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Recombinant Human Lactoferrin as a biomaterial for Bone Tissue Engineering: Mechanism of Antiapoptotic and Osteogenic Activity

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Abstract

Lactoferrin is a bioactive globular protein with unique properties towards musculo-skeletal cells and anabolic to bone *in vivo*. Even though the potent anti-apoptotic and osteogenic activity of lactoferrin has been reported, the mechanism of action has not been fully elucidated. The study demonstrated that the anti-apoptotic effect of rhLF towards MC3T3 pre-osteoblast cells is mediated by Wnt5a/PKA pathway and the stabilization of β -catenin by rhLF is dependent on PKA/LRP6 signaling pathway. The study also investigated the feasibility of developing rhLF as a biomaterial for cell delivery. The injectable rhLF cell delivery vehicles were prepared by enzymatic crosslinking of tyramine-modified rhLF in the presence of hydrogen peroxide and horseradish peroxidase. The modified rhLF showed bioactivity similar to unmodified rhLF. The rhLF gels supported encapsulated MC3T3 cell viability, proliferation and differentiation, as well as phosphorylation of signaling proteins. In conclusion, the study demonstrated the involvement of Wnt5a, LRP6 and PKA signaling in rhLF mediated bioactivity towards MC3T3 cells and the feasibility of developing an injectable cell delivery vehicle from rhLF.

Keywords

injectable hydrogels; ligand; receptor mediated cell signaling; lactoferrin; anti-apoptotic

1. Introduction

Bone possesses the intrinsic capacity for regeneration and remodeling – a constant formation and resorption of 5–15% total bone mass per year under normal physiological conditions¹. Growth factors, cytokines, hormones, bone matrix proteins and mechanical forces act as modulators of essential homeostatic cellular functions including cell survival, proliferation and differentiation through the induction of specific cell signaling pathways. The essential signaling molecules during bone regeneration include IL-1, IL-6, TNF- α , TGF- β , PDGF, BMP, FGF, IGF, MMP, VEGF and angiopoietin^{2–4}. For example, extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) family, is activated by growth factors, such as FGF2, to increase osteoblast proliferation^{5–7}. Multiple signaling pathways, including ERK, have been shown to converge and lead to the activation and phosphorylation of osteoblast-specific transcription factor, *Runt-related transcription factor 2 (Runx2)*⁸, which plays a fundamental role in osteoblast differentiation and bone formation⁹. Similarly, the precise regulation of Wnt/ β -catenin signaling plays an essential role in the facilitation of multipotent mesenchymal stem cell differentiation into osteoblasts^{10,11}.

Lactoferrin (LF) is a 78 kDa endogenous glycoprotein that serves as a ligand capable of activating various cell signaling pathways. The potent bone anabolic properties of LF have recently been demonstrated^{12–16}. Local injection of lactoferrin has shown to increase bone formation *in vivo* and bone mineral density in ovariectomized rats¹⁷. The anabolic effect of LF has been attributed to its ability to increase osteoblast proliferation, survival and differentiation^{12,13,18} and decrease osteoclast survival^{19,20}. LF can induce ERK phosphorylation in osteoblasts, mediated by low density lipoprotein receptor-1 (LRP-1) activation without ligand internalization^{12–14}. We and others have demonstrated the ability of LF to induce the synthesis of growth factors cytokines and chemokines by osteoblasts^{12–15}. These include vascular endothelial growth factor (VEGF), Insulin like growth factors (IGF), fibroblast growth factor (FGF), interleukin-6 (IL-6), CCL2 and CCL7. The upregulation of these biologically active molecules could play potential roles in modulating LF bioactivity.

Cell-based strategies are raising interest, due to the ability of transplanted cells to modulate regenerative outcomes via autocrine, paracrine and/ endocrine signaling pathways²¹. Despite encouraging results, critical challenges to clinical translation exist including the inability to deliver and retain the transplanted cells at the healing site, prevent significant cell death, and control cell functions in the unfriendly milieu of the injured tissue^{22,23}. Several injectable *in situ* forming hydrogels are being developed as cell carriers due to their mild gelation process, ability to localize the cells at the defect site and potential for minimally invasive delivery²⁴. Biofunctional injectable cell carriers that can serve as transient microenvironment to promote cell survival and support desired cellular processes may significantly improve cell engraftment and functional tissue regeneration. The use of recombinant proteins as scaffolds for tissue engineering may have the potential to offer distinct advantages to create cell instructive structures. We have previously reported the iron-independent biological activity of recombinant human lactoferrin (rhLF) towards mouse osteoblast like MC3T3 cells¹⁶.

As discussed above, LF has been shown to have cell protective and osteogenic effects, however the mechanism of action has not been fully elucidated¹⁵. The purpose of the present study is to probe the molecular mechanisms underlying rhLF's cell survival and osteogenic activity in MC3T3-E1 cells. Considering the unique biological properties of LF, another purpose of this study is to develop and characterize an injectable hydrogel from rhLF via enzymatic crosslinking.

2. Results and Discussion

2.1 Anti-apoptotic properties of rhLF

In addition to its potent mitogenic effect, LF has shown to decrease apoptosis in response to serum deprivation in primary osteoblast cells¹³. A recent study by Tang *et al.*, showed the ability of rhLF to increase cell viability and decrease cell apoptosis in primary human dermal fibroblasts²⁵. We investigated the ability of rhLF to protect MC3T3 cells against serum deprivation-induced apoptosis. MC3T3 cellular apoptosis was reduced by 100 µg/mL rhLF over the course of 3 days as evidenced from the live/dead staining (Fig. 1a). Furthermore, 24-hour rhLF treatment increased Akt phosphorylation in MC3T3 cells (Fig. 1b).

A common mechanism by which growth factors promote cell survival is PI3K-dependent Akt phosphorylation. One of the potent cell surface receptors activated by bovine LF is LRP1. However, bovine LF has been shown to increase LRP1-independent, PI3K/Akt phosphorylation¹⁸. Interestingly, studies have indicated that phosphorylation of Akt may not be required for the LF-mediated cell survival and the mechanism by which LF prevents cellular apoptosis is not clearly understood¹⁸. To further investigate the mechanism, Wnt-mediated signaling transduction pathway array was used (Fig. 2a). Wnt-family of secreted growth factors is known to regulate developmental processes, including cell survival²⁶. It has been reported that Wnt3a and Wnt5a can prevent osteoblast apoptosis induced by serum deprivation via both canonical and non-canonical pathways²⁶. Among the Wnt's, Wnt5a was stimulated 3.8-fold upon 24-hour rhLF treatment as shown in Figure 2a. A signaling pathway reported to be mediated by Wnt5a's anti-apoptotic effects is the protein kinase A (PKA)/ cAMP response element binding protein (CREB)²⁷. A PKA inhibitor (H-89), and Akt inhibitor (LY294002) were therefore used to probe these signaling pathways in rhLF treated MC3T3 cells (Fig. 2b). Wnt5a was used as the positive control. rhLF (lane 3) and Wnt5a (lane 2) increased phosphorylation of Akt relative to media control (lane 1). The data confirms that Wnt5a treatment of MC3T3 cells induces Akt phosphorylation – since cells that were co-treated with Wnt5a and LY294002 (Akt inhibitor) did not display increased phosphorylation of Akt. Similar to Wnt5a, rhLF mediated phosphorylation of Akt was inhibited by both H-89 (lane 6) and LY294002 (lane 9) implicating that the rhLF mediated phosphorylation of Akt is dependent on the PKA pathway. Caspase 3 activity, a marker of cellular apoptosis, was investigated in MC3T3 cells following serum deprivation upon rhLF treatment in combination with LY294002 and H-89. An apoptotic inhibitor, 50µM Z-FAD-FMK (lane 10) was used as the positive control for the caspase 3 assay and Wnt5a was used as the experimental control (Fig. 2c). rhLF (lane 3) and Wnt5a (lane 2) treatment decreased caspase 3 activity in MC3T3 cells relative to the media control (lane 1) demonstrating the

ability of both Wnt5a and rhLF in reducing cell apoptosis (Fig. 2c). On the other hand, rhLF (lane 9) and Wnt5a (lane 8) did not rescue cell apoptosis when concurrently inhibited with H-89 treatment (Fig. 2c). This finding confirms the dependence of PKA signaling pathway in rhLF mediated anti-apoptotic effect similar to Wnt5a. This is consistent with a previous report showing the involvement of PKA in the Wnt5a mediated anti-apoptotic activity of human dermal fibroblast²⁷. On the other hand rhLF (lane 6) and Wnt5a (lane 5) treatment partially rescued cell apoptosis of MC3T3 cells when concurrently inhibited with LY294002 treatment (Fig. 2c). This finding demonstrates the partial dependence of PI3K/Akt signaling pathway in rhLF anti-apoptotic effects. The inhibitor studies thereby demonstrate the dependence of rhLF/Wnt5a/PKA pathway for cell survival.

Grey *et al.* has reported that the anti-apoptotic effect of LF is not mediated by LRP1-induced p44/42 MAP kinase pathway in osteoblasts¹⁸, implicating that other cell surface receptors might be involved in rhLF-mediated cell survival. Since the present study demonstrated that the anti-apoptotic property of rhLF is mediated by cAMP/PKA pathway (Fig. 2c), another potential receptor that may be involved is the G protein coupled cell surface receptors (GPCRs)²⁷. Further studies are required to confirm the role of G proteins in rhLF mediated cell survival.

2.2 Activation of β -catenin by rhLF

rhLF is known to increase thymidine incorporation in primary rat osteoblast cultures²⁸. We found increased Ki67 expression, a protein that is expressed by proliferating cells in all phases of active cell cycle (G1, S, G2 and M phase), in MC3T3 cells after rhLF treatment compared to the untreated culture (Fig. 3a). Similarly, EdU incorporation, which occurs during cell proliferation, increased in the MC3T3 cells upon rhLF treatment (Fig. 3a).

The mechanisms and cell signaling pathways involved in the transduction of LF's bone anabolic properties have not yet been comprehensively studied. Being a bone anabolic molecule, LF can positively affect osteoblast survival, proliferation and differentiation²⁻⁴. The activation of the canonical Wnt/ β -catenin pathway is initiated with the activation of Dishevelled, which leads to the inhibition of glycogen synthase kinase-3 β (Gsk3 β) and subsequent stabilization of β -catenin²⁹. 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 Wnt target genes. Treatment of MC3T3 cells with rhLF has shown to increase phosphorylation of Dishevelled 2, β -catenin and Gsk3 β ¹⁶. We demonstrated using western blots, the accumulation of β -catenin in both the nuclear and cytoplasmic fractions showing the nuclear translocation of the stabilized β -catenin (Fig. 3b). Anti-tubulin was used to confirm no cross-contamination of the nuclear and cytoplasmic fractions and anti-actin was used to confirm equal loading of the samples in the western blot experiment.

The Wnt-array demonstrated a significant increase in LRP6 and Dvl2 upon 24 hours of rhLF treatment relative to control (Fig. 2a). Non-canonical Wnt5a was up-regulated, while canonical Wnts – Wnt2 and Wnt3 were down-regulated by rhLF treatment. Following the upregulation of LRP6, the intrinsic LRP6 inhibitor–Dkk1, was down-regulated (Fig. 2a). To

understand the role of PKA and LRP6 on β -catenin activation, LRP6 inhibitor (Dkk1) and PKA inhibitor (H-89), were used to inhibit the targeted pathways (Fig. 3c). In this experiment, BMP2 (lane 3) and Wnt5a (lane 2) were used as positive controls for activation of LRP6 and PKA, respectively. rhLF-treated cells (lane 4) showed increased expression of LRP6 and active β -catenin similar to BMP2-treated cells. Furthermore, rhLF-treated cells had increased expression of p-CREB similar to Wnt5a treated cells. Wnt5a-treated cells (lane 2) did not show upregulation of LRP6 or active β -catenin in MC3T3 cells. Upon H-89 inhibition, active β -catenin was inhibited in rhLF treated cells (lane 8), indicating the dependence of PKA/pCREB activation for β -catenin accumulation. The PKA inhibition in MC3T3 cells by H-89 also inhibited LRP6 expression and p-CREB expression in rhLF treated cells (lane 8) whereas, the presence of H-89 inhibitor did not affect LRP6 or active β -catenin expression in BMP-2 treated cells indicating the differences in rhLF and BMP-2 mediated cell signaling. Furthermore, upon DKK1 inhibition of rhLF treated cells (lane 12), active β -catenin was again inhibited indicating the dependence of LRP6 phosphorylation for β -catenin accumulation in the case of rhLF, similar to BMP-2. This data suggests the activation of β -catenin by rhLF to be dependent on PKA/LRP6 signaling pathway. A recent report shows the role of G protein coupled receptors (GPCRs) in LRP6 mediated cAMP generation²⁷, which further imply the possible role of GPCRs in lactoferrin mediated biological functions.

2.3 rhLF Hydrogel Preparation and Characterization

Among the various cross-linking methods used to create injectable gels, enzyme-catalyzed reactions are raising significant interest due to the mild, cell friendly gelation process³⁰. The injectable matrix from rhLF was developed via an enzymatic cross-linking of the protein that involves oxidative coupling of phenol groups catalyzed by horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2). Previous studies have shown that reactions driven by the peroxidase and peroxide³¹ at low concentrations will not adversely affect the viability of the encapsulated cells^{30,32–39}. Although rhLF contains tyrosine, the phenolic content of rhLF was found to be much lower than what is needed to develop an effectively crosslinked matrix. Treating unmodified rhLF with different concentrations of HRP and H_2O_2 did not lead to the formation of a crosslinked matrix. The phenolic content of rhLF was therefore, increased by carbodiimide mediated coupling of rhLF with tyramine. Figure 4a shows the increase in phenolic content of tyramine modified rhLF (modified-rhLF) as a function of the reaction time with tyramine and subsequent effect on the gelation time. Increase in reaction time led to increase in the phenolic content of modified-rhLF and corresponding decrease in the gelation time. The concentrations of rhLF, HRP and H_2O_2 have also been shown to have significant effect in modulating the gelation time (Fig. 4b). The standard precursor concentrations used in the present study are 10 mg/ml modified rhLF dissolved in 10 U/ml HRP and 10 μ l/mL of 0.25% H_2O_2 which yielded a gelation time of ~ 1 minute (Fig. 4c). No changes in the viability of MC3T3 cells were observed in the presence of H_2O_2 at the concentrations used in the present study (data not shown).

Another physical property investigated was the extent of water uptake (swelling) by the gels. Supplemental figure 2 shows the extent of water uptake of gels made from different polymer concentration. Although there was a trend towards increasing water uptake as the

polymer concentration increased, there was no statistically significant differences between the weights of the gels at 0 day and after 8 days of incubation in buffer (Supplemental figure 2).

Since chemical modification has the potential to reduce the biological activity of proteins, the bioactivity of the modified-rhLF was compared to rhLF. The protein expression levels of phosphorylated ERK (an established proliferation marker) and RunX2 (an established differentiation marker) in MC3T3 cells treated with modified-rhLF and rhLF was followed in 2D culture (Fig. 5a). Both unmodified and modified rhLFs showed significant increase in expression of phosphorylated ERK and RunX2 over the control. The bioactivity of the modified rhLF was further confirmed by following the alkaline phosphatase (ALP) activity and mineral deposition by MC3T3 cells cultured in the presence of modified-rhLF (+*) and rhLF in 2D culture (Fig. 5b). Both groups showed increase in ALP activity and mineral content over the control. No statistically significant differences in ALP activity/mineral content were found between the rhLF and modified-rhLF groups. These studies demonstrated that the modified-rhLF retained the bioactivity of rhLF.

The crosslinked rhLF gel prepared from 10 mg/ml modified rhLF displayed a highly porous microstructure (Fig. 6a). Irrespective of the polymer concentrations (10–50 mg/ml), the gels showed similar porous microstructure (data not shown). However, an increase in rhLF concentration led to a corresponding increase in the storage modulus, (G') (Fig. 6b). Based on the storage modulus, the gels formed from 10–50 mg/ml concentrations of rhLF can be classified as soft gels⁴⁰. Being a globular protein, LF is known to be degraded by proteolytic enzymes such as trypsin and chymotrypsin⁴¹. For a biomaterial to serve as a transient cellular microenvironment, its ability to undergo *in vivo* degradation is important. The *in vivo* biodegradability of rhLF gels was followed after subcutaneous implantation in Sprague Dawley rats. Significant cell infiltration in the gel with no obvious fibrous capsule formation around the biomaterial was observed at 4 weeks post-implantation. rhLF gel was found to fully degrade by 12 weeks post-implantation demonstrating the ability of rhLF gels to undergo degradation *in vivo* (Fig. 6c).

An injectable cell delivery vehicle should allow for homogeneous distribution of cells in the gel and promote favorable cell-matrix interactions particularly for anchorage dependent cells. Polymers such as alginic acid and chitosan do not support osteoblast cell spreading⁴², unlike polymers with adhesive epitopes, such as gelatin³⁶. The rhLF gel presented a very conducive environment to support cell adhesion and cell growth (Fig. 7a). As can be seen, the encapsulated cells show high viability and the cell density increased significantly in the gel as a function of time. The ability of cells to proliferate within rhLF gels was further confirmed by immunostaining for Ki67 protein which is present in the proliferation cycle of cell division (Fig. 7b). Since soluble rhLF has shown to increase phosphorylation of Akt and β -catenin stabilization in MC3T3 cells, studies were performed to evaluate the ability of injectable rhLF gels to activate intracellular signaling in encapsulated cells. Figs. 7 c & d show the expression of phosphorylated Akt and active β -catenin, respectively, by MC3T3 cells encapsulated in 10 mg/ml rhLF gel and cultured for 2 days. Furthermore, the cells encapsulated in rhLF gels showed expression of collagen I protein, as well as osteocalcin

(Figs. 7 e & f respectively) at 21 days post culture in mineralization media, indicating the ability of MC3T3 cells to differentiate in the rhLF gel environment.

3. Conclusions

The study demonstrated that the anti-apoptotic effect of rhLF is regulated upstream of PI3K/Akt and is mediated by Wnt5a. The role of PKA in rhLF mediated anti-apoptotic effect and β -catenin stabilization has also been demonstrated. The data shows that tyramine modification did not affect the biological activity of rhLF. The enzymatic crosslinking presented a mild process to develop rhLF-based injectable and degradable biomaterials for cell encapsulation. The gel provided a conducive environment to support cell viability, proliferation and differentiation and support phosphorylation /dephosphorylation of proteins demonstrating a potential avenue for developing cell instructive injectable materials.

4. Experimental Section

Materials

rhLF (Lacromin) was procured from Invitria (Fort Collins, CO, USA). 2-(N-morpholino)ethanesulfonic acid (MES) buffer, *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *N*-Hydroxysuccinimide (NHS), tyramine, aqueous hydrogen peroxide solution (30% w/w) and horse radish peroxidase were purchased from Sigma Chemicals (St. Louis, MO).

Mouse preosteoblast cells (MC3T3-E1 Clone 4 cells) were obtained from ATCC (Manassas, VA). MEM α medium, FBS and Pencillin/Streptomycin were purchased from Invitrogen, USA. β -glycerophosphate was obtained from Sigma Aldrich (St. Louis, MO). L-ascorbic acid was purchased from Fisher Scientific (Pittsburg, PA). Cetylpyridinium Chloride (CPC) and Alizarin Red were purchased from Sigma Aldrich (St. Louis, MO). Lithium Chloride (LiCl) was purchased from Sigma Aldrich (St. Louis, MO). Recombinant human bone morphogenetic protein and DKK1 was purchased from R&D Systems (Minneapolis, MN). Alkaline Phosphatase Substrate Kit was ordered from BioRad (Hercules, CA). Wnt Signaling Pathway PCR Array was purchased from SA Biosciences (Valencia, CA). H-89 and LY294002 was purchased from Cell Signaling (Danvers, MA). Bioluminescent Caspase-3 Assay was purchased from Promega (Madison, WI).

CellLytic M and Protease Inhibitor were purchased from Sigma Aldrich (St. Louis, MO). BCA Protein Assay Kit, Super Signal West Pico Chemiluminescent Substrate and CL-XPosure Film were purchased from Thermo Scientific (Pittsburg, PA). ReadyPrep Cytoplasmic/Nuclear Protein Extraction kit, Laemmli Sample Buffer, 4–15% Tris-HCl Ready Gels, Mini-PROTEAN Tetra System, 10x Tris/Glycine/SDS Buffer, Precision Plus Protein Dual Color Standard, 10x Tris/Glycine Buffer, Mini Trans-Blot Electrophoresis Transfer Cell, Blot Papers and 0.2 μ m Nitrocellulose Membrane, Blocking Grade Blocker Non-Fat Dry Milk, 10x Tris-Buffered Saline and 0.1% Tween-20 were purchased from BioRad (Hercules, CA).

Anti- β -Tubulin, clone AA2, anti-active β catenin, clone 8E7, anti-phospho-Dishevelled 2, anti-phospho-MAP kinase 1/2 (Erk 1/2), anti-phospho-Akt and anti-LRP6 were purchased from Millipore (Billerica, MA). Anti-phospho-CREB were purchased from Cell Signaling (Danvers, MA). Anti-Collagen 1 was purchased from Abcam (Cambridge, MA). Anti-Runx2 (C-19) was purchased from Santa Cruz (Santa Cruz, CA). Goat Anti-Rabbit IgG Conjugate and Goat Anti-Mouse IgG Conjugate were purchased from KPL (Gaithersburg, MD). Goat polyclonal Secondary Antibody to Mouse IgG – H&L (FITC) and Goat polyclonal Secondary Antibody to Rabbit IgG – H&L (FITC) were purchased from Abcam (Cambridge, MA). Vectashield Mounting Medium with Propidium Iodide was purchased from Vector Laboratories (Burlingame, CA). Live/Dead Viability/Cytotoxicity Kit was ordered from Molecular Probes (Carlsbad, CA).

RNeasy Mini RNA Isolation kit and QIAshredder were purchased from Qiagen (Valencia, CA). RT Complete cDNA synthesis kit was purchased from Clontech (Mountain View, CA). iQSupermix was purchased from BioRad (Hercules, CA). *β catenin* (Assay ID Mm01350396_g1) and *GAPDH* (Assay ID Mm99999915_g1) were purchased from Applied Biosciences (Carlsbad, CA).

Methods

1. Cell Culture—MC3T3-E1 Clone 4 cell line was selected to evaluate the osteoconductivity and the bioactivity of rhLF, tyramine-modified rhLF and rhLF gel. Basal media consisted of MEM α medium supplemented with 10% volume fraction of FBS and 1% volume fraction of Pencillin/Streptomycin. Serum-free media consisted of MEM α media supplemented with 1% volume Pencillin/Streptomycin. Mineralization media consisted of basal media additionally supplemented with 10 μ g/ml L-ascorbic acid and 3mM β -glycerophosphate.

2. 2D MC3T3 cell culture studies using soluble rhLF

2.1. Cell Differentiation Studies: MC3T3-E1 cells were plated in 24-well plate at a density of 50,000 cells/well and maintained in basal medium until 90% confluence. Cells were then maintained in mineralization media and treated $\pm$ 100 μ g/ml of rhLF or modified rhLF for 21 days and media was changed every three days. Samples were harvested at day 21 for westernblot analysis. Samples were harvested at day 14 to evaluate alkaline phosphatase activity and mineral deposition.

2.1.1. Alkaline Phosphatase Activity: Alkaline Phosphatase Substrate Kit was used in accordance to manufacture's instructions. Alkaline phosphatase levels were normalized to total protein concentration.

2.1.2. Mineral Deposition: Mineralized matrix synthesis was analyzed with Alizarin Red staining method for calcium deposition. This technique used a colorimetric analysis of the solution obtained by the dissolution of the complexed dye with CPC. Briefly, cells were rinsed twice with PBS- to remove any unattached cells and then fixed with 70% ethanol for 1 hr at 4°C. Ethanol was then removed and cells were air-dried and then rinse with distilled water. Cells were then incubated with 4M Alizarin Red for 10 minutes at room temperature.

Samples were washed five times with DDI water and then incubated with 10% (w/v) CPC for 15 minutes at room temperature. Sample absorbance was quantified at 562 nm.

2.2. Protein Expression Studies: MC3T3 cells were plated at low density and grown to 90% confluence (~1 million cells). Cells were then serum starved for 6 hours and then treated with 100 µg/ml of rhLF or modified rhLF or untreated (control) for 15 minutes. Cell lysates were prepared for western blotting as described below. Primary antibodies of interest (anti-β-Tubulin, anti-active β catenin, anti-phosphoErk, anti-Runx2) and secondary antibodies (Goat Anti-Rabbit IgG Conjugate and Goat Anti-Mouse IgG Conjugate) were used in accordance to manufacture's instructions.

2.2.1. Western Blot Analysis: CellLytic M and Protease Inhibitor was added to cells and then incubated at 4°C for 30 minutes. Cells were removed by mechanical scraping and spun down at 10,000 rpm for 5 minutes. Protein concentrations were measured using BCA Protein Assay Kit and the absorbance of the samples were measured at 562 nm after 30 minute incubation at 37°C. Each sample was prepared with Laemmli Sample Buffer and samples were boiled for 5 minutes. 25 µg of each sample were run on 4–15% Tris-HCl Ready Gels for western blot protein electrophoresis. Mini-PROTEAN Tetra System, 10x Tris/Glycine/SDS Buffer, 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 hours using 10x Tris/Glycine Buffer, Mini Trans-Blot Electrophoresis Transfer Cell, Blot Papers and 0.2 µm Nitrocellulose Membrane. Membranes were blocked for 2 hours at 4°C in 10% milk/TBS-T solution (10x Tris-Buffered Saline, 0.1% Tween-20). Membranes were washed with TBS-T solution after each incubation. Membranes were incubated overnight with primary antibodies diluted in 5% milk/TBS-T solution at 4°C. Membranes were incubated for 45 minutes with secondary antibody diluted in 5% milk/ TBS-T solution at 4°C and then washed three times with TBS-T. Super Signal West Pico Chemiluminescent Substrate was used for detection, and CL-XPosure Film was used for exposure of the membranes.

2.2.2. Serum Deprivation – Induced Apoptosis: MC3T3-E1 cells were plated in 24-well plate at a density of 50,000 cells/well and maintained in basal medium for 1 day. Cells were then cultured in serum free media and treated with or without 100 µg/ml of rhLF (on day 0) and imaged on days 1, 2 and 3. The viability was examined using Live/Dead Viability/ Cytotoxicity Kit and imaged using Zeiss LSM 510 confocal microscope.

2.2.3. Immunocytochemical Analysis: Cells were fixed in ice-cold methanol for 15 minutes and then air-dried and washed three times with PBS. 10% normal blocking serum in PBS for 20 minutes to suppress non-specific binding of IgG. Incubate with primary antibody overnight. Primary antibodies were used in accordance with the manufacture's instructions and diluted in 1% normal blocking serum. The cells were then washed with PBS three times for 5 minutes each followed by incubation for 45 minutes in dark chamber with FITC-conjugated secondary antibody diluted in 1% normal blocking serum. Samples were mounted using Vectashield Mounting Medium with Propidium Iodide and image via confocal microscopy.

3. Studies of molecular mechanisms of soluble rhLF using 2D culture

3.1. Gene expression analysis

3.1.1. Mouse Wnt Signaling Pathway Array: Quantitative real time PCR was employed to assess the influence of the 100 µg/ml rhLF (n=3) on Wnt signaling pathway in MC3T3 preosteoblast cells. Briefly, cells (~1 million) were serum starved for 6 hours and treated with rhLF (100 µg/ml) or PBS (control) and cultured for 24 hours. Total RNA was isolated using the Qiagen RNeasy Mini Kit according to the manufacturer's instructions. For cDNA synthesis, 2 µg total RNA was used as a template for Clontech Sprint RT Complete cDNA synthesis kit in a total volume of 20 µl. BioRad MyiQ2 Two-Color Real-Time PCR Detection System was utilized to measure expression profiles of 84 genes related to Wnt-mediated signal transduction on cells treated with rhLF relative to untreated control. The RT² ProfilerTM PCR Arrays include a built-in positive control elements for the proper normalization of the data, for the detection of genomic DNA contamination, for the quality of the RNA samples, and for general PCR performance.

3.2. Cell signaling and inhibitor experiments: MC3T3 cells were grown to confluence and then serum starved overnight. Cells were then treated with select inhibitors (10 µM LY294002, 30 µM H-89 or 100 ng/ml DKK1) for 2 hs, followed by treatment with 100 µg/ml rhLF, 100 ng/ml Wnt5a or 100 ng/ml BMP2 for 24 hours. Cells were then harvested and cell lysates were prepared.

3.2.1.1. Protein analysis: Western blot analysis was performed as described in Methods section 2.2.1. Primary antibodies of interest (anti-β-Tubulin, anti-active β catenin, anti-LRP6, anti-phospho-CREB, anti-phospho-Akt) and secondary antibodies (Goat Anti-Rabbit IgG Conjugate and Goat Anti-Mouse IgG Conjugate) were used in accordance to manufacture's instructions. To investigate nuclear translocation of β catenin, ReadyPrep Cytoplasmic/Nuclear Protein Extraction kit was used to prepare fractions enriched in cytoplasmic and nuclear proteins and then probed for active β catenin expression via Western blotting.

3.2.2. Caspase-3 Activity: 50 µM Z-VAD-FMK an apoptosis inhibitor was used as the positive control. Bioluminescent caspase-3 assay was used according to manufacture's instructions. 75 µg of protein lysate was used (n=3) and the absorbance was read at 405nm.

4. Formulation of rhLF Gel

4.1. Synthesis of Modified rhLF: Briefly, 500 mg rhLF was dissolved in 50 ml of 1M MES buffer. To this solution, appropriate amounts of EDC (2.61 mM), NHS (1.3 mM) and tyramine. Hydrochloride (1.73 mM) were added. The mixture was allowed to react for 24 hours under gentle stirring. The modified polymer was purified by dialysis against excess deionized water using standard regenerated cellulose dialysis tubing (MWCO 10,000) followed by lyophilization.

4.2. Quantification of Tyramine Modification: rhLF (250 mg) was modified with varying reaction times (0.5 – 24 hours) and the phenolic content was then determined as a function of modification reaction time at 275 nm using UV-Vis Spectrophotometer (Thermo Scientific

Evolution 60). A tyramine standard curve was used to determine the concentration of a phenol in the modified rhLF samples.

4.3. Preparation of rhLF gels: rhLF gels were prepared by the HRP-mediated oxidative coupling of modified rhLF molecules in the presence of hydrogen peroxide. Various concentrations of modified rhLF ranging from 10 mg/ml to 50 mg/ml were dissolved in HRP/phosphate buffer solution. Gelation was initiated by the addition of 0.25% aqueous hydrogen peroxide.

4.3.1. Gelation Time: Sol to gel time was determined via vial inversion method. The effect of modified rhLF (10 – 50 mg/ml), HRP (10 – 50 U/ml) and 0.25% H₂O₂ (1 – 10 μ l) concentrations on gelation time was investigated. 10 mg/ml modified rhLF in 100 μ l of 10 U/ml HRP + 1 μ l of 0.25% H₂O₂ represents the standard gelling conditions. Variables were individually changed and tested for their effect on gelation time.

5. Characterization of rhLF gels

5.1. Morphology: The morphology of the rhLF gels formed from modified rhLF solution was visualized by scanning electron microscope (JEOL 6335 Field Emission Scanning Electron Microscope). The gels were flash frozen by immersing in liquid nitrogen followed by lyophilization and then observed under the SEM.

5.2. Rheological Analysis: The rhLF gels were subjected to rheological measurements to assess the viscoelastic properties at varying polymer concentrations at 37°C. Briefly, the modified rhLF/HRP and H₂O₂ mixture were introduced in a parallel plate rheometer (ARES-LS, TA instruments). Stress sweeps at a constant frequency of 1 Hz were first performed to obtain the linear viscoelastic region for collecting subsequent data. Dynamic frequency sweeps were then performed in the linear viscoelastic region to determine values of the storage (G') and loss (G'') modulus to compare the mechanical strength of gels with different concentrations of rhLF.

5.3. Biomaterial Degradation *in vivo*: Sprague Dawley rats (12–16 weeks) were used. 10 mg/ml rhLF were injected subcutaneously to form the gels *in situ*. At 4 and 12 weeks, rats were sacrificed and the tissue surrounding the injection site was collected. Samples were fixed and embedded in paraffin and sectioned and stained with H&E.

6. 3D MC3T3 cell Culture Studies by Encapsulating in rhLF Gels

6.1. Cell Encapsulation in rhLF Gels: Modified rhLF 10 mg/ml was dissolved in 10 U/ml HRP in 1:1 water/PBS mixture. 500,000 cells/ml were resuspended in 100 μ l of the modified rhLF/HRP solution followed by the addition of 1 μ l of 0.25% H₂O₂ on a 35 mm glass-bottom dishes. The encapsulated cells were then maintained in basal medium at 37°C.

6.2. Cell Viability: The viability of the rhLF gel – encapsulated cells was examined using Live/Dead Viability/Cytotoxicity Kit. The MC3T3 cell encapsulated rhLF gels were incubated at 37°C in basal medium for a time course of 20 days and the cells were imaged using Live/Dead Kit. Live/Dead stain is a vital fluorescence double-stain that utilizes calcein

AM and ethidium homodimer-1 probes to measure membrane integrity and cellular esterase activity to semi-quantitatively assess cell viability. For staining, cells were incubated at 37°C for 30 min in PBS containing 2 mM calcein AM (Live) and 2 mM ethidium homodimer-1 (Dead). Cells stained green (i.e. live cells) and red (i.e. dead cells) were imaged using Zeiss LSM 510 confocal microscope.

Immunocytochemical Analysis: Cells (500,000 cells/ml) were encapsulated in 10mg/mL rhLF gel as described under section 6.1 and cultured on sterile glass bottom culture plates, 35 mm uncoated. For cell differentiation studies, the cells were grown in basal media for 1 day and then mineralization media for an additional 21 days. At the end of each time point, the gels were washed briefly with PBS and fixed using ice-cold methanol for 5 minutes. Methanol was aspirated and allowed to air dry. Samples were washed three times with PBS. Samples were incubated in 200 µl of 10% normal blocking serum in PBS for 20 minutes to suppress non-specific binding of IgG. Samples were then incubated with primary antibody overnight in 1% normal blocking serum followed by washing with PBS three times 5 minutes each. Incubated in dark chamber for 45 minutes with fluorochrome-conjugated secondary antibody diluted to in PBS with 1% normal blocking serum and washed with three changes of PBS. Mounted coverslip with Vectashield Mounting Medium with Propidium Iodide (nuclear stain) and imaged via confocal microscopy.

1. Statistical Data Analysis: All data is presented as mean $\pm$ S.D. (standard deviation) for $n=3$, unless stated otherwise. Statistical analyses were performed by t-test; $p < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

a. Live/Dead viability assay demonstrates increased live (green) cells relative to dead (red) cells after 1–3 days for MC3T3 cells treated with 100 $\mu\text{g/ml}$ rhLF relative to untreated control upon serum deprivation.

b. Immunocytochemical analysis showing the phosphorylation of Akt (FITC-labeled secondary antibody) in MC3T3 cells treated with 100 $\mu\text{g/ml}$ rhLF treatment relative to untreated control after 24 hours.

Figure 2.

a. Wnt Signalling Array was utilized to examine the effect of 100 μ g/ml rhLF treatment (y-axis) relative to untreated control (x-axis). Greater than or equal to 2-fold change over control is considered to be significantly regulated.

b. Inhibitor study to understand the role of PKA and Akt inhibitors on the phosphorylation of Akt. 30 μ M H-89 and 10 μ M of LY294002 inhibited rhLF- induced phosphorylation of Akt in MC3T3 cells after 24 hours. Wnt-5a was used as a positive control.

c. Evaluation of the dependence of rhLF/Wnt5a/PKA pathway for rhLF mediated cell survival. Caspase-3 activity was investigated after 24 hours of rhLF treatment with signaling pathway inhibitors, H-89 and LY294002. Wnt-5a was used as the experimental control and 50 μ M Z-FAD-FMK was used as anit-apopototic control.

Figure 3.

a. Immunocytochemical analysis showing the increase in proliferation of MC3T3 cells in the presence of rhLF. 100 µg/ml rhLF increases EdU incorporation and Ki67 protein expression (8 hour treatment) in MC3T3 cells relative to untreated control. Nuclei are stained with propidium iodide.

b. Increased accumulation/activation of β catenin in nucleus (N) and cytoplasm (C) of rhLF treated MC3T3 cells after 24 hours treatment. 10 mM LiCl treatment was used as the positive control for the activation of β catenin.

c. Evaluation of the dependence of β catenin/PKA/LRP6 pathway for rhLF mediated cell survival. 30 μ M H-89 and 100 ng/ml DKK1 were used to inhibit the targeted β catenin activation. Inhibitor experiments were performed for 24-hour stimulation of rhLF. Wnt-5a and BMP2 were used as positive controls.

Figure 4.

a. Effect of tyramine modification time (over 24 hours) on phenolic content and gelation time of rhLF. The standard gel precursor concentrations, 10 mg/ml modified rhLF dissolved in 100 µl of 10 U/ml HRP and 1 µl of 0.25% H₂O₂, were used to test gelation time.

b. Effect of gel precursors (modified rhLF, HRP, H₂O₂) concentration on gelation time. To study the effect of each gel component, one component was varied and the concentrations of other components were held at the standard conditions. The standard conditions for

approximately 1 minute gelation time is 10 mg/ml modified rhLF dissolved in 10 U/ml HRP plus 10 μ l of 0.25% H₂O₂.

c. Photographs showing modified-rhLF solution and the gel formed after treating with HRP and H₂O₂.

Figure 5.

a. Retention of bioactivity of modified rhLF. Bioactivity after tyramine modification was tested by the phosphorylation of Erk in monolayer (2D) MC3T3 cells after 15 minutes treatment with modified rhLF (+*) relative to unmodified rhLF (+) and untreated control (-) cultured in basal media. Samples probed for Runx2 expression were maintained in mineralization media for 21 days and treated with unmodified (+) or modified (+*) rhLF or untreated control (-).

b. Alizarin red mineralization assay and alkaline phosphatase assay were performed in 2D culture on MC3T3 cells maintained in mineralization media for 14 days and treated with soluble unmodified rhLF (+) or modified rhLF (+*) rhLF or untreated control (–). Statistical significance ($p < 0.05$) is represented as □□ for alizarin red data and # for alkaline phosphatase data relative to appropriate controls (–).

Figure 6.

- Microstructure of enzymatically crosslinked rhLF gels formed from 10mg/mL of modified rhLF. The gels were flash frozen followed by freeze-drying followed by SEM imaging.
- Effect of rhLF polymer concentration on hydrogel storage modulus. Data is expressed in Pascal (Pa).
- In vivo* degradation of 10 mg/ml rhLF gel in rat subcutaneous tissue after 4 and 12 weeks (4x objective magnification). In 4 weeks image, rhLF gel is indicated with an “X”.

Figure 7.

a. Cellular viability and proliferation upon encapsulation in rhLF gel was followed as a function of time (1 day, 10 and 20 days). MC3T3 cells (500,000 cells/ml) were encapsulated in 10mg/mL rhLF gels and cultured in basal media. The cell viability and cell density was followed by live/dead assay. Green (live cells) and red (dead cells) Immunocytochemical analysis showing **(b)** expression of Ki67 **(c)** phosphorylation of Akt **(d)** activation of β catenin (using FITC-labeled secondary antibodies) in MC3T3 cells

encapsulated in 10mg/ml rhLF gel cultured in basal media. Nuclei were stained with propidium iodide.

Immunocytochemical analysis **(e)** Collagen 1 (Col1) and **(f)** osteocalcin (OCN) expression (using FITC-labeled secondary antibodies) in MC3T3 cells encapsulated in 10mg/ml rhLF treatment after 21 days cultured in mineralization media. Nuclei were stained with propidium iodide.