Molecular cloning of lipocalin-2 into a eukaryotic vector and its expression in bovine mammary epithelial cells as a potential treatment for bovine mastitis

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Abstract: Lipocalin-2 (LCN2) is a 25-kDa protein in the lipocalin family that shows antibacterial activity. The aim of this study was to clone human LCN2 into the mammalian-specific pIRES2-AcGFP1 vector and to determine the antibacterial activity of the recombinant pIRES2-AcGFP1-hLCN2 plasmid against major bacterial pathogens that cause bovine mastitis. The amplified hLCN2 was successfully cloned into the vector, and the plasmid was transfected into bovine mammary epithelial stem cells using nucleofection. Immunochemistry, reverse transcription polymerase chain reaction, and ELISA were used to monitor and quantify hLCN2 expression in the cells. After 1 day of incubation, the concentration of hLCN2 secreted by cells in the medium (6.85 ± 0.11 µg) was higher than that stored within the cells (22 ± 2.52 ng). After 4 days of culture, the amount of hLCN2 was 300-fold higher in the medium than within the cells. The recombinant clone showed potential antibacterial activity against mastitis-causing bacteria, with greater inhibitory activity against Escherichia coli than against Staphylococcus aureus. Antibacterial activity remained strong after 4–5 h of culture. Thus, pIRES2-AcGFP1 was identified as an efficient vector for the delivery of the target gene to the mammary cells, and recombinant hLCN2 showed strong antibacterial activity against major mastitis-causing bacteria.

Key words: Recombinant human lipocalin 2, pIRES2 vector, transfection, bovine mammary epithelial stem cells, antibacterial, bovine mastitis

1. Introduction

Lipocalin-2 (LCN2, also known as neutrophil gelatinase-associated lipocalin) belongs to the lipocalin protein family. The members of this family have a barrel-shaped tertiary structure containing a hydrophobic pocket that can bind lipophilic molecules (Flower, 1994). The human lipocalin-2 gene (hLCN2) is located at the human chromosomal 9q34 location. LCN2 was initially discovered in the granules of human neutrophils (Xu and Venge, 2000) and was later shown to be expressed during inflammatory responses by certain epithelial cells (Cowland et al., 2003).

Iron is required for many essential life processes. However, excess free iron can injure cells and increase their susceptibility to bacterial infections by promoting hydroxyl radical production (Smith, 2007). Siderophores are chelators with a high affinity for iron that are utilized by bacteria to absorb iron, thereby enabling the bacteria to survive in the host (Flo et al., 2004; Raffatellu et al., 2009). These proteins are also part of the innate immune defense system (Smith, 2007) and are released by microorganisms when the lack of soluble iron becomes a limiting factor for growth.

LCN2 binds to bacterial siderophores, depriving bacteria of iron; thus, LCN2 acts as a bacteriostatic protein. LCN2 was highly expressed during acute microbial infection and during tissue involution (Miharada et al., 2005). LCN2 protein levels are increased in blood serum during infection and inflammation (Berger et al., 2005; Nairz et al., 2010). As hLCN2 has potential for treatment of infection, it is necessary to generate a recombinant hLCN2 and increase its production using animal bioreactors.

Mastitis is a devastating disease affecting dairy cattle worldwide. It is an inflammation of the mammary gland (Sharma et al., 2007) that can have serious economic repercussions (Tiwari et al., 2013). The frequent and prolonged use of antibiotics to treat mastitis has led to the emergence of antibiotic-resistant strains. Plasmid-mediated antibacterial gene transfer is a novel method that could aid in the prevention of mastitis and help control its spread. The plasmid-mediated technique enables...
mammary cells to synthesize and secrete the target protein in the udder.

The aim of the present study was to determine whether the mammalian protein hLCN2 exhibits antibacterial activity against bacteria that cause bovine mastitis. Hence, a mammalian-specific recombinant pIRES2-AcGFP1-hLCN2 vector was constructed and introduced into stable bovine mammary gland epithelial cell lines. Because hLCN2 has 67% sequence similarity with bovine LCN2, it may be used as a model to understand the molecular pathways related to innate immune defense in human beings. Therefore, bovine udders may be used as nongenetically-modified organism (non-GMO) bioreactors to produce hLCN2.

Foreign gene transfer via embryonic stem cell (McLaren, 1992) has been unsuccessful in species other than mouse, as cells cannot be generated reliably. Transgenic animals provide an alternative approach to produce hLCN2 in large quantities at relatively low cost, and animal mammary gland bioreactors are feasible tools for the large-scale production of hLCN2. However, it is vital to construct an efficient and specific expression vector in order to produce hLCN2 in a mammary gland bioreactor.

Characteristics of mammary gland development such as growth, tissue remodeling, lactation, and involution make the mammary gland one of the most dynamic organs in mammals. Milk is considered the best biological fluid medium for producing recombinant proteins from animals. In addition, the lactation capacity of some domestic mammalian species that are widely used for commercial milk production, such as cows, exceeds the nutritional needs of their offspring. Therefore, their mammary glands are potent bioreactors that can be used to produce valuable biologically active proteins.

2. Materials and methods
2.1. Bacterial strains, plasmids, and growth conditions
Plasmid cloning vector pIRES2-AcGFP1 was procured from Clontech, USA. *Escherichia coli* DH5α strain was used as the maintenance host for the propagation of the plasmid. *E. coli* DH5α was grown in Luria–Bertani broth aerobically at 37 °C. Agar plates were prepared by adding 1.5% (w/v) agar to the liquid medium. *E. coli* transformants were selected on Luria–Bertani plates containing 50 µg/mL kanamycin (Sigma, USA).

2.2. Synthesis and isolation of hLCN2 gene
The 720-bp full-length *hLCN2* (GenBank accession no.: NM_005564) was commercially constructed and cloned into the pUC57 plasmid. Then *hLCN2* was detected using quantitative polymerase chain reaction (qPCR) primers (132 bp) that were each 20-mer oligonucleotides (forward: 5′-TAG CGC TAC CGG ACTC AGAT-3′ and reverse: 5′-AGG AGA CCT AGG GGC ATGAT-3′). The supplied vector was linearized using BamHI restriction enzyme, and the targeted gene was isolated by using cloning primers.

The cloning primers (sense: 5′-ATG AAC TCT GCC ACC TCC TCT GCC ACC CC-3′ and antisense: 5′-ATG GAT CCC GGG CTG GTG CGG CAG CTG GGC GC-3′) containing restriction sites for EcoRI and BamHI in the forward and reverse primers, respectively, were designed for subcloning. PCR amplification was performed using the following program: 98 °C for 5 min followed by 32 cycles of 98 °C for 10 s, 69 °C for 30 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min. A 50-µL PCR reaction volume was used with a 10 pmol concentration of primers, using the Prime Taq DNA polymerase kit (Genet Bio, Korea). PCR products were separated on a 1.2% agarose gel and extracted by Expin Gel DNA gel extraction kit (GeneAll Biotechnology, Korea). The amplified PCR product (1 µg) and expression vector pIRES2-AcGFP1 (1 µg) were digested with 1 µL (20 U/µL) of the restriction enzymes EcoRI and BamHI. Two microliters of EZ-One buffer were added in a total reaction volume of 20 µL, incubated at 37 °C for 15 min, followed by inactivation at 65 °C for 20 min. The samples that were ready for ligation were stored at −20 °C.

2.3. Preparation of expression constructs for mammalian-specific expression vector pIRES2-AcGFP1
The strategy for the construction of the mammalian-specific expression vector pIRES2-AcGFP1 harboring recombinant *hLCN2* is shown in Figure 1. The plasmid expressing hLCN2 was constructed by cloning the full-length *hLCN2* into the pIRES2-AcGFP1 expression vector (Clontech, USA). The ligation reactions were performed using a T4 DNA ligation kit (Enzymomics, Korea). The digested PCR products (hLCN2) were added to the vector pIRES2-AcGFP1 in a 1:3 molar ratio for ligation at the EcoRI and BamHI sites after the cytomegalovirus promoter. The reaction mixture was incubated overnight (12 h) at 16 °C with 1 µL of T4 DNA ligase enzyme (400 U/µL). The reaction was stopped by incubating at 60 °C for 20 min, and the ligation mixture was immediately transformed into *E. coli* DH5α with 50 µg/mL kanamycin as the selection marker. After 12-13 h of incubation at 37 °C, a single colony was selected and grown in 3 mL of broth at 37 °C for 8 h. The positive transformants were selected by PCR screening.

The plasmid with the gene designated pIRES2-AcGFP1-hLCN2 (Figure 1) was subjected to ligation confirmation by single and double digestion. The clone (pIRES2-AcGFP1-hLCN2) was further confirmed by DNA sequencing (Cosmo Genetech, Korea). Furthermore, 1 µg of the recombinant material was subjected to single digestion with 1 µL of 4 U/µL Stul enzyme and 2 µL of EZ-One buffer in a total 20-µL reaction volume, then incubated
Figure 1. Schematic representation of molecular cloning of recombinant pIRES2-AcGFP1-hLCN2 vector. (A) Isolation of hLCN2 from pUC57 plasmid containing hLCN2 by double digestion with EcoRI and BamHI restriction enzymes; (B) targeted plasmid pIRES2-AcGFP1 cloning vector map to construct recombinant vector; (C) recombinant pIRES2-AcGFP1-hLCN2 vector (6045 bp).
at 37 °C for 15 min followed by inactivation at 65 °C for 20 min. The ligation of insert was also confirmed by using hLCN2 qPCR primers (132 bp) that were each 20-mer oligonucleotides (forward: 5′-TAG CGC TAC CGG ACT CAGAT-3′ and reverse: 5′-AGG AGA CCT AGG GGC ATG AT-3′). The PCR amplification was performed in an Eppendorf Mastercycler using the following program: 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 58.5 °C for 30 s, and 72 °C for 60 s, and final extension at 72 °C for 5 min.

The endotoxin-free, linearized, recombinant plRES2-AcGFP1-hLCN2 was subsequently used for transfection into the bovine mammary epithelial stem cells. Linearization was performed using Stal enzyme (Economics, Korea).

2.4. Generation of stable transfectants with hLCN2
Bovine mammary epithelial stem cells (bMESCs) were collected from Korean Holstein cows and maintained in a tissue culture flask in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) supplemented with 10% fetal bovine serum, 5 µg/mL of insulin, 10 ng/mL epidermal growth factor, 10 ng/mL basic fibroblast growth factor, 100 unit/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL gentamicin, and 2.5 µg/mL amphotericin B with a seeding rate of 1 × 10^5 cells/cm^2 (4000 cells/cm^2).

When the bMESCs reached approximately 90% confluence they were subcultured for 2–3 days before transfection, and fresh medium was added 24 h before transfection. Trypsinized 80% confluent cultured cells were washed two times with phosphate buffer saline (PBS, pH 7). Next, 1–2 × 10^6 cells were transfected with linearized plRES2-AcGFP1-hLCN2 plasmid (10 µg) using an Amaxa human mammary epithelial cells nucleofector kit, following the supplied protocol with some modifications. Nucleofection was performed using the U-029 program of the Amaxa Nucleofector-II machine. For the positive control, 5 µg of pmax green fluorescent protein (GFP) vector (Lonza, Amaxa) containing GFP was used. It is very difficult to transfect bMESCs; hence, dimethyl sulfoxide (DMSO; Sigma, USA) was used at an optimized concentration of 1.6% in the transfection reagent and for 24 h after pulse. After nucleofection, cells were cultured in the DME/F12 growth medium supplemented with 20% fetal bovine serum. After 48 h of transfection, cells were incubated with 250 µg/mL G418 antibiotic for 2 weeks. Pure, stable cell lines were stored for further analysis.

2.5. Detection of hLCN2 protein in bovine mammary epithelial stable cell lines by immunocytochemistry
Transfected bMESCs were seeded in 24-well plates and were used for immunostaining at 60% confluence. Cells were fixed in 4% paraformaldehyde for 30 min at 4 °C, then treated with 0.2% Triton X-100 for 30 min at 4 °C, followed by treatment with 0.3% H_2O_2 for 10 min to stop endogenous peroxidase activity. To prevent nonspecific binding, cells were incubated with 10% goat serum for 1 h at 4 °C. After they were washed 3 times with PBS, the cells were subjected to immunostaining with rabbit polyclonal anti-Lipocalin-2 (Abcam, UK) primary antibody supplemented at a dilution of 1:100 and incubated overnight at 4 °C in the dark. For visualization, fluorescent secondary antibodies for goat anti-rabbit conjugated with phcoerythrin (PE; Santa Cruz, CA, USA) were added at a dilution of 1:100. To visualize nuclei, the cells were counterstained with 0.3 µg/mL 4,6-diamidino-2-phenylindole (DAPI) (Sigma, USA) for 10 min. Immunostained cells were observed under a fluorescence microscope (Olympus, Japan).

2.6. Expression analysis of hLCN2 by reverse transcription-PCR
To confirm the insertion of hLCN2 into the transfectant (bMESCs), total RNA from transfected cells was extracted using the easy-BLUE total RNA extraction kit (Intron Biotechnology, Korea). First-strand DNA synthesis was performed using 1 µg of RNA in a 20-µL reaction volume using the SuperScript III First-Strand Synthesis system (Invitrogen, USA). The reaction mixture was incubated for 50 min at 50 °C then immediately transferred to ice, followed by inactivation of the RTase at 85 °C for 5 min, and in the final step it was treated with RNase for 20 min at 37 °C. All incubation and RT-PCR reactions were performed in the Eppendorf Mastercycler (Eppendorf, USA). First-strand DNA was used as the RT-PCR template with the following RT-PCR program: initial heating at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, 58.5 °C for 30 s, and extension at 72 °C for 60 s, followed by a final extension step for 5 min at 72 °C. The hLCN2 primer concentration was 10 pmol (forward: 5′-TAG CGC TAC CGG ACT CAGAT-3′ and reverse: 5′-AGG AGA CCT AGG GGC ATG AT-3′) in a total 50-µL PCR reaction.

2.7. Quantitation of hLCN2 secreted by transfectants
The transfected and nontransfected bMESCs (1 × 10^5 cells/well) were cultured in growth medium (DMEM/ F12) contained in 24-well plates. The cells and media from both the transfected and nontransfected cell cultures were harvested after specific intervals (i.e. on the first, second, and fourth days of culture), and hLCN2 concentrations in the cells and medium were determined using the Quantikine Human Lipocalin-2/NGAL immunoassay kit (R&D Systems, USA). The cells were lysed in dilution buffer (PBS, 1% Triton X-100, and 1% BSA) before the ELISA measurements (Cowland et al., 2003). Nontransfected cells and culture media were used as controls.

2.8. Bacterial isolates and antibacterial activity of hLCN2
Two bacteria species that cause bovine mastitis, Staphylococcus aureus and Escherichia coli, were isolated directly from clinical cases of bovine mastitis using...
standard protocols for isolation and characterization of these species. After the species were confirmed, the antibacterial activity of hLCN2 against both bacteria was evaluated by various antimicrobial assays. To test the antimicrobial activity of recombinant hLCN2, fetal bovine serum and antibiotic-free media from the recombinant pIRE2-AcGFP1-hLCN2 transfected bMESC were analyzed for antimicrobial activity by different assays. All tests were conducted in triplicate, and results are expressed as mean ± standard deviation.

2.8.1. Agar-well diffusion assay
The antimicrobial activity of recombinant hLCN2 was determined by broth microtiter dilution assay and agar plate and agar-well diffusion assays (Cappuccinno and Sherman, 1999; Espitia et al., 2013). S. aureus and E. coli were grown in brain heart infusion (BHI) broth and incubated for 24 h at 37 °C. A 100-µL aliquot of bacterial suspension containing 1 × 10^5 cfu/mL of each bacterium was spread over Mueller–Hinton agar plates containing 10-mm wells, which were filled with 100 µL of media from transfected cells. Penicillin–streptomycin (100 µg/mL) and gentamicin (50 µg/mL) were used as positive controls for S. aureus and E. coli. Media from nontransfected cells were used as negative controls in both the assays. Petri dishes containing test samples inoculated with the bacteria were incubated at 37 °C for 24 h. The antibacterial activity was determined by measuring the diameter of the zone of inhibition (in millimeters) around each well. A zone of inhibition with a diameter ≤10 mm indicated resistant bacteria (Johnson and Case, 1995).

2.8.2. Agar plate assay
Antibacterial activity of recombinant hLCN2 was also confirmed by the agar plating method. First, 1 × 10^5 cfu/mL of bacteria (S. aureus and E. coli) were incubated in BHI broth treated with nontreated or 1:2 diluted media from recombinant pIRE2-AcGFP1-hLCN2 transfected cells or nontransfected cells and incubated for 24 h at 37 °C. Next, 100 µL of suspension from each tube was spread over Mueller–Hinton agar and incubated for 15–16 h, and the resulting colonies were counted. Penicillin–streptomycin (100 µg/mL) and gentamicin (50 µg/mL) were used as positive controls for S. aureus and E. coli, respectively, while media from nontransfected bMESC were used as negative controls in both assays.

2.8.3. Kinetic study of the antimicrobial activity of hLCN2
A kinetic study of the antimicrobial activity of recombinant hLCN2 against major bovine mastitis-causing bacteria was conducted by broth microwell assay, following the protocol described by Lourenco and Pinto (2001) with some modifications. The wells in a 96-well plate were inoculated with 100 µL containing ~1 × 10^5 cfu of S. aureus and E. coli bacteria and media from transfected and nontransfected bMESC, followed by incubation at 37 °C for 24 h in BHI broth. The plates were analyzed for turbidity by measuring the optical density at 595 nm using a microplate reader (Model-680, Bio-Rad). Penicillin–streptomycin (100 µg/mL) and gentamicin (50 µg/mL) were used as positive controls for S. aureus and E. coli, respectively, while media from nontransfected cells were used as negative controls in both assays.

2.9. Statistical analysis
All experiments were performed in triplicate, and data were analyzed using SPSS. The results are reported as mean ± standard deviation. P values <0.05 were considered significant.

3. Results
3.1. Construction of hLCN2 mammalian cell expression vector pIRE2-AcGFP1
A recombinant pIRE2-AcGFP1-hLCN2 plasmid containing two mammalian expression cassettes was constructed as described in the methods section. GFP expression was visually detected as early as 8–10 h after nucleofection. As shown in Figure 2a, the pUC57 vector containing hLCN2 was identified using the hLCN2 detection primer (132 bp). The synthetic gene fragment of hLCN2 (720 bp) was amplified and detected by agarose gel electrophoresis on 1.5% agarose gel (Figure 2b). The mammalian expression vector pIRE2-AcGFP1 was digested with restriction enzymes EcoRI and BamHI. The amplified fragment of synthetic gene was ligated into the pIRE2-AcGFP1 vector. The recombinant fragment was called pIRE2-AcGFP1-hLCN2. The gene products were obtained by restriction digestion to confirm that the gene fragments were present in the vector that was used for transfection into bMESC (Figure 2c). The incorporation of the gene into the vector was confirmed by RT-PCR amplification of the gene fragments from the recombinant plasmid, using specific forward and reverse primers for the respective genes (Figure S1). Thus, we confirmed that the gene fragment was successfully inserted into the pIRE2-AcGFP1 vector.

The quality of the recombinant plasmid was confirmed by DNA sequencing, and the clone was evaluated using a sequencing chromatogram. The result was observed using Finch TV software, and the sequence was evaluated again by BLASTn (Figure S2). The sequence data showed 100% similarity to the original gene sequence (accession no.: NM_005564) with the hLCN2 insert (720 bp). Hence, the clone produced was high in quality.

3.2. Establishment of hLCN2-transfected bovine mammary epithelial stable cell lines
To produce stable cell lines, we transduced the bMESC with the Amaxa nucleofection method using the
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recombinant pIRES2-AcGFP1-hLCN2 plasmid. Pmax green fluorescent protein (GFP; Lonza, USA) was used as a positive control and showed an approximately 30%–35% transfection rate (Figure 3a). The recombinant pIRES2-AcGFP1-hLCN2 plasmid was expressed at 48 h posttransfection with 15%–20% transfection efficiency in bMESCs (Figures 3b and 3c). However, weak GFP expression was observed after 8–10 h of transfection. These results indicated that the recombinant plasmid was successfully transfected into host cells. After 48 h of transfection, cells were subjected to selection with 250 µg/mL of neomycin (G418), and within 2 weeks pure colonies were obtained (Figure 3d). Neomycin-resistant cells were selected and transferred to fresh selection medium where they multiplied, finally producing recombinant hLCN2-transfected purified cells. The purified transfected cells were used for further analysis and determination of hLCN2 production.

3.3. Analysis of hLCN2 expression in the transfected cells using RT-PCR

After the pure and stable recombinant transfected bMESCs were obtained, an hLCN2 mRNA expression study further confirmed the integration of the target insert. Strong expression of hLCN2 (132 bp) was observed in the transfected stable cell lines (Figure 4), while nontransfected bMESCs were used as negative control. These results confirmed the successful construction of a recombinant plasmid and its integration into bMESCs.

3.4. Potential of transfected bMESCs to produce recombinant hLCN2

To study the production of hLCN2 by transfected bMESCs, the concentration of hLCN2 in both the cell lysate and cell culture medium was analyzed at different time points. Nontransfected cells served as the negative control. We cultured 1 × 10^5 bMESCs per well and found 22 ± 2.52 ng of hLCN2 in the cell lysate after 1 day of culture (Figure 5a). The same number of cells secreted 6.85 ± 0.11 µg of hLCN2 into the medium over a 1-day period (Figure 5b), an amount 300-fold higher than the value of hLCN2 stored within the cells. These results indicate that hLCN2 is transported out of the cell by constitutive secretion.

Meanwhile, hLCN2 was not detected in the nontransfected cell lysate or the medium of the control cells (Figures 5a and 5b). The quantity of hLCN2 stored within the cells increased (although not significantly) during culture, perhaps due to the increase in the number of cells from the initial 1´10^5 to 4 × 10^5 ± 3.93 on the fourth day. The production of hLCN2 in the media also increased with

Figure 2. PCR detection and amplification of hLCN2 in pUC57 vector and confirmation of hLCN2 ligation in the recombinant pIRES2-AcGFP1-hLCN2 plasmid.

(A) PCR detection of hLCN2 in the pUC57 vector with qPCR primer. Lane M1: 1-kb DNA ladder; lane 1: pUC57 vector with hLCN2 gene; lane 2: PCR-detected hLCN2 (132 bp); lane M2: 100-bp DNA ladder. (B) PCR amplification of hLCN2 from pUC57 vector using cloning primer. Lane M: 100-bp DNA ladder; lane 2: PCR-amplified hLCN2 (720 bp). (C) Confirmation of hLCN2 ligation in the recombinant pIRES2-AcGFP1-hLCN2 plasmid by restriction enzymes. Lane M1: 1-kb DNA marker; lane 1: undigested recombinant pIRES2-AcGFP1-hLCN2 plasmid; lane 2: single digestion of recombinant pIRES2-AcGFP1-hLCN2 plasmid with BamHI; lane 3: double digestion of recombinant pIRES2-AcGFP1-hLCN2 plasmid with BamHI and EcoRI; lane M2: 100-bp DNA marker.
Figure 3. Transfection, expression, and establishment of bovine mammary epithelial stable cell lines with recombinant pIRES2-AcGFP1-hLCN2. (A) Expression of Pmax GFP (positive control) after 48 h of transfection (100×); (B) phase contrast image of recombinant pIRES2-AcGFP1-hLCN2 after 48 h of transfection (100×); (C) green fluorescence expression of recombinant pIRES2-AcGFP1-hLCN2 after 48 h of transfection; (D) purified recombinant hLCN2 (pIRES2-AcGFP1-hLCN2) cell lines after 2 weeks of transfection in neomycin (250 µg/mL), used as selection marker (100×). Cells showed normal proliferation rate; bovine mammary epithelial stem cells showed normal morphology, nuclei, and cytoplasm ratio.

Figure 4. Expression analysis of hLCN2 in bMESC by RT-PCR. Lane M: 100-bp DNA marker. Lane 1: Expression of hLCN2 mRNA in the bMESC transfected with recombinant pIRES2-AcGFP1-hLCN2. Lane 2: Nontransfected bMESC did not show expression of hLCN2.
time and was 350-fold higher than the amount of hLCN2 stored within the cells after 4 days of culture with the same number of cells (4 × 10^5 ± 3.93). These results suggest that the recombinant cells into which pIRES2-AcGFP1-hLCN2 was transfected may potentially be used as a source of hLCN2.

### 3.5. Determining hLCN2 expression in the stable cell lines using immunocytochemistry

The purified colonies of hLCN2-transfected cells were subjected to immunochemical analysis to further confirm cloning and transfection. Red signals of phycoerythrin fluorescence (Figure 6a) obtained after staining with primary rabbit polyclonal anti-Lipocalin-2 confirmed that hLCN2 was successfully cloned and transfected into bMESCs. Figure 6b shows green fluorescence signals but no staining with primary antibodies in the clone. These results further confirmed the successful cloning of hLCN2 and the potential of pIRES2-AcGFP to express the insert in transfected bMESCs. These results also indicate the location of recombinant hLCN2 in the bMESCs.

### 3.6. Antibacterial activity of recombinant hLCN2

Previous studies reported that LCN2 possesses strong antibacterial and anti-inflammatory properties (Hammer and Skaar, 2011; Robinson et al., 2014). Hence, antimicrobial assays were performed to evaluate the antibacterial activity of hLCN2 against major pathogens causing bovine mastitis. Results from these antibacterial assays, which were conducted by agar-well diffusion assay, are shown in Figure 7. We found that hLCN2 inhibited the growth of *E. coli* more strongly than it inhibited *S. aureus*. The diameter of the zone of inhibition resulting from hLCN2 activity against *E. coli* was 32.0 ± 0.98 mm (Figure 7a, B), and for the standard antibiotic positive control it was 38.5 ± 1.2 mm (Figure 7b, B). The diameter of the zone of inhibition against *S. aureus* was 22.02 ± 0.08 mm (Figure 7a, A), and for the standard antibiotic control it was 46.18 ± 0.18 mm (Figure 7b, A). Inhibition of bacterial growth was not observed for the negative control PBS or media from nontransfected cells (Figures 7c, 7d; A, B).

A kinetic study of the antimicrobial activity of recombinant hLCN2 was performed by broth microwell assay, and bacterial growth was determined at different
Figure 6. Confirmation of successful integration of recombinant hLCN2 into bovine mammary epithelial stem cells (bMESCs) by immunochemical detection.
(A) bMESCs transfected with recombinant pIRES2-AcGFP1-hLCN2 showing red fluorescence after immunostaining with primary rabbit polyclonal anti-Lipocalin-2 and goat anti-rabbit secondary antibody conjugated with phycoerythrin (PE) (a), 4, 6-diamidino-2-phenylindole (DAPI) nuclear staining (a'), and merged (a") (100×).
(B) bMESCs transfected with recombinant pIRES2-AcGFP1-hLCN2 and immunostained without primary antibody (control), showing only green signal fluorescence of recombinant hLCN2, while no signals of goat anti-rabbit secondary antibody conjugated with phycoerythrin (PE) are observed (b), DAPI nuclear staining (b'), and merged (b") (200×).

Figure 7. Evaluation of antibacterial activity of recombinant hLCN2 against bovine mastitis, causing gram-positive and gram-negative bacteria by agar-well diffusion assay. Plates show antibacterial activity of hLCN2 secreted by transfected bMESCs (a) against mastitis-causing bacteria S. aureus (A) and E. coli (B). A strong zone of inhibition is observed for penicillin–streptomycin (100 µg/mL) or gentamicin (50 µg/mL) as positive control (b); no zone of inhibition is observed with media from nontransfected cells (c) and normal saline (d), the negative controls.
time points. Data obtained from these assays indicated that hLCN2 has strong antibacterial activity against both S. aureus (Figure 8a) and E. coli (Figure 8b). Absorbance values obtained for the hLCN2-treated group were compared with those obtained for the group treated with a standard antibiotic. The results clearly show strong antibacterial activity from 4–5 h onward. Recombinant hLCN2 antibacterial activity against E. coli was stronger than activity against S. aureus.

The ability of hLCN2 to suppress bacterial proliferation is one of its most important properties. Hence, the antibacterial activity of recombinant hLCN2 against the major mastitis-causing bacteria S. aureus (Figure 9, A) and E. coli (Figure 9, B) was tested by agar plate method. The presence of nondiluted media from recombinant cells into which pIRES2-AcGFP1-hLCN2 had been transfected significantly slowed the proliferation of both types of bacteria (Figure 9a, A; Figure 9a, B), compared with untreated controls (Figure 9c, A; Figure 9c, B). Antibacterial activity of hLCN2 in a 1:2 diluted medium (Figure 9b, A and B) was lower than activity in a nondiluted medium (Figure 9a, A and B). hLCN2 had a stronger antibacterial effect on E. coli than on S. aureus. After treatment with nondiluted media from hLCN2-transformed cells, S. aureus and E. coli grew at 366.00 ± 7.94 cfu/mL and 51.00 ± 3.60 cfu/mL, respectively, with significantly higher cell densities (P < 0.05) in the 1:2 diluted media-treated groups (1814 ± 13.05 cfu/mL) and S. aureus (917 ± 15.72 cfu/mL). Inhibition of bacterial proliferation was observed from 4 h onward. Thus, the results obtained from different antibacterial assays clearly indicate that hLCN2 exhibits antibacterial activity against major mastitis-causing pathogens and can be used as an antimicrobial agent to manage bovine mastitis.

4. Discussion
To our knowledge, this is the first study in which pIRES2-AcGFP1 containing hLCN2 was expressed in cattle mammary epithelial cells that were then used as bioreactors to produce the antibacterial protein hLCN2. For the past several decades, research has focused on investigating novel antimicrobial agents to control challenging diseases, including bovine mastitis. Numerous antimicrobial peptides and proteins, most of which are components of the acute innate immune response to infection, have been tested for clinical applications. The production of these proteins is induced by pattern recognition receptors, as well as cytokines of the innate and acquired immune pathways, that play important roles in infection control and immunomodulatory homeostasis (Chan et al., 2009).

Lipocalin 2, also known as neutrophil gelatinase-associated lipocalin (NGAL), siderocalin, or uterocalin, is a 25-kDa protein that belongs to the lipocalin protein family (Kjeldsen et al., 2000). It exhibits antimicrobial activity by sequestering iron and improves host defense against pathogens, such as E. coli (Flo et al., 2004; Hammer and Skaar, 2011) and S. aureus (Robinson et al., 2014). The LCN2 gene is highly expressed in various epithelial tissues (Cowland and Borregaard, 1997). Previous studies suggested that NGAL is upregulated in response to inflammation (Nielsen et al., 1996; Friedl et al., 1999). The properties of LCN2 prompted us to investigate hLCN2 as a potential antibacterial agent for the treatment of bovine mastitis and to examine the expression of hLCN2 in bovine mammary epithelial cells after transfection under normal growth conditions.

In the present study, hLCN2 was successfully cloned into the pIRES2-AcGFP1 vector. The pIRES2-AcGFP1-hLCN2 clone was transfected into bMESCs in vitro and analyzed through GFP expression, immunocytochemistry, and
RT-PCR. Strong GFP expression and immunochemistry results showed that the gene had been successfully integrated into the genome of the transgenic cells and was expressed at a high level. Indeed, GFP-positive cells could be detected 8 to 10 h after transfection of bMESCs with the Amaxa human mammary epithelial cell nucleofector kit. No obvious toxic effects on host cells were detected after microscopic examination of transduced bovine mammary epithelial stem cells. However, the efficiency of transfection was very low, even after 48 h of transfection (Figure 3).

Figure 9. Inhibitory activity of recombinant hLCN2 against bovine mastitis-causing bacteria (S. aureus/E. coli). Nondiluted and 1:2 diluted media from recombinant pIRES2-AcGFP1-hLCN2 transfected cells or nontransfected cells were incubated for 24 h with 1 × 10⁵ cfu/mL bacteria (S. aureus/E. coli) in BHI broth. A 100-μL aliquot of suspension from each tube was spread over Mueller–Hinton agar and incubated for 15–16 h.

(A) S. aureus bacteria cultured from bovine mastitis cases. (a) Few bacterial colonies are seen on nondiluted media; (b) approximately twice as many bacterial colonies are seen on 1:2 diluted media compared to nondiluted media; (c) confluent bacterial growth is seen on media from nontransfected cells (negative control); and (d) no bacterial growth is observed upon addition of standard antibiotic penicillin–streptomycin (100 μg/mL) to S. aureus culture (positive control).

(B) E. coli bacteria cultured from bovine mastitis samples. (a) Few bacterial colonies are seen on nondiluted media; (b) approximately twice as many bacterial colonies are seen on 1:2 diluted media compared to nondiluted media; (c) media from nontransfected cells showing confluent growth of bacteria (negative control); and (d) no bacteria growth is seen upon addition of standard antibiotic gentamicin (50 μg/mL) to E. coli culture (positive control).
DMSO enhances the uptake of DNA by augmenting the permeability of the cell membrane and reducing osmotic shock (Kawai and Nishizawa, 1984). The DMSO concentration required for efficient transfection varies from cell to cell. In fact, the concentration of DMSO that is optimal for one cell line may be toxic for another. This finding has also been confirmed by a recent study conducted by Hashemi et al. (2012), who observed a 60% and 50% decrease in the electroporation efficiency and survival rate, respectively, of Huh-7. In the present study, we used 1.6% DMSO to enhance transfection efficiency, whereas 2% DMSO showed more than 90% toxicity in bMESCs. Melkonyan et al. (1996) recommended 1.25% DMSO for high transfection efficiency of mammalian cells.

A recent report by Maurisse et al. (2010) indicated that electroporation or nucleofection were superior to chemical methods for the delivery of DNA into primary and transformed mammalian cells. Like those of Maurisse et al. (2010), our results showed that electroporation was the best method for use with primary bovine mammary epithelial stem cells. This method resulted in the successful generation of the pRES2-AcGFP1-hLCN2 transduced stable bovine mammary epithelial stem cells in the present study.

In order to produce a novel antibacterial protein, a recombinant pRES2-AcGFP1-hLCN2 vector was designed. The gene was inserted between the BamHI and EcoRI sites, and the recombinant expression vector for the heterologous expression of pRES2-AcGFP1-hLCN2 in E. coli DH5α was obtained. Recombinant hLCN2 was subjected to antibacterial assays to determine its activity against major bovine-mastitis–causing organisms. Data obtained from antimicrobial activity assays, conducted by broth microwell, agar plate, and agar-well diffusion methods, confirmed that hLCN2 showed potent antibacterial activity against S. aureus and E. coli bacteria.

LCN2 prevents the growth of bacteria that rely on catechol-type siderophores for iron acquisition (Flo et al., 2004). Siderophores are small iron-chelator molecules that are produced both by E. coli (Bryan et al., 2010) and S. aureus (Dale et al., 2004; Hammer and Skaar, 2011) and are essential for bacterial survival and virulence (Dale et al., 2004). Many gram-negative outer-membrane transporters and gram-positive lipoprotein siderophore-binding proteins have been characterized, and their binding abilities with outer-membrane transporters and siderophore-binding proteins for Fe-siderophores have been determined (Fukushima et al., 2013). Dale et al. (2004) identified and characterized a nine-gene iron-regulated operon, designated sbn, that is situated between sirABC and galE on the S. aureus chromosome and that is involved in the production of a siderophore.

Vertebrates sequester excess iron within proteins in order to alleviate toxicity and restrict the amount of free iron available for invading pathogens. Restricting the growth of infection-causing microorganisms by sequestering essential nutrients is referred to as nutritional immunity (Hammer and Skaar, 2011). LCN2 binds catecholate-type siderophores such as enterobactin (made by E. coli). LCN2 can arrest E. coli growth by depriving bacteria of iron, which distinguishes LCN2’s mode of action from the conventional pore-forming mechanisms of cationic antimicrobial peptides (Chan et al., 2010). Our results agree with those of Chan et al. (2010). We observed higher antibacterial activity against E. coli than against S. aureus isolates, which may be due to the greater iron requirements of E. coli.

Immune system cells produce LCN2, which scavenges the E. coli siderophore-iron complex, thereby preventing iron uptake in bacteria such as E. coli (Flo et al., 2004) and S. aureus (Robinson et al., 2014). This activity inhibits E. coli growth. Berger et al. (2005) reported that LCN2-deficient mice exhibit increased sensitivity to E. coli infection. Flo et al. (2004) reported that staphylococcal siderophores are not scavenged by LCN2, and that growth of S. aureus is not inhibited by LCN2-containing serum; however, our data indicate that hLCN2 has antibacterial activity against E. coli and S. aureus, conflicting with the results obtained by Flo et al. (2004).

LCN2 protein levels in serum increase during infection and inflammation (Kjeldsen et al., 2000; Berger et al., 2005; Nairz et al., 2010). Hence, LCN2 has potential as an antibacterial agent to manage bovine mastitis. The failure of antibiotic therapy and vaccination to control mastitis poses a major challenge. Therefore, researchers are investigating novel options, such as plasmid-mediated antimicrobial gene transfer to the mammary gland cells, nutritional approaches to enhance the immunity of the udder, and development of an antibacterial system in the udder to fight the invading microorganisms. Zhang et al. (2007) demonstrated the use of the plasmid-mediated gene transfer technique to enable mammary gland cells to synthesize and secrete bovine lactoferricin, as well as bovine tracheal antimicrobial peptides, in order to prevent bovine mastitis.

In conclusion, our work showed that pRES2-AcGFP1 was an efficient vector that delivered the target gene into the bovine mammary epithelial stem cells. Moreover, the recombinant cells into which pRES2-AcGFP1-hLCN2 was transfected may be used as a source of hLCN2 to protect mammary glands from major mastitis-causing bacteria.
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Figure S1. Confirmation of hLCN2 ligation in the recombinant pIRES2-AcGFP1-hLCN2 plasmid by PCR. 
Lane M1: 1-Kb DNA marker. 
Lane 1: recombinant pIRES2-AcGFP1-hLCN2 plasmid. 
Lane 2: PCR product of hLCN2 (132 bp). 
Lane M2: 100-bp DNA marker.
Figure S2. Authentication of cloned amplicon by sequencing. Recombinant pIRES2-AcGFP1-hLCN2 sequencing similarity results (A) and chromatogram (B).
Figure S2. (Continued).