miR-214 Regulates Lactoferrin Expression and Pro-Apoptotic Function in Mammary Epithelial Cells

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Abstract
Lactoferrin (Lf) is an abundantly expressed protein in human milk. Lactoferrin exhibits several important biological functions, and its expression is regulated by multiple environmental factors. Cellular endogenous factors, however, have not been extensively studied with regard to lactoferrin gene expression. In this study, we showed that lactoferrin gene expression and function are directly targeted by miR-214 in HC11 and MCF7 cells. In the lactoferrin mRNA 3′ untranslated region (UTR) of human, mouse, rat, pig, bovine, camel, and goat species, there is a conserved region that perfectly matches the seed region of miR-214. Transfection of miR-214 mimic in HEK293 cells dose-dependently inhibited the activity of pGL3-control vector containing lactoferrin mRNA 3′UTR downstream of the luciferase gene. In HC11 cells, miR-214 overexpression inhibited the induction of lactoferrin expression by β-estradiol (E2) and dexamethasone-prolactin-insulin (DPI). Furthermore, in MCF7 cells, overexpression of miR-214 markedly decreased lactoferrin expression (P < 0.05), and inhibition of endogenous miR-214 expression increased lactoferrin expression and cellular apoptotic activities (P < 0.05). In summary, our data showed that miR-214 is directly involved in lactoferrin expression and lactoferrin mediated cancer susceptibility (proapoptotic activities) in mammary epithelial cells. J. Nutr. 140: 1552–1556, 2010.

Introduction
Lactoferrin (Lf) is an 80 kDa iron-binding glycoprotein of the transferrin family. Lf is found in most biological fluids, including secondary granules of neutrophils and bodily secretions such as breast milk, tears, saliva, and bile, etc., with highest concentrations in breast milk (7–8 g/L in colostrum, 1–2 g/L in mature milk) (1). Lf has multiple functions for newborn infants and their mothers, such as being a major component of the innate immune system, which is involved in stimulating the host immune system. It also acts as a growth factor to stimulate cell proliferation and differentiation at different concentrations [see reviews (2–4)]. Notably, Lf also has antioxidant and anticancer activities. Lf regulates apoptosis related genes (5) and G1 cyclin kinases in cancer cells causing cell cycle arrest (6); in addition, Lf was found to be constitutively downregulated in human breast cancer cells and was suggested as a prognostic marker (7).

Because of the wide array of important biological functions being discovered, regulation of lactoferrin gene expression is under active investigation. It has been shown that Lf is regulated by both extrinsic (nutrition, development, and growth factors) and intrinsic factors (transcriptional factors and nuclear receptors), such as estrogen, retinoic acid, tumor necrosis factor α, Sp1 transcription factor, and lipopolysaccharides [see review (8)], however, the post-transcriptional regulation of Lf expression, has not been fully characterized.

MicroRNAs (miRNA), typically ~22 nucleotides (nt) in size, are a large family of noncoding RNA molecules that can repress gene expression post-translationally (9). Studies have shown dysregulated expression of miRNA in different cell types, disease states, and during developmental stages, indicating roles for miRNA as important regulators in development, cell cycle regulation, and apoptosis, etc. [see reviews (9–11)]. It has been predicted that the lactoferrin mRNA 3′ untranslated region (UTR) could be a target for 37 miRNA (12). Notably, among these miRNA, miR-214 contains a seed region that perfectly matches a highly conserved sequence in the lactoferrin mRNA 3′UTR, indicating that miR-214 may functionally regulate cellular lactoferrin expression and functions. miR-214, with the mature sequence 5′-ACAGCGGACAGAGAGGAGCU-3′ (13), was expressed in 23 species (14). miR-214 has been implicated in cervical cancer and regulates cell growth and apoptosis in HeLa cells (15,16); in the progenitor fate and developmental timing of the retina (17); and in mediating the process of angiogenesis in human umbilical vein endothelial cells (HUVEC) (18). However, the role of miR-214 in mammary epithelial cells is not fully understood.

1 Supplemental Figure 1 is available with the online posting of this paper at jn.nutrition.org.
2 Author disclosures: Y. Liao, X. Du, and B Lönnérdal, no conflicts of interest.
3 Abbreviations: DPI, dexamethasone prolactin insulin; E2, estradiol; EGF, epidermal growth factor; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells; Lf, lactoferrin; miRNA, microRNA; RIPA, radioimmunoprecipitation assay; UTR, untranslated region.
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First published online July 7, 2010; doi:10.3945/jn.110.124289.
Our objective in this study was to unravel the potential roles of miR-214 in regulating Lf and Lf mediated functions in mammary gland epithelial cells.

Materials and Methods

Bioinformatics analysis. The mature sequence of miR-214 (5'-ACACGAGGCACAGCACGGACGGACGU-3') was retrieved from the miRBase Sequence Database release 14.13. Lf mRNA 3'UTR from human, mouse, rat, bovine, pig, camel, and goat were aligned with the miR-214 nt 2172–2360 of GenBank Accession number NM_002343) was PCR amplified from a pDNR-LIB vector containing Lf full-length cDNA (Open Biosystems); the PCR product was cloned into pGL3-control luciferase reporter vector (Promega) via the XhoI restriction site.

Vector construction. The 189 bp Lf mRNA 3’UTR (corresponding to nt 2172–2360 of GenBank Accession number NM_002343) was PCR amplified from a pDNR-LIB vector containing Lf full-length cDNA (Open Biosystems); the PCR product was cloned into pGL3-control luciferase reporter vector (Promega) via the XhoI restriction site.

Cell culture and treatments. HEK293 cells were maintained as described previously (20). The human breast cancer cell line MCF7 was a gift from Dr. Fumio Matsumura at University of California, Davis, and maintained in DMEM supplemented with 500 units/L penicillin, 500 μg/L streptomycin, and 10% fetal bovine serum (FBS). The mouse mammary epithelial cell line HC11 was maintained as described previously (21).

Luciferase activity was measured as described previously (20). Luciferase expression construct (4.5 mg/L) and 30–150 nmol/L miR-214 mimic (Dharmacon) were used.

For cell differentiation stimulation, HC11 cells were seeded in 6-well plates at 20% confluency per well in growth media without EGF (yet containing insulin) for 24 h. Cells were then treated with 1 μmol/L dexamethasone and 1 mg/L prolactin (both from Sigma) to differentiate into a secretory cell type with or without the transfection of 25 nmol/L miR-214 mimic. At 48 h after treatment, cells were collected in RIPA buffer for immunoblotting analysis.

For β-estradiol (E2) treatment, HC11 cells were maintained in phenol-red free growth media. Cells were seeded in 6-well plates at 40% confluency per well in serum free media for 30 h, and treated with 100 nmol/L E2 with or without transfection of 25 nmol/L miR-214 mimic. After 24 h treatment, cells were collected in RIPA buffer for immunoblotting analysis.

Immunoblotting analysis. HC11 cells and MCF7 cells were homogenized in RIPA buffer (25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS) containing 1 × complete EDTA-free protease inhibitor (Roche), and subjected to 10% SDS-PAGE. Antibody against mouse Lf was purchased from Millipore. Bands were detected using Super Signal Femto chemiluminescent reagent (Pierce) and quantified using the Chemi-doc gel quantification system (Bio-Rad). All data were normalized to β-actin.

Quantitative real-time PCR. Total RNA from HC11 cells and MCF7 cells was isolated using the miRNeasy mini kit (Qiagen) and diluted to 2 μg in 1 μL in DEPC-treated water (Ambion).

For Lf mRNA assays, cDNA was generated from 2 μg RNA using a high capacity cDNA reverse transcription kit (Applied Biosystems). Relative expression of Lf and β-actin were determined by real-time Q-PCR using a SYBR Green detection system (Applied Biosystems). The primers used for HC11 cells were Lf forward 5'-GGAGGACGGGTATTTGAGGA-3', reverse 5'-CCAGGTGGCACCCTCTGTAT-3'; and β-actin forward 5'-ACTGCTCTGCTCTCCAGC-3', reverse 5'-ACATCTGCTGGAAGTGCG-3'. The primers used for MCF7 cells were Lf forward 5'-CTTGACGGCGGAATGCTAC-3', reverse 5'-CAAGGTTGGCATGTTGAC-3'. The reaction was performed according to (20).

Data analysis. Statistical analyses were carried out with the GraphPad Prism software. All data were tested by one-way ANOVA, and if any significant differences were detected between 2 individual groups (P < 0.05), differences were identified using Tukey’s test. Data are shown as means ± SEM of 3 independent experiments. A P-value of <0.05 was considered significant.

Results

Lf mRNA 3' UTR contains a sequence that 100% matches the miR-214 seed region. Sequence inspection of the following Lf mRNA 3'UTR revealed that there is a specific region remarkably well conserved between 7 species and that 100% matches the seed region of miR-214: human (corresponding to nt 2305–2311 of GenBank Accession number NM_002343), mouse (corresponding to nt 2248–2354 of GenBank Accession number NM_008522), rat (corresponding to nt 2244–2350 of GenBank Accession number NM_001106864), pig (corresponding to nt 2531–2537 of GenBank Accession number NM_214362), bovine (corresponding to nt 2402–2408 of GenBank Accession number NM_180998), camel (corresponding to nt 2282–2288 of GenBank Accession number AJ131674.1), and goat (corresponding to nt 2301–2307 of GenBank Accession number FM875929.1) (Supplemental Fig. 1).

miR-214 directly binds to Lf mRNA 3' UTR. The ability of miR-214 to physically bind the mRNA 3'UTR of Lf was evaluated using a luciferase reporter assay. We cloned the full length Lf mRNA 3'UTR into luciferase expression vector. Dose-

![FIGURE 1](https://academic.oup.com/jn/article-abstract/140/9/1552/4600219)
dependent repression ($P < 0.05$) of the luciferase activity was observed when increasing concentrations of miR-214 mimic were cotransfected with luciferase reporter vector (Fig. 1). The data indicate that there is a direct binding between the miR-214 mimic and the cloned Lf mRNA 3’UTR sequence, through which miR-214 exerts its inhibitory effects on the upstream luciferase gene. In addition, the repression was dependent on Lf mRNA 3’UTR, because miR-214 did not affect the luciferase reporter activity of pGL3-control vector (data not shown). cel-miR-67 served as a negative control for miR-214 transfections.

**miR-214 regulates Lf protein synthesis and mRNA expression in HC11 cells.** The regulation of Lf by miR-214 was examined in mammary epithelial HC11 cells. E2 significantly stimulated Lf mRNA expression and protein synthesis relative to control, and miR-214 mimic transfection significantly blocked the E2 stimulation both for protein (Fig. 2A) and mRNA (Fig. 2B).

During differentiation of HC11 cells stimulated by DPI, Lf mRNA expression and protein synthesis were significantly induced. When miR-214 mimic was transfected before DPI treatment, Lf mRNA (Fig. 2C) and protein (Fig. 2D) expression were significantly decreased compared with cel-miR-67 transfected cells. cel-miR-67 served as a negative control for miR-214 transfections.

**miR-214 regulates Lf expression in MCF7 cells.** The regulation of Lf in mammary cancer cells was investigated in MCF7 cells. miR-214 mimic transfection time-dependently affected the protein abundance (Fig. 3A) as well as the lactoferrin mRNA expression of endogenous Lf compared with the level before treatment (Fig. 3B). cel-miR-67 served as a negative control for miR-214 transfections.

**miR-214 is involved in Lf induced apoptosis in MCF7 cells.** The role of miR-214 in the functional regulation of Lf in mammary epithelial cells was explored in MCF7 cells. miR-214 inhibitor time-dependently increased endogenous Lf protein (Fig. 4A) and mRNA (Fig. 4B). In addition, caspase-3 activities were also elevated significantly in response to miR-214 inhibitor transfection (Fig. 4C). cel-miR-67 served as a negative control for miR-214 transfections.

**Discussion**

Lf is a secreted multifunctional protein synthesized during normal functional development of mammary epithelium and during lactation in response to hormones such as prolactin. Lf is a major protein in breast milk, and its beneficial effects for the newborn are unquestionable. The expression of Lf during normal functional development of the mammary epithelium has been documented (22–24), and has been suggested to be an important parameter of mammary differentiation (25). On the other hand, in malignant mammary epithelial cells, Lf has been suggested to have antiproliferative effects to protect from local and distant metastasis, and downregulation of Lf expression has been reported in breast cancer (7,23). Lf expression is further suggested to be associated with breast cancer susceptibility (26) and is a positive prognostic indicator of breast cancer with the lobular histotype (7). Targeting Lf is potentially an effective approach for cancer therapeutics; therefore, examination of Lf expression regulation in cancer cells helps to understand the pathophysiology of tumor formation and may suggest novel cancer therapies.
Cellular Lf expression may be affected by a multitude of external factors in mammary cells, such as nuclear receptors, transcriptional factors, prolactin, estrogen, epidermal growth factor, IgA immune complexes, and retinoic acid [see review (8)]. It is also crucial to examine the intracellular regulatory mechanisms of Lf gene expression under normal conditions and malignancy to better understand how nutrition intervention targeting the Lf gene can be beneficial to breast-fed infants, as well as finding strategic approaches for cancer prevention.

The results in this study provide the first indication that miR-214 participates in regulation of Lf expression and is further involved in its secretion and apoptosis-inducing effects in normal and cancerous mammary cells, respectively.

miRNA are small nonprotein coding regulatory RNA that regulate mRNA translation by ribosome inhibition, mRNA 5'-cap structure decapping, mRNA poly(A) deadenylation, and mRNA degradation (27). miRNA are endogenously expressed by mammalian cells and implicated in the pathogenesis of most human malignancies (28). Therefore, RNA therapeutics that mimic or repress miRNA activity in human cancers have attracted considerable attention over the past few years [reviewed in (29)]. In breast cancer stem cells, a screen of 460 miRNA identified that miR-214 was among the 37 miRNA differentially expressed by the CD44+CD24-/-low lineage- human breast cancer stem cells and the lineage+ non-tumorigenic breast cancer cells, and miR-214 was upregulated in all 11 human breast cancer samples (30).

In this study, we demonstrated that miR-214 directly targets Lf expression. Li et al. (31) found that estrogen treatment significantly induces Lf expression in normal mouse mammary epithelial HC11 cells. Moreover, as Lf is a secreted protein in mammary cells, its expression is stimulated in response to prolactin, which differentiates the cells into a secretory cell type (32). We therefore transfected a miR-214 mimic together with estrogen or prolactin treatments and found that miR-214 blocked the upregulation of Lf gene and protein expression, indicating a direct relationship between miR-214 and the physiological Lf expression and secretion. Previous findings in cancerous mammary gland epithelial cells, such as MCF7 cells, T47D cells, BT-20 cells, and MDA-MB-231 cells, showed that Lf expression is lower in normal breast epithelial cells (7,23), indicating a role of Lf in cancer pathogenesis. In our study, miR-214 transfection of MCF7 cells repressed Lf expression over a 48 h period, suggesting that endogenous expression of Lf in cancerous cells may also be regulated by miR-214.

We then examined whether miR-214 could further regulate Lf mediated functions in cancerous mammary gland epithelial cells. Apoptosis plays a key role in the development and pathology of mammary cells, and a dysregulated balance between proliferation, differentiation, and apoptosis ultimately leads to mutations and tumor growth (33,34). In clinical studies, therapeutic interventions including chemotherapy and radiotherapy could induce measurable apoptosis (35,36), and apoptosis stimulation sensitizes tumor cells to various anticancer therapies (37). It has been reported that Lf has antiproliferative and apoptosis inducing effects in cancer cells (5,38,39). Caspase-3 is one of the biomarkers that correlates with apoptosis in breast cancer (40,41). Our data show that the miR-214 inhibitor markedly promotes caspase-3 expression in MCF7 cells, which was
associated with inhibition of cellular growth and increased levels of Lf. The decreased cell population may be due to apoptotic cell death and/or cell growth inhibition. miR-214 may be particularly associated with the sensitivity of cells to anticancer therapy because of its involvement in Lf induced apoptosis.

In summary, our study demonstrates that the translation of Lf mRNA is under direct regulation of miR-214 in mammary gland epithelial cells. miR-214 may further regulate Lf-mediated functions, and miR-214 may be a potential therapeutic target in breast cancer prevention and therapeutic interventions.

Acknowledgments
We thank Dr. Fumio Matsumura and Dr. Chris Vogel for help with breast cancer cell culture and Dr. Christina Teng for helpful suggestions with HCl1 cell culture and experiments. B.L. and Y.L. designed research; Y.L. and X.D. conducted research; B.L. provided essential reagents; Y.L. and X.D. analyzed data; Y.L. and B.L. wrote the paper. B.L. had primary responsibility for final content. All authors read and approved the final manuscript.

Literature cited