

Generation of β -lactoglobulin-modified transgenic goats by homologous recombination

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β -Lactoglobulin (BLG) is a dominant allergen present in the milk of goats and other ungulates, although it is not found in human breast milk. Thus, the presence of BLG restricts the consumption of goat's milk by humans. In the present study, we examined whether the disruption of the *BLG* gene in goats by homologous recombination (HR) reduced BLG content in goat's milk and decreased the allergic response to milk. In one approach, exon 2 of the *BLG* gene was efficiently targeted using HR with a *BLG* knockout vector. In a second approach to disrupt *BLG* gene expression and drive exogenous human α -lactalbumin (*hLA*) gene expression, two *hLA* knock-in constructs were used to target exons 1–4 of the *BLG* gene via HR, and expression of *hLA* was then confirmed in goat mammary epithelial cells *in vitro*. The recombinant clones from both approaches were then used for somatic cell nuclear transfer, generating two transgenic goats possessing a *BLG* knockout allele or site-specific *hLA* integration allele. Milk assays demonstrated a reduction in BLG levels in both the *BLG* knockout and *hLA* knock-in goats; furthermore, hLA was present in the hLA knock-in goat's milk. Allergenic analysis in mice indicated that the transgenic goat's milk was less allergenic than wild-type goat's milk. These results support the development of gene-targeted animals as an effective tool for reducing allergic reactions to milk and improving nutrition.

Introduction

β -Lactoglobulin (BLG), which does not exist in human milk, is a major whey protein in goat's milk and is considered as one of the primary milk allergens for a number of people, especially infants and young children [1]. The demand for goat milk is growing as a result of its special value for human nutrition and wellbeing [2] and a variety of biochemical approaches are being investigated to reduce BLG allergenicity [3–5]. However, these strategies can only alleviate the allergic reaction but cannot completely eliminate the

BLG allergenic effect, and they may even introduce new antigenic sites [1].

As a primary whey protein in most mammalian milk, α -lactalbumin (α -LA) accounts for 25–35% of total human milk protein, which is higher than the proportion in goat milk. The amino acid composition of human α -lactalbumin (hLA) is very balanced and is rich in essential amino acids of tryptophan, lysine and cysteine. hLA can increase brain serotonin synthesis and affect various related behaviors, such as appetite,

Abbreviations

BLG, β -lactoglobulin; FCAF, Freund's complete adjuvant; GEF, goat adult fibroblasts; GFFs, goat fetal fibroblasts; GMECs, goat mammary epithelial cells; hLA, human α -lactalbumin; HR, homologous recombination; SCNT, somatic cell nuclear transfer.

mood, sleep and cognition [6]. Thus, it is often added to infant formula milk. Furthermore, complexes of α -LA with β -1,4 galactosyltransferase can promote lactose synthase, and the accumulation of lactose within the Golgi apparatus plays an important role in the assembly of the aqueous phase of milk [7,8]. More recently, studies have shown that hLA has an important role in preventing neonatal gastrointestinal infections [9,10], inducing tumor cell apoptosis [11] and treating skin warts caused by the human papilloma virus [12].

DNA damage can spontaneously induce chromosomal double-strand breaks, which can be precisely repaired by homologous recombination (HR) based on the presence of homologous sequences elsewhere in the genome (e.g. a homologous chromosome, a sister chromatid, or homologous construct) [13]. This HR repair may introduce short or long DNA fragment insertion at a specific locus, resulting in the function loss of the target gene as a result of a reading frame shift mutation or base replacement. HR-mediated gene targeting technology is a directional modification method for changing the apparent livestock traits at the DNA level. It can target a gene at a particular locus in the genome at the same time as not only avoiding position effects or ectopic expression by random integration [14–16], but also exploiting endogenous regulatory elements to achieve efficient gene expression. Subsequent to the first production of transgenic sheep with a therapeutic transgene inserted at the ovine α 1(I) procollagen (*COL1A1*) locus by HR [17], efficient and reproducible gene targeting strategies with fetal fibroblasts and somatic cell nuclear transfer (SCNT) have been widely applied in transgenic research.

In the present study, we report the production of *BLG* knockout and *hLA* knock-in transgenic goats by conventional HR. The *BLG* gene was chosen as a suitable target site for establishing gene targeting in goat fetal fibroblasts (GFFs). We first transfected the knockout or knock-in vectors to GFFs to assess the targeting efficiency via HR. We then generated gene-targeted goats with reduced BLG content or hLA present in animal milk. This offers a direct platform for evaluating the levels of serum total IgE and BLG-specific IgE, which represent milk allergenic sensitization *in vivo* [18,19].

Results

Construction of targeting vectors

The goat BLG protein is specifically expressed in mammary epithelial cells and can be secreted into

goat's milk. Therefore, *hLA* expression driven by *BLG* promoter in goat mammary epithelial cells (GMECs) is appropriate. For the first construct pBLG2-neo (Fig. 1a), exon 2 of the *BLG* gene was deleted and replaced by the neo cassette (Fig. 1b). For a second construct, pBLG1-hLA-neo (Fig. 1a), the endogenous BLG 5'-UTR was used to initiate the translation of the *hLA* gene (Fig. 1c). A third construct, pBLG1M-hLA-neo (Fig. 1a), was prepared for comparison of the targeting efficiency with pBLG1-hLA-neo when containing different homologous arms (Fig. 1c).

Gene targeting with pBLG2-neo

The optimal concentration for G418 selection and the conditions for electroporation were assessed before conducting the experiment (Fig. 2a,b). The GFF cell lines were then electroporated with pBLG2-neo. After the G418 ($600 \mu\text{g}\cdot\text{mL}^{-1}$) and GCV ($1.8 \mu\text{M}$) selection for 10–12 days, 20 out of 10 213 drug-resistant colonies were analyzed with targeting by nested-PCR (Fig. 2c and Table 1). A frequency of targeting of 0.20% was observed in PCR analysis. Karyotyping analysis of the targeted cell clones revealed a normal karyotype and displayed a full complement of 60 chromosomes (Fig. 2d-A). These three clones were frozen in small aliquots and used as nuclear transfer donors.

Gene targeting with pBLG1-hLA-neo and pBLG1M-hLA-neo

Results from targeting with pBLG1-hLA-neo and pBLG1M-hLA-neo are presented in Fig. 2c and Table 1. A nested PCR technique was used to analyze a total of 9773 and 7000 drug-resistant colonies from transfections with pBLG1-hLA-neo and pBLG1M-hLA-neo, respectively (Table 1). Twelve colonies were detected as targeted by nested-PCR based on the transfection of pBLG1-hLA-neo constructs (Fig. 2c), leading to a targeting efficiency of 0.12% in all drug resistant clones. However, for the targeting construct, pBLG1M-hLA-neo, only four targeted clones were detected, even though 7000 cell clones were analyzed (Fig. 2c), resulting in a integration frequency of 0.05%. Targeted cell clones generated from targeting event with pBLG1-hLA were analyzed by karyotype analysis (Fig. 2d-B) and used as nuclear transfer donors.

BLG promoters can direct hLA expression in recombinant adenovirus vector *in vitro*

To ensure that hLA could be promoted by the endogenous BLG regulator, we constructed three

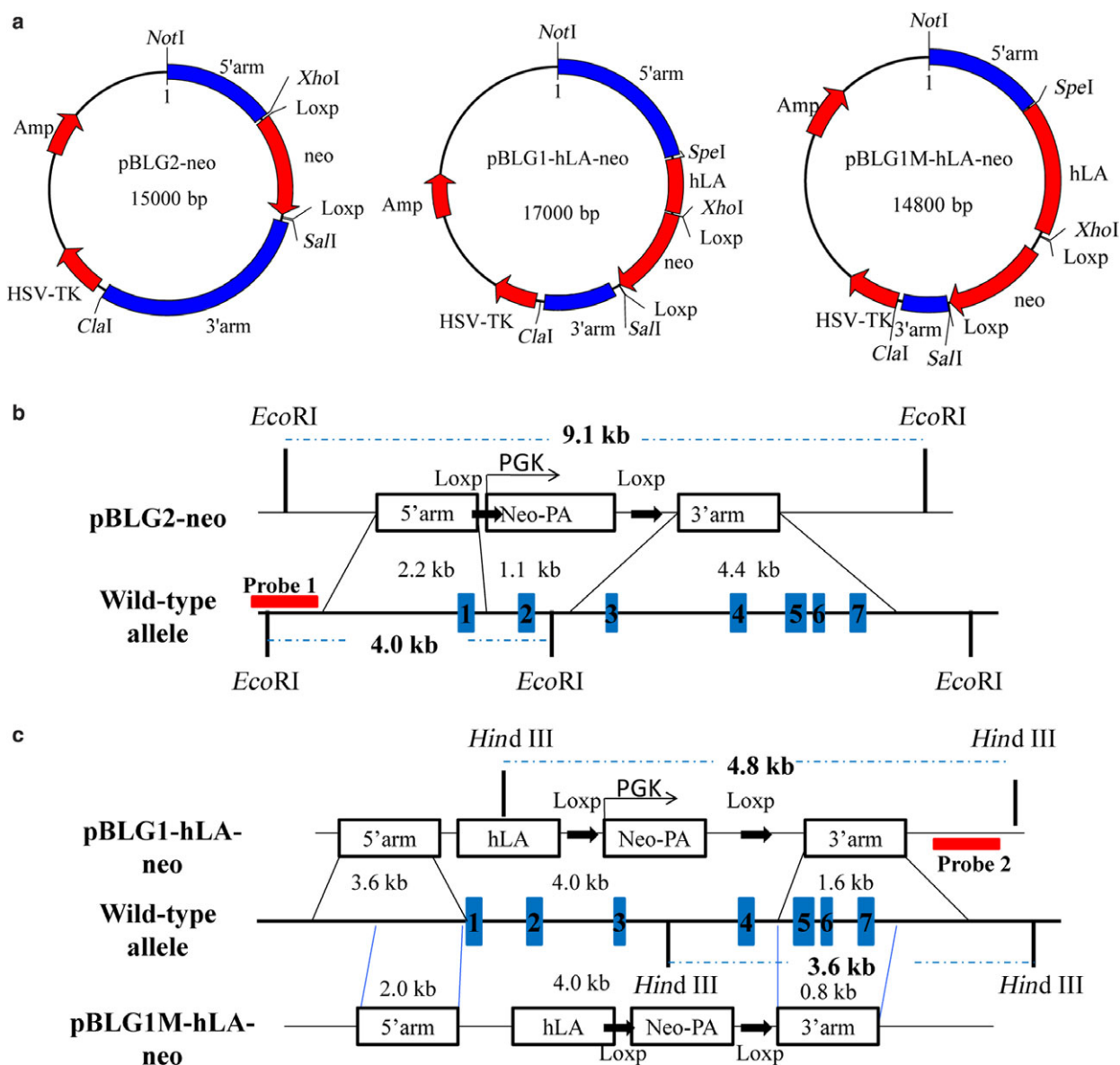


Fig. 1. Schematic overview depicting the targeting strategy for the *BLG* locus. (a) Map of three targeting constructs. (b) Targeting strategy with the construct pBLG2-neo. Blue boxes: exons of *BLG*; red boxes: probes used for Southern blot analysis. (c) Targeting strategy with the constructs pBLG1-hLA-neo and pBLG1M-hLA-neo. Blue boxes: exons of *BLG*; red boxes: probes used for southern blot analysis. Both (a) and (b) indicate the predicted size of southern hybridization bands with *EcoRI* or *HindIII* digestion for both the endogenous *BLG* locus and the *BLG* targeted locus.

recombinant adenovirus vectors of pAd-B51-RFP, pAd-B52-RFP and pAd-B53-RFP containing three different *BLG* regulatory elements (Fig. 3a). Linear reconstructed *BLG* adenovirus vectors were transfected to HEK293 cells for packaging, and the final titers for the three vectors were 7.1×10^7 , 3.6×10^8 , 2.9×10^8 GFU $\cdot$ mL $^{-1}$, respectively. The titers of three vectors were adjusted in the same way and used to infect GMECs. Then, 12 h later, the GMECs were

cultured for 48 h with induction medium (DMEM/F12 + 10 ng $\cdot$ mL $^{-1}$ EGF + 5 μ g $\cdot$ mL $^{-1}$ prolactin + 1% ITS + 1 μ g $\cdot$ mL $^{-1}$ hydrocortisone). The infected GMECs all showed red fluorescent protein expression (Fig. 3b). Subsequently, the RFP region was replaced with the *hLA* gene, and the packaged pAdTrack-B52-hLA vector was seeded in the medium for infecting the GMECs (Fig. 3c-A). After 24 h, the GMECs showed green fluorescent protein expression with an

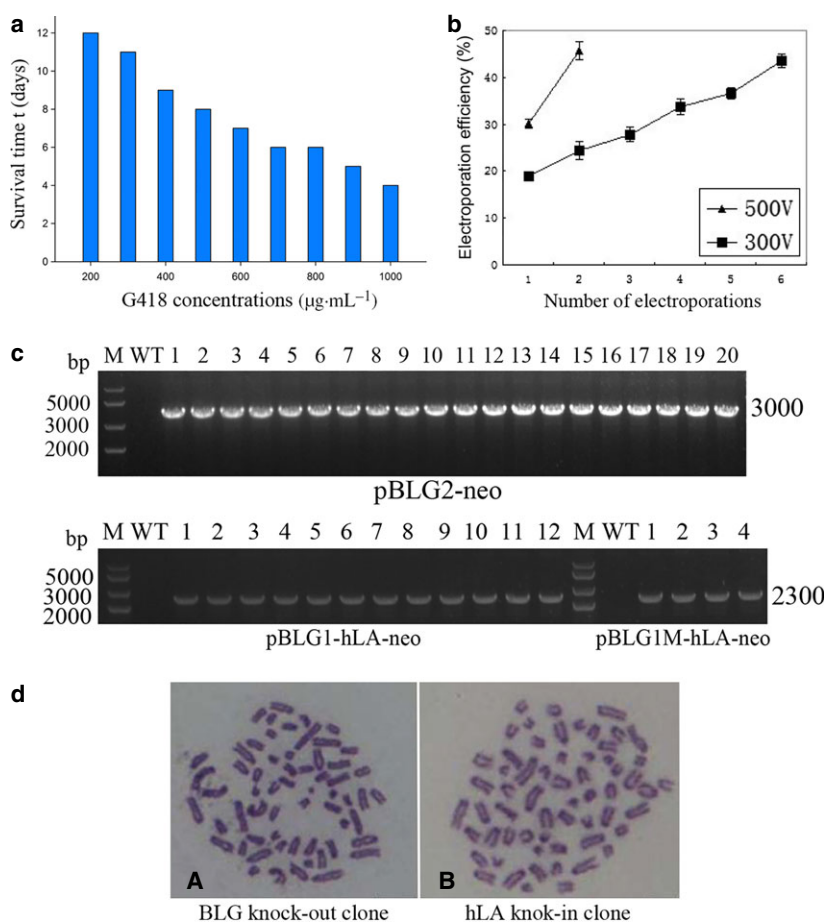


Fig. 2. Analysis of drug-resistant clones transfected with the pBLG2-neo, pBLG1-hLA-neo and pBLG1M-hLA-neo targeting constructs. (a) Survival time of GFF cells under different G418 concentrations. (b) Transfection efficiency with different shocking times. The data points and the error bars show the mean $\pm$ SD of four experiments. (c) Nested PCR analysis. Panels show the 20, 12 and 4 gene targeted cell clones transfected with pBLG2-neo, pBLG1-hLA-neo and pBLG1M-hLA-neo, respectively. Lane WT, nontransfected cells as negative control. Lane M, Trans2k plus II DNA marker. (d) Karyotype analysis. A (left) represents *BLG* knockout clone. B (right) represents the hLA knock-in clone.

Table 1. Selection and detection of the *BLG* gene targeted cells.

Targeting constructs	Number of transfections	Number of drug-resistant clones	Number of targeted clones (%)	Number of clones suitable for nuclear transfer
pBLG2-neo	29	10 213	20 (0.20)	3 (T229F2, T1041F1, T7963F6)
pBLG1-hLA-neo	36	9773	12 (0.12)	2 (B412F1, B7732F6)
pBLG1M-hLA-neo	30	7000	4 (0.05)	0

infection efficiency of 53.6%. After Hoechst 33258 staining of the infected GMECs, the cells exhibited a normal nucleus without significant apoptosis (Fig. 3c-B). Following hormonal induction, the liquid supernatants were collected and processed for western blot analysis, and the results demonstrated that hLA was promoted and secreted in the supernatants (Fig. 3d).

Production and identification of *BLG* gene-targeted goats

The fused efficiency of oocytes and donor cells was evaluated 2–3 h after electrofusion. The results showed that the fusion rate for transgenic embryos was significantly lower than that of nontransgenic embryo fibroblasts (62.8% versus 91.9%; $P < 0.01$) (Table 3).

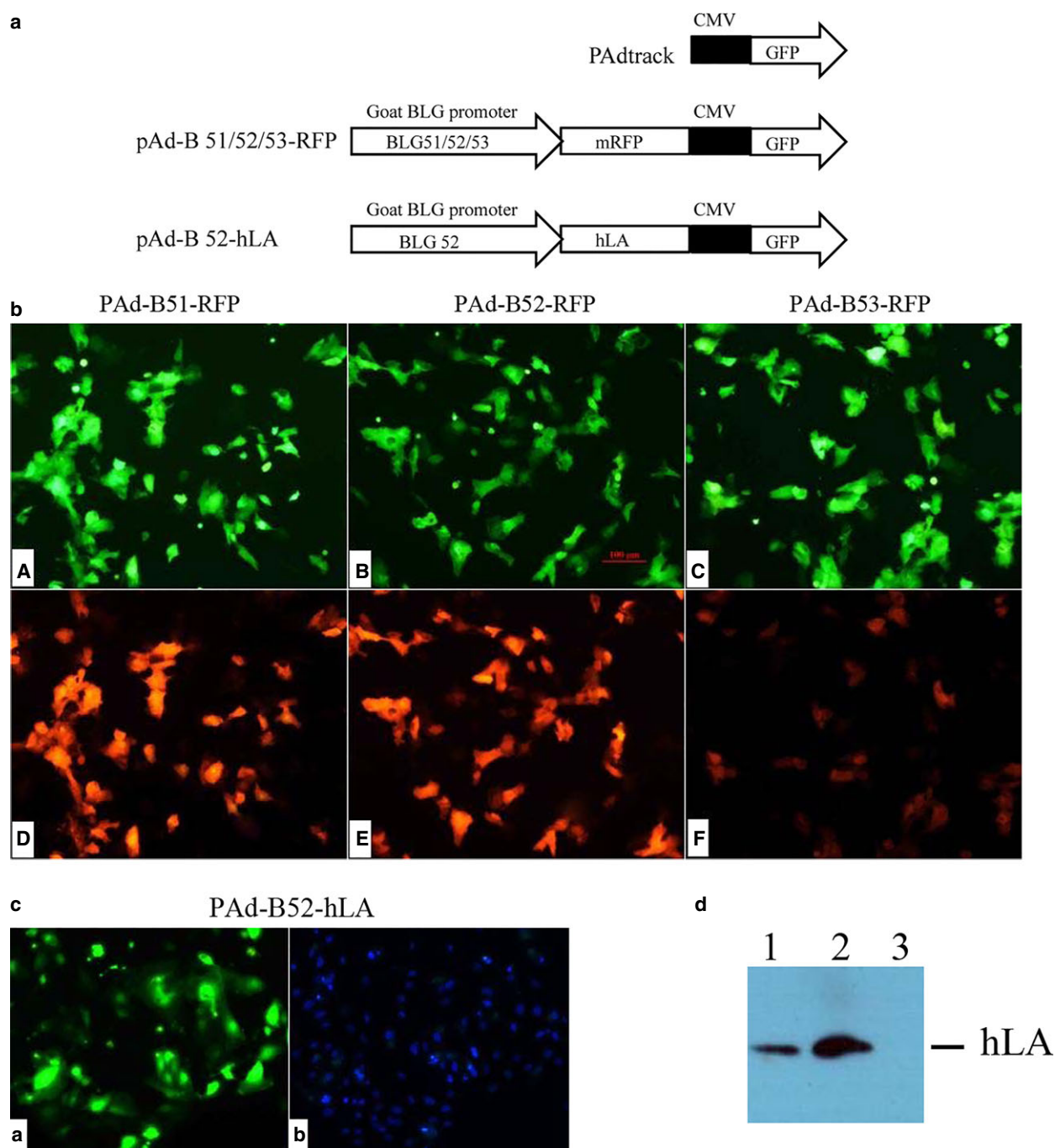


Fig. 3. Evaluation of *hLA* expression mediated by adenovirus vector transfection in GMECs *in vitro*. (a) Schematic representation of BLG51, BLG52, and BLG53 fragments integrase and the *hLA* expression cassettes in adenovirus vector. (b) Evaluation for the BLG promoters in GMECs *in vitro*. (A, D), (B, E) and (C, F) show fluorescence expression in GMECs infected with vectors pAd-B51-RFP, pAd-B52-RFP and pAd-B53-RFP, respectively. (c) The infection of packaged pAd-B52-hLA on GMECs *in vitro*. (A) Green fluorescent expression in GMECs after infection with pAd-B52-hLA vector. (B) GMEC nucleus staining with Hoechst 33258. (d) Western blot analysis of *hLA* expression in supernatant of GMECs with hormonal induction. Lane 1, culture medium of GMECs infected with pAd-B52-hLA. Lane 2, human whey protein. Lane 3, culture medium of GMECs without transfection.

After culturing the gene targeted cloned embryos for 7 days *in vitro* (Fig. 4a), the blastocyst rate for transgenic embryos was calculated. The result reveals an insignificant difference from that of non-transgenic embryos (18.9% versus 22.4%; $P > 0.05$) (Table 2).

The five cell lines T229F2, T1041F1, T7943F6, B412F1 and B7732F6 were used as donors for nuclear transfer. A total of 410 nuclear transfer embryos were transferred to 18 recipient goats (mean of 23 embryos/recipient; range 18–30 embryos/recipient) (Table 3). Eight pregnancies were detected by ultrasonography at day 45, and three were carried to term, resulting in three live births. Modification of the *BLG* gene in goat genomes was confirmed by PCR (Fig. 4b). Southern blot analysis revealed that fetuses GB1 and GBA1 were *BLG* mono-allelic targeted goats (Fig. 4c). Goat GB2 exhibiting a negative Southern blot result may demonstrate that the T1041F1 cell line contains non-targeted cells (Fig. 4c).

The three goats died soon after birth and we cultured the goat adult fibroblasts (GEFs) of GEF1 and GEF2 from the ear tissue of goats GB1 and GBA1. The recovered cells were used for SCNT again, which led to the production of two live goats of GB1O1 and GBA1O1 (Fig. 5a). The transgenic goats

have survived for more than 16 months, and the behaviors of the targeted goats and the wild-type goats are similar.

Detection of *BLG* and *hLA* expression in transgenic goat's milk

Six-month-old prepubertal gene-targeted goats (GB1O1 and GBA1O1) were induced to lactate as described previously [20]. We expected that the *BLG* expression could be decreased greatly in the milk of *BLG* knockout or *hLA* knock-in goats and that *hLA* expression could mimic that of endogenous *BLG* in the milk of *hLA* knock-in goat. Indeed, *BLG* protein was present in the milk from two transgenic goats, and there was a clear reduction in *BLG* expression in transgenic goats compared to the nontransgenic goat according to Coomassie Blue staining of the milk whey (Fig. 5b). Quantitative PCR analysis for *BLG* expression in milk cells demonstrated that *BLG* mRNA levels were decreased by 57–61% in targeted goats (Fig. 5c). Moreover, western blot analysis illustrated a obvious *BLG* reduction in targeted goats and also showed that *hLA* protein was detected in the milk of *hLA* knock- in goats (Fig. 5d).

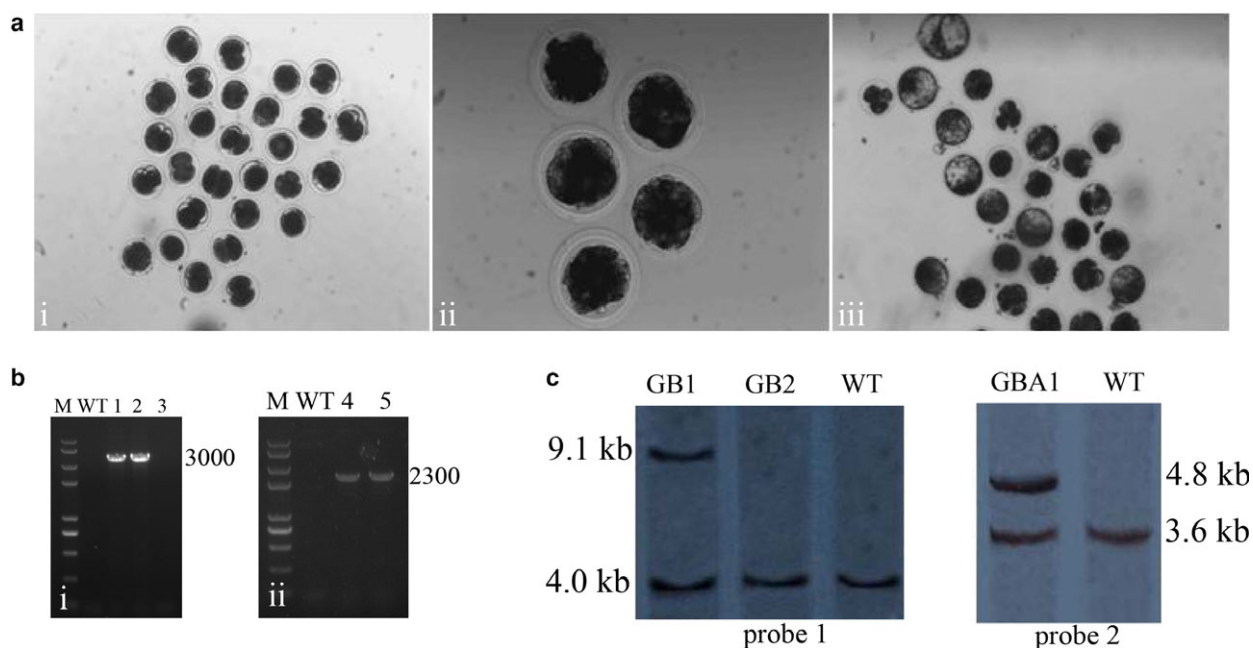


Fig. 4. Production of gene-targeted goats via nuclear transfer. (a) Goat gene-targeted cloned embryos developed *in vitro*. 'i', 'ii' and 'iii' represent the reconstructed embryos at the two-cell stage, morular stage and blastocyst stage, respectively. (b) Identification of cloned goats by nested PCR analysis. Lanes 1–3, cell clones T229F2, goat GB1 and goat GB2. Lanes 4–5, gene-targeted clone B412F1 and goat GBA1. Lane WT, wild-type goat as negative control. Lane M, Trans2k plus II DNA marker. (c) Southern blot analysis of cloned goats. Lane WT, wild-type goat genomic DNA. Lanes GB1 and GB2, *EcoRI*-digested genomic DNA from goats GB1 and GB2. Lane GBA1, *HindIII*-digested genomic DNA from goat GBA1.

Table 2. Development of reconstructed embryos cloned from gene targeted cells. Within a column, values with different superscript letters are significantly different from each other ($P < 0.05$).

Donor cells	Number of embryos reconstructed (%)	Number of fused embryos (%)	Number of cleavages (%)	Number of blastocysts (%)	Number of hatching blastocysts (%)
Targeted cells	258	162 (62.8 ± 3.1) ^c	95 (59.6 ± 3.9) ^b	18 (18.9 ± 2.3) ^a	8 (44.4 ± 1.7) ^a
Nontransgenic cells	247	227 (91.9 ± 2.4) ^a	156 (68.7 ± 2.6) ^a	35 (22.4 ± 0.7) ^a	17 (48.6 ± 2.8) ^a

Table 3. Summary of nuclear transfer results from the gene-targeted goat fetal fibroblast cells.

Donor cells (sex)	Number of embryos transferred	Number of recipients	Number of pregnancies at day 45 (%) ^a	Number of goats at birth (%) ^b
T229F2 (male)	76	3	2 (66.7)	1 (GB2, 1.3)
T1041F1 (female)	93	4	3 (75.0)	1 (GB1, 1.1)
T7943F6 (male)	39	2	0 (0)	0
B412F1 (female)	95	6	3 (50)	1 (GBA1, 1.1)
B7732F6 (female)	107	3	0 (0)	0

^aPregnancy rate = number of pregnant recipients/number of recipients used.^bCloning efficiency = number of kids born/number of embryos transferred.

Transgenic goat milk induced a weaker allergenic reaction in Balb/C mice

Balb/C mice were divided into five groups (C, CF, GM, TGM1 and TGM2; 10 per group). Groups GM, TGM1 and TGM2 were initially sensitized with wild-type goat milk plus Freund's complete adjuvant (FCA) to prepare the milk allergenic mice models. The C and CF groups designated as negative control received PBS and PBS plus FCA, respectively.

The mice were finally given wild-type goat milk (C, CF and GM groups) or transgenic goat milk (TGM1 and TGM2 groups) intragastrically. Hypersensitivity symptoms [21] became evident within 1 h after the first challenge (Table 4). The number of mice with hypersensitivity symptoms in TGM groups was lower than that in GM group. The mice in TGM groups had a more mild milk reaction than that in the GM group. However, the numbers of mice with hypersensitivity symptoms in two TGM groups were close to each other.

One hour after the second administration of milk, blood and intestines were acquired from the mice. The levels of serum total IgE and BLG-specific serum IgE were measured by ELISA. TGM groups exhibited significantly lower serum total IgE levels than the GM group ($P < 0.01$) (Fig. 6a). The BLG-specific IgE levels in TGM groups were also significantly lower than that in the GM group ($P < 0.01$) (Fig. 6b). However, no obvious differences in BLG-specific IgE levels were found between the C, CF and TGM groups. Hemotoxylin and eosin and toluidene blue staining for the intestines demonstrated that the inflammatory cells

infiltration and the mast cell degranulation were less severe around the intestines in the TGM groups and that in the GM group (Fig. 6c).

Discussion

Generally, goat milk has more advantages than the milk of other species with respect to its composition, biological traits and nutritive value [2,22]. However, milk allergen has limited the prevalence of goat milk production for humans. Goat β -lactoglobulin has reportedly participated in milk allergy, which often affects young children [23,24]. Thus, we generated *BLG* gene knockout cloned goats by transferring the targeting vectors containing different genes and homologous arms. The results obtained suggested the feasibility of HR-mediated gene knockout or knock-in at endogenous goat *BLG* loci. In addition, the milk allergenic reaction in Balb/C mice suggested that transgenic goat milk is less allergenic than wild-type goat milk.

In the present study, the targeting frequencies by knockout vector pBLG2-neo and knock-in vectors pBLG1-hLA-neo and pBLG1-hLA-neo are 0.2%, 0.12% and 0.05%, respectively. This is in agreement with previous studies indicating that a shorter distance between two arms or longer homologous arms could result in higher targeting efficiency in livestock. Although engineering nucleases such as zinc-finger nucleases, transcription activator-like effector nucleases and RNA-guided endogenous nucleases could increase the targeting frequencies, off-target cutting and genotoxicity would occur in the genome. Recently, Khan *et al.* [25] and Rogers *et al.* [26] obtained high

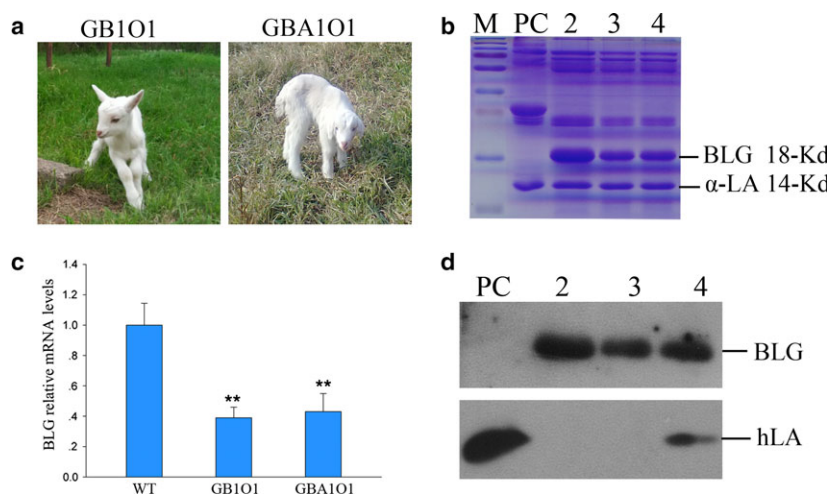


Fig. 5. Milk analysis of gene-targeted goats. (a) Gene-targeted goats GB1O1 and GBA1O1 derived from ear fibroblast cells of goats GB1 and GBA1, respectively. (b) Milk whey protein analysis of targeted goats by Tricine-SDS/PAGE and Coomassie Blue staining. Milk samples (5 μ L) were loaded and analyzed. Lane PC, human milk as positive control. Lanes 2–4, milk samples from wild-type goat, targeted goats GB1O1 and GBA1O1, respectively. (c) BLG mRNA levels via quantitative PCR. These tests were repeated three times; values are the mean $\pm$ SD. The results were compared using Student's *t*-test. Values with asterisks are significantly different compared to the WT group (**P* < 0.05; ***P* < 0.01). (d) Western blot analysis of BLG and hLA in goat milk whey. Milk samples (3 μ L) were loaded and analyzed. Lane PC, human milk as positive control. Lanes 2–4, milk samples from wild-type goat, targeted goats GB1O1 and GBA1O1, respectively.

Table 4. Number of mice showing hypersensitivity symptoms.

Hypersensitivity symptoms	Number of C group	Number of CF group	Number of GM group	Number of TGM1 group	Number of TGM2 group
Scratching and rubbing around the nose and head	1	2	7	3	4
Decreased activity with increased respiratory rate	0	0	4	1	2
Wheezing and labored respiration	0	0	1	0	0
Cyanosis around the mouth and the tail	0	0	0	0	0
Death	0	0	0	0	0

frequencies of approximately 1% by exploiting adeno-associated virus (AAV) for gene targeting [25,26]. Their results demonstrate that AAV could mediate accurate and efficient gene targeting with minimal cytotoxicity in the absence of chromosomal breaks attributed by the single-stranded nature of AAV.

Because the homologous recombination efficiency in mammalian cells is very low [27], an appropriate method must be used to enrich positive cells. In the present study, we choose the positive (Neo) and negative (TK) screening methods to construct the targeting vector. However, positive clone enrichment methods with G418 and GCV drugs accelerated cell aging and apoptosis. Studies show that long-term cell culture with both G418 and GCV screening will inevitably affect cell viability and reprogramming, which may be result in fetal death. Although pregnancies were

established with embryos reconstructed from targeted cell lines used for cloning, the transgenic goats died soon after birth for no apparent reason. Therefore, we suggest that single drug selection with G418 and subsequent PCR analysis may improve the cloning efficiency. Additionally, some researchers consider that fetal death or malformations are mainly a result of incomplete reprogramming of oocytes and the donor nucleus. Studies are now improving reprogramming efficiency by adding exogenous agents [28,29].

The efficiency of homologous recombination ranged from 0.05% to 0.2% in the present study. The low gene targeting efficiency in domestic animal cells aroused the *in vitro* evaluation of *hLA* gene expression under BLG regulators. GMECs are an ideal donor for evaluating exogenous gene expression because they can largely maintain milk synthesis and secretion

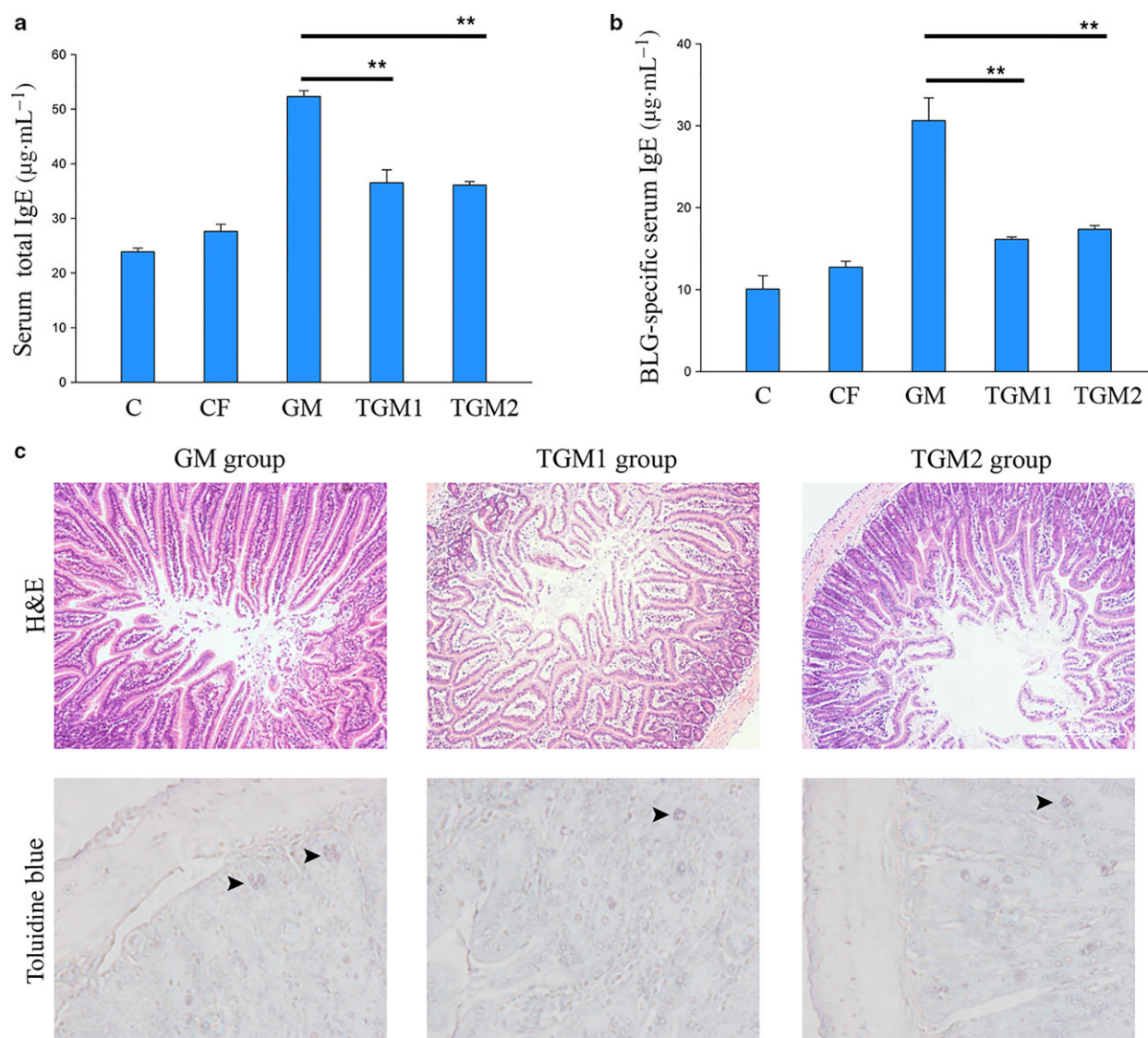


Fig. 6. *In vivo* evaluation of milk allergenic reactions. (a) The serum levels of total IgE in mice after receiving milk was evaluated. Mice in TGM1 and TGM2 groups receiving transgenic goat milk show significant lower IgE levels than GM group receiving wild-type goat milk. (b) BLG-specific IgE levels in mice serum, showing that transgenic goat milk induce almost the same levels with control groups. (c) Hematoxylin and eosin and toluidine blue staining for mice intestines. The arrows indicate the mast cells. These experiments were repeated three times; Values are the mean $\pm$ SD. The results were compared using Student's *t*-test. Asterisks indicate values that are statistically significantly different compared to controls (* $P < 0.05$; ** $P < 0.01$).

mechanisms similar to that reported *in vivo* [30]. However, senescence of GMECs tends to increase after several passages, and the cryopreservation would cause irreversible damage to GMECs. Furthermore, the transfection efficiency in GMECs is lower than that of other cells. In recent years, the adenovirus vector has been broadly applied in the fields of transient gene expression as a result of its larger capacity, higher transfection efficacy and biological safety [31]. In the present study, the recombinant adenovirus vectors

containing goat BLG promoters were constructed to mediate transfection and assess the expression of *hLA* in GMECs. Compared with the highest reported transfection efficiency of approximately 66% in GMECs [32], our approach of exploiting adenovirus vectors reached a comparable transfection efficiency of up to 53.6%, and the GMECs maintained a good condition without senescence. Furthermore, the detection of *hLA* demonstrated that BLG promoters could stimulate *hLA* gene translation, which represents a good

foundation for further studies of mammary gland bioreactors or gene targeting.

Although we have only knocked out one allele of the *BLG* gene, the results show that *BLG* expression in animal milk has been decreased greatly (Fig. 5). To mimic human food allergy, we chose an identical food allergy animal model of Balb/C mice [33] to compare the type I hypersensitivity reaction induced by milk from transgenic goats and wild-type goats. IgE can mediate type I hypersensitivity and activate mast cells and basophils when reacting against a specific antigen, thus increasing the biologically active medium participating in the allergenic reaction. The lower levels of serum total IgE and BLG-specific IgE, as well as the integrity of mast cells in the intestines in transgenic groups, demonstrate that the milk from the transgenic goats provoked a lesser allergenic response than that of wild-type goats.

The present study is the first to show that conventional HR can be performed to generate knockout or knock-in goats by nuclear transfer. Because it is difficult to remove the allergen of BLG from goat milk, the production of BLG knockout and hLA knock-in goats provides a powerful background for reducing milk allergies and improving milk nutrition.

Experimental procedures

Ethics statement

All animals were handled in strict accordance with good animal practice defined by the relevant national and local animal welfare bodies. The experimental procedure was conducted under an animal use protocol approved by the Care and Use of Animals Centre, Northwest A&F University. Goat ovaries were collected from the Tumen abattoir, a local slaughterhouse in Xi'an, PR China. Fetuses and recipient goats were obtained from Yangling Keyuan Cloning Co., Ltd (Yangling, Shaanxi, China). The breed of the fetuses and the recipient goats was Xinong Sannen dairy goat. Female Balb/C mice aged 3–4 weeks old were purchased from the Laboratory Animal Center of Xi'an Jiao Tong University (Xi'an, Shaanxi, China) and housed in a light-controlled environment (12 : 12 h light/dark cycle) at 22 ± 2 °C. All efforts were made to minimize the number of animals used, as well as their suffering.

Oocyte and fetal fibroblast collection

Goat ovaries were transported to the laboratory within 5 h in sterile saline at 26 °C. The ovary follicles were pierced, and the cumulus–oocyte complexes were placed in a maturation medium (TCM-199; Gibco, BRL, Grand Island, NY, USA) for 22–24 h.

The goat fetal fibroblast cells were obtained from 40-day-old fetuses. Each fetus was processed separately. The head, limbs and viscera were dissected and removed, and the remaining tissue was cut into approximately 1-mm pieces. Tissue pieces from each fetus were cultured for fibroblast outgrowth in 60-mm dishes in DMEM/F12 medium supplemented with 15% fetal bovine serum and 1% penicillin/streptomycin. Genomic DNA was isolated from each fetus and analyzed by PCR for sex determination. Cells were trypsinized after reaching 80–90% confluence. They were then frozen overnight in a freezer at -80 °C in 90% FBS containing 10% dimethyl sulfoxide and subsequently stored in liquid nitrogen until use.

BLG targeting constructs

Three targeting constructs were generated to target the *BLG* gene. To disrupt the *BLG* locus, targeting construct pBLG2-neo was designed. A neomycin-bovine growth hormone poly(A) (neo) sequence cassette driven by PGK promoter was inserted between two homologous arms (5' arm: 2.3 kb, contains sequence from partial 5'-UTR to intron 1; 3' arm: 4.4 kb, contains sequences from exon 3 to exon 7) (Fig. 1b). Primers PL01/PL02 and PR01/PR02 were used to amplify the 5' arm and 3' arm, respectively (Table S1).

To replace the *BLG* gene, the targeting construct pBLG1-hLA-neo was generated. The *hLA* gene and neo cassette are flanked by two homologous arms (5' arm: 3.6 kb, contains partial 5'-UTR and exon 1; 3' arm: 1.6 kb, contains sequences from partial intron 5 to all 3' regulatory fragment) and were inserted adjacent to and in frame with the endogenous BLG regulatory element upon targeting (bases 28 of exon 1) (Fig. 1c). The following 4000 bases were deleted upon targeting, including partial exon 1 to partial intron 5. The *hLA* gene contains human endogenous signal peptide, a Kozak consensus sequence and the hLA ORF sequence. Primers BLG5-1F/BLG5-1R and BLG3-1F/BLG3-1R were used to amplify the 5' arm and 3' arm, respectively (Table S1).

Construct pBLG1M-hLA-neo is a variant of pBLG1-hLA-neo obtained by replacing the 5' and 3' arms with fragments with lengths of 2.0 kb (5' arm, contains partial 5'-UTR and exon 1) and 0.8 kb (3' arm, contains the sequence from intron 5 to exon 7 and partial 3'-UTR) (Fig. 1c). The primers BLG5-2F/BLG5-2R and BLG3-2F/BLG3-2R were used to amplify the 5' arm and 3' arm, respectively (Table S1). All PCR fragments were cloned into PMD-19T/PMD-18T (Takara, Dalian, China) and sent to GenScript for sequencing (GenScript Co., Ltd, Nanjing, China).

Fibroblast transfection and drug selection

In total, 29, 36 and 30 transfections were carried out with constructs pBLG2-neo, pBLG1-hLA-neo and pBLG1M-hLA-neo, respectively (Table 1). Fetal fibroblasts frozen at passage 0–2 were thawed and cultured in 60-mm dishes for

72 h in DMEM/F12 supplemented with 15% FBS at 37 °C in an atmosphere of 5% CO₂ in air. Then, cell fibroblasts were harvested by trypsinization, and approximately 1×10^6 cells were electroporated with 15 μ g of linearized DNA at optimal conditions of 500 V, 1 ms and two pulses using a BTX ECM2001 manipulator (Harvard Apparatus, Inc., Holliston, MA, USA).

Electroporated cells were cultured in 90-mm tissue culture plates (2×10^5 cells/plate) for 48 h and then selected in a culture medium containing G418 (Sigma, St Louis, MO, USA) with an optimal concentration of 600 μ g·mL⁻¹. Three days later, the cells were selected with a culture medium containing both G418 (600 μ g·mL⁻¹) and GCV (1.8 μ M) (Sigma). Selection was carried out for 10–12 days and drug-resistant colonies were transferred to 48-well plates. Partial cells from each clone were used for DNA isolation and PCR analysis for targeting events. Possible targets from the 48-well plates were expanded to one hole of 24-well plates and then three holes of 24-well plates. Wells from two plates were used for PCR and karyotyping analysis, and cells in the third plate were frozen as described above in small aliquots for nuclear transfer.

The analyses of drug-resistant colonies

DNA was isolated from drug-resistant colonies in 20 μ L of lysis buffer (40 mM Tris-HCL, pH 8.9, 0.9% Triton X-100, 0.9% Nonidet P-40, 0.4 mg·mL⁻¹ proteinase K) [34]. Nested PCR analysis was performed using 2–3- μ L templates and two sets of primers (6NU/6ND and 1NU/1ND) (Table S1) for preliminary screening of targeted events based on the transfections of pBLG2-neo. For targeting with pBLG1-hLA-neo and pBLG1M-hLA-neo, primers 3PF6/3PR6 and PF1/PR1 were used in the two rounds of PCR analysis (Table S1). All PCR products were recovered and cloned to PMD18-T (Takara) and sent to GenScript Co., Ltd, for sequencing.

Karyotype analysis of the targeted cells was performed as described previously [35]. Briefly, the actively proliferating cultures of positive cell clones were treated with colchicine at 37 °C for 5 h, and then the cells were trypsinized, spun down and incubated in 0.75 M KCL at 3 °C for 30 min. Cells were subsequently fixed with ice-cold acetic methanol/acid (3 : 1, v/v) for 1 min and fixed with new reagent overnight at 4 °C. Thereafter, the cells were collected by centrifugation, and the dispersed cell suspension was smeared on a cold slide, air dried, stained with Giemsa, and observed under microscopy in oil.

Recombinant adenovirus construct harboring BLG promoter

RFP was double digested with *KpnI* and *SalI* and was inserted into the vector pAdTrack (Clontech, Palo Alto,

CA, USA). The positive recombinant vector was named pAdTrack-RFP. Three BLG 5' regulatory fragments, BLG51 [sited in *BLG* locus: (–3588) to (–13); GenBank database: [DQ417346](#) and [Z33881](#)], BLG52 [sited in *BLG* locus: (–2159) to (–13); GenBank database: [Z33881](#)] and BLG53 [sited in *BLG* locus: (–752) to (–13), GenBank database: [Z33881](#)], were amplified and inserted into pAdTrack-RFP just prior to RFP region (Fig. 3a). The positive plasmids were named pAd-B51-RFP, pAd-B52-RFP and pAd-B53-RFP, respectively. Linearized recombinant adenoviral vectors pAd-B51-RFP, pAd-B52-RFP and pAd-B53-RFP were transfected to HEK293 cells with Lipofection 2000 (Invitrogen, Carlsbad, CA, USA) for packaging. Particles were collected during 48–72 h and were concentrated for infecting GMECs. Vector pAdTrack-B52-hLA was constructed by replacing RFP with the *hLA* gene to evaluate hLA expression *in vitro*. Rabbit anti-human α -lactalbumin antibody (dilution 1 : 1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to detect the hLA in the supernatant using western blot analysis.

Nuclear transfer

The SCNT procedure was conducted as described previously [36]. Briefly, the polar body and metaphase plate were sucked out, and a single round donor cell was injected into the perivitelline space of the enucleated oocytes. The karyoplast–cytoplasm couplets were fused by electrofusion and incubated for 2–3 h in TCM-199 supplemented with 10% FBS and 7.5 mg·mL⁻¹ cytochalasin B. The fused embryos were treated with 5 mM ionomycin for 5 min and then 2 mM 6-dimethylaminopurine for 4 h, and then the embryos were then activated. Embryos were washed extensively and cultured in 200 mL of mSOF supplemented with 10% FBS when covered with mineral oil at 38.5 °C in a humid atmosphere with 5% CO₂. One to four-cell stage embryos were transferred into recipients synchronized on day 1 of estrus (day 0 = estrus) 23 embryos per recipient). Pregnancy was determined by ultrasonography at 45 days of gestation.

Southern blotting

Genomic DNA isolated from cloned goats was analyzed by Southern blotting in accordance with standard procedures using a Dig High Prime DNA Labelling and Detection Starter Kit II (Roche Molecular Biochemicals, Mannheim, Germany). GB1 and wild-type goat genomes were digested with *EcoRI*. GBA1, GBA2 and wild-type genomes were digested with *HindIII*. Approximately 40 μ g of genomic DNA was separated on 0.8% agarose gel and blotted on a positively-charged nylon membrane. Probe 1 (760 bp) and probe 2 (213 bp) were generated to identify the targeted integration mediated-by constructs pBLG2-neo and pBLG1-hLA-neo (Fig. 1). Membranes were subsequently

hybridized overnight at 42 °C with the labelled probes in a hybridization solution and exposed to film (Kodak, Rochester, NY, USA) for 20 min.

Analysis of goat milk

Lactation was hormonally induced in 6-month-old goats according to a previously described protocol [20]. Milk samples were centrifuged at 10 000 *g* for 15 min to remove the fat fractions and then diluted with the same amount of PBS (*v/v* = 1 : 1). Thereafter, the diluted samples were adjusted to pH 3.8–4.6 with 1 M HCL to eliminate the casein fraction.

To extract the total RNA from the animal milk cells, EDTA was added to 5 mL of milk at a concentration of 0.5 mmol·L⁻¹ to prevent the precipitation of casein. The cell pellets were collected from the milk precipitate after centrifuging at 200 *g* for 15 min at 4 °C and then re-suspended in PBS for centrifugation at 200 *g* for 5 min. The, Trizol reagent was immediately added to the cell pellet to extract total mRNAs.

The milk whey samples were then separated by 15% SDS/PAGE and transferred onto a polyvinylidene fluoride membrane. After blocking in blocking buffer (Beyotime, Jiangsu, China) at room temperature for 2 h, the membranes were incubated at 4 °C overnight with the primary antibodies. Rabbit anti-human α -lactalbumin antibody (dilution 1 : 1000) (Santa Cruz Biotechnology) and rabbit anti-BLG antibody (dilution 1 : 500) (Bioss, Beijing, China) were used for detecting the hLA and goat BLG proteins, respectively. After washing the membranes three times with Tris-buffered saline and Tween (TBST; 10 min each time), the membranes were incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (Beyotime) for 2 h at room temperature and washed with TBST for 1 h (20 min each time). The blots were finally analyzed using chemiluminescence reagents (Beyotime) to detect protein expression.

Preparation of allergenic mice models and oral administration of goat's milk

Balb/C mice were maintained on a regular mouse chow diet under pathogen-free conditions. The C and CF groups were designated as negative control. The GM, TGM1 and TGM2 groups were used for comparing the allergenic reactions induced by milk from the wild-type goats and the transgenic goats. Animals in the GM and TGM groups were firstly sensitized via intraperitoneal injection with wild-type goat milk plus FCA (Sigma) and boosted four times at weekly intervals. Mice received 1 mg goat milk·g body weight⁻¹ with the same volume of FCA in accordance with a previous study [21]. The C and CF groups only received the PBS and alum in PBS, respectively.

Five weeks after the first feeding and injection, the mice were fasted overnight, and milk was given intragastrically. The C, CF and GM groups received two doses of wild-type goat milk (50 mg per mouse) given 50 min apart, whereas the TGM1 and TGM2 groups received two doses of transgenic goat milk (50 mg per mouse) of GT101 and GAT101. One hour after the last administration, the mice blood was collected for obtaining the serum. The mice intestines were removed for hemotoxylin and eosin and toluidine blue staining.

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Author contributions

YPJ and YZ designed the research. LYH, HMZ, JL, CCC and YJS performed most of the experiments. HTC contributed to the plasmid. LYH and HMZ analyzed the data. HMZ and LYH wrote the paper.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. PCR primers used in the generation of BLG modified goats.

Generation of β -lactoglobulin-modified transgenic goats by homologous recombination

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Table S1. PCR primers used in the generation of BLG knockout goats. Letters underlined are the restriction enzyme cutting sites.

Primer name	Primer sequence
PL01	5'-ATTT <u>GCGGCCG</u> CTCACAGGAAGTTGGGAGACC-3'
PL02	5'-CCGCTCGAGCCTAGCTGACTGATGCGAAC-3'
PR01	5'-GCTCTAGAAAATGAACAAGATGTCCTGGAAACG-3'
PR02	5'-ACGCGT <u>CGACC</u> CTGGTGGCACAGTGGTAAAGAAT-3'
BLG5-1F	5'-TCAGCGGCCGCTTCAAATCCGTGAGCATGATGT-3'
BLG5-1R/5-2R	5'-ACA <u>ACTAGT</u> GGTCGTGCTTCTGAGCTCTG-3'
BLG5-2F	5'-TCAGCGGCCGCTCTCTCTCTCTGGATGGT-3'
BLG3-1F/3-2F	5'-AAGGTCGACCTTCCTGAGGTGTCCAGTCCCAT-3'
BLG3-1R	5'-GGAATCGATGGCTACCCACTCCAGTATTCTTGC-3'
BLG3-2R	5'-GCAATCGATGAGTCCCTCCCTGGTTCCTG-3'
3PF6	5'-TGCTCCTGCCGAGAAAGTATCCATC-3'
3PR6	5'-GGTGTTCCCAGGTCACAGTCTACCC-3'
PF1	5'-ATCAGGACATAGCGTTGGCT-3'
PR1	5'-GGTGGCACAGTGGTAAAGAA-3'
6NU	5'-AAAGCCCACATACAGCAGGGAAGAC-3'
6ND	5'-CAGGAGCAAGGTGAGATGACAGGAG-3'
1NU	5'-TGAAGGGCATGGTTTATGGT-3'
1ND	5'-GCAGCCGATTGTCTGTTGTG-3'