



Knocking-in of the Human Thrombopoietin Gene on Beta-casein Locus in Bovine Fibroblasts*

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ABSTRACT : Animal bioreactors have been regarded as alternative tools for the production of limited human therapeutic proteins. The mammary glands of cattle are optimal tissues to produce therapeutic proteins that cannot be produced in large amounts in traditional systems based on microorganisms and eukaryotic cells. In this study, two knock-in vectors, pBCTPOKI-6 and pBCTPOKI-10, which target the hTPO gene on the bovine beta-casein locus, were designed to develop cloned transgenic cattle. The pBCTPOKI-6 and pBCTPOKI-10 vectors expressed hTPO protein in culture medium at a concentration of 774 pg/ml and 1,867 pg/ml, respectively. Successfully, two targeted cell clones were obtained from the bovine fibroblasts transfected with the pBCTPOKI-6 vector. Cloned embryos reconstructed with the targeted nuclei showed a lower *in vitro* developmental competence than those with the wild-type nuclei. After transfer of the cloned embryos into recipients, 7 pregnancies were detected at 40 to 60 days of gestation, but failed to develop to term. The results are the first trial for targeting of a human gene on the bovine milk protein gene locus, providing the potential for a large-scale production of therapeutic proteins in the animal bioreactor system. (**Key Words** : Animal Bioreactor, Bovine Beta-casein Gene, Knock-in, Human Thrombopoietin (hTPO) Gene, Bovine Fibroblasts, Somatic Cell Nuclear Transfer (SCNT))

INTRODUCTION

Advances in animal genomics have accelerated production of genetically-modified domestic animals. So far, many transgenic domestic animals have been generated as animal bioreactors; in these systems, human proteins for disease therapy could be produced not only at lower cost, but also on a relatively large scale. The general strategy for

protein production in animal bioreactor systems is to target transgenic gene expression in the mammary gland using milk protein genes (Clark, 1998). These transgenic livestock have been developed by a universal pronuclear injection method. However, the method for producing transgenic animals has some limitations such as low transgene expression and unpredictable genetic events (e.g., silencing of developmental genes) by random integration (Eyestone, 1994). Moreover, ectopic expression of the transgenes might cause early embryonic lethal (Gao et al., 1999). Thus, one strategy to address these challenges is to develop an efficient system that can induce transgene expression strictly in the mammary gland and only during lactation. Beta-casein is an abundantly expressed milk protein that accounts for more than 20% of the total milk protein in mice (Kumar et al., 1994). Despite its high concentrations, deficiency in the gene encoding beta-casein did not affect viability of the transgenic animals or their ability to lactate and rear offspring. Therefore, the beta-casein gene has been regarded as an optimal candidate to direct the expression of foreign genes to be secreted into and harvested from the milk of transgenic animals. The specificity of the endogenous milk protein gene in the mammary gland indicate that its regulatory elements could

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be used to direct the expression of foreign genes in the tissue without lethality in embryonic or post-natal development, as observed with unregulated expression of other transgenes (Clark, 1998). Gene-targeting, defined as the introduction of site-specific modifications in the genome, is a powerful tool for tissue-specific expression of recombinant proteins. Application of this technology in mice is dependent on the ability to isolate, maintain, and genetically manipulate embryonic stem cells. However, despite multiple attempts for more than a decade, similar cell lines have not been isolated in domestic species (Piedrahita, 2000). Instead of, somatic cell nuclear transfer (SCNT) using differentiated donor cells can generate physiologically normal clones, circumventing the need to isolate the elusive ES cells (Cibelli et al., 1998). The SCNT technique using somatic cells also allows generation of gene-targeted livestock. Generation of the first knock-in sheep opened up a new field of therapeutic protein production in transgenic large animals (McCreath et al., 2000).

TPO is one of the major hematopoietic regulators that function in megakaryocytopoiesis, or the proliferation and differentiation of megakaryocytes resulting in platelet production. Recombinant TPO is shown to ameliorate thrombocytopenia in animal models, suggesting the potential for its use in therapeutic applications (Grossmann et al., 1996; Fanucchi et al., 1997).

This study was to develop expression vectors for production of hTPO in the milk. For generating knock-in constructs to replace a mammary gland-specific gene locus, the bovine beta-casein gene which transcribes specifically in milk during late pregnancy and lactation was targeted. The hTPO gene with antibiotic selection marker gene was replaced between exon 2 and intron 4 of the bovine beta-casein gene, generating pBCTPOKI-10 and pBCTPOKI-6 vectors which contain 10 kb and 6 kb long arms, respectively. Two targeted-clones were obtained from pBCTPOKI-6 vector after transfection into bovine fibroblasts. Reconstructed embryos containing the beta-casein gene targeted genome could develop to the blastocyst stage. After embryo transfer, 7 pregnancies were sustained to gestational day, 60 days, but failed to develop to the term. The pBCTPOKI-6 vector established in this study will be useful for the production of valuable proteins in animal bioreactors.

MATERIALS AND METHODS

Construction of knock-in vectors

Bovine beta-casein promoter has been employed to express human genes (Kim et al., 1999). Schematic depictions of the targeting constructs are shown in Figure 1A and B. The 10-kb beta-casein gene from the pBC10

vector (Sohn et al., 2003) was used as the long arm of the pBCTPOKI-10 vector (Figure 1A). The sequence spanning introns 4 to 8 of the bovine beta-casein gene (sequence 4,676 to 7,898) was inserted as the short arm in the pBCTPOKI-10. The short arm region was amplified from bovine genomic DNA using primers (5'-attcagtcga gtggaacataaactttcagcc-3' and 5'-catatgtcgactgtgagattgta ttttgact-3') and ligated into the pGEM-T vector (Promega); the fragment was subcloned into the Sall site of pBluescriptII SK(+) (Stratagene). A part of *neo* gene (sequence 2423 to 5111) was amplified from the pMAMneo vector (CLONTECH) using primers (5'-cgtaggatccgat ccgctg-3') and (5'-cgatgatatccagacatga-3') and ligated to the BamHI and EcoRV of the pBC10 vector. The short arm was ligated into the EcoRV and Sall sites of the pBC10 vector harboring the *neo* gene fragment. The hTPO cDNA encoding the full-length gene (1 kb) flanked by a 300 bp poly(A) addition signal sequence of the bovine growth hormone gene (Kim et al., 1999) was selected as a transgene. The 1.3 kb transgene was ligated into the SacII and NotI sites between the long arm and the *neo* gene, resulting in the pBCTPOKI-10 vector construct (Figure 1A). The pBCTPOKI-10 vector was ligated to the AatII and Sall sites of the pGEM-7Zf vector (Promega), generating the pBCTPOKI-6 vector (Figure 1B). For construction of a control vector used as a control for long-range PCR analysis, 591 bp DNA fragment harboring intron 8 and exon 9 corresponding to sequence 7,888 to 8,479 of bovine beta-casein gene was amplified by PCR from genomic DNA using primers (5'-ctgctcgagacagtcagatattggacttaa-3' and 5'-ttgactcgagtggtaggaaatagattcttaa-3'). The PCR products were ligated into Sall sites at 3' short arm locus of the pBCTPOKI-10 vector. The pBCTPOKI-6 and pBCTPOKI-10 vectors were purified using QIAfilter Plasmid Midi kits (Qiagen) and then, linearized by Sall and AatII digestion, respectively.

Preparation and culture of bovine fibroblasts

Experiments were conducted according to the Animal Care and Use Committee guidelines of the National Livestock Research Institute of Korea. Bovine fibroblasts were isolated from ear skins of a 2 year-old cow that produced over 12,000 kg milk a year. After washing twice with PBS (Gibco, Invitrogen), the tissues were minced with a surgical blade on a 100 mm culture dish and were incubated as previously described (Koo et al., 2000).

Transfection

A total of 3.6×10^5 bovine fibroblast cells at passage 2 or 3 were plated in 6-well culture dishes and transfected with 2 μ g of linearized DNAs using the LipofectamineTM 2000 reagent (Gibco) in 2 ml of OPTI-MEM (Gibco) following the procedures recommended by the manufacturer. After 24

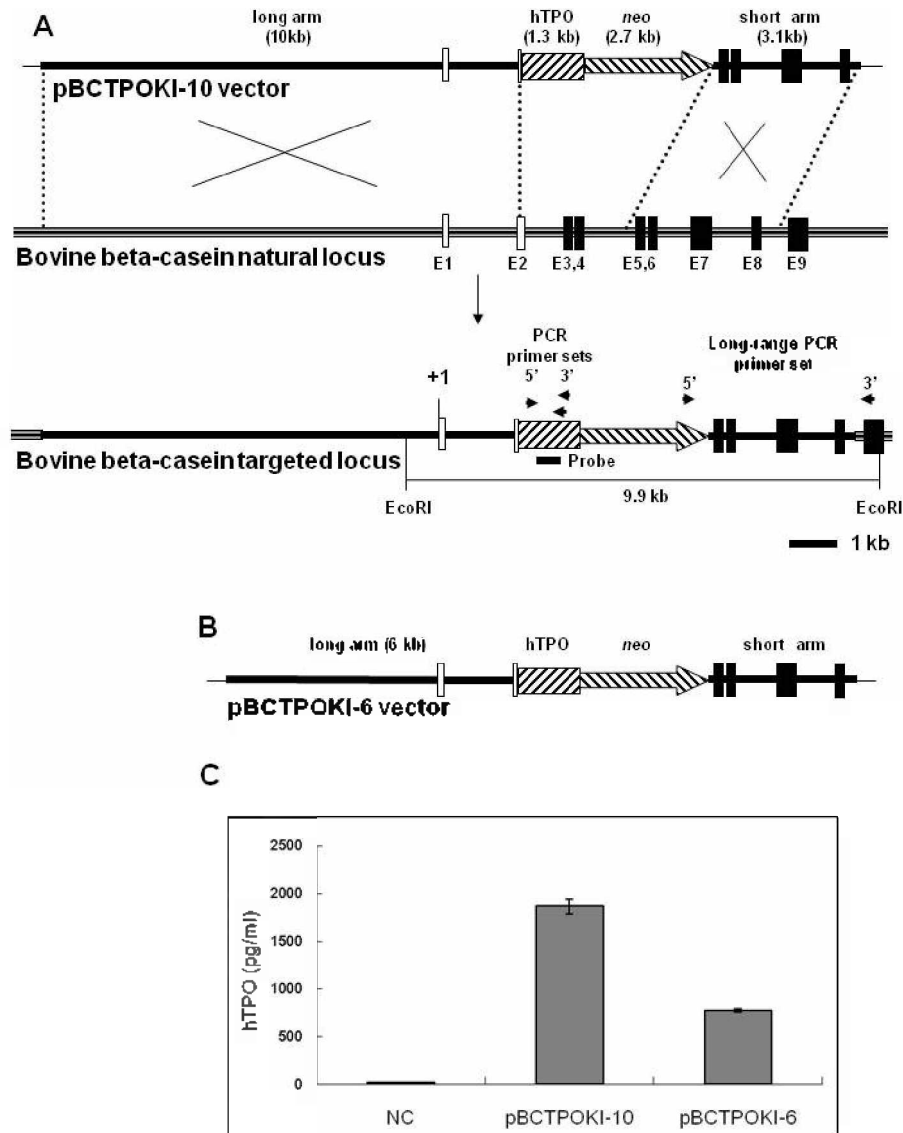


Figure 1. Construction of bovine beta-casein gene knock-in vectors. (A) A schematic depiction of the pBCTPOKI-10 vector containing a long arm (10 kb), hTPO gene (1.3 kb), *neo* gene (2.7 kb), and a short arm (3.1 kb). The long and short arms of the pBCTPOKI-10 vectors are homologous to the beta-casein gene in the bovine genome and double crossover results in replacement of the endogenous beta-casein gene with the sequence in the vector, resulting in knock-in of the hTPO gene. The untranslated exons (E1 and E2) and translated exons (E3 to E9) of the beta-casein gene were shown as white and black boxes, respectively. PCR primer positions for transgenic and targeting (long-range PCR) analyses were indicated as arrows. Location of a probe, EcoRI sites and the size of EcoRI genomic fragments for Southern analysis were indicated. (B) The pBCTPOKI-6 vector consists of a long arm (6 kb) and the same hTPO gene, *neo* gene and a short arm as pBCTPOKI-10 vector. (C) Expression of hTPO protein in culture medium of HC11 cells transfected with pBCTPOKI-6 or pBCTPOKI-10 vectors. Expression levels of hTPO protein secreted to culture media of pBCTPOKI-6 and pBCTPOKI-10 transfected cells were 1,867 pg/ml and 774 pg/ml, respectively. Each column represents the mean \pm standard error (N = 9). The negative control (NC) was untransfected cells subjected to hormone induction.

h, the transfected cells were split into two 100 mm culture dishes, and the following day, 0.6 mg/ml G418 (Gibco) was added to the culture medium. Four to six days later, drug-resistant cell clones of 2-3 mm in diameter were formed. Ten days later, the cell clones were isolated using cloning cylinders and replated into individual wells of 96-well culture dishes. Each cell clone was cultured until confluent.

Prior to transferring cell colonies in 6-well dishes, half of each colony was subjected to PCR analysis.

ELISA

HC11 mammary epithelial cells were cultured as previously described (Burdon et al., 1994). Approximately 1 μ g of linear pBCTPOKI-10 and pBCTPOKI-6 vectors

was transfected overnight into hormonally responsive HC11 cells grown to 90-95% confluence on 100 mm culture dishes using Lipofectamine™ 2000 reagent, respectively. After 14 days of selection, cell clones were trypsinized and expanded as a pool of clones. For hormone induction, cells grown to confluence were further cultured in RPMI 1640 medium containing 1% fetal bovine serum and 5 µg/ml insulin for 2 days, and subsequently cultured in the low serum medium with 5 µg/ml insulin, 5 µg/ml ovine prolactin (Sigma) and 1 µM dexamethasone (Sigma) for 4 days. Quantification of recombinant hTPO secreted in the medium was measured by using hTPO-specific ELISA following the manufacturer's instructions (R & D systems).

Screening of gene-targeted cells

Transgenic cell clones were screened among the G418-resistant colonies by PCR. Genomic DNA was extracted from half of the cells cultured in 6-well dishes using the AccuPrep Genomic DNA Extraction Kit (Bioneer), and the AccuPower PCR Premix (Bioneer) was used for the PCR reactions. Primers for the hTPO cDNA were as follows: 5'-ggagctgactgaattgctcctctgt-3' and 5'-cctgacgcagagggtggaccctcc-3'; thermal cycle conditions were as follows: 94°C, 2 min; 30 cycles of 94°C for 1 min, 65°C for 30 s, and 72°C for 45 s; 72°C, 10 min. Long-range PCR was carried out to detect gene-targeted cell clones using the AccuPower HL PCR Premix (Bioneer); one PCR primer (5'-ccacacagcatagagtgctctgc-3') binds to the 3' end of the *neo* gene within the transfected vector constructs, and the other (5'-ccacagaattgactgcgactgg-3') to a region in intron 8 of the bovine beta casein, which is outside of the vector sequence (Figure 1A). The thermal cycle conditions were as follows: 92°C, 2 min; 35 cycles of 92°C for 20 s, 65°C for 45 s, 68°C for 3 min; 68°C, 10 min.

Southern blot analysis

Cell clones were expanded into two 100 mm culture dishes; genomic DNA was extracted from one dish, and at least 10 µg DNA from each clone was digested with EcoRI overnight at 37°C, and subsequently separated on a 0.75% agarose gel in TAE buffer overnight. The DNA was transferred onto a positively charged nylon membrane (Roche) and hybridized with the DIG-labeled hTPO cDNA fragments. For random primed DIG-labeling, the blots were then probed with primers following the guidelines provided by the manufacturer (Roche), and PCR was performed using the PCR DIG labeling mix (Roche), Taq DNA polymerase (QIAGEN) and the primers 5'-ggagctgactgaattgctcctctgt-3' and 5'-ctgacgcagagggtggaccctcc-3'. The thermal cycle conditions were as follows: 94°C, 3 min; 30 cycles of 94°C for 45 s, 52°C for 30 s, 72°C for 1 min; 72°C, 10 min.

Nuclear transfer

SCNT was performed as described previously (Koo et al., 2002). After 7 day culture of the reconstructed embryos, blastocyst formation was observed. The resultant blastocysts were transferred to recipients by a non-surgical method. Pregnancies were monitored by ultrasonography (7-4 MHz; SonoSite®, Bothell, USA).

PCR was used to screen transgenic embryos. The embryos were lysed in lysis buffer comprising 20 mM Tris pH 8.5, 0.9% Nonidet P-40 (BIOSESANG), 0.9% Tween 20 (Sigma), and 0.4 mg/ml Proteinase K (QIAGEN) at 55°C for 1 h, followed by heat treatment at 100°C for 5 min. Using the AccuPower PCR Premix, nested PCR was conducted. The first PCR primers for the hTPO cDNA were as follows: 5'-ggagctgactgaattgctcctctgt-3' and 5'-cctgacgcagagggtggaccctcc-3'. The nested PCR primers were as follows: 5'-ggagctgactgaattgctcctctgt-3' and 5'-gagacggacctgtccagaaagctg-3'. Thermal cycle conditions were as follows: 94°C, 2 min; 30 cycles of 94°C for 1 min, 65°C for 30 s, and 72°C for 45 s; 72°C, 10 min.

RESULTS

Generation of knock-in vector constructs and expression of a therapeutic protein

We constructed two knock-in vectors to direct expression of the hTPO gene under control of the endogenous beta-casein promoter at the bovine beta-casein locus (Figure 1A and B). Both of the pBCTPOKI-6 and pBCTPOKI-10 knock-in vectors contain hTPO cDNA and genomic sequences homologous for the bovine beta-casein gene. They are different in the length of 5'- long arm (6 kb and 10 kb), respectively. Both knock-in vectors were designed to replace exons 2, 3, and 4 of the endogenous beta-casein gene downstream of the untranslated exon 1 with the hTPO and *neo* gene as a selection marker. To test activity of the vectors, both knock-in vectors were transfected into an HC11 cell line derived from a mouse mammary gland, respectively. After selection with G418, resistant colonies were cultured to confluence and induced with lactogenic hormones. ELISA assay showed that the transfectants secreted hTPO protein in media at the level of 774 pg/ml (pBCTPOKI-6) and 1,867 pg/ml (pBCTPOKI-10), respectively (Figure 1C). Only background levels of expression were detected in the negative control. The different levels of hTPO protein expression by pBCTPOKI-10 and pBCTPOKI-6 vectors may be related to sequences of long arms consisted of beta-casein gene promoter regions. The results provide a hint that the knock-in vectors designed in this study could express hTPO in the mammary gland system.

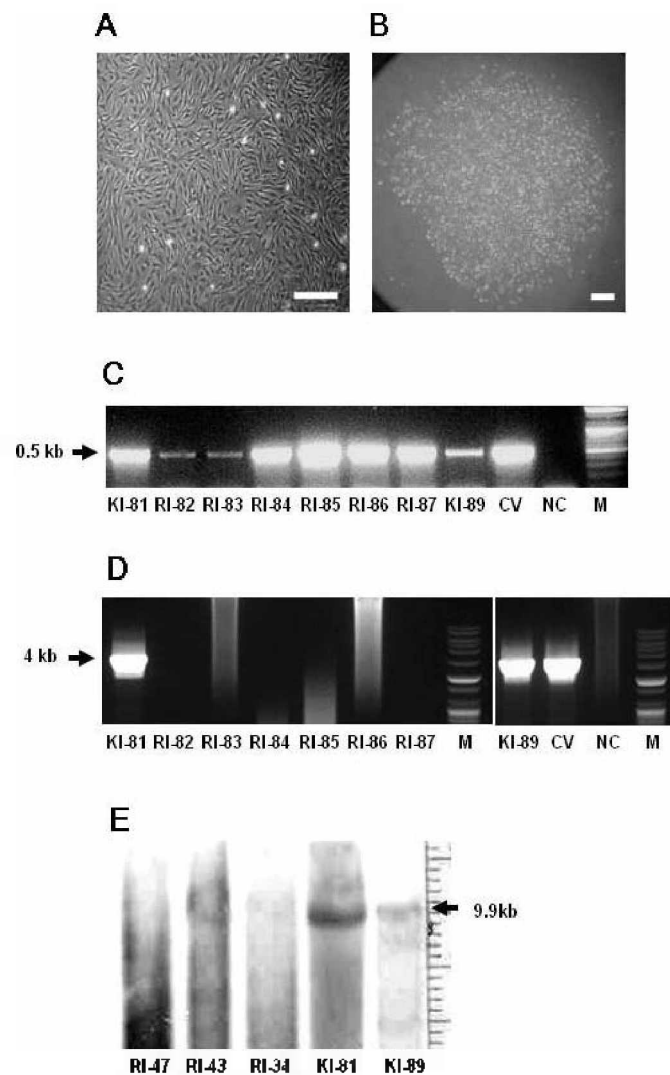


Figure 2. Analysis of cell clones from bovine fibroblasts transfected with targeting vectors. Morphologies of non-transfected bovine fibroblasts (A) and a selected bovine fibroblast cell clone (B). The bars represent 200 μ m. (C) Bovine fibroblasts transfected with pBCTPOKI-6 vector were identified as transgenic (KI-81, RI-82 to 87 and KI-89) by PCR analysis. (D) KI-81 and KI-89 cell clones were confirmed as targeted by long range PCR. (E) The KI-81 and KI-89 cell clones were confirmed as targeted by Southern blot analysis, showing specific 9.9 kb signals. CV, a control vector; NC, non-transfected bovine fibroblasts; M, a marker.

Production of targeted cell clones

To obtain targeted cell clones, bovine fibroblasts cultured to 90% confluence (Figure 2A) were transfected with pBCTPOKI-6 and pBCTPOKI-10 vectors using LipofectamineTM 2000 transfection reagent, respectively. Following G418 selection, distinct cell clones were observed (Figure 2B). Although 165 (94%) of 176 pBCTPOKI-10 clones were confirmed as transgenic as

indicated by the presence of a 0.5 kb PCR product, no targeted clone was detected (Table 1, Figure 2C). In the pBCTPOKI-6 vector, 155 (95%) of 163 cell clones were confirmed as transgenic by PCR analysis (Figure 2C and Table 1). Exact targeting of pBCTPOKI-6 vector at the endogenous beta-casein locus was identified in two cell clones (KI-81 and KI-89), as indicated by the presence of a 4 kb PCR product (Figure 2D and Table 1). Two targeted

Table 1. Transgenic and targeting rates of bovine fibroblast cell clones

Vector types	No. analyzed clones	No. transgenic clones (%)	No. targeted clones (%)
pBCTPOKI-10	176	165 (94% ^a)	0 (0% ^b)
pBCTPOKI-6	163	155 (95% ^a)	2 (1.3% ^b)

^a Transgenic rate (%); No. transgenic cell clones/ No. analyzed cell clones \times 100.

^b Targeting rate (%); No. targeted cell clones/No. transgenic cell clones \times 100.

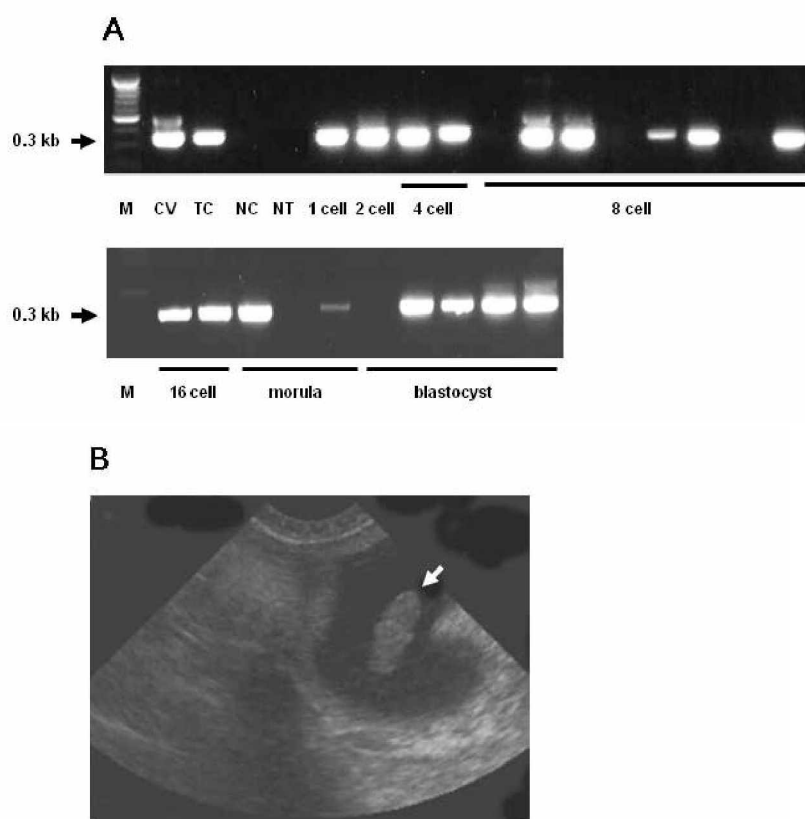


Figure 3. (A) PCR analysis of embryos reconstructed with the KI-81 cell clone. Of the 22 reconstructed embryos on various developmental stages, seventeen were confirmed as a transgenic. M, a marker; CV, a control vector; TC, the targeted cell clone (KI-81); NC, non-transfected bovine fibroblasts; NT, no template. (B) The cloned fetus at day 60 of pregnancy was monitored by ultrasonography. The arrow indicates the location of the fetus.

clones were further confirmed by Southern blot analysis; a specific 9.9-kb signal band was detected after hybridization using a probe from the hTPO cDNA (Figure 2E).

Generation of cloned embryos

Next experiments were carried out to examine whether the targeted cells could be used as donor cells in the SCNT. Transgenes of cloned embryos were analyzed at various developmental stages by PCR amplification for hTPO. Twenty-three (88%) of 26 cloned embryos derived from KI-81 cells and 43 (83%) of 52 from KI-89 cells were positive for the transgene (Figure 3A and Table 2). This result indicates that most of the single cells consisting of the cell

clones contain targeted alleles. Next, *in vitro* developmental competence of cloned embryos with the targeted nuclei was investigated (Table 3). Whereas Fifty-nine (27.3%) of 216 cloned embryos reconstructed with wild-type cell nuclei were developed to the blastocyst stage, forty-two (14%) of 190 embryos cloned with the KI-81 targeted nuclei and forty-four (18%) of 168 embryos cloned with the KI-89 targeted nuclei were developed to the blastocyst stage, showing lower developmental rates than those with wild-type nuclei. The cloned blastocysts were transferred into 21 (KI-81) and 25 (KI-89) recipients by a non-surgical method (Table 4). Among these recipients, total 7 recipients were detected as pregnant at 40 to 60 days of gestation by

Table 2. Transgenic rates of embryos reconstructed with targeted cells

	Cell clones	Developmental stages							Total (%)
		1 cell	2 cell	4 cell	8 cell	16 cell	Morula	Blastocyst	
Analyzed	KI-81	1	1	4	17	-	3	-	26
Transgenic		1	1	4	15	-	2	-	23 (88%*)
Analyzed	KI-89	3	3	6	28	2	4	6	52
Transgenic		3	3	6	22	2	3	4	43 (83%*)

* Transgenic rate (%); No. transgenic cloned embryos/No. analyzed cloned embryos \times 100.

Table 3. *In vitro* development rates of reconstructed embryos with targeted cells

Cell types	No. reconstructed embryos	No. cleaved embryos	No. blastocysts (%)
Wild-type bovine fibroblasts	216	166	59 (27.3%*)
KI-81	190	104	42 (14%*)
KI-89	168	111	44 (18%*)

* Developmental rate (%); No. blastocysts/No. reconstructed embryos×100.

Table 4. Pregnancy rates of cloned embryos transferred into recipients

Cell clones	No. embryos transferred	No. recipients	No. pregnant (%)	No. live-born calves
KI-81	31	21	2 ^a (9.5*)	0
KI-89	35	25	5 ^b (20*)	0

^a Two pregnancies were confirmed at day 40 and 50.

^b Two of five pregnancies were confirmed at day 40, and the others were confirmed at day 60.

* Pregnancy rate (%); No. fetuses at pregnancy/No. embryos transferred×100.

ultrasonography (Figure 3B and Table 4).

DISCUSSION

The animal bioreactor system that could secrete valuable proteins in milk has been considered to be an optimal system in that the system allows the production of high levels of therapeutic proteins without sacrifice or detriment of animals. However, few studies have reported successful knock-in of a therapeutic gene into the tissue-specific gene in bovine. In this report, we successfully achieved, for the first time, knock-in of the hTPO gene on the beta-casein locus of bovine fibroblasts.

In large animals, gene-targeting events by homologous recombination are extremely inefficient. Furthermore, it has been reported that transcriptionally silent and tissue-specific genes are less efficient for gene-targeting compared to transcriptionally active genes, as they show a lower frequency of homologous recombination (Thomson et al., 2003). In this study, we targeted the transcriptionally silent beta-casein gene and produced 2 (1.3%) targeted clones among 155 transgenic cell clones transfected with pBCTPOKI-6 vectors; however, no targeted clone of 165 transgenic cell clones transfected with pBCTPOKI-10 vectors was detected (Table 1). This difference may be responsible for the length of homologous targeting sequences. It has been reported that the frequency of homologous recombination may be dependent on the length of homologous regions, and that successful gene targeting requires optimal length of the homologous regions in targeting vectors (Thomas and Capecchi, 1987; Scheerer and Adair, 1994). Here, it was shown that the pBCTPOKI-6 vector with a 6 kb long arm is more efficient in homologous recombination events than the pBCTPOKI-10 vector with a 10 kb long arm, indicating that optimization of targeting vector construction may be required for successful gene-

targeting of transcriptionally silent genes in the somatic cells.

Two targeting vectors secreted hTPO protein in HC11 cell culture medium. Interestingly, the expression level of the hTPO protein in the pBCTPOKI-10 was higher than that in the pBCTPOKI-6 (Figure 1C). The pBCTPOKI-10 vector contains longer beta-casein promoter sequences than the pBCTPOKI-6 vector. The difference of the promoter lengths affected the hTPO protein expression levels.

In this study, we successfully developed the knock-in vector by which valuable human gene could be placed on the endogenous bovine beta-casein gene locus. The knock-in vector was inserted into the target site, beta-casein locus, in bovine fibroblasts. Moreover, embryos cloned with the targeted nuclei developed to the pre-implantation stage and early gestation. Therefore, our knock-in system could be used to produce therapeutic proteins in animal bioreactors.

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