Lactoferrin Promotes the Autophagy Activity During Osteoblast Formation via BCL2-Beclin1 Signaling

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Research Article

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Abstract

Lactoferrin, as the main component of milk, can maintain osteoblast formation, which is conducive to the prevention and treatment of osteoporosis. Lactoferrin also serves as an autophagy regulator, especially in osteoblasts. This study aimed to explore the significance of autophagy in osteoblast formation regulated by Lactoferrin and the internal mechanism. Our results showed that Lactoferrin enhanced the autophagy activity of primary osteoblasts. Importantly, Lactoferrin inhibited BCL2 expression and the co-immunoprecipitation of BCL2 and Beclin1 in osteoblasts. Moreover, Lactoferrin-promoted autophagy was reversed by BCL2 overexpression or Beclin1 inhibition with spautin-1 in osteoblasts. In conclusion, Lactoferrin can inhibit BCL2 expression in osteoblasts, further enhancing Beclin1-dependent autophagy activation.

Introduction

Lactoferrin is a kind of iron-binding glycoprotein, which exists in milk, breast milk secretion and so on [1]. Lactoferrin can promote the osteoblast formation and subsequent osteogenesis, whereby improving pathological bone loss [2–4]. Chen *et al.* and Li *et al.* proved that bovine lactoferrin significantly improved bone mass and bone microstructure in ovariectomized rats [5, 6]. Furthermore, both bovine lactoferrin and human lactoferrin can efficiently promote the proliferation of osteoblast cell line, MC3T3-E1 [7]. Lactoferrin can also improve the inhibition of osteogenesis induced by aging through IGF1 signaling [5]. Lactoferrin promotes bone formation of primary osteoblasts, MC3T3-E1 cells in vitro and C57BL/6J mice in vivo [6]. Similar results were reported in other studies [4, 8, 9].

Autophagy, as a highly conserved intracellular mechanism, maintains cell homeostasis by degrading damaged or aged organelles, breaking down dispensable macromolecules or pathogens, and releasing nutrients and energy. Autophagy also exerts a significant effect on the formation of osteoblasts. The knockdown of autophagic molecule, ATG5, can suppress the proliferation and differentiation of osteoblasts [10]. Moreover, autophagy activation also contributes to the osteogenic differentiation of human gingival mesenchymal stem cells [11]. Accordingly, the positive effect of autophagy has an important responsibility for osteoblast formation. Other investigations have also clarified the similar theory [12–14]. Lactoferrin is an important regulator of autophagy. Most studies have elucidated the role of lactoferrin in promoting autophagy. Lactoferrin can improve the pathological cardiac hypertrophy of aging mouse heart by enhancing lysosome-dependent autophagy [15]. The protective effect of lactoferrin on hepatocytes is also related to the promotion of autophagy activity of damaged hepatocytes [16]. In addition, lactoferrin can induce the autophagy in human kidney proximal tubular cells and suppress oxidative stress-induced cell death by upregulating the autophagy activity [17]. Furthermore, lactoferrin suppresses renal fibrosis through the induction of autophagy [17]. At present, only one study has described the role of lactoferrin in osteoblast autophagy. However, the study only focused on the effect of lactoferrin on the autophagic activity of osteoblast precursors [9]. Therefore, there is still a lack of evidence for the direct effect of lactoferrin on osteoblast autophagy.

BCL2-Beclin1 signaling is a classic pathway of autophagy activation. BCL2 prevents Beclin1 from dissociating from BCL2-Beclin1 complex by conjugation, which leads to less Beclin1 entering autophagy flux and autophagy inactivation [18]. Under various stresses, BCL2-Beclin1 complex dissociates, leading to Beclin1-dependent autophagy activation [19–22]. Previous studies also lack the relevant reports regarding the regulatory effect of lactoferrin on BCL2 protein expression in osteoblasts. Therefore, the significance of BCL2-Beclin1 signaling in the autophagy and formation of osteoblasts regulated by lactoferrin is worthy of further study.

In this study, we explored the direct effect of lactoferrin on the autophagy of primary osteoblasts from rat calvaria. The experimental data showed that lactoferrin can enhance the autophagic activity of primary osteoblasts. In addition, a potential mechanism related to osteoblast formation dominated by lactoferrin, BCL2-Beclin1-autophagy signaling pathway, has also been revealed for the first time.

Materials And Methods

Cell isolation and culture

Primary osteoblasts were prepared from the calvaria of Sprague Dawley (SD) rats (Animal center of Gem Pharmatech Co., Ltd; Nanjing, China). In short, the calvarias of rats (2 weeks after birth) were dissected, washed with PBS and digested in fresh 0.1% collagenase type II in alfa-minimal essential Eagle's medium (α-MEM) at 37℃ for 40 minutes (repeated for 5 times). After digestion, the supernatant was mixed and centrifuged to pellet cells. The cells were then maintained in α-MEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin sulfate, at 37℃ with 5% CO_2. Next, the medium was replaced with α-MEM containing 1% bovine serum albumin (BSA), and the cells were cultured for 16 hours before preparing for subsequent experiments.

ALP activity analyses

Osteoblasts were evaluated by measuring ALP activity.ALP activity was measured using a commercial kit in accordance with manufacturer's protocols (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

Alizarin red staining

The capability of mineralization of corresponding cells was assessed in 6-well plates using Alizarin red staining. The indicated cells were fixed with ice-cold 70% ethanol and stained with Alizarin red staining kit (Sigma-Aldrich, MO, USA) to detect the calcification according to the manufacturer's protocols. ImageJ 1.8.0 software was applied to detect the percentages of positive areas, and then quantify the mineralized areas.

Quantitative real-time PCR (qRT-PCR) assays

Total RNA was extracted and purified by Trizol method. cDNA synthesis and quantitative real-time PCR (qRT-PCR) assays were carried out in accordance with manufacturer's protocols (Takara, Tokyo, Japan). The designed primer sequences are as following:

Specific primer sequences for qPCR

Gene	Forward（5'-3'）	Reverse（5'-3'）
PCNA	ACTCGCATTGGCTGGCATGG	TGACTACCGCTTTGTGGCTTTGG
Col1	CACCTGGCGTCTTACTTCGTCTTC	GTTGGGCGTGGGCAGTTCAG
BGLAP	CACTCCTCGCCCTATTGGC	CCCTCCTGCTTGGACACAAAG
OPN	AGCAGCTTGGCCCAGACCTA	TAGCGCCGGAGTCTGTTCACTAC
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

Western Blotting assays

Total cellular protein was extracted using RIPA buffer (Beyotime, Jiangsu, China) and quantified using BCA protein assay kit (Beyotime, Jiangsu, China). The proteins were loaded and electrophoresed separately through a 15% SDS-PAGE gel. The separated proteins were subsequently transferred to the polyvinylidene fluoride membranes (PVDF) membrane and incubated with primary antibodies (rabbit anti-LC3, BCL2, Beclin1 and GAPDH; Cell Signaling Technology, Boston, USA) at 4°C overnight. After washing, the membrane was incubated with the secondary antibody at room temperature for 60 minutes. The immunoreactive bands were visualized using an ECL kit (Millipore, MA, USA) and were quantified using a Chemi-Doc image analyzer (Bio-Rad).

Lentiviral Transduction

Recombinant lentiviruses encoding the wild-type BCL2 or shRNA against Beclin1 were constructed by homologous recombination between the expression vector (pEX-Puro-Lv105) and cDNA/shRNA in 293 cells using the lentivirus construction kit in accordance with manufacturer's protocols. The same method was used to construct and package the corresponding control vector. After 2 days, supernatants were harvested, and primary osteoblasts were incubated in medium containing lentiviruses and 5 μg/ml polybrene at a multiplicity of infection (MOI) of 40 for 2 d. The infected cells were selected using puromycin (10 μg/ml). The overexpression efficiency of viral gene was detected using qPCR analysis.

Coimmunoprecipitation (Co-IP) assays

The total protein was extracted by RIPA Lysis and Extraction Buffer (ThermolFisher Scientific, USA). Subsequently, we rinsed the beads with 100 μL iced buffer, added 100μL antibody-binding buffer to revolve the antibody and magnetic beads for 30 min, and then rinsed the beads 3 times using 200 μL buffer for 5 min each time. Cell lysates and antibody-bound magnetic beads were incubated for 1

hours at room temperature and washed using 200 μL buffer for 5 min each time. 20 μL eluent was used to rinse the beads once and the supernatant was removed. The cell lysates were extracted for Co-IP with anti-BCL2 antibody (Cell Signaling Technology), and subsequently, precipitates were examined using Western Blotting with anti-Beclin1 antibody.

Transmission electron microscopy (TEM) analyses

After treatment with the indicated interventions, the preparation of cell sections, staining, and TEM assays were performed according to manufacturer's protocols (Servicebio, Wuhan, China). The cell ultrastructures were observed under TEM (Hitachi, Tokyo, Japan).

Statistical analysis

Data are expressed as mean±SEM. Statistical analyses were performed using SPSS19.0. For comparisons, one-way ANOVA or Student's t-test was performed. Tukey test was used for Post-Hoc Multiple Comparisons of one-way ANOVA. Differences were considered signifcant at a threshold of $P<0.05$.

Results

Lactoferrin promoted the autophagy activity of osteoblasts

We first observed the effect of lactoferrin on the autophagy activity of primary osteoblasts. As shown in Fig.1a-c, lactoferrin increased ALP activity and alizarin red-positive areas of osteoblasts in a concentration-dependent manner, which showed the effectiveness of lactoferrin in this experimental system. It was detected that lactoferrin can enhance the LC3 transformation rate (the ratio of LC3II to LC3I) in the presence or absence of lysosomal protease inhibitor (E64D + Pepstain A). It could be observed that the administration of lysosomal protease inhibitor upregulated the LC3 transformation of osteoblasts, which indicated that autophagy flux is unobstructed and our experimental system is reliable. In addition, lactoferrin increased the number of autophagosomes in osteoblasts. These results supported that lactoferrin could stimulate the autophagy activity of osteoblasts.

Lactoferrin inhibited the BCL2 protein expression in osteoblasts

Then, we examined the regulatory ability of lactoferrin on BCL2 protein expression in osteoblasts. It was observed that under the intervention of different concentrations of lactoferrin, BCL2 protein expression decreased in a concentration-dependent manner, while Beclin1 protein level increased in a concentration-dependent manner. However, BCL2 mRNA expression was not affected by lactoferrin administration. These results suggested that lactoferrin can inhibit BCL2 protein production in osteoblasts. As shown in Fig.2C, the BCL2 protein level of osteoblasts inhibited by lactoferrin is reversed by chloroquine administration, supporting that autophagy plays an important role in the BCL2 protein expression in osteoblasts regulated by lactoferrin. Consistently, it was found that lactoferrin inhibited the

coimmunoprecipitation level of BCL2 and Beclin1 in osteoblasts, indicating the dissociation effect of lactoferrin on BCL2-Beclin1 complex in osteoblasts.

BCL2 overexpression reversed lactoferrin-promoted osteoblast autophagy

We documented that lactoferrin could stimulate the autophagy activity and inhibit BCL2 protein expression in osteoblasts. We further investigated the role of BCL2 in lactoferrin-regulated osteoblast autophagy. First, we upregulated BCL2 expression level in osteoblasts by using Gene-overexpressing technology. The overexpression efficiency of BCL2 was verified via Western Blotting. As shown in Fig.3B, lactoferrin enhanced the LC3 transformation in osteoblasts, which was reversed by BCL2 overexpression. In addition, the osteogenic parameters enhanced by lactoferrin (ALP activity, alizarin red-positive areas and mRNA expression of PCNA, OCN and Col1) were recovered by BCL2 overexpression. These results indicated the important role of BCL2 in lactoferrin-regulated osteoblast formation and autophagy.

Beclin1 inhibition reversed lactoferrin-promoted osteoblast autophagy

We documented that lactoferrin could promote Beclin1 protein expression and dissociate BCL2-Beclin1 complex in osteoblast. We further explored the significance of Beclin1 in lactoferrin-regulated osteoblast autophagy. First, we downregulated Beclin1 expression level in osteoblasts by Gene-silencing technology. The silencing efficiency of Beclin1 was verified via Western Blotting. As shown in Fig.4B, the LC3 transformation in osteoblasts enhanced by lactoferrin was recovered by Beclin1 silencing. In addition, the osteogenic parameters enhanced by lactoferrin (ALP activity, alizarin red-positive areas and mRNA expression of PCNA, OCN and Col1) were reversed by Beclin1 silencing. These results suggested the significance of Beclin1 in lactoferrin-regulated osteoblast formation and autophagy.

Discussion

Lactoferrin is an anti-inflammatory factor derived from milk, which can promote the osteogenesis and subsequent bone remodeling [2–10]. In addition, as an important autophagy regulator, lactoferrin can promote the autophagy activity of multiple cells [15–17]. Autophagy plays an indispensable role in the osteoblast formation [10–14], which provides us with an interesting scientific question, how lactoferrin affect the autophagy of osteoblasts. There is no effective evidence for the above problem. Our study is the first to reveal a scientific discovery: lactoferrin regulates the autophagy and formation of osteoblasts through BCL2-Beclin1 signaling.

First, we confirmed that lactoferrin not only directly promoted the growth of primary osteoblasts but also enhanced the autophagy activity of osteoblasts. Therefore, the direct effect of lactoferrin on osteoblast autophagy was proved for the first time, which acts in osteoblast formation promoted by lactoferrin. In addition, it was found that lactoferrin also downregulated BCL2 protein expression and the coimmunoprecipitation ability of BCL2 and Beclin1. It is well known that BCL2-Beclin1 complex plays a key role in autophagy activation [18–22]. This study is the first to elucidate that lactoferrin can promote Beclin1 into autophagy flux by reducing BCL2 protein level, thus activating autophagy. Remarkably,

lactoferrin had no effect on BCL2 mRNA level. Furthermore, the application of chloroquine, an autophagy inhibitor, reversed the reduced BCL2 protein level by lactoferrin. Chloroquine, as a late autophagy inhibitor, can prevent the fusion of autophagosome and lysosome and the digestion of autolysosome on the substances contained. These results suggest that lactoferrin may promote the autophagic degradation of BCL2 through autophagy activation. Accordingly, there may be a cycle in lactoferrin-regulated osteoblast formation, BCL2-autophagy-BCL2. Obviously, overexpression of BCL2 gene or pharmacological inhibition of Beclin1 can recover the autophagy and formation of osteoblasts enhanced by lactoferrin, further identifying the significance of BCL2-Beclin1 signaling in the autophagy and formation of osteoblasts treated by lactoferrin.

As an effective component of dairy products, lactoferrin is beneficial to bone formation and integrity. Our data elucidate the intrinsic mechanism underlying the direct effect of lactoferrin on osteoblast formation from the perspective of autophagy. The current results not only unmask a novel signal transduction pathway existing in lactoferrin-regulated osteoblast formation: BCL2-Beclin1-autophagy activation signaling, but also provide an important reference for the clinical application of lactoferrin in pathological bone loss.

Declarations

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of interest

None of the authors disclosed potential conflicts of interest.

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Author’s contributions

Dianshan Ke, Yinquan Lin and Shengwang Wei conceived and designed the overall experiments; Dianshan Ke and Shengwang Wei carried out experiments, analyzed data, and prepared figures; Xinwen Wang assisted in experimental preparation and data analysis; Dianshan Ke and Shengwang Wei wrote the manuscript.

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Figures

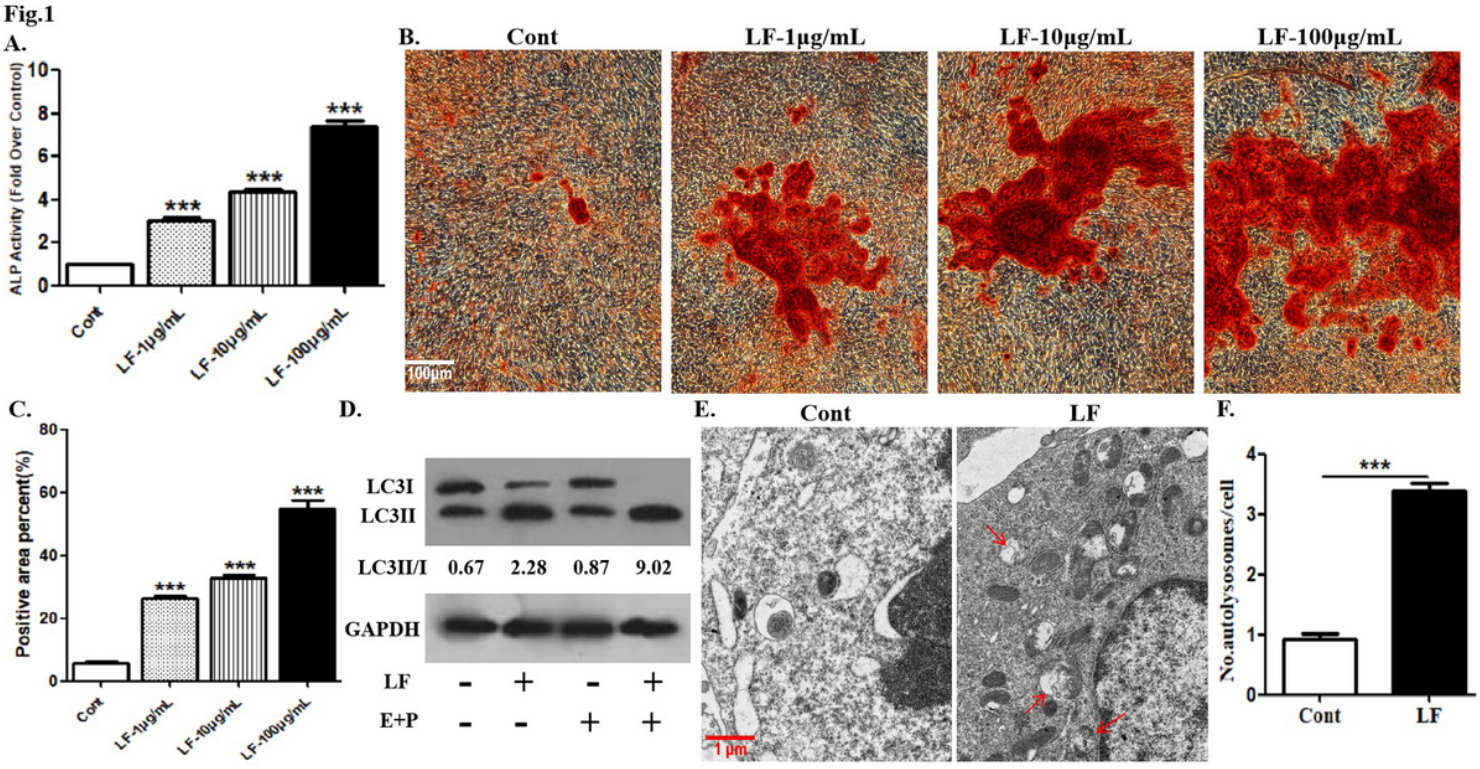

Figure 1

Lactoferrin promotes the autophagy activity of osteoblasts. (A) After treatment with different levels of lactoferrin for 5 days, ALP activity of osteoblasts was measured using a commercial kit. (B-C) After treatment with different levels of lactoferrin for 5 days, mineralized nodules were detected by Alizarin red staining and quantified by ImageJ 1.47 software (5 visible areas per group). Scale bar, 100μm. (D) The ratio of LC3II/I in osteoblasts treated with lactoferrin (100 μg/mL) for 8 hours in the presence or absence

of E64D plus Pepstain A was examined using Western Blotting assays. (E) After treatment with lactoferrin for 12 hours, the autolysosomes (Red arrows) were imaged under TEM. Scale bar, 1 μm. (F) The histogram showing the quantitative results of autolysosomes in (E) (60 cells from three independent experiments). Data are presented as mean±SEM from three independent experiments. ***P<0.001. LF, lactoferrin; E, E64d; P, Pepstantin A; Cont, control group.

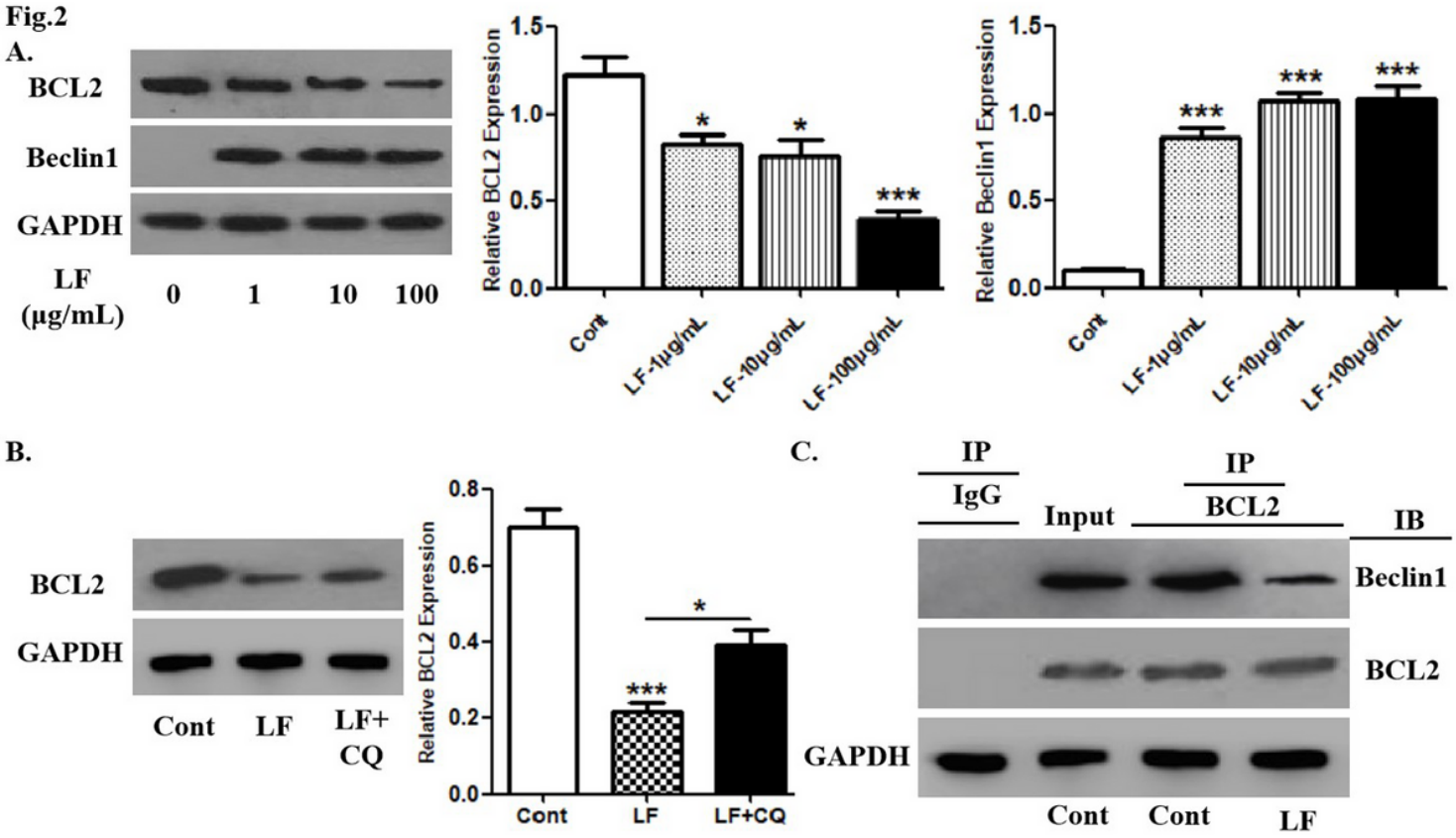

Figure 2

Lactoferrin inhibits the BCL2 protein expression in osteoblasts. (A) After treatment with different levels of lactoferrin for 8 hours, the protein expressions of BCL2 and Beclin1 were examined using Western Blotting assays. (B) After treatment with lactoferrin for 8 hours in the presence or absence of chloroquine (5 μM), BCL2 protein expressions was examined using Western Blotting assays. (C) After treatment with lactoferrin for 8 hours, the lysates were extracted for Co-IP with anti-BCL2 antibody, and subsequently, the precipitates were examined using Western Blotting assays with anti-Beclin1 antibody. Data are presented as mean±SEM from three independent experiments. *P<0.05, ***P<0.001. LF, lactoferrin; CQ, chloroquine; Cont, control group; IP, the antibody for immunoprecipitation; IB, the antibody for immunoblot.

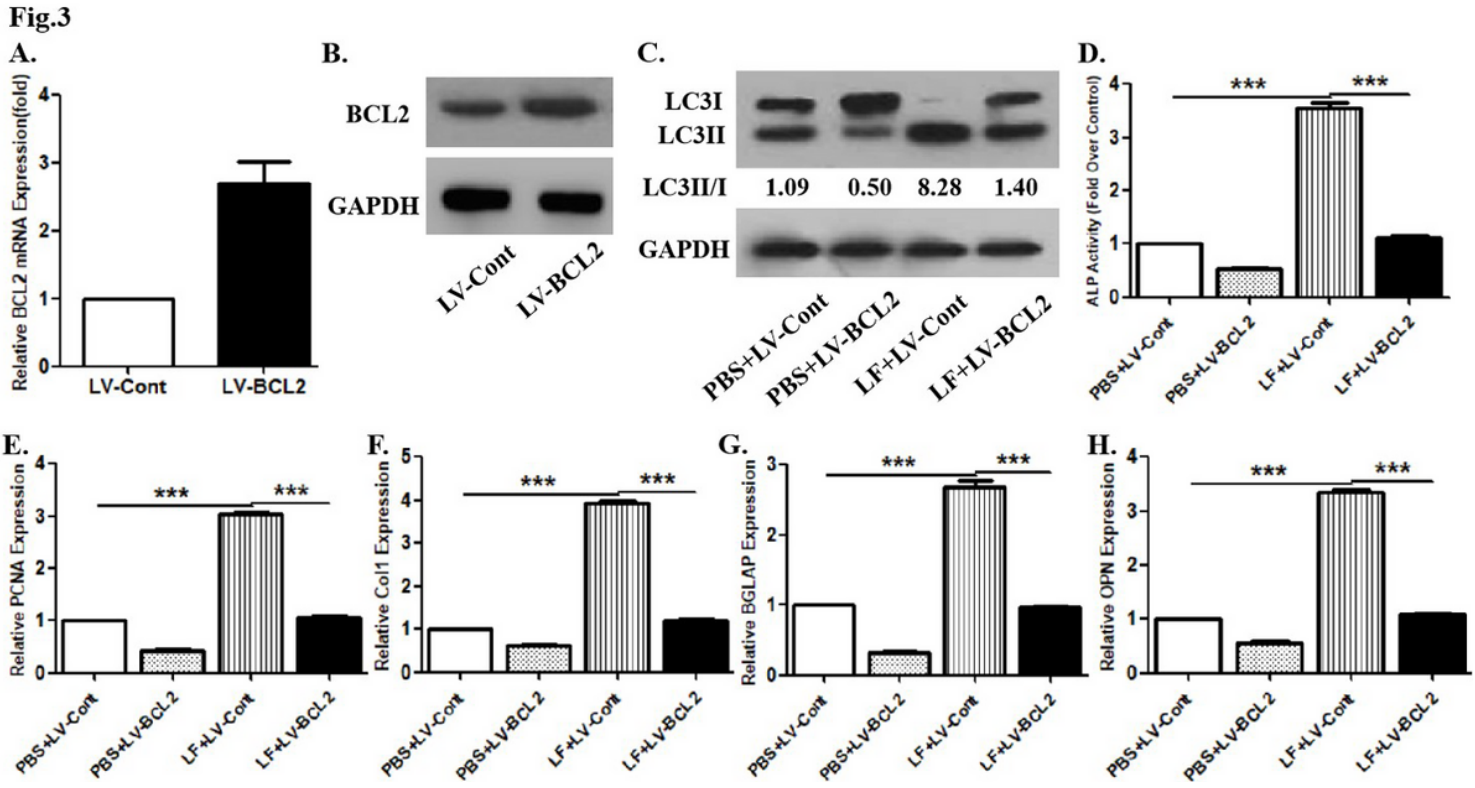

Figure 3

BCL2 overexpression reverses lactoferrin-promoted osteoblast autophagy. (A) mRNA level of BCL2 in osteoblasts infected with lentiviruses encoding BCL2-cDNA (LV-BCL2) or control viruses (LV-Cont) were detected using qRT-PCR assays. (B) The protein level of BCL2 in osteoblasts infected with lentiviruses encoding BCL2-cDNA (LV-BCL2) or control viruses (LV-Cont) were detected using Western Blotting assays. (C) Following lentiviral transduction, the ratio of LC3II/I in osteoblasts treated with lactoferrin for 12 hours was detected using Western Blotting assays. (D) Following lentiviral transduction, ALP activity in osteoblasts treated with the same reagents as (C) for 5 days was measured using a commercial kit. (E-H) Following lentiviral transduction, mRNA levels of PCNA, Col1, BGLAP and OPN in osteoblasts treated as described in (D) were detected using qPCR assays. Data are presented as mean±SEM from three independent experiments. *P<0.05, ***P<0.001. LF, lactoferrin.

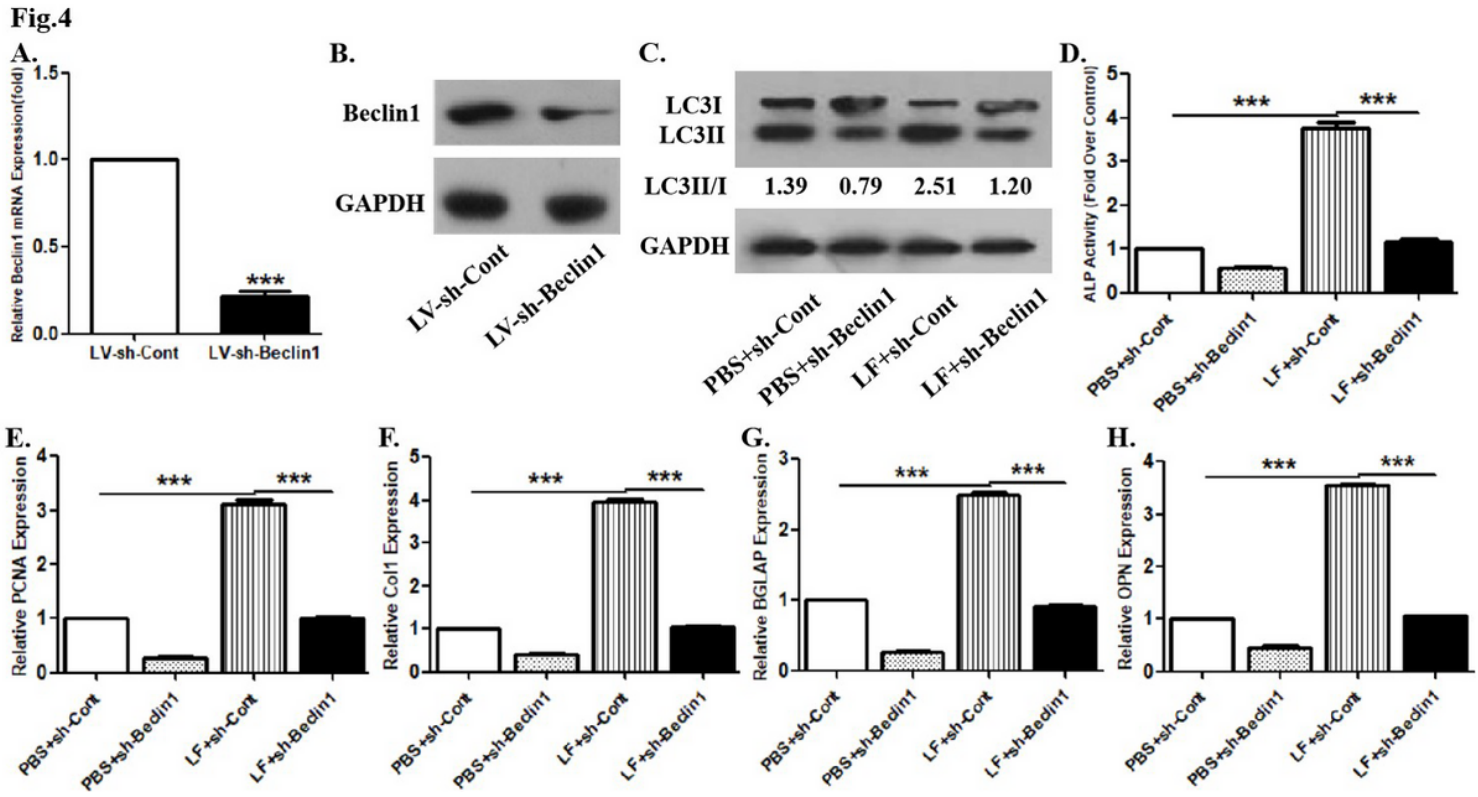

Figure 4

Beclin1 inhibition reverses lactoferrin-promoted osteoblast autophagy. (A) mRNA level of BCL2 in osteoblasts infected with lentiviruses encoding Beclin1-shRNA (LV-sh-Beclin1) or control viruses (LV-sh-Cont) were detected using qRT-PCR assays. (B) The protein level of BCL2 in osteoblasts infected with lentiviruses encoding Beclin1-shRNA (LV-sh-Beclin1) or control viruses (LV-sh-Cont) were detected using Western Blotting assays. (C) Following lentiviral transduction, the ratio of LC3II/I in osteoblasts treated with lactoferrin for 12 hours was detected using Western Blotting assays. (D) Following lentiviral transduction, ALP activity in osteoblasts treated with the same reagents as (C) for 5 days was measured using a commercial kit. (E-H) Following lentiviral transduction, mRNA levels of PCNA, Col1, BGLAP and OPN in osteoblasts treated as described in (D) were detected using qPCR assays. Data are presented as mean±SEM from three independent experiments. *P<0.05, ***P<0.001. LF, lactoferrin.

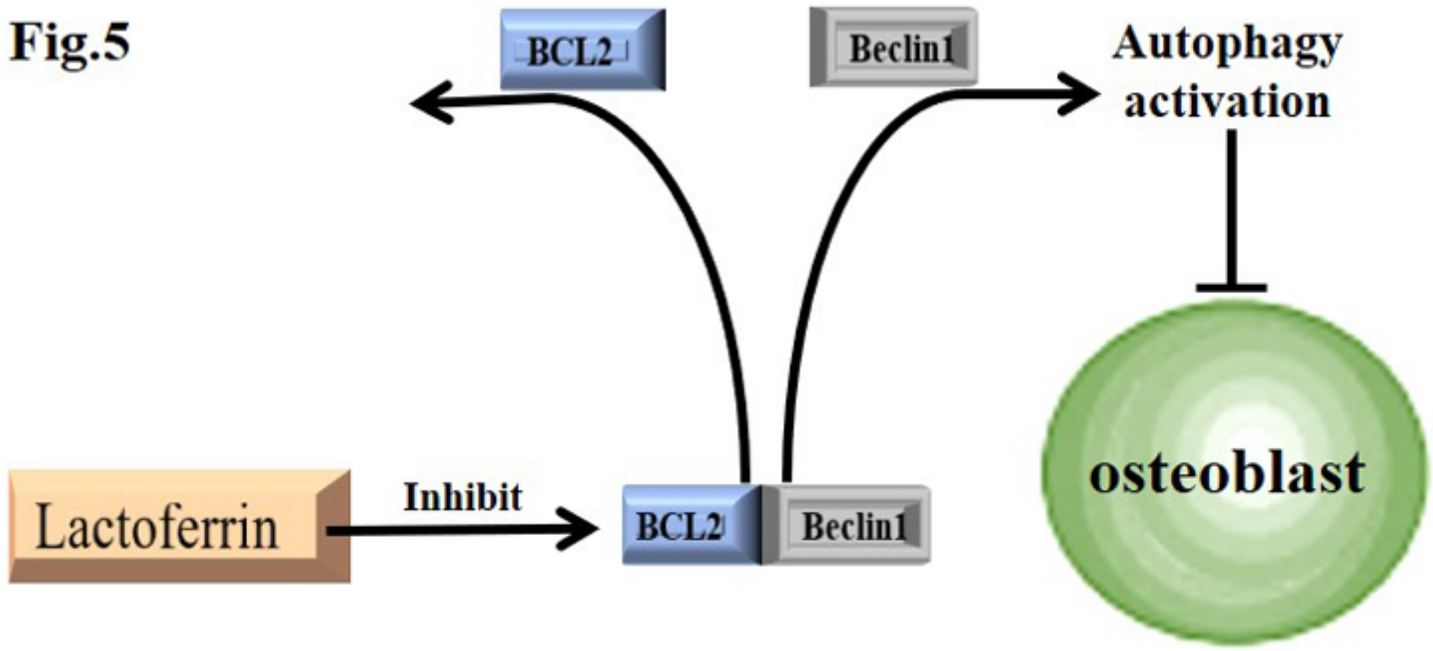

Figure 5

The working model regarding the role of BCL2-Beclin1 signaling in lactoferrin-regulated osteoblast autophagy.