



Knock-in of Enhanced Green Fluorescent Protein or/and Human *Fibroblast Growth Factor 2* Gene into β -Casein Gene Locus in the Porcine Fibroblasts to Produce Therapeutic Protein

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ABSTRACT: Transgenic animals have become important tools for the production of therapeutic proteins in the domestic animal. Production efficiencies of transgenic animals by conventional methods as microinjection and retrovirus vector methods are low, and the foreign gene expression levels are also low because of their random integration in the host genome. In this study, we investigated the homologous recombination on the porcine β -casein gene locus using a knock-in vector for the β -casein gene locus. We developed the knock-in vector on the porcine β -casein gene locus and isolated knock-in fibroblast for nuclear transfer. The knock-in vector consisted of the neomycin resistance gene (neo) as a positive selectable marker gene, *diphtheria toxin-A* gene as negative selection marker, and 5' arm and 3' arm from the porcine β -casein gene. The secretion of enhanced green fluorescent protein (EGFP) was more easily detected in the cell culture media than it was by western blot analysis of cell extract of the HC11 mouse mammary epithelial cells transfected with EGFP knock-in vector. These results indicated that a knock-in system using β -casein gene induced high expression of transgene by the gene regulatory sequence of endogenous β -casein gene. These fibroblasts may be used to produce transgenic pigs for the production of therapeutic proteins via the mammary glands. (**Key Words:** Knock-in, Homologous Recombination, Therapeutic Proteins, Porcine β -casein Gene)

INTRODUCTION

Many transgenic animals are produced by microinjection, retroviral vectors, sperm mediated vectors and nuclear transfer methods (Houdebine, 2000; Robl et al., 2007; Houdebine, 2009). Microinjection is the method of injecting a foreign gene into the pronucleus of a fertilized egg using a micro-manipulator, for the expression of a foreign gene, which is the most widely used method

(Gordon and Ruddle, 1981; Palmiter et al., 1982; Hammer et al., 1985; Clark, 1998; Houdebine, 2000).

Animal cloning by nuclear transfer using somatic cells was first successfully applied in sheep (Wilmut et al., 1997). The production of clone animals by nuclear transfer using somatic cells can be used to produce transgenic animals. In this method, the transgenic vector is introduced into the somatic cells before they are used for nuclear transfer. The fundamental principles of the nuclear transfer method were experimentally verified by the production of a human factor IX transgenic sheep using transfected fetal fibroblasts (Schnieke et al., 1997). The generation of transgenic domestic animals by nuclear transfer using transfected cells provides a number of advantages over the classic DNA microinjection technique. One major advantage of this

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method is that integration and expression of the transgene can be investigated in transfected cells before they are used as a nuclear donor (Wolf et al., 2000). Also, transgenesis by nuclear transfer offers a route for random or targeted genetic modification in domestic animals (Schnieke et al., 1997; Robl et al., 2007; Houdebine, 2009).

The production of therapeutic proteins from transgenic animals is one of the most important and significant successes of animal biotechnology. However, the production efficiency of transgenic animals by conventional methods is only 2%-3% and the level of foreign gene expression is also very low, because the foreign genes are integrated at random sites throughout the host animals' genome (Chan, 1999; Wolf et al., 2000).

To overcome the above problems, gene targeting methods using homologous gene recombination techniques have been developed. Gene targeting is a technique which utilizes homologous recombination between an engineered exogenous DNA fragment and the genome of the donor cells (Clark et al., 2000; Piedrahita, 2000; Denning and Priddle, 2003; Wang and Zhou, 2003). Recombination between identical regions contained within the introduced DNA fragment and the native chromosome will lead to the replacement of a portion of the chromosome with the engineered DNA. There are two methods used in gene targeting; the knock-out and knock-in methods. In knock-out gene targeting, recombination occurs within the gene. This results in the insertion of a foreign sequence within the middle of the gene, which disrupts the gene. With its sequence interrupted, the altered gene in most cases will be translated into a nonfunctional protein. In knock-in gene targeting, a specific endogenous gene is removed and an exogenous gene is inserted in the specific endogenous locus. Expression of the inserted exogenous gene is then controlled by the gene regulatory sequence of the endogenous gene (Clark et al., 2000; Piedrahita, 2000; Denning and Priddle, 2003; Wang and Zhou, 2003). Gene-targeted sheep created by nuclear transfer were generated using ovine fetal fibroblasts which targeted the $\alpha 1$ -antitrypsin gene into the $\alpha 1(I)$ procollagen locus for mammary gland specific expression (McCreath et al., 2000). Using this method, they reported that transgenic sheep were hormonally induced to lactate, and had a high expression (650 $\mu\text{g/mL}$) of $\alpha 1$ -antitrypsin in their milk. These results indicate that gene targeting is a powerful method to produce animal bioreactors.

When the β -casein gene was knocked-out by gene targeting in a mouse, the knock-out mouse survived normally and could even become pregnant and nurse its young (Kumar et al., 1994). This result indicates that when the β -casein gene is knocked-out by gene targeting and a specific foreign gene is inserted in its place, the foreign

gene can be expressed by the β -casein gene regulatory sequence. However, the application of gene targeting to the porcine β -casein gene locus for the production of bioactive substances in porcine milk has not yet been reported.

Therefore, the aim of this study was to develop a knock-in gene targeting system, using the β -casein gene of porcine to produce bioactive substances.

MATERIALS AND METHODS

Construction of knock-in vector for expression of enhanced green fluorescent protein or human fibroblast growth factor 2

Porcine β -casein genome DNA was cloned previously by our group (Lee et al., 2012). The β -casein genome DNA was used as a template for the construction of the knock-in vector in this study. The 5.1 kb 5' arm fragment of porcine β -casein gene was cloned by polymerase chain reaction (PCR) amplification using a forward primer with an additional NotI site (GCGGCCGCGATATCTAGGGTCTCTTCTAGT) and a reverse primer with an additional NcoI site (CCATGGCGATCAAGTCCTGTGAATGGGGAA). To clone the 3' arm of porcine β -casein gene, genome DNA containing exon 2 to exon 9 was digested by PstI and XbaI restriction enzyme, and then the 2.6 kb PstI/XbaI fragment was subcloned into the PstI-XbaI site of pBluscript SK+ (pBSK+) vector to produce the pBSK-PstI/XbaI 3' arm plasmid. The pBSK-PstI/XbaI 3' arm plasmid was digested with PstI and XbaI enzyme, blunt-ended with Klenow, and ligated to SalI and XhoI linker (Takara Co., Shiga, Japan). SalI/XhoI fragments were ligated into the SalI-XhoI site in the pBluscript SK- (pBSK-) to produce the pBSK-SalI/XhoI 3' arm plasmid. Human fibroblast growth factor 2 (FGF2) cDNA was cloned by the real-time-PCR, using a forward primer with an additional NcoI site (CCATGGCAGCCGGGAGCATCAC) and a reverse primer with an additional SalI site (GTCGACCCATTTAAATCAGCTC), from cDNA of human hepatocellular liver carcinoma cell line (HepG2) cell. Also, EGFP gene was cloned by PCR, using the pEGFP-N3 vector (Clontech Co., Palo Alto, CA, USA) as a template, using a forward primer with an additional NcoI site (CCATGGTGAGCAAGGGCGAGGA) and a reverse primer with an additional SalI site (GTCGACCGGCCGCTTACTTGT) primer. SV40 polyA signal (0.2 kb) was cloned by PCR, using pCMV-Tag1 vector (Stratagene Co., La Jolla, CA, USA) as template, using a forward primer with an additional XhoI site (CTCGAGACTCGATCGCCCTT) and a reverse primer with an additional EcoRV site (GATATCAATTTACGCGTTAA) primer. The nuclear localization signal (NLS) sequence was cloned by PCR, using pEGFP-N3 vector (Clontech, USA) as a

template, using sense (GCGGCCGCGATTCG GATTCG GAGTTA) and antisense (ACTAGTCCAGCTGT GGAATG) primers. To the cloned *PGK-neo* gene, pKJ2 neo cassette plasmid (Gibco BRL, Grand Island, NY, USA) was digested with EcoRI and BamHI enzyme, blunt-ended with Klenow, and ligated to EcoRV and Sall linker (Takara Co., Japan). EcoRV/Sall fragments were ligated into the EcoRV-Sall site in the pBSK- to produce the pBSK-PGKneo plasmid. All PCR amplified fragments were subcloned into the pGEM T-easy vector (Promega Co., Madison, WI, USA) and the sequence was conformed. Knock-in vectors were constructed as follows: NotI/NcoI 5.1 kb fragment and NcoI/Sall hman FGF2 or EGFP fragments were inserted into the NotI-Sall site of pBSK+ by 3 fragment ligation to produce pBSK-5' arm-hFGF2 or EGFP plasmid. T-easy NLS plasmid was digested with SpeI enzyme, blunt-ended with Klenow, and digested with NotI. NotI/blunt NLS fragment was ligated into the NotI-EcoRV site of pBSK-5' arm-hFGF2 or EGFP plasmid to produce pBSK-NLS-5' arm-hFGF2 or EGFP plasmid. SV40 polyA fragment (XhoI-EcoRV) and NLS-5'arm- hFGF2 or EGFP fragment

(NotI-Sall) were ligated into the NotI-EcoRV site of pBSK+ by 3 fragment ligation to produce pBSK-NLS-5' arm-hFGF2 or EGFP-polyA plasmid. PGK-neo fragment (EcoRV-Sall) and 3'arm fragment (Sall-XhoI) were subcloned into the EcoRV-XhoI site of pBSK+ by 3 fragment ligation to produce pBSK-PGKneo-3'arm plasmid. PGKneo-3'arm fragment (EcoRV-XhoI) was inserted into EcoRV-XhoI site of pBSK-NLS-5' arm-hFGF2 or EGFP-polyA plasmid to produce pBSK-NLS-5' arm-hFGF2 or EGFP-polyA-neo-3' arm plasmid. Finally, to produce the knock-in vector, NotI-XhoI fragments of pBSK-NLS-5' arm-hFGF2 or EGFP-polyA-neo-3' arm plasmid were ligated into the NotI-XhoI site in the pMCDT-A plasmid (Gibco BRL, USA). These knock-in vectors consisted of NLS sequence, porcine β -casein 5' arm (5.1 kb), human FGF2 or EGFP cDNA, SV40 polyA, neomycin resistance gene (neo) as a positive selectable marker gene, porcine β -casein 3'arm (2.6 kb), and the diphtheria toxin A fragment (*DT-A*) gene as a negative selectable marker gene (Figure 1A). We named these knock-in vectors EGFP or hFGF2 knock-in vectors for expression of EGFP or hFGF2 on the

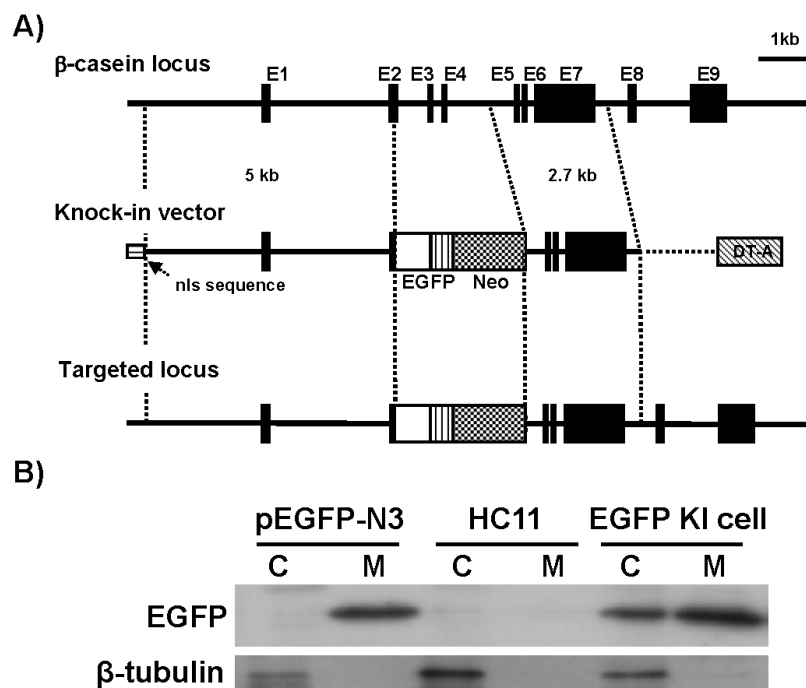


Figure 1. The knock-in strategy in the porcine β -casein gene locus. (A) Diagram indicating homologous recombination of knock-in vector at the porcine β -casein gene locus. Solid black box indicates exon of porcine β -casein gene. Open box, vertical line box, and dotted box indicates cDNA of *EGFP* or *hFGF2* gene, poly A signal sequence and *PGK-neo* gene without polyA signal sequence, respectively. The PCR primer pairs used for detecting homologous recombinations are shown as primer A, located in the *Neo* gene, and primer B, located in the out site from the homologous 3' arm. (B) Secretion of EGFP protein in the cell culture media of the HC11 cell transfected with EGFP knock-in vector. C indicated total protein from media of each cells. M indicates protein isolated from media of each cells. The β -tubulin antibody was used to distinguish between cell and media protein. EGFP, enhanced green fluorescent protein; hFGF2, human fibroblast growth factor 2; PGK, phosphoglycerate kinase promoter; PCR, polymerase chain reaction; HC11, mouse mammary epithelial cell line; pEGFP-N3, plasmid vector enhanced green fluorescent protein-N3.

porcine β -casein locus, respectively. For transfection of linearized knock-in vectors to porcine fibroblasts, knock-in vectors were digested with NotI enzyme.

Cell culture of HC11 cell, Transfection and lactogenic hormone induced differentiation

The HC11 cell was cultured in RPMI1640 (HyClone Co., Logan, UT, USA) containing 10% fetal bovine serum (HyClone Co., USA), 100 unit/mL penicillin and 100 μ g/mL streptomycin (HyClone Co., USA). Transfection of knock-in vector was performed as follows. The HC11 cells were inoculated on 35 mm dishes at a density of 2.4×10^5 cells/dish, and culture medium was replaced with 1.8 mL of fresh medium the next day. The cells were then transfected using a jetPEI transfection reagent (Polyplus-transfection, Illkirch, France) according to the manufacturer's instruction using 4 μ g of Knock-in vector and pEGFP-N3 vector (Clontech Co., USA) as a control. After 24 h, selection was performed for 10 days using 300 μ g/mL of G418 (Gibco BRL, USA). For lactogenic hormone induction, the HC11 cells as a negative control and HC11-EGFP cells were cultured in growth media (RPMI1640 [HyClone Co., USA] containing 10% FBS [HyClone Co., USA], 100 unit/mL penicillin, 100 μ g/mL streptomycin, 5 μ g/mL insulin [Sigma Chemical Co., St Louis, MO, USA] and 10 μ g/mL epidermal growth factor [Invitrogen Co., Carlsbad, CA USA]). The lactogenic hormone induction of HC11 cells was described previously (Doppler et al., 1989). Briefly, cells were grown to confluence and were maintained for 3 days in growth media. The growth media was removed and washed with PBS three times to remove the remaining serum. The cells were then cultured with induction media (RPMI1640 [HyClone Co., USA] containing 100 unit/mL penicillin, 100 μ g/mL streptomycin, 5 μ g/mL insulin [Sigma Chemical Co., USA], 1 μ M DEX [Sigma Chemical Co., USA], 5 μ g/mL prolactin [Sigma Chemical Co., USA] and 10% FBS [HyClone Co., USA]) during 3 days without changing the medium.

Culture of porcine ear fibroblasts and transfection

Porcine ear fibroblasts were prepared from ear tissues of three-way crossbreds (LWD) obtained at the slaughterhouse, according to a previously reported method (Ahn et al., 2011). The cells were cultured with growth medium (DMEM, 15% FBS, 1 \times non-essential amino acids, 1 \times sodium pyruvate, 10^{-4} M β -mercaptoethanol, 100 unit/mL penicillin, 100 μ g/mL streptomycin) for electroporation. The transfection was conducted according to a previously reported method (Kim et al., 2012), as follows. The cells were trypsinized and resuspended at a concentration of 1.25×10^7 cells/mL in F10 nutrient mixture. The cell suspension (400 μ L) was electroporated in a 4 mm cuvette using 10 μ g knock-in vectors with four-1 ms pulses using

400 V capacitive discharges by a BTX Electro-cell manipulator (ECM 2001, BTX, Holliston, MA, USA). After electroporation, the cuvette was placed on ice for 10 min. The cells in the cuvette were resuspended in 10 mL of medium, distributed into a 48-well plate, and further cultured. After 24 h, selection was performed for 11 days using 300 μ g/mL of G418 (Gibco BRL, Grand Island, NY, USA). After selection, the single colonies were passaged into 24-well plates, and were further cultured for the analysis of positive colonies.

Preparation of protein and Western blot analysis

Protein from culture media was isolated by acetone precipitation as follows. Cultured medium from lactogenic hormone induced cells was collected in a 15 mL tube and was centrifuged at 800 rpm, 4°C for 5 min to remove floating matter. Four volumes of acetone (Merck Co., Darmstadt, Germany) was added in the collected medium and was maintained at -20°C overnight. The collected medium was then centrifuged at 13,000 rpm, 4°C for 30 min. After centrifugation, the protein pellet was dissolved in 100 μ L of sodium chloride-tris-EDAT (TEN) buffer (40 mM Tris HCl [pH 7.4], 1 mM cobalt ethylenediamine-tetraacetate [EDTA], 150 mM NaCl, 0.05% Triton X-100) containing inhibitor cocktail and phenylmethylsulfonyl fluoride (PMSF). Protein from cells was prepared by TEN buffer (40 mM Tris HCl [pH 7.4], 1 mM EDTA, 150 mM NaCl, 0.05% Triton X-100) containing inhibitor cocktail and PMSF in cells. The protein concentration was measured using a Bio-Rad protein assay kit as per the manufacturer's instructions.

For western blots, equivalent amounts of protein (50 μ g) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (10% gel) and were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Co., Hercules, CA, USA). The membranes were blocked in 5% skim milk in tris buffered saline with tween 20 (TBST) for 2 h at room temperature. The membrane was subsequently incubated for 2 h at room temperature with monoclonal anti-green fluorescent protein (1:2,000 dilution; Ab Frontier, Seoul, Korea) in TBST solution containing 0.1% bovine serum albumin (BSA, Sigma Chemical Co., USA). After three times washing with TBST, the membranes were then incubated for 2 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:2,000 dilution; SantaCruz Co., Santa Cruz, CA, USA) in TBST containing 0.1% BSA (Sigma Chemical Co., USA). The protein bands were visualized by incubation with enhanced chemiluminescence (Amersham Co., Arlington Heights, IL, USA).

Polymerase chain reaction analysis of knock-in colony

Preparation of genome DNA from G418-resistant

colonies was performed according to a previously reported method (Kim et al., 2012). When the cells were sub-cultured from 24-well plates to 12-well plates, 200 μ L of cell suspension from the 24-well plates were recovered by centrifugation and suspension with 40 μ L of deionized water containing 0.05 mg/mL proteinase K (Roche, Foster City, CA, USA). These cells were incubated at 55°C for 130 min and were then heated to 98°C for 10 min in order to inactivate the proteinase K.

The 1st PCR was performed with 20 μ L genome extract, neo sense (GCCTGCTTGCCGAATATCATGGTGAAAT) and 3' β C antisense (GGCATGTGGGAAATAATTGCACATAAGGA) primers, 1 \times PCR buffer, 0.5 U Ex Taq polymerase (Takara Co., Japan) and 200 μ M of dNTP mixture as follows; pre-denaturation at 94°C for 2 min, denaturation at 94°C for 30 s, annealing at 66°C for 30 s, and extension at 72°C for 5 min, 35 cycles from denaturation to extension, and final extension at 72°C for 10 min. The PCR products were confirmed by electrophoresis on 0.8% agarose gel. As a result, a band of approximately 3.2 kb was confirmed.

For accurate analysis of knock-in colonies by 2nd PCR, the genome DNA were isolated from the positive cells of the 1st PCR analysis, and 2nd PCR amplification was conducted with the same conditions of the 1st PCR. The PCR products (20 μ L) were confirmed by electrophoresis on 0.8% agarose gel.

Southern blot analysis

Final confirmation of knocked-in cells was performed by southern blot analysis. Ten μ g of the genomic DNA was digested with *EcoRV* (Takara Co., Japan), followed by purification. The DNA was electrophoresed on 0.8% agarose gel, and was then transferred on nitrocellulose membrane (Bio-Rad Co., USA). The membrane was washed with 2 \times saline-sodium citrate (SSC) buffer for 30 s. The DNA was cross-linked onto the membrane by using a UV crosslinker, followed by hybridization in the solution containing 5 \times saline-sodium phosphate EDTA (SSPE), 5 \times Denhardt's (Sigma Chemical Co., USA), 1% sodium dodecyl sulfate (SDS) (w/v) and 50% formamide (w/v) (Sigma Chemical Co., USA) at 42°C for 15 h. A probe was prepared by using a 600 bp/*SpeI-EcoRI* DNA fragment containing the porcine β -casein exon 9, random labeling kit (Amersham, Amersham, England) and (α -32P) dCTP (110 TBq/mmol, Amersham, England). A neo probe was also prepared by using 600 bp/*PstI* DNA fragment from pKJ2 plasmid. The concentration of the probe for hybridization was one million cpm/mL hybridization solution. After hybridization, the membrane was washed three times with 0.2% SSC and 0.1% SDS (w/v) at 68°C for 20 min, exposed on X-ray film (Sigma Chemical Co., St Louis, MO, USA) at -80°C for 4 days, and then developed.

RESULTS

Construction of knock-in vector for expression enhanced green fluorescent protein or human fibroblast growth factor 2 on the porcine β -casein locus

Diagram of knock-in vector for expression of EGFP or hFGF2 on the porcine β -casein locus was shown in Figure 1A. The knock-in vector was constructed using 5' and 3' homologous arms from β -casein genomic DNA with human FGF2 or EGFP, polyA signal, positive and negative selection marker. The 5' homologous arm of the knock-in vector was 5 kb containing a promoter, exon 1, and intron 1, and the 3' homologous arm was 2.7 kb containing exons 5, 6, and 7. The start codon (ATG) site of exon 2 of porcine β -casein gene was replaced with ATG of human basic FGF2 or EGFP, which could be secreted protein by the porcine β -casein promoter. *Neo* gene was used as a positive selection marker and diphtheria toxin A was used as a negative selection marker. These selection markers did not contained a polyA signal. A NLS was inserted in the 5' region of the knock-in vector to easily enter cells. In this study, the knock-in vector was designed using a positive-negative selection marker and a poly A trap strategy. Homologous recombination depending on the knock-in vector is shown in Figure 1A.

Expression of porcine β -casein knock-in vector in mouse mammary gland HC11 cells

Because, knock-in vectors constructed in this study contained about 3 kb of porcine β -casein promoter and were designed for the insertion of ATG of EGFP or hFGF2 instead of ATG of porcine β -casein gene, we analyzed the expression of EGFP protein by western blot using protein isolated from cells and media of HC11 mouse mammary epithelial cell transfected EGFP knock-in vector. The EGFP expression was detected in the cells and media of cells transfected with the EGFP knock-in vector. However, EGFP protein was more expressed into the media than HC11 cells (Figure 1B).

Efficiency of gene targeting in porcine somatic cells

Primary porcine ear fibroblasts were used for electroporation with the knock-in vector. G418-resistant colonies were screened by 3' PCR (1st PCR) with neo sense (primer A, a sequence from the 3' end of *neo* gene) and porcine β -casein anti-sense (primer B, a sequence from the intron 7 in sequence located outside the 3' recombination arm) primers (Figure 1A). Thus, only through successful targeting of the porcine β -casein locus would the expected 3.0 kb PCR products be obtained. The PCR analysis was performed using 387 colonies in the fibroblasts transfected FGF knock-in vector. Also, 405 G418 resistant colonies were analyzed by PCR in the fibroblasts transfected with

Table 1. Efficiency of gene targeting at the β -casein gene locus with knock-in vector for EGFP and human FGF2 expression

Knock-in vector	Source of cells	No. of cells transfected	No. of G418 ^R colonies analyzed by PCR	No. of PCR positive colonies	No. of southern blot-positive colonies (%)
hFGF2	Male	5×10 ⁶	387	4	2
EGFP	Male	5×10 ⁶	405	2	1
Total		1×10 ⁷	792	6	3 (0.38)

EGFP, enhanced green fluorescent protein; FGF2, fibroblast growth factor 2; PCR, polymerase chain reaction.

the EGFP knock-in vector. As a result, four positive colonies were identified in fibroblasts transfected with the FGF knock-in vector. Finally, two positive colonies were confirmed by southern blot analysis in male ear fibroblasts (Table 1). Moreover, when the EGFP knock-in vector was introduced into ear fibroblasts, only one colony was identified by PCR and southern blot analysis (Table 1).

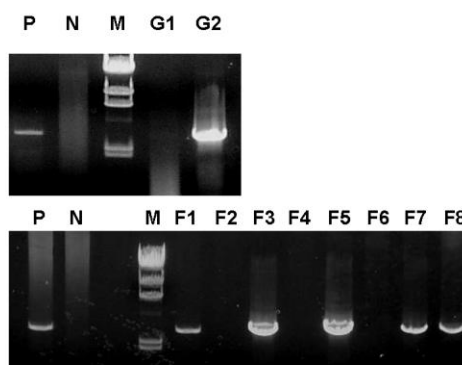
Figure 2A shows the 3' PCR results for a representative group of G418-colonies. In the fibroblast transfected FGF knock-in vector, the G2 colony showed a strong band (upper panel). Therefore, two colonies (F3 and F5) showed a strong band and other colonies (F1, F7, and F8) presented a weak band in the fibroblast transfected FGF knock-in vector (Figure 2A lower panel). Therefore, to further confirm successful targeting at the porcine β -casein locus, we carried out southern blot analysis using porcine β -casein

exon 9 and neo probe in the FGF knock-in fibroblast. As a result of southern blot analysis, a mutant allele was identified in F3 and F5 colonies (Figure 2B). When the Exon9 - probe was used, 11.5 kb of wild type allele and 6.1 kbp of mutant allele were identified in FGF Knock-in fibroblasts (Figure 2B upper). Also, 6.1 kb of mutant allele was identified in the same Knock-in fibroblasts (Figure 2B under) hybridized with neo-probe. The efficiency of gene targeting at the porcine β -casein locus was 0.38%.

DISCUSSION

In this study, knock-in vectors were constructed to express human FGF2 and EGFP using the porcine β -casein gene. Expression of EGFP in the knock-in vector was analyzed in the mouse mammary gland cell line, HC 11

A) PCR analysis



B) Southern blot analysis

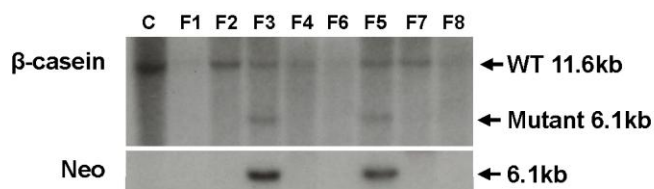


Figure 2. Analysis of knock-in fibroblasts by PCR and southern blot analysis. (A) Representative EtBr stained agarose gel of PCR amplicons from primer A and B of G418-resistant colonies, selected from a transfection with linear knock-in vector. M, size marker (γ /HindIII); N, negative control; P, positive control; Number, G418-resistant colonies. The upper panel indicates the analysis of hFGF2 knock-in fibroblasts and the lower panel indicates the analysis of hFGF2 knock-in fibroblasts. (B) Analysis of hFGF2 knock-in fibroblasts by southern blot. The wild type allele was detected with a 11.6 kb band and mutant alleles, depending on the homologous recombination, which was detected with a 6.1 kb band. Control hybridization with Neo probe is only detected in the knock-in fibroblasts. PCR, polymerase chain reaction; EtBr, ethidium bromide; EGFP, enhanced green fluorescent protein; hFGF2, human fibroblast growth factor 2.

cells. Also, we successfully isolated knock-in fibroblast for nuclear transfer.

Previously, it was reported that the expression of transgenes in transgenic animals was dependent on the insertion site of the transgene, structure of the expression vector, and other related factors (Houdebine et al., 2000). In previous studies, knock-in mice that targeted replacement of the mouse apolipoprotein E gene with the human apolipoprotein E (human APOE) allele were produced, and the human *APOE* gene was expressed under the natural regulation of this protein by the gene regulatory sequence of the mouse *APOE* gene (Sullivan et al., 1997; Sullivan et al., 1998; Hamanaka et al., 2000). In addition, it has been reported that the exogenous *htPam* gene was targeted to the β -casein locus in a goat somatic cell using a knock-in system (Shen et al., 2007).

Therefore, we examined whether knock-in vector containing the porcine β -casein gene could be used to target the porcine β -casein locus for the stable expression of EGFP or hFGF2 using the gene regulation sequence of the porcine β -casein locus. In this experiment, the knock-in vector was constructed using the porcine β -casein genome locus, neo gene as the positive selection marker and diphtheria toxin A as the negative selection marker. G418 was then used to select the somatic cells in which homologous recombination with the target vector occurred, since only the cells containing the target vector can survive under these conditions due to the expression of the negative marker, diphtheria toxin A. A gene targeting vector that contained the neo gene as a positive selection marker and diphtheria toxin A as a negative selection marker was previously used in mouse gene targeting with a mouse embryonic stem cell line (Yanagawa et al., 1999). Therefore, the knock-in vector constructed in this study may function normally in porcine somatic cells.

The EGFP expression was observed in the mouse mammary gland HC11 cells that had been transfected with the knock-in vector. Also, EGFP protein was secreted into the cell culture media more than the cell. The EGFP was under the control of the 3kb promoter in the 5' arm of the knock-in vector, since the promoter in the 5' arm of the knock-in vector may have basic transcriptional components. These results indicated that the expression of *EGFP* gene was regulated by the gene regulatory sequence of porcine β -casein gene in the mammary gland epithelial cells. A similar result was reported for green fluorescent protein (GFP) expression in somatic cells transfected with knock-in vector, where the cells containing the knock-in vector stably expressed GFP (Shen et al., 2005). In addition, Shen et al. constructed a knock-in vector containing the human tissue plasminogen activator mutant gene, and somatic cells were successfully transfected with this vector via homologous recombination. They then made cloned embryos using the somatic cells containing this knock-in vector, and GFP

expression was observed in the embryo. They used the neo gene as a positive selection marker and thymidine kinase as a negative selection marker. Using gene-targeted cell lines as donor cells for nuclear transfer, a total of 600 reconstructed embryos were obtained, and 146 developed cloned embryos were transferred to 16 recipient goats, three of which became pregnant at day 90 (Shen et al., 2007).

In this study, knock-in somatic cells, which may be used to produce a mammary gland bioreactor, were generated by gene targeting to the β -casein gene locus using a human FGF and EGFP knock-in vector. Previously, knock-in somatic cells were introduced into goat to produce recombinant protein from mammary glands (Shen et al., 2004). An efficient strategy to promote homologous recombination is required to target specific genes in the somatic cells of domestic animals. The targeting frequency in somatic cells varied from 9×10^{-5} cell to 1.6×10^{-7} per electroporated cells (Denning and Priddle, 2003). Several knock-in modifications have been reported in goat using the β -casein gene locus and somatic cells (Shen et al., 2004; Shen et al., 2005; Li et al., 2006; Shen et al., 2007). In this study, we isolated 3 colonies from 875 G418 resistance colonies. The targeting efficiency was 5×10^{-6} per electroporated cells. These results indicated that this knock-in system can be used for the development of knock-in cells for the expression of interesting genes on the β -casein gene locus. In the study, two of five colonies in the FGF knock-in fibroblast were detected by southern blot analysis. Three PCR positive colonies were not detected by southern blot analysis. We thought that these results indicated that some PCR-positive signal may come from PCR artifacts. However, these southern results strongly indicated that knock-in fibroblast had insertions of targeting vectors on the porcine β -casein gene locus.

CONCLUSION

These experiments indicate that a human FGF2 and EGFP expressing knock-in vector was constructed and transfected into mouse mammary gland HC11 cells. Thus, the expression of FGF2 and EGFP in mammary glands can potentially be accomplished using HC11 cells. Also, we isolated knock-in cells that introduced these knock-in vectors. These results indicated that knock-in cells were established for efficient and reproducible gene targeting in porcine ear fibroblasts by inserting the exogenous human FGF and EGFP cDNA into the β -casein gene locus. Knock-in cells may be used in the production of transgenic porcine that expressed EGFP or hFGF2 on the porcine β -casein gene locus.

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