Supplementary

Materials and methods

Creation of targeting construct for recombineering

The 4.8-kb hLZ genomic sequence, starting from the ATG start codon to the TAA stop codon and flanked by two homology arms, was obtained by PCR using the BAC clone RP11-1143G9 (Genome Systems Inc., St. Louis, Mo.) as a template and using the following primers: hLF-hLZ-F (5′-CTAGCTAGCAAAAGCCCTGAATAAAAGGGGCGCAGGCGCAAGTGGCAGAGCCTTCGTTTGCCAAGTCGCCTCCAGACCGCAGACATGAAGGCTCTCATTGTTCTG-3′) and hLF-hLZ-R (5′-CTAGCTAGCAAGGGAGGCCAAGCCCACACACACCTGGGAGAAGAGCTGGGGGCTAGTAATGGCTGAGGCTTTCTTGGGGAGCTGGGCCATCTTCTTC TTACACTCCACAACCTTGAAC-3′), where homology arms to the hLF gene are in bold, and NheI restriction sites are underlined. The PCR product was cloned into the pMD19-T cloning vector (Takara, Dalian, China) and confirmed by sequencing. A zeocin (Zeo) cassette flanked by two FRT sites for positive selection was blunt-end ligated into the HpaI site in intron 2 of the hLZ gene. The resulting targeting construct, named pMD19-hLZ-Zeo, was linearized with NheI, and the DNA fragments containing hLZ-Zeo were purified for recombineering.

Modification of BAC clones by recombineering

Recombineering was performed as described (Sharan et al. 2009) (see also http://web.ncifcrf.gov/research/brb/recombineeringInformation.aspx). The procedure consisted of three distinct recombineering steps. In step 1, the hLF BAC clone (GenBank accession number: U95626), which contains the entire hLF genomic DNA plus 90-kb 5′ and 30-kb 3′ flanking regions
was introduced into *Escherichia coli* strain SW102. The linear targeting substrate DNA, the hLZ-Zeo, was electroporated into competent SW102 cells containing hLF BAC. The electroporation parameters were a 0.1-cm cuvette, 1.8 kV, 25 μF capacitance, and 200 Ω. The transformed bacteria were plated on LB plates with zeocin and incubated at 32°C for 20-24 h. Selected clones were picked and subjected to PCR to confirm successful recombination. In step 2, a selectable marker neomycin (Neo) for eukaryotic cells was obtained by PCR using the PL452 plasmid as a template and the primers hLF-Neo-F (5′-GTCTCCTCCTACCTTACCTGGACTGACACATGGACTCTCATTTGAAGGACTTTCTCAGT) and hLF-Neo-R (5′-CTCAAGCCCAATTCGATCATA-3′), where homology arms to the hLF gene are in bold. The Neo cassette was flanked by two loxP sites and was designed to be inserted into the 5′ genomic DNA of hLF BAC for G418 selection of cultured somatic cells. The second recombineering step was performed as described above. In step 3, the Zeo cassette was excised by transformation of a helper plasmid 707-Flpe tetR (Gene Bridges, Dresden, Germany) that encodes FLP recombinase.

Generation of transgenic mice

The pBAC-hLF-hLZ-Neo construct was purified using a NucleoBond Xtra Midi kit (Macherey Nagel, Germany) and diluted to 2 to 3 ng/μL in TE buffer for microinjection. Circular BAC DNA was microinjected into the pronuclei of fertilized Kunming White eggs (Hogan et al. 1994). Genomic DNA was extracted from tail biopsies of mice, and P1 primers (Table S1) were used to screen for positive transgenic founders. Mice positive by PCR were confirmed by Southern blotting with the DIG High Prime DNA Labeling and Detection Starter kit II (Roche). Generally, genomic DNA (10 μg) was
digested with EcoRI and hybridized with digoxigenin (DIG)–labeled probes, which were the amplification products of the 637-bp hLZ fragment using P4 primers (Table S1). The hybridized probe was immunodetected with alkaline phosphatase–conjugated anti-DIG and visualized with the chemiluminescence substrate CSPD.

Reverse transcription (RT)-PCR

Total RNA was extracted from the mammary glands and other tissues during lactation using Trizol (Tiangen, China). First strand cDNA was synthesized with oligo-dT (Promega, USA). RT-PCR primers were designed on the basis of the hLZ coding sequences, and the upstream primer (Exon1-2-F) was designed across one intron (Table S1). The predicted 322-bp fragment was amplified. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an RT-PCR internal control. The primers for mouse GAPDH (Table S1) and amplified a 530-bp fragment.

SDS-PAGE and western blotting

Milk samples from transgenic mice were collected on postnatal days 9 and 16, diluted ten-fold with phosphate-buffered saline (PBS), and centrifuged (10,000 × g, 20 min, 4°C) to remove the whey from the fat layer and insoluble precipitates. The cleared fraction lacking whey was mixed with SDS-PAGE sample buffer and subjected to 15% SDS-PAGE under both reducing and non-reducing conditions. Protein bands were visualized by staining with Coomassie brilliant blue R-250.

For western blot analysis, proteins resolved by SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane (Amersham Pharmacia, UK) that was then blocked overnight at 4°C with 3% bovine serum albumin in PBS containing 0.05% (w/v) Tween 20 (PBS-T). Polyclonal rabbit anti-hLZ (1:1000) (US Biological Inc., Swampscott, MA) and horseradish peroxidase–conjugated goat anti–rabbit IgG (1:5000) (Sino-American Co., Beijing, China) were used to detect rhLZ. Milk samples
from wild-type mice served as negative controls. Blots were developed by enhanced chemiluminescence and autoradiography.

ELISA

The amount of rhLZ in the milk of transgenic mice was quantified using a Human Lysozyme EIA kit (Biomedical Technologies, Inc., MA). Each sample was analyzed at least three times, and the results represent mean ± SD.

Lysozyme activity assays

Lysozyme activity was evaluated by two techniques (Maidment et al. 2009). For the lysoplate or gel diffusion assay, a suspension of Micrococcus lysodeikticus cells (China General Microbiological Culture Collection Center, Beijing) was prepared in nutrient broth medium containing agar. After solidification of the medium, 6-mm-diameter circles of quantitative filter paper were spotted with milk samples to be tested for lysozyme activity and placed on the agar plates. After 36 hours of incubation at 28°C, zones of transparency appeared around the filter paper circles due to lysis of M. lysodeikticus cells by lysozyme produced by the transgenic mice. The experiment was repeated three times.

For the turbidimetric assay, the enzymatic activity of lysozyme was determined by monitoring the reduction in turbidity of a suspension of M. lysodeikticus cells at 450 nm. First, 2.5 ml of M. lysodeikticus cell suspension (OD_{450} 0.80 to 0.85) in 66 mM potassium phosphate, pH 6.24, was placed in a 4-mL cuvette at room temperature. The reaction was initiated by adding 100 μL of 1:100 dilutions of mouse milk samples or 100 μL purified chicken egg lysozyme at concentrations of 1000, 2000, 3000, 4000, 5000, 6000, and 8000 U/μL. Absorbance at 450 nm (A_{450}) was recorded every 15 s over a 3-min period. The activity of rhLZ was calculated from the standard curve. All samples were measured in triplicate.
References


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<th>Primer name</th>
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<th>Utility</th>
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<td>P1-F</td>
<td>5'-AAGGCGATCTTCAAGTAAAG-3’</td>
<td>BAC verification in step1 and PCR detection</td>
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<tr>
<td>P1-R</td>
<td>5'-TCGGGTATCTCTGGTTATAAAA-3’</td>
<td>of transgenic founders (703bp)</td>
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<td>P2-F</td>
<td>5'-TGCTTTGTGTATGGAGGGTC-3’</td>
<td>BAC verification in step2 (PC, 2541bp; NC, 1960bp)</td>
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<td>P2-R</td>
<td>5'-CCAGGAACAAACTTACCGAG-3’</td>
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<tr>
<td>P3-F</td>
<td>5'-CCGCTACTGGTGTAATGATG-3’</td>
<td>BAC verification in step3 (PC, 1129bp; NC, 2093bp)</td>
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<td>5'-ATGTTCTTTATGGGAATTT-3’</td>
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<td>P4-F</td>
<td>5'-TTATACACACGGCTTTAC-3’</td>
<td>Primers for PCR digoxigenin-labeled probe synthesis (637bp)</td>
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<td>RT-PCR for hLZ (322bp)</td>
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<td>Exon4-R</td>
<td>5'-CTCCACAACCTTGAACATAC-3’</td>
<td>RT-PCR for GAPDH control (530bp)</td>
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PC, positive control; NC, negative control; RT-PCR, reverse transcriptase PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase
Supplementary figures legends

**Fig. S1** Verification of the pBAC-hLF-hLZ-Neo construct by PCR analysis

(a) PCR detection of the modified BAC in step 1 with the P1 primers. The amplified product of the hLF-hLZ hybrid gene is 703 bp. (b) PCR detection of the modified BAC at step 2 with the P2 primers. The amplified products are 2541 bp for BAC clones that recombination occurred at the correct location and 1960 bp for the negative control (NC). (c) PCR detection of the modified BAC at step 3 with the P3 primers. The amplified products are 1129 bp for the PC and 2093 bp for the NC because of the absence or presence of the zeocin cassette, respectively. M, 100-bp or 1-kb DNA ladder.