAMINE PLUS, FuGENE 6 and ExGen500) according to the manufacturer's instructions to optimize transfection conditions. Adult fibroblast and oviduct cells were transfected with pEGFP-N1 under optimized conditions to determine the donor cell effects on transfection efficiency. The cells were cultured for 2 to 3 days until confluent and passaged twice to achieve stable integration of the gene into chromosomes before use for SCNT.

In vitro maturation

Ovaries were obtained from prepubertal gilts at a local abattoir and transported to the laboratory in 0.9% (w/v) NaCl solution at 30 to 35°C. Follicular fluid and cumulus-oocyte complexes (COC) from 3 to 6 mm follicles were aspirated using an 18 gauge needle attached to a 5 ml disposable syringe. Compact COCs were selected and washed three times in Hepes-buffered North Carolina State University NCSU-23 medium before being transferred to NCSU-23 medium supplemented with 10 ng/ml epidermal growth factor (Sigma), 4 IU/ml of pregnant mare chorionic gonadotropin (Intervet, Seoul, Korea), 4 IU/ml of human chorionic gonadotropin (hCG, Intervet) and 10% (v/v) porcine follicular fluid (pFF) (Carballada et al., 2000). Porcine follicular fluid was aspirated from superficial follicles from prepubertal gilts, pooled and centrifuged at 1,600×g for 30 min and filtered sequentially through 1.2 µm and 0.45 µm syringe filters (Gelman Sciences, Ann Arbor, MI). The prepared pFF was aliquoted and stored at -20°C until use. A group of 50 COCs was cultured in 500 µl of NCSU-23 medium in a 4-well dish (Nunc, Roskilde, Denmark) at 39°C in a humidified atmosphere of 5% CO₂ and 95% air. After culturing for 22 h, COCs were washed three times and then cultured in NCSU-23 medium without hormones for another 20 h. At the end of the maturation culture, the oocytes were incubated in Hepes-buffered

NCSU-23 medium containing 0.5 mg/ml hyaluronidase (Sigma) for 1 min and the cumulus cells were subsequently removed by gentle pipetting.

Somatic cell nuclear transfer

After denuding, oocytes were incubated in NCSU-23 containing 7.5 µg/ml cytochalasin B (Sigma) for 30 min at 39°C in a humidified atmosphere of 5% CO₂ and 95% air. The zona pellucidae of oocytes were partially dissected using a fine glass needle. The first polar body and adjacent cytoplasm, presumptively containing metaphase II chromosomes, were extruded by squeezing with the same needle. After enucleation, oocytes were stained with 5 µg/ml bisbenzimidazole (Hoechst 33342, Sigma) for 5 min and observed under an inverted microscope equipped with epifluorescence. Oocytes still containing DNA materials were excluded from the experiments. Trypsinized nontransfected or EGFP-transfected single cells with a smooth surface were selected under an inverted microscope equipped with a GFP filter (wavelength: exciting 489 nm and emission 508 nm) and were transferred into the perivitelline space of enucleated oocytes. Couplets were equilibrated with 0.26 M mannitol solution containing 0.5 mM HEPES, 0.1 mM CaCl₂ and 0.1 mM MgCl₂ for 4 min and transferred to a chamber consisting of two electrodes that were overlaid with fusion and activation solution. The couplets were fused and activated simultaneously with a single DC pulse of 1.8 kV/cm for 30 µsec using a BTX Electro cell Manipulator 2001 (BTX Inc., San Diego, CA). Activated oocytes were washed three times with NCSU-23 supplemented with 4 mg/ml BSA, placed in 25 µl microdrops (10 to 15 oocytes per drop) of NCSU-23 under mineral oil and cultured at 39°C in 5% CO₂, 5% O₂ and 90% N₂. The reconstructed embryos were cultured for 7 days after activation. The rates of fusion, cleavage and blastocyst formation were monitored under a stereomicroscope at 1, 48 and 168 h, respectively.

Differential staining

The quality of blastocysts was assessed by differential staining of the inner cell mass (ICM) and the trophectoderm (TE) cells according to a procedure described previously (Hardy et al., 1989). Briefly, the zona pellucidae of blastocysts on Day 7 were removed by treating with 0.25% pronase solution (Sigma) for 2 to 3 min followed by washing in NCSU-23 at least 3 times. Zona-free blastocysts were placed at 4°C for 30 min in 15 mM trinitrobenzene sulfonic acid (TNBS, Sigma) and then exposed to goat anti-dinitrophenol-BSA (ICN Biomedicals Inc., Irvine, CA) for 20 min at 39°C. After rinsing three times in NCSU-23 medium, blastocysts were incubated for 2 h at 39°C in a solution containing 0.01 mg/ml of propidium iodide (Sigma) and 15% (v/v) guinea pig complement (Sigma).

transferred into absolute alcohol containing bisbenzimidazole and stored overnight at 4°C. Blastocysts were then placed in absolute alcohol for at least 1 h, mounted on a glass slide under a coverslip and examined under an inverted microscope (Nikon Corp., Tokyo, Japan) equipped with epifluorescence. ICM cell nuclei labeled with bisbenzimidazole appeared blue and trophectoderm labeled with both bisbenzimidazole and propidium iodide appeared red under the microscope.

Fluorescence activated cell sorting

FACS analyses were carried out on a two-laser FACStar Plus platform (Becton Dickinson Biosciences, San Jose, CA). Forward scatter, side scatter, and green fluorescence were collected in the list mode. List mode data were analyzed using Cell Quest (Becton Dickinson Biosciences). Positive cells were determined as the percent of cells expressing green fluorescence above the mock control. Mean fluorescence intensity was used as an indication of the level of GFP expression by the cells. To optimize detection of GFP fluorescence by FACS, the standard fluorescent filter was replaced with a 515/40-nm interference filter. Furthermore, to allow subtraction of the autofluorescence background signal, a detector with a 630/30 bandpass filter was used for autofluorescence compensation. On the FACS, the same emission fluorescence can be collected from two different laser excitations because the laser-stream intercepts are separated and the light emitted from each laser excitation follows a separate light path. Multiparameter data were collected and analyzed by using FACS-DESK.

Experimental designs

In Experiment 1, GFP fluorescence levels were determined in porcine fetal fibroblasts, transfected with pEGFP-N1 gene by three types of transfection reagents including LipofectAMINE PLUS, FuGENE 6 and ExGen500. In Experiment 2, fetal fibroblasts were transfected with pEGFP-N1 gene by three types of transfection reagents and used for SCNT to validate the effects of transfection reagents on *in vitro* SCNT embryo development. In Experiment 3, using FuGENE 6 as a carrier, pEGFP-N1 gene was transfected into fetal or adult fibroblasts or oviduct cells. In Experiment 4, effects of serum starvation on donor cells were investigated. Fetal fibroblasts were cultured in serum-fed DMEM with 10% FBS or serum-starved DMEM with 0.5% FBS for 3 to 5 days before SCNT. The pEGFP-N1 gene was transfected into serum-fed or serum-starved cells using FuGENE 6.

Statistical analysis

SCNT embryos were randomly distributed in each experimental group and experiments were replicated at least three times. The differences in embryo development among

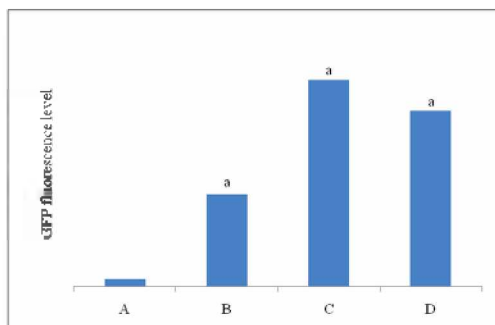


Figure 1. Comparison of the GFP fluorescence levels in porcine fetal fibroblasts transfected with LipofectAMINE PLUS® (B), FuGENE 6® (C), and ExGen500® (D). Results are given in the histogram as the relative activity of the transfected gene, following the subtraction of the values obtained from samples of the appropriate porcine fetal fibroblast-only (A). Bars with different superscripts within each transfection reagent differ significantly (a: $p < 0.05$).

experimental groups were analyzed using one-way ANOVA after arcsine transformation to maintain homogeneity of variance. *Post hoc* analyses to identify between group

differences were performed using the LSD test. The same test was used to determine the statistical significance in the cell number of blastocysts among experimental groups without arcsine transformation. All analyses were performed using SAS (SAS Institute, version 8.1). Significant difference among the treatments was determined where the p value was less than 0.05.

RESULTS

Comparison of the GFP fluorescence levels

Results are given in the histogram as the relative activity of the transfected gene, following the subtraction of the values obtained from samples of the appropriate porcine fetal fibroblast-only. GFP fluorescence intensity in transfected fetal fibroblasts determined by microplate reader and FACS is shown in Figure 1 and 2 respectively. As shown in Figure 1, the levels of GFP fluorescence in transfected fetal fibroblasts exhibited no significant differences among transfection reagents. However, transfection efficiency of GFP was significantly ($p < 0.05$)

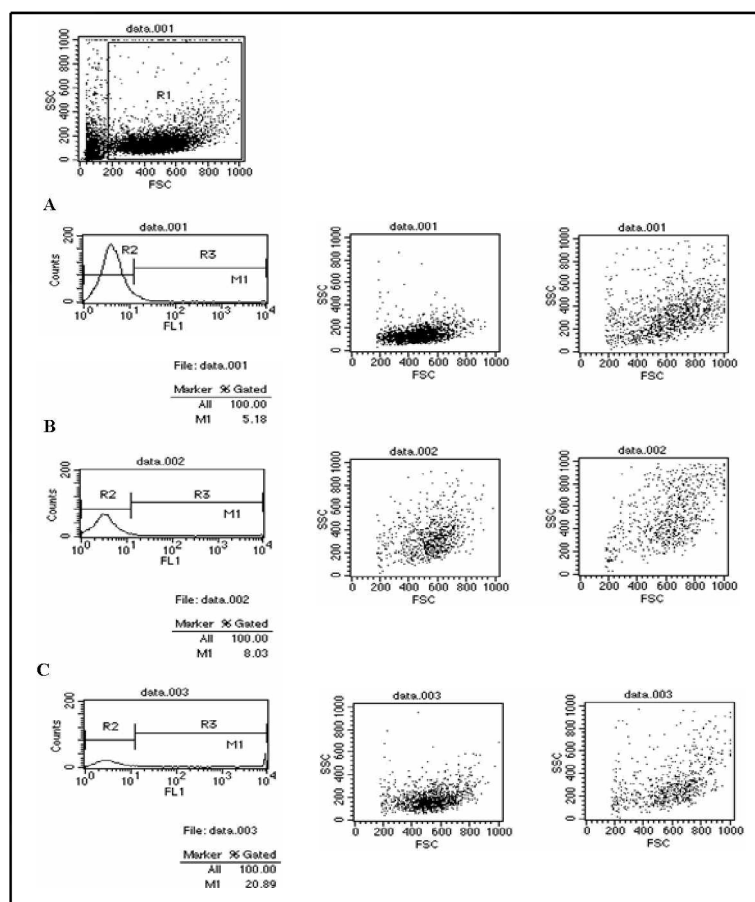


Figure 2. Comparison of the GFP fluorescence levels in porcine fetal fibroblasts. DNA was introduced into porcine fetal fibroblasts by using several commercial reagents such as LipofectAMINE PLUS® (A), ExGen500® (B) and FuGENE 6® (C). List mode data was analyzed using Cell Quest (Becton Dickinson Biosciences). Multiparameter data were collected and analyzed using FACS-DESK (Becton Dickinson Biosciences).

Table 1. Comparison of transfection efficiency among various transfection reagents

Transfection method	Final cell number ($\times 10^6$)	Transfection efficiency (%)
LipofectAMINE PLUS	4.01 \pm 1.02	7.95 \pm 3.92 ^b
FuGENE 6	3.25 \pm 1.98	26.52 \pm 7.96 ^a
ExGen500	4.33 \pm 1.30	8.84 \pm 2.94 ^b

Model effects of the treatment in each parameter, which was indicated as a p value, were 0.6994 and 0.0100 in the number of cell and transfection efficiency, respectively.

^{a,b} Within the same parameter, values with different superscripts differed significantly, $p < 0.05$.

Table 2. Comparison of development competence among various treatments

	No. (%) ^a of oocytes		No. (%) ^b of clone embryos		No. (%) ^c of GFP-expressing blastocysts	Cell number of blastocysts**			ICM:TE
	Nuclear transferred	Successfully fused	Cleaved	Developed to blastocysts		(mean \pm SE)			
						ICM	TE	Total	
Control (non-transfected)	155	108 (69.7)	75 (69.4)	14 (18.7)	0 (0)	16.3 \pm 4.8	36.7 \pm 6.1 ^{de}	53.0 \pm 9.4	0.446 \pm 0.110 ^d
Lipofect-AMINE PLUS	160	103 (64.4)	72 (69.9)	12 (16.7)	3 (25.0) ^d	9.0 \pm 4.8	29.4 \pm 10.0 ^e	38.4 \pm 14.6	0.297 \pm 0.066 ^e
FuGENE 6	145	98 (67.6)	71 (72.4)	14 (19.7)	10 (71.4) ^e	13.4 \pm 3.5	41.8 \pm 5.1 ^d	55.1 \pm 8.5	0.316 \pm 0.047 ^e
ExGen500	153	107 (69.9)	74 (69.2)	12 (16.2)	5 (41.7) ^{de}	13.0 \pm 6.5	34.1 \pm 8.1 ^{de}	47.1 \pm 14.2	0.365 \pm 0.109 ^{de}

GFP = Green fluorescence protein; ICM = Inner cell mass; TE = Trophectoderm.

^a Percentage of the number of oocytes provided for nuclear transfer. ^b Percentage of the number of oocytes successfully fused.

^c Percentage of the number developed to blastocysts. ^{d,e} Within the same parameter, values with different superscripts differed significantly, $p < 0.05$.

Model effect of the treatments on the cell number of ICM, TE and total blastomeres and the ratio of ICM to TE, which was indicated as a p value, was 0.0855, 0.0295, 0.0589 and 0.0158, respectively.

** Counted at 168 h after reconstruction.

higher from fibroblasts transfected with FuGENE 6[®] (26.52 \pm 7.96) than from those transfected with LipofectAMINE PLUS[®] (7.95 \pm 3.92) or ExGen500[®] (8.84 \pm 2.94) (Table 1).

Effect of transfection on development of embryos

The effects of transfection reagents on *in vitro* development of SCNT embryos are shown in Figure 3 and Table 2. There was no difference in rate of blastocyst formation among different transfection reagents (16.2 to 19.7%). Also, no differences in rates of blastocyst formation among nontransfected (18.7%) and transfected groups (16.2 to 19.7%) were observed. No significant differences were found in rates of fusion and cleavage among nontransfected and transfected groups or among different transfection reagents. However, higher numbers of GFP-expressing blastocysts (71.4%) and higher cell number in blastocysts (55.1 \pm 8.5) were observed in the FuGENE 6 group than in LipofectAMINE PLUS (25.0% and 38.4 \pm 14.6, respectively) or ExGen 500 groups (41.7% and 47.1 \pm 14.2, respectively) (Table 2).

Effect of donor cell types on development of porcine cloned embryos

The three different types of donor cells (fetal or adult fibroblasts and oviduct cells) were transfected with pEGFP-N1 using FuGENE 6 and embryo development was monitored after SCNT. As shown in Table 3, SCNT embryos reconstructed with fetal fibroblasts had significantly higher numbers of successfully fused embryos (72.2%) compared to adult fibroblasts (64.5%) and oviduct

cells (61.4%). Higher numbers of cleaved embryos (58.7%) and blastocysts (19.6%) in SCNT embryos reconstructed with fetal fibroblasts were observed compared to adult fibroblast (52.0 and 13.6%, respectively) and oviduct cell groups (47.4 and 6.8%, respectively). Significantly higher numbers of GFP-expressing blastocysts were found in the fetal fibroblasts (70.4%) compared to the other two cell types (0 to 64.3%). No differences in cell numbers of blastocysts among the three donor cell types were observed.

Effect of serum starvation on development of embryos

When fetal fibroblasts were used as donor cells for SCNT with/without transfection, serum starvation had no effect on embryo development and cell number in blastocysts. As shown in Table 4, no differences were observed in the rates of fusion (65.1 to 69.3%), cleavage (62.9 to 67.9%) and blastocyst formation (16.4 to 20.8%) among experimental groups regardless of transfection, serum supplementation or starvation. Also, no difference was found in the number of GFP-expressing blastocysts (71.4% versus 66.7%) between serum-fed or starved groups. As shown in Figure 4, total cell numbers of blastocysts were not significantly different among the experimental groups (51.4 \pm 10.5 to 54.0 \pm 8.3).

DISCUSSION

The purpose of gene transfer is to produce animals with a stable incorporation of foreign DNA in the germ line that will serve as founder stock to produce offspring carrying a desirable gene. Transgenic animals are of great value for

Table 3. Effects of donor cell types on the developmental competence and cell number of porcine somatic cell nuclear transfer embryos reconstructed with transfected cells with pEGFP-N1 gene using a FuGENE 6 as a carrier

Donor cell type	No. (%) ^a of oocytes		No. (%) ^b of clone embryos		No. (%) ^c of GFP-expressing	Cell number of blastocysts**			ICM:TE
	Nuclear transferred	Successfully fused	Cleaved	Developed to blastocysts		ICM	TE	Total	
Fibroblasts, fetal	320	231 (72.2) ^d	138 (58.7) ^d	27 (19.6) ^d	19 (70.4) ^d	16.3±5.2	42.3±6.3	58.7±11.1	0.380±0.085 ^{de}
Fibroblasts, adult	307	198 (64.5) ^e	103 (52.0) ^e	14 (13.6) ^{de}	9 (64.3) ^e	11.6±2.1	39.0±7.0	50.6±8.8	0.299±0.031 ^e
Oviduct cells	254	156 (61.4) ^e	74 (47.4) ^e	5 (6.8) ^e	0 (0) ^f	13.8±4.4	32.0±7.4	45.8±9.5	0.427±0.082 ^d

GFP = Green fluorescence protein; ICM = Inner cell mass; TE = Trophectoderm.

^a Percentage of the number of oocytes provided for nuclear transfer. ^b Percentage of the number of oocytes successfully fused.

^c Percentage of the number developed to blastocysts. ^{d,e,f} Within the same parameter, values with different superscripts differed significantly, $p < 0.05$.

Model effect of the treatments on the cell number of ICM, TE and total blastomeres and the ratio of ICM to TE, which was indicated as a p value, was 0.2155, 0.0526, 0.1338 and 0.0412, respectively.

** Counted at 168 hours after reconstruction.

Table 4. Effects of serum starvation on the developmental competence and cell number of porcine somatic cell nuclear transfer embryos reconstructed with non-transfected or transfected fetal fibroblasts by FuGENE 6

Donor cell treatment	No. (%) ^a of oocytes		No. (%) ^b of cloned embryos		No. (%) ^c of GFP*-expressing blastocysts	Cell number of blastocysts (mean±SE)	
	Nuclear transferred	Successfully fused	Cleaved	Developed to blastocysts			
Serum-fed	Transfected	150	103 (68.7)	69 (67.0)	14 (20.3)	10 (71.4)	55.3±11.9
	non-transfected	153	106 (69.3)	72 (67.9)	15 (20.8)	-	54.0±8.3
Serum-starved	Transfected	145	96 (66.2)	62 (64.6)	12 (19.4)	8 (66.7)	53.0±9.0
	non-transfected	149	97 (65.1)	61 (62.9)	10 (16.4)	-	51.4±10.5

* GFP = Green fluorescence protein.

^a Percentage of the number of oocytes provided for nuclear transfer. ^b Percentage of the number of oocytes successfully fused.

^c Percentage of the number developed to blastocysts. ^{d,e} Within the same parameter, values with different superscripts differed significantly, $p < 0.05$.

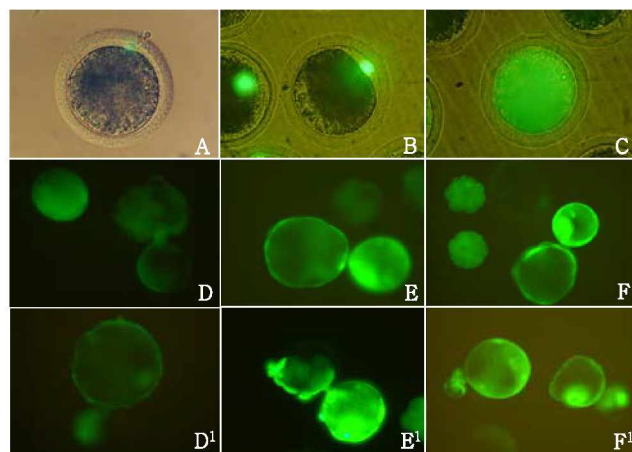


Figure 3. Expression of green fluorescence protein (GFP) in porcine transgenic SCNT embryos from fused to blastocyst stage were examined by fluorescent microscopy using a standard fluorescein isothiocyanate (FITC) filter set (excitation wavelength; 450-490 nm). (A-B) A single transfected fetal fibroblast was transfer into the perivitelline space of an enucleated oocyte (C) After fusion, GFP was detected under fluorescent microscopy (D¹) Reconstructed with fetal fibroblast and adult fibroblast transfected with LipofectAMINE PLUS[®] (E¹) Reconstructed with fetal fibroblast and adult fibroblast transfected with FuGENE 6[®] (F¹) Reconstructed with fetal fibroblast and adult fibroblast transfected with ExGen500[®], respectively.

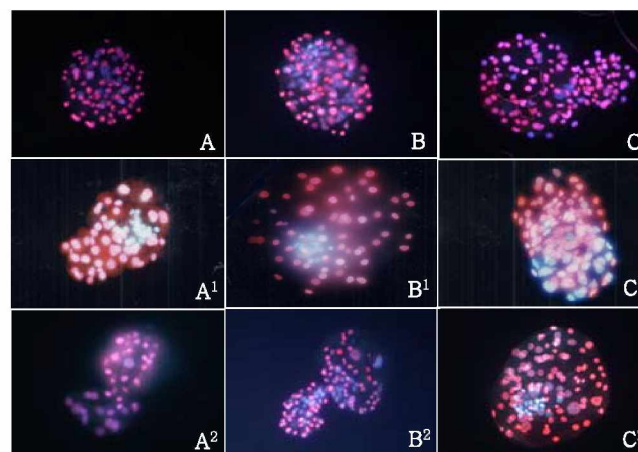


Figure 4. Differential staining of transgenic SCNT blastocysts (Day 7). Each blastocyst was derived from three different transfection reagents and cell types. (A-A²) Fetal fibroblasts transfected with LipofectAMINE PLUS[®], FuGENE 6[®], and ExGen500[®], respectively. (B-B²) Adult fibroblasts transfected with LipofectAMINE PLUS[®], FuGENE 6[®], and ExGen500[®], respectively. (C-C²) Oviduct cells transfected with LipofectAMINE PLUS[®], FuGENE 6[®], and ExGen500[®], respectively. Trophoblasts (red color) and inner cell mass (blue color) of the blastocyst stained with immunofluorescent dyes observed with an inverted microscope with epifluorescence at 400× magnification.

research and commercial purposes, and animal donors such as pigs could provide an alternative source of organs for transplantation. In this study, to establish an efficient transfection system for production of transgenic cloned pigs, different methods for the transfection and types of donor cell were evaluated. As the results demonstrated, porcine SCNT embryos reconstructed with fetal fibroblasts transfected with FuGENE 6 yield better developmental competence than other cell types and transfection reagents.

In addition, it was demonstrated that serum starvation of donor cells had no effect on the development and cell number of SCNT embryos.

A desired gene can be transferred into the somatic cell by infection with a viral vector or by electroporation with lipid (liposome) or nonlipid (polymer) reagents carrying DNA. In previous reports of transfection methods, it was suggested that retroviral vectors are capable of stably transducing up to 100% of the target cells in tissue-culture experiments (Miller et al., 1993; Schnieke et al., 1997; Cibelli et al., 1998; Uhm et al., 2000). However, replication of the target cells is necessary for efficient transduction and proviral integration into the host cell genome to occur; this feature limits the *in vivo* utility of the vectors. In addition, injected volume and titers of retroviral particles have a critical role in transgenesis (Chan et al., 2001). In previous studies, lipid-mediated transfection methods had effects on development of fish, chick and mouse embryos (Petters and Wells, 1993; Szelei et al., 1994; Rosenblum and Chen, 1995). This method could also be used to determine early embryonic development by transfection of cultured cells, *in vivo* organs (Ledley, 1995; Scheule and Cheng, 1996) and sperm cells (Bachiller et al., 1991). Carballada et al. generated transgenic mice by using a lipid mediated transfection. However, few studies of liposome-mediated transfection methods are available in porcine embryogenesis. In this study, we compared the efficiencies of different chemical-based methods for the transfection of porcine fetal fibroblasts. Biochemical transfection methods do not seem to cause detrimental effect on *in vitro* development of SCNT embryos according to our results. Transfection with FuGENE 6 into porcine fetal fibroblasts was an easy application, with high efficiency and low toxicity compared to other reagents (LipofectAMINE PLUS and ExGen 500). This result indicated that nuclear transfer using GFP gene-transfected somatic cells provides a non-invasive method for the production of transgenic porcine embryos. In agreement with our results, Jacobsen et al. (2004) demonstrated that FuGENE 6 transfection reagent had minimal to no cytotoxicity and a high level of transfection in many different cell lines.

The donor cell is one of the most important factors affecting the efficiency of the SCNT technique and reports have compared the effectiveness of different types of donor

cells for promoting embryo development after reconstruction in different species. Cho et al. (2002) reported that, using adult somatic cells in the bovine, embryos reconstructed with cumulus or ear fibroblast cells had better competence for blastocyst formation than embryos reconstructed with uterine or oviductal cells. However, overall comparison showed that adult cells of any type are inferior to fetal fibroblasts in terms of activating preimplantation development. Lee et al. (2003) demonstrated that in porcine SCNT, use of fetal fibroblasts as a donor somatic cell might be one of the best choices for improving SCNT outcome in pigs. In the present study, we demonstrated that transgenic fetal fibroblasts transfected with a GFP gene also showed higher developmental competence and GFP gene expression in blastocysts than adult fibroblasts and oviductal cells. It is suggested that fetal fibroblasts are highly undifferentiated cells (Kubota et al., 2000; McCreath et al., 2000; Polejaeva et al., 2000) compared with other cells retrieved from adult tissue. The superiority of fetal fibroblasts shown in this study and by Lee et al. (2003) suggest that undifferentiated cells are more amenable to reprogramming after reconstruction than differentiated cells. Previous reports in different species support this hypothesis (Rideout et al., 2001; Yamazaki et al., 2001).

In this study, we compared the developmental competence of SCNT embryos reconstructed with serum-fed and serum-starved donor cells with or without transfection of a GFP gene. There was no significant difference in the number of fused, cleaved or developed blastocysts, GFP-expressing blastocysts and cell number of blastocysts regardless of transfection of a GFP gene. Wilmut et al. (1997) demonstrated that mammary cells at G0 stage helped in the reprogramming of NT embryos, whereas Cibelli et al. (1998) showed that cycling cells could be used for bovine SCNT. The present study showed that most of the cells in G2/M cycle stage can be reprogrammed in enucleated *in vitro* matured oocytes and probably produce viable cloned piglets with normal ploidy (Lai et al., 2001). The synchronization of donor cells may not be necessary in either transfected or non-transfected cell lines for the SCNT procedure, according to our study.

GFP was detected in SCNT embryos from 1-2 h post-activation to blastocyst stage. After the insertion of the transfected donor cells into cytoplasts, GFP protein is supposedly dispersed into the cytoplasm of oocytes and GFP is produced in the cytoplasm as well (Petters and Wells, 1993; Kato et al., 1999; Chafie, 1994; Park et al., 2001). Since the percentage of blastocysts expressing GFP was lower than that of retroviral vector-mediated transfection, we performed detection of GFP gene in non GFP-expressed along with GFP-expressing SCNT blastocysts by RT-PCR amplification, followed by Southern blot analysis. The

results of PCR assay showed that GFP-expressing as well as GFP non-expressing blastocysts had integration of GFP gene. Furthermore, GFP expression level in blastocysts was highly varied. This fact implies that the GFP protein was not expressed in an epigenetic mechanism such as methylation or transcriptional regulation. Expression level was affected by promoter and enhancer regions. Therefore, the stable expression vector and higher and more consistent level of gene expression could be designed to produce the transgenic animals.

In summary, the use of nonliposomal proprietary lipid (FuGENE 6) as a carrier to introduce foreign DNA into porcine fetal fibroblasts could be a useful method in studies of transgenic embryonic development and generating transgenic animals.

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