Gene Transfer into Pig and Goat Fetal Fibroblasts by Co-transfection of tPA Transgene and Neo^r Gene

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ABSTRACT

The transfection efficiency of a transgene into pig and goat fetal fibroblast cells (PFF and GFF, respectively) was tested using co-transfection of a human tissue-type plasminogen activator (tPA) transgene and neomycin-resistant (Neo^r) gene, followed by G418 selection. To initially test G418 resistance, GFF and PFF were incubated in culture medium containing different concentration of G418 for 2 weeks, and cell survival was monitored over time. Based on the obtained results, the concentrations chosen for G418 selection were 800 ug/ml and 200 ug/ml for GFF and PFF, respectively. For co-transfection experiments, the pBC1/tPA and Neo^r vectors were co-transfected into GFF and PFF (1×10^6 cells in each case) using the FuGENE6 transfection reagent, and resistant colonies were obtained following 14 days of G418 selection. We obtained 96 and 93 drug-resistant colonies of GFF and PFF, respectively, only 54 and 39 of which, respectively, continued proliferating after drug selection. PCR-based screening revealed that 23 out of 54 analyzed GFF colonies and 5 out of 39 analyzed PFF colonies contained insertion of the tPA gene. Thus, the experimentally determined transfection efficiencies for tPA gene co-transfection with the Neo^r gene were 42.6% for GFF and 12.8% for PFF. These findings suggest that co-transfection of a transgene with the Neo^r gene can aid in the successful integration of the transgene into fetal fibroblast cells.

(Key words : Co-transfection, tPA gene, Neo^r gene, Goat fetal fibroblasts, Pig fetal fibroblasts)

INTRODUCTION

Transgenic animals can be generated by nuclear transfer (NT) with genetically modified somatic cells. Briefly, animal somatic cells are transfected with DNA vectors containing a transgene plus a drug resistance gene that is used for drug selection *in vitro*. The transgene-containing cells may then be used for NT into enucleated oocytes, which are transferred into the oviducts of recipient females, where they grow to produce cloned transgenic animals (Schnieke *et al*., 1997; Cibelli *et al*., 1998; Park *et al*., 2001; Zakhartchenko *et al*., 2001). Animals produced by NT with genetically modified cells were almost transgenic, which greatly improved upon the traditional inefficiency of pronuclear microinjection (McCreath *et al*., 2000; Keefer *et al*., 2001; Park *et al*., 2001; Bordignon *et al*., 2003). Gene targeting, which is a powerful tool for genetic manipulation in animals that has been successfully performed in cloned pigs and sheep (McCreath *et al*., 2000; Lai *et al*., 2002; Dai *et al*., 2002; Denning *et al*., 2001), requires essentially the same procedure as NT, i.e. the transfection of DNA into somatic cells followed by drug selection. In particular, G418 selection is commonly used for gene targeting in mammalian primary cells, including knockouts designed to nullify a particular locus without random integration of the transgene at other loci.

Most somatic cells used for NT in animals are fetus-derived primary cells, which differ from stem cells in terms of culture conditions, strength of passing, and drug resistance. One of the key steps in gene transfer to somatic cells is the choice of a proper system for selecting cells in which the transgene has been properly integrated into the chromosomal DNA. However, the use of drug selection in gene transfer procedures often causes primary cells to grow slowly or stop proliferating, resulting in a low transfection efficiency (Han *et al*., 1993; Hwang *et al*., 2006). Single-drug selection with G418, resistance to which is conferred by the neomycin phosphotransferase (Neo^r) gene, is commonly used during gene transfer into somatic cells. For produc-

* This work was supported by grant #20070401034031 from the BioOrgan Program, grant #20070101034010 from the BioGreen 21 Program of the Rural Development Administration, Republic of Korea, and grant #R11-2002-100-05001-0 from the ERC program of the Korean Science & Engineering Foundation.

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tion of transgenic animal by NT, the transgene vector should include both the transgene (plus its promoter) and the drug resistance gene in the same plasmid vector, in order to achieve enrichment of transgene-inserted cells. However, construction of such NT vectors can be difficult, especially when restriction enzyme sites for the transgene compete with those of the drug resistance gene. During co-transfection, cells are transfected with two separate DNA vectors, such as a transgene vector and a drug resistance vector. Both DNA vectors enter the cell and are integrated into the cellular genome, and drug resistance can be used for the selection of cells harboring proper transgene integration. Studies in stable cell lines have shown that co-transfection facilitates the simultaneous integration of both the target and drug resistance genes (Chesnut *et al*., 1996; Muff *et al*., 1999; Brophy *et al*., 2003; Sugita, 2004; Yeh and Lee, 2006). For NT-mediated gene transfer, co-transfection methods can avoid the necessity of constructing complex single vectors harboring both the transgene and the drug resistance gene.

The objective of this study was to estimate the overall efficiency of co-transfecting vectors encoding human tissue-type plasminogen activator (tPA) and Neo^r into goat and pig fetal fibroblast cells.

MATERIALS AND METHODS

Construction of pBC1/tPA and pBlue/Neo^r

To construct a vector in which the human tPA cDNA was fused to the β-casein promoter sequence, the tPA cDNA was PCR cloned from a lung cDNA library and ligated into the XhoI site of the pBC1 vector (Invitrogen, USA). The Neo^r DNA sequence was derived from the pPNT knock-out vector (Tybulewicz *et al*., 1991) and fused into the XhoI and EcoRI sites of the pBluescriptⅡKS(+)vector. For ligation, the relevant DNA fragments were dephosphorylated with 20 units of calf-intestinal alkaline phosphatase (Takara, Japan) at 37 ℃ for 5 hrs, and then ligated at room temperature for 5 minutes in ligation buffer (Takara) at a molar ratio of 1 to 3 (vector:DNA; 20 ul total volume). The ligated DNA fragments were transformed into *E. coli* HB101, and the correct recombinant vectors were recovered and named pBC/tPA and pBlue/Neo^r, respectively. Both vectors were linearized with single-cut restriction enzymes (SalI and XhoI, respectively) before use.

Preparation of Goat and Pig Fetal Fibroblasts

Goat fetal fibroblast cells (GFF) and pig fetal fibroblast cells (PFF) were isolated from day 30 and 50 fetuses recovered from a Saanen goat and a Landrace pig, respectively. 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. This procedure was repeated once more, and the isolated cells were pelleted by centrifugation at 500 g for 10 min. The supernatant was aspirated off, and the cells were resuspended in DMEM. The cells were plated to culture dishes and cultured at 37℃, in a 5% CO_2 incubator. The cells that divided into a monolayer were considered to be P_0, and cells of the following passage were designated P_1, and so forth. P_2- or P_3-stage cells were used for the transfection experiments. The cells were cryopreserved in liquid nitrogen using freezing medium (11% DMSO, 50% FBS, 39% DMEM) until use.

To test the GFF and PFF for neomycin, cells were exposed to culture medium containing different concentrations (200, 400, 600, 800 and 1,000 ug/ml) of G418 (Gibco, USA) for 2 weeks, and cell survival was monitored.

Co-transfection

When the cells reached 70 to 80% confluence, transfections were performed using a solution containing 97 ul of serum-free DMEM, 3 ul of FuGENE 6 (Roche, USA), 0.5 ug of pBC1/tPA and 0.5 ug of pBlue/Neo^r. The mixture was incubated at room temperature for 15 minutes and then added to the cell cultures. The transfected cells were cultured for 24 hours with no selection, and were thereafter selected with G418 (GFF, 800 ug/ml; PFF, 200 ug/ml) for 10 to 14 days. Selected colonies were cultured in medium containing 10% FBS.

PCR-based Transgene Detection

To determine integration of the tPA transgene, genomic DNA was extracted from selected GFF and PFF, and PCR amplification was performed in a 50 ul reaction volume containing 100 ng/ul of DNA, 2 mM deoxyNTP, 5 ul 10X EX Taq buffer, 1 unit EX Taq polymerase (Takara, Japan) and 10 pM primers (forward: 5'-ATTTCAGCTGCAGCAGCGCAATG-3'; reverse: 5'-ATCGAGACTCAAAGCCCTGGT-3'; yielding a predicted product of 649-bp). The cycling conditions consisted of denaturation at 94℃ for 5 minutes, followed by 35 cycles of 94℃ for 1 minute, 56℃ for 1 minute, and 72℃ for 1 minute, with a final elongation step of 7 minutes at 72℃. The amplified PCR products were electrophoresed in a 2% agarose gel.

RESULTS

The Recombinant pBC1/tPA and pBlue/Neo^r Vectors

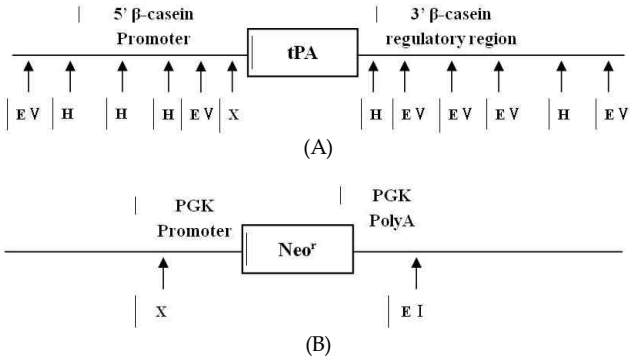

Fig. 1. Schematic representation of transgene constructs and restriction enzyme sites. A) The pBC1/tPA vector. The tPA cDNA was inserted into the pBC1 vector. B) The pBlue/Neo^r vector. The Neo^r gene fragment was isolated from the pPNT vector and ligated into the pBluescriptⅡKS(+) vector (H: HindⅢ, X: Xho Ⅰ, E Ⅰ: EcoR Ⅰ, E Ⅴ: EcoR Ⅴ).

The pBC1 vector and human tPA cDNAs were ligated and selected by digestion with restriction enzymes (Fig. 1A). When the pBC1 vector was digested with EcoR Ⅴ, six fragments (10.5, 5, 2.7, 2.3, 0.96 and; 0.2 kb) were produced. When the pBC1 vector was digested with HindⅢ, five fragments (8.15, 4.5, 4.4, 3.3 and 1.2 kb) appeared. Double digestion with Xho Ⅰ and Sal Ⅰ was used to confirm the correct orientation of the inserted tPA gene. The expected 14.7-kb and 8.6-kb fragments were obtained following double-digestion with Xho Ⅰ and Sal Ⅰ, confirming the correct orientation of the pBC1/tPA vector.

The Neo^r gene was derived from the pPNT vector, which contains the PGK promoter, the Neo^r gene and the PGK poly(A) signal. To clone the Neo^r gene into the pBluescript vector, pBluescriptⅡKS(+) and the Neo^r gene were ligated at the Xho Ⅰ and EcoR Ⅰ restriction sites. Proper construction of the pBlue/Neo^r vector was confirmed by double-digestion with EcoR Ⅰ and Xho Ⅰ, which produced the expected 2.9-kb and 2.1-kb fragments (Fig. 1B).

Estimation of G418 Resistance for Goat and Pig Fetal Fibroblast Cells

Intact GFF and PFF were first tested for neomycin resistance. Cells were exposed to culture medium containing different concentration of G418 (200, 400, 600, 800 and 1,000 ug/ml) for 2 weeks, and cell survival over time was monitored (Fig. 2). GFF cells were relatively resistant to G418, tolerating up to 600 ug/ml G-418. In contrast, PFF cells were sensitive to 200 ug/ml of G418, beginning to die within 1 week. Therefore, the optimum concentrations of G418 for the selection of cells expressing Neo^r gene were estimated to be 800 ug/ml and 200 ug/ml for GFF and PFF, respectively.

Efficiency of DNA Co-transfection into Goat and Pig Fetal Fibroblast Cells

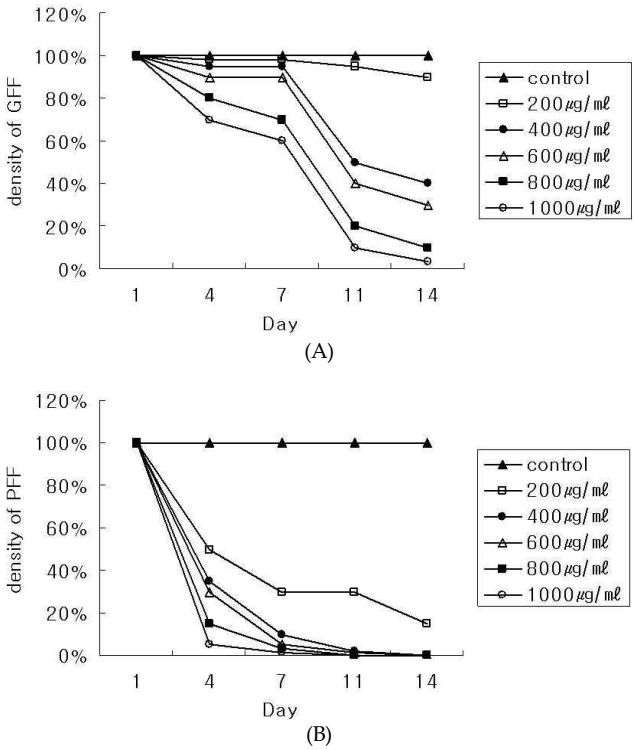

Fig. 2. The neomycin (G418) resistance of intact goat fetal fibroblast cells (A) and pig fetal fibroblast cells (B).

To determine the efficiency of co-transfection of the test vectors into GFF and PFF, 1×10^6 cells were co-transfected with the constructed tPA- and Neo^r-encoding DNA vectors, and the cells were cultured in medium containing G418 for 10 to 14 days, in order to select for cells carrying the Neo^r gene. Resistant colonies were counted and transferred to 24-well plates for expansion, and the transfected cell clones were PCR screened for the presence of the tPA gene (Fig. 3). The results of the co-transfection experiments are summarized in Table 1.

Co-transfection of 1×10^6 cells yielded approximately 96 and 93 drug-resistant colonies of GFF and PFF. However, only 54 of 96 selected GFF colonies and 39 of 93 selected PFF colonies continued to proliferate following G418 selection. PCR-based screening of these G418-resistant GFF and PFF colonies revealed that 23 out of 54 GFF colonies and 5 out of 39 PFF colonies were positive for the tPA gene. These results indicate that the transfection efficiencies for the tPA gene co-transfected with the Neo^r gene were 42.6% and 12.8% for GFF and PFF, respectively.

DISCUSSION

The experiments presented here demonstrated the

Table 1. Transfection efficiency of GFF and PFF co-transfected with tPA and Neo^r vectors followed by G418 selection.

Cell	No. cells treated	No. colonies selected	No. colonies proliferating (%)	No. colonies transfected	Transfection efficiency (%)
GFF	1,000,000	96	54 (56.3)	23	42.6
PFF	1,000,000	93	39 (41.9)	5	12.8

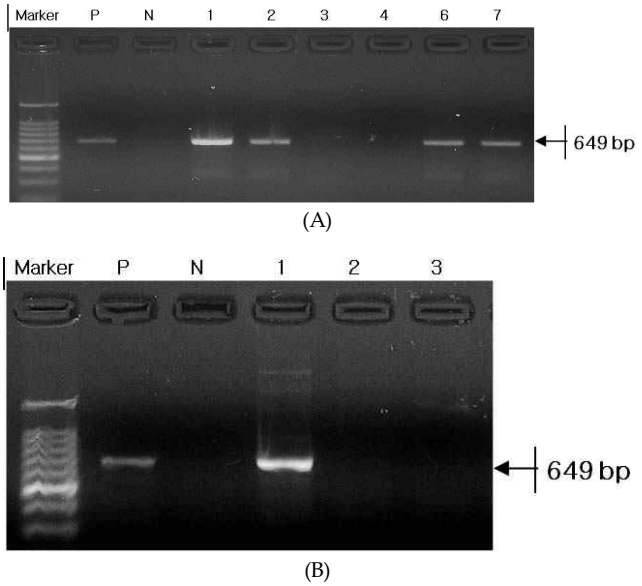

Fig. 3. PCR amplification of tPA sequences from co-transfected GFF (A) and PFF (B) (Marker: 100 bp marker, P: positive control, N: negative control).

novel use of a co-transfection system for transgene insertion into animal primary somatic cells. The production of a transgenic animal by NT requires integration of the transgene into a somatic cell that will be used as the nuclear donor; this aim can be achieved using an NT vector containing both the transgene and a drug resistance gene that can be used to select for successfully transfected cells. However, when the transgene is very long or includes complex regulatory sequences, it may be difficult to construct a single NT vector that fulfills both needs. In this case, co-transfection with two separate vectors, one containing the transgene and the other harboring the drug resistance gene, would seem to be a possible alternative for NT- mediated transgenic animal production.

Co-transfection has been previously used to transfer reporter and drug resistance genes into stable cell lines (Chesnut *et al*., 1996; Muff *et al*., 1999; Sarkar and Das, 2003). For example, human breast carcinoma cells and human kidney 293 cells were co-electroporated with pHook-1 and pCR3lacZ (a reporter gene directing expression of β-galactosidase), yielding selected cells that stained 95% positive for β-galactosidase activity (Chesnut *et al*., 1996). Similarly, rabbit aortic endothelial cells were successfully co-transfected with mammalian expression vectors encoding HAMP (human receptor activity modifying protein)-1, -2 and -3 together with the hCTR2 gene (Muff *et al*., 1999). The tetracycline-inducible system is a widely used co-transfection system, in which a plasmid expressing the tetracycline repressor is transfected into cells along with a vector containing a tetracycline response element with a minimal promoter directing expression of the target gene (Sarkar and Das, 2003).

Gene insertion into cells is an *in vitro* process that requires intensive drug-selection and numerous cell passages. However, primary cells (e.g. fetal fibroblasts) are very sensitive to drug exposure, which can cause the cells to senesce. It has been reported that many colonies ceased proliferation following drug selection during gene targeting procedures (Denning *et al*., 2001). In our experiment, about half of the colonies selected with G418 showed cessation of cell division following selection (Table 1). In the future, the overall efficiency of gene transfection into primary somatic cells could be increased by efforts to avoid drug-induced senescence. In the present work, we first determined the optimum concentration of G418 for selection of fetal fibroblast cells following transfection with a vector encoding the Neo^r gene. Intact GFF and PFF were found to have different sensitivities to G418, with GFF being relatively resistant to G418 compared with PFF (Fig. 2). The concentrations of G418 chosen for selection were 800 ug/ml and 200 ug/ml for GFF and PFF, respectively. Previously reported transfection experiments with PFF used 100~200 ug/ml of G418 (Park *et al*., 2001; Lai *et al*., 2002; Dai *et al*., 2002), which is similar to the dosage used in our experiment. In the case of GFF, however, previous studies used 400~600 ug/ml of G418 (Baguisi *et al*., 1999; Wang *et al*., 2008), which is lower than the optimum level determined herein. Sub-optimal drug selection following transfection of a transgene vector into primary cells may result in lower transfection efficiency. The frequency of successful transfection in somatic cells will also depend on the transfection conditions and the amount of vector DNA. Proper adjustment of these conditions will be required to support efficient gene transfection into somatic cells.

In conclusion, our results indicate that co-transfection of a transgene with a vector encoding the Neo^r gene can be an efficient method for inserting target genes into fetal fibroblast cells.

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(Received: 15 June 2009/ Accepted: 19 June 2009)