Evaluation of the Bioactivity of Recombinant Human Lactoferrins Toward Murine Osteoblast-Like Cells for Bone Tissue Engineering

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Lactoferrin (LF), which belongs to the iron-binding transferrin family, is an important regulator of the levels of free iron in the body fluids. LF has raised significant interest as a bioactive protein due to its wide array of physiological effects on many different cell types, including osteoblasts and osteoclasts. The glycoprotein's degree of iron saturation has a pivotal influence on its physical structure. The objective of this study is to investigate the biological effects of apo (low iron saturation), pis (partially iron saturated), and holo (high iron saturation) recombinant human LF (rhLF) on MC3T3-E1 cells to identify the suitable candidate for bone tissue engineering application. Our studies demonstrated a dose-dependent mitogenic response of MC3T3 to rhLF treatment irrespective of the iron concentration. Furthermore, rhLF induced the cells to produce transcription factors, chemokines, and cytokines as determined by β -catenin activation, phosphorylation of Akt, vascular endothelial growth factor, and interleukin (IL-6) expression. The iron saturation of rhLF did not have any significant effect on these biological activities of MC3T3 cells. In addition, the overall pattern of gene regulation in MC3T3-E1 cells upon rhLF treatment was followed by a global microarray analysis. Among the 45,200 genes tested, only 251 genes were found to be regulated by rhLFs of different iron concentrations. Of these, the transferrin receptor (*Tfrc*) was the only gene differentially regulated by the iron saturated and iron depleted (apo) rhLFs. In conclusion, the study demonstrated that rhLF is a bioactive protein and that the iron saturation of rhLF may not play a significant role in modulating osteoblast functions.

Introduction

LACTOFERRIN (LF) IS A 78 kDA GLYCOPROTEIN, which belongs to the iron-binding transferrin family. It is present in exocrine secretions and is an important regulator of the levels of free iron in the body fluids.¹ LF has raised significant interest as a bioactive protein due to its wide array of physiological effects on many different cell types.² The diverse list of LF's multifunctional roles includes immunomodulatory, anticancer, antibacterial, and antiviral properties.^{3,4} Some of the recent studies have demonstrated LF as an effector molecule in the skeleton,^{5,6} due to its ability to increase osteoblast proliferation, survival, and differentiation⁶⁻⁸ and decrease osteoclast survival.^{9,10} Many of the biological activities of LF have been attributed to its ability to induce signal transduction pathways through cell surface receptors. The low-density lipoprotein receptor-1 (LRP1) has been established as one of the putative receptors in

osteoblasts. The activation of this receptor by bovine LF (bLF) has been shown to induce MAPK-mediated osteoblast mitogenesis.¹¹ However, the antiapoptotic properties of this glycoprotein have been attributed to a PI3K/Akt-independent, and LRP1-independent pathway.⁷ The anabolic effect of bLF⁶ has also been attributed to bLF's possible positive effects on osteogenic differentiation.¹² Yagi *et al.* demonstrated the ability of bLF to push the differentiation of pluripotent mesenchymal stem cells toward the osteoblastic or chondroblastic lineage, and prevent the differentiation toward myeloblastic and/or adipocytic lineages.¹² Recent studies indicate the ability of bLF to promote fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF) synthesis by osteoblasts via the p44/p42 MAP kinase pathway.^{13,14} Additionally, bLF has been shown to inhibit osteoclastogenesis in primary culture of murine bone cells at a concentration as low as 10 μ g/mL.⁹ Collectively, these studies substantiate LF as a positive regulator of the skeleton.

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LF harbors two iron-binding motifs, which are each responsible for the sequestration of a ferric Fe^{3+} molecule.^{15,16} The glycoprotein's degree of iron saturation has a pivotal influence on its physical structure.¹⁷ When fully iron saturated (holo), LF presents as a stable closed structure, as opposed to its open iron-free state (apo).^{18,19} Furthermore, the structure of this protein, although differs throughout species,^{16,20} retains essentially identical high-affinity ($K_D \sim 10^{-22}$ M) iron-binding sites.²¹

The role of iron concentration and the resulting changes in protein conformation in modulating the bioactivity of LF is not clearly understood. Jiang *et al.* investigated the effect of apo and holo human lactoferrin (hLF) on Caco-2 cell proliferation under standard cell culture conditions. The study demonstrated that even though both the proteins were internalized by the LF receptor they differentially affected ERK-signaling and cell proliferation. Apo-hLF showed a significant increase in cell proliferation and activation of ERK cascade compared to holo-hLF.²² Similarly, only apo-hLF was capable of activating the ERK-signaling pathway and both apo and holo-hLFs were capable of activating the PI3K/AKT pathway to modulate crypt cell proliferation.²³ Francis *et al.* studied the iron-dependent effect of hLF on neutrophil survival²⁴ and concluded that only apo-hLF and not holo-hLF is capable of inhibiting neutrophil apoptosis. These studies indicate the possibility that iron concentration may play a significant role in LF bioactivity.

Cornish *et al.* investigated the effects of bLF's structure-activity relationship²⁵ and reported that the degree of bLF iron saturation did not significantly affect the proliferation of primary rat osteoblasts. Furthermore, the substitution of bLF's iron with cations of similar size (i.e., magnesium and chromium) did not significantly change the extent of cell proliferation compared to iron.²⁵ Compared to the previous studies, this data indicates that the iron content of bLF may not significantly affect the biological activities of this glycoprotein toward osteoblast. However, most of the studies reported thus far to understand the effect of LF on osteoblasts and osteoclasts were performed using LF isolated from bovine milk (bLF).

Transgenic, rice-derived recombinant human LF (rhLF) has recently been made commercially available in three different iron saturation forms, ranging from apo-rhLF (iron depleted, <10% iron), pis-rhLF (partially iron saturated, ~50% iron), and holo-rhLF (>90% iron saturated).²⁶ Biochemical and biophysical analyses indicate that rhLF is similar to native hLF and supports mammalian cell proliferation.^{26,27}

The objective of this study is to investigate the biological effect of apo-, pis-, and holo-rhLFs on MC3T3-E1 cells to identify the suitable polymer to develop injectable hydrogels for bone tissue engineering application.^{28,29} The MC3T3-E1 (subclone 4) cell line was selected to evaluate the bioactivities of rhLFs since it is a well-characterized murine osteoblast cell line³⁰ that has been used extensively as a model for osteoblasts *in vitro*. We evaluated the dose- and iron concentration-dependent mitogenic activity of rhLF using thymidine assay. The ability of rhLF to induce MC3T3-E1 cells to activate osteoblast relevant transcription factors, signaling molecules, chemokines, and cytokines were determined by following β -catenin activation, phosphorylation of Akt, VEGF, and interleukin (IL-6) expression. rhLFs at a

concentration of 100 $\mu\text{g}/\text{mL}$ was used to evaluate the differences in bioactivity, based on previous studies using bLF and the present proliferation study using rhLF demonstrating bioactivities in this concentration range.^{9,10} The regulation of cell signaling molecules, such as VEGF and IL-6, were investigated since they are known to play important roles in the bone regenerative process as early stage regulators of angiogenesis and fracture healing.^{31,32} Protein and phosphorylation levels were followed using Western blot and immunocytochemical analysis. Moreover, a global microarray analysis was performed to study the overall pattern of gene regulation in MC3T3-E1 cells when cultured in the presence of different rhLFs for 24 h.

Materials and Methods

Cell culture

Murine osteoblast-like cells (MC3T3-E1 Clone 4 cells) were obtained from ATCC (Manassas, VA). The minimum essential medium-alpha (MEM α) medium, fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco (Carlsbad, CA). Apo-, pis-, and holo-rhLF (endotoxin level: <1.5 EU/mg) were procured from Invitria (Fort Collins, CO). Basal media consisted of the MEM α medium supplemented with 10% volume fraction of FBS, and 1% volume fraction of penicillin/streptomycin.

Cell proliferation

The effect of varying concentrations of apo-, pis-, and holo-rhLF on MC₃T₃-E1 cell proliferation was measured through thymidine incorporation. MC3T3 cells (50,000/well) were plated in 24-well plate ($n=4$). Cells were maintained in the basal MEM α medium for 6 h to allow proper cell adhesion and maintained in a serum-free medium overnight. The cell culture was then performed upon supplementation with 0, 10, 100, and 1000 $\mu\text{g}/\text{mL}$ of apo-, pis-, and holo-rhLFs and [³H] thymidine for 24-h incubation at 37°C. Incorporation of [³H] thymidine radioactivity was counted for 30 s in a liquid scintillation counter and expressed as disintegrations per minute per microgram of DNA. Data were then normalized to untreated control sample and expressed as fold change over control.

Western blot analysis

Western blot protein analysis studies were repeated in triplicate and data shown are representative of the three studies. MC3T3-E1 cells (200,000/10 cm dish) were grown to 90% confluence in basal media, and then serum starved for 6 h. Cells were stimulated with 100 $\mu\text{g}/\text{mL}$ of apo-, pis-, or holo-rhLF or untreated (control) for 24 h. Positive control samples for β -catenin activation includes MC3T3 cells treated with 100 ng/mL Wnt 3a (R&D, Minneapolis, MN) for 6 h and 10 mM LiCl treated for 15 min. CellLytic M and Protease Inhibitor (Sigma, St. Louis, MO) were added to cells, and then incubated at 4°C for 30 min. Cells were removed by mechanical scrapping and spun down at 10,000 rpm for 5 min. Protein concentrations were measured using the BCA Protein Assay Kit and the absorbance of the samples was measured at 562 nm after 30-min incubation at 37°C. Each sample was prepared with the Laemmli Sample Buffer and samples were boiled for 5 min. 25 μg of each sample were run

FIG. 1. Effect of recombinant human lactoferrin (rhLF) concentration and iron saturation on MC3T3 cell proliferation after 24-h treatment measured by thymidine incorporation. Data are expressed relative to the control group and astrices represent statistical significance ($*p < 0.05$) relative to control. The data show significant increase in cell proliferation in presence of 100 and 1000 µg/mL rhLF over the control group irrespective of iron concentration.

on 4%–15% Tris-HCl Ready Gels for Western blot protein electrophoresis. Mini-PROTEAN Tetra System, 10× Tris/Glycine/SDS Buffer, and Precision Plus Protein Dual Color Standard were used and gels were run at a constant 100 volts. Gels were transferred at a constant 100 volts for 2 h using the 10× Tris/Glycine Buffer, Mini Trans-Blot Electrophoresis Transfer Cell, Blot Papers, and 0.2 µm Nitrocellulose Membrane. Membranes were blocked for 2 h at 4°C in 10% milk/TBS-T solution (10× Tris-buffered saline, 0.1% Tween-20). Membranes were washed with the TBS-T solution after each incubation. Membranes were incubated overnight with primary antibodies diluted in 5% milk/TBS-T solution at 4°C. Primary antibodies, anti-β-Tubulin (clone AA2), anti-active β-catenin (clone 8E7), anti-phospho-Dishevelled 2, and anti-phospho-Akt, were purchased from Millipore (Billerica, MA) and diluted to 1:1000 in 5% milk/TBS-T solution. Membranes were incubated for 45 min with a secondary antibody diluted in 5% milk/TBS-T solution at 4°C, and then washed three times with TBS-T. Secondary antibodies, goat Anti-Rabbit IgG Conjugate and Goat Anti-Mouse IgG Conjugate, were purchased from KPL (Gaithersburg, MD) and were diluted at 1:3000. The SuperSignal West Pico Chemiluminescent Substrate was used for detection, and CL-XPosure Film was used for exposure of the membranes. All reagents and materials for Western blot analysis were purchased from BioRad (Hercules, CA) unless otherwise indicated.

Immunocytochemistry

MC3T3 cells were grown on glass-bottom tissue culture plates to 90% confluence in basal media, and then serum starved for 6 h. Cells were either untreated (control) or stimulated with varying iron saturations of 100 µg/mL rhLF for 24 h ($n=3$). Cells were fixed in -10°C methanol for 15 min, and then air-dried and washed in phosphate-buffered saline (PBS). Ten percent normal blocking serum in PBS was then added to each sample for 20 min to suppress nonspecific binding of IgG. Samples were then incubated in a primary antibody diluted in 1% normal blocking serum for 60 min—primary antibodies were diluted 1:100 and used in

accordance with the manufacturer's instructions. After PBS washing, samples were incubated for 45 min in a dark chamber with an FITC-conjugated secondary antibody diluted 1:200 in 1% normal blocking serum. Samples were mounted using the Vectashield Mounting Medium with Propidium Iodide (nuclear staining) and image via confocal microscopy. The analysis was performed in triplicate and data shown are representative of the three samples.

Microarray gene expression analysis

MC3T3 cells were grown to 90% confluence in basal media, and then serum starved for 6 h. Cells were stimulated with 100 µg/mL of apo-, pis-, or holo-rhLF or untreated (control) ($n=3$) for 24 h. The RNeasy Mini RNA Isolation Kit and QIAshredder (Qiagen, Valencia, CA) were used to isolate and purify the RNA. The IlluminaTotalPrep RNA Amplification Kit (Ambion, Carlsbad, CA) was used for RNA amplification. RNA Nano Chip on Agilent 2100 Bioanalyzer was used to detect quality of RNA and labeled cRNA. Samples were hybridized to Illumina MouseWG-6 v2.0 Expression BeadChip. The multisample format includes 45,200 transcripts and six samples simultaneously on a single BeadChip, which dramatically increases throughput, while decreasing experimental variability. The MouseWG-6 beadchip exhibits a dynamic range of >3 logs; detectable fold change <1.35 -fold and reproducibility CV $<10\%$. The ratio of the signal intensity of each experimental sample (100 µg/mL apo-, pis-, holo-rhLF treatment) and the signal intensity of untreated control was represented as the fold change relative to control (Supplementary Table ST1a–c; Supplementary Data are available online at www.liebertpub.com/tea).

Statistical data analysis

All data are presented as mean $\pm$ standard deviation for $n=3$, unless stated otherwise. Statistical analyses were performed by the t -test; $p < 0.05$ was considered statistically significant.

FIG. 2. Effect of rhLF iron concentration on (A) Akt phosphorylation and (B) Dishevelled 2, Gsk3 β phosphorylation, and β -catenin activation in MC3T3 cells after 24-h treatment. Anti-tubulin was used as the gel loading control. The data shows upregulation of all these proteins by rhLF irrespective of the iron concentration. (C) Activation of β -catenin by 100 ng/mL Wnt 3a and 10 mM LiCl included as positive controls.

Results

Effect of LF iron concentration on the proliferation of MC3T3-E1 cells

Figure 1 shows the effect of rhLF concentration and iron saturation on the proliferation of MC3T3-E1 cells after 24-h treatment. Irrespective of the iron saturation, rhLF did not show significant increase in MC3T3-E1 cell proliferation compared to control culture at a protein concentration of 10 μ g/mL. However, significant increase in cell proliferation was observed when cells were cultured in rhLFs at concentrations of 100 and 1000 μ g/mL relative to untreated control. At any of the protein concentrations studied, no significant differences in cell proliferation was observed when cells were treated with apo-, pis-, or holo-rhLF.

Effect of LF iron concentration on MC3T3-E1 cell signaling

LF is a pleiotropic protein with multiple functions and is known to activate various cell signaling pathways in cells, including osteoblast cells. In addition to the mitogenic effects, LF is known to increase cell survival, cause significant

phosphorylation of Akt, and support osteogenic differentiation.² Our previous studies have demonstrated the ability of bLF to increase the accumulation of active β -catenin in MC3T3-E1 cells (data not shown). Figure 2A and B show the effect of iron saturations on the expression of various signaling proteins in MC3T3-E1 cells after 24h of treatment with 100 μ g/mL of the proteins. All the rhLFs, irrespective of iron saturation showed a significant increase in the expression of phosphorylated Akt compared to the control showing that iron saturation does not affect the phosphorylation process. Similarly, irrespective of the iron saturation, all the rhLFs showed a significant increase in phospho-Dishevelled 2, phospho-GSK3, and active β -catenin, key transcription factors that are potentially involved in osteogenic pathways. To confirm the upregulation of active β -catenin in the presence of rhLF, the expression was compared to MC3T3-E1 cells exposed to Wnt3a and LiCl, two molecules known to upregulate the expression of active β -catenin (Fig. 2C).

Immunocytochemistry was also used to confirm the accumulation of β -catenin in MC3T3-E1 cells cultured in the presence of apo- and holo- rhLF. Figure 3A shows the significant accumulation of β -catenin (indicated in green) in MC3T3-E1 cells irrespective of the iron saturation. In addition to being a bone anabolic factor, LF is known to induce the synthesis of angiogenic factors and pleiotropic cytokines by osteoblasts.^{14,15,33} Immunocytochemical studies were performed to confirm the production of IL-6 and VEGF by MC3T3-E1 cells in the presence of apo- and holo-rhLF to evaluate whether the expression of the cytokines is dependent on iron saturation. Figure 3B and C show the immunofluorescence images of MC3T3-E1 cells exposed to 100 μ g/mL of apo- and holo-rhLF indicating positive stains for both IL-6 and VEGF. The data demonstrates that rhLF is capable of inducing the expression of IL-6 and VEGF in MC3T3-E1 cells and the effect is independent of the protein iron saturation level.

Global gene expression profiles of MC3T3-E1 cells exposed to rhLFs

Microarray gene expression analysis was performed on MC3T3 cells treated with 100 μ g/mL of rhLF with three different iron saturations (apo, pis, and holo) for 24 h (Fig. 4). Data quality and reproducibility are supported by the use of three biological replicates per sample and also high level of bead-type redundancy (up to an average of 30 beads per probe) on each array. Of the 45,200 gene transcripts present in the gene array, only 251 genes were significantly regulated by rhLF. We compared the relative expression of 251 regulated genes by rhLF of different iron saturations normalized to untreated control. Approximately, 94% of the 251 genes were regulated similarly by rhLFs irrespective of the iron saturation. Only 6% genes of the 251 statistically significant genes were found to be affected by rhLF iron saturation. For example, *Ccl-7* was upregulated 1.77-, 4.75-, and 3.48-fold over control, respectively, by apo-, pis-, and holo-rhLFs. *Ccl-2*, which is under the control of the nuclear factor κ B (NF κ B) and expressed by mature osteoclasts and osteoblasts,³⁴ was upregulated 1.46-, 3.69-, 2.74-fold over control, respectively, by apo-, pis-, and holo-rhLFs. *Nfkbiz*, a gene that encodes for the NF κ B inhibitor zeta, was also positively regulated by rhLF. Interestingly, this cluster of genes—*Ccl-7*, *Ccl-2*, and

FIG. 3. Immunocytochemical (20 $\times$) analysis of (A) active β -catenin, (B) interleukin (IL-6), and (C) vascular endothelial growth factor- α (VEGF- α) after 24-h treatment of 100 μ g/mL of apo- or holo-rhLF or untreated (control). Nuclei were detected using propidium iodide (red) and protein expression was detected using FITC-labeled (green) secondary antibodies. The data demonstrate the significant increase in protein expression when cultured in presence of apo- and holo-rhLF over the control. Color images available online at www.liebertpub.com/tea

Nfkbiz—were all more upregulated upon treatment with rhLFs with a higher iron saturation (pis- and holo-) relative to the form with the lowest iron saturation (apo-). *Tfrc*, a gene that encodes for the transferrin receptor (*Tfrc*), was the only gene found to be differentially regulated by the rhLFs with high (pis- and holo-) and low (apo-) iron concentration. Iron-deficient rhLF (apo-) upregulated *Tfrc* 1.32-fold, whereas pis- and holo-rhLF downregulated the gene by 1.20- and 1.26-fold, respectively. Supplementary Table S1a–c presents a comprehensive list of genes regulated in MC3T3-E1 by 100 μ g/mL of rhLFs of three different iron saturations after 24 h in culture.

Discussion

In this study, the effect of iron saturation on the biological activity of rice-derived rhLFs toward MC3T3-E1 cells was examined. LF is a known pleiotropic factor with unique antimicrobial, antiviral, and immune-modulatory properties as well as anabolic effects in bone at physiological concentrations. The molecular mechanisms behind the favorable pleiotropic properties of LF are largely unknown; however, the role of various cell surface receptors in mediating the LF biological response has been identified. One of our long-term goals is to develop bioactive biomaterials using unique ligand molecules that can activate specific receptor-mediated signaling pathways.³⁵ We have previously demonstrated the feasibility of developing LF-based biomaterials via an enzymatic process irrespective of the iron content of the protein.^{28,29} The goal of the present study was to evaluate whether the iron content of the protein can make a

significant difference in the bioactivity toward osteoblast-like cells to determine the most effective protein to form rhLF-based biomaterials to support bone regeneration. The effect of apo-, pis-, and holo-rhLF on MC3T3-E1 cell proliferation, regulation of osteoblast relevant transcription factors, expression of chemokines and cytokine were followed to understand the effect of iron concentration in modulating osteoblast functions.

rhLF derived from rice has shown to have mitogenic effects toward a variety of cells types, such as intestinal cells, hybridoma cells, osteoblast cells, and embryonic kidney cells.²⁶ Based on LF's mitogenic activity on different cells types, two possible mechanisms have been suggested. The possibility of iron acting as a nutrient to support cell proliferation and LF regulated gene expression in cells through putative cell surface receptors. Statistically significant dose-dependent proliferation of MC3T3-E1 cells cultured in the presence of 100 and 1000 μ g/mL of apo-, pis-, and holo-rhLFs relative to untreated control demonstrated the mitogenic effect of rhLF toward MC3T3-E1 cells. However, lack of difference in cell proliferation when treated with apo-, pis-, and holo-rhLFs at all the concentrations studied indicates that the mitogenic effect of rhLF toward MC3T3-E1 is independent of iron saturation and therefore potentially mediated by modulation of gene expression via cell surface receptors. The observed results show a significant difference in the behavior of rice-derived rhLFs toward HT-29 intestinal cells, which might be due to the different receptors involved in osteoblast and HT-29 cell signaling cascade.²⁶ The mitogenic effect of bLFs toward osteoblasts is known to be mediated at least partially via LRP1, whereas in the intestinal

FIG. 4. Global expression profiles in MC3T3 murine osteoblast-like cells. Cultures were treated for 24 h with apo- (lane 1), pis- (lane 2), and holo-rhLF (lane 3). Data are expressed as fold change over 24-h untreated control sample. Red signifies upregulation, and green signifies downregulation. 251 genes had statistically significant regulation by the three iron saturated rhLFs relative to the untreated control sample. For example, *Bglap2*, *Ptgis*, *Ibsp*, and *Adamts2* were downregulated by all forms of rhLF. Whereas *Mmp9*, *Nfkbiz*, *Ccl2*, and *Ccl7* were upregulated by all forms of rhLF. *Tfr1* was upregulated by apo-rhLF, but downregulated by pis- and holo-rhLF. Color images available online at www.liebertpub.com/tea

cells, the ERK1/2 signaling cascades are mediated via the LF receptor.²³

Previous studies using osteoblasts have demonstrated the ability of bLF to induce phosphoinositide 3-kinase-dependent Akt signaling independent of LRP1 activation.⁷

MC3T3-E1 cells cultured in the presence of rhLFs showed a significant increase in Akt phosphorylation indicating that as in the case of mitogenic activity, Akt phosphorylation of rhLF is mediated by a mechanism independent of iron concentration and potentially mediated through a similar receptor-mediated signaling process.

The β -catenin-dependent signaling pathway has been documented as a potential osteogenic signaling pathway.³⁶ The phosphorylation of Dishevelled (Dsh) leads to the destabilization of the β -catenin destruction/ubiquitination complex, which includes a series of events, including Gsk3 β phosphorylation. These actions ultimately lead to the stabilization of cytoplasmic β -catenin. Accumulation of β -catenin in the cytoplasm prompts its translocation to the nucleus, where it interacts with members of the T cell-specific transcription factor (TCF)/lymphoid-enhancer binding factor (LEF) family of transcription factors and induces the transcription of target genes. We demonstrated for the first time that LF, irrespective of the iron saturation can increase phosphorylation of Dishevelled 2, Gsk3 β , and activation (dephosphorylation) of β -catenin relative to untreated control. The stabilization of β -catenin has been known to occur via Wnt-mediated as well as Wnt-independent pathways. Wnt and growth factor signaling can act through convergent pathways and possibly synergistically on GSK3 β and β -catenin.³⁷ Among Wnt proteins, Wnt3a has been extensively investigated as a potent molecule to induce stabilization of β catenin, which then cooperatively regulates gene expression with LEF/Tcf transcription factors.^{38–40} Our recent study, however, showed the downregulation of Wnt3a by rhLF (unpublished data), indicating the involvement of other potential mechanisms. Signaling pathways affecting GSK3 β (e.g., PI3K/Akt) can also modulate the β -catenin transcriptional activity. LRP6 phosphorylation by various protein kinases is considered another crucial factor for β -catenin stabilization. The mechanism of rhLF-mediated β -catenin stabilization and the transcriptional activity will be discussed elsewhere. The data demonstrate the ability of rhLF to stabilize β -catenin in osteoblast-like cells and that the effect is independent of the iron saturation. Moreover, apo-, pis-, and holo-rhLF similarly regulate several key upstream proteins and transcription factors of β -catenin indicating potential similarity in their osteogenic activity.

The pleiotropic functions of LF have also been attributed to its ability to induce the synthesis of growth factors and cytokines by the cells. Cell signaling molecules, such as growth factors and cytokines, play an important role in the bone regenerative process. IL-6, a pleiotrophic cytokine, has been demonstrated to be an important regulator for bone maturation during development.³¹ IL-6 is also known to be involved in initiating signaling cascades required for bone regeneration following injury.³² The immunofluorescence study demonstrated the expression of the IL-6 protein in MC3T3-E1 cells after 24 h in culture in the presence of 100 μ g/mL apo- and holo-rhLFs. The results concur with the upregulation of IL-6 mRNA in MC3T3-E1 cells treated with 50 μ g/mL bLF after 2 h.³³ Collectively, the results demonstrate that rhLF is capable of inducing IL-6 expression in MC3T3-E1 cells irrespective of the iron concentration.

VEGF- α is a major angiogenic factor that has been demonstrated to be an essential component of skeletal development and repair. Recent study demonstrated the ability of

bLF in inducing the synthesis of the angiogenic factors VEGF and FGF-2 in MC3T3 cells via the p44/p42 MAP kinase pathway.¹⁵ Other studies demonstrated the ability of hLF in stimulating VEGF α -mediated endothelial cell proliferation and migration.⁴¹ It has been demonstrated that exposure of human umbilical vein endothelial cells to human LF significantly increased VEGF-induced ERK/MAPK phosphorylation.⁴¹ Similarly, recent studies have demonstrated that increased skeletal VEGF can enhance the β -catenin activity and results in excessively ossified bones.⁴² The present study demonstrates that rhLF is capable of inducing VEGF- α expression in MC3T3 cells and the expression is independent of rhLF iron saturation.

The global whole-genome array study was performed to understand the effect of iron saturation on the overall gene expression profile of MC3T3-E1 cells. The regulated genes cover a broad range of functional activities of LF ranging from immunity, cell cycle progression, and bone metabolism. The data reported to our knowledge are the first genetic profile of rhLF-treated MC3T3-E1 cells. The data may provide better understanding of the molecular mechanism behind the pleiotropic effect of rhLF toward osteoblasts. Only a limited number of genes from the Supplementary Table S1a–c with known osteoblast relevant functions are discussed here. A number of known genes regulated during osteoblast differentiation were found to be downregulated in MC3T3-E1 cells at 24 h when cultured in basal media in the presence of rhLFs irrespective of the iron saturations (Supplementary Table S1a). These include mature osteoblast markers, osteocalcin (*Bglap*), and bone sialoprotein (*Ibsp*), transcription factors parathyroid hormone receptor 1 (*Pthr1*), and prostaglandin 12 synthase (*Ptgis*). *Adamts2* (A disintegrin and metalloproteinase with thrombospondin motifs 2), which is responsible for processing several types of procollagen proteins,⁴³ was also downregulated approximately 1.28-fold by all iron saturation forms. Interestingly, FGF2-treated MC3T3 cells also showed similar results of significant downregulation of osteoblast differentiation-associated genes (i.e., *BMP-2*, *osteocalcin*, *Runx2*, and *collagen 1*) at 24 h in culture.⁴⁴ On the other hand, matrix metalloproteinase-9 (*MMP9*), a key regulator in the development of the growth plate during endochondral bone formation,⁴⁵ was stimulated by all iron saturation forms (apo-, pis-, holo-) by 1.30-, 1.67-, 1.43-fold, respectively (Supplementary Table S1a–c).

The gene array, however, demonstrated an upregulation of early osteoblastic induction and proliferation markers. One example is the chemokine (C-X-C motif) ligand 12 (*Cxcl12*) or stromal cell-derived factor-1 (*SDF-1*), which is an early marker of osteoblastic induction modulated by growth factors, which include VEGF, PDGF, and PTH.⁴⁶ Cell division cycle associated 3 (*Cdca3*), which is required for entry into mitosis, was also upregulated an average of 1.25-fold across the three rhLF saturations above untreated control.

Three genes that are significantly stimulated by rhLFs are *Ccl-7*, *Ccl-2*, and *Nfkbiz* (Supplementary Table S1b). Studies have demonstrated *Nfkbiz* to be an activator of IL-6 production and also involved in the induction of inflammatory genes activated through TLR/IL-1 receptor signaling.⁴⁷ *Nfkbiz* is a gene that encodes for a family of proteins known to play a role in the inflammatory response to LPS by their interaction with NF κ B proteins.⁴⁸ *Ccl2* is expressed by mature osteoblasts and is under the control of NF κ B.⁴⁹ Other

studies demonstrated that *Ccl2* mediates fibroblast survival through IL-6.⁵⁰ As discussed before, IL-6 expression was found to be stimulated by 100 μ g/mL of both apo- and holo-rhLF treatments relative to untreated control.

The only gene that was found to be differentially regulated by the iron concentration of rhLF is *Tfrc* (Supplementary Table S1c). Interestingly, previous studies using cells, such as monocytes and intestinal cells, have demonstrated a binding site for LF different from the *Tfrc*. Only with high molar excess of both ligand proteins (LF and transferrin), did a small percentage of the ligands cross-react with the receptor for the other. This most likely occur due to the structural similarity of the two glycoproteins.^{51–53} With regard to transferrin and iron, the transferrin family is known to be potent regulators of iron homeostasis,⁵⁴ and *Tfrc* 1 is the primary target of transferrin in the iron transport system. It has been reported that low iron concentrations can lead to upregulation of this protein,⁵⁵ which may explain why *Tfrc* was differentially regulated by the iron unsaturated and saturated forms.

Conclusions

The present study investigated the effect of rhLF iron saturation on murine osteoblast-like cells. rhLF was found to have a significant effect on MC3T3 cell proliferation, Akt phosphorylation, β -catenin activation, Dishevelled 2, and Gsk3 β phosphorylation at all iron concentrations studied over untreated control after 24-h treatment. Upregulation of chemokines and cytokines, such as VEGF and IL-6, was induced by rhLF in MC3T3 cells independent of the iron saturation. Microarray analysis of MC3T3 cells treated with rhLFs of different iron saturations demonstrated only one gene, *Tfrc*, to be differentially regulated by the iron concentration of rhLF. In summary, the study demonstrated the bioactivity of rhLFs of different iron saturations toward MC3T3-E1 cells and that iron saturation may not play a significant role in modulating osteoblast functions.

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Disclosure Statement

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