Chapter 23

Generating Goat Mammary Gland Bioreactors for Producing Recombinant Proteins by Gene Targeting

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Abstract

Exogenous genes can be site-specifically integrated into the genomic DNA of animals by homologous recombination, generating transgenic animals. These animals have a clear hereditary background, although position effects of the exogenous genes and potential functional disruption of host genes can be caused by the genetic inserts. Therefore, the generation of mammary gland bioreactors via gene-targeting methods is a great asset for producing recombinant proteins in milk. Here, we describe a method of generating gene-targeted goats with the human alpha-lactalbumin gene (*hα-LA*) integrated into the beta-lactoglobulin gene (*BLG*) locus. The milk from these goats will be less allergenic and will be enriched with components of human milk protein.

Key words Mammary gland bioreactors, Gene targeting, Recombinant proteins, Goat, Human alpha-lactalbumin

1 Introduction

Homologous recombination (HR) is a type of genetic recombination in which nucleotide sequences are exchanged between two identical or similar molecules of DNA. HR functions to accurately repair harmful double-strand DNA breaks in cells and is also utilized for gene targeting for site-specific modification of genomic DNA [1]. Gene targeting is a genetic technique that is used to alter or replace an endogenous gene. Exogenous genes can be site-specifically integrated into the genomic DNA of animals via HR via the gene-targeting process. To target genes in mice, a gene-targeting construct is transfected into mouse embryonic stem cells in culture. After cells with the correct site-specific modification in the genomic DNA have been selected, these cells can then be injected into a mouse embryo to facilitate contribution to the genetic makeup of the animal [2]. Finally, chimeric mice, in which modified cells have made up the reproductive organs, are selected for breeding and propagation of the genetic modification. As a

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result, the entire body of the offspring will reflect the gene-targeted genetic modification based on the previously selected embryonic stem cells.

In livestock, germline competent embryonic stem cells are not currently available. Thus, gene-targeted offspring of livestock cannot be generated as a chimeric animal as described above for mice. Therefore, somatic cell nuclear transfer (SCNT) has been found to offer an efficient genetic engineering strategy for the production of genetically modified animals [3]. In this procedure, the nuclear donor cells—primary cultured somatic cells—are modified via HR to target the gene of interest. Then, cloned livestock are generated by SCNT using the gene-targeted modified cells [4, 5]. Gene-targeted animals are not affected by any positional effects of the exogenous genes in the host genome or by an potential functional disruption of the host genes due to genetic inserts [6]. Additionally, these animals with site-specific gene insertions have an advantage over breeding selection since the hereditary background of the gene-targeted animals is known [7]. Gene-targeted cattle [8–10], pigs [11–13], sheep [14], and goats [15, 16] have been generated; however, it remains difficult to produce transgenic domestic animals with site-specific gene modifications. A gene-targeting strategy can also be used to produce a mammary gland bioreactor for producing recombinant proteins by these animals [17]. Important applications of this gene-targeted mammary gland bioreactor include the modification of milk proteins to eliminate allergens and/or enhance nutritional value and the expression of various recombinant proteins in the mammary gland. In our previous work, we successfully produced gene-targeted goats that the beta-lactoglobulin gene (*BLG*) replaced with the human alpha-lactalbumin gene (*hα-LA)* so that the goat mammary glands will produce *hα-LA* instead of *BLG*. Here, we describe a procedure for the production of goat mammary gland bioreactors via gene targeting.

2 Materials

2.1 Reagents

1. pGEM-T vector.
2. Porf-hav1tk.
3. Pyrobest DNA polymerase.
4. Restriction endonucleases *Sal*I, *Not*I, *Xho*I, *Dra*I.
5. T4 DNA ligase.
6. QIAquick gel extraction kit.
7. DMEM/F12.
8. Ham F-12 medium.

9. FBS.
10. Collagenase I.
11. Transferrin.
12. Hydrocortisone.
13. Sodium acetate.
14. Insulin.
15. Hyaluronidase.
16. Ethanolamine.
17. Prolactin.
18. Trypsin.
19. Mineral oil.
20. EDTA.
21. Hoechst 33342.
22. Cytochalasin B.
23. Ionomycin.
24. N-6 dimethylaminoprine.
25. M2 medium.
26. M16 medium.
27. BSA.
28. Geneticin® Selective Antibiotic (G418).
29. Penicillin-Streptomycin.
30. Cloprostenol.
31. FSH.
32. LHRH.
33. Rabbit anti human hα-LA monoclonal antibody.
34. Goat anti rabbit IgG polyclonal antibody.
35. DIG-high prime DNA labeling and detection starter kit I.

2.2 Preparation of the Medium and Solution

1. Goat mammary gland cell culture medium: DMEM/F12 containing 10% FBS, 1.2 mg/mL $NaHCO_3$, 5 mmol sodium acetate, 5 μg/mL transferrin, 0.5 mmol ethanolamine, 10 μg/mL insulin, 5 μg/mL hyaluronidase, 100 U/mL penicillin, and 100 μg/mL streptomycin (pH 7.2–7.4).
2. Goat fetal fibroblast culture medium: DMEM/F12 containing 15% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (pH 7.2–7.4).
3. D-Hank's solution: 8 g/L NaCl, 0.4 g/L KCl, 0.05 g/L Na_2HPO_4, 0.06 g/L KH_2PO_4, 0.35 g/L $NaHCO_3$ (PH 7.2~7.4).

4. Collagenase digestion solution:DMEM/F12 containing 200 U/mL collagenase I, 100 U/mL hyaluronidase, 100 U/mL penicillin, and 100 μg/mL streptomycin (pH 7.2–7.4).
5. Trypsin digestion solution: Hank's solution supplemented with 0.5 g/L trypsin and 0.4 g/L ethylenediamine tetraacetic acid (EDTA) (pH 7.2–7.4).
6. Cell freezing medium: DMEM/F-12 supplemented with 10% dimethyl sulfoxide (DMSO) and 20% FBS.
7. Expression induction medium: goat mammary gland cell culture medium supplemented with 5 μmol prolactin.
8. Cell lysis buffer: 40 mM Tris/HCl pH 8.9, 0.9% Triton X-100, 0.9% Nonidet P-40, and 0.4 mg/mL proteinase K.
9. Fusion buffer: 0.3 M mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, 0.5 mM HEPES, and 3 mg/mL BSA.

2.3 Supplies and Instruments

1. 200 μL PCR tube.
2. 1.5 mL Eppendorf tube.
3. 15 mL centrifuge tube.
4. 50 mL centrifuge tube.
5. 48-well cell culture plate.
6. 24-well cell culture plate.
7. 6-well cell culture plate.
8. Water bath.
9. 2 mm gap electroporation cuvettes.
10. Multiporator (Eppendorf AG.).
11. Eppendorf Mastercycler.
12. ECM-2001 (BTX).
13. Fluorescence microscopy with micromanipulator apparatus.

3 Methods

3.1 Construction of the Gene-Targeting Vector

3.1.1 Amplification of hα-LA Genomic DNA by PCR

1. The genomic coding sequence of human *α-LA*, including the signal peptide (+26 to +82), four exons and introns (0–2361), and the polyA (+2339 to +2344) sequences were amplified by PCR. The primers LA1 and LA2 (Table 1) were designed according to sequence information obtained from GenBank (*hα-LA*, accession no: X05153.1), and an *Xho*I restriction endonuclease site was added to the 5′-end of the primers to allow for easy tracking of the cloned gene. The 2351-bp DNA fragments were amplified using Pyrobest DNA polymerase.

Table 1
PCR primers for gene amplification and detection

Primer name	Primer sequences	TM (°C)	PCR product size (bp)
LA1 LA2	GCTCGAGATGAGGTTCTTTGTCCCTC GCTCGAGTGACTTCAAAGTGGGACC	67.8 67.4	2351
pA F pA R	TGTTCCCATAGTAACGCCAAT AGCGAAACTCCACCTCAAA	57.5 55.4	843
tA F tA R	CTTGCTCCTGCCGAGAAAGTATCCATC GGAGGGTGTTCCCAGGTCACAGTCTAC	67.5 67.2	3538
BIN	GGTCACTGAATCTAACAGCCGTTGT	65.4	3868[a]
Neo 1 Neo 2	GCTATTCCAGAAGTAGTGAGG CAGAAGAACTCGTCAAGAAG	51.2 49.6	896

[a]Primers tA R and BIN were used to amplify wild-type *BLG3′* sequences to confirm gene targeting only at monoallelic sites

2. PCR products were cloned into the pGEM-T vector to yield the plasmid pGEM-LA.
3. Cloned sequence was confirmed by DNA sequencing.

3.1.2 Construction of the Gene-Targeting Vector pBla5-TK

1. The hα-LA gene was cloned into pgBLC/LF14 vector (available from our lab) at the *Xho*I site. This vector is a mammary gland-specific expression vector under the control of the *BLG5′* regulatory region, cytomegalovirus (CMV) promoter and enhancer, and *BLG3′* regulatory region [18]. This step yielded the expression vector pBlac.
2. The vector containing the Porf-hav1tk-encoded negative screening gene HSV-TK was recombined with pBlac to create the replacement targeting vector pBla5-TK (Fig. 1), which can be screened by a positive-negative selection strategy.

3.2 Analysis of the Plasmid Expression in Goat Mammary Gland Cells (GMGECs)

3.2.1 Culture of Primary GMGECs

1. GMGECs were isolated from lactating Sannen goats. Approximately 5 g of secretory tissue was removed from one of the two udders and was trimmed of visible fat and connective tissue.
2. After washing with Ca^{2+} and Mg^{2+}-free D-Hank's solution, the tissue sections were minced finely and digested in collagenase digestion solution with agitation at 37 °C for 1 h.
3. The digestion was stopped by the addition of one-fourth volume of FBS. Samples were subjected to centrifugation at 800 × *g* for 5 min to recover CMGECs.
4. The cells were then washed three times with D-Hank's solution. The isolated CMGECs were then cultured in goat mammary gland cell culture medium at 37 °C in a humidified atmosphere containing 5% CO_2.

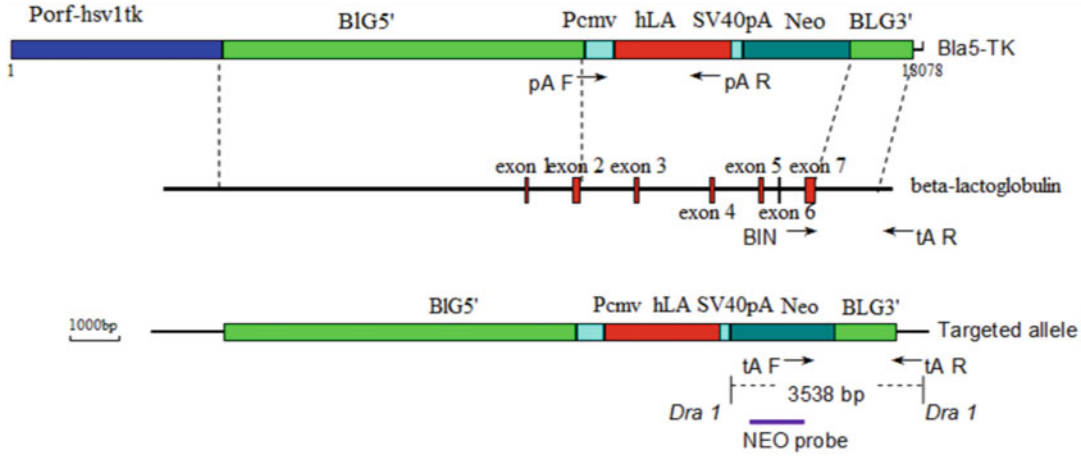

Fig. 1 pBla5-tk gene-targeting vector and detection strategy. The primers pA F and pA R were used to detect *hα-LA* integration, and primers tA F, tA R, and BIN were used to detect homologous recombination. The amplified 3538 bp region is indicated. The targeted allele contains sites for *Dra*I digestion and can be detected with a *NEO* probe

5. CMGECs that became attached to the culture dish were washed several times with D-Hank's solution after 24 h to remove dead cells. The cells were continuously cultured in goat mammary gland cell culture medium for 4–7 days.
6. For subculturing, the CMGECs were digested in trypsin digestion solution at 37 °C until the cells detached from the plastic dish. Then, the cells were recovered by centrifugation at 800 × *g* for 5 min and resuspended in fresh medium. The cell suspension was plated in culture dishes.
7. The epithelial cell population was enriched by selective detachment and attachment methods.
8. The purified cells were frozen in freezing medium or used for transfection.

3.2.2 Transfection of GMGECs

1. When GMGECs reached 80% confluence, the cells were transfected with pBlac plasmid, which was linearized by *Sal*I and *Not*I digestion. Transfection was performed by electroporation. The cells (2×10^6 cells) were mixed with 15 μg/mL pBlac DNA in hypoosmolar buffer (Eppendorf AG., Hamburg, Germany) and transferred into 2 mm gap electroporation cuvettes (Eppendorf AG.).
2. Electroporation was completed using a Multiporator (Eppendorf AG.) at 270 V for 100 μs.
3. Transfected cells were plated in 48-well culture plates at extra-low cell densities that each well plated no more than ten cells.

4. Antibiotic drug selection in mammary gland cell culture medium with 400 μg/mL G418 began 48 h after transfection and continued for 8–12 days.
5. Cell colonies were expanded into 6-well culture plates via subculture in mammary gland cell culture medium with 200 μg/mL G418.

3.2.3 Induction and Detection of hα-LA Expression in GMGECs

1. Transfected GMGECs were treated with 5 μmol prolactin to induce hα-LA expression. Cell culture medium was collected after 48 and 72 h for the detection of hα-LA expression.
2. 50 μL cell culture medium were used in an ELISA assay using standard laboratory western blotting protocols. The primary antibody was rabbit anti-human hα-LA monoclonal antibody (1:1000, Santa Cruz), and the secondary antibody was goat anti-rabbit IgG polyclonal antibody (1:2000, Santa Cruz).
3. To further assess the hα-LA expression, 24 μL cell cultured medium that was positive for hα-LA in the ELISA assay was analyzed by western blotting. Western blotting was performed using standard laboratory western blotting protocols. Recombinant hα-LA protein, human whey, induction medium from non-transfected GMGECs, and culture medium without prolactin from transfected GMGECs were used as controls (*see* **Note 1**).

3.3 Generation of Gene-Targeted Cloned Goats

3.3.1 Transfection and Selection of Goat Fetal Fibroblasts

1. The gene-targeting vector pBla5-TK was linearized with *Not*I. DNA fragments were purified using the QIAquick gel extraction kit.
2. Goat fetal fibroblasts were isolated from a 35-day-old female fetus recovered surgically from a Sannen goat [19]. Fetal fibroblasts were separated and propagated in goat fetal fibroblast culture medium.
3. When cultured fetal fibroblasts reached 80% confluency, the cells (2×10^6 cells) were mixed with 15 μg/mL pBla5-TK DNA fragments in hypoosmolar buffer (Eppendorf AG., Hamburg, Germany) and transferred into 2 mm gap electroporation cuvettes (Eppendorf AG.).
4. Electroporation was performed using a multiporator (Eppendorf AG.) at 270 V for 100 μs. Transfected cells were plated in 60 mm cell culture dishes.
5. DMEM/F12 supplemented with 10% FBS and 600 μg/mL G418 was begun 36 h after transfection and continued for 8–12 days in order to select for recombinant cells.
6. Healthy stable colonies were collected using a cloning ring and expanded via subculture in DMEM/F12 supplemented with 10% FBS and 400 μg/mL G418.

7. A portion of the cells were collected for cryopreservation, and the remaining cells were used for PCR screening for gene recombination at the BLG locus (*see* **Note 2**).

3.3.2 Screening Gene Targeting in Transfected Cells by PCR

1. Drug-resistant cells (1000 cells per 10 μL) were lysed in cell lysis buffer at 65 °C for 30 min, and then proteinase K was inactivated by incubation at 96 °C for 10 min.
2. 4 μL cell lysate were used as the DNA template in PCR. First, integration of exogenous genes was determined using primers pA F and pA R (Table 1). Next, PCR amplification was performed to screen for HR events using primer tA F and tA R, which amplified the sequence from the *NEO*^{r} gene to the outside of the BLG3′ homologous arm (Table 1). PCR were performed according to the following protocol: pre-denaturing at 95 °C for 5 min, 32 cycles of denaturing at 94 °C for 40 s and annealing and extension at 68 °C for 7 min, and a final extension at 72 °C for 5 min.
3. PCR products were analyzed by agarose gel electrophoresis, and product sequences were confirmed by direct sequencing.

3.4 Production of Cloned Fetuses by SCNT

3.4.1 SCNT and Activating Reconstructed Embryos

1. The cells containing the genomic-targeted modification were used as donor cells for SCNT. Donor cells were cultured with low-serum medium (DMEM/F12 + 0.5% FBS) in 24-well cell culture plates and incubated at 38 °C in an atmosphere with 5% CO_2 for 48 h. Prior to SCNT, donor cells were collected by trypsin digestion and resuspended in M2 medium plus 10% FBS.
2. Oocyte donor goats (Yangtze River Delta white goats) were synchronized, and superovulation was induced [20]. Oocytes were recovered surgically by flushing the oviducts with Ham F-12 medium containing 1% FBS.
3. Cumulus cells were removed from the oocytes by vortexing the cumulus-oocyte complexes in M2 medium containing 1 mg/mL hyaluronidase.
4. SCNT was performed as follows. Oocytes were incubated in M2 medium containing 2 mg/mL Hoechst 33342 and 7.5 mg/mL cytochalasin B at 38 °C with an atmosphere of 5% CO_2 for 20 min. A group of oocytes (20–30) were enucleated in M2 medium containing 7.5 mg/mL cytochalasin B by aspiration of the first polar body and the MII chromosomes lying beneath the polar body under fluorescence microscopy. After each individual oocyte was enucleated, a donor cell was injected directly into the perivitelline space using a glass pipette.
5. The karyoplast-cytoplast couplets were equilibrated briefly in M16 medium for 30 min before fusion. The karyoplast-

cytoplast couplets were washed briefly in fusion buffer and fused by the application of two direct current pulses (1.7 kV/cm for 40 ms) delivered by ECM-2001 (BTX). Cell fusion was evaluated after 30 min, and a second fusion pulse was administered to unfused couplets.

6. Fused couplets were washed extensively in M16 medium and then incubated for 5 h in M16 medium, before incubation in M16 medium containing 7.5 mg/mL cytochalasin B and 5 mM ionomycin for 5 min.
7. The constructed embryos were washed and then cultured in M16 medium containing 7.5 mg/mL cytochalasin B and 2 mM N-6 dimethylaminopurine for 5 h.
8. Finally, the reconstructed embryos were washed and cultured in M16 medium until embryo transfer (*see* **Note 3**).

3.4.2 Embryo Transfer and Ultrasonography

1. Reconstructed embryos were surgically transferred into synchronized recipient dairy goats. Each oviduct received five to seven cloned embryos.
2. Recipients were subjected to a transvaginal ultrasonographic evaluation on days 30, 60, and 90 of gestation.
3. Pregnant recipients were separated and placed in groups of two females per pen. For pregnancies that continued beyond 149 days, parturition was induced with prostaglandin F2a (Lutalyse, 4 mg im). Cotyledon number and birth weights were recorded.

3.5 Identification of Cloned Offspring

1. Genomic DNA was extracted from the umbilical cords or ear tissue of newborn cloned offspring 30 days after birth (Fig. 2).

3.5.1 PCR Analysis of the Genomic DNA of Cloned Offspring

2. For the detection of the integration of the exogenous gene, PCR was performed with primers pA F and pA R. For determination of HR events, the primers tA F, tA R, and BIN were used to amplify the genomic-targeted modification and wild-type *BLG3′* sequences (Fig. 1).

3.5.2 Southern Blot Analysis of the Genomic DNA of Cloned Offspring

1. To further confirm the site-specific gene modification, the genomic DNA of cloned offspring was subjected to southern blot analysis. The genomic DNA was digested with *Dra*I. The digested DNA was separated by 0.8% agarose gel electrophoresis, capillary transferred to a positively charged nylon membrane, and hybridized with a DIG-labeled probe for the NEO gene, which had been amplified from the pBlac-TK construct with primers neo 1 and neo 2 (Fig. 1).
2. Hybridization results were visualized with NBT/BCIP (DIG-high prime DNA labeling and detection starter kit I; Roche diagnostics, Indianapolis, IN, USA).

Fig. 2 Four transgenic cloned goats (**a**: TC-102, **b**: TC-108, **c**: TC-113, **d**: TC-114)

4 Notes

1. This procedure includes the methods for gene-targeting vector construction, analysis of the expression from the vector, SCNT to generate gene-targeted cloned goats, and detection of the expression of the recombinant protein. It is important to confirm the constructed vector has the ability to express the recombinant protein. Analysis of the expression function of the vector in GMGCs is a valid method to achieve this purpose.
2. The efficiency of gene targeting in somatic cells is low, usually $<10^{-4}$. Thus, in order to obtain a sufficient number of cell lines of gene-targeting modified cells for SCNT, transfection and selection of cells must be performed on a larger scale, e.g., 1×10^{7} cells or more.
3. Gene-targeted cells undergo lengthy (>30 days) selection and culture expansion, potentially inducing cell differentiation and lack of activity. The efficiency of SCNT may be decreased if these cells are used. To overcome this barrier, a second round of cloning can be performed, in which the donor cells are separated from the 30-days cloned fetus. With this approach, the cloned fetus can be assessed to determine whether it carries the gene-targeted genotype, its karyotype, and the growth activity of the cells. This method improves the efficiency of generation of healthy cloned offspring.

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