Original Research

Inhibiting myostatin signaling prevents femoral trabecular bone loss and microarchitecture deterioration in diet-induced obese rats

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Abstract

Besides resulting in a dramatic increase in skeletal muscle mass, myostatin (MSTN) deficiency has a positive effect on bone formation. However, the issue about whether blocking MSTN can inhibit obesity-induced bone loss has not been previously investigated. In the present study, we have evaluated the effects of MSTN blocking on bone quality in high-fat (HF), diet-induced obese rats using a prepared polyclonal antibody for MSTN (MsAb). Twenty-four rats were randomly assigned to the Control, HF and HF + MsAb groups. Rats in the HF + MsAb group were injected once a week with purified MsAb for eight weeks. The results showed that MsAb significantly reduced body and fat weight, and increased muscle mass and strength in the HF group. MicroCT analysis demonstrated that obesity-induced bone loss and architecture deterioration were significantly mitigated by MsAb treatment, as evidenced by increased bone mineral density, bone volume over total volume, trabecular number and thickness, and decreased trabecular separation and structure model index. However, neither HF diet nor MsAb treatment had an impact on femoral biomechanical properties including maximum load, stiffness, energy absorption and elastic modulus. Moreover, MsAb significantly increased adiponectin concentrations, and decreased TNF-α and IL-6 levels in diet-induced obese rats. Taken together, blocking MSTN by MsAb improves bone quality in diet-induced obese rats through a mechanotransduction pathway from skeletal muscle, and the accompanying changes occurring in the levels of circulating adipokines and pro-inflammatory cytokines may also be involved in this process. It indicates that the administration of MSTN antagonists may be a promising therapy for treating obesity and obesity-induced bone loss.

Keywords: Myostatin, obesity, trabecular bone microarchitectures, adipokines, pro-inflammatory

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Introduction

The view that obesity may be beneficial to bone and a protective factor for osteoporosis has recently been challenged.[1] New insights view obesity as an important risk factor for osteoporosis.[2–4] Lean mass and fat mass are important components of body weight. Especially in men, body mass index (BMI) as a protective indicator of bone mineral density (BMD) was strongly associated with elevated muscle mass, but not fat mass.[5] A recent study on a pair of twins from Finland revealed that lean mass was a stronger determinant of BMD than fat mass.[6] Moreover, excessive fat mass, especially visceral adipose tissue, was reported to be detrimental to bone.[7,8] The potential mechanisms may involve the abundant adipokines and pro-inflammatory cytokines produced by adipose tissue, which can regulate bone metabolism.[9–11] Furthermore, marrow adipogenesis may be inversely related to osteoblastogenesis,[12,13] since adipocytes and bone-forming osteoblasts are derived from a common multipotential mesenchymal stem cell.[14]

Myostatin (MSTN), a member of the transforming growth factor-β superfamily, plays an essential role in negatively regulating skeletal muscle growth.[15] MSTN mutations in animals or humans result in a dramatic increase in skeletal muscle mass and strength.[16,17] The physiological effects of MSTN deletion are not restricted to strengthened skeletal muscle, but also include enhanced bone formation. MSTN-knockout mice show increased BMD compared to their wild-type counterparts.[18] Moreover, the increased whole-body BMD and bone mineral content were found in MSTN-knockout mice to persist into old age.[19]

These data are further supported by genetic studies in human populations showing that MSTN gene polymorphisms play a role in attainment of peak BMD variation.[20] Furthermore, inhibiting MSTN signaling by transgenic overexpression of MSTN propeptide increased BMD in mice.[21] Interestingly, higher circulating MSTN levels were found in both obese animals and obese humans.[22,23] Therefore, we wondered if blocking of MSTN would prevent obesity-induced bone loss. Despite the positive effects of MSTN deficiency or blocking on bone in non-obese conditions, the issue about whether inhibiting MSTN signaling will be of benefit to bone quality in obese conditions has not been investigated previously.

In the present study, we examined the effects of MsAb, which had previously been shown to suppress MSTN signaling, on bone quality in diet-induced obese rats. Analyses included studies of serum markers of bone metabolism, measures of bone mechanical strength, and microCT scans of bone architecture. In addition, circulating levels of adipokines (leptin and adiponectin) and pro-inflammatory cytokines (TNF-α and IL-6) were also evaluated to provide insight into possible molecular mechanisms by which MsAb affects bone health in obese rats.

Materials and methods

Preparation of MsAb

The preparation and effectiveness of MsAb were demonstrated in detail in our previous study.[24] Briefly, the prokaryotic expression vector pQE30 was constructed to express C-terminal mature MSTN encoding the TT epitope. The recombinant plasmid pQE-TT-Ms that was transformed into host cell DH5α could highly express fusion protein His-TT-MSTN as inclusion bodies. Purification of inclusion bodies was completed by affinity chromatography using a nickel-charged nitrilotriacetic column. Rats were immunized with purified recombinant protein His-TT-Ms and successfully produced MsAb. The purified MsAb was determined and identified by ELISA and immunoblotting, and was proven effective in suppressing MSTN protein expression in muscle.

Animals and MsAb treatment

Eight-week-old male Sprague-Dawley rats (177.7 ± 10.7 g) were purchased from the Laboratory Animal Breeding and Research Center of Xi'an Jiaotong University (Xi'an, China) and were housed in a controlled room (22 ± 2°C, 60 ± 5% humidity, and 12-h light/dark cycle). After five days of acclimation, rats were randomly assigned to either the normal diet group (Control, $n = 8$) or the HF diet group (HF, $n = 16$). Rats in the Control group were fed with standard rodent chow (12% fat, 28% protein, and 60% carbohydrate, as a percentage of total kcal) for eight weeks. Rats in the HF group were fed with HF diet (58% fat, 25% protein, and 17% carbohydrate, as a percentage of total kcal) for eight weeks.

After eight weeks, animals in the Control group continued on a normal diet for additional eight weeks, whereas those in the HF group were randomly divided into two groups ($n = 8$ for each group): with (HF + MsAb group) or without MsAb treatment (HF group), in addition to an HF diet for another eight weeks. HF + MsAb rats were injected once a week in the tail vein with 0.2 mL (20 mg/mL) purified MsAb in PBS. Rats in the HF group were injected with 0.2 mL PBS once a week. The two treatments were administered for eight weeks. All experiments were conducted with approval of the ethics committee and in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Grip test

During the last week, grip tests were performed on the forelimbs with a grip-strength meter (YLS-13A, Anhui Zhenghua Bioinstrumentation Co., Ltd., Huaibei, Anhui, China). Rats were tested three times in succession without rest. The values of three peak grip strength were averaged for each rat.

Body weight and sample preparation

After eight weeks of treatment, rats were euthanized with an overdose of diethyl ether, and the final body weight was recorded. Abdominal fat pad tissue and a portion of quadriceps (rectus femoris), gastrocnemius, and pectoralis were dissected and weighted. These muscles were selected because they are relatively large in rats. They are thus more easily isolated and weighted, providing measurements with smaller errors than other muscles. Taken together, any effects on their weight better reflect effects on total muscle mass than any individual muscle. Blood samples were obtained via abdominal aorta puncture, and then centrifuged at $1500 \times g$ for 20 min at 4°C, and the serum was separated and stored at −70°C until analysis. The left and right femurs were harvested, cleaned of adhering soft tissues (including other muscles, tendons, joint capsules and ligaments) with scissors and scalpel, wrapped in saline-soaked gauze on ice, and then stored at −20°C for use in biomechanical and MicroCT analyses, respectively.

Serum analysis

Serum calcium (S-Ca) concentrations, inorganic phosphorus (S-P) concentrations, and tartrate-Tresistant acid phosphatase (TRACP) activity were determined by standard colorimetric methods using commercial kits (Nanjing Jiancheng Bioengineering Inst., Nanjing, Jiangsu, China). Serum levels of adiponectin, leptin, tumor necrosis factor (TNF-α), interleukin (IL)-6 were assessed using ELISA kits (Cell Signaling Technology Inc., Danvers, MA, USA). Absorbance was measured on a Model 680 microplate reader (Bio-rad Corp, Philadelphia, PA, USA).

MicroCT analysis

The left distal femurs were scanned to evaluate trabecular microarchitecture using microCT (μCT-Sharp, ZKKS-MCT, Guangzhou, Guangdong, China). The scanning system was set to 50 kV, 50 W. The scanning resolution was 35 μm/slice. Scanned images were reconstructed and analyzed using 3D

Med analysis software (version 4.5) (Key Laboratory of Molecular Imaging, Chinese Academy of Sciences, Beijing, China). A volume of interest (VOI) was selected for the analysis of trabecular bone microarchitecture, which started at a distance of 0.35 mm (10 slices) from the lowest end of the growth plate and extended to the proximal end of the femur with a distance of 2.1 mm (60 slices). Bone morphometric parameters including bone mineral density (BMD), bone volume over total volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and structure model index (SMI) were obtained by analyzing the VOI.

Biomechanical examination

The right femurs were subjected to a three-point bending test using a materials testing system (858 Mini Bionix II, MTS, Eden Prairie, MN, USA) as shown in Figure 1. The femurs were immobilized on a fixed support with two loading points with a 20-mm interval distance. The upper loading point was located at the midpoint between the two lower loading points. Then, the load was applied at a constant speed of 2 mm/min until bone fracture occurred. The inner and outer width and height of the femur at the point of fracture were measured with a vernier caliper. The maximum load, the stiffness, and the energy absorption were directly determined from the load–deformation curve. Elastic modulus was calculated according to the formula: $E = FL^3/48dI$, where F is the maximum load, L is the distance between supporting points, d is the displacement, and I is the moment of inertia of the cross-section in relation to the horizontal axis.

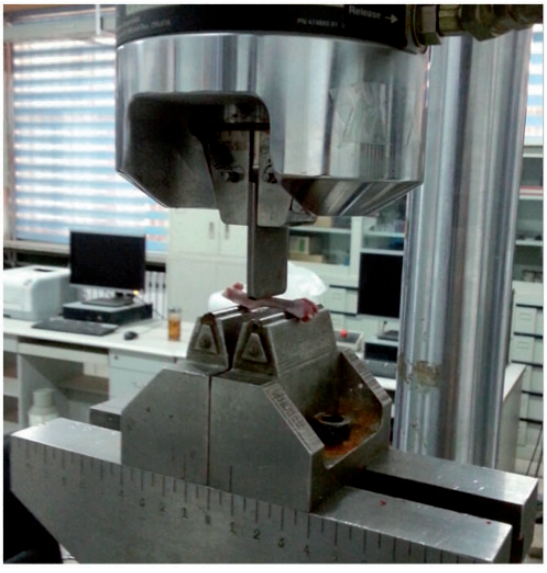

Figure 1 Three-point bending test using a materials testing system. The femurs were immobilized on a fixed support with two loading points with a 20-mm interval distance. The upper loading points were located at the midpoint between the two lower loading points. Then, the load was applied at a constant speed of 2 mm/min until bone fracture occurred. (A color version of this figure is available in the online journal.)

Statistical analysis

The results are expressed as mean ± SD. Statistical analyses were performed using SPSS version 13.0 (SPSS Institute, Chicago, IL, USA). One-way analysis of variance was employed for evaluating the existence of differences among the three groups and once a significant difference was detected, Student's *t*-test was used to determine the significance between every two groups. A $p < 0.05$ was considered statistically significant.

Results

Body weight, fat weight, muscle mass, and strength

Figure 2 summarizes the results of body weight, fat weight, and muscle mass and strength. Generally, HF diet-induced increases in body and fat weight were decreased by the MsAb treatment, and HF diet-induced decreases in muscle mass and strength were also raised by the MsAb treatment. Compared with the Control group, HF diet induced 17.4% weight gain, 59.5% elevated fat weight, and decreased muscle mass (quadriceps 12.0%, gastrocnemius 9.7%, and pectoralis 18.8%) and strength by 10.9% of rats in the HF group. Compared with the HF group, the average body weight and abdominal fat pad weight were decreased by 12.7% and 33.9%, respectively, in the HF + MsAb group. Quadricepses, gastrocnemii, and pectoralis in the HF + MsAb group were 12.2%, 10.2%, and 16%, respectively, heavier than those in the HF group. Moreover, average peak grip strength of the HF + MsAb rats was 8.8% greater than that in the HF group.

Bone metabolism

The effects of MsAb on serum bone metabolism indices in diet-induced obese rats are shown in Figure 3. It seems that higher serum TRACP in the HF group was downregulated by the MsAb treatment and there were no changes in either S-Ca or S-P among the three experimental groups. Statistical results show that neither HF diet nor MsAb significantly affect S-Ca and S-P compared with the Control group ($P > 0.05$). In addition, serum TRACP was increased in the HF group compared with the Control group, and decreased in the HF + MsAb group compared with the HF group, but there is no significance ($p > 0.05$).

MicroCT analysis

The effects of HF diet and MsAb on morphological changes of bone are shown in Figure 4. In general, HF diet-induced bone loss and microarchitecture deterioration were prevented by the MsAb treatment. The statistical results for the femoral trabecular MicroCT analysis show that HF diet caused significant decrease in BMD, Tb.N, Tb.Th and BV/TV ($p < 0.05$), and increase in Tb.Sp and SMI ($p < 0.05$) in the HF group. However, MsAb (HF + MsAb group) significantly reversed HF-induced bone microarchitecture deterioration, as evidenced by increased BMD, Tb.N, Tb.Th, BV/TV ($p < 0.05$), and decreased Tb.Sp and SMI ($p < 0.05$) compared with the HF group. The preventive effects of MsAb on trabecular bone mass and microarchitecture

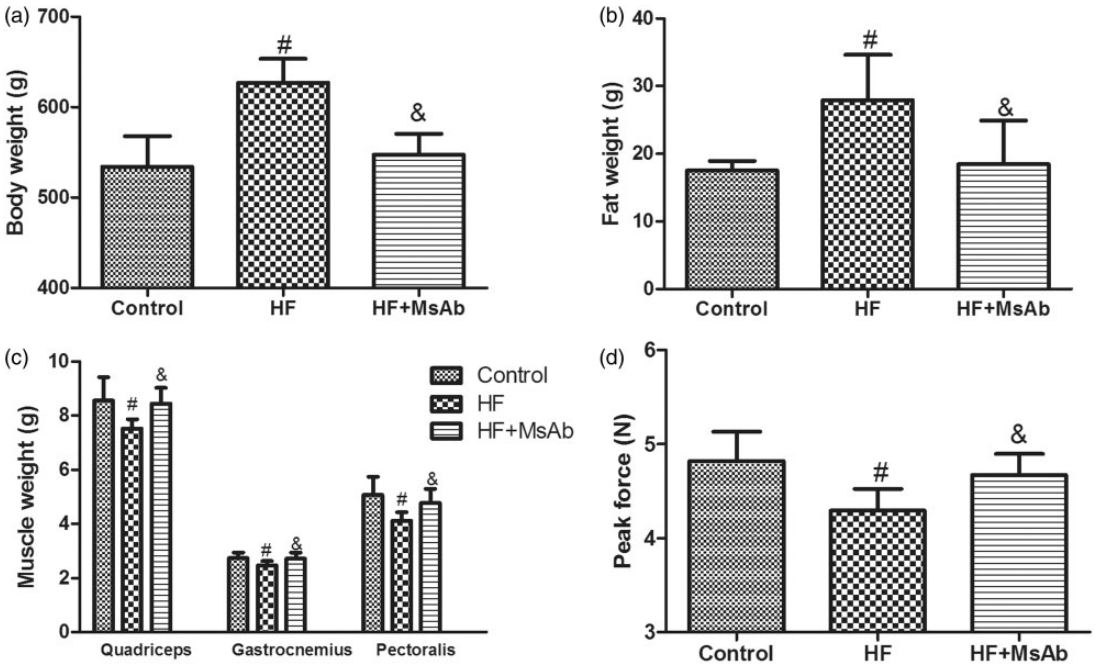

Figure 2 Effects of MsAb on (a) body weight, (b) fat weight, (c) skeleton muscle mass and (d) grip strength in obese rats. Data are expressed as mean ± SD ($n = 8$ per group). $^{\#}p < 0.05$ vs. control; $^{\&}p < 0.05$ vs. HF diet group (HF)

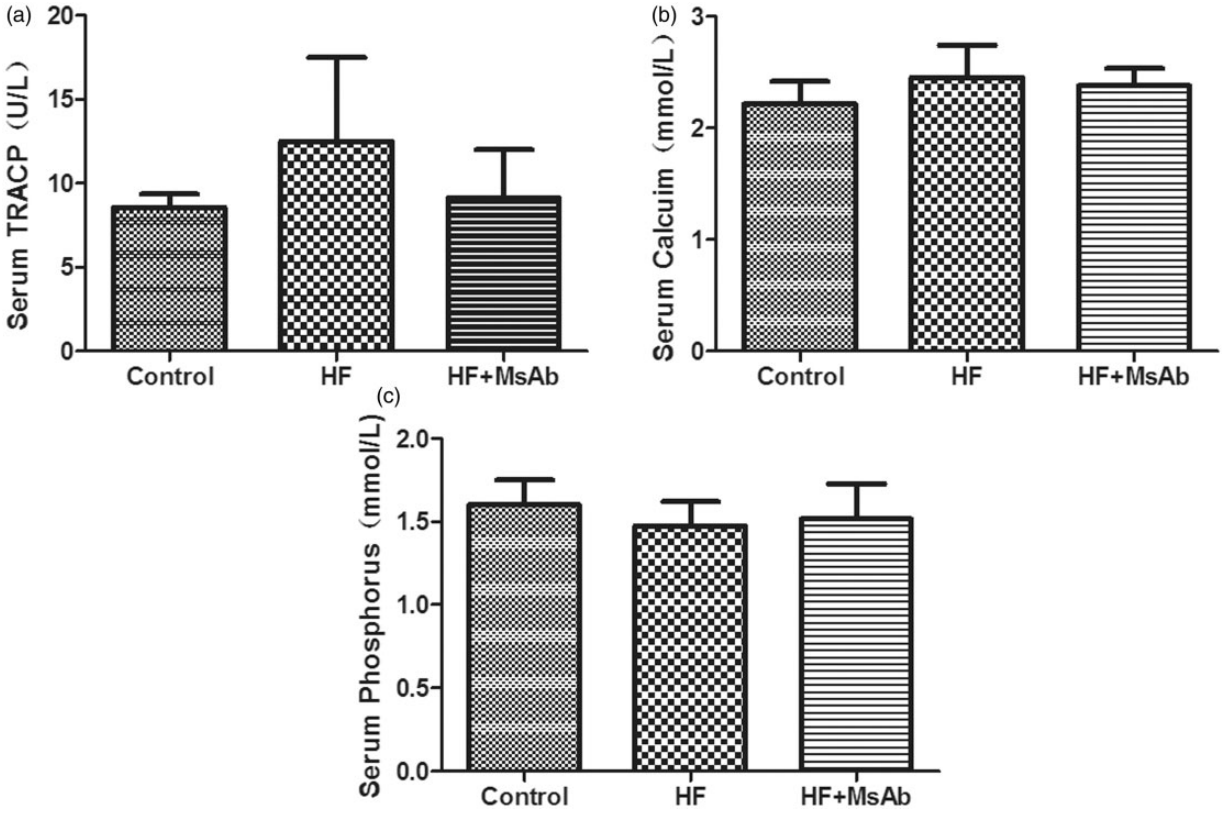

Figure 3 Effects of MsAb on serum bone metabolism indices (a) serum tartrate-resistant acid phosphatase (TRACP), (b) serum calcium, and (c) serum phosphorus concentrations in obese rats. Results are showed as mean ± SD ($n = 8$ per group)

deterioration in diet-induced obese rats are further supported by 3D-microCT images shown in Figure 5. Compared with the Control group, femurs in the HF group presented obvious reduction in the trabecular number and trabecular area. However, MsAb (HF + MsAb group) significantly preserved the trabecular bone mass and bone microarchitecture compared with the HF group.

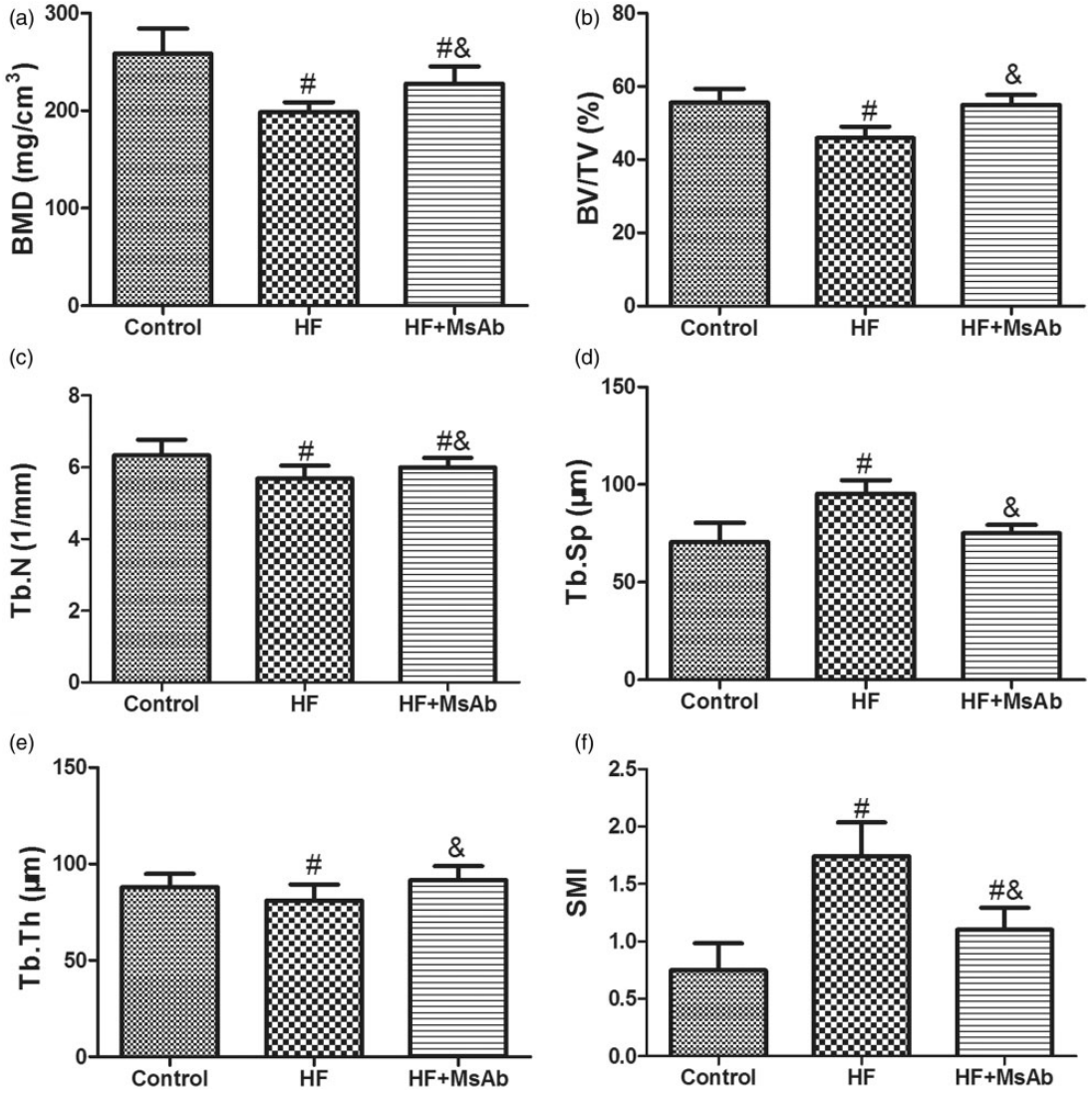

Figure 4 Effects of MsAb on femoral trabecular microCT indices: (a) BMD, (b) BV/TV, (c) Tb.N, (d) Tb.Sp, (e) Tb.Th, and (f) SMI in obese rats. Data are expressed as mean ± SD ($n = 8$ per group). $^{\#}p < 0.05$ vs. Control; $^{\&}p < 0.05$ vs. HF diet group (HF)

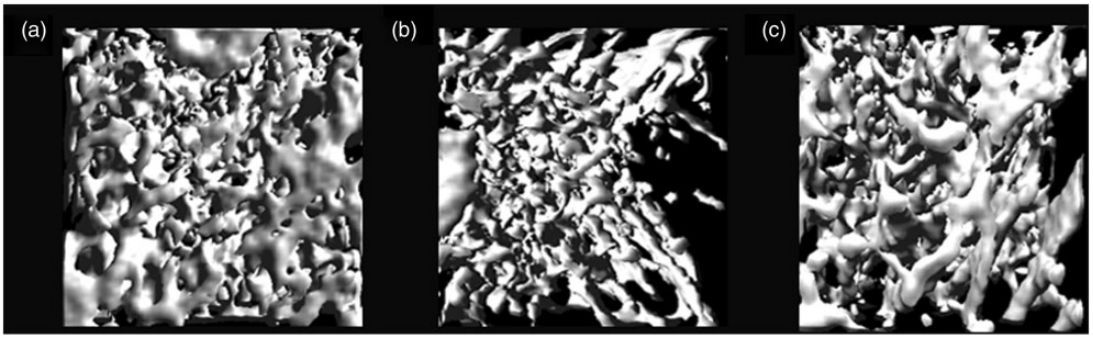

Figure 5 Representative MicroCT images of trabecular bone microarchitecture in the distal femurs. (a) Control group, (b) HF group, and (c) HF + MsAb group

Biomechanical examination

The results of three-point bending experiments are shown in Figure 6. It seems that the changes in mechanical properties are not as obvious as those in morphological characteristics. Bones in the HF group displayed similar maximum load, stiffness, energy absorption and elastic modulus compared with the Control group ($p > 0.05$). Meanwhile, the MsAb treatment for

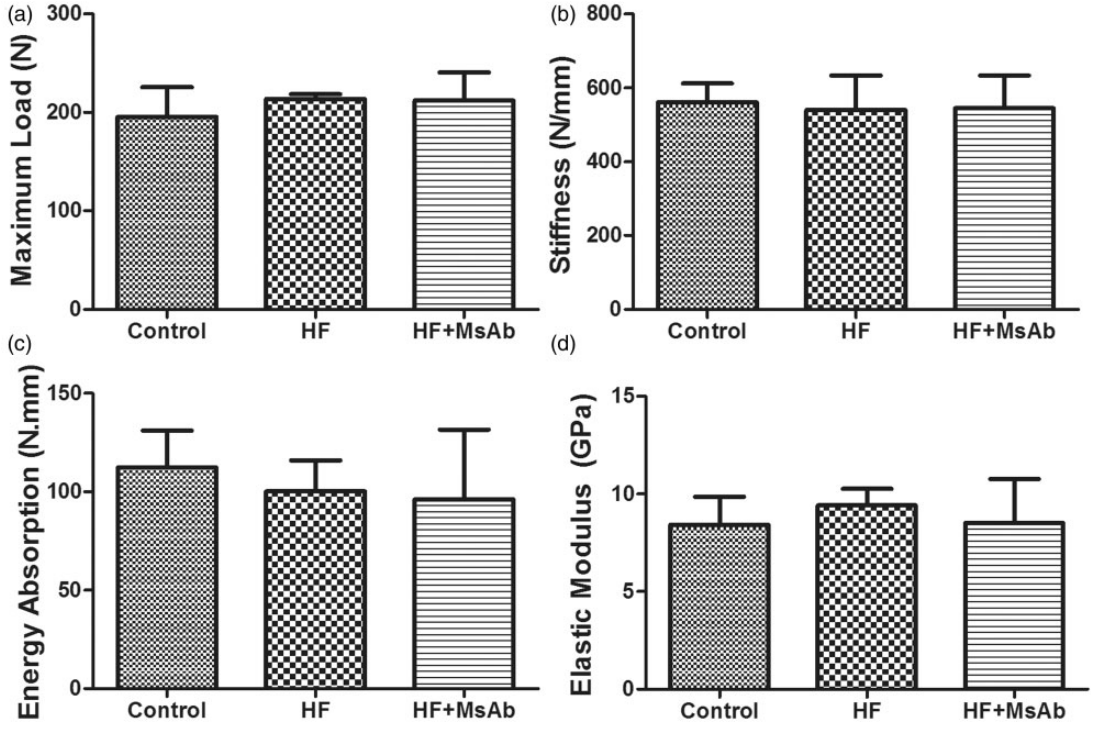

Figure 6 Effects of MsAb on biomechanical indices of femurs: (a) maximum load, (b) stiffness, (c) energy absorption, and (d) elastic modulus in obese rats. Data are expressed as mean ± SD ($n = 8$ per group)

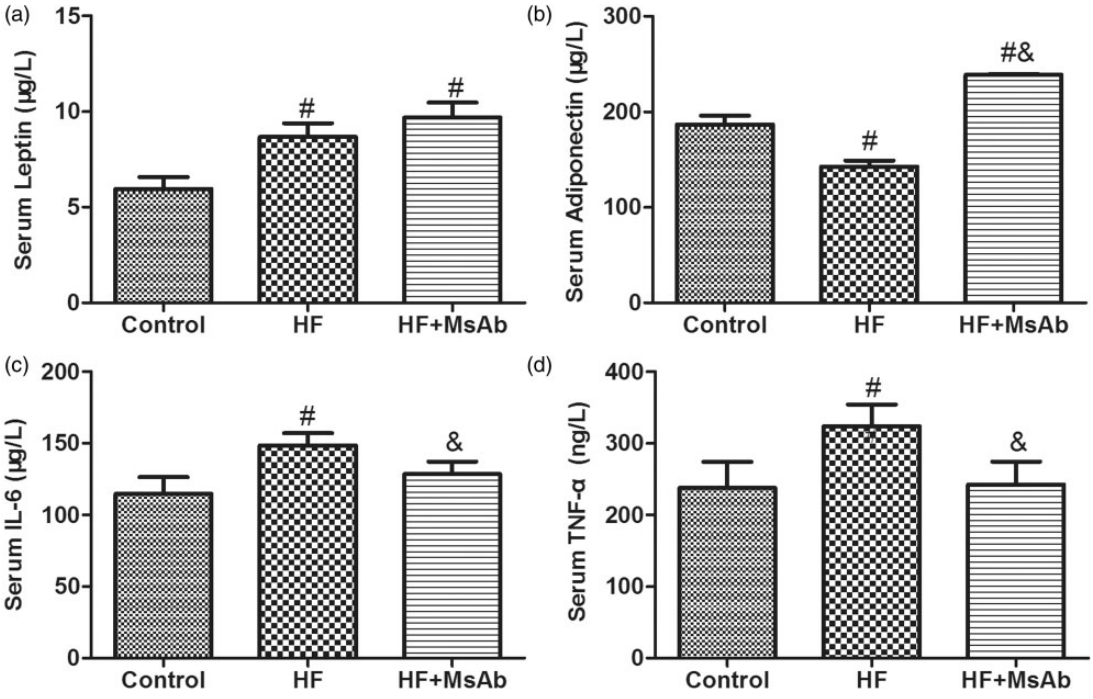

Figure 7 Effects of MsAb on adipokines and pro-inflammatory cytokines in obese rats. (a) Leptin concentrations, (b) adiponectin concentrations, (c) IL-6 levels, (d) TNF-α levels. Data are expressed as mean ± SD ($n = 8$ per group). $^{\#}p < 0.05$ vs. Control; $^{\&}p < 0.05$ vs. HF diet group (HF)

eight weeks in the HF + MsAb group exerted no significant impact on maximum load, stiffness, energy absorption, and elastic modulus compared with the HF group ($p > 0.05$).

Adipokines and pro-inflammatory cytokines

The effects of MsAb on adipokines and pro-inflammatory cytokines in diet-induced obese rats are shown in Figure 7. MsAb seems to play a role in regulating adipokines and

pro-inflammatory cytokines secreted by excessive adipose tissue. Compared with the Control group, the HF group had significantly lower serum adiponectin and higher leptin concentrations ($p < 0.05$). The rats in the HF + MsAb group had higher serum adiponectin concentrations when compared with those in the HF group ($p < 0.05$). There was no statistical difference in serum leptin concentration between the HF and HF + MsAb groups ($p > 0.05$). In terms of serum pro-inflammatory cytokines, both IL-6 and TNF-α were significantly increased in the HF group ($p < 0.05$). However, compared with the HF group, MsAb treatment caused a significant decrease in serum IL-6 and TNF-α ($p < 0.05$).

Discussion

Recently, accumulating evidence shows that a relationship exists between obesity and poor bone quality, as determined by decreased microarchitecture and biomechanical properties.[25–27] On the other hand, MSTN blocking or deficiency has been shown to enhance bone formation.[28,29] However, we still have much to learn regarding whether inhibiting MSTN can reduce obesity-induced bone loss, and if so, what possible mechanisms might be responsible. Our findings demonstrated that blocking MSTN by MsAb preserves bone microstructure and that this effect may involve the accompanying changes occurring in the levels of circulating adiponectin and pro-inflammatory cytokines.

MSTN, as a key negative muscle-regulatory factor, is known to play an inhibitory role in controlling muscle mass. However, MSTN also plays a role in adipose tissue. MSTN deficiency leads to decreased fat mass and MSTN promotes adipogenesis in multipotent mesenchymal stem cells.[15,30] Consistent with these findings, in the present study, MsAb not only increased skeletal muscle mass and strength, but also decreased body weight and fat mass in diet-induced obese rats as suggested by our previous study.[24] These results confirm that MsAb is effective in reducing fat mass and favors a healthy body composition in obesity.

Trabecular bone microarchitecture is generally considered an ideal predictor of bone mass loss and bone structure deterioration.[31] MicroCT, as a new high resolution digital imaging technique, has been widely used to provide detailed quantitative nondestructive analysis of 3D microscopic bone architecture.[32] Normal trabecular bone structure parameters could be reduced in HF-induced obesity as reported previously.[1,33] In accordance with these findings, our results also demonstrated notable trabecular bone deterioration in the HF group, evidenced by decreased trabecular BMD, Tb.N, Tb.Th and BV/TV, as well as increased Tb.Sp and SMI. However, the MsAb treatment partly prevented HF-induced increases in Tb.Sp and SMI, and decreases in BMD, Tb.Th, Tb.N and BV/TV. The preventive effects of MsAb on trabecular bone loss and microarchitecture deterioration are further supported by 3D microCT images. The HF diet induced a notable reduction in the trabecular number and trabecular area, which was partially prevented by treatment with MsAb. The MicroCT scan results suggest that MsAb could protect against obesity-induced bone loss and maintain the trabecular bone mass and microarchitecture.

Biomechanical strength is an important factor reflecting bone fragility and fracture risk.[34] In contrast to MicroCT analysis results, HF diet treatment did not detectably affect bone biomechanical properties including maximum load, stiffness, energy absorption and elastic modulus. These seemingly paradoxical results are not surprising. The distal femur used to assess trabecular bone microarchitecture using MicroCT scans is rich in cancellous bone, while the midshaft femur used to assess mechanical strength is a site characterized primarily by dense cortical bone. In general, cancellous bone is more susceptible than cortical bone to diet or drug treatments, because the cancellous bone is more actively remodeled than cortical bone due to the larger surface to volume ratio. Thus, changes in the biomechanical properties of the femoral diaphysis usually lag behind the alteration of bone microstructure in the epiphyses. Maybe this factor is a part of the reason for the prior view that obesity was not negatively impacting bone. In other words, a longer period of HF diet treatment may be required to affect cortical bone strength in the femur. This possibility is supported by two studies. Ionova-Martin et al.[27] showed that 19 weeks on a HF diet significantly reduced femoral bone strength. Shen et al.[35] showed that eight months on a similar diet had a similar effect. Additionally, eight weeks of MsAb also have no impact on femur bone strength, although the improvement of trabecular microarchitecture was observed. Our results demonstrated that neither 16 weeks on HF diet nor 8 weeks of MsAb treatment in these rats had a significant effect on the biomechanical properties of femoral shafts. A longer period of HF diet and MsAb treatment will be considered in future studies to confirm their effects on cortical bone biomechanical properties. Moreover, the biomechanical studies on epiphyses at both the shorter and longer time periods should be also considered in future studies, which would be more sensitive than those on diaphysis.

Adipokines play an important role in bone response to obesity. In particular, leptin and adiponectin are heavily influenced by obesity and interfere with bone metabolism. Leptin has been found in higher concentrations in obese mice and was shown to be a potent inhibitor of bone formation.[36,37] Moreover, Fujita et al.[38] demonstrated that serum leptin may be a useful indicator of risk for osteoporosis associated with diet-induced obesity. In contrast to leptin, adiponectin, which was reported to preserve bone both *in vivo* and *in vitro*,[39] has been reported to decrease in obese mice.[40] The observation that relative to the Control group, the HF group increased fat mass and leptin, decreased adiponectin, and reduced trabecular bone mass and microarchitecture seems to support the relationships among leptin, adiponectin, fat mass, and skeleton health.

Obesity is also associated with a state of chronic inflammation and elevated production of pro-inflammatory cytokines,[41,42] particularly TNF-α and IL-6, which can increase osteoclast formation and function.[9,43] Consistent with these studies, our results showed that the HF diet induced significantly higher TNF-α and IL-6 concentrations in serum along with a trend toward elevated TRACP. TRACP is an

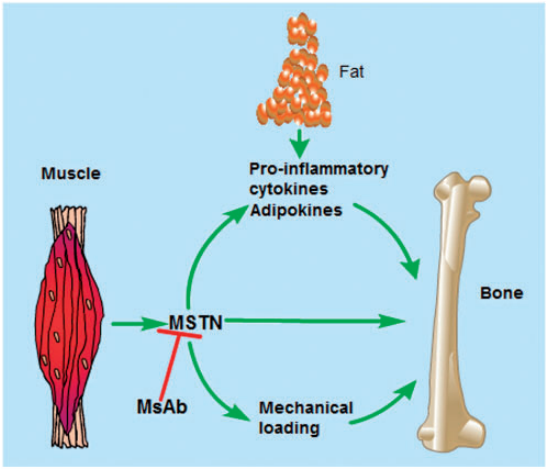

Figure 8 Schematic diagram summarizing the possible mechanisms for MsAb-mediated blocking of MSTN-related bone loss. First, blocking the effects of MSTN using MsAb may enhance bone quality of obese rats through a mechanotransduction pathway. Second, MsAb may directly improve bone formation through increasing osteogenic differentiation by suppressing MSTN binding to its receptor, type IIB activin receptor, on bone marrow-derived mesenchymal stem cells. Third, the regulation of adipokines and pro-inflammatory cytokines secreted by excessive adipose tissue may also be a possible pathway. (A color version of this figure is available in the online journal.)

osteoclastic bone resorption marker that has been reported to increase in mice fed a HF diet.[26] However, in the current study, MsAb treatment in diet-induced obese rats significantly increased circulating adiponectin concentrations, decreased TNF-α and IL-6 levels, but had no significant effect on circulating leptin levels, while causing a trend toward decreased serum TRACP. Our findings suggest that the regulation of adiponectin, TNF-α, and IL-6 is involved in preventing bone loss in diet-induced obese rats when treated with MsAb to block MSTN signaling.

We confirmed that HF diet-induced obesity causes trabecular bone loss and microarchitecture deterioration. However, MsAb treatment protected against the negative effects of obesity on bone microarchitecture. Possible mechanisms (Figure 8) may involve increased mechanical loading on bone due to increased muscle mass and strength by inhibiting MSTN production.[44] In other words, blocking the effects of MSTN using MsAb may enhance the bone quality of obese rats through a mechanotransduction pathway. An alternative explanation is that MSTN has a direct effect on bone maintenance by regulating osteoblasts. This possibility is supported by the findings that the MSTN receptor, the type IIB activin receptor, has been identified in bone marrow-derived mesenchymal stem cells and that increased osteogenic differentiation was reported in MSTN-deficient mice.[45] Moreover, our results indicate that regulating the levels of adipokines and suppressing the release of pro-inflammatory cytokines may also participate in this process. Nevertheless, much more work will be needed to prove the exact role of changes of adipokines and pro-inflammatory cytokines in reducing obesity–bone loss by suppressing MSTN signaling.

In conclusion, while a number of strategies have been employed to block the effects of MSTN with related outcomes,[46–50] this is the first report demonstrating that blocking MSTN with a polyclonal anti-MSTN antibody preparation improves trabecular bone microstructure, but has no detectable effect on cortical bone strength in diet-induced obese rats. This study supports the idea that MSTN may serve as a novel and effective therapeutic target for both obesity and obesity-induced bone loss and suggests that MsAb may serve as a useful antagonist in further studies of MSTN activity and of potential benefit in treating obesity and improving bone quality in such patients.

Authors' contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; LT and LS designed the experiments, XY and XG conducted the experiments and performed analysis, HD, YH, DZ, and ZW participated in the establishment of animal model, and LT and LS wrote the manuscript.

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