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Lactoferrin ameliorates aging-suppressed osteogenesis via IGF1 signaling

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Abstract

Lactoferrin (LF) is an iron-binding glycoprotein that plays an important role in promoting bone formation and inhibiting bone resorption; however, its effects on senile osteoporosis remain unknown. This study aimed to investigate the effects and mechanism of LF intervention using a senile osteoporosis model (SAMP6 mice) and senescent osteoblasts. Micro-CT and hematoxylin and eosin staining demonstrated that the intragastric administration (2 g/kg/day) of LF could improve the bone mass and microstructure of SAMP6 mice. Furthermore, LF treatment improved bone metabolism and increased insulin-like growth factor 1 (Igf1) mRNA expression and activated phosphorylation status of AKT. Using osteoblasts passaged for ten generations as an in vitro senescence model, various markers associated with osteoblast formation and differentiation, as well as related indices of oxidative stress were analyzed. Our results revealed that after multiple generations, osteoblasts entered senescence, in conjunction with increased oxidative stress damage, reduced bone metabolism and enhanced expression of aging-related markers. While inhibiting oxidative stress, LF improved osteoblast proliferation by promoting the expression of osteogenesis markers, including alkaline phosphatase (ALP) activity, Igf1, bone gla protein (Bglap) and osteoprotegerin/receptor activator of nuclear factor-kB ligand (Opg/Rankl) mRNA and delayed senescence by decreasing the level of p16 and p21 expression. RNAI-mediated downregulation of IGF1 attenuated the effect of LF on osteogenesis. Therefore, the findings of the present study indicate that LF may promote osteogenesis via IGF1 signaling, thereby preventing senile osteoporosis.

Key Words
- lactoferrin
- senile osteoporosis
- SAMP6 mice
- oxidative stress
- IGF1 signaling

Introduction

Senile osteoporosis is a type of primary osteoporosis characterized by a substantial loss of bone mass, micro-architectural deterioration of bone tissue and reduction of bone quality (Zhou et al. 2016). According to data from the World Health Organization (WHO), osteoporosis affects approximately 75 million people throughout Europe, the USA, Japan and China (Eastell et al. 2009). Currently, there is a lack of effective clinical treatment
due to the limited ability of existing drugs to recover bone mass, the presence of various side effects (Ke et al. 2016) or high cost. Therefore, the identification of safer and more effective anti-osteoporosis agents is required.

Lactoferrin (LF) is a non-heme iron-binding glycoprotein that is strongly expressed in human and bovine milk (Mayeur et al. 2016). Under normal physiological conditions, LF is primarily expressed in mucosal epithelial and immune cells during a state of inflammation. Moreover, LF is known for its physiologically pleiotropic properties, such as antioxidant (Kruzel et al. 2002) anti-inflammatory and anti-tumor effects, as well as the regulation of bone metabolism. Indeed, Bharadwaj et al. found that following an oral supplementation of LF, bone formation in postmenopausal women was significantly increased, whereas bone resorption decreased (Bharadwaj et al. 2009). Similar effects of LF have also been found in other reports (Cornish et al. 2004, Guo et al. 2009), particularly for postmenopausal osteoporosis. Our previous studies also confirm these findings, as we found that an intragastric administration (1 g/kg/day) of LF significantly increased the bone mass and improved the bone microstructure of ovariectomized rats (Hou et al. 2012). However, there have been no studies on the effects of LF in senile osteoporosis to date.

The insulin/IGF1 signaling pathway was the first cell signaling mechanism that has been clearly shown to influence aging (Kenyon et al. 2010). IGF1 is a critical polypeptide required for skeletal growth, which promotes bone cell growth and differentiation and cell cycle progression and increases the activity of pre-existing bone cells (Ardawi et al. 2013). It was found that LF inhibited apoptosis and promoted the proliferation and differentiation of primary osteoblasts by increasing the level of Igf1 gene expression, and these effects may be related to the IGF1/PI3K/AKT pathway (Hou et al. 2014, 2015). In addition, aging places the body in a state of oxidative stress (OxS). Several studies have shown that reactive oxygen species (ROS) may be responsible for senile osteoporosis (Manolagas et al. 2010, Almeida et al. 2012, 2013). Nevertheless, antioxidant therapy has been shown to have a bidirectional effect on the disease, excessive antioxidant may have negative effects on bone health (Fujita et al. 2012). Researchers have found that antioxidants (e.g., vitamins C and E and β-carotene) can reduce the risk of senile osteoporosis (Chai et al. 2008). However, the antioxidant effect of LF on IGF1 remains unknown. Thus, we hypothesize that LF may improve OxS damage through an IGF1-related pathway, thereby delaying the process of senile osteoporosis.

In this study, we used SAMP6 mice as an animal model and primary osteoblasts passaged for ten generations as an in vitro model of senile osteoporosis. We aimed to examine changes in bone mass and the ultrastructure of senescent osteoblasts, changes in bone metabolic indicators and OxS levels and the role of the IGF1 signaling pathway on these changes in response to LF.

Materials and methods

Animals, treatments and specimen collection

A total of 15 four-month-old male SAMR1 mice and 30 four-month-old SAMP6 male mice (body weight: 30.0±5.0 g) were obtained from the Laboratory Animal Center of Peking University (Certificate of Conformity: No. SCXK 2011-0001) (Beijing, China). Previous studies have demonstrated that the low bone mass of SAMP6 mice occurs as early as 2 months of age and is maintained at 12 months of age and is primarily caused by low bone acquisition (Chen et al. 2012). Since SAMP6 mice display a small decrease in bone mass from 5 months to 12 months of age, we chose 4-month-old SAMP6 mice as our subjects (Chen et al. 2009). Ethical approval for the use of animals in this study was granted by the Institutional Animal Care and Use Committee of Fujian Medical University (Fuzhou, China), and all experimental procedures were performed strictly in accordance with Legislation Regarding the Use and Care of Laboratory Animals of China. All mice were housed at 22°C (air conditioning) under a 12-h light/dark cycle with free access to distilled water and standard mouse chow. The weight of the animals was recorded weekly during the experimental period. According to our previous study (Hou et al. 2012), the optimal intragastric intervention doses of LF in Sprague–Dawley rats was 1 g/kg/day. Following the Rat Mouse Equivalent Dose Conversion Principle, the equivalent dose of LF used in mice was 2 g/kg/day. After 7 days of acclimatization, 30 SAMP6 male mice were randomly divided into two groups (n=15 per group): (1) SAMP6 with saline group and (2) SAMP6 with 2 g/kg/day LF (bovine LF, purity >90%, New Zealand, purchased from Hubei Widely Chemical Technology CO., Ltd., Wuhan, China). The SAMR1 mice (n=15) treated with saline were used as a control group. At the end of 8 weeks of treatment, all mice were killed via an intraperitoneal injection of 3% pentobarbital sodium (100 mg/kg). Sera, femora and humeri were harvested and stored at −80°C for analysis.
Micro-CT imaging

The left femur of 18 mice (six per group) was selected to evaluate the trabecular microarchitecture of the distal femoral metaphysis by micro-computed tomography (Micro-CT, Viva CT 40, Scanco Medical, Switzerland). Scanning was performed from the distal femur to the distal femoral metaphysis (15 µm/slice) for every selected femur sample. A total of 350 images were obtained from this region of each femur. The volume of interest (VOI) was selected as a cross-sectional area spanning 50–100 slices from the distal femur. This scanning generated 3D images of the microarchitecture that were examined and displayed. Bone morphometric parameters obtained from the micro-CT, including bone mineral density (BMD), bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp) and trabecular thickness (Tb.Th), were analyzed for the VOI.

Histological analysis

Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) were used to evaluate the extent of osteoporosis and involvement of related key signaling pathways. The left femora were obtained and fixed using formalin (10%) for 72 h. The femora were then decalcified in EDTA (10%) for 4 weeks and embedded in paraffin. The samples were cut into serial 4 µm thick longitudinal sections, stained with H&E and used for histopathological examination. The percent trabecular area (%) was analyzed by Image Pro Plus Software 6.0 (Media Cybernetics, CA, USA), as previously described (Parfitt et al. 1987). For the IHC analysis, the sections were dewaxed and dehydrated. Following antigen retrieval in citrate buffer, the sections were blocked overnight at 4°C. The sections were then probed with rabbit anti-p-AKT (anti-phosphorylation-AKT) antibody (1:400, #4060, Cell Signaling Technology) and mouse anti-4-hydroxynonenal (4-HNE) antibody (1:500, ab 48506, Abcam). All of the above assays were performed by Wuhan Goodbio Technology Co., Ltd. (Wuhan, China) according to a standard protocol. The results were observed in an inverted microscope (Olympus), and the images were captured using Cell Sens software (Olympus). Image Pro Plus Software 6.0 was used to semi-quantitatively measure the concentration of p-AKT and 4-HNE (Luo et al. 2017).

Biochemical analysis

The serum ALP concentration was measured using a commercial kit (Shanghai Genmed Scientifics Pharmaceutical Technology Co., Ltd.) with standard colorimetric methods. The concentration was measured on an automatic biochemical analyzer (Unicel Dxc 800, Beckman Coulter, Inc.). The serum lactoferrin concentration was measured using a mouse lactoferrin ELISA kit (Shanghai Enzyme-linked Biotechnology Co., Ltd, Shanghai, China) following the manufacturer’s protocol. The experiment was repeated three times.

Cell culture

Primary osteoblasts were isolated from the calvaria of eight newborn (<48 h) Sprague-Dawley male rats (Fujian Medical University Laboratory Animal Center, Certificate of Conformity: No. SCXK 2016-0002, Fuzhou, China). Briefly, neonatal rat calvariae were dissected from the adherent soft tissue and periosteum and washed in PBS (Hyclone, 2 mL) until the calvariae turned white. The tissue was cut into pieces approximately 1 mm³ and then sequentially digested with 3 mL 0.25% trypsin-EDTA (Genview, Houston, TX, USA) for 15 min, followed by 4 mL 0.1% type I collagenase (Gibco BRL) mixed with 1 mL 0.25% trypsin-EDTA for 60 min to release the cells. The cell culture medium consisted of Dulbecco’s minimum essential medium (DMEM, Hyclone) containing 10% FBS (Gibco BRL), l-glutamine (Beyotime Biotechnology) and antibiotics (Penicillin/streptomycin, Genview, Houston, TX, USA). The cells were seeded at a density of 3 × 10⁴/cm² in a 25 cm² T flask and cultured overnight in an incubator with 5% CO₂ at 37°C. During the initial seeding and several passages, ‘differential attachment’ was used to achieve purification of the osteoblasts. In the third generation, the purification rate of the osteoblasts was 95%. The cells were subcultured every 3–4 days in fresh medium after release with a 0.25% trypsin-EDTA solution. The medium was changed every other day. When the cells reached the tenth passage, they were used for experiments.

Transmission electron microscope

Cells at passage 3, passage 10 and passage 10 + 100 µg/mL LF (purity >95%, Woka, Japan) were initiated at a seeding density of 3 × 10⁴/cm² in a 25 cm² flask for 5 days. After washing with PBS, the cells were scraped with a cell scraper and centrifuged at 1000 rpm (~175 g) for 10 min. The ultrastructure of the samples fixed in 2.5% glutaraldehyde was observed by transmission electron microscopy (Philips, Amsterdam, Netherlands). The number of lysosomes (N.Lysosomes) per square micron were evaluated in a blinded fashion (n = 9 independent samples/group).
Senescence-associated-β-galactosidase (SA-β-gal) staining

β-Galactosidase staining was performed using a senescence-associated β-Galactosidase Staining Kit (Beyotime, China) 24 h after treatment with 100 μg/mL LF. The cells were washed three times with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were then stained with 0.05 mg/mL X-gal solution overnight at 37°C in the dark and without carbon dioxide. The population of SA-β-gal-positive cells was determined by counting 400 cells per dish and photographs were taken using a phase-contrast microscope at 100× magnification (Olympus). The proportion of cells that were positive for SA-β-gal activity were given as the percentage of the total number of cells counted in each dish (n=9 independent samples/group).

Lentiviral transduction

Based on our previous findings (Hou et al. 2014), we used the shRNA sequence to knock down the Igf1 gene (Table 1). The shRNA sequence was synthesized by Hanbio Biotechnology Co., Ltd. (Shanghai, China). The shRNA vectors were co-transduced into HEK293T cells with the lentiviral packaging plasmids, and the recombinant lentiviral particles were used to infect osteoblasts at passage 10. The cells were transduced with the lentiviral vectors for 48 h.

Combined shRNA and LF treatment

The osteoblasts were divided into the following groups: (1) P3, the control group (passage 3 osteoblast group); (2) P10, the senescence group (passage 10 osteoblasts group); 3) the LF group (passage 10 osteoblasts with 100 μg/mL LF treatment); (4) an LF+shIGF1 group (passage 10 osteoblasts transduced with shIGF1 and treated with 100 μg/mL LF) and (5) an LF+NT group (passage 10 osteoblasts with negative transduction with a scrambled sequence and 100 μg/mL LF treatment). The cells were transduced with the lentiviral vectors for 48 h and the culture medium was replaced with medium containing 100 μg/mL LF.

Table 1 Igf1 RNA interference sequence.

<table>
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<th>Gene</th>
<th>Target sequence</th>
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<tr>
<td>Igf1</td>
<td>GATCCGCACCTCTGCTTGCACCTTTCAAGAGAAGG</td>
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Determining the effect of lentiviral transduction on cellular proliferation

The cells obtained from the transduction experiment were inoculated in a 96-well plate at a density of 3×10^4 cells/well. Each group contained five repeats. After 3 days of 100 μg/mL LF intervention, 20 μL MTT (5 mg/mL, Sigma) was added to the cells and cultured for 4 h. After the incubation, 100 μL of dimethyl sulfoxide (DMSO, Sigma) was added to each well, and the plate was oscillated for 10 min until the crystals had fully dissolved. The optical density (OD) at a wave length of 490 nm was determined using a microplate reader (Bio Tek, Vermont, USA) and a blank control was introduced. The experiment was repeated at least three times.

Determination of the effect of lentiviral transduction on ALP activity

Five groups of cells were inoculated in a 96-well plate at a density of 5×10^4 cells/well. Each group had five repeats. After culturing with 100 μg/mL LF treatment for five days, 100 μL 0.1% Triton-X100 (Beijing dingguo changsheng Biotech, Beijing, China) was added to the cells and the plate was oscillated for 30 min until the cells were lysed. The ALP activity was measured using an Alkaline Phosphatase Assay Kit (Beyotime Biotechnology, Jiangsu, China) according to the manufacturer's protocol. The OD at a wave length of 405 nm was determined using a microplate reader, and a blank control was introduced. The experiment was repeated at least three times.

Measurement of superoxide dismutase (SOD) and malondialdehyde (MDA)

OxS refers to the imbalance of oxidative and anti-oxidative effects in the body and the propensity for OS. SOD is the primary free radical scavenger in an organism. Its activity is closely related to the degree of antioxidant stress in the body. The level of MDA can indicate the degree of lipid peroxidation in the body and indirectly reflects the severity of the cellular damage caused by free radicals. Thus, the anti-oxidative marker, SOD, and the oxidative injury markers, MDA, were evaluated. The level of cellular SOD activity and MDA content were measured using corresponding assay kits (Nanjing Jiancheng Biotechnology Co., Ltd. Shanghao, China) 24 h after treatment with 100 μg/mL LF treatment for five days, 20 μL X-gal solution (Beyotime, China) was added to the cells and cultured for 4 h. After the incubation, 100 μL of X-gal solution (Beyotime, China) was added to each well, and the plate was oscillated for 10 min until the crystals had fully dissolved. The optical density (OD) at a wave length of 490 nm was determined using a microplate reader (Bio Tek, Vermont, USA) and a blank control was introduced. The experiment was repeated at least three times.
Bioengineering Institute) according to the manufacturer’s protocols.

**qRT-PCR measurements of mRNA expression**

The total RNA of the femur was isolated using an EASYspin Plus Bone Tissue RNA Kit (RNS4, Aidlab Biotechnologies Co., Ltd., Beijing, China) according to the manufacturer’s protocol. The total RNA of the osteoblasts was extracted using an RNA prep pure Cell/Bacteria Kit (DP430, Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer’s protocol. The concentration and purity of the total RNA was calculated by measuring the absorbance at 260 nm and 280 nm. Total RNA (1 μg) was used for the synthesis of the first strand of cDNA (cDNA synthesis kit, DRR037A TaKaRa, Japan). PCR primers (Tables 2 and 3) were designed and synthesized by TaKaRa. Real-time PCR was performed using 2 μL of cDNA in a 25 μL reaction volume that included the SYBR PremixEx Taq II (2×) (DRR820A, TaKaRa, Japan) and diluted gene-specific primers on a Thermal Cycler Dice™ Real-Time System (Light Cycler, Roche). Melt curves were performed after primer sequences for qRT-PCR to verify a single transcript. All reactions were performed in triplicate and analyzed using the 2^{-ΔΔCT} method. Gapdh was used as an internal control for the in vivo experiments and β-actin for the in vitro experiments. The experiment was repeated at least three times.

**Western blot analysis**

Cells obtained from the transduction experiment were cultured with LF for 2 days. The total cellular protein was extracted using RIPA buffer (Beyotime Biotechnology, Jiangsu, China) and quantified by a BCA protein assay kit (Beyotime Biotechnology, Jiangsu, China). A total of 30 μg protein was loaded and electrophoresed separately through an 8–15% SDS-PAGE gel. The separated proteins were subsequently transferred to a PVDF membrane and incubated with primary antibody at 4°C overnight. The following primary polyclonal antibodies were used: mouse anti-IGF1 (1:1000, ab36532, Abcam), rabbit anti-p-PI3K (phosphorylated-PI3K,1:1000, #4228, Cell Signaling Technology), PI3K (1:1000 #4292, CST), p-AKT (phosphorylated-AKT, 1:1000, #4060, CST), AKT (1:1000, #9272, CST) and GAPDH (1:1000, #5174, CST). After washing, the membrane was incubated with the secondary antibody (BA1054/BA1050, Boster Biological Technology Co., Ltd., Wuhan, China) at room temperature for 1 h. The signals were developed and immunoreactive bands were visualized using an ECL kit (SAB, Maryland, USA) according to the manufacturer’s instructions and were quantified using a Chemi-Doc image analyzer (Bio-Rad). The experiment was repeated at least three times.

**Statistical analysis**

The results are reported as the means ± s.d. Data analyses were performed using the Statistical Package for the Social Sciences (SPSS version 20.0) software package. The differences between the groups were determined using a one-way ANOVA model, followed by an LSD (least significant difference) post-test. Differences were considered significant at a threshold of P<0.05.

### Table 2 Primer sequences for qRT-PCR in vivo.

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<th>Primer sequences</th>
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<td>Forward</td>
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<td>Bglap</td>
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<td>Opg</td>
<td>NM 008764.3</td>
<td>5′-TTCCCTGGAGAAGGTAATGCTG-3′</td>
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<td>Rankl</td>
<td>NM 011611.3</td>
<td>5′-CGACGATGGCTCTGTCTGTA-3′</td>
</tr>
<tr>
<td>Tgf1</td>
<td>NM 00111274.1</td>
<td>5′-TCCTCGCCTTCTGCTTGA-3′</td>
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<tr>
<td>Gapdh</td>
<td>NM 008084.2</td>
<td>5′-AAATGTGAAGGCCCTGACT-3′</td>
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### Table 3 Primer sequences for qRT-PCR in vitro.

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<td>Bglap</td>
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<td>NM 057149.1</td>
<td>5′-CGACGATGGCTCTGTCTGTA-3′</td>
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<td>Tgf1</td>
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<td>5′-TCCTCGCCTTCTGCTTGA-3′</td>
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<td>P16</td>
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<td>P21</td>
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<td>β-actin</td>
<td>NM 031144.2</td>
<td>5′-AAATGTGAAGGCCCTGACT-3′</td>
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</table>
Results

LF increases bone mass and ameliorates osteoporosis in SAMP6 mice

Micro-CT 3D imaging demonstrated that in the SAMP6 group, the bone microstructure of the femur was severely impaired sparse bone trabeculae when compared to the SAMR1 group (Fig. 1A). Moreover, the SAMP6 group displayed a significant decrease in the BMD, BV/TV, Tb.N and Tb.Th (Fig. 1B, C, D and F), but Tb.Sp (Fig. 1E) was significantly higher; however, treatment with 2 g/kg/day LF alleviated the negative structural properties of the trabecular bone (Fig. 1A), including BMD, BV/TV and Tb.Th (Fig. 1B, C and F); however, there were no changes in the trabecular number or separation.

In addition to the observed changes in the trabecular regions of the femur, the histomorphology was examined using H&E staining (Fig. 1H). The trabecular area percentage was substantially reduced in the SAMP6 group compared to the SAMR1 group and the percentage of trabecular area was increased to 98% after LF treatment (Fig. 1G). These results illustrate that LF attenuates the impairment in bone mass and the bone trabecular microstructure of SAMP6 mice.

LF promotes bone differentiation and increases the Opg/Rankl ratio and the expression of other bone metabolism-related genes

The results showed that the levels of serum LF and serum ALP in the SAMP6 group were significantly lower than those of the SAMR1 group (Fig. 2A and B). Following LF intervention, both groups displayed an increasing trend.

To evaluate the level of metabolism-related factor gene expression in vivo, we analyzed mouse femora for the expression of Bglap, Opg and Rankl mRNA. The Opg/Rankl ratio is a marker for the formation and absorption of bone tissue. We found that Bglap, Opg and Rankl mRNA levels were decreased in the SAMP6 group compared with the SAMR1 group. LF intervention significantly induced Bglap mRNA expression and the ratio of Opg/Rankl (Fig. 2C, D, E and F). These data indicate that the effects of senile osteoporosis on bone metabolism-related gene expression could be reversed by LF.

LF activates the IGF1/PI3K/Akt signaling pathway and alleviates OxS in SAMP6 mice

As mentioned above, LF plays a role in the IGF1/PI3K/Akt pathway in primary osteoblasts. Thus, the effects of LF on the IGF1/PI3K/Akt osteogenic signaling pathway were also assessed. The levels of Igf1 mRNA and p-AKT in the SAMP6 group were substantially decreased compared with the SAMR1 group (Fig. 3A, B and D); however, LF substantially increased the levels of Igf1 mRNA and p-AKT. These results indicate that LF stimulated Igf1-related signaling in the bones of SAMP6 mice.

4-HNE is found throughout animal tissues, and in higher quantities during OxS due to the increase in the lipid peroxidation chain reaction, due to the increase in stress events. The levels of 4-HNE were increased by 70% in the SAMP6 group compared to the SAMR1 group. LF decreased 4-HNE levels, suggesting that LF alleviates OxS in SAMP6 mice.

LF improves the subcellular structure and reduces the SA-β-gal activity of senescent osteoblasts

Passage 3 osteoblasts displayed a plump cytoplasm with abundant organelles, a well-developed Golgi complex and obvious dilation of the rough endoplasmic reticulum with a large amount of ribosomes (Fig. 4A). However, passage 10 cells became elongated with more secondary lysosomes in the cytoplasm with additional heterochromatin in the nucleus (Fig. 4B). The cells treated with 100μg/mL LF exhibited a plump cytoplasm with abundant organelles, expanded rough endoplasmic reticulum and fewer secondary lysosomes (Fig. 4C). The number of lysosomes was semi-quantitatively counted under the electron microscope, and the number of lysosomes in the passage 10 group was significantly increased, approximately four times greater than that in the passage 3 group. After treatment with lactoferrin, the number of lysosomes decreased to 1/3, compared to the passage 10 group. These results suggest that following LF treatment, the number of age-associated organelles, lysosomes, decreased to some extent.

Detectable β-gal activity at pH 6 was found to be increased during the replicative senescence of fibroblast cultures in vitro and was absent in immortal cell cultures. This activity was termed senescence-associated β-galactosidase or SA-β-gal activity (Dimri et al. 1995). Our study revealed strong SA-β-gal activity in the passage 10 group, which indicated that the cells had entered a senescence-like state through continuous passaging. LF was found to effectively suppress the SA-β-gal activity (Fig. 4E, F, G and H).
Figure 1
Effects of 2 g/kg/day lactoferrin treatment on the parameters of bone microarchitecture and H&E staining in SAMP6 mice. (A) 3D reconstructions of the femoral metaphyseal trabecular structure as determined by micro-CT. (B, C, D, E and F) Analysis of bone mineral density (BMD), bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular separation/spacing (Tb.Sp) and trabecular thickness (Tb.Th) in mice in the SAMR1, SAMP6 and SAMP6 + LF groups. (G) Bone histomorphometric analysis of the percent trabecular area (%) (n = 6 independent samples/group). **P < 0.01; ***P < 0.001 vs the SAMR1 group; #P < 0.05; ##P < 0.01 vs the SAMP6 group. (H) Hematoxylin and eosin (H&E) staining for the analysis of bone structure in mice in the SAMR1, SAMP6 and SAMP6 + LF groups (magnification: 200×). TB, trabecular.
LF promoted proliferation and differentiation and enhanced the expression of markers of bone formation through \( Igf1 \) in senescent osteoblasts

MTT staining revealed that a 100\( \mu \)g/mL concentration of LF significantly promoted proliferation compared to the P10 senescent group (Fig. 5A). ALP activity represents the degree of osteoblast differentiation. Similar to cellular proliferation, the level of ALP activity showed a consistent trend (Fig. 5B). In addition, LF treatment significantly increased \( Igf1 \) and \( Bpg \) mRNA expression and the \( Opg/Rankl \) ratio, compared with the senescent group (Fig. 5C, D, E and F). Following lentiviral transduction, sh\( IGF1 \) silencing significantly suppressed the LF-induced proliferation, ALP activity, \( Bglap \) mRNA expression and the \( Opg/Rankl \) ratio. No significant difference in the above indicators was detected between the LF + sh\( IGF1 \) group and the LF + NS group. These results indicate that LF improves senescent osteoblast proliferation, ALP activity and the \( Opg/Rankl \) ratio through induction of \( Igf1 \).

LF alleviates aging-related gene expression, improves antioxidant enzyme activity and reduces oxidative injury in vitro through \( Igf1 \)

The expression of \( p16 \) and \( p21 \) mRNA were increased in the senescent group, but was decreased following LF treatment. Due to the instability of ROS, the anti-oxidative marker, SOD, and the oxidative injury marker, MDA,
were evaluated in senescent osteoblasts. These results demonstrated that extensive passaging in osteoblasts led to the inhibition of SOD and an obvious enhancement of MDA; however, these effects were significantly reversed by LF (Fig. 6A, B, C and D).

When Igf1 was knocked down, the effects of LF to decrease p21 mRNA expression and SOD activity were reversed. The level of MDA accumulation was reduced following lentiviral transduction with Igf1 shRNA. P16 mRNA expression remained the same after both lentiviral
study, we further confirmed that LF can ameliorate age-related osteoporosis in SAMP6 mice. In addition, in senescent osteoblasts, LF increased cellular proliferation, ALP activity and the Opg/Rankl ratio and reduced OxS damage, all of which may be related to the activation of IGF1/PI3K/AKT-related pathways.

In the present study, we selected SAMP6 mice and senescent osteoblasts as a model to study senile osteoporosis. SAMP6 mice are a sub-strain of senescence-accelerated mouse model that was developed as an animal model for senile osteoporosis (Chen et al. 2004). Chen et al. found that BMD, femoral weight, serum calcium and phosphorus levels were significantly reduced in SAMP6 at 2 and 5 months of age compared with SAMR1 mice (Supplementary Fig. 2, see section on supplementary data given at the end of this article). The number of osteoblasts present in trabecular bone was also significantly reduced. Additionally, the microstructural properties of bone in SAMP6 mice were remarkably heterogeneous throughout the skeleton, which is analogous to changes that occur in human bones (Chen et al. 2009). According to Niimi’s finding (Niimi et al. 2009), unlike SAMR1 mice, SAMP6 mice are obese and exhibit hyperlipidemia. A common pathogenic factor may underlie the osteoporosis and hyperlipidemia of SAMP6 mice. What’s more, Faridvand et al. (2019) found that bLF was effective against OxS by its ability to reduce the lipid peroxidation in high-cholesterol-fed rats. There is an effect of LF on these metabolic abnormalities could have had a secondary effect on the bone phenotype of the SAMP6 mice, but further research is needed.

In cellular models, after continuous passaging, osteoblasts were closer in both form and function to senescent physiological conditions, which is unmatched by any other method (Kveiborg et al. 2001, Yu et al. 2002, Peterson et al. 2004). In addition, we found that passage 10 cells were identified as osteoblasts (Supplementary Fig. 1) and were larger than passage 3 cells, displaying an increased number of secondary lysosomes in the cytoplasm by electron microscopy and higher SA-β-gal expression (Fig. 4). These findings were consistent with the study by Kassem et al. in serially passaged long-term cultures of human trabecular osteoblasts (Kassem et al. 1997).

This is the first study to measure the level of LF in the serum of different groups of mice in vivo. The results of this study illustrate that the expression of LF in SAMP6 mice is downregulated compared with that of the SAMR1 group. LF also can increase the levels of serum Ca, Fe and ALP activity in SAMP6 mice, whereas the level of serum P was

**LF stimulates the IGF1/PI3K/AKT signaling pathway in senescent osteoblasts**

To further elucidate how IGF1 functions in the mechanism of LF, we explored its direct interaction in the IGF1/PI3K/AKT signaling pathway. Western blot analysis indicated that the senescent group exhibited low levels of IGF1/PI3K/AKT activation. LF intervention at a concentration of 100 μg/mL significantly induced both p-PI3K and p-AKT expression. Moreover, LF-treated shIGF1-transduced cells exhibited significantly decreased levels of p-PI3K and p-AKT (Fig. 7).

**Discussion**

Previous studies have found that LF improves the bone mass of ovariectomized rats and stimulates osteoblast formation and differentiation through the IGF1/PI3K/AKT and PKA and p38 pathways (Zhang et al. 2014). However, the molecular mechanism by which LF alleviates senile osteoporosis remains poorly understood. In the present

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**Figure 6**

Effects of lactoferrin on aging-associated gene expression, antioxidant enzyme activity and oxidative injury in vitro. (A and B) Analysis of p16 and p21 mRNA expression following shIGF1 transduction. (C and D) Analysis of SOD activity and the levels of MDA following shIGF1 transduction. ***P < 0.001 vs the P10 group; **P < 0.01, ***P < 0.001 vs the LF group. NT, negative transduction with a scrambled sequence.

and negative transduction. No significant difference in the above indicators was detected between the LF+shIGF1 and the LF+NS group. These results indicate that LF decreased p21 mRNA expression and led to a substantial inhibition of MDA and an obvious enhancement of SOD; however, these LF-mediated effects were significantly reversed by the knockdown of IGF1.
decreased. These findings indicate that LF can balance the levels of calcium and phosphorus in vivo (Supplementary Fig. 2).

There is a close relationship between senile osteoporosis and OxS. OxS modulates the osteoblastic differentiation of bone cells and ROS can increase osteoblast apoptosis and inhibit bone formation. Elderly male patients with osteoporosis have a significant increase in MDA content (Yalin et al. 2005) and a lower level of SOD compared with that of healthy individuals (Zhang et al. 2010). To probe the potential mechanisms of the antioxidant effects of LF in bone formation, the levels of SOD and MDA, which are closely related to OxS injury was examined in vitro. Our results indicate that LF suppressed oxidative injury thereby reducing the accumulation of MDA and improved SOD activity in senescent osteoblasts. These effects changed following the downregulation of Igf1. It is widely known that the polypeptide protein, IGF1, can bind to the IGFR1, resulting in the phosphorylation of insulin receptor substrate 1 (IRS-1) and activation of AKT, thereby causing osteoblast differentiation, mineralization and proliferation in vitro (Zhang et al. 2012, Castilla-Cortázar et al. 2015). However, in our previous research (Hou et al. 2015), we had used shRNA to knock down the IGFR1 receptor expression. Lactoferrin-treated shIGF1-IR cells displayed a significantly higher level of p-PI3K and p-RAS compared with shIGF-1R. According to this phenomenon, we hypothesized that LF may promote the expression of IGFR1 and directly activate the downstream PI3K/AKT pathway through an IGF-1R-independent mechanism. In addition, IGFR1 activates PI3K/AKT, which in turn stimulates NF-xB signaling via the IKK complex (Tilstra et al. 2011). Given the role of NF-xB in the stress response, as well as cellular survival and proliferation, NF-xB may be a link between IGF1 signaling and OxS. Similar effects have also been found in Parkinson’s disease, for which Ayadi et al. found that IGF1 protects dopaminergic neurons against OxS (Ayadi et al. 2016). Therefore, the findings of our study validate the feasibility of the antioxidant therapeutic approach of LF in senile osteoporosis.

With respect to cellular senescence, we chose to investigate the effect of LF on the mRNA levels of p16 and p21. In particular, the expression of p16 in mouse osteocytes increases markedly after approximately 18 months of age in both sexes (Farr et al. 2017). In addition, alterations in p16 and p21 expression have been reported to be involved in various forms of OxS (Yan et al. 2011). The results of the expression of aging-related genes in the present study revealed that LF decreased the mRNA expression of both p16 and p21. However, the expression of p16 mRNA remained unchanged following lentivirus transduction, which indicates that p16 mRNA levels were independent of IGF-1. Moreover, we found that following RNA interference, p21 mRNA expression increased, indicating that this effect may be related to Igf1. In contrast to our findings, Lee et al. found that LF facilitates the phosphorylation of p21 by activating the PI3K/AKT pathway and upregulates the expression of p21 protein in cervical cancer and breast cancer so as to achieve the anti-tumor growth effect of cell cycle stagnation (Lee et al. 2009). We hypothesize that this difference may be due to the slow proliferation of senescent osteoblasts compared with tumor cells. Following LF treatment, p21

Figure 7
Effects of lactoferrin treatment on IGF1 downstream signaling in aging osteoblasts. (A, B and C) Analysis of the level of IGF1, p-PI3K, p-AKT expression following shIGF1 transduction. **P < 0.01; ***P < 0.001 vs the P10 group; ###P < 0.001 vs the LF group. NT, negative transduction with a scrambled sequence.
was downregulated, active cell division returned and the proliferative activity of the senile osteoblasts improved, which may be related to Igf1.

Fan et al. found that LF suppresses the RANKL/OPG ratio in ovariecomized mice, which is similar to our findings. However, Fan et al. focused on the effect of LF in the context of osteoimmunology. They also examined the effect of LF on IFN-γ, IL-5 and IL-10 levels (Fan et al. 2018). The relationship between LF and these immune indicators in bone in senile osteoporosis remains to be further tested. It has also been found that OxS can stimulate the production of a large amount of advanced oxidation protein products (AOPP) in an osteoblast-like progenitor cell line, which can inhibit the proliferation and differentiation of osteoblast-like cells (Zhong et al. 2009). Since the level of AOPP is closely correlated with bone destruction, the body may stimulate the production and release of inflammatory factors through OxS, and the effects on osteoblasts and osteoclasts may disrupt the balance between bone repair and bone destruction, leading to the occurrence and progression of osteoporosis (Wang et al. 2015). However, the effects of AOPP levels in the mechanism of LF’s effects on senile osteoporosis remain unexplored.

In conclusion, in vivo treatment of LF was found to improve the bone mass and microarchitecture, increase ALP activity and the expression of Igf1, Bglap and the Opg/Rankl ratio, as well as the levels of p-AKT protein and reduce the expression of 4-HNE in SAMP6 mice. In line with the in vivo findings, in vitro treatment of LF induced similar changes in bone metabolism-related genes. Moreover, lentiviral shRNA transduction to decrease the expression of Igf1 suggested that LF works through Igf1 and activation of the PI3K/AKT signaling pathway to improve bone. Such results indicate that LF can alleviate age-related osteoporosis and inhibits osteoblast senescence. In addition, LF was found to downregulate the expression of genes related to aging, increase antioxidant enzyme activity and reduce oxidative injury, all of which may be related to Igf1. The present study suggests that LF treatment may represent a promising therapeutic strategy for the prevention of senile osteoporosis and could provide underlying benefits for such patients.

Supplementary data
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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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