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Efficient Gene Targeting using Nuclear Localization Signal (NLS) and Negative Selection Marker Gene in Porcine Somatic Cells

Hye Min Kim[1,2], Sang Mi Lee[1], Hyo Young Park[1,3] and Man-Jong Kang[1,†]

[1]*Department of Animal Science, College of Agriculture & Life Science, Insti. of Ag. Sci. and Tech., Chonnam National University, Gwangju 500-757, Korea*

[2]*Alternative Toxicological Methods Research Center, Division of Predictive Toxicology, Korea Institute of Toxicology, Daejeon 350-343, Korea*

[3]*Laboratory of Reproductive Medicine, Creation and Love Women's Hospital, Gwangju 502-800, Korea*

ABSTRACT

The specific genetic modification in porcine somatic cells by gene targeting has been very difficult because of low efficiency of homologous recombination. To improve gene targeting, we designed three kinds of knock-out vectors with α1,3-galactosyltransferase gene (α1,3-GT gene), DT-A/pGT5′/*neo*/pGT3′, DT-A/NLS/pGT5′/*neo*/pGT3′ and pGT5′/*neo*/ pGT3′/NLS. The knock-out vectors consisted of a 4.8-kb fragment as the 5′ recombination arm (pGT5′) and a 1.9-kb fragment as the 3′ recombination arm (pGT3′). We used the neomycin resistance gene (*neo*) as a positive selectable marker and the diphtheria toxin A (DT-A) gene as a negative selectable marker. These vectors have a *neo* gene insertion in exon 9 for inactivation of α1,3-GT locus. DT-A/pGT5′/*neo*/pGT3′ vector contain only positive-negative selection marker with conventional targeting vector. DT-A/NLS/pGT5′/*neo*/pGT3′ vector contain positive-negative selection marker and NLS sequences in upstream of 5′ recombination arm which enhances nuclear transport of foreign DNA into bovine somatic cells. pGT5′/*neo*/pGT3′/NLS vector contain only positive selection marker and NLS sequence in downstream of 3′ recombination arm, not contain negative selectable marker. For transfection, linearzed vectors were introduced into porcine ear fibroblasts by electroporation. After 48 hours, the transfected cells were selected with 300 μg/ml G418 during 12 day. The G418-resistant colonies were picked, of which 5 colonies were positive for α 1,3-GT gene disruption in 3′ PCR and southern blot screening. Three knock-out somatic cells were obtained from DT-A/NLS/ pGT5′/*neo*/pGT3′ knock-out vector. Thus, these data indicate that gene targeting vector using nuclear localization signal and negative selection marker improve targeting efficiency in porcine somatic cells.

(Key words : Porcine somatic cells, Gene targeting, α1,3-Galactosyltransferase gene, Nuclear localization signal)

INTRODUCTION

Gene targeting defined as the genetic manipulations of animal genome using homologous recombination for the altering of gene activity. The technology of homologous recombination allows the precise modification such as replacement and deletion of certain alleles in the genome (Muller, 1999). In domestic animals, other than the mouse, gene targeting has been extremely difficult because cloning of ES cells from mammalian species has made only limited progress. Although embryo-derived pluripotent cell lines have been reported in pig (Wheeler, 1994) and cattle (Stcie *et al.*, 1996), successful germ-line chimeric offspring has not been reported (Cibelli *et al.*, 1998; Piedrahita *et al.*, 1998). Alternatively, gene targeting in domestic animals has been accomplished in primary somatic cells, which were then subjected to somatic cell nuclear transfer (Denning *et al.*, 2003).

The efficiency of homologous recombination is a critical parameter for the success of gene targeting. However, the efficiency of homologous recombination in somatic cells is lower than in mouse ES cells (Templeton *et al.*, 1997). In pigs, two groups reported that pigs deleting one allele of the α1,3-galactosyltransferase

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† Corresponding author : Phone: +82-62-530-2113, E-mail: mjkang@chonnam.ac.kr

(GT) gene through nuclear transfer using gene-targeted fetal fibroblasts (Dai *et al.*, 2002; Lai *et al.*, 2002). However, gene targeting of the α1,3-GT had low frequency of 1.5% and 5%, respectively (Denning *et al.*, 2003).

Improvement of gene targeting methods for somatic cells would greatly increase the production of gene-targeted domestic animals. The most commonly used targeting vector is the replacement vector, which contains a positive selection marker for selection of gene-targeted cells. Moreover, a negative selection marker is added outside of the region of the homology to select the random integrated cells (Muller, 1999). The positive-negative selection strategy was developed by Mansour *et al.* (1988) to enrich for clones that have undergone homologous recombination in mouse ES cells.

Some laboratories have researched for increasing gene targeting efficiency, including addition of nuclear localization signals (Mir *et al.*, 2004). A nuclear localization signal (NLS) reported that increases both the nuclear transport and expression of transfected plasmid DNA (Dean *et al.*, 1999). Also, Mir *et al.* (2004) demonstrated that the efficiency of gene targeting in bovine primary fetal fibroblasts has enhanced by NLS and cell synchronization.

In this study, we cloned the exon 8-9 of α1,3-GT gene by PCR using genomic DNA isolated from miniature pig, constructed three α1,3-GT knock-out vectors using positive-negative selection system and NLS, and transfected into ear primary fibroblasts, finally established to porcine ear primary fibroblast knocked out α1,3-GT locus. Also, we investigated the targeting efficiency when NLS sequence added to the knock-out vector and treated in thymidine to the ear primary fibroblasts.

MATERIAL AND METHODS

Cloning of α1,3-GT Gene and Nuclear Localization Signal (NLS)

Genomic DNA was extracted from blood of Chicago miniature pig and used for PCR amplification. PCR was performed with forward primer (EX8 5′: AGGTCGTGACCATAACCAGATGGAAGGCTC) and reverse primer (EX9 3′: CTTGCACCATGAAGTCTCTGCACTCCAGAA) which were designed using the complementary sequence to *Sus scrofa* α1,3-galactosyltransferase gene exon 8 (GenBank accession no. AF221516) and exon 9 (GenBank accession no. AF221517). The PCR reaction was conducted in 200 ng of genomic DNA, 10 pmole of each primer (Bioneer, Korea), 1×PCR buffer, 2.5 mM of each dNTP and 2.5 U i-MAX II DNA polymerase (Intron, Korea) with a total volume of 20 μl. PCR amplification was repeated for 30 cycles of 40 sec at 94℃, 40 sec at 68℃ and 10 min at 72℃. Nuclear localization signal sequence was obtained from SV40 enhancer of pEGFP-N3 (Clontech, USA). The PCR products were estimated for electrophoresis on 0.8% agarose gel stained with EtBr and cloned into pGEM T-easy vector (Promega, USA) for DNA sequencing determination. Nucleotide sequences were determined using ABI PRISM 377 Sequencer (Applied Biosystems), and sequences of each clone were analyzed by Genetyx-win (Version 4.0).

Construction of α1,3-GT Knock-Out Vectors

The 7.8-kb α1,3-GT genomic fragment cloned by PCR amplification was used for construction of knock-out vectors. These fragments were cut by NotI within pGEM T-easy vector and subcloned into NotI site of pBluescriptII SK (−) vector (Stratagene, USA). For the 5′ and 3′ arm of knock-out vectors, these plasmid DNAs were digested with NotI and EcoRV. And then 5.4-kb and 2.5-kb fragments were subcloned into a pBluescriptII SK (−) vector, respectively. For the 5′ arm of the knock-out vectors, a 5.4-kb NotI-EcoRV fragment was digested with SmaI and blunt-ended with Klenow and ligated SalI linker. Also, The EcoRV site was converted into NotI site by linker insertion. These 4.8-kb SalI-NotI fragments were subcloned into a pBluescriptII KS (+) vector. For the 3′ arm of the knock-out vectors, 2.5-kb EcoRV-NotI fragments were cut by PstI and converted into XhoI site by linker insertion. And then, these fragments were digested with EcoRV-XhoI and the 1.9-kb EcoRV-XhoI fragments were subcloned into a pBluescriptII SK (−) vector. In this study, the neomycine resistance (*neo*) gene was used for positive selection marker. The pKJ2-*neo* plasmid DNAs were digested with EcoRI-BamHI and converted into NotI and EcoRV site by linker ligation, respectively. For ligation of 1.9-kb 3′ arm and *neo* gene, the NotI-EcoRV *neo* fragments and EcoRV-XhoI 3′ arm fragments were subcloned into pBluescriptII SK (−) vector by 3 fragment ligation. This plasmid, named pBlue-*neo*-3′ arm, contained the *neo* gene and 1.9-kb 3′ arm. For ligation of 4.8-kb 5′ arm, SalI-NotI fragments were ligated into SalI and NotI site in the pBlue-*neo*-3′ arm plasmid. This plasmid named pBlue-5′ arm-*neo*-3′ arm plasmid. Finally, SalI-XhoI fragments from pBlue-5′ arm-*neo*-3′ arm plasmid were inserted into the SalI and XhoI site of Diphtheria toxin A (DT-A) cassette, pMCDT-A(A+T/pau) vector, yielding the conventional knock-out vector (DT-A/pGT5′/*neo*/pGT3′). This vector was linearized by SalI restriction enzyme for transfection. Furthermore, two knock-out vectors were additionally constructed using NLS sequence. DT-A/NLS/pGT5′/*neo*/pGT3′ was inserted the 190-bp XhoI-SalI NLS fragment in upstream of 5′ arm of

SalI-linearized conventional knock-out vector. pGT5′/*neo*/pGT3′/NLS vector was inserted the SalI-SalI NLS fragment in downstream of XhoI-linearized pBlue-5′ arm-*neo*-3′ arm plasmid. These vectors were linearized by SalI and ApaI restriction enzyme for transfection, respectively.

Preparation and Culture of Porcine Ear Fibroblast

A small ear tissue was obtained from a 10-day-old male Chicago miniature pig. After removing fur, the tissue was washed three times in PBS. Under a stereo microscope, the tissue was incised with a surgical knife to exposure basal epidermis and cut into size of 2×2 mm segments. The minced tissues were washed in DMEM (WelGene, Korea) containing 15% fetal bovine serum (Hyclone, USA), 100 unit/ml penicillin (Sigma, USA), 100 μg/ml streptomycin (Sigma, USA). Seven or eight pieces of tissues were placed in 60 mm culture dish containing 2 ml of media, cultured in a 5% CO_2 incubator at 37℃, allowed to attach and add 3 ml of media after 2 day. The cells were grown to confluence on culture dish and passaged 1:2 in 75 cm^2 culture flask. The ear fibroblasts were then trypsinized and cryopreserved in liquid nitrogen using freezing media containing 10% DMSO (Sigma, USA). The frozen cells were thawed, expanded for two passages, and karyotyped by GTG banding at the GenDix (Korea). For estimation of cell cycle synchronization in ear fibroblasts, cells were thawed and cultured in DMEM containing 20% defined FBS, 1×non-essential amino acid, 1×sodium pyruvate, 10^{-4} M β-mercaptoethanol, 100 unit/ml penicillin and 100 μg/ml streptomycin. And then, cells were plated at 1×10^6 cells per 100 mm culture dish and grown to 60% confluence. These cells were treated and not treated with media containing 2 mM thymidine (Sigma, USA) for 24 h. Cells were then harvested at 5×10^5 cells by trypsinization and washed in PBS. And then, cells were fixed in 70% ethanol at 4℃ for 1h. Fixed cells were separated by centrifugation and washed in PBS and resuspended in 500 μl of propidium iodide staining solution containing 20 μg/ml propidium iodide (Sigma, USA), 30 μg/ml RNase A (Sigma, USA), 0.2% Triton X-100, and then incubated at 37℃ for 30 min. The stained cells were analyzed by cytomics FC-500 cytometer (Beckman Coulter, USA) to determine DNA content.

Transfection and Selection

Ear fibroblasts were thawed and cultured in DMEM containing 20% defined FBS, 1×non-essential amino acid, 1×sodium pyruvate, 10^{-4} M β-mercaptoethanol, 100 unit/ml penicillin and 100 μg/ml streptomycin. At 60% confluence, cells were treated and not treated with 2 mM thymidine for 24 h. And then, cells were trypsinized and resuspended at a concentration of 1.25×10^7 cells per milliliter in F10 nutrient mixture (WelGene, Korea). Four hundred microliters of the cell suspension were electroporated in a 4 mm cuvette with four pulses of 1 ms duration using 400 V capacitive discharge using 10 μg DNA and the BTX Electro-cell manipulator (ECM 2001, BTX, USA). Electroporated cells were mixed with 10 ml of fresh medium and plated in 100 mm culture dishes. Two days later, selection was carried out for 12 days using culture medium containing 300 μg/ml G418 (Gibco BRL Co., USA) and G418-resistant colonies were isolated using cloning cylinder (Sigma, USA). And then, colonies were transferred to 24-well plates and passaged to 12-well plates, 6-well plates, 60 mm culture dishes and 100 mm culture dishes for expansion.

PCR Analysis of Targeted Cells

For screening of G418-resistant colonies, 200 μl of cell suspension from 24-well plate were recovered by centrifuge. The cells were resuspended in 50 μl of distilled water containing 0.05 mg/ml proteinase K (Roche, Germany). To extract of genomic DNA, the cells incubated at 55℃ for 130 min and heated to 98℃ for 10 min to inactive the proteinase K. To amplify targeted allele, 25 μl of genomic extract was added to a sterile 0.5-ml tube containing 5 μl of 10×PCR buffer, 4 μl of 10 mM dNTP mixture, 1 μl of forward primer (10 pmol/μl, Bioneer, Korea), 1 μl of reverse primer (10 pmol/μl, Bioneer, Korea) and 1.25 U of EX Taq DNA polymerase (5 U/μl, TaKaRa, Japan) and the volume was adjusted to 50 μl with distilled water. Forward primer was derived from the *neo* gene, TCGTGCTTTACGGTATCGCCGCTCCCGATT, and reverse primer from the α1,3-GT sequence that was not include in the knock-out vector, GACACAAATGACCTAAACTGGAACACAAGC. The PCR was carried out for 38 cycles in thermal cycle (Bioneer, Korea). Each cycle consisted of denaturation for 30 sec at 94℃, annealing for 30 sec at 68℃, and extension for 2.5 min at 72℃. The reaction samples were run in 0.8% agarose gel by standard procedures.

Southern Blot Analysis of Targeted Cells

PCR-positive ear primary fibroblasts were grown in 100 mm culture dish until they reached confluency, harvested by trypsinization and the genomic DNA was isolated using proteinase K and phenol extraction method. 10 μg of genomic DNA was digested overnight with EcoRV (TaKaRa, Japan) and the digested DNA was loaded 0.7% agarose gel, electrophoresed at 25 voltages for 16 hr. After electrophoresis, the digested DNA was transferred onto zeta-probe membrane (Bio-Rad Co., USA)

by blotting with 10×SSC. The membrane was prehybridized with the prehybridization solution (5×SSPE, 5×Denhardt's solution, 1% SDS, 50% formamide, 100 μg /ml of Salmon sperm DNA) at 42℃ for 3 hr 30 min. A 491-bp DNA probe corresponding to exon 8 of the α 1,3-GT gene just outside the 5′ end of the targeting vector was produced by PCR. Moreover, the genomic DNA was performed the digestion of BstEII (Intron, Korea) and an 800-bp *neo* fragment used for probe. The probe was made by random primer labeling method using rediprimeII random prime labeling system kit (Amersharm Co., UK) and [α-^{32}P]dCTP(110TBq/mmol, Amersharm Co., UK) according to the manufacturers. The prehybridization solution was discarded and added to fresh hybridization solution with 100 μg/ml salmon sperm DNA and the labeled probe to hybridization bottle. The hybridization was performed at 42℃ for 16 hr with shaking. The membrane was washed one times in 2×SSC/0.1% SDS (w/v) at room temperature for 10 min, and then washed three times in 0.3×SSC/0.1% SDS (w/v) at 68℃ for 15 min to increase the levels of stringency. The washed membrane was exposed to Kodak BioMAX XAR film with an intensifying screen at −80℃ for 7 days.

RESULTS

Porcine α1,3-galactosyltransferase gene consisted of 9 exon and 8 intron. The translation start and stop codons are located in exon 4 and 9, respectively. To isolate the isogenic α1,3-GT exon 8-9 fragment, PCR was performed by genomic DNA isolated from the blood of Chicago miniature pig using each primers corresponding to exon 8 and 9. The 7.8-kb genomic fragment was amplified from genomic DNA. The 5′ and 3′ sequence of this fragment showed striking homology to porcine α1,3-GT exon 8 and 9 (GenBank accession No. AF221516 and AF221517). Also, the restriction site required for constructing vector was checked through restriction enzyme mapping (data not shown).

The α1,3-GT knock-out vector was designed using positive-negative selection marker and poly A trap strategy (Fig. 1). The knock-out vector consisted of a 4.8-kb fragment as the 5′ recombination arm, a 1.3-kb fragment of the neomycin resistant gene as positive selectable marker, a 1.9-kb fragment as the 3′ recombination arm, and a 4.0-kb fragment containing the Diphtheria toxin A (DT-A) gene as negative selectable marker (DT-A/pGT5′/*neo*/pGT3′).

For high efficiency of gene targeting, we also constructed two knock-out vectors by addition of nuclear localization signal, DT-A/NLS/pGT5′/*neo*/pGT3′ and pGT5′/*neo*/pGT3′/NLS (Fig. 1). Nuclear localization signal that is two 72-bp tandem repeat enhancer sequences containing the SV40 genome was obtained from pEGFP-N3 vector by PCR (data not shown). DT-A/NLS/pGT5′/*neo*/pGT3′ vector was inserted the NLS fragment in upstream of 5′ arm of conventional knock-out vector. pGT5′/*neo*/pGT3′/NLS vector was inserted NLS fragment in downstream of 3′ arm, but not contained DT-A cassette. In vector construction, the *neo* gene was placed in EcoRV site of exon 9 including the sequence encoding catalytic domain. Gene targeting using these vectors is expected to result in functional inactivation of the α1,3-GT gene by disruption of catalytic domain.

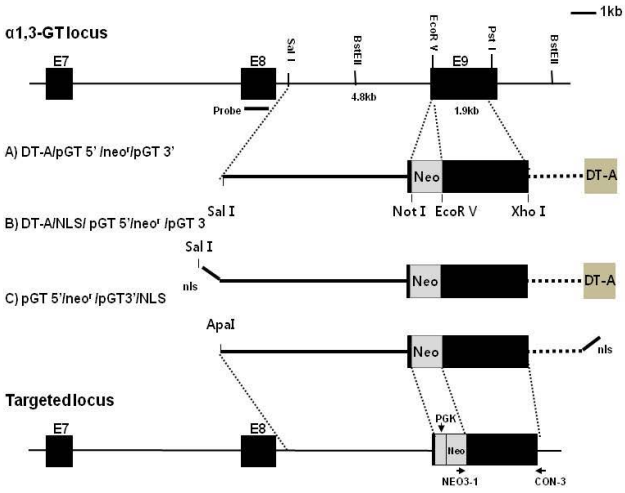


Fig. 1. The knock-out strategy for porcine α1,3-GT gene and three types of α1,3-GT knock-out vectors. Diagram of α1,3-GT locus, α 1,3-GT knock-out vector and targeted locus after homologous recombination. The 5′ arm was 4.8-kb fragment including intron 8 and 3′ arm was 1.9-kb fragment including exon 9. The *neo* gene was used as positive selection marker and DT-A used as the negative selection marker. NLS sequence was cloned into 72-bp tandem repeats of SV40 enhancer. (A) DT-A/pGT5′/*neo*/pGT3′ is conventional targeting vector containing only positive-negative selection marker. (B) DT-A/NLS/pGT5′/*neo*/pGT3′ contains positive-negative selection marker and NLS sequence. (C) pGT5′/*neo*/pGT3′/NLS contains only positive selection marker and NLS sequence. The positions of the primers used for amplify of targeted allele are indicated by short arrow. The short bar indicates the probes used for southern blot analysis.

In this study, porcine ear fibroblasts were isolated from ear tissue of a 10-day-old Chicago miniature male pig and shown typical fibroblast morphology and normal karyotype (data not shown). To confirm the transfection condition, pEGFP-N3 vector was transfected into porcine ear fibroblasts synchronized S phase by treatment of thymidine. Fig. 3 shows the transfection efficiency obtained in thymidine treated and not treated cells using electroporation. The percentage of S phase in thymidine not treated cells is 20.7%, and that in thy-

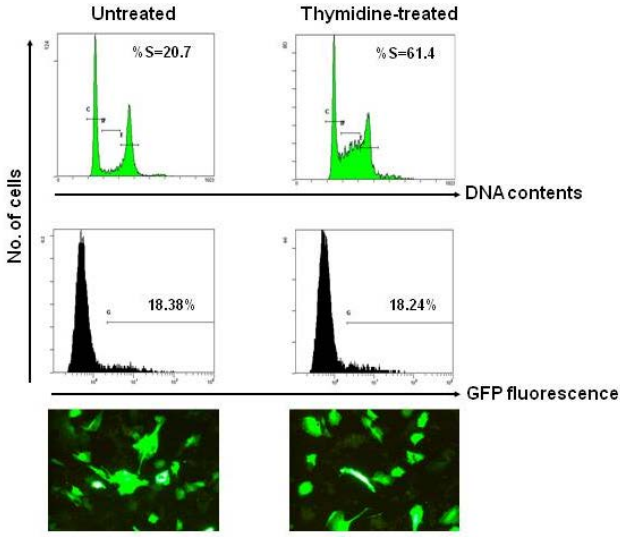


Fig. 2. Efficiency of transfection into porcine ear fibroblasts using electroporation. Cells were treated and not treated in 2 mM thymidine for 24 h to synchronize. The percentage of S phase in thymidine not treated cells is 20.7%, and that in thymidine treated cells is 61.4%. After transfection, cells were analyzed for GFP expression by FACS. The percentage of expression is indicated in each histogram. Transfected cells were visualized by GFP, respectively.

midine treated cells is 61.4% (Fig. 2A). The transfection efficiency was 18.38% in thymidine not treated cells and 18.24% in thymidine treated cells, respectively (Fig. 2B). These results indicate that transfection efficiency was not difference between the thymidine treated and not treated cells.

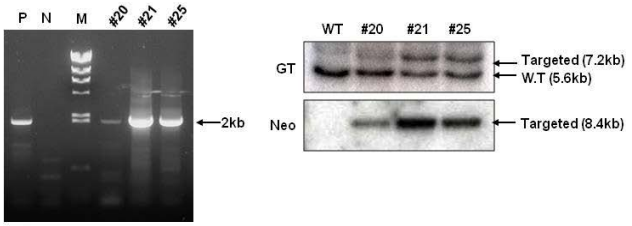
To isolate the knock-out somatic cells, three α1,3-GT knock-out vectors was transfected into thymidine treated and not treated porcine ear fibroblasts. And then, transfected cells selected with 300 μg/ml G418 for 12 days. G418-resistant colonies were obtained, and analyzed that homologous recombination event was occurred in α1,3-GT locus. Table 1 summarized the results of gene targeting efficiency by different types of knock-out vectors. In thymidine treated and not treated cells, 239 G418-resistant colonies were picked, of which 5 colony was positive for α1,3-GT gene disruption. The number of G418-resistant colony was not different between the cells treated with or without thymidine treatment.

The PCR for confirmation of gene targeting was performed with NEO 3′-1 (a sequence from the 3′ end of *neo* gene) and CON-3 (a sequence from the 3′ end of exon 9 located outside the 3′ arm) as forward and reverse primers. The 2.0-kb positive band was amplified from 15 G418-resistant colonies (Fig. 3A). 8 colonies confirmed that porcine ear fibroblasts transfected with DT-A/NLS/pGT5′/*neo*/pGT3′ vector.

To ensure that our PCR data were accurate, we carried out southern blot analysis using genomic DNA isolated from 3′ PCR-positive colonies. Targeted colony should have a expected 7.2-kb fragment due to the presence of an additional 1.3-kb PGK-*neo* cassette in the α1,3-GT locus. In contrast, the wild-type allele should have a 5.6-kb fragment, when southern blot of genomic DNA digested with EcoRV restriction enzyme were hybridized with the probe containing exon 8 and part of intron 8. Also, southern blot of genomic DNA digested BstEII probed by *neo* fragment should have an 8.4-kb fragment. In these results, 5 of 15 3′ PCR-positive colonies were positive for southern blot analysis (Fig. 3B). Moreover, 3 colonies confirmed that porcine ear fibroblasts transfected with DT-A/NLS/pGT5′/*neo*/pGT3′ vector. These colonies that verified knock-out of α1,3-GT gene were originated from all cells regardless of thymidine. These results show that thymidine does not influence homologous recombination in porcine ear fibroblasts. As a result, the frequency of targeted colonies per total G418-resistant colonies is 2.09%, but targeting efficiency in colonies using thymidine treated cells transfected with DT-A/NLS/pGT5′/*neo*/pGT3′ is 5.71% (Table 1). To check availability for nuclear transfer with these cells, the karyotype was analyzed at passage 12,

Table 1. Efficiency of targeting at the α1,3-GT locus

Targeting vector	Thymidine treated	No. of cells transfected	No. of $G418^R$ colonies	No. of PCR-positive colonies	No. of southern blot-positive colonies
DT-A/pGT5' /*neo*/pGT3'	-	1.5×10^7	52	1 (1.92%)	0
	+	1.5×10^7	39	5 (12.8%)	1 (2.56%)
DT-A/NLS/ pGT5'/*neo*/pGT3'	-	1.5×10^7	35	4 (11.4%)	1 (2.85%)
	+	1.5×10^7	35	4 (11.4%)	2 (5.71%)
pGT5'/*neo* /pGT3'/NLS	-	1.5×10^7	45	0	0
	+	1.5×10^7	33	1 (3.03%)	1 (3.03%)
Total		9.0×10^7	239	15 (6.27%)	5 (2.09%)



(A) (B)

Fig. 3. PCR and southern blot analysis for G418-resistant colonies. (A) Approximately 1,000 cells were used for PCR, with NEO3′-1 as the forward primer and CON-3 as the reverse primer indicated by diagram. P, 1fg of α1,3-GT control vector as the positive control; N, genomic DNA of wild-type ear primary cells. (B) GT Probe, Southern blot using EcoRV-digested genomic DNA. The 7.2-kb band represents the targeted α1,3-GT locus and 5.6-kb band represents the endogenous α1,3-GT locus. Neo Probe, Southern blot was used BstEII-digested genomic DNA and probed the *neo* gene. The 8.4-kb band represents the targeted α1,3-GT locus. WT, the normal ear primary cell DNA as a negative control. No. 20, 21, 25 colonies were obtained from the DT-A/NLS/pGT5′/*neo*/pGT3′ vector.

and resulted that cells have normal karyotype (data not shown). Therefore, targeting vector using positive-negative selection marker and nuclear localization signal may improve gene targeting efficiency in porcine somatic cells.

DISCUSSION

Gene targeting is a genetic technique that uses homologous recombination to change an endogenous gene. The method can be used to delete a gene, remove exons, and introduce point mutations. For gene targeting of porcine α1,3-GT gene, some groups only used *neo* gene as positive selection marker into the targeting vector (Dai *et al.*, 2002; Lai *et al.*, 2002). Low efficiency of gene targeting in porcine somatic cells has been reported (Denning *et al.*, 2003).

In this study, knock-out somatic cells for porcine α 1,3-GT gene by gene targeting were obtained from three knock-out vectors used positive and negative selection system and nuclear localization signal. Targeting events were detected at a frequency of 1.09% (1 of 91) in DT-A/pGT5′/*neo*/pGT3′ vector. Also, we constructed two α1,3-GT knock-out vectors containing NLS sequence and confirmed that targeting efficiency is 4.28% (3 of 70) in DT-A/NLS/pGT5′/*neo*/pGT3′ vector and 1.28% (1 of 78) in pGT5′/*neo*/pGT3′/NLS vector. As results, DT-A/NLS/pGT5′/*neo*/pGT3′ vector has an efficient gene targeting in porcine somatic cells.

Low efficiency of delivering targeting constructs into the nucleus is major problems limiting the success of gene targeting. Dean *et al.* first demonstrated that the 72-bp enhancer repeat from the SV40 genome supported nuclear import (Dean *et al.*, 1997, 1999). In support of these findings, Greassman *et al* reported that SV40 enhancer lead to increased transcription of a herpes TK promoter-derived gene, compared to plasmids lacking the enhancer (Graessmann *et al.*, 1989). Moreover, in bovine fibroblasts, this enhancer that was termed nuclear localization signal (NLS) has increased the frequency of homologous recombination by addition of NLS into targeting vector, whereas the random integration decreased (Mir *et al.*, 2004).

Diphtheria toxin A (DT-A) used as negative selection marker and *neo* gene used as positive selection marker. Diphtheria toxin A fragment inactivates elongation factor 2 (EF-2) in the cytoplasm, thereby inhibiting protein synthesis, and killing the cells (Yamaizumi *et al.*, 1978). The poly A signal was absent from these selectable marker genes in the knock-out vector. When the vector was inserted into homologous site, *neo* gene was expressed using poly A of homologous gene whereas DT-A gene was not expressed for loss of DNA. However, when the vector was inserted into random site, integration occurred in two types. One was integration in the position that can not use poly A, resulting in cell death from no expression of *neo* gene during G418 selection. The other was insertion to genome position that can use poly A, resulted that the cells died from expression of DT-A gene. But, neo gene not expressed because RNA polymerase II pausing signal artificially inserted into the behind site on this gene. Yagi *et al.* (1993) reported that positive-negative selection system using DT-A gene as negative selection marker provides stable and effective isolation of homologous recombinant in mouse embryonic stem cells. Therefore, these results suggested that homologous recombinant could be efficiently isolated from knock-out vector used positive-negative selection system and nuclear localization signal.

In this study, an efficient transfection condition of porcine ear fibroblasts was established by electroporation method. Also, effects of thymidine in the transfection examined. Homologous recombination occurs mainly at late S/G2 phase (Takata *et al.*, 1998) and thymidine efficiently arrests cells in the S phase by depletion of one or several deoxyribonucleoside triphosphate precursors of DNA synthesis (Lundin *et al.*, 2002). Also, Mir *et al.* reported that thymidine treatment into bovine fetal fibroblasts enhances gene targeting (Mir *et al.*, 2004). In this study, expression of GFP was 18.38% or 18.24% in the cells treated with or without thymidine treatment, respectively. These results show that expression of GFP in the porcine ear fibroblasts has not effects with thymidine. Also, knock-out somatic cells obtained from both thymidine not treated cells and

thymidine treated cells.

In this report, the knock-out vectors for porcine α 1,3-GT gene have been constructed by positive/negative selection marker and nuclear localization signal. The porcine ear fibroblasts established from Chicago miniature pig and the knock-out somatic cells for α1,3-GT locus obtained by gene targeting. This approach will have an efficient gene targeting of somatic cells for production of transgenic animals by somatic cell nuclear transfer.

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