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Expression of recombinant human lysozyme in bacterial artificial chromosome transgenic mice promotes the growth of *Bifidobacterium* and inhibits the growth of *Salmonella* in the intestine

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ABSTRACT

Targeted gene modification is a novel intervention strategy to increase disease resistance more quickly than traditional animal breeding. Human lysozyme, a natural, non-specific immune factor, participates in innate immunity, exerts a wide range of antimicrobial activities against pathogens, and has immuneregulatory effects. Therefore, it is a candidate gene for improved disease resistance in animals. In this study, we successfully generated a transgenic mouse model by microinjecting a modified bacterial artificial chromosome containing a recombinant human lysozyme (rhLZ) gene into the pronuclei of fertilized mouse embryos. rhLZ was expressed in serum, liver, spleen, lung, kidney, stomach, small intestine, and large intestine but not in milk. rhLZ protein concentrations in the serum of transgenic mice ranged from 2.09 to 2.60 mg/l. To examine the effect of rhLZ on intestinal microbiota, total aerobes, total anaerobes, *Clostridium*, *Enterococcus*, *Streptococcus*, *Salmonella*, *Escherichia coli*, *Staphylococcus*, *Bifidobacterium*, and *Lactobacillus* were measured in the intestines of transgenic and wild type mice. Results showed that *Bifidobacteria* were significantly increased ($p < 0.001$), whereas *Salmonella* were significantly decreased ($p < 0.001$) in transgenic mice compared to wild type mice. Our study suggests that rhLZ expression is a potential strategy to increase animal disease resistance.

1. Introduction

Progress in transgenic technologies has made it possible to change the genetic traits of animals to create specific research models with various applications that benefit humans. Compared to traditional breeding methods, transgenic animal models have the advantages of being targeted and having shorter breeding times. The enhancement of disease resistance in animals is one important application in animal husbandry. In the last few decades, genetic modification has been used to increase disease resistance in several animal models. For example, prion protein (PRNP) knockout has been used in cattle to increase resistance to prion diseases (Richt et al., 2007), human lysozyme (hLZ) expression in goats and cows has been used to improve mastitis resistance(Wall et al., 2005; Maga et al., 2006a), genetic modification of chickens has reduced transmission of avian influenza (Lyall et al., 2011), and *SP110* gene knock-in has been used to increase resistance to tuberculosis in cattle (Wu et al., 2015). Therefore, generation of genetically modified animals is an effective strategy to combat disease and has great potential for further development.

Lysozyme is a non-specific immune protein with a wide range of antimicrobial properties, including the hydrolysis of *N*-acetylglucosaminyl–*N*-acetylmuramic acid linkages in the cell walls of susceptible bacteria (Salton, 1957). Due to its functions and benefits, lysozyme has been widely used in pharmaceuticals, nutritional supplements, and feed additives. Furthermore, due to the disadvantages of conventional antibiotics, lysozyme may be an alternative to treat bacterial infections, as has been demonstrated in swine (Oliver and Wells, 2015) and poultry (Humphrey et al., 2002). hLZ is present in many healthy tissues in the human body (Mason and Taylor, 1975) and has higher lytic activity than that of other species. hLZ plays an important role in host defense against gastrointestinal pathogens and has been shown to decrease the rate of gastrointestinal illness in breastfed infants

Abbreviations: BAC, bacterial artificial chromosome; CPSF6, cleavage and polyadenylation specific factor 6; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; hLF, human lactoferrin; hLZ, human lysozyme; LB, lysogeny broth; PRNP, prion protein; rhLZ, recombinant human lysozyme; RT-PCR, reverse transcription PCR; SOC, super optimal broth with catabolite repression; TE, Tris-EDTA; WT, wild type

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(Lonnerdal, 2003).

It has been shown that consumption of goat milk containing recombinant human lysozyme (rhLZ) alters the gut microbiota and improves the gastrointestinal health of pigs (Maga et al., 2012; de Sa Carneiro et al., 2017). Goat rhLZ milk was also shown to improve the recovery of piglets with bacterial-induced diarrhea (Cooper et al., 2013; Garas et al., 2017). We have shown that expression of high levels of rhLZ in the mammary glands of transgenic pigs enhances resistance to diarrhea in sucking piglets and absorption of rhLZ in milk has a positive effect on the intestinal morphology and gut microbiota of piglets (Lu et al., 2014). However, expressing rhLZ during the suckling period does not protect piglets after weaning and adding rhLZ as a feed supplement requires significant human and material resources. Therefore, we propose that expressing rhLZ in animals, not only in the mammary gland, may improve disease resistance of animals throughout the lifespan. Therefore, we explored the feasibility and effectiveness of this strategy by generating a rhLZ transgenic mouse model.

Remote regulatory elements, such as enhancers, insulators, locus control regions, and matrix associated regions are typically located approximately 50 kb from the genes that they regulate and are important for expression and regulation of tissue-specific genes (McKnight et al., 1992; Giraldo and Montoliu, 2001). However, because DNA cloning vectors have a limited capacity, some of these important elements could be lost, leading to low expression and position effects. Bacterial artificial chromosomes (BAC) are artificial chromosomes based on the *Escherichia coli* functional fertility plasmid and have an average insert size of approximately 150 kb (O'Connor et al., 1989; Shizuya et al., 1992). BACs can contain all necessary regulatory elements and have minimal chromosomal position effects on gene expression (Stinnakre et al., 1999; Giraldo and Montoliu, 2001). Moreover, BACs are more stable and easier to operate than yeast artificial chromosomes, which are difficult to purify and lead to a high frequency of chimeras and rearrangements (Monaco and Larin, 1994).

In this study, we modified a hLZ BAC by homologous recombination to construct the rhLZ expression plasmid pBAC-rhLZ-Neo in which the human lysozyme genomic gene is flanked by 155 kb of 5′ sequence and 6 kb of 3′ sequence. We successfully generated a transgenic mouse model by microinjecting this BAC into the pronuclei of fertilized mouse embryos. We then determined the rhLZ expression pattern and the influence of rhLZ on the gut microbiota of transgenic mice.

2. Materials and methods

2.1. Ethics statement

This study protocols were approved by the Institutional Animal Care and Use Committee of the China Agricultural University and the approved number is SKLAB-2012-04-05. We performed all the procedures in strict accordance with the Guide for the Care and Use of Laboratory Animals.

2.2. Construction of pBAC-rhLZ

The *E. coli* strain SW102 (Warming, Costantino et al., 2005), which carries a defective λ prophage and harbors the recombination genes *exo*, *bet*, and, *gam*, was used for BAC modification. BAC clone RP11-72P21 was obtained by screening a human BAC library (Genome Systems Inc., St. Louis, MO, USA). The BAC (named pBAC-rhLZ) contains a 170 kb insert that includes the full-length hLZ gene and the cleavage and polyadenylation specific factor 6 (CPSF6) gene. To ensure that only rhLZ, and not CPSF6, was expressed, we first knocked out the CPSF6 gene. pBAC-rhLZ was introduced into SW102 cells by electroporation and cells were grown in lysogeny broth (LB) containing chloramphenicol (25 mg/ml). The neomycin/kanamycin resistance cassette was amplified from pBR322-loxP-Neo using primers 5′-*TCT CTG AGG ATG CAG AAA CAA GAC ATT GTT TTT GGT CAT ACG* CCG TTC ACA GAA TGC CCG CCG CCT CCA TCC AGT CTA TT -3′ and 5′-*TTA TCT GTG GCT CCA AAA GTC AAA GGA CTT GTT GCA AAT GAG CCC GCA CCT CAG TCG CCT* GTC GCC GCA TAC ACT ATT CT-3′, where the homology arms to the exon 1 of CPSF6 are in italics. SW102 cells containing pBAC-rhLZ were induced and made electrocompetent as previously described (Giraldo and Montoliu, 2001). Briefly, 10 ml of cells were grown at 32 °C to an optical density at 600 nm of 0.6 and induced at 42 °C for 15 min. After chilling on ice for 15 min, cells were washed with ice-cold water three times, resuspended in 50 ml of ice-cold, sterile water, and immediately used for electroporation. The parameters for electroporation were: 1.8 kV, 25 μF capacitance, and 200 Ω. Purified PCR products (5 μl) were electroporated into 50 μl of electrocompetent SW102 bacteria containing the pBAC-rhLZ in a 0.1 cm cuvette. Transformed cells were suspended in 600 μl super optimal broth with catabolite repression (SOC) medium and incubated for 1.5 h at 37 °C before plating on LB agar plates containing kanamycin (50 μg/ml). Positive clones were analyzed by PCR using bacteria as the template. The PCR products were gel purified and sequenced.

2.3. Generation and Identification of Transgenic Mice

The pBAC-hLF-Neo construct was purified using a NucleoBond Xtra Midi kit (Macherey Nagel, Germany) and diluted to 2–3 ng/μL in TE buffer for microinjection. Circular BAC DNA was microinjected into the pronuclei of fertilized Kunming White eggs. Genomic DNA was extracted from tail biopsies of mice, and P-hLZ-637 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, 10 μg of genomic DNA digested by EcoRI was used to detect transgenic mice by southern blot. Non-transgenic porcine genomic DNA was used as a negative control and pBAC-hLZ-Neo BAC as a positive control. A DIG-labeled probe (Roche, Mannheim, Germany) was generated by PCR using P-hLZ-637 primers and the positive hybridization signal was a 3.3 kb fragment.

2.4. RT-PCR analysis

we extracted total RNA from multiple tissues (heart, liver, spleen, lung, kidney, stomach, intestine and muscle) using Trizol reagent (Tiangen, Beijing, China). Primers P-HLZ-322 designed across one intron was used to amplify 322-bp fragment. The primers for internal control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to amplified a 530-bp fragment.

2.5. Western blot analysis

Total protein was extracted from transgenic mice and non-transgenic mice using the IP lysis buffer (Beyotime, Beijing, China), and the protein was quantified using Coomassie brilliant blue G250. For each sample, 50 μg total protein were loaded. Blood samples were obtained from puncture of the ocular oribit using glass capillaries, collected in tubes containing a clotting activator, allowed to clot at room temperature, and centrifuged at 3000 × *g* for 10 min to obtain serum. The serum samples were stored at −80 °C. For western blot analysis, 10 μL serum samples were loaded. Milk samples from transgenic mice and wild type mice collected during mid-lactation (days 9–14) were diluted three-fold with distilled water and defatted by centrifugation (10,000 × *g*, 15 min, 4 °C). 15% sodium dodecyl sulfate polyacrylamide gel was used for electrophoresis, and samples were electrophoretically transferred to a nitrocellulose membrane (Amersham Pharmacia UK, Ltd., Buckinghamshire, UK) and blocked overnight at 4 °C. rhLZ was detected with polyclonal rabbit anti-hLZ (1:2000) (US Biological Inc., Swampscott, MA, USA) and horseradish peroxidase-conjugated goat anti-rabbit IgG (1:20000) (Sino-American Co., Beijing, China), as described previously(Lu, Li et al. 2014).