

In vitro evaluation of a mammary gland specific expression vector encoding recombinant human lysozyme for development of transgenic dairy goat embryos

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Abstract A vector expressing human lysozyme (pBC1-hLYZ-GFP-Neo) was evaluated for gene and protein expression following liposome-mediated transformation of C-127 mouse mammary cancer cells. Cultures of G418-resistant clones were harvested 24–72 h after induction with prolactin, insulin and hydrocortisone. Target gene expression was analyzed by RT-PCR and Western blot and recombinant human lysozyme (rhLYZ) bacteriostatic activity was also evaluated. The *hLYZ* gene was correctly transcribed and translated in C-127 cells and hLYZ inhibited gram-positive bacterial growth, indicating the potential of this expression vector for development of a mammary gland bioreactor in goats. Guanzhong dairy goat skin fibroblasts transfected with pBC1-hLYZ-GFP-Neo were used to construct a goat embryo transgenically expressing rhLYZ by somatic nuclear transplantation with a blastocyst rate of 9.0 ± 2.8 %.

These data establish the basis for cultivation of mastitis-resistant hLYZ transgenic goats.

Keywords Cancer cells · Human lysozyme · Mammary gland bioreactor · Mouse mammary cancer cells

Introduction

Human lysozyme (hLYZ) forms part of the defensive machinery of organisms in preventing infection and tumors and immunological regulations and thus, has been widely used for medical and industrial purposes (Ibrahim et al. 2001). Natural hLYZ is extracted from human breast milk, neutrophils or urine. However, these sources are limited and the safety of the product cannot be guaranteed. Therefore, this approach is unsuitable for large-scale industrial production. Transgenic animals as mammary bioreactors provide high levels of expression of an active product that can be purified without contamination, thus representing a novel method for large-scale production of recombinant proteins. However, this approach has yielded limited success to date.

The choice of the genetic vector used for generation of transgenic animals requires preliminary verification of expression performance. Direct injection of mammary glands is a convenient approach in small animals, although it is associated with sampling problems and

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has proved unreliable in large animals. Analysis of transgenic mice is a commonly used and reliable method that generally reflects the expression characteristics of the vector (Higashibata et al. 2004). However, using current techniques, exogenous genes are randomly integrated in the genome which can then lead to positional effects on target gene expression (Aoyama et al. 2005). Thus a transgenic mouse line needs to be established to confer statistical significance on the results. In vitro mammary epithelial cell transfection produces large numbers of transduced cells, avoids positional effects and allows accurate artificial control of culture conditions. However, preparation of primary cultures of mammary epithelial cells from large animals is tedious and differentiation cultured in vitro is difficult. As a carcinoma cell line derived from RIII mouse mammary gland epithelium (Tollefson et al. 1991), C-127 cells can be obtained commercially and easily manipulated. Moreover, C-127 cells have been used to validate mammary specific expression constructs after transient transfection (Yi and Li 2010). However, to our knowledge, utilization of C-127, integrated stably with transgene(s) to validate mammary specific transgenic vector(s), is still lacking. In this study, the goat β -casein promoter driven mammary cell specific expression vector harboring the *hLYZ* gene was introduced into C-127 mammary gland carcinoma cells. Expression of hLYZ was hormonally induced and investigated at the mRNA and protein levels by RT-PCR and Western blotting analysis, respectively. A hLYZ expressing stable cell line was generated and investigated for potential of this expression vector in the development of a mammary gland bioreactor in goats using transgenic technology.

Materials and methods

Reagents

The C-127 cell line was purchased from Shanghai Institutes for Biological Sciences (Shanghai, China). All cell culture medium reagents were purchased from invitrogen. DNA and RNA isolation assay kits were obtained from Qiagen (Germany). Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich.

Mammary-specific vector extraction and purification

The mammary expression vector pBC1-hLYZ-GFP-Neo plasmid kindly provided by China Agricultural University (Beijing, China) contained the *hLYZ* gene fragment and its regulatory elements (Gong et al. 2004). The plasmid was purified using an endotoxin-free plasmid mega purification kit, digested with SaII, concentrated and sterilized using the anhydrous alcohol precipitation method. The plasmid fragment was dissolved in double-distilled water and the concentration and purity were confirmed to be suitable for transfection by the $A_{260/280}$ ratios and the A_{260} values. The plasmid was stored at -20°C .

Mouse C-127 mammalian cell line transfection, screening and identification

C-127 cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 5 % (v/v) fetal bovine serum (FBS) at 37°C in 5 % CO_2 . After 2–3 passages, 2.5×10^5 cells were seeded into a six-well plate and cultured for a further 24–36 h (85–95 % confluence) prior to Lipofectamine 2000 transfection with the linearized vector. Positively-transfected clones were selected with G418 (500 $\mu\text{g}/\text{ml}$) and grown to confluence when the cultures were divided for genomic DNA extraction and further amplification. Clones were maintained in medium containing G418 (250 $\text{ng}/\mu\text{l}$). The hLYZ insert was identified in genomic DNA by PCR using the P1/P2 primer pair (Supplementary Table 1). Positive, negative and blank controls were represented by the pBC1-hLYZ-GFP-Neo plasmid, untransfected cells and water, respectively, in all experiments.

hLYZ expression in C-127 cells under exogenous hormone stimulation

G418-resistant C-127 cells were cultured in 60 mm dishes (80–90 % confluence), washed twice with PBS and cultured in serum-free medium (DMEM/F12 +10 μg insulin/ml + 5 μg prolactin/ml + 10 μg hydrocortisone/ml) for 72 h. Supernatants were harvested at 12 h intervals on six occasions, centrifuged at $450 \times g$ for 10 min before being transferred to a super-filter (3 kDa cut-off, millipore) for concentration by

further centrifugation at $1,800\times g$ for 20 min and then divided into 50 μl lots and stored at $-80\text{ }^{\circ}\text{C}$.

Detection of hLYZ mRNA by RT-PCR

Transfected cells were induced for 24, 48 and 72 h. Untransfected cells were induced for 72 h. Total RNA was isolated using the RNeasy mini-isolation kit (Qiagen) and processed using RNase-free DNase I (Qiagen) to eliminate genomic DNA. cDNA was synthesized from total RNA (0.5 μg) in 20 μl using a QuantiTect Reverse Transcription PCR kit (Qiagen) and used as a template for detection of *hLYZ* gene expression using the P3/P4 primer pair (Supplementary Table 1). β -actin was amplified as an internal control using the P5/P6 primer pair (Supplementary Table 1).

Bacteriostatic assay of hLYZ activity

The bacteriostatic activity of hLYZ in culture supernatants was evaluated using *Micrococcus luteus* and *Staphylococcus aureus* as gram-positive bacterial substrates in paper disc diffusion assays. Bacteria were grown in nutritional broth at $37\text{ }^{\circ}\text{C}$ with shaking at 100 rpm (6–8 h) to an OD_{600} of 0.5 (*S. aureus*) or 0.1 (*M. luteus*). Bacteria were spread uniformly onto sterile Mueller–Hinton (MH) solid agar plates and dried for 15 min. Sterile filter paper (6 mm diameter) was evenly placed on the agar plate after complete absorption. Culture supernatants (20 μl) for detection of hLYZ were added to these filter papers and the plates were kept at $4\text{ }^{\circ}\text{C}$ for 30 min for complete absorption and spreading. The diameter of inhibition zones at the maximal sizes (12–16 h) was recorded using precision vernier calipers and then photographed.

Generation of hLYZ transgenic goat embryo

Transgenic goat embryos were generated by somatic cell nuclear transplantation (SCNT) (see Tao et al. 2009). Goat transgenic somatic donor nuclei were transfected, screened and enriched using the same protocol previously described for obtaining hLYZ transgenic C-127 cell lines. Transgenic donor cells of either female or male goat fetal fibroblast cells that tested positive for hLYZ in PCR were introduced into in vitro matured oocytes by microinjection. Reconstructed eggs were transferred into TCM199 + 10 % FBS (T10) and equilibration at $38.5\text{ }^{\circ}\text{C}$, 5 % CO_2 for

0.5 h prior to electrofusion and were rinsed (3–5 times) in $38.5\text{ }^{\circ}\text{C}$ pre-warmed fusion solution (0.3 M mannitol supplemented with 0.05 mM CaCl_2 , 0.1 mM MgSO_4 , and 0.01 % polyvinyl alcohol) before being transferred into the BTX fusion slot (Harvard Apparatus, MA, USA) filled with fusion buffer solution with a 1 mm gap between the electrodes. Electrofusion of the donor cell and the oocyte was carried out using the protocol described by Tao et al. (2009). Fused embryos were then rinsed (3–5 times) with T10 and incubated in G1 medium (G-1 V⁵, Vitrolife, Sweden) for 1 h before examination under a stereomicroscope.

Cloned transgenic hLYZ embryo activation

After fusion, the reconstructed embryo was activated by treatment with 5 μM ionomycin and 2 mM 6-dimethylamino purine for 2 h. The embryo was then rinsed (3–5 times) and cultured with pre-equilibrated G1 medium, 10–15 embryos/50 μl , for 48 h. The embryos were then transferred into G2 (Vitrolife, Sweden) medium and cultured for a further 72 h. The development of the cloned embryos was observed and recorded at 48 and 168 h during in vitro culture.

Results

Confirmation of hLYZ expression in transfected C-127 cells

G418-resistant clones of C-127 cells transfected with the mammary expression vector pBC1-hLYZ-GFP-Neo were screened for GFP expression by fluorescence microscopy. Integration of the plasmid into the genomic DNA of GFP expressing resistant clones was confirmed by PCR amplification of hLYZ (638 bp) using the P1/P2 primer pair (data not shown). Expression of hLYZ in transfected C-127 cells following hormonal induction as stated in materials and methods for 24, 48 and 72 h was detected by RT-PCR amplification of a 540 bp product (Fig. 1a). The target gene was not detected in identically treated untransfected cells after 72 h. Amplification of the β -actin gene (353 bp) was carried out as an internal reference and was detected in both transfected and untransfected cells (Fig. 1b).

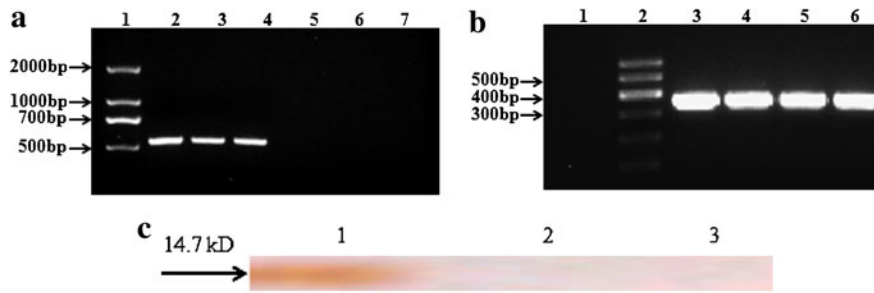


Fig. 1 RT-PCR detection of hLYZ mRNA and protein expression by transgenic cells after hormonal induction. **a** RT-PCR amplification of the target gene (*hLYZ*); 1: Marker D2000; 2/3/4: induction of transgenic cells for 24/48/72 h; 5: induction of non-transgenic cells for 72 h; 6: total RNA from the transgenic cells after induction; 7: sterile water. **b** Amplification

of the internal reference gene (β -actin); 1: sterile water 2: Marker I; 3/4/5: induction of the transgenic cells for 24/48/72 h; 6: induction of non-transgenic cells. **c** Western blot analysis of the secretion of hLYZ protein in cell culture supernatant. *Lane 1* transgenic cell after induction, *Lane 2* normal culture solution of transgenic cells, *Lane 3* normal cells after induction

Expression of hLYZ protein (14.7 kDa) was detected in culture supernatants of hormonally induced transfected C-127 culture supernatants by Western blot analysis, while expression was not detected in culture supernatants of identically treated untransfected cells (Fig. 1c). These data confirmed goat β -casein promoter driven expression of the *hLYZ* gene following hormonal induction and secretion of the mature protein after intracellular processing.

Bactericidal activity of rhLYZ

The biological activity of rhLYZ was analyzed using the paper disc diffusion method. Growth of *M. luteus* was inhibited in the presence of hormonally-induced transfected cell cultures with inhibition zone diameters reaching ~19 mm (Fig. 2). Inhibition was not observed in untransfected groups. (The inhibition of *S. aureus* was less pronounced; data not shown.)

Establishment and identification of stably transfected cell clones from C-127

Large numbers of G418-resistant clones obtained following culture at high antibiotic concentrations (500 μ g/ml) for 16–18 days were examined for GFP expression. Variable expression was observed (data not shown) and five clones exhibiting good morphology and relatively strong green fluorescence were selected and expanded for analysis of hLYZ expression by RT-PCR and Western blotting. Expression of the *hLYZ* gene was detected by RT-PCR amplification using genomic DNA as a template and the P1/P2

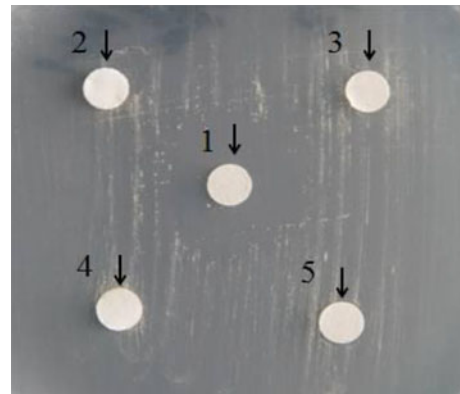


Fig. 2 Lytic activity of rhLYZ against *M. luteus* tested by the Mueller–Hinton agar disc diffusion method. The small circles (6 mm diameter) consist of quantitative filter paper with 20 μ l samples each, and the larger circles are the inhibition zones. 1 rhLYZ at 5.6 μ g/ml concentrated from culture supernatants of transfected C-127 cells after lactogenic hormone induction, 2 culture supernatants concentrated from transfected C-127 cells without induction, 3 culture supernatants concentrated from non-transfected C-127 cells after induction, 4 culture supernatants concentrated from non-transfected C-127 cells without induction 5 lactogenic induction medium only

primer pair (Supplementary Table 1). The pBC1-hLYZ-GFP-Neo plasmid was used as a positive control. Positive hLYZ expression was confirmed for all five clones (designated hLYZ1-5) (Fig. 3).

In vitro development of the transgenic hLYZ embryos

The embryos derived from goat transgenic fibroblast cells expressing hLYZ-1 were capable of developing to blastocyst stage (Fig. 4) and the cleavage rate of the

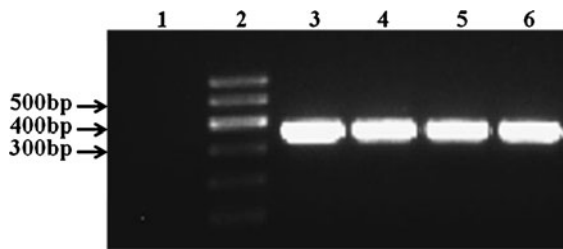


Fig. 3 PCR identification of the *hLYZ* gene in dairy goat fibroblasts transfected with using a genomic DNA template. 1: Marker D2000; 2: Positive control with pBC1-*hLYZ*-Neo-GFP plasmid as the template); 3: Genomic DNA of non-transfected fibroblasts from Guanzhong dairy goat as the template (negative control); 4/5/6/7/8: Clones 1/2/3/4/5, genomic DNA samples were used as the templates

transgenic embryos did not significantly differ from the non-transgenic cloned embryos (66 vs. 73.5 %). However, the blastocyst rate of transgenic clones was significantly lower than that of the somatic cloned embryos (9 % vs. 18.5 %) (Table 1).

Discussion

hLYZ functions as a bactericide by catalyzing the hydrolysis of β -1,4 glucosidic bond in mucopolysaccharides in the bacterial cell wall, directly in the case of Gram-positive bacteria and indirectly the via the effects of secretory immunoglobulin A and complement in gram-negative bacteria (Woods et al. 2011). Thus, this protein has been widely used for both medical and industrial purposes. However, natural resources of lysozyme are limited. Expression of the recombinant *hLYZ* in bacterial (Muraki et al. 1986) and yeast (Yoshimura et al. 1988) systems is associated with problems such as incomplete processing and post-translational modification, host cell lysis and low expression levels, thus making this approach unsuitable for industrial production.

The mammalian mammary gland functions as an external secretion organ for natural and highly efficient synthesis and secretion of proteins and, therefore, is considered as an ideal “bioreactor” for production of recombinant proteins. This technique requires the construction of mammary-specific expression vectors for highly efficient expression of exogenous genes in mammary glands of transgenic animals. To date, this aim has been impeded by

limitations in the elucidation of gene regulation mechanisms. Mammary cell models have been used as a convenient and reliable method for characterization and verification of the suitability of expression vectors prior to generation of transgenic animals. However, preparation of primary mammary epithelial cells is complex and these cells cannot be cultured long-term. Furthermore, expression of exogenous genes requires mammary epithelial cells to form three-dimensional alveolar structure (Tanaka et al. 2003).

In this study, the mammary carcinoma cell line (C-127) was used to detect soluble and active recombinant proteins in the supernatant after transient transfection with mammary-specific expression vector following hormone induction. This system has several advantages in terms of ease of culture, absence of requirements for exogenous extracellular media or co-cubation with fibroblasts and adipose cell and also efficient β -casein promoter driven expression induced by prolactin and other hormones. Furthermore, exogenous proteins expressed by C-127 cells are secreted into the culture media and are easily detected.

In contrast to the approach of Yi and Li (2010), large numbers of G418-resistant transfected clones were detected by antibiotic screening. Clones were cultured to 80 % confluence before being transferred for further culture and induction of exogenous gene expression in order to avoid the influences of chromosome position effects and to increase the percentage of positive transgenic cells and thus, to facilitate protein expression following induction.

Expression of *hLYZ* mRNA was detected by RT-PCR at 24, 48 and 72 h after hormonal induction in transfected C-127 cells, and Western blot analysis revealed secreted expression of the recombinant protein (14.7 kDa) in transfected C-127 cells. Analysis of the biological activity of the recombinant *hLYZ* protein was analyzed in bacteriostatic assays using *M. luteus* and *S. aureus* as substrates. Obvious growth inhibition of the substrate *M. luteus* was observed in the presence of hormonally induced transfected cell cultures with inhibition zone diameters reaching \sim 19 mm, thus indicating the biological efficacy of the *hLYZ* expressed in the transgenic mammary cancer cells against growth of Gram-positive bacteria. Inhibition of *S. aureus* was less pronounced (data not shown), which may be related to the substrate specificity of bacterial structures and the action of lysozyme.

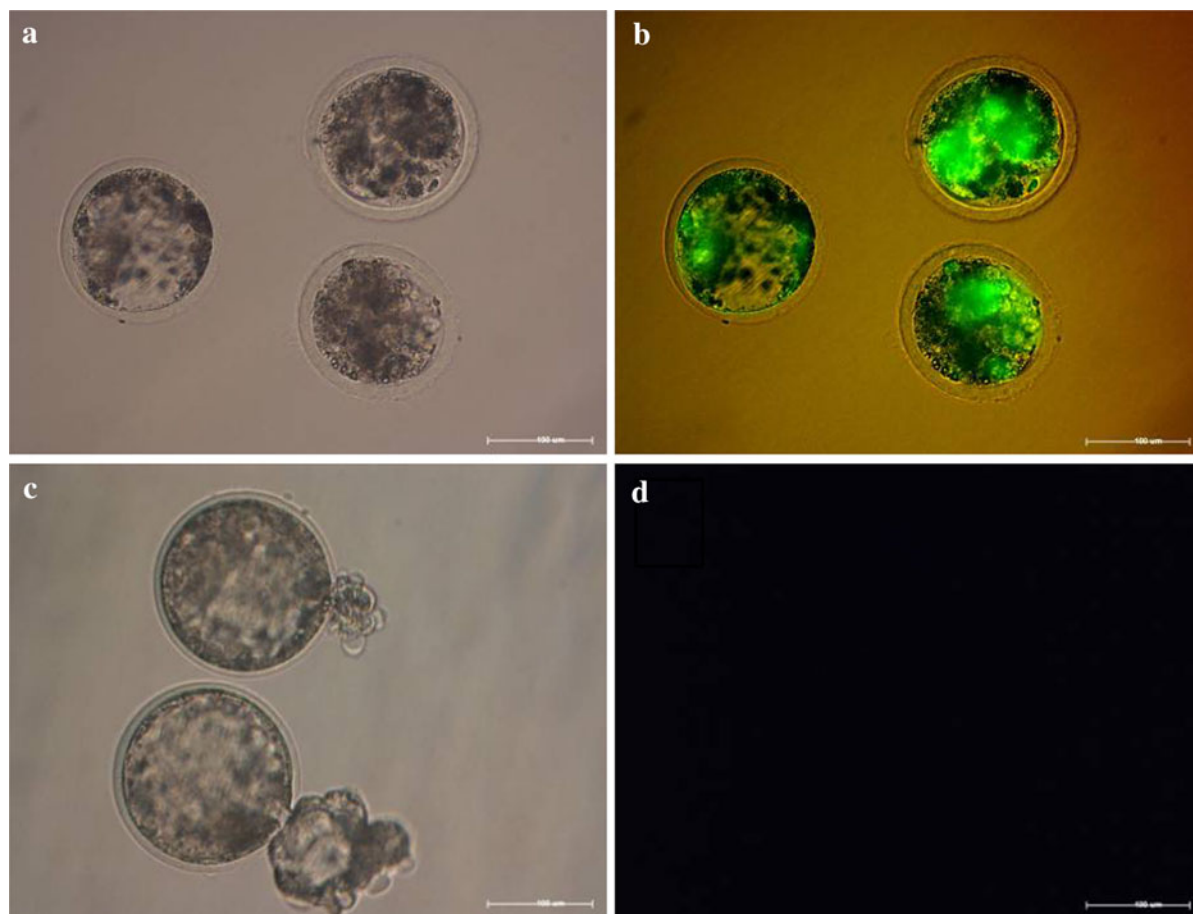


Fig. 4 Green fluorescent protein expression in transgenic embryos. **a** bright field image of transgenic cloned embryo, **b** fluorescent field image of transgenic cloned embryo, **c** bright

field image of non-transgenic cloned embryo, **d** fluorescent field image of non-transgenic cloned embryo. Scale bars 100 μm

Table 1 hLYZ (♀) cell line early embryo development

Cell line	No. of embryos cultured	No. of embryos cleaved (%; mean \pm SEM) ^a	No. of embryos blastocyst (%; mean \pm SEM) ^b
hLYZ-1	107	72 (66.1 \pm 6.7) ^c	9 (9.0 \pm 2.8) ^d
Non-transgenic cells	124	92 (73.6 \pm 4.3) ^c	23 (18.5 \pm 1.8) ^e

Different superscript letters in the same column indicate significant difference ($P < 0.05$); experiments were repeated on more than three occasions

^a Cleavage rate = no. embryos cleaved/no. embryos cultured

^b blastocyst rate = no. blastocyst/no. embryos cultured

These data confirmed correct and efficient transcription and translation of the *hLYZ* gene in C-127 cells transfected with the mammary-specific expression vector pBC1-hLYZ-GFP-Neo and furthermore, demonstrated recombinant protein secretion. Verification of the bactericidal activity of the recombinant

protein against gram-positive bacteria demonstrated the suitability of this vector for investigation of the generation of mammary biological reactors in goats.

Somatic cell nuclear transplantation (Wilmot et al. 1997) provides a highly efficient method for producing transgenic animals. Viable offspring are obtained from

a number of donor cell types (Campbell et al. 2007), although fibroblasts have been widely used for this purpose in many studies (Rius et al. 2010) and have exhibited higher cloning efficiency after SCNT in comparison to other types of somatic cells (Liu et al. 2007). Therefore, fibroblasts derived from transgenic dairy goats were used as donor cells in the generation of transgenic animals in this study.

Transfection of donor cells and screening of positively transduced cells are critical procedures during transgenic operations. Frequently used methods for exogenous gene transfection of somatic cells include electroporation and viral and liposomal transfection, each of which is associated with significantly different efficiencies. Liposomal transfection is simple and safe and has been widely applied in various mammals including pigs, cow, goats and rabbits. After confirmation of the efficacy of the pBC1-hLYZ-GFP-Neo vector in the C-127 cell in vitro model, the liposomal method was used in this study to transfect the fibroblasts of goat embryos. G418 was used to screen for positive transduction. A cell line harboring the *hLYZ* gene was generated and used as the donor cell for nuclear transplantation to obtain a cloned transgenic embryo. Statistical analysis revealed no significant differences in the cleavage rate of the reconstructed embryo and the normal somatic cells used as a control. In contrast, the blastocyst rate of the reconstructed embryo was significantly lower than that of the normal somatic cells, which is in accordance with results reported by Xiong et al. (2011) in the production of bovine transgenic hLYZ embryos. The aim of this study was to investigate the use of exogenous gene expression vector transfected mammary epithelial cells in the generation of mammary biological reactors. This study demonstrated that the *hLYZ* gene was efficiently expressed in the C-127 cells under the control of milk protein regulating genes. Furthermore, the recombinant protein was shown to be correctly transcribed by RT-PCR and correct translation and folding was detected at the protein level by Western blot analysis. Subsequently, positive transgenic cells were used as nuclear donors for the generation of reconstructed dairy goat embryos. This technique represents a highly efficient and accurate approach for the generation of transgenic animals and establishes the basis for cultivation of novel mastitis-resistant transgenic goats and expressing rhLYZ.

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