Construction of expression vector of human lactoferrin and its expression in bovine mammary epithelial cells

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ABSTRACT. Lactoferrin has multiple biological roles, including inflammation and immune modulation, iron absorption in newborns, non-iron-dependent antimicrobial activity, regulation of the level of retinoblastoma protein, protection against cancer development and metastasis, transactivation of the p53 tumor suppressor gene. Moreover, the ubiquitous expressed human lactoferrin (hLF) is species, tissue, and cell-type specific. To facilitate further studying of the biological function of hLF, we hereby constructed and expressed a recombinant vector containing exogenous hLF gene and bovine mammary gland ß-lactoglobulin (BLG) gene. cDNAs of both hLF gene (2259bp) and 5' flank regulatory fragment of BLG (1449bp) consisting of the promoter region, exon 1 and intron 1 of the gene were obtained respectively. Then the translation initiation codon of 5' flank regulatory fragment was mutated from ATG to AAG and renamed as mblg. hLF and mblg were recombined through PCR method and was named as BL, which was then cloned into pEGFP-C1 vector and renamed pMBL. Later on, the pMBL vector was transferred into a cell line of bovine mammary epithelia by liposomal transfection and cultured with G418 drug for 3 weeks to obtain positive transgene cell clones. Then the expression of hLF was detected in the positive cell clones by Immunocytochemistry and Western Blotting assays. The results showed that the exogenous hLF gene had been successfully integrated into the chromosome of the positive cell clones, which highly expressed hLF.

KEY WORDS : Bovine β-lactoglobulin (BLG) gene, point mutation, Human lactoferrin (hLF) gene, Recombinant PCR

INTRODUCTION

Lactoferrin gene is highly conserved among human, mouse, bovine and porcine species. Human lactoferrin (hLF) gene, at the human chromosomal 3p21 location (KLINTWORTH et al., 1998; YANG et al., 2003), is a non-haem iron-binding glycoprotein of 80kDa that belongs to transferrin family. Mature hLF protein contains 692 amino acids and its three-dimensional structure reveals a globular protein folded into two highly homologous parts (N-lobe and C-lobe), each of which can tightly bind a single ferric ion (Fe^{3+}) separately (ANDERSON et al., 1987). The genomic gene of hLF comprises 17 exons and spans about 35kb in genome (SEYFERT et al., 1994; KIM et al., 1998). It was first discovered in milk and later found in the wet surface mucosa epithelium (BAVEYE et al., 1999; VORLAND, 1999). Further studies showed that the expression of hLF is both ubiquitous and species, tissue, and cell-type specific (TENG, 2002). In the adult, hLF is mainly secreted by mammary gland and neutrophils. The highest levels of hLF are detected in colostrums and milk, with lower levels detected in tears, nasal fluids, saliva, pancreatic, gastrointestinal and reproductive tissue secretions (LEVAY & VILJOEN, 1995; TENG et al., 1989). Extensive in vitro and in vivo evidence has suggested that lactoferrin has multiple biological roles, including inflammation and immune modulation, iron absorption in newborns, non-iron- dependent antimicrobial activity, and regulation of the level of retinoblastoma protein (CONNEELY, 2001; BERLUTTI et al., 2006; ALYSHEV & VALYSHEVA, 2006; SON et al., 2006; WARD & CONNEELY, 2004; WARD et al., 2002). Moreover lactoferrin is also involved in protection against cancer development and metastasis, transactivation of the p53 tumor suppressor gene (TP53) and regulation of other gene expression (VORLAND, 1999; BEZAULT et al., 1994; ARTYM, 2006; SHIMAMURA et al., 2004; WANG et al., 2000). It can also promote bone growth and possibly modulates behavior in human and animals (NAOT et al., 2005; SACHARCZUK et al., 2005). hLF is a bioactive, versatile protein, and has large potential in nutritional and therapeutic applications, therefore the need for a recombinant source of hLF protein has increased and production of it using animal bioreactors has been studied widely to satisfy its large requirement (BAVEYE et al., 1999; VORLAND, 1999).

Transgenic animals provide an alternative approach to supply human lactoferrin in large quantity with relative low cost and animal mammary gland bioreactors are supposed to be the feasible tools for production of hLF in large scale. However, construction of an efficient and specific expression vector is the key link for the production of a mammary gland bioreactor. Several researchers have reported that hLF can be expressed in bovine, tobacco, rice plant, rat and other expression systems (KIM et al., 1998; ZAKHAROVA et al., 2005; TAKASE et al., 2005; CHOI et al., 2003; KUMURA et al., 1998; KRIMPENFORT, 1993) and transgenic animals can produce hLF in milk (KRIMPENFORT et al., 1991; PLATENBURG et al., 1994; KIM et al., 1999; PATRICK et al., 2002), but the expression level is not high enough to meet the needs. Therefore we

constructed a new specific expression vector containing the hLF gene and transfected it into bovine mammary epithelial cells, in this way, we have created a solid foundation for making transgenic animals.

MATERIALS AND METHODS

Bacterial strains and plasmids

Vector pMD 18-T and pEGFP-C1 were purchased from Clontech and TaKaRa respectively. *E. coli* DH5α, bovine mammary epithelial cells were preserved in our lab.

Primer sequences

Primers were designed based on the sequence of hLF (GenBank NOX53961; Rey et al., 1990) and bovine ß-lactoglobulin (BLG) (GenBank NOX14710; Jamieson et al, 1987) in GenBank using the software Prime Premier 5.0. The hLF sense primer, LFS1,5-CAGACCGCAGA-CATGAAACT -3 and antisense primer, LFA1, 5'-GCAATCCCCACCTTCAGCAG-3'. The BLG mutant primer is 5'-GC TGCAGCCAAGAAGTGCCT-3'. Ligation primers are as follows: BF2: -AGTTTCATGTCT-GCGGT CTGGGGAGGGACCTTGAGCTG-3' and BF3: 5'-CAGCTCAAGGTCCCTCCCCAGACCGCAGAC ATGAACT-3'. The detection sense primer, JCS1, 5'-CCT-CAGGGTGCCGAGTTGG-3' and anti-sense primer, JCA1, 5'-TTCAAGAATGGACGAAGTGT-3'.

Cloning and sequencing of human lactoferrin gene

The 2259bp full-length of human lactoferrin cDNA (GenBank accession No. AY165046) was cloned by reverse transcription polymerase chain reaction (RT-PCR) from human mammary carcinoma tissue obtained from Yangling Model District Hospital (Shannxi, China) after consent of the informed patients. Total RNA was extracted using Trizol Reagent (Invitrogen) according to the manufacturer's instructions and was reverse transcribed into cDNA with SuperScript™ II reverse transcriptase (Invitrogen) and oligo (dT) primer. Then the cDNA was amplified by PCR with primers LFS1 and LFA1. Thirty cycles of PCR were performed under the following conditions: denaturalization at 94 for 1min, annealing at 55 for 1min, and extension at 72 for 2min. The PCR product was verified by agarose gel electrophoresis and purified by gel extraction kit (Qiagen). Then the full-length hLF cDNA was ligated into pMD 18-T vectors and subjected to DNA sequence analysis.

Construction of bovine β-lactoglobulin (BLG) gene expression vector pBLG and point mutation of the 5' flanking fragment of BLG gene

Plasmid pBLG containing the 5' flanking fragment was constructed as previously described (Higuchi et al., 1988; Carl & Gabriela, 2003). Mutant site was introduced to BLG mutant primer when it was synthesized in vitro. The translation start codon of the 5' flanking fragment of bovine β-lactoglobulin (BLG) gene was mutated from ATG to AAG through point mutation with a primer-mediated mutagenesis procedure (Higuchi et al., 1988) The mutagenesis reaction was carried out following the method of references for creating point mutations (Carl & Gabriela, 2003). The mutant fragment was named mblg and cloned into vector pMD 18-T, and the mutant nucleotide acid in the pMBLG plasmid was confirmed by DNA sequencing and analyzing.

Construction and identification of hLF expression vector pMBL

The strategy of the construction of the mammary-specific expression vector of the recombinant hLF gene is shown (Fig. 1). First two ligation primers BF2 and BF3 were designed. The fragments mblg and hLF were ligated by recombinant PCR method (Carl & Gabriela, 2003). The PCR products were named BL with the ends containing the *Ase* I and *Hind* III restriction sites and ligated into the pMD 18-T vector as plasmid pBL. Later BL was excised and subcloned into the multiple cloning site of the pEGFP-C1 vector at *Ase* I and *Hind* III sites after the CMV promoter and GFP gene of vector pEGFP-C1 had previously been removed with restriction enzymes *Ase* I and *Hind* III. The recombined plasmid was designated as pMBL (Fig. 2), which is a mammary gland-specific expression vector. The pMBL was digested by *Mlu* I and the digested products were evaluated by agarose gel electrophoresis.

Cell culture and transfection

The plasmid pMBL was prepared by Endofree Plasmid Extraction Kit (Promega, USA) according to the manufacturer's instructions, and diluted in endotoxin-free PBS (Sigma). Bovine mammary epithelial cells were cultured in a 1:1 mixture of Ham's F12: Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS), hydrocortisone (5μg/mL; Sigma), epidermal growth factor (10ng/mL; Sigma), insulin- transferrin- selenium sodium (5μg/mL; Sigma) and insulin-like growth factor (10ng/mL; Sigma) and maintained at 37 in 5% CO_2. For transfection procedure, cells at 50% confluence in 6-well plates were transfected with 4μg pMBL using the Lipofectamine 2000 reagent according to the manufacturer's instruction (Invitrogen). Cells were selected in growth medium containing 200μg/mL G418. After 3 weeks of selection, G418-resistant clones were selected randomly from the surviving colonies.

Detection of hLF gene stably integrated into chromosome of bovine mammary epithelial cells by PCR

To demonstrate whether hLF gene stably integrated into chromosome of bovine mammary epithelial cells, genomic DNA was extracted from the positive cloning cells according to the conventional method. The PCR amplification was performed using primers that amplified the specific 605bp fragment in pMBL plasmid DNA (sense primer JCS1 locates in gene BLG; antisense primer JCA1 is located in gene hLF).

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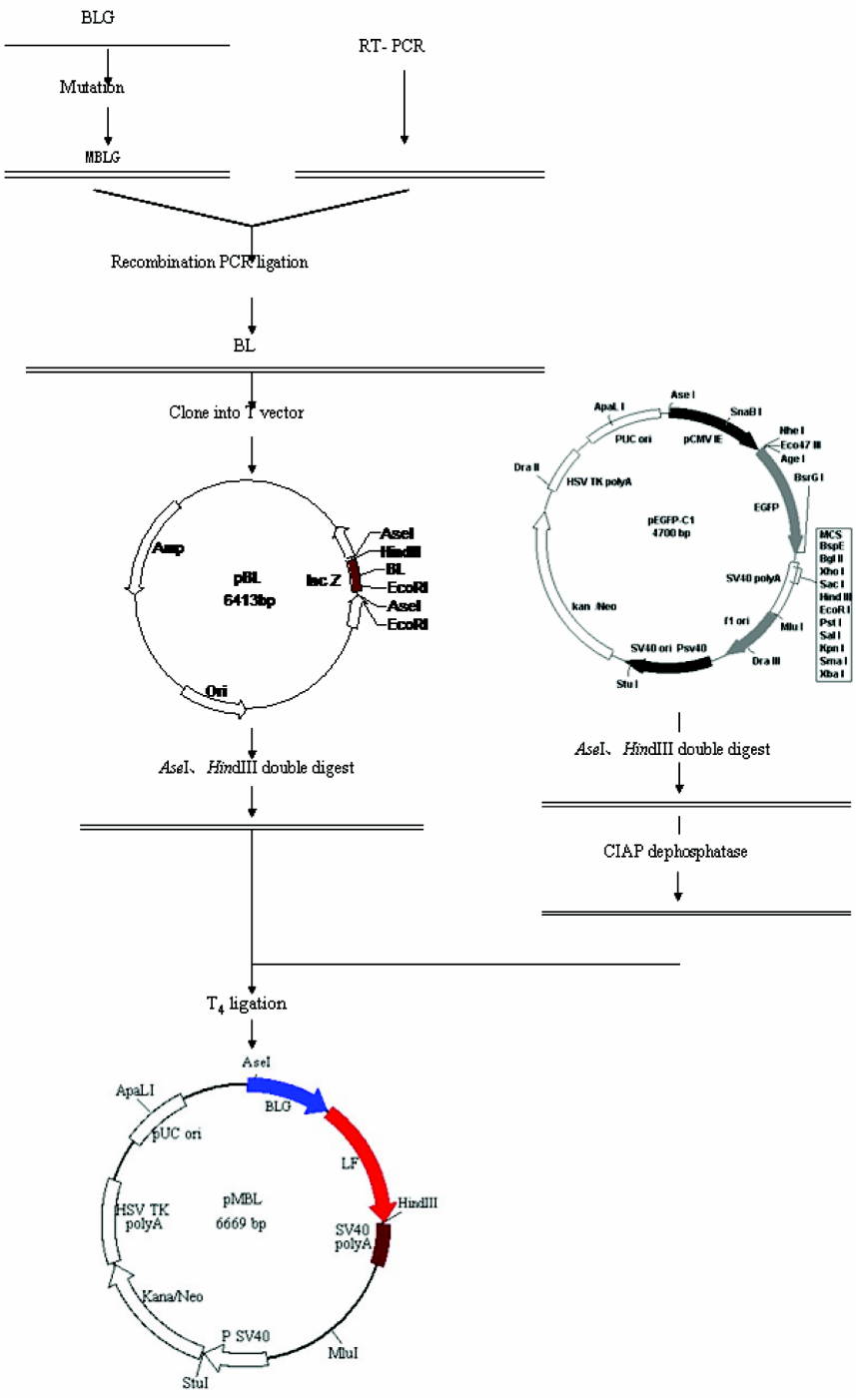

Fig. 1. – Strategy of the construction of the spedifically mammary expression vector of hLF gene.

Fig. 2. – Structure of human lactoferrin mammary specific expression vector pMBL
BLG P: bovine ß-lactoglobulin (BLG) promoter, E1: the exon of BLG gene, I1: the intron exon of BLG gene, hLF cDNA: cDNA of human lactoferrin gene, SV40 polyA: SV40 polyadenylation signal.

Detection of hLF stable expression by immunocytochemistry

The transfected cells were seeded on glass slides at 60-80% confluence, fixed in 4% paraformaldehyde for 10 minutes, blocked in sheep serum for 10 minutes, washed in 0.2% Triton X- 100 for 2 minutes and then immuno-stained with affinity-purified polyclonal rabbit antibody (Abcam) to human lactoferrin (1:200). For visualization, FITC-conjugated mouse anti-rabbit secondary antibodies (Sigma) were applied. Cellular nuclei were visualized by staining with 4,6-diamidino-2-phenylindole (Sigma) at 0.1μg/mL. Finally the cells were detected with a laser confocal microscopy (Leica).

Detection of hLF stable expression by western blotting

Western blotting was done according to the method of Kim (Kim et al., 1999). The cells were homogenized and the supernatant was separated by 15% PAGE (50μg total protein/lane). After transferred to nitrocellulose membranes, the membranes were blocked with 5% nonfat milk/PBS, followed by incubation at room temperature for 1h with polyclonal human lactoferrin antibody derived from rabbit (1:400) in 5% milk/PBS. After three 5min washes at room temperature, the membranes were incubated with HRP-conjugated secondary antibody (goat anti-rabbit IgG, 1:6000) in 5% milk/PBS. After three washes in PBS with tween-20, then followed by 5min of incubation with SuperSignal West Pico substrate (Pierce), the membranes were exposed on x-ray film and the signal was detected with Western Blotting Detection System (Bio-Rad), following the manufacturer's instructions. The expression of beta-actin protein was detected as normal expression control.

RESULTS

Construction of hLF expression vector pMBL

To construct the hLF expression vector pMBL, the translation initiation codon of the 5' flanking fragment of BLG gene was mutated from ATG to AAG (at positon 81) by PCR point mutation firstly. The mutant fragment was named mblg and cloned into pMD 18-T vector. The result was confirmed by DNA sequence analysis (Fig. 3). Secondly, the cDNA of hLF gene was cloned by RT-PCR. By agarose gel electrophoresis, it demonstrated that the cDNA was successfully amplified and the sizes of PCR products were correct (Fig. 4A). The PCR products were purified and cloned into pMD 18-T vectors to sequence and analyze the change of gene sequence. The sequence was aligned with the sequence registered in the GenBank based on Basic Local Alignment Search Tool (BLAST) analysis, and it showed that the sequence had 99.73% sequence identity and six point mutations: 100 (G to A), 155 (A to G), 458 (C to T), 1279 (T to G), 1752 (G to C) and 1909 (C to T). By Compared with human lactoferrin cDNA and its amino acids sequences, it showed the first five point mutations were allele mutation sites and the sixth point mutation was the change of synonyms codon. Thirdly, to demonstrate whether the BL fragment derived from mblg and hLF fragments connected together was inserted into the multi-clone sites of pEGFP-C1 vector correctly, the expression vector pMBL was digested by Mlu I and evaluated with agarose gel electrophoresis. The PCR product was a single approximate 6.6kb-fragment as showed in Fig. 4 B, it suggested that the BL fragment was successfully inserted into pEGFP-C1 vector.

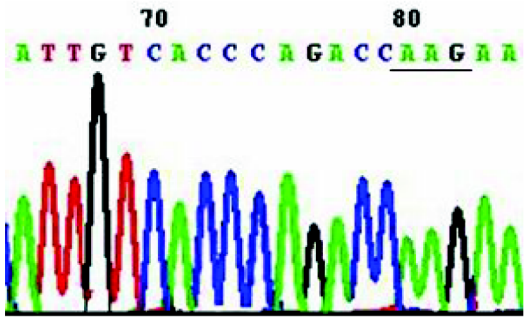

Fig. 3. – The sequencing chromatogram figure of the bovine BLG translation start code Note: The mutant nucleotide acids marked as underline.

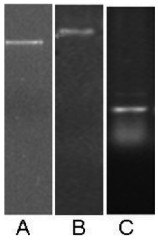

Fig. 4. – The figures of agarose gel electrophoresis.
Lane A: The PT-PCR product of hLF cDNA; Lane B: The expression vector pMBL digested with Mlu I; Lane C: Detection of exogenous hLF gene integrated into the genomes of the pMBL plasmid DNA transfected cells.

hLF gene stably integrated into chromosome of bovine mammary epithelial cells by PCR detection

To detect whether exogenous hLF gene was integrated into the genomes of the bovine mammary epithelial cells, the specific 605bp fragment was amplified by PCR. PCR analysis showed that specific fragment was amplified only from pMBL plasmid DNA transfected cells, but not from the cells without transfected (Fig. 4C).

hLF stably expressed in transfected cells by immunocytochemistry and western blot assays

Exogenous hLF gene expression in bovine mammary epithelial cells was detected by immunocytochemistry and western blot assays. After positive single clone cells were isolated and picked out by cultured with G418 drug for three weeks, the cells were coated with hLF antibody

and fluorescent antibody. Under confocal microscopes, it was observed that the fluorescence staining was positive in the cytoplasm of transfected cells and negative in the untransfected control cells (Fig. 5). Results of western blot also indicated that human lactoferrin, a 76kD protein, expressed highly in positive cells but negative in the controls (Fig. 6).

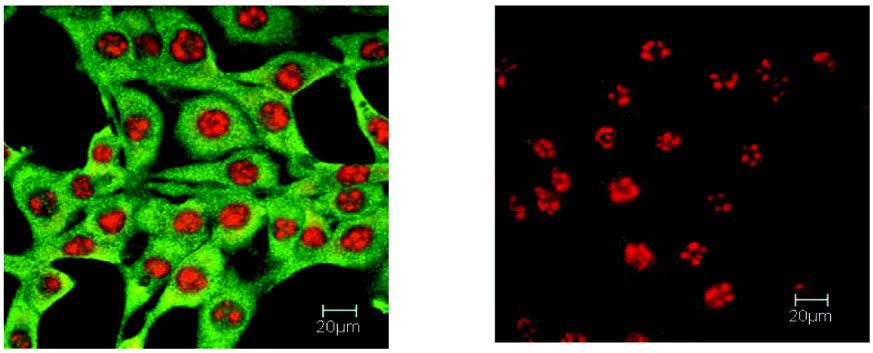

Fig. 5. – Detection of exogenous hLF gene expression in bovine mammary epithelial cells by immunocytochemistry. A: Transfection group and the fluorescence staining in the cytoplasm was positive, B. Negative control group of no transfection.

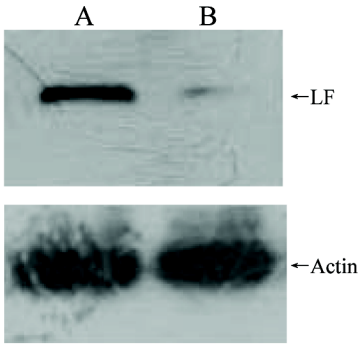

Fig. 6. – Detection of exogenous hLF gene expression in bovine mammary epithelial cells by western blot, the expression of actin detected as endogenous expression level. A: Transfection group, B: Negative control group of no transfection.

DISCUSSION

The key step of generation mammary gland bioreactor is to select appropriate regulatory elements. These regulatory elements enable high and specific expression of exogenous gene in the mammary gland of transgenic animal with subsequent secretion of the desired proteins in the milk. In this research, to evaluate the rationalization and expression efficiency of the expression vector, we transfected bovine epithelial cells cultured *in vitro* with the pMBL plasmid DNA and detected the expression level of exogenous hLF gene by immunocytochemistry and western blot assays. The results demonstrated that hLF gene had been integrated into the genome of transgenic cells and expressed at a very high level. As a result, it not only provides donor cell for somatic cell nuclear transfer to produce transgenic animal, but also takes the foundation of theory and practice for making transgenic animals and constructing efficient expression vector. We are attempting to prepare transgenic donor cell of hLF gene for producing transgenic calves by somatic cell nuclear transfer in the present study.

The 5' regulatory region of BLG gene is widely used to regulate exogenous gene expressed specifically in mammary epithelial cells (Van Kuik-Romeijn et al., 2000; Wright et al., 1991). In preparation for expression vector, because the regulatory sequence involves the first exon, expression of the recombinant gene would generate the BL fusion protein, resulting in decreased biological activity of lactoferrin. Therefore we mutated the translation initiation codon of the BLG gene from ATG to AAG. This mutation not only avoids the production of fusion protein but also mimics the natural transcription pattern of the bovine BLG gene through the first intron of the 5' flanking regulatory sequence. Several studies have confirmed that the first intron plays an important role in foreign gene transcription and correct splicing (Palmiter et al., 1982; Palmiter et al., 1992). In many studies the 5' regulatory sequence flanking the BLG gene of sheep or goat is used as the promoter of expression vector for producing transgenic animal. But few studies used the bovine 5' flanking regulatory sequence as promoter.

To construct an expression vector, we tried to connect the 5' flanking regulatory sequence of BLG with hLF gene using many methods. The conventional methods have many deficiencies when blunt ends are connected using T4 DNA ligase, such as low ligation efficiency and a high number of pseudopositive result**s.** This does not facilitate screening of positive clones. Using recombinant PCR to link two fragments is efficient and easy, but it requires the design of ligation primers and the optimization of the PCR reaction conditions. To avoid of mutation generated by PCR amplification, we used high faithful Platinum Taq DNA polymerase and reduced cycles of

PCR procedures (about 25 cycles). We selected unmutanted positive clones from lots of clones by sequencing and analysis. The result proved that the recombinant PCR was effective. In domestic reports, we have not found reports about using hLF as exogenous gene to construct mammary specific expression vector and transfect mammary epithelial cell cultured in vitro, also not found reports about transferring hLF gene in big transgenic domestic animals. Previous reports only used mouse, insect and plant to express hLF gene. In external reports, the reports about transferring hLF gene in big transgenic domestic animals are few.

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REFERENCES

Alyshev AV & Valysheva IV (2006). The role of anti-lactoferrin activity of bacteria in their persistence. Zh Mikrobiol Epidemiol Immunobiol, 4: 23-25.

Anderson BF, Baker HM, Dodson EJ, Norris GE & Rumball SV (1987). Structure of human lactoferrin at 3.2-A resolution. Proc Natl Acad Sci U S A, 84: 1769-73.

Artym J (2006). Antitumor and chemopreventive activity of lactoferrin. Postepy Hig Med Dosw (Online), 60: 352-369.

Baveye S, Elass E, Mazurier J, Spik G & Legrand D (1999). Lactoferrin: a multifunctional glycoprotein involved in the modulation of the inflammatory process. Clin Chem Lab Med, 37: 281-286.

Berlutti F, Schippa S, Morea C, Sarli S, Perfetto B, Donnarumma G & Valenti P (2006). Lactoferrin downregulates pro-inflammatory cytokines upexpressed in intestinal epithelial cells infected with invasive or noninvasive *Escherichia coli* strains. Biochem Cell Biol, 84: 351-357.

Bezault J, Bhimani R, Wiprovnick J & Furmanski P (1994). Human lactoferrin inhibits growth of solid tumors and development of experimental metastases in mice. Cancer Res, 54: 2310-2312.

Carl WD & Gabriela SD (2003). PCR Primer: A laboratory Manual. Cold Spring Harbor Laboratory Press: 451-475.

Choi SM, Lee OS, Kwon SY, Kwak SS, Yu DY & Lee HS (2003). High expression of a human lactoferrin in transgenic tobacco cell cultures. Biotechnol Lett, 25: 213-218.

Conneely OM (2001). Antiinflammatory activities of lactoferrin. J Am Coll Nutr, 20: 389-397.

Higuchi R, Krummel B & Saiki RK (1988). A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res, 16: 7351-7367.

Jamieson AC, Vandeyar MA, Kang YC, Kinsella JE & Batt CA (1987). Cloning and nucleotide sequence of the bovine beta-lactoglobulin gene. Gene, 61(1): 85-90.

Kim SJ, Sohn BH, Jeong S, Pak KW, Park JS & Park IY (1999). High-level expression of human lactoferrin in milk of transgenic mice using genomic lactoferrin sequence. J Biochem (Tokyo), 126: 320-325.

Kim SJ, Yu DY, Pak KW, Jeong S, Kim SW & Lee KK (1998). Structure of the human lactoferrin gene and its chromosomal localization. Mol Cells, 8: 663-668.

Kim SJ, Yu DY, Han YM, Lee CS & Lee KK (1998). Cloning of human genomic lactoferrin sequence and expression in the mammary glands of transgenic animals. Adv Exp Med Biol, 443: 79-83.

Klintworth GK, Sommer JR, Obrian G, Han L, Ahmed MN, Qumsiyeh MB, Lin PY, Basti S, Reddy MK, Kanai A, Hotta Y, Sugar J, Kumaramanickavel G, Munier F, Schorderet DF, Matri LEl, Iwata F, Kaiser-Kupfer M, Nagata M, Nakayasu K, Hejtmancik JF & Teng CT (1998). Familial subepithelial corneal amyloidosis (gelatinous drop-like corneal dystrophy): exclusion of linkage to lactoferrin gene. Mol Vis., 4: 31.

Krimpenfort P (1993). The production of human lactoferrin in the milk of transgenic animals. Cancer Detect Prev, 17: 301-305.

Krimpenfort P, Rademakers A, Eyestone W, van der Schans A, van den Broek S, Kooiman P, Kootwijk E, Platenburg G, Pieper F, Strijker R & de Boer H (1991). Generation of transgenic dairy cattle using 'in vitro' embryo production. Biotechnology (N Y), 9: 844-847.

Kumura H, Hiramatsu Y, Ukai Y, Mikawa K & Shimazaki K (1998). Expression of human lactoferrin in transfected rat mammary epithelial cells. Adv Exp Med Biol, 443: 85-89.

Levay PF & Viljoen M (1995). Lactoferrin: a general review. Haematologica,80: 252-267.

Naot D, Grey A, Reid IR & Cornish J (2005). Lactoferrin--a novel bone growth factor. Clin Med Res, 3: 93-101.

Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birnberg NC & Evans RM (1982). Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature, 300: 611-615.

Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birnberg NC & Evans RM (1992). Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Biotechnology, 24: 429-433.

Rey MW, Woloshuk SL, deBoer HA & Pieper FR (1990). Complete nucleotide sequence of human mammary gland lactoferrin. Nucleic Acids Res, 18 (17): 5288.

van Berkel PHC, Welling MM, Geerts M, van Veen HA, Ravensbergen B, Salaheddine M, Pauwels EKJ, Pieper F, Nuijens JH & Nibbering PH (2002). Large scale production of recombinant human lactoferrin in the milk of transgenic cows. Nat Biotechnol, 20: 484-487.

Platenburg GJ, Kootwijk EPA, Kooiman PM, Woloshuk SL, Nuijens JH, Krimpenfort PJA, Pieper FR, de Boer HA & Strijker R (1994). Expression of human lactoferrin in milk of transgenic mice. Transgenic Res: 399-108.

Sacharczuk M, Zagulski T, Sadowski B, Barcikowska M & Pluta R (2005). Lactoferrin in the central nervous system. Neurol Neurochir Pol, 39: 482-489.

Seyfert HM, Tuckoricz A, Interthal H, Koczan D & Hobom G (1994). Structure of the bovine lactoferrin-encoding gene and its promoter. Gene, 143: 265-269.

Shimamura M, Yamamoto Y, Ashino H, Oikawa T, Hazato T, Tsuda H & Iigo M (2004). Bovine lactoferrin inhibits tumor-induced angiogenesis. Int J Cancer, 111: 111-116.

Son HJ, Lee SH & Choi SY (2006). Human lactoferrin controls the level of retinoblastoma protein and its activity. Biochem Cell Biol., 84: 345-350.

Takase K, Hagiwara K, Onodera H, Nishizawa Y, Ugaki M, Omura T, Numata S & Akutsu K (2005). Constitutive expression of human lactoferrin and its N-lobe in rice plants to confer disease resistance. Biochem Cell Biol, 83: 239-249.

Teng CT (2002). Lactoferrin gene expression and regulation: an overview. Biochem Cell Biol, 80: 7-16.

Teng CT, Pentecost BT, Chen YH, Newbold RR, Eddy EM & McLachlan JA (1989). Lactotransferrin gene expression in

the mouse uterus and mammary gland. Endocrinology, 124: 992-999.

Van Kuik-Romeijn P, de Groot N, Hooijberg E & de Boer HA (2000). Expression of a functional mouse-human chimeric anti-CD19 antibody in the milk of transgenic mice. Transgenic Research., 9(2): 155-159.

Vorland LH (1999). Lactoferrin: a multifunctional glycoprotein. Apmis, 107: 971-981.

Wang WP, Iigo M, Sato J, Sekine K, Adachi I & Tsuda H (2000). Activation of intestinal mucosal immunity in tumor-bearing mice by lactoferrin. Jpn J Cancer Res, 91: 1022-1027.

Ward PP & Conneely OM (2004). Lactoferrin: role in iron homeostasis and host defense against microbial infection. Biometals, 17: 203-208.

Ward PP, Uribe-Luna S & Conneely OM (2002). Lactoferrin and host defense. Biochem Cell Biol, 80: 95-102.

Wright G, Carver A, Cottom D, Reeves D, Scott A, Simons P, Wilmut I, Garner I & Colman A (1991). High level expression of active human alpha-1-antitrypsin in the milk of transgenic sheep. Biotechnology, 9: 830-834.

Yang Y, Li JF, Szeles A, Imreh MP, Kost-Alimova M, Kiss H, Kholodnyuk I, Fedorova L, Darai E, Klein G & Imreh S (2003). Consistent downregulation of human lactoferrin gene, in the common eliminated region 1 on 3p21.3, following tumor growth in severe combined immunodeficient (SCID) mice. Cancer Lett., 191: 155-164.

Zakharova ES, Pryzhkova MV, Kibardin AV, Ermolkevich TG, Kadulin SG, Gnuchevan V & Kiselev SL (2005). Transcription and mRNA splicing of the human lactoferrin gene controlled by the regulatory region of the bovine alphaS1 casein gene in the mammary gland of transgenic mice and in mouse embryonic stem cells. Genetics, 41: 299-306.

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