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Theriogenology 72 (2009) 535–541
Theriogenology
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Removal of selectable marker gene from fibroblast cells in transgenic cloned cattle by transient expression of Cre recombinase and subsequent effects on recloned embryo development

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Received 22 November 2008; received in revised form 20 March 2009; accepted 9 April 2009

Abstract

Introduction of selectable marker genes to transgenic animals could create an inconvenience to further research and may exaggerate public concerns regarding biological safety. The objective of the current study was to excise loxP flanked neo^R in transgenic cloned cattle by transient expression of Cre recombinase. Green fluorescent protein gene (GFP) was incorporated to monitor Cre expression; therefore, Cre-expressed cells could be selected indirectly by fluorescence-activated cell sorting (FACS). The neo^R was removed and Cre expressed transiently in GFP-positive colonies; excision of neo^R was confirmed by single-blastocyst PCR in recloned blastocysts, with neo^R-free fibroblast cells as donors. There was no difference ($P > 0.05$) in rates of cleavage (76.0% vs. 68.8%) or blastocyst formation (56.6% vs. 52.9%) between recloned embryos with neo^R-free or neo^R-included donors. The differential staining of recloned blastocysts were similar ($P > 0.05$) in terms of total cell number (124 vs. 122) and the ratio of ICM (Inner Cell Mass) to the total cell number (38.1% vs. 38.2%). Furthermore, pregnancy and calving rates were not different ($P > 0.05$) from those of the control. In conclusion, we successfully excised neo^R from transgenic cloned cattle; the manipulation did not affect the developmental competence of recloned preimplantation embryos. This approach should benefit bioreactor and transgenic research in livestock.
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Keywords: Cattle; Cre recombinase; Neo^R; Recloning; Transgenic cloning

1. Introduction

Transgenic domestic animals are of great importance to biomedical studies such as bioreactor and agricultural research, including improvement of animal product quality, growth rate, disease resistance, reproductive performance, and so forth [1]. Since the first transgenic livestock was born in 1985 [2], microinjection of exogenous DNA into pronuclei of zygotes has been the favored method. However, due to lower intrinsic

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doi:10.1016/j.theriogenology.2009.04.009

efficiencies, along with cost and time, it was generally replaced with a more efficient approach based on somatic cell nuclear transfer (SCNT). With use of this method, it becomes possible to apply precise genomic modification technologies to livestock, such as gene knock-out and knock-in [3]. One of the most critical steps of this approach is obtaining donor cells into which the exogenous genes have been integrated. Accordingly, selectable marker genes were required to detect positive cells and were therefore permanently introduced to the host genome, along with a foreign DNA stable insertion. However, their introduction is undesirable because (1) the existence of foreign marker gene may interfere with the expression of neighbor endogenous gene and generate inconveniences to phenotypical and genetical analysis [4,5]; (2) the small number of available positive selectable marker genes in mammalian cells restricts multiple genetic manipulation in the same host [6]; and (3) it could exaggerate public concerns regarding biological safety. Hence, marker-free technologies are needed.

Bacteriophage P1 Cre, 38-kDa recombinase, could specifically recognize two direct oriented loxP sites and catalyze the deletion of a targeted gene flanked by them in mammalian cells [7]. It is well known that the system of gene excision mediated by Cre-loxP has been universally applied in producing mice with tissue-specific gene targeting, as well as marker gene excision. Traditionally, marker genes are removed by the transient expression of the Cre recombinase in mouse embryonic stem (ES) cells. However, given the low transfection efficiency of ES cells, negative selectable marker genes such as herpes simplex virus thymidine kinase (HSV-tk) have to be used in order to enrich marker-free cells, which may compromise pluripotency by extending the culture period of ES cells [8]. Another method is to infect morulae with replication-deficient recombinant adenoviruses expressing the Cre recombinase [9] or directly introducing transient Cre-expression vectors into fertilized eggs by pronuclear injection [10]. Additionally, it has been demonstrated to be successful in eliminating selective marker genes from transgenic livestock [11,12]. However, these methods, especially adenovirus packaging and pronuclear injection, may be troublesome and technically demanding in transgenic domestic animals.

The objective of the current study was to establish an efficient and convenient approach to eliminating selectable marker genes from transgenic cloned cattle and to investigate the effects of genetic manipulations on in vitro and in vivo developmental potential of recloned embryos.

2. Materials and methods

Chemicals and reagents were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA) unless stated otherwise.

2.1. Vector construction

The coding region of Cre recombinase gene was amplified by employing commercial plasmid PBS185 (Gibco BRL, Grand Island, NY, USA; catalog no. 10347-011), which is designed for transient expression of Cre recombinase in mammalian cells. Then, the cassette was subcloned into the multiple cloning site of pIRESthyg3 (BD Biosciences Clontech, Palo Alto, CA, USA; catalog no. 631620), which contains the internal ribosome entry site (IRES), followed by the hygromycin B phosphotransferase gene as antibiotic gene. Finally, the NotI-IRES EGFP-XbaI fragment amplified by pIRES2-EGFP (BD Biosciences Clontech, catalog no. 6029-1) was harbored to replace the IRES-hyg fragment in order to construct the Cre and GFP (Green Fluorescent Protein) expression vector pCRE-IRES-EGFP (Fig. 1).

2.2. Cell culture and transfection

Bovine transgenic fibroblast (BTF) cells were collected from the ear of a neonatal transgenic cloned calf, bearing human lysozyme (hLys). These cells and human embryonic kidney 293 cells (HEK-293T) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, catalog no. 12800-017) supplemented with 10% fetal bovine serum (FBS; Thermo Scientific HyClone, Logan, UT, USA; catalog no. SH30396.03) at 37.5 °C under 5% CO_2. Cells were seeded (concentration, 4×10^5 to 6×10^5 per well) in 6-well Plates 24 h before transfection and were cultured until achieving 70% to 90% confluence. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen Corporation, Carlsbad, CA, USA; catalog no. 11668-027) according to the manufacturer's instructions.

2.3. Western blotting

Forty-eight hours after transfection, HEK-293T cells were washed twice with PBS (Phosphate Buffered Saline) and then lysed with immunoprecipitation (IP) cell lysis buffer (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China; catalog no. P0013). The BTF cells transfected with pCRE-IRES EGFP were sorted by fluorescence-activated cell sorting (FACS; DakoCytomation, Inc., Ft. Collins, CO, USA), and thereby GFP-

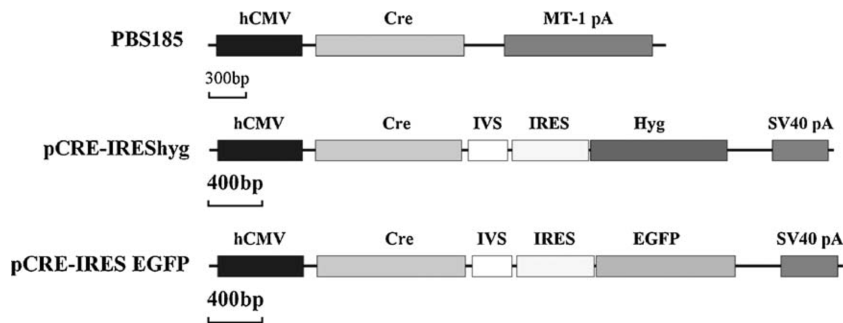

Fig. 1. Construct of the transient Cre and GFP expression vector. IVS, synthetic intron; IRES, internal ribosome entry site; Hyg, hygromycin B phosphotransferase gene.

positive (GFP^+) cells were enriched. After being lysed with IP cell lysis buffer (100 μL/10^6 cells) and incubation on ice for 30 min, cell lysates were centrifuged at 13,000 × g for 5 min at 4 °C. The supernatants were collected for protein separation by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to Amersham Hybond-P PVDF (Polyvinylidene Fluioride) membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK; catalog no. RPN303F). The membranes were incubated with mouse anti-Cre recombinase monoclonal antibody (Abcam Inc., Cambridge, MA, USA; catalog no. ab24607) at room temperature for 1.5 h and stained by the secondary HRP (Horseradish Peroxidase)-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; catalog no. sc-2031) for 1 h. Then, the membranes were visualized using SuperSignal West Dura Extended Duration Substrate (Pierce Manufacturing, Inc., Appleton, WI, USA; catalog no. 34075), with β-actin used as an internal positive control.

2.4. DNA analysis

Genomic DNA was extracted from BTF cells by phenol/chloroform extraction, followed by ethanol precipitation. To detect hLys and floxed neo^R insertions, PCR was done with primer pairs LysF, LysR and SM14, SM1922.

To identify neo^R excision, GFP^+ BTF cells were sorted by FACS (DakoCytomation, Inc.) after transfection and replated into 100-mm dishes (1000 cells/100 mm dish) with DMEM (Gibco BRL) supplemented with 10% FBS (Thermo Scientific HyClone) and 30% to 50% media, which were conditioned by wild-type bovine fibroblast cells (unpublished data). Seven to 9 d later, cell colonies were picked and expanded in 24-well plates. Upon passaging, 2000 to 3000 cells were separated and incubated in 20 μL embryonic lysis buffer (40 mM Tris/HCl [pH 8.0], 0.9% Triton X-100, 0.9% Nonidet P-40, 0.4 mg/mL proteinase K) at 65 °C for 30 min, followed by 10 min at 95 °C. Then the lysate was used as template in a 50-μL PCR reaction using SM14 and SM1922 primer pair as above, with GAPDH (Glyceraldehyde Phosphate Dehydrogenase) amplified as an internal positive control.

To ensure that no Cre or GFP was inserted into the genome of the marker-free cells, negative PCR was performed using primer pairs GFPF, GFPR, creF, and creR. Existence of hLys was confirmed by primer pair LysF, LysR. Primer pairs SM1274, SM2175 and LysF, LysR were used to identify neo^R excision and hLys gene presence in single recloned embryos. All PCR conditions are shown in Table 1. The PCR products were verified by DNA sequencing.

2.5. In vitro maturation

Bovine ovaries were collected from a local abattoir and transported to the laboratory in a vacuum bottle filled with physiologic saline at 25 to 35 °C. Cumulus-oocyte complexes (COCs) were aspirated from follicles (2 to 8 mm in diameter), and oocytes enclosed by at least two to three layers of compact cumulus cells were selected. The maturation medium consisted of tissue culture medium (TCM-199) supplemented with 10% FBS (Thermo Scientific HyClone), 0.01 U/mL follicle-stimulating hormone (FSH), 0.01 U/mL luteinizing hormone (LH), 1 μg/mL β-estradiol, and 1% (v/v) penicillin/streptomycin. Selected COCs were cultured at 38.5 °C in 5% CO_2 for 18 to 20 h.

2.6. Nucleus transfer

Cloned blastocysts were produced as described previously [13]. Briefly, matured oocytes were enucleated by a glass needle to remove the polar body and

Table 1
Primers used for PCR.

Primers	Primer sequences($5' \rightarrow 3'$)	Size (bp)	PCR conditions
LysF	TTATACACACGGCTTTAC	657	35 cycles of 94 °C for 30 sec, 53 °C for 30 sec, and 72 °C for 30 sec
LysR	CAGCATCAGCGATGTTATCT		
SM14	AACCTACTCGAGATAACTTC	1929	35 cycles of 94 °C for 30 sec, 58 °C for 30 sec, and 68 °C for 1.5 min
SM1922	GATGGTCGATAGATAACTTC		
GFP-F	TGCAGTGCTTCAGCCGCTAC	422	40 cycles of 94 °C for 30 sec, 62 °C for 30 sec, and 72 °C for 30 sec
GFP-R	CTCAGGTAGTGGTTGTCGGG		
creF	ACATTTGGGCCAGCTAAAC	411	40 cycles of 94 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 30 sec
creR	CGGAAATCCATCGCTCGACC		
GAPDH-F	TCTCAAGGGCATTCTAGGCTAC	308	35 cycles of 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec
GAPDH-R	TGTGAAGGGCTGTTTACCGA		
SM1274	GCGTTGGCTACCCGTGATATTG	927	40 cycles of 94 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 50 sec
SM2175	CGGAAATGTTGAATACTCATAC		

F, forward primer; R, reverse primer.

surrounding cytoplasm. Successful enucleation was confirmed by Hoechst 33342 staining. Donor cells were then individually transferred into enucleated oocytes. Reconstructed embryos were electrically fused in a chamber filled with Zimmerman cell fusion medium [14] and with two stainless steel electrodes, 24 h after the start of maturation. Cell fusion was induced with two DC pulses (250 V/mm for 10 μs) spacing 1 sec, delivered by a BTX2001 Electro Cell Manipulator (BTX, San Diego, CA, USA). Activation was induced by incubation in 10 μg/mL cycloheximide and 2.5 μg/mL cytochalasin-D in CR1aa medium [15], supplemented with 0.1% (w/v) BSA (Bovine Serum Albumin) for 1 h and cycloheximide (10 μg/mL) alone for 4 h. Then the embryos were further cultured in CR1aa medium supplemented with 0.1% (w/v) BSA for 48 h under 5% O_2, 5% CO_2, and 90% N_2. Cleaved embryos were then selected and cultured for an additional 5 d in CR1aa supplemented with 5% (v/v) FBS (Thermo Scientific HyClone) on cumulus cell monolayers under 5% CO_2 in air.

2.7. Differential staining

Blastocysts were incubated in 5 mg/mL pronase to remove zona pellucida. Then, zona-free blastocysts were immediately transferred to PBS-PVA (Polyvinyl Alcohol), and subjected to three 2-min washes. Thereafter, the blastocysts were recovered in an incubator for 1 h. After recovery and three washes in PBS-PVA, they were transferred to 0.1% Triton-X 100 solution to incubate for 20 sec. Subsequently, these blastocysts were incubated in PI (Propidium Iodide) and Hoechst 33342 (1 and 15 min, respectively). Stained blastocysts were mounted onto a glass microscope slide in one drop of glycerol after three washes. The slide was gently flattened with a coverslip and observed under a fluorescent microscope (Nikon E800; Nikon, Tokyo, Japan) with ultraviolet filter. Blue and pink colors were counted as ICM and TE (Trophectoderm) cells, respectively.

2.8. Embryo transfer

Seven days after activation, blastocysts were selected and transferred to synchronized recipients (two or three embryos/recipient) [16]. To verify pregnancy status, transrectal palpation was done 90, 120, and 240 d after embryo transfer.

2.9. Statistical analysis

All experiments were replicated at least three times. Percentage data, including rates of cleavage and blastocyst formation in the embryo culturing experiment, were analyzed by chi-square analysis. Cell numbers in the control and treatment groups were compared by Student's t-test. All data were expressed as mean ± SEM across the three replicates. All analyses used SAS (Version 9.1; SAS Institute, Cary, NC, USA), and $P < 0.05$ was deemed significant.

3. Results

3.1. Production of hLys gene transgenic cloned cattle with floxed neo^R gene as selectable marker gene

Human lysozyme gene transgenic cloned cattle were produced based on SCNT using oviductal epithelial cells transfected with the hLys gene expression vector as donors (unpublished data). The construct of the hLys expression vector is shown in Fig. 2A. Four transgenic cloned cattle were born in February 2006, all of them

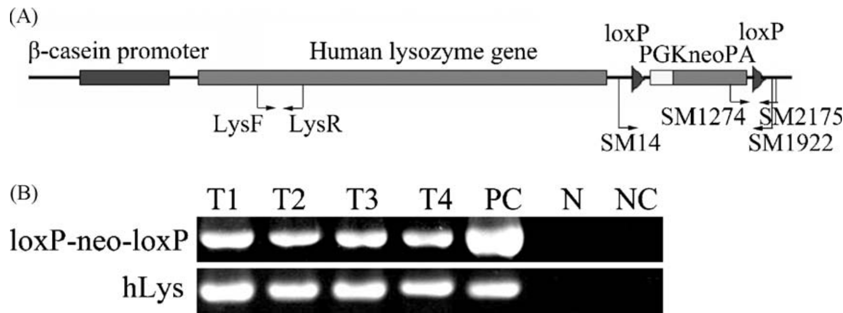

Fig. 2. (A) Schematic representation of the hLys and neo^R gene expression vector. Primer pairs LysF, LysR and SM14, SM1922 were used to identify four transgenic cloned cattle carrying both hLys and floxed neo^R gene, whereas SM1274 and SM2175 were used for single recloned blastocyst PCR (see Fig. 4C). (B) Identification of hLys and floxed neo^R in four transgenic cloned cattle by PCR. The HLys expression vector was set as a positive control (PC), whereas the genome of normal cattle (N) and distilled water (NC) were negative controls. The hLys and floxed neo^R genes were present in the genomes of all four transgenic cloned cattle (T1,T2,T3,T4).

carrying both hLys and selectable marker gene neo^R (Fig. 2B). Sequencing the PCR products showed that neo^R existed between two direct oriented loxP sites.

3.2. Expression of Cre recombinase in mammalian cells

The GFP$^+$ cells expressed Cre recombinase after mammalian cells were transfected with pCRE-IRES EGFP plasmid (Fig. 3).

3.3. Neo^R excision in BTF cells and recloned embryos

Forty-eight hours after transfection, approximately 20% of the BTF cells appeared green, and most of the randomly picked colonies grown from the sorted GFP$^+$ cells were neo^R eliminated (Fig. 4A). The similar efficiency of neo^R deletion was demonstrated among four BTF cell lines. Unexpectedly, one third of the marker-free cell colonies had Cre or GFP inserted into the genome (Fig. 4B). It was noteworthy that 43% of the randomly picked colonies grown from sorted GFP$^+$ cells were free of neo^R, along with no Cre or GFP insertion. As shown in Fig. 4C, floxed neo^R was efficiently removed in single recloned blastocyst from marker-free cells.

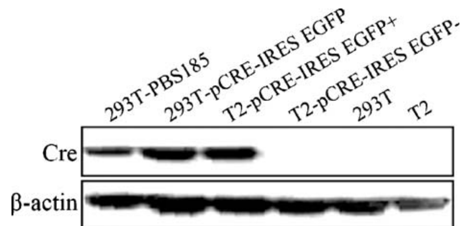

Fig. 3. Expression of Cre recombinase in mammalian cells. Protein extracts from 293T cells transfected with PBS185 plasmid was deemed as a positive control, whereas those from 293T cells and BTF cells were analyzed as a negative control. The Cre recombinase was present in GFP-positive cells after 293T cells and BTF cells were transfected with pCRE-IRES EGFP. In GFP-negative cells, Cre recombinase was absent. Its replica blot was probed with anti-β-actin antibody and served as an internal positive control.

3.4. Development competence of marker-free cells

There were no remarkable differences in terms of the cleavage and blastocyst rates when using marker-free

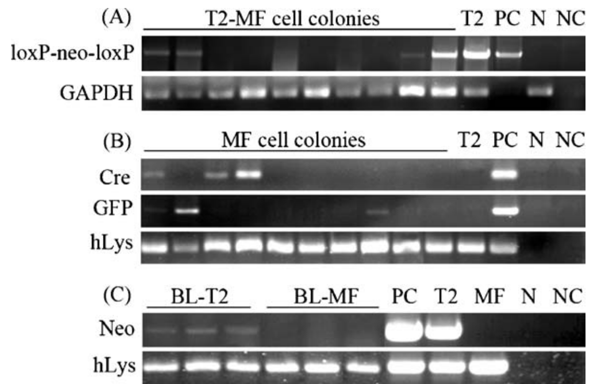

Fig. 4. (A) Verification of neo^R excision. T2-MF cell colonies: cell colonies from a single GFP$^+$ cell that were transiently transfected with pCRE-IRES EGFP. T2, genome of transgenic cloned cattle T2; PC, hLys expression vector; N, genome of normal cattle; NC, embryonic lysis buffer control. (B) Analysis of Cre or GFP integration. MF cell colonies: cell colonies with neo^R excision. T2, genome of transgenic cloned cattle T2; PC, pCRE-IRES EGFP plasmid; N, the genome of normal cattle; NC, water control. (C) Verification of neo^R excision in single recloned blastocyst with marker-free cells as donors. Positive control: recloned single blastocyst using transgenic cloned cattle T2 fibroblast cells as donors (BL-T2). BL-MF: recloned single blastocyst using marker free cells as donors. PC, hLys expression vector; T2, genome of transgenic cloned cattle T2; MF, genome of marker-free cell colonies; N, genome of normal cattle; NC, embryonic lysis buffer control.

cells as donors in comparison with non–marker-free cells ($68.8 \pm 6.8\%$ vs. $76.0 \pm 2.1\%$, $P = 0.2$ for cleavage rate; $52.9 \pm 10.9\%$ vs. $56.6 \pm 11.2\%$, $P = 0.7$ for blastocyst rate). In addition, no significant influence of the marker-free procedure was found on the total cell number (122 ± 23 vs. 124 ± 22, $P = 0.7$) or on the ratio of ICM to the total cell number ($38.2 \pm 5.1\%$ vs. $38.1 \pm 6.4\%$, $P = 0.9$) compared with that of the control, which used the same cell line without marker-free manipulation. After embryo transfer, similar pregnancy and calving rates were also observed compared with that of the non–marker-free control (data not shown).

4. Discussion

As the original material for the current study, hLys transgenic cloned cattle were produced in the laboratory, and Western blotting analysis confirmed that human lysozyme could be expressed in certain tissues of the cattle (unpublished data). However, there were problems when we tried to purify human lysozyme, as selectable marker neo^R was also incorporated into the genome. Moreover, it also creates potential concerns regarding biological safety. Hence, in the current study, we started our attempts to remove the marker gene neo^R in fibroblast cells derived from the above transgenic cloned cattle and then used marker-free cells as donors to generate recloned transgenic cattle. Hereby, in the current study, we established a convenient and efficient approach to produce marker-free cells that would potentially be beneficial to study recloning in transgenic large animals.

Four types of the Cre-mediated excision methods have been established to eliminate marker genes flanked by loxP sites in mice. First, a negative selectable marker gene (for example, tk) floxed by two loxP sites was introduced along with target DNA. With the Cre-mediated excision, FIAU (Fialuridine) was used to select expected marker-free colonies [8,17], which has been demonstrated to be possible for producing marker-free cattle [11]. However, the disadvantage is that negative drug selection is required and more than one marker gene is needed in sequential transgenic research. Second, it is reported that direct introduction of transiently expressed Cre to fertilized eggs by pronuclear injection can excise floxed DNA segment in mice [10,18]. Nevertheless, it is inefficient and expensive to apply in livestock. Third, 16-cell-stage morulae are able to be infected with replication-deficient recombinant adenoviruses expressing Cre [9]. Despite the high efficiency (68%, 15 of 22) in mice, it was troublesome in adenovirus packaging and infection of embryos at the same stage in livestock. The last approach was similar to the current study [19]. The GFP-Cre fusion gene was constructed and driven by EF1α promoter. Then, ES cells were transfected with the EF1α-GFPcre vector, and FACS was used to enrich GFP^+ cells that were going to undergo Cre-mediated marker gene excision. Among 15 randomly picked colonies from the sorted population of GFP^+ cells, 12 (80%) were marker-free, which was higher than that of the other three methods. Regardless, no other study has been reported, except in ES cells or imperishable cell lines, while there is still a lack of data regarding the proportion of Cre or GFP integrated.

In large animals, it is generally acknowledged that one of the most difficult procedures in producing marker-free transgenic animal is selecting Cre-mediated marker-free cells and propagating cells. In contrast with studies noted above, we used GFP to track Cre expression; thereby, Cre-mediated marker-free cells could be sorted conveniently by FACS, regardless of transfection efficiency. Afterward, conditional media, which were collected from media conditioned by wild-type bovine fibroblast cells, were supplemented to promote the formation of colonies without any selective antibiotic. In the current study, out of 1000 cells in one 100-mm dish, 70 to 90 individual colonies could be formed, and 10% to 15% of the colonies could be propagated to 100% confluence in 1 well of a 6-well plate, which is sufficient for molecular analysis and subsequent SCNT. Besides, PCR was conducted to identify the cell colonies and discard those into which Cre or EGFP was integrated, so as to remove their potential adverse effects to individuals [20].

Surprisingly, one-third of sorted colonies were either Cre or GFP integrated, although more than 60% were neo^R excised among randomly picked colonies from four fibroblast cell lines We inferred that further studies are needed to optimize the method.

In conclusion, we established a practical, economical, and efficient approach to produce marker-free transgenic animals. It is noteworthy that there was no obvious influence of our manipulation on preimplantation and in vivo development capability of recloned bovine embryos. Therefore, it is expected that transgenic research and gene manipulation in livestock would be facilitated with this approach.

Acknowledgments

We thank Dr. Zhang Yunhai for help in manuscript preparation, Ms. Fei Jing for technical assistance, Mr.

Wang Gang for help in ovary collection and transportation, and Dr. Yang Xiao for the generous gift of the PBS185 plasmid. This research was funded by the National High-tech Research and Development Program of China (grant no. 2007AA02Z168).

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