Interleukin-6 gene knockout antagonizes high-fat-induced trabecular bone loss

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Abstract

The purpose of the study was to determine the roles of interleukin-6 (IL6) in fat and bone communication. Male wild-type (WT) mice and *IL6* knockout (*IL6*^{−/−}) mice were fed with either regular diet (RD) or high-fat diet (HFD) for 12 weeks. Bone mass and bone microstructure were evaluated by micro-computed tomography. Gene expression related to lipid and bone metabolisms was assayed with real-time quantitative polymerase chain reaction. Bone marrow cells from both genotypes were induced to differentiate into osteoblasts or osteoclasts, and treated with palmitic acid (PA). HFD increased the body weight and fat pad weight, and impaired lipid metabolism in both WT and *IL6*^{−/−} mice. The dysregulation of lipid metabolism was more serious in *IL6*^{−/−} mice. Trabecular bone volume fraction, trabecular bone number and trabecular bone thickness were significantly downregulated in WT mice after HFD than those in the RD ($P < 0.05$).

However, these bone microstructural parameters were increased by 53%, 34% and 40%, respectively, in *IL6*^{−/−} mice than those in WT mice on the HFD ($P < 0.05$). *IL6*^{−/−} osteoblasts displayed higher alkaline phosphatase (ALP) activity and higher mRNA levels of Runx2 and Col1a1 than those in WT osteoblasts both in the control and PA treatment group ($P < 0.05$). *IL6*^{−/−} mice showed significantly lower mRNA levels of *PPAR* γ and *leptin* and higher mRNA levels of *adiponectin* in comparison with WT mice on HFD. In conclusion, these findings suggested that *IL6* gene deficiency antagonized HFD-induced bone loss. *IL6* might bridge lipid and bone metabolisms and could be a new potential therapeutic target for lipid metabolism disturbance-related bone loss.

Key Words

- ▶ interleukin-6
- ▶ lipid metabolism
- ▶ bone metabolism
- ▶ osteoblasts
- ▶ osteoclasts
- ▶ adipocyte

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Introduction

Obesity and osteoporosis (OP) are interrelated disorders. Generally, it is considered that body mass index (BMI) or body weight (BW) is beneficial for bone formation and low BMI or low BW is negatively correlated with bone formation (Cao 2011). However, recent studies suggested that excess fat mass is a risk factor for bone loss in humans (Pollock *et al.* 2007), visceral fat is an independent

predictor of bone density (Russell *et al.* 2010) and high-fat diet feeding reduces bone mineral density (BMD) in mice (Halade *et al.* 2010).

Obesity affects bone metabolism through several potential mechanisms. It is well known that both adipocytes and osteoblasts are derived from the same progenitor cells: bone marrow mesenchymal stem

cells (BMSCs). Obesity increases the differentiation of adipocytes, whereas it decreases the differentiation of osteoblasts, which leads to fat accumulation and bone mass reduction. Obesity is usually accompanied with abnormal adipokine secretion, such as leptin and adiponectin, which might exert a direct effect on bone formation or indirect effect on bone resorption. Furthermore, obesity is related to a chronic inflammation condition. Increase in circulating and tissue proinflammatory cytokines, such as tumor necrosis factor alpha (TNF- α), interleukin-6 (IL6) and IL1, might enhance osteoclast differentiation and bone resorption. However, the precise mechanisms underlying the relationship between obesity and skeleton metabolism are still not clear.

IL6, an important and multifunctional cytokine, is produced by a variety of cells, such as B-cells, T-cells, adipocytes and osteoblasts (Naka *et al.* 2002, Hassan *et al.* 2014). IL6 shows extensive biological activities, including immune responses, lipid metabolism and bone metabolism (Mihara *et al.* 2012). IL6 shows antiadipogenic effects to regulate body weight, which is similar as that of leptin. However, intracerebroventricular injection of IL6 reduces body fat in rat and increases energy expenditure in mice (Wallenius *et al.* 2002). On the other hand, interleukin-6-deficient (IL6^{-/-}) mice usually show mature-onset obesity, higher levels of triglyceride (TG) and very-low-density lipoprotein (VLDL) than wild-type (WT) mice (Wallenius *et al.* 2002). IL6 soluble receptor and IL6 receptor double transgenic mice show a decrease in body weight, total cholesterol (TC) and TG (Peters *et al.* 1997). Additionally, IL6 increases fat oxidation and accelerates fat decomposition, whereas inhibits lipid synthesis, which eventually reduces serum lipid levels.

IL6 also plays a key role in bone metabolism. Yang and coworkers found that IL6^{-/-} mice showed delayed callus mineralization and remodeling compared with those in WT mice during fracture healing (Yang *et al.* 2007). However, the effects of IL6 on osteoblasts and osteoclasts are not always consistent, and sometimes, even opposite (Blanchard *et al.* 2009, Mihara *et al.* 2012). In osteoclasts, IL6 was reported to stimulate osteoclast precursor cells to differentiate into mature and active osteoclasts and regarded as osteoclastogenesis promoter (Udagawa *et al.* 1995, Yokota *et al.* 2014). However, another study showed that IL6 had an inhibitory role on osteoclast formation and bone resorption by inhibiting RANKL signaling pathway (Duplomb *et al.* 2008). IL6-null mice had decreased osteoblast numbers (Yang *et al.* 2007). IL6 has been reported to stimulate osteoblast differentiation in stem cells (Erices *et al.* 2002). On the other hand, IL6 has

been shown to impair osteoblast maturation both *in vitro* and *in vivo* (Peruzzi *et al.* 2012). These contradictory findings suggested that IL6 may have a double role in bone metabolism.

Furthermore, the action of IL6 on the signal communication between fat and bone metabolism is not clear. In this study, we aimed to determine the pathological roles of IL6 in the high-fat diet (HFD)-induced bone loss.

Materials and methods

Animals and diets

Male wild-type (WT) C57BL/6J mice and IL6 knockout (IL6^{-/-}) mice (C57BL/6J background), aged 8 weeks, were obtained from the Jackson laboratory (Bar Harbor, ME, USA). All mice were housed in Plexiglas ventilated cages with ALPHA-Dri bedding within a pathogen-free facility and at a constant temperature (22°C) under a 12/12-h light/darkness cycle with free access to water. IL6^{-/-} and WT mice were randomly separated into regular diet group or high-fat diet group. Mice were fed with a high-fat diet (HFD, 60% lipid, 20% carbohydrate, 20% protein, 5.2 Kcal/g) (Table 1) or a regular diet (RD, 13% lipid, 65% carbohydrate, 22% protein, 3.4 Kcal/g) for 12 weeks. Both diets were purchased from TROPHIC Animal Feed High-tech Co., Ltd (Nantong, Jiangsu, China). Body weight was recorded weekly. After 12 weeks of feeding, tissue and blood were collected for the experiments described below. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal

Table 1 Composition and fatty acid profiles of HFD.

HFD	g (%)	Kcal (%)
Protein		20
Carbohydrate		20
Fat		60
Total Kcal/g	5.2	
Ingredient	g	Kcal
Casein	258	1032
Corn starch	0	0
Maltodextrin	162	648
Sucrose	89	356
Soybean oil	32	288
Lard	317	2853
Cellulose	65	
Mineral mix, M1020	58	
Vitamin mix, V1010	13	52
L-Cystine	4	16
Choline bitartrate	3	
TBHQ	0.069	
Total	1000	5245

Care and Use Committee at the West China Hospital, Sichuan University.

Serum parameters for lipid metabolism

Serum TG and TC levels were measured by enzyme method (Beijing North Kangtai reagent CO., Beijing, China). Low-density lipoprotein cholesterol (LDL-C) levels were detected by the selective precipitation method (Beijing North Kangtai reagent CO.).

Micro-CT for bone mass and microstructure

Distal ends of left femora were fixed with 4% paraformaldehyde for 24h and then scanned on the vivaCT 40 microCT scanner (Scanco Medical, Basserdorf, Switzerland). Bone microstructure parameters including trabecular bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp) were analyzed according to our early protocol (Zhou *et al.* 2014). Sanco software, version 5.0 was used for three-dimensional (3D) reconstruction in the region of interest (ROI) (Zhang *et al.* 2015).

Bone histological analysis

The femurs were fixed in 4% paraformaldehyde solution for 24h and then decalcified in 20% ethylene diamine tetraacetic acid (EDTA) buffer. The buffer was changed every 4 days until the femurs were completely decalcified. The samples were dehydrated and paraffin blocks were prepared by the standard histological procedure. Longitudinal sections about 5µm from the middle of femur were used for tartrate-resistant acid phosphatase (TRAP) staining (Sigma-Aldrich, kit 387-A) and histological analysis. For each tissue sample, from the region 2mm beneath the growth plate, cells were collected. TRAP-positive cells with three or more nuclei were counted as osteoclasts to evaluate the formation of osteoclasts.

Real-time quantitative RT-PCR

Total RNA was extracted from epididymal white adipose tissue (WAT), distal metaphyses of the right femurs and cultured osteoblasts with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA quality was evaluated by A260/A280 ratio. Complementary DNAs (cDNAs) were synthesized from 1µg total RNA using 20µL reverse transcription

Table 2 RT-PCR primer sequences.

Primer	Sequence 5'-3'
Runx2	Forward: 5'-GTGGTAGGCAGTCCCACTTT-3' Reverse: 5'-GAGCACTCACTGACTCGGT-3'
Col1a1	Forward: 5'-GCGAAGGCAACAGTCGCT-3' Reverse: 5'-CTTGGTGGTTTTGTATTCGATGAC-3'
OPG	Forward: 5'-TACCTGGAGATCGAATTCTGCTT-3' Reverse: 5'-CCATCTGGACATTTTTTGCAAA-3'
RANKL	Forward: 5'-CCTGAGGCCAGCCATT-3' Reverse: 5'-CTTGGCCCAGCCTCGAT-3'
Leptin	Forward: 5'-GGAAAATGTGCTGGAGACCC-3' Reverse: 5'-TACCGACTGCGTGTGTGAAAT-3'
Adiponectin	Forward: 5'-TGACGACACCAAAAGGGCTC-3' Reverse: 5'-GAGTGCCATCTCTGCCATCA-3'
PPAR γ	Forward: 5'-TTCGCTGATGCATCTGCTAT-3' Reverse: 5'-TGATCGCACTTTGGTATTCTTGG-3'
GAPDH	Forward: 5'-TGCACCACCAACTGCTTAG-3' Reverse: 5'-GGATGCAGGGATGATGTTTC-3'

reaction solution with the PrimeScript RT reagent kit (Takara Bio). Real-time qPCR reaction was performed with SYBR Premix Ex Taq II kit (Takara Bio) in cycler PCR machine (LightCycler 96, Roche), and 2µL cDNA were amplified following the universal protocol: 94°C for 3 min, then, 94°C for 20s and 60°C for 20s for 45 cycles. The primer sequences are listed in Table 2. GAPDH was used as an endogenous control. The relative mRNA expression levels were normalized to the GAPDH in the same sample. The data analysis was performed with the $2^{-\Delta\Delta CT}$ method as in our previous report (Yu *et al.* 2005).

Bone marrow cells culture and PA intervention

Bone marrow cells were obtained from tibias and femurs and cultured in 24-well culture plates at a density of 1×10^6 cells/well in α -MEM medium (Invitrogen) containing 10% fetal bovine serum (FBS), 1% penicillin, 1% streptomycin and 1% β -glutamine for 7 days. Culture medium was then changed to osteoblastic differentiation medium, which consisted of regular medium plus 50µg/mL ascorbic acid and 10mM β -glycerophosphate. Medium was changed every day for the entire duration of culture. After further culture for 7 days, cells were near-serum-starved for 12h, using 0.2% FBS supplemented medium, to minimize the effects of growth factors in serum. Cells were treated with 4×10^{-4} M palmitic acid (PA) in 0.3% bovine serum albumin (BSA) or just 0.3% BSA for 24h. Alkaline phosphatase (ALP) staining and qRT-PCR were performed to determine osteoblast differentiation and gene expression.

Bone marrow cells were also induced to differentiate into osteoclasts. The bone marrow cells were plated in 24-well culture plates at a density of 1×10^6 cells/well

in the osteoclastic differentiation medium, which consisted of regular medium, 25 µg/mL receptor activator of nuclear factor kappa-B ligand (RANKL) and 25 µg/mL macrophage colony-stimulating factor (M-CSF). Medium was changed every 3 days. On day 8, cells were near-serum-starved for 12 h, using 0.2% FBS supplemented medium, to minimize the effects of growth factors in serum. Then, the cells were treated with 4×10^{-4} M PA for 8 h. TRAP staining (Sigma) was performed to determine the osteoclast formation.

Statistical analysis

Statistical analysis was performed with SPSS statistic 17.0 software. Data were expressed as mean $\pm$ standard error of the mean (S.E.M.). Analysis of variance (ANOVA) was applied for statistical analysis to assess the differences between genotypes, diet treatment and the interactions between genotype and diet treatment. $P < 0.05$ was considered significant.

Results

Body weight and serum lipid levels

On the regular diet, the body weight of IL6^{-/-} mice was significantly higher than that of WT mice at all time points tested (Fig. 1A). Body weight in both genotypes on HFD tended to increase in comparison with the corresponding mice on RD (Fig. 1A). Body weight of

WT mice on HFD increased by 18.9% compared with that of WT mice on RD after 12 weeks (32.68 ± 0.87 vs 27.49 ± 0.58 g, $P < 0.01$). However, body weight of IL6^{-/-} mice on HFD exhibited no significant difference compared with that of IL6^{-/-} mice on RD (30.91 ± 0.97 vs 29.21 ± 0.77 g). Under high-fat feeding, IL6^{-/-} mice exhibited 11.9% less body weight than WT mice (29.21 ± 0.77 vs 32.68 ± 0.87 g). Furthermore, high-fat diet feeding induced abnormal accumulation of the epididymal fat pad in both genotypes, but the gain of fat pad weight was 33.3% less in IL6^{-/-} mice than that in WT mice (Fig. 1B).

On the regular diet, IL6^{-/-} mice showed significantly higher level of TC (1.38 ± 0.09 vs 0.91 ± 0.06 mmol/L, $P < 0.01$) than WT mice. After high-fat feeding, the levels of TC and LDL-C increased in both genotypes. TC level increased by 90% (1.72 ± 0.07 vs 0.91 ± 0.06 mmol/L, $P < 0.01$) and 40% (2.06 ± 0.11 vs 1.38 ± 0.09 mmol/L, $P < 0.01$) in WT mice and IL6^{-/-} mice, respectively (Fig. 1C). LDL-C level increased by 85% (0.39 ± 0.05 vs 0.21 ± 0.04 mmol/L, $P < 0.05$) in WT mice and 69% (0.44 ± 0.07 vs 0.26 ± 0.09 mmol/L, $P = 0.069$) in IL6^{-/-} mice (Fig. 1D). Furthermore, only the TC level in IL6^{-/-} mice was 19% significantly higher than that in WT mice (2.06 ± 0.11 vs 1.72 ± 0.07 mmol/L, $P < 0.05$) on HFD group. These data revealed that HFD feeding worked successfully in the genotypes, but IL6^{-/-} mice showed weaker response compared with WT mice.

Figure 1

IL6^{-/-} mice showed weaker response in body weight and serum lipid levels after high-fat diet feeding. IL6^{-/-} mice and WT mice were fed with a high-fat diet (HFD) or a regular diet (RD), body weight was recorded each week (A); after 12 weeks of feeding, the epididymal fat pad weight was measured (B); the serum levels of TC (C) and LDL-C (D) were assessed with commercial kits. $n = 6$; * $P < 0.05$, ** $P < 0.01$, compared with the same genotype; # $P < 0.05$, ## $P < 0.01$, compared with the same diet.

Figure 2

IL6 knockout protected HFD-induced femoral trabecular bone loss. IL6^{-/-} mice and WT mice were fed with RD or HFD for 12 weeks. Bone microstructure parameters of the distal femur metaphysis were analyzed with micro-CT. Trabecular bone mass (A), trabecular number (B), trabecular thickness (C), trabecular separation (D), 3D reconstruction (E). $n=6$, * $P<0.05$, ** $P<0.01$, compared with the same genotype; # $P<0.05$, ## $P<0.01$, compared with the same diet. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-16-0076>.

Changes in bone mass and microstructure

On regular diet, IL6^{-/-} mice exhibited higher trabecular bone mass (volume fraction) (Tb.BV/TV) (Fig. 2A), trabecular number (Tb.N) (Fig. 2B) and trabecular thickness (Tb.Th) (Fig. 2C) and lower trabecular separation (Tb.Sp) (Fig. 2D) than those in WT mice. Only the difference in Tb.Th was significant. Under HFD feeding, Tb.BV/TV and Tb.N were decreased, and Tb.Sp was increased in both genotypes. However, only the changes in WT mice were significant. IL6^{-/-} mice on HFD exhibited significantly higher values of Tb.BV/TV, Tb.N and Tb.Th, but significantly lower value of Tb.Sp than WT mice on the same diet. Three-dimensional reconstruction assay also supported the fact that the HFD induced more trabecular bone loss in WT mice than that in IL6^{-/-} mice (Fig. 2E). These results suggested that IL6 knockout protected HFD-induced femoral trabecular bone loss.

Changes in osteoblastogenesis

On regular diet, mRNA expression of *Runx2* was higher in IL6^{-/-} mice than that in WT mice; mRNA expression of *Col1a1* was similar between IL6^{-/-} and WT mice. After high-fat diet for 12 weeks, mRNA levels of *Runx2* and *Col1a1* were downregulated in WT mice, but the changes in IL6^{-/-} mice were not significant. Furthermore, IL6^{-/-} mice demonstrated higher mRNA expression of *Runx2* and *Col1a1* than WT mice ($P<0.01$) (Fig. 3A and B).

To study the direct effect of fatty acid on osteoblastogenesis, PA was used to treat osteoblasts

from both the genotypes. ALP staining showed that there was no notable difference in ALP activity in osteoblasts between WT mice and IL6^{-/-} mice under vehicle treatment. After treatment with PA, ALP activity was reduced in osteoblasts from both genotypes, but IL6^{-/-} osteoblasts showed higher ALP activity than WT osteoblasts (Fig. 4A). The mRNA levels of *Runx2* and *Col1a1* were significantly higher in IL6^{-/-} osteoblasts

Figure 3

HFD downregulated osteoblastic gene expressions in WT mice, but not in IL6^{-/-} mice. IL6^{-/-} mice and WT mice were fed with RD or HFD for 12 weeks. mRNA levels of *Runx2* (A) and *Col1a1* (B) in the distal femur metaphysis were determined with qRT-PCR. $n=5$; * $P<0.05$, ** $P<0.01$, compared with the same genotype; # $P<0.05$, ## $P<0.01$, compared with the same diet.

Figure 4

IL6^{-/-} mice showed less reduction in osteoblastogenesis after PA treatment. Bone marrow stromal cells from IL6^{-/-} mice and WT mice were induced to differentiate into osteoblasts *in vitro*. Osteoblasts were treated with 4×10^{-4} M palmitic acid (PA) for the last 24 h. ALP staining, 10 $\times$ magnification (A); mRNA levels of *Runx2* (B) and *Col1a1* (C) in osteoblasts were determined with qRT-PCR. $n=6$; * $P<0.05$, ** $P<0.01$, compared with the same genotype; # $P<0.05$, ## $P<0.01$, compared with the same treatment.

than those in WT osteoblasts under basal condition (Fig. 4B and C). After treatment with PA, mRNA levels of *Runx2* and *Col1a1* were downregulated in both IL6^{-/-} and WT osteoblasts in comparison with their corresponding controls. Similar to the data from *in vivo* study,

IL6^{-/-} osteoblasts showed higher mRNA expressions of *Runx2* and *Col1a1* than those in WT osteoblasts after treatment with PA.

As osteoblastogenesis is usually negatively associated with adipocyteogenesis, we studied gene expressions related to adipocyteogenesis with qRT-PCR. On regular diet, mRNA level of *PPAR γ* in IL6^{-/-} mice was 0.79-fold higher than that in WT mice ($P<0.05$) (Fig. 5A), whereas mRNA levels of *leptin* and *adiponectin* were similar between IL6^{-/-} mice and WT mice (Fig. 5B and C). On HFD, mRNA expressions of *PPAR γ* and *leptin* were upregulated by 2.34-fold and 3.24-fold, respectively, in WT mice ($P<0.05$), whereas no significant change was found in IL6^{-/-} mice. However, IL6^{-/-} mice showed significantly lower mRNA levels of *PPAR γ* and *leptin* than WT mice on HFD. In contrast, HFD reduced mRNA level of *adiponectin* in WT mice but increased it in IL6^{-/-} mice, but both the changes were not significant. mRNA expression of *adiponectin* was 1.24-fold higher in IL6^{-/-} mice than that in WT mice on HFD (1.21 ± 0.25 vs 0.54 ± 0.09 , $P<0.05$).

Figure 5

HFD upregulated adipocyteogenesis gene expressions in WT mice, but not in IL6^{-/-} mice. IL6^{-/-} and WT mice were fed with RD or HFD for 12 weeks. mRNA levels of *PPAR γ* (A), *leptin* (B) and *adiponectin* (C) in the distal femur metaphysis were determined with qRT-PCR. $n=6$; * $P<0.05$, ** $P<0.01$, compared with the same genotype; # $P<0.05$, ## $P<0.01$, compared with the same diet.

Figure 6

HFD and PA induced less reduction in osteoclastogenesis in IL6^{-/-} mice. (A and B) IL6^{-/-} and WT mice were fed with RD or HFD for 12 weeks. Longitudinal sections from distal femur metaphysis were stained with TRAP to assay osteoclast formation, 20× magnification. (C and D): Bone marrow cells from IL6^{-/-} and WT mice were induced to differentiate into osteoclasts *in vitro*. Osteoclasts were treated with 4×10^{-4} M palmitic acid (PA) for the last 8 h and stained with TRAP to assay osteoclast formation, 10× magnification. N=4; *P<0.05, **P<0.01, compared with the same genotype; #P<0.05, ##P<0.01, compared with the same treatment.

Changes in osteoclastogenesis

TRAP staining showed that IL6^{-/-} and WT mice exhibited no significant difference in the number of TRAP-positive osteoclasts under regular diet feeding. HFD increased the number of TRAP-positive osteoclasts in both genotypes, but only the change in WT mice was significant (Fig. 6A and B). Although the number of osteoclasts was not different between IL6^{-/-} and WT mice under RD or HFD feeding, the numbers of osteoclasts in IL6^{-/-} mice tended to be less than those in WT mice under HFD feeding.

To further study the direct effect of fatty acid on osteoclasts, PA was used to treat osteoclasts *in vitro*. TRAP staining showed that the number of TRAP-positive osteoclasts was similar between WT and IL6^{-/-} osteoclasts on vehicle treatment (Fig. 6C and D). After treatment with PA, the number of TRAP-positive osteoclasts reduced significantly in WT and IL6^{-/-} osteoclasts. Furthermore, the number of TRAP-positive osteoclasts was 33.6% less in PA-treated IL6^{-/-} osteoclasts than in PA-treated WT osteoclasts.

Discussion

On regular diet, IL6^{-/-} mice showed higher body weight, serum level of TC and mRNA level of *Runx2* in bone. However, except higher Tb.Th in IL6^{-/-} mice, there was no significant difference in Tb.BV/TV and Tb.N between IL6^{-/-} mice and WT mice. These results indicated that *IL6* gene knockout did not change skeletal phenotype significantly in mice. Kopf and Yang and coworkers

also reported that IL6 knockout mice exhibited normal skeletal phenotype (Kopf *et al.* 1994, Yang *et al.* 2007). On high-fat diet, we observed that body weight, epididymal fat pad weight and serum levels of TC and LDL-C were upregulated in IL6^{-/-} mice and WT mice. This result is consistent with previous obese model induced by high-fat diet (Lin *et al.* 2000, Jones *et al.* 2005). Our findings also showed that HFD significantly decreased Tb.BV/TV, Tb.N and Tb.Th, and increased Tb.Sp in WT mice. Our study and the studies from Halade and Cao suggested that the murine model of trabecular bone loss can be established by the high-fat diet-induced obesity (Cao *et al.* 2009, Halade *et al.* 2010). In this study, we found that the HFD-induced obesity failed to induce trabecular bone loss in IL6^{-/-} mice, which indicated that IL6 played a critical role in the pathological process of obesity-related trabecular bone loss.

Adipocytes and osteoblasts are derived from the same progenitor cells – BMSCs. HFD has been reported to induce BMSCs to differentiate into adipocytes rather than osteoblasts, which may be one of the reasons for HFD-related bone loss. We found that HFD feeding significantly enhanced *PPARγ* expression and epididymal fat pad accumulation in WT mice, which was disadvantageous to bone metabolism. However, the increased level of *PPARγ* in IL6^{-/-} mice on HFD was much lower than that in WT mice on the same diet. Consistent with lower level of *PPARγ*, the accumulation of body weight and epididymal fat pad weight was lower in IL6^{-/-} mice on HFD than that in WT mice on the

same diet. On the other hand, we found lower mRNA expression of *Runx2* and *Colla1* in WT mice after high-fat diet feeding, as well as lower ALP activity and mRNA expression of *Runx2* and *Colla1* in WT osteoblasts after treatment with PA. However, we found higher mRNA level of *Runx2* in IL6^{-/-} mice after HFD and PA-treated IL6^{-/-} osteoblasts than that in WT mice and osteoblasts on the same treatment. Moreover, PA-treated IL6^{-/-} osteoblasts showed higher ALP activity than PA-treated WT osteoblasts. These results indicate that knocking out *IL6* gene protects abnormal adipocyteogenesis and osteoblastogenesis induced by the HFD. IL6 has been reported to inhibit *Runx2* expression and ALP activity through SHP2/MEK2, SHP2/AKT2 and insulin-like growth factor 5 (IGFBP5) signal pathway (Peruzzi *et al.* 2012, Kaneshiro *et al.* 2014). This may be the mechanism for better osteoblastogenesis in IL6^{-/-} mice on HFD.

It is well known that leptin and adiponectin are two important adipokines produced by adipocytes. Obesity is usually accompanied with higher level of leptin and lower level of adiponectin in the serum (Matsubara *et al.* 2002). Our research also confirmed that obesity induced by the high-fat diet was accompanied with the upregulation of leptin and downregulation of adiponectin in WT mice. In the obese state, IL6 is usually upregulated and acts as a positive regulator for leptin expression (Sarraf *et al.* 1997, Gualillo *et al.* 2000) and an inhibitor for adiponectin expression in adipocytes (Fasshauer *et al.* 2003, Ouchi & Walsh 2007). In this study, we found that HFD failed to downregulate adiponectin in IL6^{-/-} mice, and the change in leptin in IL6^{-/-} mice was much lower than that in WT mice on HFD. Our data further confirmed the regulating roles of IL6 on the expressions of leptin and adiponectin. Injecting leptin intracerebroventricularly leads to bone loss, whereas *leptin* knockout mice show higher bone mass (Eleftheriou *et al.* 2004). Contrary to leptin, adiponectin promotes osteoblast differentiation and inhibits osteoclast formation (Oshima *et al.* 2006). Our data suggested that *IL6* gene knockout may reduce adipocyte differentiation and accumulation, enhance the expression of adiponectin while inhibiting the expression of leptin, thus preventing bone loss induced by the high-fat diet.

Obesity belongs to a chronic inflammatory state in which the levels of proinflammatory cytokines, such as IL1, IL6 and TNF- α , are upregulated, which may enhance osteoclast proliferation and bone resorption (Cao 2011). Delamata and coworkers reported that IL6 enhanced the expression of parathyroid hormone-related protein (PTHrP), which stimulated the differentiation

and maturation of osteoclast precursors (Delamata *et al.* 1995). Our study revealed that HFD feeding significantly increased the number of the TRAP-positive osteoclasts. In contrast, knockout of *IL6* attenuated abnormal osteoclast formation induced by the high-fat diet or PA treatment. These data implicated that deletion of *IL6* decreased osteoclastogenesis. Moreover, normal bone remodeling depends on the dynamic balance between bone formation and bone resorption. HFD decreased bone formation but enhanced bone resorption, thus resulting in trabecular bone loss. Knockout of *IL6* can weaken the negative impact of HFD on bone metabolism. Therefore, it resists the trabecular bone loss induced by the high-fat diet.

Both IL6 and TNF α are important proinflammatory cytokines. Our early study showed that TNF α ^{-/-} mice are protected from trabecular bone loss induced by high-fat feeding (Zhang *et al.* 2015). In this study, we further confirmed that knockout *IL6* can antagonize HFD-induced trabecular bone loss. Combining the results from our previous study (Zhang *et al.* 2015) and this study, we found that the changes in bone phenotypes were distinctive among TNF α ^{-/-} mice, IL6^{-/-} mice and WT mice. On regular diet, IL6^{-/-} mice exhibited significantly higher Tb.Th, but TNF α ^{-/-} mice exhibited significantly higher Tb.N than WT mice. After HFD feeding, Tb.BV/TV, Tb.N and Tb.Th were downregulated, whereas Tb.Sp was upregulated in WT mice. These changes were similar in IL6^{-/-} mice, whereas they were reversed in TNF α ^{-/-} mice. Consistent with those changes, IL6^{-/-} mice and TNF α ^{-/-} mice exhibited higher *Runx2* and *Colla1* mRNA levels than those in WT mice both on the regular diet and high-fat diet, with the highest levels in TNF α ^{-/-} mice. These results indicated that both IL6 and TNF α play a significant role in HFD-induced trabecular bone loss, but they regulate fat and bone metabolism differently.

In conclusion, we have identified that HFD induced trabecular bone loss by reducing osteoblastogenesis and enhancing osteoclastogenesis and adipocyteogenesis. However, *IL6* gene knockout resists osteogenic, adipogenic and osteoclast gene abnormalities, thus preventing HFD-induced femoral trabecular bone loss. IL6 and TNF α were two important factors linking fat and bone metabolism, and both played a critical role in the HFD-related trabecular bone loss, but their roles were different.

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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