The goat β-casein/CMV chimeric promoter drives the expression of hLF in transgenic goats produced by cell transgene microinjection

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Abstract. There is growing interest in the application of lactoferrin (LF) as a drug or food additive for animals and humans. The objective of this study was to produce transgenic cloned goats that would serve as living bioreactors, expressing high levels of recombinant human LF (rhLF) in their milk. We designed a pCL25 expression vector containing goat β-casein/CMV chimeric promoter in order to facilitate rhLF expression. This pCL25-rhLF-Neo vector was microinjected into goat fetal fibroblasts. G418 selection and PCR analysis were used to identify transgenic donor cells suitable for somatic cell nuclear transfer (SCNT). After SCNT and embryo transplantation, goats harboring the *hLF* gene were produced, as confirmed via PCR and southern blotting. The average rhLF concentration in milk from this transgenic goat was 3.89 mg/ml as determined via ELISA. We also used an optimized buffer in order to effectively elute high-purity (95.8%) rhLF from a cation-exchange column, with the recovered rhLF exhibiting high biological activity. Findings from this study demonstrated that it is possible to generate a transgenic goat harboring the hLF transgene driven by the goat β-casein/CMV chimeric promoter. It represents an initial step towards the production of rhLF, potentially allowing for industrialized purification in the future.

Introduction

There is an increasing need for the production of recombinant therapeutic proteins generated via a range of transgenic techniques, with the optimal approach to such expression being to produce these recombinant proteins in bioreactors such as bacteria, yeast, plants, mammalian cells, or transgenic animals (1-7). Among these bioreactors, mammary gland bioreactors in transgenic animals offer the advantage of being fully compatible with humans and being approved by the FDA (8). Such mammary gland-derived recombinant proteins have already been implemented for clinical use (9) For example, recombinant human antithrombin III (Atryn®) is produced from the milk of transgenic goats (10). Such mammary gland bioreactors are highly advantageous for the production of those proteins that require post-translational modifications in order to mediate their stability or activity (11). Producing recombinant proteins in mammary glands therefore represents a more profitable approach to the production of recombinant human proteins. Pantano *et al* suggested that relatively few mammary cells in transgenic animals ultimately express recombinant proteins (12), underscoring that there is an urgent need to determine how to bolster the *in vivo* expression of these recombinant proteins using optimized expression vector systems.

Achieving high-level production of recombinant proteins within the milk of transgenic animals depends upon ensuring high-level transcription of the introduced cDNA. This makes it essential to select appropriate cis-acting elements, including promoters and enhancers, for the introduced genes. Large quantities of β-casein proteins are produced in goats during lactation in response to hormonal stimulation with β-casein concentrations being 43% higher in goat milk relative to bovine milk (13,14). It is thought to be a binding site for STAT5a in the -300 bp region of the goat β-casein promoter, and this binding site serves to mediate responses to lactogenic hormone stimulation (15). This lays the theoretical foundation for the selection of a goat β-casein promoter, thus allowing for the efficient expression of proteins in mammary glands.

Although the goat β-casein promoter has previously been widely used to drive the transcription of many recombinant proteins in transgenic goats, the expression of these proteins has not been sufficiently high for commercial applications. A variety of approaches have been employed in an effort to boost

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mammary expression of these recombinant proteins, including the use of distal regulatory elements/large genomic DNA fragments (16), insulators (17), matrix-attached regions (18), and targeted site integrations (19). The cytomegalovirus (CMV) promoter is a high-efficiency promoter/enhancer widely used for transgene expression in cells. Zarrin *et al* found this promoter to be more efficient than alternatives such as the SV40, Rous sarcoma virus (RSV), and Vλl promoters for certain B-cell lines (20). There are few reports, however, regarding the use of a goat β-casein/CMV chimeric promoter to facilitate protein production in the mammary glands of medium and large transgenic animals.

The properties of a given protein determine its protein purification strategy. LF is a cationic protein, thus making it well suited to purification via cation-exchange chromatography (21,22). This approach is widely used for bovine LF purification by bLF-producing companies. Concanavalin A affinity chromatography or metal ion affinity chromatography are also viable strategies for purifying LF owing to its glycosylation and Fe^{3+}-binding activity (23,24).

In this study, we generated a transgenic goat harboring the human lactoferrin transgene driven by a chimeric goat β-casein/CMV promoter. This animal was generated using goat fetal fibroblasts microinjected with the pCL25-rhLF-Neo vector as SCNT donor cells, allowing for mammary gland-specific transgene expression (25,26), while retaining the biological characteristics necessary for better efficacy as a drug or food additive. We additionally conducted ELISAs, western blot, and antibacterial activity assays to confirm that human lactoferrin was efficiently expressed in transgenic goat milk while retaining its normal biological activity.

Materials and methods

Ethics statement. Animal experiments and procedures were performed in accordance with the guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of the People's Republic of China) and approved by the animal care and use committee of Yangzhou University, Yangzhou, China [license no. SYXK(Su)2017-0044]. A total of 50 female dairy goats (45-60 kg, 13-18 months old; Jiangsu Academy of Agricultural Sciences, Nanjing, China) used in the current study were raised at room temperature (25±2˚C), with a 12 h day/night cycle, and allowed free access to food and water. All animals were anesthetized using xylazine hydrochloride injection (0.001-0.002 ml/kg) purchased from Huamu Animal Health Products Co., Ltd. during surgery, with all possible effort being made to reduce their pain, distress, and suffering.

Lactoferrin expression vector construction. Human lactoferrin (GenBank: KT006756.1) cDNA was synthesized by Genscript (China), using cDNA containing 5' and 3' terminal *Xho*I sites. The sequence encoding the mature lactoferrin peptide was fused to both the goat β-lactoglobulin signal peptide as well as the Kozak translation initiation sequence.

The synthesized lactoferrin gene was cloned into the pCL25 vector (generated internally) containing the goat β-casein/CMV chimeric promoter and a neo-selectable cassette in the goat β-casein 3' genomic region near the vector *Not*I site (Fig. 1). *Not*I and *Sal*I were used for vector digestion, and a QIAquick Gel Extraction kit (28704, Qiagen, Germany) was used to purify the resultant fragments.

Cell culture and transgene expression. A 30-day old fetus was surgically removed from a Sannen dairy goat and used to generate fibroblasts. Briefly, fetal tissue was cut into small fragments following the removal of the internal organs, head, and limbs, and these fragments underwent 0.05% trypsin-EDTA-mediated digestion. Fibroblasts were then isolated from the supernatant portion of this digestion and grown using DMEM/F12 (SH30023.01; Hyclone) containing 10% FBS (SH30406.01; Hyclone), and 1% penicillin-streptomycin (SV30010; Hyclone) at 37˚C in a 5% CO_2 humidified incubator. Cells underwent passaging at 80% confluency, and after the second passage cells were aliquoted and frozen in freezing media containing 10% DMSO (D2650; Sigma) and 20% FBS.

Aliquots of cells were frozen, and once they grew to 80% confluency they were microinjected with 5 ng/μl of the purified pCL25-rhLF-Neo DNA fragment using an Eppendorf InjectMan (NI2; Eppendorf) and then cultured as above. After 24 h, the cells were grown in selective medium containing 800 ng/μl G418 (SV3006801; Hyclone) for approximately 10 days. A cloning ring was used to isolate and expand healthy colonies following selection, and these clones were subcultured as above. Some of these subcultures were frozen for long-term storage, while the rest were screened for expression of the transgene via polymerase chain reaction (PCR).

Generation of a transgenic goat via SCNT. Somatic cell nuclear transfer (SCNT) was conducted after identifying transgene-positive clones. Enucleated oocytes served as recipients for transgenic cell nuclei, with a super electro cell fusion generator (EGFE21; Nepa Gene) being used for the SCNT procedure. Next, 5 μmol/l ionomycin (I0634; Sigma) and 7.5 μg/ml cytochalasin B (C6762; Sigma) in M16 medium (M7292; Sigma) was used to activate these reconstructed embryos for 5 min, and then cells were treated with M16 containing 2 mmol/l 6-dimethylaminopurine (D2629; Sigma) and 7.5 μg/ml cytochalasin B for 5 h. After activation, the embryos were implanted into recipient goats, and after a 1-month period these animals were assessed via ultrasound to confirm pregnancy. Approximately 150 days later, kids were delivered naturally. For all kids, a small portion of the ear was taken as a biopsy sample from which DNA was isolated and used to assess transgene incorporation by PCR and southern blotting. DL2000 DNA marker (3427A; Takara) was purchased from Takara Biotech (Dalian) Co., Ltd.

Confirmation of transgene integration in cloned goats. Genomic DNA from the transgenic donor cells and ear tissues of cloned goats was prepared with an Easy Pure Genomic DNA kit (EE101-1; TransGen). A pair of primers specific for human LF was used to determine which donor cells had incorporated the transgene. Primer sequences used were: CMV-crhLF-1: ATGGGCGTGGATAGCGGTTTGAC and CMV-crhLF-2: CCACCATCAAGGGTCACAGCATCG. To identify transgenic goats, the following primers were instead used: CMV-grhLF-1: ATAGTAACGCCAATAGGGA and CMV-grhLF-2: GGTCGCAGTTTGTAGGG. The following

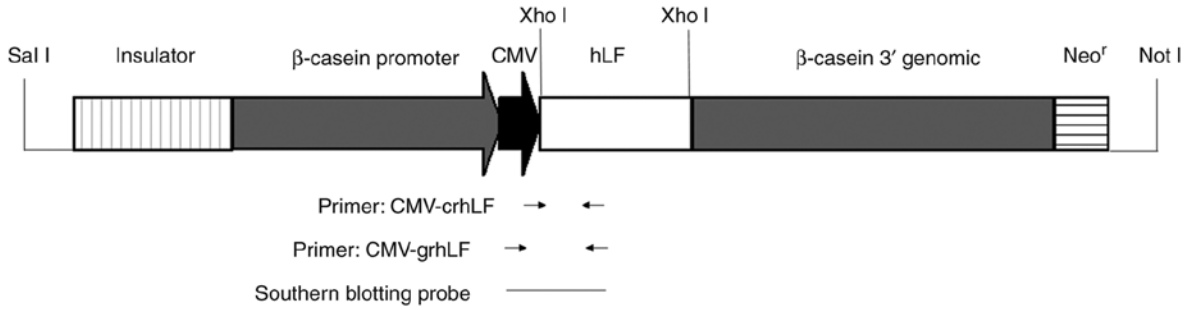

Figure 1. Schematic of pCL25-rhLF-Neo. Insulator: Chicken β-globin insulator (2X); β-casein promoter: Goat β-casein promoter; CMV: Human cytomegalovirus immediate-early promoter/enhancer; rhLF: Optimized rhLF coding region; β-casein 3' genomic: β-casein 3' genomic fragment; Neor: Neomycin resistance gene. Regions identified by Southern blotting probes and primers are also shown.

conditions were used for all PCR reactions: 94˚C for 5 min, 33 cycles at 94˚C for 1 min, 56˚C for 1 min, then 72˚C for 38 sec, and finally held at 72˚C for 10 min. Product sizes for the two primer pairs were 450 and 775 bp, respectively. Sequencing analysis was performed by Sangon Biotech (Shanghai) Co., Ltd.

Southern blotting was next employed to confirm specific transgene DNA integration in goats. Ear biopsy-derived DNA from transgenic and wild-type (WT) goats underwent overnight *Bam*HI digestion, with the PCL25-CMV-rhLF-Neo plasmid serving as a positive control. A digoxigenin-labeled probe then underwent PCR amplification with the CMV-grhLF-1 and CMV-ghLF-2 primer pair. Samples underwent 4-h agarose gel electrophoresis, after which DNA was transferred to a nylon membrane (11417240001; Roche) for blotting. This membrane next underwent probe hybridization for 18 h, followed by incubation with biotin-labeled mouse anti-Digoxin for 30 min. A positive band was expected to be approximately 9.1 kb in size. Southern blotting reagents were purchased from Boster Co. (Wuhan).

ELISAs. Milk samples collected from lactating transgenic and WT goats were centrifuged at 10,000 x g for 30 min at 4˚C for whey isolation. The samples were diluted at 1:10 with PBS, and were used for ELISA reactions with a rabbit-anti-lactoferrin polyclonal primary antibody (dilution 1:2,000, 4% FBS/PBS; Sangon; D121815-0025). After incubation at 37˚C for 1 h and being washed three times with PBS-T (PBS containing 0.05% Tween-20), wells were probed with an HRP-conjugated goat-anti-rabbit secondary antibody (dilution 1:1,000, 4% FBS/PBS; Sangon) at 37˚C for 1 h. The samples then underwent a colorimetric reaction via adding TMB substrate to each well, after which absorbance at 450 nm was measured via microplate reader (Rayto). Protein standards (SRP6519; Sigma) were used for standard curve generation, and sample rhLF concentrations were determined based on this standard curve.

Purification of rhLF from the transgenic cloned goat. Fat and other undissolved substances were removed from milk via centrifugation at 12,000 x g for 30 min at 4˚C, after which the pH was reduced to 4.0 in order to facilitate casein precipitation. Milk was then centrifuged at 4˚C at 100,000 x g for 1 h. Supernatant pH was adjusted to 6.0 using acetic acid, after which the samples were centrifuged again as in the previous step. A protein purification system (ÄKTAprimeTM PLUS; GE Healthcare) was used for all purification reactions. First, after equilibration in a column containing Buffer A (0.07 mol/l HAc, pH 3.1), samples were loaded onto a HiTrap Capto S cation exchange column (1 ml; GE Healthcare) and the bound proteins were eluted via a step gradient of 30 and 100% Buffer B (0.5 mol/l NaCl, 0.07 mol/l Tris-HAc, pH 7.5). The eluate containing 100% Buffer B was collected and desalted via a Bestdex G-25 column (1.6x2.5 cm; BestChrom) for use in downstream experiments. SDS-PAGE analysis was then used to assess protein purity.

Western blotting. Whey was isolated as above and then boiled in SDS loading buffer for 10 min, after which samples were electrophoretically separated using 12% polyacrylamide Tris-glycine gels. Afterwards, these gels were stained using Coomassie Brilliant Blue G-250, and the sample purity and concentrations were determined using Tanon Gis software (Bio-Tanon). For western blotting, separated proteins were then transferred onto PVDF membranes (F019531; Sangon). The membranes were blocked using 5% BSA/TBST overnight at 4˚C, and then probed using a polyclonal rabbit-anti-LF antibody (1:2,000, 10% FBS/TBST; Sangon) at 37˚C for 1.5 h. Next, an HRP-conjugated secondary goat-anti-rabbit IgG (1:1,000, 10% FBS/TBST; Sangon) was used to probe blots at 37˚C for 1 h. The blots were then washed three times in TBST (20 mM Tris-base, 137 mM NaCl, 0.05% Tween-20), and protein bands were detected with an ECL substrate solution (Millipore Corporation) based on provided directions.

Bacteriostatic activity assessment. Lactoferrin has been shown to be able to inhibit the growth of both Gram-positive and -negative bacteria, including important pathogenic species such as *Helicobacter pylori*, *Staphylococcus aureus*, *Shigella flexneri*, enteropathogenic *Escherichia coli* (EPEC) and *Salmonella enterica* serovar *typhimurium* (2,27-31). We therefore selected *E. coli* K88 grown on LB plates as a model strain to test the bacteriostatic activity of transgenic goat milk.

A single *E. coli* K88 colony was transferred into 15 ml LB culture medium, shaken and kept overnight at 37˚C. The resultant bacteria were then streaked evenly across an LB agar plate using a cotton swab.

To test bacteriostatic activity of the milk, sterile filter papers (8 mm in diameter) were placed onto the plate surface, and 100 μl sample aliquots were added on top of this paper.

Table I. Nuclear transfer outcomes.

Cell lines	Oocytes	Reconstructed embryos	Transferred embryos	Recipients	Pregnancies at day 30	No. of newborns
No. 4	95	70	65	5	1	1

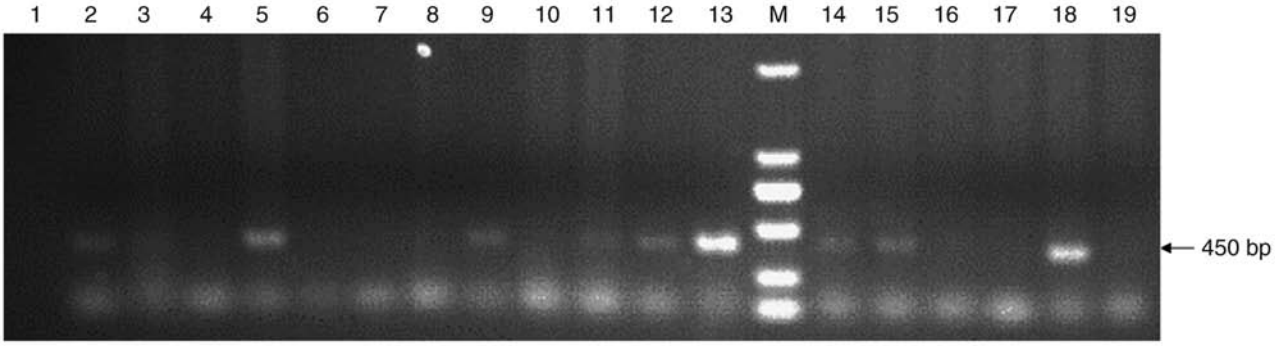

Figure 2. Identification of the rhLF gene in transgenic fibroblasts. 2-17: DNA samples from 16 clonal fetal fibroblast cell lines; M: The DL-2000 DNA marker; 1: ddH_2O as a blank; 18: A positive control of pCL25-rhLF-Neo plasmid mixed with WT genomic DNA l; 19: A negative control of WT genomic DNA.

After a 4-h incubation at 37˚C, the size of the growth inhibition area surrounding a given sample was used to assess bacteriostatic activity.

Results

Lactoferrin expression vector construction. We successfully inserted the LF cDNA fragment into the pCL25 vector, thereby producing a pCL25-rhLF-Neo recombinant vector that was found to be of appropriate size based on restriction enzyme digestion and sequencing. Sequencing confirmed that the rhLF coding region was fused in-frame upstream of pCL25.

Fetal goat fibroblast transfection. Goat fetal fibroblasts were microinjected with 5 ng/μl of the purified pCL25-rhLF-Neo DNA fragment, and were selected using G418. A total of 16 G418-resistant transgenic cells were obtained by single cell amplification. Of these, 9 were determined to express the hLF transgene via PCR using the CMV-crhLF-1 and CMV-crhLF-2 primers (Fig. 2). In total, 56.25% (9/16) of the cell clones had confirmed pCL25 integration. Clone no. 4 cells served as SCNT donors as they were found to exhibit the best viability and quality.

SCNT-mediated transgenic cloned goat generation. SCNT was used to produce transgenic goats as previously identified (27). We transferred 65 reconstructed embryos into 5 recipient goats, leading to the birth of a single female kid that was found by PCR to harbor the pCL25-rhLF-Neo transgene (Table I). The female kid was designated as LF-1 (Fig. 3), and upon reaching sexual maturity underwent mating with a WT ram.

Confirmation of transgene integration in cloned goats. PCR and Southern blotting were used to confirm that the transgenic goat integrated the rhLF transgene. Human LF-specific primers (CMV-grhLF-1 and CMV-grhLF-2) were used to identify the cloned goats by PCR, while digoxigenin-labeled versions of these primers were used as probes for Southern blotting.

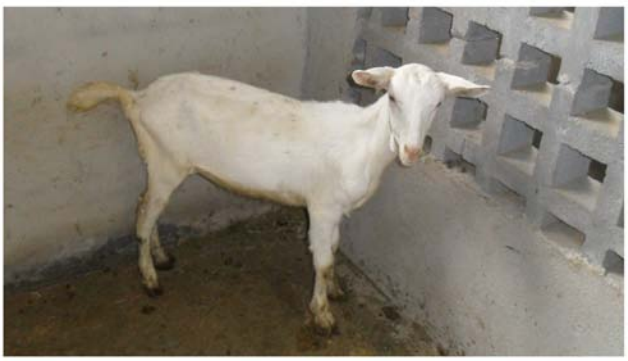

Figure 3. Founder goat LF-1. In this image the LF-1 founder goat was 20 months old.

Following PCR, we were able to amplify a 775 bp product, confirming successful rhLF transgene integration into this cloned goat (Fig. 4A). Southern blotting further confirmed this finding (Fig. 4B).

Assessment of milk rhLF expression in transgenic goats. Expression of rhLF in WT and transgenic goat milk samples was next assessed via ELISA. Milk was collected during days 1-30 of lactation following delivery. We found that the rhLF concentration reached a peak of 4.7 mg/ml on day 4, with an average concentration of 3.89±0.82 mg/ml from days 1-30 of lactation.

To assess the possible ectopic expression of rhLF in this transgenic goat, rhLF levels in the serum and saliva of lactating goat were measured via ELISA. There was no indication of rhLF expression in the serum or saliva of this transgenic goat (data not shown).

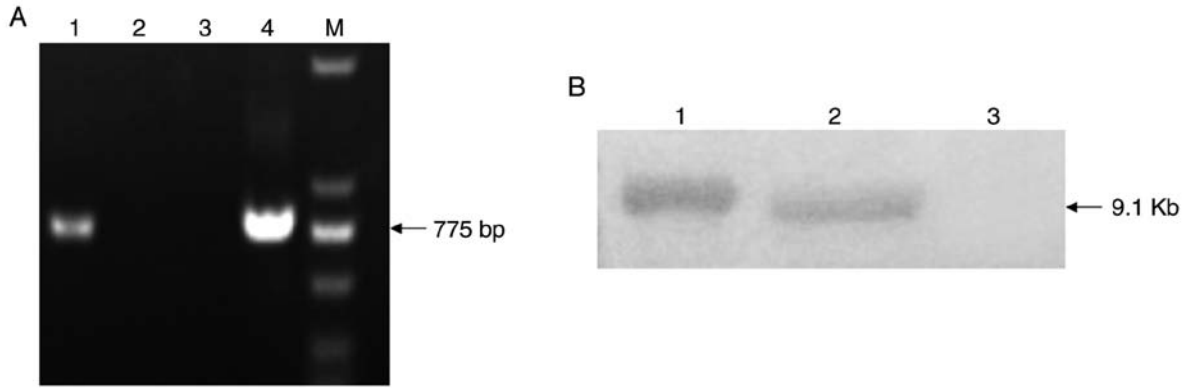

Figure 4. Identification of the rhLF gene in transgenic cloned goats. (A) PCR detection of the rhLF gene in transgenic cloned goats; 1: Cloned goat DNA; 2: Water; 3: WT goat DNA; 4: A positive control of pCL25-rhLF-Neo plasmid combined with WT goat genomic DNA; M: DNA marker. (B) Identification of rhLF in transgenic cloned goats by Southern blot analysis; 1: A positive control of pCL25-rhLF-Neo following *Bam*HI digestion; 2: Cloned goat DNA following *Bam*HI digestion; 3: A negative control of WT goat DNA digested with *Bam*HI.

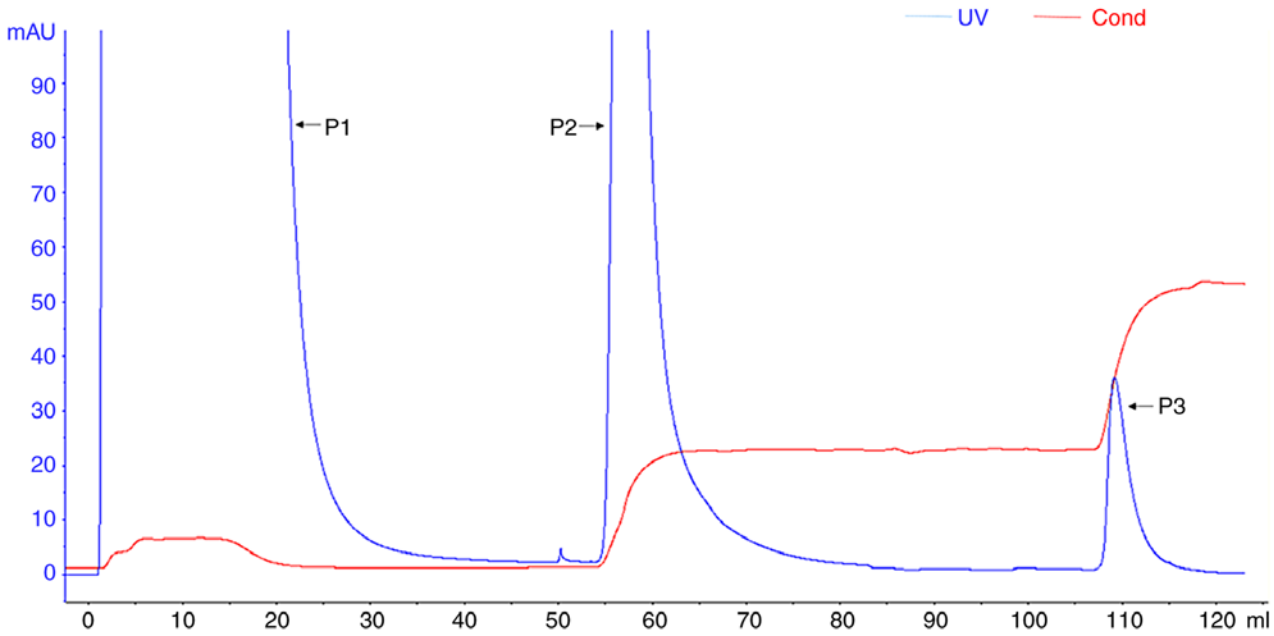

Figure 5. Purification of rhLF via cation-exchange chromatography with a HiTrap Capto S cation exchange column. P1: The flow through; P2: The elution peak 1; P3: The elution peak 2.

Purification of rhLF from the transgenic cloned goat. Cation exchange chromatography can be used to separate lactoferrin from milk, as lactoferrin has a net-positive charge. In order to explore the optimal elution conditions for separation and purification of rhLF via cation exchange chromatography, we first assessed the optimal solution conductivity for rhLF. Stepwise elution was used to achieve one-step elution and separation of the target protein.

Two elution peaks were obtained from the HiTrap Capto S cation exchange column eluted with a step gradient of 30 and 100% Buffer B. SDS-PAGE and western blotting revealed that high-purity rhLF was successfully collected in peak P3 of the eluent (Fig. 5), with a size of 80 kDa. The concentration of the purified rhLF was found to be 1.25 mg/ml by spectrophotometry (One Drop 1000+; OneDrop Technologies, Inc.). The purity was determined to be 95.8% based on densitometric scanning of the SDS-PAGE gel.

Western blotting confirmed that samples in the transgenic goat were identical to native hLF control samples, with a size of approximately 80 kDa (Fig. 6). Bands were absent in the WT control goat sample, as expected.

Assessment of transgenic goat milk bacteriostatic activity. The bacteriostatic activity of the rhLF in the transgenic goat milk was assessed via an agar disc diffusion method in order to allow for observation of bacteriostatic activity *in vitro*. Sterile filter paper was placed onto agar plates containing *E. coli* K88, and bacteriostatic activity was estimated based on inhibition zone sizes surrounding the sterile filter papers following a 4 h incubation at 37˚C. These results revealed that rhLF from transgenic milk exhibited comparable bacteriostatic activity to that of hLF (inhibition zone diameters of 17 and 19 mm, respectively). WT goat milk served as a negative control, with no inhibition zone being evident. We also found that rhLF

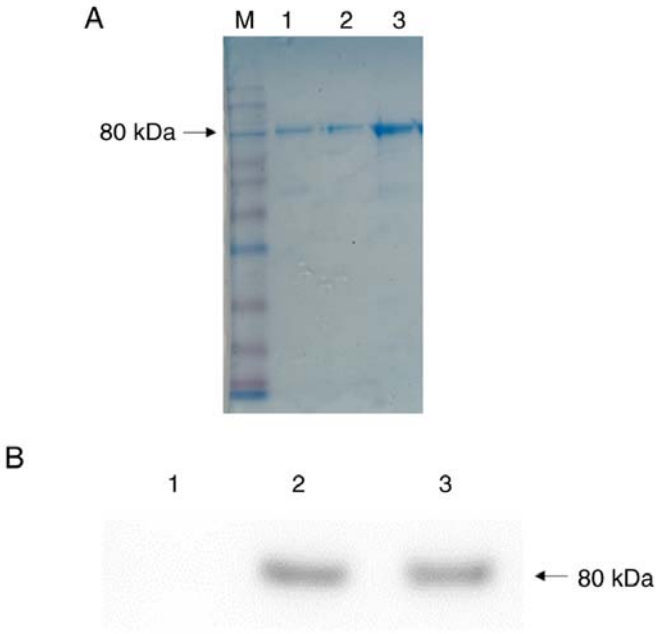

Figure 6. Identification of protein fractions eluted from the HiTrap Capto S cation exchange column. (A) Identification of purified rhLF by SDS-PAGE. 1: Lactoferrin from human milk (SRP6519; Sigma); 2: The elution peak 2 from the HiTrap Capto S cation exchange column; 3: The concentrated protein sample from the elution peak 2 to show purity; M: RealBand Pink Blue Protein Marker (Sangon Biotech). (B) Identification of purified rhLF by western Blotting. 1: 40 μl whey sample from a WT as a negative control; 2: 10 μl sample of purified rhLF from the transgenic goat; 3: 10 μg of lactoferrin from human milk (SRP6519; Sigma) as a positive control.

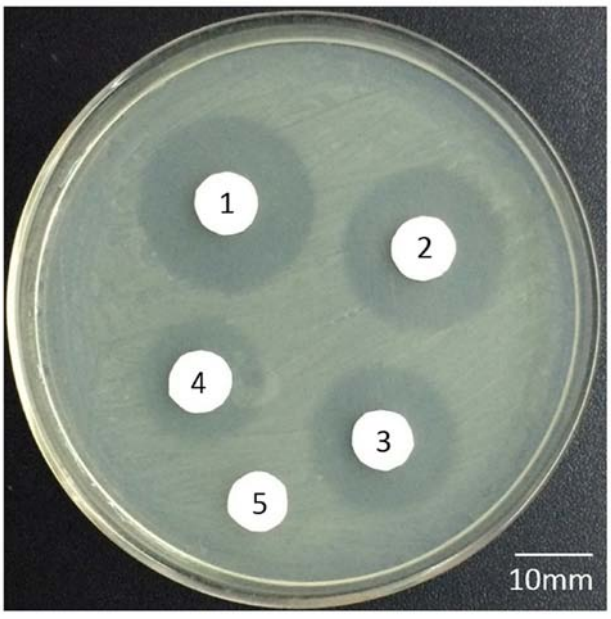

Figure 7. Inhibition of *E. coli* K88 bacterial growth by purified rhLF. 1: 0.4 million units of penicillin and 0.25 million units of streptomycin, circle diameter=22 mm; 2: The whey of human colostrum (x20 concentrated), circle diameter=19 mm; 3: The whey of rhLF transgenic milk (x20 concentrated), circle diameter=17 mm; 4: 1 mg purified rhLF, circle diameter=13 mm; 5: The whey of wild-type goat milk (x20 concentrated), circle diameter=0 mm.

purified by cation-exchange chromatography exhibited similar bacteriostatic activity (an inhibition zone diameter of 13 mm) (Fig. 7).

Discussion

In this study, we successfully used SCNT as a means of generating a transgenic goat producing rhLF in mammary cells, using transgenic goat fetal fibroblast cells as donor cells. To date, there have been no previous reports of using fetal fibroblasts microinjected with rhLF gene as donor cells for SCNT. We detected no abnormalities in the founder transgenic goat or its offspring, indicating no effect of the vector on goat biology. To determine whether the rhLF transgene could be stably transmitted to offspring, the female founder transgenic goat was mated with a wild-type ram and a single male lamb was birthed. A subsequent PCR assay demonstrated that it was transgenic for rhLF (data not shown), indicating that the rhLF transgene can be inherited by offspring.

There are many reports regarding high-level rhLF expression in transgenic mice, rabbits, and cows (32-34). Mice and rabbits, however, are not suitable for large-scale commercial rhLF production due to their limited milk production and short life span (2-3 years for mice, 8-10 years for rabbits). Cows also are not appropriate models for producing rhLF because bovine milk has more allergenic protein than does goat milk (35,36). Therefore, goats are more suitable as a biologic mammary reactor for the large-scale production of rhLF given that goat milk has been reported to contain smaller fat globules and a distinct casein composition relative to bovine milk, making it less allergenic (37,38). At present, many transgenic animals are produced via SCNT or pronucleus microinjection, including sheep (39), goats (40,41), cows (42), mice (43) and rabbits (44). The success rate of SCNT remains low and varies based upon factors such as the vector used, the source of recipient and donor cells, the exact SCNT protocol employed, and the influence of exogenous genes on embryonic development (45-47). The quality of donor cells is critical for producing transgenic animals via SCNT. Preparation of transgenic animals using electroporation-mediated transfection requires optimization of transfection conditions and is often associated with a high rate of cell death. However, cell microinjection avoids these challenges, instead offering a high integration rate while remaining suitable for genetic engineering and the establishment of transgenic animals. In this study, we improved upon the process of preparing transgenic goats using goat fetal fibroblasts microinjected with the rhLF gene as donor cells for SCNT.

In previous reports, we constructed various mammary gland-specific vectors containing a CMV enhancer and a chimeric promoter [goat β-casein, bovine αs1-casein, and goat β-lactoglobulin (BLG)] based on milk protein promoter sequences. These vectors allowed for 1.17-8.10 mg/ml hLF levels in transgenic murine milk-roughly 100,000-fold higher than the levels produced from control promoters (7-40 ng/ml). We also found that the inclusion of the CMV enhancer significantly increased hLF expression in these mice. Use of hLF cDNA did not achieve expression levels as high as those from hLF genomic DNA in these mice (25). Many factors can influence recombinant milk protein expression levels, including copy number, site of chromosomal insertion, and species-specific differences in expression patterns (48,49). rhLF expression levels in transgenic goats can be as high as 4.7 mg/ml-levels which are markedly higher than the levels

observed in transgenic goats without a CMV enhancer (50). There were no indications of rhLF expression in the serum or saliva of the transgenic goat, as the goat β-casein promoter is specifically expressed only in lactating mammary tissue and not at other ectopic sites. This means that there is no potential risk of transgenic animals expressing heterologous proteins in the mammary gland when using this goat β-casein/CMV chimeric promoter. Using western blotting we further confirmed that the size of the rhLF expressed in transgenic goats was roughly 80 kDa, which is comparable to the size of hLF.

The secretion of lactoferrin in milk is directly related to the nutritional status and environmental conditions of the mother, and as such the secretion of lactoferrin in milk can be improved by improving maternal housing conditions and other factors. However, for transgenic animals, in addition to these growth conditions and environmental factors, improving the inheritance and stability of foreign genes remains a major challenge. In this study, our aim was to produce transgenic-cloned goats as living mammary bioreactors that exhibited a high-level of rhLF expression in their milk. An optimized construct is essential in order to achieve a high level expression of recombinant proteins. We used ELISA to confirm the expression of rhLF in transgenic goat milk on days 1-30 of lactation following delivery, revealing that rhLF was continuously expressed in goat milk during this 30-day period. There were no clear decreases in rhLF expression during the lactation period. These results thus clearly show that a transgenic goat carrying the pCL25-rhLF-Neo mammary gland-specific expression vector encoding goat β-casein/CMV chimeric promoter can express rhLF stably in the mammary gland.

At present, phosphoric buffer (PB) is widely used as an eluent when extracting lactoferrin via cation-exchange chromatography (23). However, PB easily associates with common Ca^{2+} ions, Mg^{2+} ions, and heavy metal ions to form precipitates, and it can also inhibit certain biochemical processes as well as the activity of most enzymes. PB is thus not an ideal eluent choice when purifying lactoferrin by cation-exchange chromatography. In order to achieve superior purified rhLF activity, we therefore used a commercially available HiTrap Capto S cation exchange column for its effective purification from the milk of a transgenic goat, using the Tris-HAc buffer as an eluent. Similarly to the rhLF purified in other previous reports (24,51), rhLF purification efficiency in transgenic goats was high (≥95.8%). When we assessed the bacteriostatic activity of this rhLF, we found it to be comparable to that of natural hLF, penicillin, and streptomycin, which suggests that rhLF may be an effective antibiotic for future use.

In conclusion, we have successfully used SCNT to produce a transgenic goat, with goat fetal fibroblast cells serving as donor cells microinjected with the expression vector pCL25. Our results conclusively demonstrate that the pCL25 vector, which contains goat β-casein/CMV chimeric promoter, can drive transgenic goats to stably express a biologically active form of rhLF. This study offers an initial strategy for rhLF production for incorporation into drugs or food products, thereby facilitating future studies of this protein.

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Availability of data and materials

All data and materials are available from the authors.

Authors' contributions

TZ, YY, RL, SX, MZ, TY, YL, and KY performed the studies and analyzed the data. TZ and YC wrote and revised the manuscript. YC conceived and guided the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of the People's Republic of China) was followed for all surgical studies described herein, which received prior approval from the Yangzhou University Animal Care and Use Committee (license no. SYXK(Su)2017-0044). All animals were anesthetized during surgery, with all possible effort being made to reduce the pain, distress, and suffering of the study animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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