

Full Length Research Paper

Targeting the human lysozyme gene on bovine α 1-casein gene locus in fibroblasts

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Targeting an exogenous gene into a favorable gene locus and for expression under endogenous regulators is an ideal method in mammary gland bioreactor research. For this purpose, a gene targeting vector was constructed to targeting the human lysozyme gene on bovine α 1-casein gene locus. In this case, the expression of human lysozyme could be regulated by the endogenous *cis*-element of α 1-casein gene in bovine mammary glands. In order to analyze the bioactivity of the vector, the targeting vector was stably transfected and randomly integrated into mouse mammary epithelial cells. Reverse-transcription-polymerase chain reaction (RT-PCR) and western blot results showed that, the bovine α 1-casein promoter in the 5' arm was able to direct the efficient expression and secretion of human lysozyme in mammary epithelial cells. Turbidimetric assay showed that the antibacterial activity of lysozyme in transfected cells culture medium was 180 U/ml. To obtain the gene targeted cells line, bovine fetal fibroblasts were isolated and transfected with linear targeting vector (21.9 kb) using nucleofector device, which the transfection rate was about 25%. After seven rounds of independent cell transfection, a total of 8×10^7 cells were transfected, 118 colonies were expanded and analyzed by PCR, but none were found to be targeted. However, the targeted events were detected in the mixed cells which did not formed obvious colonies in five 10 cm dishes. Thus, these results indicate that, the 8.2 kb exogenous genes could be site specifically integrated into the transcriptionally silent α 1-casein gene locus in fibroblasts, but the unfavorable chromatin structure in such loci may have a disadvantage to targeted colonies formation in expansion stage. We suggest that minimizing the length of *in vitro* culture time and relax selection as soon as colonies become evident might prevent such loss of targeted cells.

Key words: Gene targeting, human lysozyme, bovine α 1-casein, C127 cells, fetal fibroblasts.

INTRODUCTION

Lysozyme is one of the most important antibacterial factors in human milk (Mathur et al., 1990). Expression of recombinant human lysozyme in bovine milk will improve human intestinal immunity to resist the invasion of foreign bacteria and reduce the incidence of bovine mastitis.

However, there are some disadvantages in traditional transgenic research, including limited regulatory elements, random insertion and repeated nature of the multiple copy number integration, which results in low level expression, even with ectopic expression of recombinant proteins (al-Shawi et al., 1990; Clark et al., 1994; Dobie et al., 1996; Kim et al., 2007). In addition, the random integration of exogenous genes into the host genome may inadvertently affect the expression of endogenous genes by disabling them or activating them at an inappropriate time, which is dangerous for the transgenic animal (Seggewiss et al., 2006). These potential problems in transgenic research are conflicts with the Guidance of Codex Alimentarius Commission and Food and Drug Administration (CAC 2008; FDA January 15,

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Abbreviations: PCR, Polymerase chain reaction; RT-PCR, reverse-transcription- polymerase chain reaction; HSV-TK, herpes simplex virus thymidine kinase; GANC, ganciclovir; IP, immunoprecipitation.

2009). Gene targeting is a genetic technique that uses homologous recombination to delete endogenous genes or knock exogenous genes into a specific genome locus. Compared with traditional transgenic research, the site specific integration of the exogenous gene into a favorable gene locus could overcome the position effect and reduce the potential risks of insert mutation in important genes. Thus, the gene targeting technology has advantages over other methods in transgenic research (McClenaghan et al., 1991; McCreath et al., 2000).

Due to this, we constructed a targeting vector which knock the human lysozyme genomic DNA into α s1-casein gene locus. In this case, the expression of human lysozyme could be expressed and regulated by the endogenous *cis*-element of α s1-casein gene in bovine mammary glands, which may overcome the disadvantage of random integration in traditional transgenic research. The α s1-casein is the most abundant protein in bovine milk, making it an excellent candidate locus for the production of biologically active protein in mammary bioreactor research (Rijnkels et al., 1997; Rijnkels et al., 2003).

MATERIALS AND METHODS

Construction of the gene targeting vector

To construct the gene targeting vector LYZ-k-in, the PLOX vector containing a neomycine expression cassette between two loxp sites and a herpes simplex virus thymidine kinase (HSV-TK) expression cassette was used as a backbone. This positive and negative selection strategy relies on the principal that, those cells in which random integration has occurred will retain the HSV-TK gene and thus, will be specifically eliminated by the antiviral agent ganciclovir (GANC). The 5' and 3' homologous arms of the α s1-casein gene were amplified from bovine fetal fibroblasts. The 5' homologous arm was a 2586 bp fragment that included the promoter, exon I, intron I and partial exon II sequence of the α s1-casein gene before the translation start site (9354 to 11939) (GeneBank accession no. X59856). The 3' homologous arm was a 5591 bp fragment that included the sequence from part of exon II to intron VII (11940 to 17530). Sequencing results indicate that, the homologous arms were above 99.7% identity to the published sequence of bovine α s1-casein gene. The 6237 bp fragment of human lysozyme gene (GeneBank accession no. X14008, 544-6780) including the translation start site and the polyadenylation signal was amplified from Hela cells. All PCR fragments were T-A cloned (PMD19-T vector, TaKaRa, Japan) and sequenced. The 3' homologous arm with a Sall restriction enzyme site was inserted into the Sall site of the PLOX vector and designated the R-PLOX vector. Then, the 5' homologous arm was subcloned into the NotI/XhoI site of the R-PLOX vector and was named R-PLOX-L. The final vector was completed by insertion of the human lysozyme genomic sequence into XhoI site of R-PLOX-L vector. All the primer sequences are synthesized by Beijing Sangon Biotechnology Co. Ltd (Beijing, China) (Table 1).

C127 cells culture and transfection

Mouse mammary epithelial C127 cells (Cell Bank, Chinese Academy of Sciences, Shanghai, China) were used to analyze

whether the bovine α s1-casein promoter in 5' arm could regulate the correct expression of lysozyme in mammary cells. C127 cells were cultured in Dulbecco's modified eagle medium (DMEM, Neuronbc, China) with 10% fetal bovine serum (FBS, Hyclone, USA) and maintained in a humidified environment with 5% CO₂. Cells at 90% confluence in 6-well plates were transfected with 4 μ g NotI linearized targeting vector in the presence of 10 μ l lipofectamin 2000 reagent according to the manufacturer's instruction (Invitrogen, USA). 48 h post-transfection, cells were passaged at 1:20 dilution into 10 cm dishes with culture medium containing 800 μ g/ml G418 (Sigma-Aldrich, USA). After 10 days drug screening, the cells colonies were mixed and expanded for subsequent analysis.

Integration analysis and cell induction

Confirmation of the presence of the inserted targeting vector in the polyclonal cells genome was carried out by the PCR and GANC toxicity analysis. Genomic DNA was isolated from polyclonal cells and C127 cells, then, analyzed by PCR for both the 3' arm and lysozyme sequences using primers G-3' arm and G-lyz. The polyclonal cells were treated with 1, 2, 6, 8, 15 and 30 μ M GANC (Sigma-Aldrich, USA), to verified the function of the HSV-TK expression cassette as a negative selection marker.

Cells at 70% confluence in 6-well plates were placed in the induction medium, lacking serum, supplemented with prolactin 2 μ g/ml, insulin 10 μ g/ml and hydrocortisone, 15 μ g/ml (Sigma-Aldrich, USA). After 24 h of induction, RNA was isolated utilizing the trizol extraction method for RT-PCR analysis. The culture medium and cytoplasmic protein were recovered after incubation for 48 h in the induction medium.

RT-PCR analysis of lysozyme transcription

Total RNA was isolated from C127 cells or polyclonal cells after induction as described earlier. cDNA was synthesized using an oligo(dT) primer and M-MLV reverse transcriptase according to the manufacturer's instructions (Invitrogen, USA). The expression of human lysozyme was analyzed by RT-PCR, using the lyzRT1 and lyzRT2 primers. The PCR products generated with lyzRT2 primer were analyzed by sequencing. Mouse GAPDH gene was used as an internal control, the RNA samples and H₂O as negative controls.

Western blot

The induced culture medium from polyclonal cells and C127 cells were ultrafiltrated and concentrated about 50-fold according to the manufacturer's instructions (3 KD, 4 ml; Millipore, USA). To collect the cytoplasmic protein, the cells were lysed with immunoprecipitation (IP) lysis buffer (100 μ l/10⁶ cells; Beyotime Institute of Biotechnology, China). Western blotting was carried out using a standard western blotting procedure. Briefly, approximately 20 μ g of total protein was separated by SDS-PAGE (15% gels). The separated protein was transferred onto a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, UK). Polyclonal rabbit anti-human lysozyme antibody (US Biological, USA) and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Zhongshan Goldenbridge, China) were used to detect lysozyme expression. The mouse β -actin was used as an internal control.

Assays for lysozyme activity

Lysozyme activity was analyzed by monitoring the reduction in

Table 1. Primers used in this study.

Primer	Primer sequences (5' - > 3')	size (bp)	PCR reaction condition
5' arm	ATTTGCGGCCGCTAATGTTCTGTGCATACAACCTGTGAAT CCCTCGAGGTCAAGATCTATGTAAGAAAATAAAATAG	2586	35 cycles of 94°C for 30 s, 59°C for 30 s and 68°C for 3min 30 s
3' arm	ACGCGTTCGACAACCATGAACTTCTCATCCTTAC ACGCGTTCGACACATTCTTGGGTATGATAGCACTGC	5591	35 cycles of 94°C for 30 s, 58°C for 30 s and 68°C for 6min 30 s
Lyz	CCGCTCGAGAACATGAAGGCTCTCATTGTTTC CCGCTCGAGTAGAAGTGAATATGAGGCCAG	6237	35 cycles of 94°C for 30 s, 57°C for 30 s and 68°C for 7 min
G-3' arm	CACCAAGGACTCCCTCAAGT TGCTGTCTGGCTCATTCTGT	477	35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s
G-lyz	TCTCCAGTACATTCCGTTCTTT TTCAGGCAGTCTGTTTCTATCA	581	35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s
LyzRT1	TGCAAAGAGGGTTGTCCGTGAT CAATACTTGTAAGCTCATCTGCCTC	1049	35 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 1 min 30 s
LyzRT2	CTTGCTGCTTCTTCCCAGTC TGATAAGAACTGAATGTGGC	1088	35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min 30 s
GAPDH	CAGTGGCAAAGTGGAGATTGTTG CAGTCTTCTGGGTGGCAGTGAT	491	35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s
3' HR	CTATCGCCTTCTTGACGAGTTCT TTTTGAGTCGGTTGTTTCATTTT	6297	35 cycles of 94°C for 30 s, 58°C, for 30 s and 68°C for 7 min 30 s

turbidity of a suspension of *M. lysodeikticus* cells at 450 nm (Huang et al., 2002). In this study, 80 µl of an optical density (OD) 450 nm = 0.8 *M. lysodeikticus* cells suspension was made in phosphate buffer saline (PBS) buffer. The cell suspension was equilibrated at room temperature and the reaction was initiated by adding 20 µl of samples containing chicken lysozyme with concentrations of 100, 150, 200, 250, 300 and 350 U/ml. Recombinant human lysozyme activity was determined in the kinetic mode for 1 min at 450 nm (Tecan infinite 200, Germany). The activity of recombinant human lysozyme was calculated relative to a standard curve and the human lysozyme activity of the suspension was calculated according to the regression equation deduced from the standard curve.

Isolation and transfection of primary bovine fetal fibroblasts

Adult Holstein milk cattle were housed in an animal facility. Fibroblasts were isolated from early stage of fetuses and cultured in DMEM medium with 10% FBS in a humidified environment with 5% CO₂ for 3 days. Fetal fibroblasts were electroporated according to the manufacturer's instructions (Amaxa, Germany). Transfected cells were cultured in DMEM medium with 15% FBS for 48 h, then trypsinized and passaged at 1: 40 to 50 dilution into 10 cm dishes under selection with G418 (500 µg/ml) and GANC (10 µM). After about 10 to 14 days screening, drug resistant colonies were recovered and cultured in 48 well plates. Once colonies became 90% confluence, they were split into two wells and cultured for freezing and DNA analysis. The Pmax-GFP vector (Amaxa,

Germany) and LYZop-GFP vector (constructed in our laboratory) were used for transfection efficiency detection.

Homologous recombination analysis of transfected fibroblasts

Genomic DNA was isolated from G418/GANC resistant cells. The homologous recombination event was identified using the 3'HR primer, which the forward primer (3'HRF) is located at the 3' end of the neomycin region and the reverse primer (3' HRR) is located outside the 3' arm (17651 to 17672 bp of *as1-casein* gene) (Figure 6a). PCR amplification was performed in a 25 µl reaction volume and the PCR products of positive colonies were sequenced and enzyme digested to further confirm the targeted events.

RESULTS

Transfection and Integration analysis of gene targeting vector in C127 cells

The gene targeting vector *lyz-k-in* was constructed as described in methods (Figure 1), verified by sequencing and enzyme digestion analysis. In order to analysis the bioactivity of targeting vector, the targeting vector was transfected and screened with G418 in C127 cells. The 488 and 571 bp expected PCR products amplified by G-

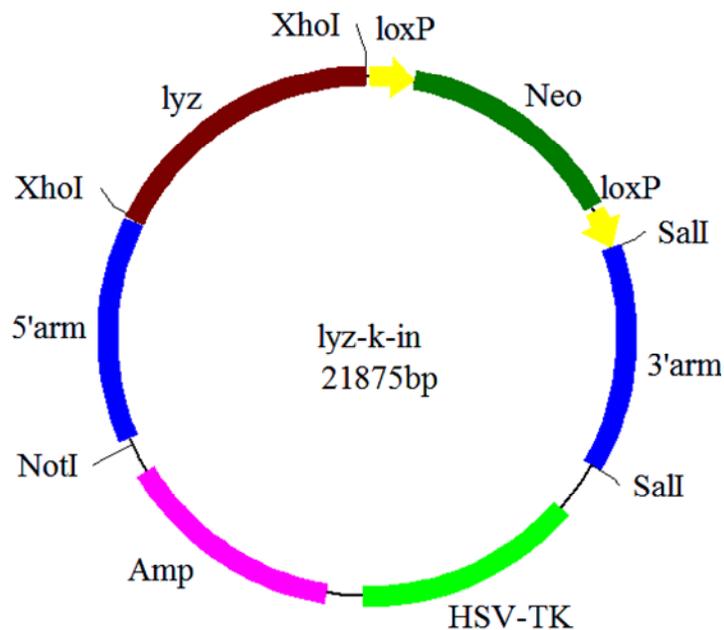


Figure 1. A schematic representation of the gene targeting vector.

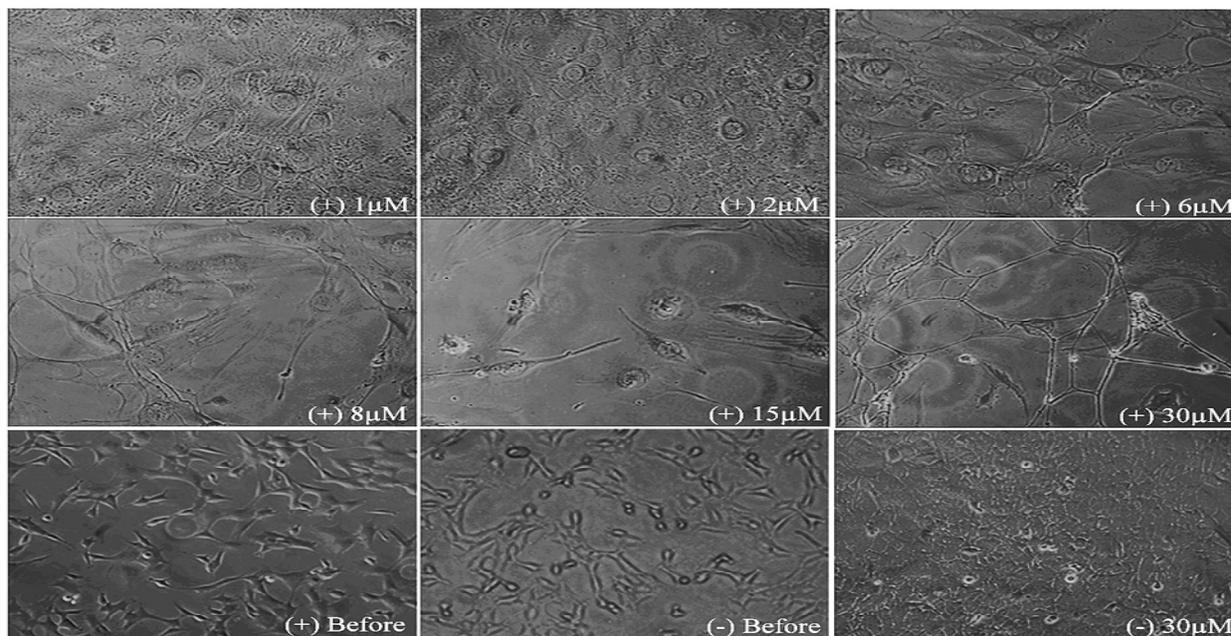


Figure 2. The polyclonal cells treated with different concentrations of GANC. (+) Represents the polyclonal cells (100 ×); (-) Represents C127 cells (100 ×); (+) Before, (-) Before, represents cells before treatment (40 ×).

3'arm and G-lyz primers showed that, the targeting vector had randomly integrated into polyclonal C127 cell genome (data not shown). Because of polyclonal, C127 cells had integrated with targeting vector and HSV-TK gene randomly; the cells will be specifically eliminated when treated with the antiviral agent GANC. As shown in Figure 2, cytotoxicity increased with increasing GANC

concentration and about ninety percent of the polyclonal cells died when treated with 8 μM for approximately 10 days. In contrast, The C127 cells as a negative control were not affected by drug treatment. These results demonstrated that, the gene targeting vector had randomly integrated into the genome of C127 cells. And the HSV-TK gene can be functionally as a negative

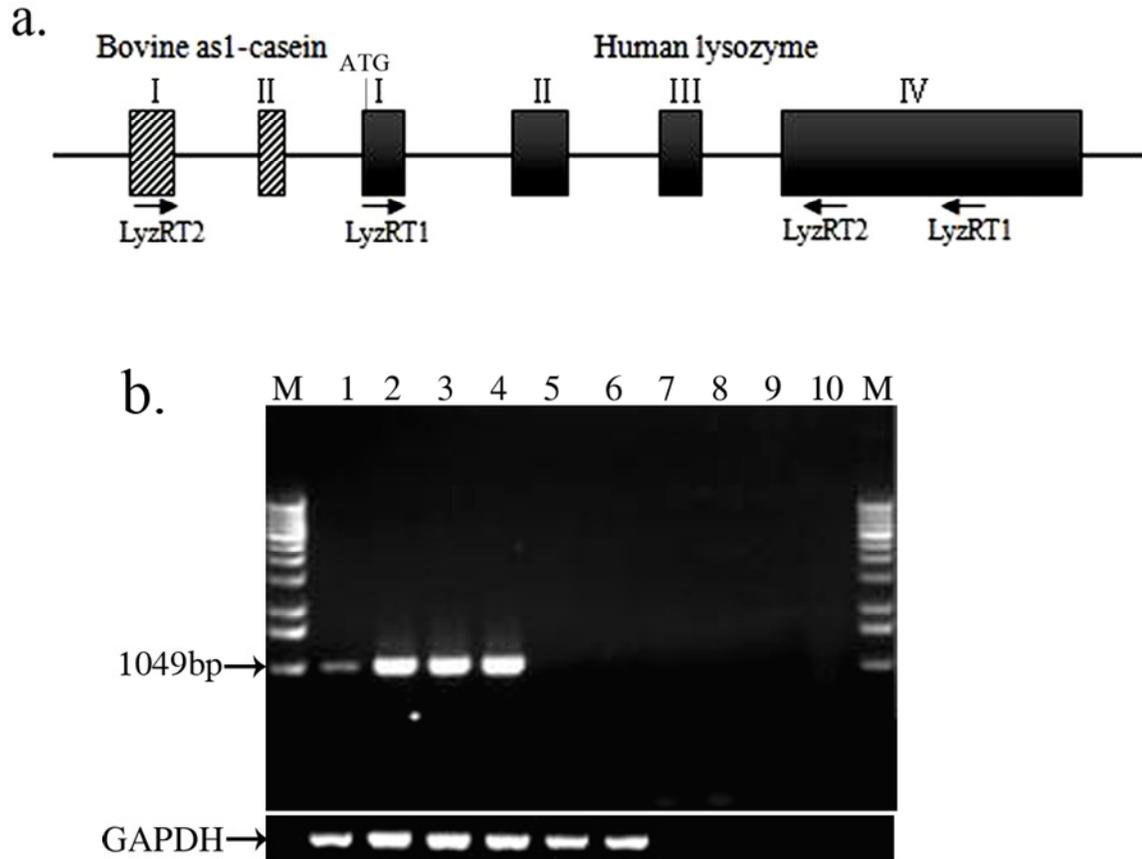


Figure 3. (A) The schematic diagram of α s1-casein and human lysozyme fusion genes showing the position of RT-PCR primers lyzRT1 and lyzRT2. (B) RT-PCR products of induced expression of human lysozyme transgene. 1 to 4, transgenic polyclonal cells with induction (cDNA samples as a template); 5 to 6, non-transgenic C127 cells with induction as a negative control (cDNA samples as a template); 7 to 8, RNA samples of polyclonal cells, which eliminate the genomic DNA contamination; 9, H₂O as template; 10, cDNA; M, 1 kb marker; Mouse GAPDH was used as an internal control.

selection marker.

Expression of human lysozyme in transfected C127 cells after induction

RT-PCR was performed to detect the expression of human lysozyme in polyclonal cells. The lyzRT1 primer, designed from the internal region of lysozyme, was used for RT-PCR (Figure 3a). As expected, 1049 bp of the amplified lysozyme band was visualized in the polyclonal cells after induction. However, neither non-transgenic cells (C127 cells) with induction nor polyclonal cells without induction can produce lysozyme mRNA (Figure 3b). In addition, the lyzRT2 primer designed to detect the fusion mRNA, also detected human lysozyme expression after induction. DNA sequencing showed that the 5' untranslated region of α s1-casein and human lysozyme gene was fused correctly and there is no mutation in the human lysozyme coding region (data not shown).

Recombinant human lysozyme was detected in the

protein samples from supernatants of culture medium and cell lysates (Figure 4). A single band of human lysozyme was detected in the supernatants of polyclonal cells after induction for 48 h, but was not detected in samples of cytoplasmic protein. This may be due to the efficient secretion of lysozyme into culture medium, which was concentrated about 50-fold through ultrafiltration, whereas, the cytoplasmic were not.

To detect the human lysozyme activity in concentrated supernatants of culture medium, a standard curve was established using chicken lysozyme to lyse *M. lysodeiktu*s. One minute of lysis was plotted for a range of different lysozyme concentrations. One unit of lysozyme activity was defined as the change in unit absorbance per minute per milliliter of reaction mixture at 450 nm. Based on the standard curve, a regression equation was deduced as $y = 0.0002x - 0.004$, where y is the difference of OD₄₅₀ and x is lysozyme activity (U/ml); $r^2 = 0.9954$. We observed that, the antibacterial activity of the polyclonal cells was enhanced because of the expression of human lysozyme. The antibacterial activity

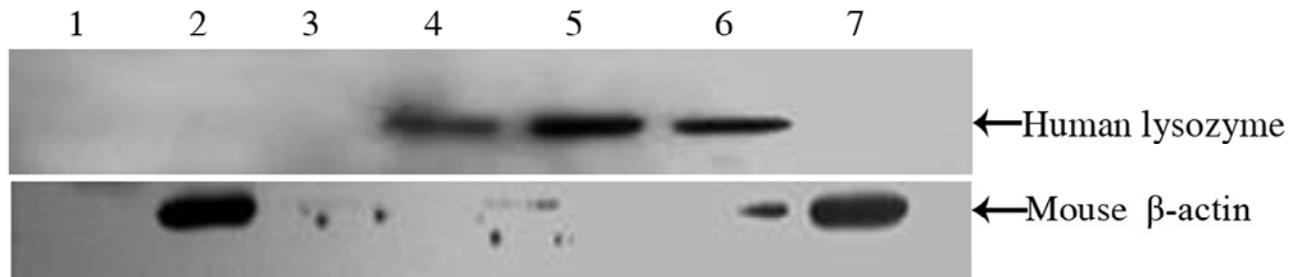


Figure 4. Western blot analysis of expression of recombinant human lysozyme in polyclonal cells. (1) Supernatants of induction culture of C127 cells; (2) Cell lysates of the polyclonal cells without induction; (3) Supernatants of the polyclonal cells without induction; 4 to 6. Supernatants of induction culture of the polyclonal cells; (7) Cell lysates of polyclonal cells after induction. The non-induced polyclonal cells and induced C127 cells were used as negative controls. Mouse β -actin was used as an internal control.

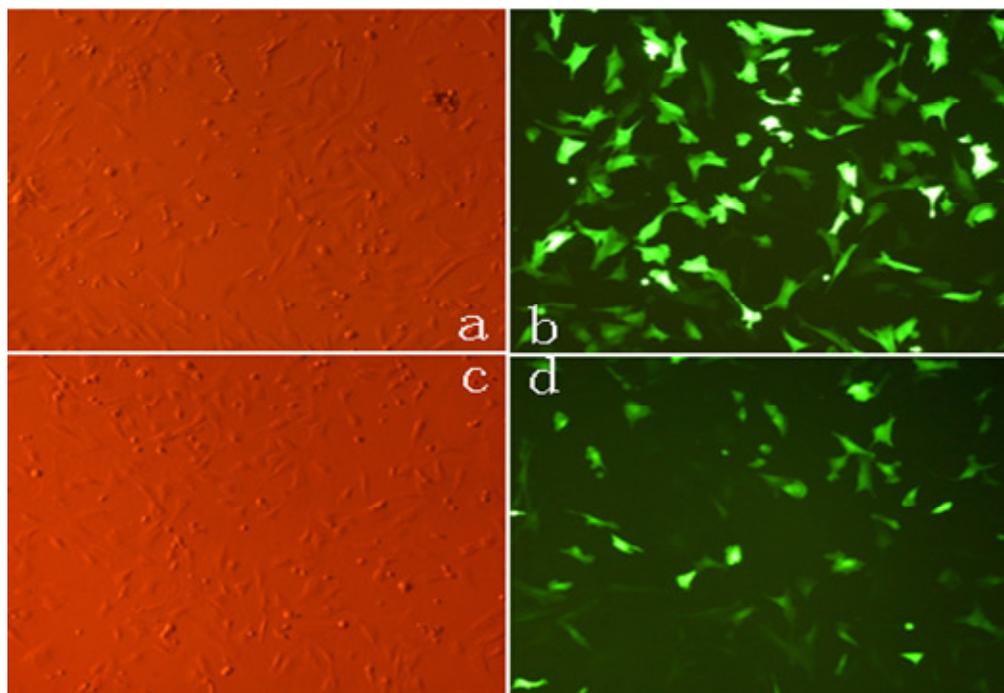


Figure 5. Transfection efficiency of fibroblasts with control vector. a. Pmax-GFP vector under visual light (3.5 kb, 100 \times); b. Pmax-GFP vector under blue light (3.5 kb, 100 \times); c. Lyzop-GFP vector under visual light (20.5 kb, 100 \times); d. Lyzop-GFP vector under blue light (20.5 kb, 100 \times).

of the supernatants of culture medium was 180 U/ml and almost undetected in culture medium from C127 cells. These results indicate that the expressed human lysozyme in C127 cells have antibacterial activity.

Knock-in of the human lysozyme genomic DNA into the *as1*-casein gene locus

The LYZ-k-in vector and control vector (Pmax-GFP, LYZop-GFP) were transferred to fetal fibroblast by electroporation. The transfection efficiency of LYZop-GFP

vector (20.5 kb), which had similar size with LYZ-k-in vector (21.8 kb), was about 25% (Figure 5). After seven rounds independent cell transfection with targeting vector, a total of 8×10^7 cells were transfected and 239 drug resistant colonies were picked, of which 121 did not expand enough to warrant genomic DNA isolation and these were discarded. The remaining 118 colonies were characterized by PCR analysis using primer 3' HR, but none of them were targeted. However, in the second round independent experiment of cell transfection (a total of 8×10^6 cells were transfected), targeted events were detected in the mixed cells which not formed obvious

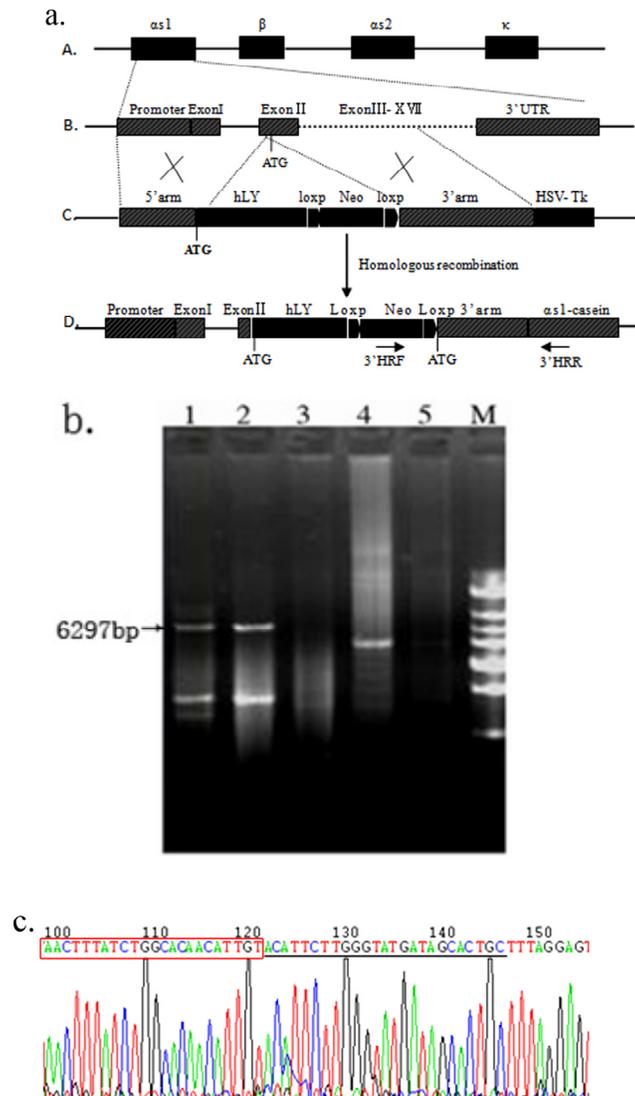


Figure 6. (a) The schematic diagram of homologous recombination. (a) Bovine casein gene locus; (b) The structure of as1-casein gene; (c) The structure of gene targeting vector; (d) Structure of the targeted locus after homologous recombination. (b) PCR analysis of mixed colonies. 1 to 2, Mixed colonies, the 6297 bp PCR products generated with 3' HR primer; 3 to 5, Fibroblasts genomic DNA, gene targeting vector and H₂O as a control, respectively; M, 1 kb marker. (c) DNA sequencing analysis of homologous recombination region. Sequences in the box showed the flanking sequence of 3' arm. Sequences underlined showed the site of 3' arm reverse primer. The results indicate that successful homologous recombination between the targeting vector and as1-casein gene locus.

colonies in five 10 cm dishes (Figure 6b). The expected 6297 bp PCR products was bidirectional sequenced using primer 3' HR primer to exclude a false positive result. Sequencing results were consistent with our expectation; the Sall restriction site and HSV-TK cassette which located outside 3' arm were not integrated into this locus because of homologous recombination (Figure 6c).

DNA digestion using restriction enzymes HindIII and Sall also generated expected fragments (Figure 6d). These results provide good evidence of targeting vector that successful targeted human lysozyme genomic DNA into the bovine as1-casein gene locus.

In summary, in order to overcome the disadvantage of random integration in traditional transgenic animal re-

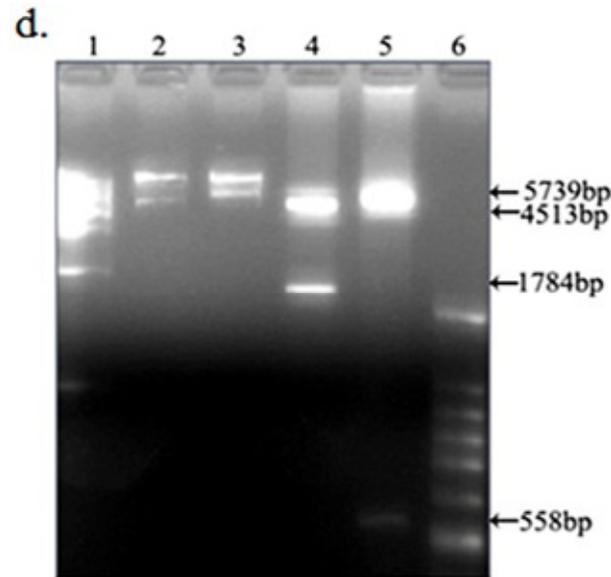


Figure 6. (d) Enzyme digestion analysis of PCR products (6297 bp). (1) 1 kb marker; (2) Gene targeting vector digested by HindIII (4474+17401 bp), as a negative control; (3) Gene targeting vector digested by Sall (5597+16278 bp), as a negative control; (4) The 6297 bp PCR products digested by HindIII (1784+4513 bp); (5) The 6297 bp PCR products digested by Sall (5739+558 bp); (6) 100 bp marker.

search, a gene targeting vector which targeting the human lysozyme genomic DNA on bovine α 1-casein gene locus was constructed. Bioactivity analysis of targeting vector indicates that, the α 1-casein gene promoter in 5' arm could direct human lysozyme gene expressed and secreted correctly in mouse mammary cells. Bovine fibroblasts were isolated and transfected with targeting vector, homologous recombination analysis of G418 and GANC resistant fibroblasts showed that, we had succeeded in targeting the 8.2 kb exogenous gene (including the human lysozyme gene and neomycine gene) into the transcriptionally silent locus.

DISCUSSION

In animal mammary bioreactor research, targeting an exogenous gene into a favorable gene locus in order to regulate recombinant protein expression is a most promising approach. In this study, we used gene targeting technology to target the human lysozyme gene on α 1-casein gene locus, so that the human lysozyme would be expressed and regulated by endogenous *cis*-element of α 1-casein gene in bovine mammary gland. This will overcome the position effect and reduce the potential risks of insert mutation in traditional transgenic research. α 1-Casein gene locus is a most active candidate locus to regulate exogenous gene expression in bovine milk. Although, the α 1-casein protein was considered to have

a function on other milk protein transport in mammary gland (Chanat et al., 1999), the heterozygous inactivation of α 1-casein gene would not influence its function, because of gene dosage compensation effect by another allele of α 1-casein gene (Kumar et al., 1994).

At the moment, gene targeting is still full of challenges in somatic cells because the homologous recombination rate in somatic cells is about 10^{-7} , with two orders of magnitude lower than that in mouse ES cells (Waldman 1992; Hanson et al., 1995). Gene targeting in somatic cells has proven difficult to achieve, particularly if the target gene is silent (Thomson et al., 2003; Rogers et al., 2008). In the present study, after seven rounds of independent transfection experiments, a total of 8×10^7 cells were transfected and 118 single colonies were analyzed by polymerase chain reaction (PCR). However, only in the second round transfection we detected targeted events happened in mixed cells with not formed obvious colonies. There are previous descriptions of unsuccessful targeting experiments as a result of the failure of cells to survive the drug selection when the transgene was inserted within an inactive chromosomal locus (Smithies et al., 1985). Kuroki et al. (2004) reported successful targeting of the silent immunoglobulin- μ gene in bovine fibroblasts and the targeting frequency was 0.45% when compared with 6.4% of the transcriptionally active prion protein locus of the same cells. This supports difficult to target. In addition, Kuroiwa the idea that the transcriptionally silent genes are more et al. (2004) found

that the selection marker genes were not expressed in regenerated IGHM-targeted fibroblasts, possibly due to the methylation of marker gene at silent locus. Similar results has been reported by Kong et al. (2009) that, the CMV promoter methylation level increased 3-fold when transgenic fibroblasts cultured *in vitro* and the GFP expression level decreased significantly. Combine previous studies and our results, we hypothesize that, the progressively silenced of marker gene which newly integrated in the silent casein gene locus may lead to the loss of the targeted cell colonies in the expansion stage and the chromatin structure at such loci may inhibit the expression of the marker gene, lead to low targeting efficiency at such loci. Recently, Hockemeyer et al. (2009) using zinc finger achieved high gene targeting frequency in human ESCs and iPSCs. However, the targeting frequency of transcriptionally silent PITX3 gene was 11 and 8% when compared with 49 and 51% of the transcriptionally active AAVS1 gene locus. These results indicate that, the low targeting efficiency at transcriptionally silent loci when compared with transcriptionally active loci, even though the zinc finger nuclease can achieve high targeting efficiency (Hockemeyer et al. 2009).

Improvement of gene targeting efficiency for somatic cells would greatly increase the applicability of transgenic farm animals in bioreactor. Modifying the level of methylation or addition of boundary elements, might reasonably be expected to improve the efficiency of gene targeting at silent loci, but may have undesirable effects on the targeted loci and reprogramming of somatic cells. The data of Kuroiwa et al. (2004) which succeeded in targeting at transcriptionally silent loci showed that, minimizing the length of *in vitro* culture time and relax selection as soon as colonies become evident maybe a favored strategy to avoid the silencing of selectable cassette, which may improved the targeting efficiency at such transcriptionally silent loci.

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