Analysis of Transgene Intergration Efficiency into Porcine Fetal Fibroblast using Different Transfection Methods

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ABSTRACT

Animals produced by somatic cell nuclear transfer (SCNT) using genetically modified cells are almost always transgenic, implying that this method is more efficient than the traditional pronuclear microinjection method. Most somatic cells for SCNT in animals are fetus-derived primary cells and successful gene integration in somatic cells will depend on transfection condition. The objective of this study is to evaluate the efficiency of electroporation (Microporator) and liposome reagents (F-6, F-HD, W-EX, W-Q, W-M) for tissue-type plasminogen activator (tPA) gene transfection and to estimate the overall efficiency of transfection of Korean native pig fetal fibroblast cells (KNPFF). Electroporation showed significantly higher transfection efficiency than liposome reagents with regard to the transfection of *in vitro* cultures in the early stages of development (41.7% with Microporator vs. 18.3% with F-6, 20.0% with F-HD 18.5% with W-EX, 5.0% with W-M and 6.3% W-Q,). Colonies identified as tPA-positives were treated once more with G418 for 10 to 14 days and growing colonies were selected again. When the cells of newly selected colonies were subjected to single-cell PCR, reselection of colonies following second round of G418 selection increased the rate of transgene integration per each colony. These results suggest that transfection with electroporation is the most efficient and the second rounds of G418 selection may be an effective method for transfection of porcine fetal fibroblast cells.

(Key words : Pig fetal fibroblast cells, Transfection, Electropotration, Liposome reagents)

INTRODUCTION

Since long, pronuclear DNA microinjection has been the most reliable method of producing transgenic animals. However, this method has low efficiency with regard to the production of transgenic livestock animals. In 1997, the successful transfer of the nuclei of somatic cells into enucleated oocytes resulted in the birth of the first cloned animal, a sheep (Wilmut *et al.*, 1997). Since then, scientists have cloned cattle, goats, mice, rabbits, and pigs using somatic cell nuclear transfer (SCNT) (Cibeli *et al.*, 1998; Baguisi *et al.*, 1999; Wakayma *et al.*, 1998; Chesne *et al.*, 2002; Betthauser *et al.*, 2000). Transgenic animals can also be generated by SCNT with genetically modified somatic cells. Cloned animals produced by SCNT.

SCNT with genetically modified cells are almost always transgenic, indicating that this method is much more efficient than traditional pronuclear microinjection. (Park *et al.*, 2001; Lai *et al.*, 2002; Bordignon, 2003). Transgenic goats, cattle, and pigs have been generated by SCNT with DNA-transfected fetal fibroblasts (Keefer *et al.*, 2001; Zakhartchenko *et al.*, 2001; Park *et al.*, 2001). Gene-targeted pigs and sheep have also been produced using SCNT (Lai *et al.*, 2002; Dai *et al.*, 2002; McCreath *et al.*, 2000; Denning *et al.*, 2001). Transgenic animals could be used as bioreactors for producing therapeutic proteins. Such animals produce valuable pharmaceutical proteins or internal human organs. Gene targeting is an integral tool for genetic manipulation, requiring procedures nearly identical to those used for transfection. Gene targeting is the process in which a cell's recombination machinery integrates a piece of foreign DNA into its own genome at a target locus homologous to the foreign donor. Gene targeting also can be used to produce recombinant proteins by using a tissue-specific promoter (Clark, 1998).

The transfer of recombinant genes into various eukaryotic cells is known as transfection. Several strategies have developed for DNA delivery into mammalian cells in order to achieve gene expression. The strategies include physical (gene gun and electroporation), biological (virus-based vectors), and chemical (cationic lipids, liposomes, and dendrimers) methods (Fromm *et al.*, 1984; Guenzburg and Salmons, 1997; Fraley *et al.*, 1980)

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Tissue-type plasminogen activator (tPA) is synthesized by vascular endothelial cells and secreted into the bloodstream (Rijken *et al.*, 1980). tPA is involved in plasmin generation, a physiological proteolytic process that causes localized and directed proteolysis in tissues and cells (Laiho and Keski-Oja, 1989). tPA has a kringle modular structure for protein binding and a carboxyl-terminal region. tPA is also a serine protease that converts zymogen and plasminogen into plasmin. tPA has wide-ranging specificity for degradation of the fibrin network of the thrombus and it can promote transformation *in vitro* (Patthy, 1985; Laiho and Keski-Oja, 1989).

The objective of this study is to compare the efficiency of electroporation and liposome reagents for transfecting Korean native pig fetal fibroblast (KNPFF) cells with the human tPA gene. PCR analysis was used to detect the transgene in the cells.

MATERIALS AND METHODS

Preparation of Porcine Fetal Fibroblast Cells

Korean native pig fetal fibroblast cells (PFF) were isolated from day 30 fetuses. After removal of the head and viscera, the fetuses were washed twice with phosphate buffered saline (PBS; pH 7.2). The red and brain tissues of each fetus were removed and minced, treated with trypsin (0.25%), and shaken for 30 min at 37℃. Each supernatant was poured into a 50 ml centrifuge tube, mixed with an equal volume of Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), and put on ice. The supernatant was aspirated off, and the cells were resuspended in DMEM. The cells were plated to culture dishes and cultured at 38.5℃, in a 5% CO_2 incubator. The cells were cryopreserved after they reached confluence. A vial of frozen cells was removed from the liquid nitrogen tank and thawed in a water bath at 37℃. After thawing, the cells were seeded into 100-mm cell culture plates in Dulbecco's modified eagle medium (DMEM; Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% antibiotics (penicillin, 10,000 U/ml; streptomycin, 10,000 μg/ml; Gibco BRL), and 1% glutamine (29.2 mg/ml; Gibco).

Transgenes

Human tPA cDNA was used with the β-casein promoter sequence, by ligation into pBC1 vector (Invitrogen, USA). The Neo^r DNA sequence derived from the pPNT vector (Tybulewicz *et al.*, 1991) was fused into the pBluescript II KS(+)vector. Both vectors were named pBC/tPA and pBlue/Neo^r, respectively and linearized with single-cut restriction enzymes (SalI and XhoI, respectively) before use.

Transfection with a Liposome Reagent

Three 1.5-ml microcentrifuge tubes (tubes 1, 2, and 3) were prepared. When the cells reached 50~80% confluence, 50 μl serum-free medium, 0.5 μg of PBC/tPA, and 0.5 μg of pBlue/Neo^r mix solution were added to tube 1. The tube was tapped gently to facilitate mixing. In tube 2, 50 μl of serum-free medium, 1 μl of Enhancer-Q or 1 μl Enhancer-Gold or 1 μl of Enhancer-Q solution were added. The tube was tapped gently to facilitate mixing. The contents of tube 2 were added to prediluted tube 1 and then tapped gently to facilitate mixing. The mixture was then incubated at room temperature for 15 min. In tube 3, we added 50 μl serum-free medium and 1.5 μl of Welfect-EX (W-EX) or Welfect-M (W-M) or Welfect-Q (W-Q) (Welgene, Korea). The tube was then tapped gently to facilitate mixing. The contents of tube 3 were added to prediluted tube 2 and then tapped gently to facilitate mixing. The mixture was incubated at room temperature for 15 min and poured into cells. The cells were cultured for 24 h. Transfected colonies were selected with G418 (KNPFFs; 400 μg/ml). Serum-free medium (97 μl) was added to each tube and then 3 μl of FuGENE 6 (F-6) or FuGENE HD (F-HD) (Roche, Indianapolis, IN, USA) was added The tubes were tapped gently to facilitate mixing. Next, a solution with 0.5 μg pBC/tPA and 0.5 μg pBlue/Neo^r was added to the tube with prediluted F-6, and it was gently tapped. The mixture was incubated at room temperature for 15 min and poured into cells. The cells were cultured for 24 h. Transfected colonies were selected with G418. Selected colonies were cultured in a culture medium containing 15% FBS.

Transfection by Electroporation

When the cells reached 50~80% confluence, they were aspirated and cultured in medium in a 24-well dish. Before culturing, the cells were washed with PBS buffer. The cells were passaged using 0.04% typsin-EDTA and then transferred into a 1.5-ml microcentrifuge tube. They were then centrifuged for 2 min at 12,000 rpm at room temperature. The supernatant was aspirated, and the cell pellets were resuspended in solution-R (resuspension buffer) at a final density of 1×10^5 cells/12 μl. The solution containing 0.5 μg pBC/tPA and 0.5 μg pBlue/Neo^r was added to the tube containing the prediluted complex mixture, and the tube was gently tapped. Then, 3 ml solution-E (electrolytic buffer) was added using an E-tube. The E-tube was inserted into the pipette station using a microporator pipette. Samples were stimulated with 1 or 2 DC pulses of 1,100~1,600 V for 10~40 ms using a Microporator. Then, the samples were transferred into the pre-

pared 24-well dish. The cells were cultured for 24 h. Transfected colonies were selected with G418 for 10 to 14 days. The selected colonies were cultured in a culture medium containing 15% FBS.

Detection of the Transgene

In order to rapidly detect the tPA gene in the cells, the cells were passaged using 0.04% typsin-EDTA. Each cell was transferred into a PCR tube containing 1 mg/ml proteinase K solution and incubated at 55℃ for 1 h. Proteinase K was inactivated by heating at 100℃ for 2 min. Single-cell PCR amplification was performed. Single-cell PCR was executed in a reaction volume of 30 μl, which contained 2～10 cells, 10 mM deoxy-NTP, 10 pM primers (sense and antisense primer), 3 μl 10× Reaction buffer, and 0.5 U HS Prime Taq (GENET BIO, Korea). The following primer pair was used to detect the tPA gene: forward primer, 5'-ATTTCAGCTGCAGCAGCGCAATG-3' and reverse primer, 5'-ATCGAGACTCAAAGCCCTGGT-3'.

The PCR protocol was as follows: initial denaturation at 94℃ for 5 min followed by 38 cycles of denaturation at 94℃ for 1 min, annealing at 59℃ for 1 min and extension at 72℃ for 1 min, and final elongation for 7 min at 72℃. The amplified PCR product, i.e., the tPA gene, was electrophoresed through a 2% agarose gel.

Data Analysis

The selection efficiency were subjected to the Chi-square test (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSION

Efficiency of Transfection Liposome Reagents

KNPFFs were treated with 5 transfection reagents and grouped according to the reagent used for transfection. To determine the difference in transfection efficiency, The pBC1/tPA and pBlue/Neo^r were co-transfected initially into 2×10^6 KNPFFs with F-6, F-HD, W-EX, W-M, and W-Q. Colonies were selected following exposure to 400 μg/μl G418 for 10 to 14 days. The total number of selected colonies was 70, 74, 77, 56, and 61 colonies from the F-6, F-HD, W-EX, W-M and W-Q groups, respectively. Some colonies stopped growing (Table 1). Of the 70 selected colonies transfected with F-6, 62 continued to grow, while 65 of the 74 colonies transfected with F-HD continued to proliferate following extensive selection with G418. Of the 77 colonies transfected with W-EX, 67 continued growing, and 40 of the 56 colonies transfected with W-M continued to divide. Of the 61 colonies transfected with W-Q, 50 continued to proliferate following extensive selection with G418. After DNA analysis for detection of the tPA gene, 11 of the 60 colonies that continued to grow after transfection with F-6 were found to contain the tPA gene. 12 of the 60 colonies transfected with F-HD had the tPA genes. Of the 65 colonies that continued to proliferate after transfection with W-EX, 12 had the tPA gene, and 2 and 3 of the 40 and 48 colonies that continued to grow after transfection with W-M and W-Q, respectively, had the tPA gene. Transfection efficiencies for the tPA gene with F-6, F-HD, W-EX, W-Q, and W-M were 18.3%, 20.0%, 18.5%, 5.0%, and 6.3%, respectively. High levels of transfection can be obtained with F-6, F-HD and W-EX with fewer steps than if traditional reagents and methods are used (Jacobsen *et al.*, 2004).

Electroporation

pBC1/tPA and pBlue/Neo^r were co-transfected into KNPFFs with an electrical Microporator (Seoryn Bio, Korea). KNPFF colonies were selected following exposure to G418 for 21～28 days. Of the 82 selected colonies, 75 continued to proliferate following extensive selection with G418 (Table 1). After DNA analysis for

Table 1. KNPFF colonies that continued growing after G418 selection

Transfection method	No. of colonies after selection	No. of colonies growing	Growing efficiency (%)
F-6	70	62	89.0
F-HD	74	65	87.8
W-EX	77	67	87.0
W-M	56	40	71.4
W-Q	61	50	81.9
Microporator	82	75	91.4

Table 2. Transfection efficiency among growing colonies following G418 selection

Transfection method	No. of colonies picked up	No. of colonies Transfected	Transfection efficiency (%)
F-6	60	11	18.3^a
F-HD	60	12	20.0^a
W-EX	65	12	18.5^a
W-M	40	2	5.0^b
W-Q	48	3	6.3^b
Microporator	60	25	41.7^c

$^{a-c}$ Values within columns with different superscripts are significantly different ($p<0.05$).

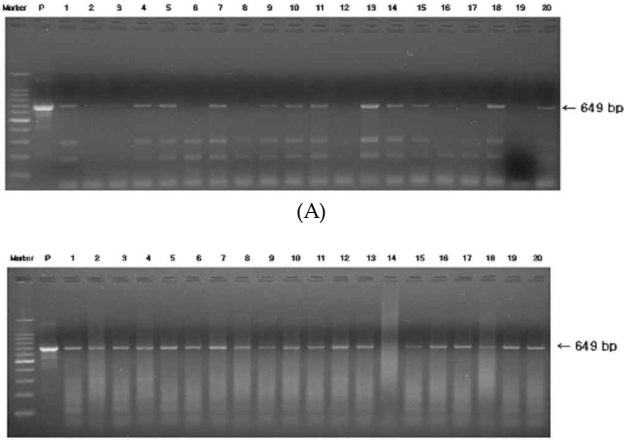

Fig. 1. Single-cell PCR amplification of the tPA gene in Korean native pig fetal fibroblasts (KNPFFs). Marker: 100-bp marker. P: positive control, Lanes 1~20: cells screened. A) Cells derived from colony selected with G418 (Table 3). B) Cells of colony selected twice with G418 (Table 4).

Table 3. Transgene integration efficiency of cells derived from colonies selected against G418

Colony #	No. of cells screened	No. of tPA-positive cells	Efficiency (%)
1	20	16	80.0
2	20	19	95.0
3	20	20	100.0
4	20	17	85.0
5	20	19	95.0
6	20	20	100.0

detection of the tPA gene, 25 of the 60 colonies that continued to proliferate were found to have the tPA gene. Thus, the transfection efficiency of the tPA gene with an electrical Microporator is 41.7%. Some of the advantages of electroporation are that it is simple and offers high transfection efficiency (Baron *et al.*, 2000).

Reselection of Transfected Colonies

To evaluate what percentages of cells among selected colonies contained tPA gene, single-cell PCR was performed using cells derived from colonies identified as tPA-positives. Single cell was isolated from growing colonies and applied to PCR analysis (Fig. 1A). As show in Table 3, transgene integration rates were variable among colonies (80% to 100%), suggesting transfected cells did not completely contained tPA gene following one time selection with G418.

Colonies identified as tPA-positives were treated with G418 for 10 to 14 days, and selected again growing colonies. And the cells of newly selected colonies were subjected to single-cell PCR (Fig. 1B). Reselection of colonies following second round of G418 selection increased the rate of transgene integration per each colony (Table 4). Our results suggested that 2 rounds of G418 treatment may be an effective method for selecting transfected cells. Treatment of the transfected somatic cells with G418 twice did not affect the colonies or enhance degradation. Single-cell PCR is an efficient method for detect tranfection in a single fetal fibroblast cell or a blastomere of embryos to check if they have the transgene (Hahn *et al.*, 1999).

Table 4. Transgene integration efficiency of cells derived from reselected colonies with G418

Colony #	No. of cells screened	No. of tPA-positive cells	Efficiency (%)
1	20	19	95.0
2	20	20	100.0
3	20	20	100.0
4	20	20	100.0
5	20	18	90.0
6	20	20	100.0

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