Targeting efficiency of α-1,3-galactosyl transferase gene in pig fetal fibroblast cells

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Abstract

Animal cloning technology with somatic cells provides an alternative tool to conventional methods for producing transgenic animals. Gene targeting in animals is made feasible using somatic cells with homologous recombination procedure that is a major technique in embryonic stem cells for knocking-out genes. Homologous recombination events in somatic cells are relatively inefficient as compared to those in ES cells, suggesting the need for establishment of efficient gene targeting system in somatic cells. To investigate the efficiency of positive and negative selection for gene targeting in pig fetal fibroblast cells, pig α-1,3-galactosyl transferase (13-GT) gene was used for gene targeting. The neomycin phosphotransferase (Neo^r) and herpes simplex virus-thymidine kinase (HSV-tk) genes were used as positive and negative selection markers in this experiment. Following transfection with targeting DNA construct, the pig fetal fibroblast cells were selected against resistance of G418 and gancyclovir. In DMEM medium containing 5 to 10% serum, Pig fetal fibroblast cells failed to proliferate during drug selection. Increasing serum concentration to 15% of medium yielded less senescent colonies of pig fetal fibroblast cells following drug selection that allowed enough cell colonies to screen genomic DNA. The frequency of gene targeting in pig fetal fibroblast cells with double drug selection was more than 10-fold efficient compared to that with G418 single selection. Double selection method with Neo^r and HSV-tk genes could be useful for gene targeting in somatic cells for production of cloned animals carrying targeted endogenous genes.

Keywords: animal cloning; galactosyltransferases; gene targeting; swine

Introduction

Recent development of animal cloning technology with somatic cells provides an alternative tool to conventional methods for modifying genes in animal (Wilmut et al., 1997). DNA-inserted somatic cells can be selected in dishes and then nuclear transfer can be done with those cells (Schnieke et al., 1997; Cibelli et al., 1998; Zakhartchenko et al., 2001). Gene targeting in animals is feasible using somatic cells with homologous recombination procedure that has been a major technique in embryonic stem cells for knocking-out genes. Nuclear transfer of somatic cells in pigs is the most promising procedure to achieve knockout of α-1,3-galactosyl transferase (13-GT) gene for pig-to-human xenotransplatation (Betthanser et al., 2000; Onishi, et al., 2000; Polejaeva, et al., 2000; Harrison et al., 2002). Production of viable pig and sheep with targeted insertion at several gene loci following nuclear transfer with gene-targeted cells has been reported (McCreath et al., 2000; Denning et al., 2001; Dai et al., 2002; Lai et al., 2002; Phelps et al., 2003; Ramsoondar et al., 2003).

Most somatic cells for nuclear transfer in animals are fetus-derived primary cells that are different from stem cells in terms of culture condition, strength of passing or drug selection. For gene targeting, extensive drug selection is required that makes primary cells to stop proliferation or to grow slow. One of the key steps in gene targeting is the selection system to enrich for somatic cells in which correct homologous recombination has occurred. The single selection with neomycin phosphotransferase (Neo^r) gene

has been a popular method in gene targeting of somatic cells probably due to weakness of proliferation ability following drug selection of primary somatic cells (McCreath *et al.*, 2000; Denning *et al.*, 2001; Dai *et al.*, 2002; Lai *et al.*, 2002). The double (positive/negative) selection procedure developed by Mansour *et al.* (1988) and Capecchi (1989) has been effectively used for gene targeting in mouse ES cells. The herpes simplex virus (HSV) thymidine kinase (tk) gene allows for selection against random insertion events with the selective drug, gancyclovir that eliminates HSV-tk gene-integrated cells during selection. In ES cell system, the single positive selection results in a 1,000-fold enrichment for targeted cell line and the double selection procedure achieves an additional 10- to 1,000-fold enrichment (Riele, *et al.*, 1992). The double selection method could have been much more effective in somatic cells over single selection procedure when strength of cell proliferation is sustained during drug selections. To date, there have been no other reports of successful gene targeting in somatic cells with the double positive-negative selection procedures. G418 and gancyclovir that have been used during positive-negative selection could be detrimental for proliferation of primary sometic cells.

Therefore, to determine whether positive-negative gene targeting could be achieved in pig fetal fibroblasts, pig genomic 13-GT gene was cloned from pig genomic library and Neo[r] plus HSV-tk genes were used for the construction of targeting vector in this experiment. Following transfection with targeting vector DNA, the pig fetal fibroblast cells were selected against resistance of G418 and gancyclovir. Our results indicate that the gene targeting in pig fetal fibroblasts can be achieved by using double selection procedure with high targeting efficiency.

Materials and Methods

Isolation of 13-GT DNA

Pig genomic DNA library (BD Biosciences Clontech) was screened with 560-bp fragment of exon 9 amplified by PCR of pig cDNA library. Screening was performed using nylon membranes and a genomic library of 3×10^5 plaques per each membrane was used with ^{32}P-radiolabelled probe. Positive plaques were confirmed by spot testing and clonally purified by titration of phage plug dilutions onto *E. coli* KW251 lawns. Positive phage DNA was isolated from 50 ml cultures of lysed *E. coli* KW251 using RNase A/D Nase I digestion of bacterial nucleotides and precipitation of phage particles with polyethylene glycol. A 11-kb 13-GT hybridizing fragment from a recombination phage was subcloned into the pBluescript(+) and transformed into *E. coli* XL1-Blue. A 13-GT frag-

ment was partially sequenced by using the Sequenase sequencing kit (United States Biochemical, Cleveland, OH). Primers used were the pBluescript(+) forward primer and revere primer. The distribution of restriction enzyme sites in 13-GT fragment was identified with several restriction enzymes.

Targeting vector construction

Knock-out plasmid, pPNT that has Neo[r] and HSV-tk genes was used for construction of 13-GT targeting vector. The targeting vector consisted of a 8 kb *NotI-XhoI* fragment from part of intron 7, exon 8 and part of intron 8 as the long arm, a 1.9-kb neomycin-resistant gene, a 0.75-kb *XbaI-BamHI* fragment derived from exon 9 as the short arm, and a 3.4-kb fragment containing the herpes simplex virus thymidine kinase gene. *NotI*-linearized targeting vector was used for electroporation into pig embryonic fibroblast cells.

Preparation and transfection of porcine fetal fibroblasts

Pig fetal fibroblast cells were isolated from 8 fetuses of the pregnancy at day 25 of gestation. After removing the head and viscera, fetuses were washed twice with PBS. The red tissues and brain tissues of each fetus were removed and cut as small as possible. And then tissues were treated with trypsin (0.25%) and shaken for 30 min in 37°C incubator. The only supernatant was poured into a 50 ml centrifuge tube mixed with an equal volume of DMEM with 10% FBS and put in ice. This procedure was repeated once more and cells were pelleted following centrifugation at 500 *g* for 10 min. The supernatant was aspirated off, and cells were resuspended with DMEM. The cells were planted into culture dishes and cultured at 37°C, 5% CO_2 incubator. The only cells dividing into monolayer were considered to be P_0 and cells with next passing were numbered to be P_1. The cells were cryopreserved in liquid nitrogen using freezing medium (11% DMSO, 50% FBS, 39% DMEM). For transfection experiments, P_2- or P_3-stage cells were used in this experiment.

For transfection of DNA, 10 μg of linearized vector DNA was introduced into 2 million cells by electroporation. The cell-DNA mixture were loaded into an electorporation chamber, and exposed to a 1 s electrical pulse from 250 F capacitator charged to 300 V. Cells were immediately plated into 100-mm culture dishes. Forty-eight hours after transfection, the transfected cells were selected with 200 μg/ml of G418 (Gibco-BRL) and 3 μM of gancyclovir for 10-14 d.

Identification of targeted cells

Cells were resuspended in lysis buffer (40 mM Tris,

pH 8.9, 0.9% Triton X-100, 0.9% Nonidet P-40, 0.4 mg/ml proteinase K), incubated at 55°C for 5 h and heated to 95°C for 10 min to inactivate the proteinase K. For PCR analysis, genomic DNA was amplified using the PCR machine (PE applied Biosystems) in 50 μl reaction volume with the following parameters; 30 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C and a final 8-min extension period. PCR with the following NEO^r primer and exon 9 3' primer (CR3'); Neo-pgk primer: 5'-CGAGATCAGCA-GCCTCTGTTCC-3' CR3': 5'-CCAACTCCATGCCCTG-TG-3'.

Southern blot analysis was performed using 10 μg of genomic DNA isolated form PCR-positive pig fetal fibroblast cells was digested with *Eco*RI to separate normal and targeted alleles. The DNA was subjected to 1% agarose gel electrophoresis followed by blotting onto nylon membranes. The membrane was probed with a ^{32}P-labeled exon 9 fragment of 13-GT by random priming, and then exposed at -70°C for 48 h to x-ray film.

Statistical analysis

Data were pooled from 4 replicates that had been conducted on the same treatment. Statistically signi-ficant differences between treatment groups were analyzed using analysis of variance and LSD test (Snedecor and Cochran, 1967). Differences where the *P* value was less than 0.05 were regarded as statistically significant.

Results

13-Gal targeting strategy

To construct targeting vector, the pig α-1,3-galactosyl transferase (13-GT) gene was cloned from swine genomic library and characterized. The targeting vector was designed to be able to use positive-negative selection described by Mansour *et al.* (1988). The vector was constructed from 8-kb 13-GT genomic DNA fragment containing part of intron 7 through part of intron 8 as a long arm and 0.75 kb of exon 9 as a short arm for homologous recombination events to remove part of intron 8 and exon 9 and replacing it by neomycin phosphotransferase (Neo^r) gene (Figure 1). In addition, the herpes simplex virus TK gene was placed at the 3' end of the construct. The linearized targeting vector was introduced into pig fetal fibroblast cells by electoporation and the cells were
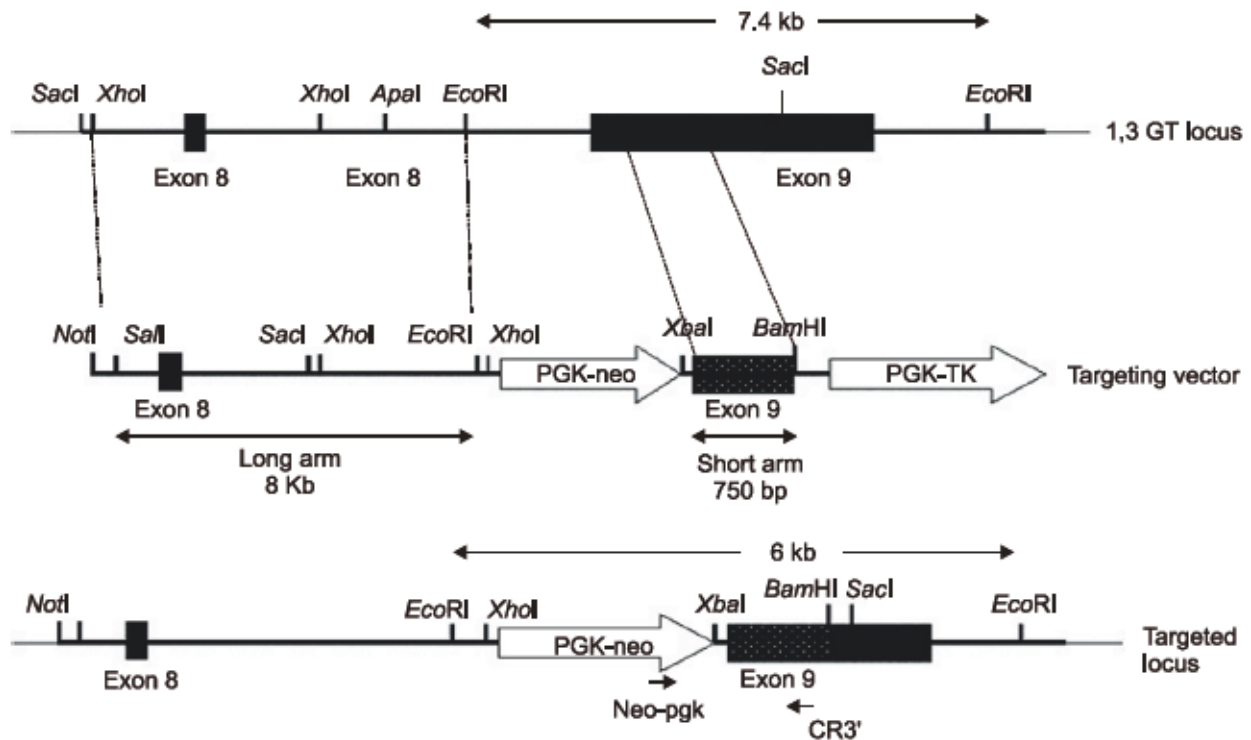

Figure 1. Targeting vector construction. Diagram of the porcine 13-GT locus, the corresponding 13-GT genomic sequences used as 5' and 3' arms in the 13-GT knockout vectors, and the structure of the targeted locus after homologous recombination. The names and positions of the primers used for PCR are indicated by short arrows. Neo-pgk: 5'-CGAGATCAGCAGCCTCTGTTCC-3'; CR3': 5'-CCAACTCCATGCCCTGTG-3'. The predicted size of bands with *Eco*RI digestion, for both the endogenous 13-GT locus and the 13-GT targeted locus, is indicated.

cultured in medium containing G418 plus gancyclovir to enrich for recombinants carrying the neo[r] gene targeted into one of the endogenous 13-GT loci.

Correctly targeted clones were primarily detected by PCR analysis using forward primer in the Neo[r] gene and reverse primer in outside of short arm fragment unique to the targeted locus (Neo-pgk primer and CR3' primer). For PCR analysis, the positive control vector was constructed using neo[r] gene and the whole exon 9 fragment.

Targeting analysis

Cell colonies surviving positive-negative selection with G418 and gancyclovir were assayed by PCR amplification with target locus-specific primers to amplify DNA fragment from 3' end of neo[r] gene through inside of exon 9 resulting in amplification of 1.3 kb DNA fragment (Figure 2A). Positive control vector and correctly targeted locus allow this fragment amplification following PCR. Pig fetal fibroblast cells (PFF) that gave positive PCR signal were potential recombinants in 13-Gal locus.

To confirm the occurrence of the targeted recombination, Southern blots of genomic DNA isolated form PCR-positive PFF clones or normal untargeted PFF, digested with EcoRI restriction enzyme were probed with exon 9 DNA fragment. Identification of PFF correctly targeted with targeting vector is illustrated in Figure 2B. The exon 9 probe hybridizes to 7.4 kb of the wild type allele and 6 kb of fragment of correctly targeted allele for EcoRI digestion. The targeted cells gave a normal wild type allele (7.4-kb band) and a disrupted allele (6-kb band), while intact cells had wild type alleles.

Targeting efficiencies

The proliferation of pig fetal fibroblast cells was sensitive to exposure of selection drugs. Following trans-

fection with targeting vector DNA and drug selection, the formation of colonies was severely suppressed in the medium containing G418 or G418 plus gancyclovir. Furthermore, many colonies following drug selection could not proliferate in normal medium enough to expand cells for DNA analysis. Increase of serum
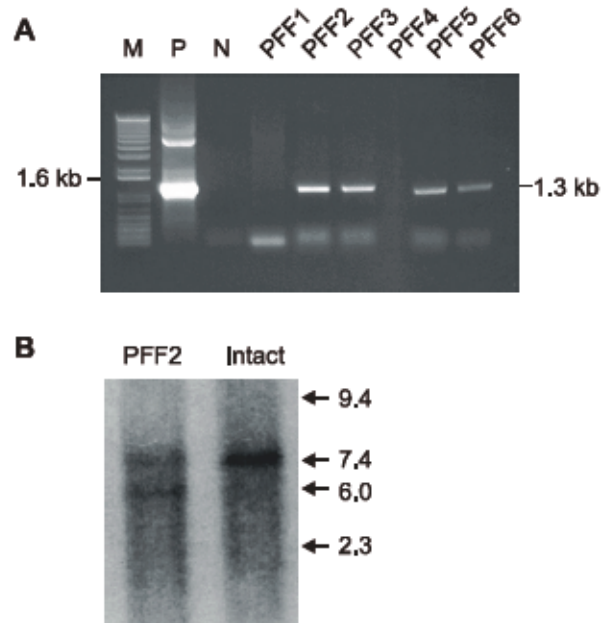

Figure 2. PCR analysis of targeted 13-GT Locus using neo-side primer and primer of 3' 13-GT (A) and Southern blot analysis of 13-GT targeted locus in pig fetal fibroblast cells (B). Approximately 1,000 cell DNA were used for PCR, with Neo-pgk as the forward primer and CR3' as the reverse primer (A). Two negative controls, water, and wild-type (PFF1) cells, were used to ensure that the PCR reaction was not contaminated. PFF2, PFF3, PFF5 and PFF6 were individually targeted colonies. A positive-control vector was included, at dilutions from 100 copies. Southern blotting with PFF2 genomic DNA represented two different sizes of alleles following EcoRI digestion (B). Only one band showed up in wild-type cell lane (Intact lane).

Table 1. Colony growth of pig embryonic fibroblast cells with serum concentration following transfection with targeting vectors and then drug selection.

Serum concentration	No. cells picked up following selection	Colonies with senescence[*]	
		G418[r]	G418[r] + Ganc[r]
5%	120	19.3±4.28[a]	53.5±6.08[a]
10%	120	20.5±4.31[a]	45.2±9.18[a]
15%	120	7.3±2.72[b]	15.3±2.29[b]
20%	120	10.7±3.98[a,b]	20.7±4.71[b]

[a,b]Values within columns with different superscripts are significantly different ($P < 0.05$).
[*]Colonies could not proliferate enough to screen genomic DNA following drug selection.

Table 2. Targeting efficiency in pig fibroblast cells.

Selective marker genes	No. cells treated	Colonies		No. colonies targeted
		G418^r	G418^r+Ganc^r	
Neo^r	2,000,000	345	-	1
Neo^r+TK	2.000.000	336	68	4

concentration in medium could reduce the number of senescent colonies after drug selection (Table 1). Fifteen percent or higher of serum yielded significantly less senescent colonies than lower serum concentration in medium containing G418 only or G418 plus gancyclovir.

To determine the difference in targeting efficiency of single selection (G418 only) and double selection (G418 plus gancyclovir), 2×10^6 PFF were initially electroporated with targeting vectors that contained neo^r gene only or neo^r gene plus TK gene followed by drug selection for 10-14 d. Resistant colonies were counted and transferred to 24-well plate for expansion. The results of two selection experiments are summarized in Table 2. The electroporation of 2×10^6 PFF yielded an estimation of 300 colonies which survived the G418 selection. The 345 G418-resistant colonies in transfectants with targeting vector containing neo^r gene only were screened for targeting by PCR and one colony was identified targeted in 13-Gal locus. A total of 68 colonies originated from cells transfected with a targeting vector carrying neo^r gene plus TK gene were doubly resistant to G418 and gancyclovir, and 4 of these colonies were identified to contain targeted alleles. The targeting frequency in this experiment was about 1/345 of the G418-resistant colonies (0.28%) and 4/68 of G418 puls gancyclovir-resistant colonies (5.9%).

Discussion

The experiments presented here demonstrated the novel use of positive-negative selection system for gene targeting in somatic cells. Recent studies that disrupted genes in somatic cells for nuclear transfer have used positive selection only using Neo^r gene (McCreath *et al.*, 2000; Denning *et al.*, 2001; Dai *et al.*, 2002; Lai *et al.*, 2002; Ramsoondar *et al.*, 2003). Large variations in the number of targeted colonies versus the number of random-integrated colonies (1/20 to 1/500) for gene targeting in somatic cells have been reported (Denning *et al.*, 2001; Dai *et al.*, 2002; Ramsoondar *et al.*, 2003). In our experiments, the efficiency of single selection with G418 was 1 in

354 that was similar to that of reports. However, double selection in our experiments contributed to cut down number of colonies to screen and gave better efficiency for gene targeting. One of the advantages of positive-negative selection is the elimination of cells with correctly targeted alleles plus random integration of targeting vectors that have the possibility to disrupt other locus in the genome. Those cells probably cannot be effectively excluded by single selection with Neo^r gene. Only Southern blotting can sort out correctly targeted cells with proper restriction enzymes among those cells. Dai *et al.* (2002) pointed out that the number of targeted somatic cells identified with PCR was dramatically reduced following Southern blot analysis. Double selection could probably diminish the discrepancy between results of PCR and Southern blot analysis following drug selection procedures. Promoter-trap targeting vector strategy is another positive-negative selection system without using gancyclovir for the elimination of random integration events (Harrison *et al.*, 2002).

It has been shown that many colonies were senescenced following drug selection during gene targeting procedure (Denning *et al.*, 2001). Genetic modifications in cells *in vitro* require intensive drug selection and cell passages. Primary cells are very sensitive to exposure into selection drug, causing cell division crisis following selection. Increase of serum concentration in growth medium could overcome proliferation problem of fetal fibroblast cells following drug treatment during drug selection (Table 1). It could contribute to improve the overall efficiency for gene targeting in somatic cells by rescuing senescent cells with correctly targeted alleles.

The frequency of successful gene targeting in somatic cells will also depend on transfection condition of targeting vector DNA and amount of vector DNA for transfection. Proper adjustment of these conditions would be required to achieve efficient gene targeting in somatic cells. Ultimately, both allele targeting in somatic cells before nuclear transfer would accelerate the practical application of gene targeting in animals. Positive-negative selection may be especially useful in knockout procedure of both alleles at a particular locus without random integration at other

loci for fine genetic modification in mammalian cell system.

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