MDP regulates BMSC differentiation and bone formation through miR-384-5p/Lepr

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Original Research

MDP regulates BMSC differentiation and bone formation through miR-384-5p/Lepr

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Abstract

The main components of bacterial flora are closely related to bone metabolism. Peptidoglycan-based muramyl dipeptide (MDP) is present in bacterial cell walls. We found an association of energy metabolism with MDP in mice fed different diets. Leptin receptor (Lepr) expression in alveolar bone and the femur was increased drastically in the high-fat-diet group with MDP (H-MDP) compared with that in the high-fat-diet control (H-NaCl) group and low-fat-diet (LFD) group. The bone mass of mice was decreased after MDP intervention in the LFD group whereas, in the high-fat-diet (HFD) group, it was increased upon MDP intervention. Micro-RNA (miR)-384-5p targeted and inhibited Lepr expression *in vitro*. We extracted the supernatants of osteocytes cultured under different conditions with MDP intervention to act on bone marrow stromal cells (BMSCs) and found that miR-384-5p could regulate epr expression and osteogenic differentiation in BMSCs. Our findings indicate a possible mechanism of periodontitis-associated alveolar bone changes caused by the main components of microflora.

Keywords: MDP, BMSC differentiation, bone formation, miR-384-5p, Lepr

Abbreviations

MDP	metabolism. Peptidoglycan-based muramyl dipeptide
Lepr	Leptin receptor
BMSCs	bone marrow stromal cells
cAMP	cyclic adenosine monophosphate
ATP	adenosine triphosphate
BAT	brown adipose tissue
scWAT.	subcutaneous white adipose tissue
vWAT	visceral white adipose tissue
PTHrP	PTH-related peptide
Irs-1	insulin receptor substrate-1
HG	high glucose
LG	low glucose
ALP	Alkaline phosphatase
H&E	hematoxylin and eosin
MLO-Y4	Murine osteocyte-like cells
HEK293T	Human embryonic kidney 293T
ANOVA	Analysis of variance

1. Introduction

Periodontal disease is a chronic inflammatory disease characterized by the destruction of connective tissue and alveolar bone which, eventually, results in loss of gingival tissue, the periodontal ligament, cementum, and alveolar bone [1]. An epidemiological study involving 291,170 participants estimated that periodontitis was the sixth-most prevalent disease, affecting 743 million people, worldwide [2]. In the USA, periodontitis affects 47% of adults [3]. Periodontitis is the main cause of tooth loss in adults, which can affect their masticatory dysfunction, nutrition, and self-esteem [4]. Evidence does not support the notion that periodontitis has a simple linear relationship between bacterial accumulation and the initiation and severity of periodontal disease [5]. Oral bacteria induce bone loss by inducing gingival injury and immune stimulation [6]. Little is known about the specific bacterial components that may affect bone metabolism [7]. Therefore, additional research is needed to understand the role of microflora-associated factors in periodontitis.

Importantly, bacteria can change the host's metabolism. Muramyl dipeptide (MDP) is a peptidoglycan and is the main component of bacterial cell walls [8]. MDP can regulate expression of receptor activator of nuclear factor kappa-B ligand in osteoblasts through nucleotide-binding oligomerization domain-containing protein-2 (Nod-2), so it may play a key part in bone resorption in inflammatory diseases such as periodontitis [9]. In addition to causing oral diseases such as periodontitis, microflora can regulate disorders of the systemic metabolism. Studies have shown that microflora are involved in regulation of energy metabolism in the host [10]. Indigestible carbohydrates are fermented into short-chain fatty acids that can be utilized by the host. These short-chain fatty acids can affect the feeding behavior and energy metabolism of the host directly [11]. Butyric acid promotes intestinal gluconeogenesis in a free fatty acid receptor 2-independent way, which results in higher adenosine triphosphate (ATP) levels and, in turn, higher levels of cyclic adenosine monophosphate (cAMP) [12]. Conversely, microflora are thought to inhibit expression of lipoprotein lipase by affecting the level of angiogenin-like protein-4 in the circulation, thereby reducing the absorption of fatty acids by human tissues [13]. Moreover, the components of bacterial cell walls may play an important part in metabolism. Host detection of the components of bacterial cell walls is sufficient to promote the immune response, metabolic inflammation, lipolysis, and insulin resistance [14]. MDP injection has been shown to reduce adipose inflammation and glucose tolerance in obese mice, and MDP could reduce hepatic insulin resistance in obese mice and mice suffering from endotoxemia. Activating MDP of Nod1 can aggravate glucose tolerance [15].

Systemic energy metabolism is closely related to bone metabolism and maintenance of bone health [16]. Clinical studies have shown that the high-energy metabolism of hyperthyroidism can lead to secondary osteoporosis and reduction in bone mineral density, which is based on accelerated bone turnover and bone resorption [17]. However, observational studies have provided evidence that people with type-2 diabetes mellitus tend to have higher bone mineral density than people not suffering from diabetes mellitus [18]. The energy metabolism of bone is closely related to bone-marrow stromal cells (BMSCs). The latter are pluripotent and can differentiate into various cell types needed for tissue regeneration, including osteoblasts and adipocytes [19]. As cells related to energy metabolism, adipocytes are rich in mitochondria. They can regulate energy metabolism through the brown adipose tissue (BAT)-specific gene uncoupling protein-1 [20].

The main nutrient of BMSCs is glucose, which can be transported through glucose transporter-1. Glucose intake is beneficial to osteoblast differentiation and bone formation, thereby revealing the key regulatory role of metabolic requirements [21]. Studies have shown that osteocytes can regulate BMSC differentiation [22, 23]. By extracting the supernatants of osteocytes, we found that the exosomes from osteocytes carrying microRNA (miR)-124-3p could regulate galectin-3 expression in osteoblasts [24].

The leptin receptor (Lepr) belongs to a family of cytokine receptors [25]. In response to diet and obesity, the leptin receptor acts as a systematic “sensor” for energy homeostasis in BMSCs, which can promote fat formation and inhibit osteogenesis [26]. Liu et al. showed that subcutaneous injection of exogenous PTH-related peptide (PTHrP) can promote fracture repair in *Lepr*^{-/-} mice largely because of upregulation of expression of the genes and proteins in osteoblasts [27].

miRs are small regulatory non-coding RNAs of length ~22 bp. miRs regulate expression of post-transcriptional genes by identifying specific messenger (m)RNA sequences [28]. Abnormal expression of miRs during bone remodeling leads to pathologic bone resorption and bone diseases [29]. Previously, we showed that type-I collagen plays an important part in osteogenesis regulation through insulin receptor substrate-1 (*Irs-1*)/miR-342 and increases osteogenesis. This phenomenon is due to the differentiation of immature osteoblasts into mature osteoblasts and increased synthesis of collagen fibers [30].

In this study, we established a mouse model to study the effect of MDP on energy metabolism under different dietary conditions. We aimed to examine the mechanism of *Lepr* changes after MDP intervention. Hence, we measured *lepr* expression in BMSCs under high glucose (HG) and low glucose (LG) conditions, and its effect on osteogenic differentiation.

2. Materials and methods

Ethical approval of the study protocol

The study protocol was approved by the Ethics Committee, the Second Xiang-Ya Hospital of Central South University (Changsha, China), conformed to the ARRIVE guidelines, and followed the *Guiding Principles in the Care and Use of Animals* endorsed by the China Physiological Society.

Animals

Twelve male C57BL/6J mice (~4 weeks; 20–25 g; Hunan SJA Laboratory Animals, Hunan, China) were housed in the Animal Experiment Center of Central South University under specific pathogen-free conditions; the animals had *ad libitum* access to food and water.

After 1 week of acclimatization, mice were randomly assigned into four groups (n=3 each): (a) low-fat diet control (L-NaCl), (b) low-fat diet with MDP (L-MDP), (c) high-fat diet control (H-NaCl), and (d) high-fat diet with MDP (H-MDP).

For the high-fat-diet groups, separate cohorts of male mice were fed randomly for 8 weeks (60% kcal from fat, 20% kcal from protein, 20% kcal from carbohydrate; catalog number, TP23300; Trophic Animal Feed High-Tech, Nantong, China). For low-fat-diet groups, separate cohorts of male mice were fed randomly for 8 weeks (45% kcal from fat, 20% kcal from protein, 35% kcal from carbohydrate; TP23302; Trophic Animal Feed High-Tech).

The bodyweight of mice was monitored every day. The glucose level was determined by tail-vein blood sampling using a handheld glucometer (Sinocare, Changsha, China). In the last 10 days, MDP was injected (i.p.) every day. After the last injection of MDP, all mice were killed by anesthesia overdose. Then, blood, adipose tissue, and bone tissue were collected for further testing.

Micro-computed tomography (micro-CT)

The bone tissue of mice was fixed overnight in 10% paraformaldehyde solution at 4°C and stored in 75% ethanol until use. Then, maxillary tissues were scanned by micro-CT (Siemens Medical Solutions, Knoxville, TN, USA). The sagittal plane of the mandible and femur of different groups of mice were taken to observe bone mass. Trabecular morphometry was characterized by measuring the fraction of bone volume/total volume (BV/TV), bone surface/bone volume (BS/BV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp).

Histology, staining, and immunohistochemistry

Adipose tissue and bone tissue were fixed with 4% paraformaldehyde solution for 2 days and decalcified in 10% EDTA (pH 7.4) for 21 days at 4°C. Decalcified adipose tissue and bone tissue were embedded in paraffin, sectioned longitudinally at 5-μm thickness, and mounted on glass slides. The paraffin sections of tissue were stained with hematoxylin and eosin (H&E). Alkaline phosphatase (ALP) staining was undertaken with an ALP assay kit (Sigma–Aldrich, Saint Louis, MO, USA). Oil Red O staining was undertaken with an Oil Red O staining kit (Solarbio Life Sciences, Beijing, China) according to manufacturer instructions. Immunohistochemical staining for LepR was undertaken using goat anti–mouse Lepr antibody (R&D Systems, Minneapolis, MN,

USA). Antibody complexes were detected by diaminobenzidine (MaxVision™ HRP-Polymer Anti-goat IHC Kit; Fuzhou Maixin Biotech, Fujian, China) and counterstained with hematoxylin before observation under a microscope (Zeiss, Oberkochen, Germany).

RNA-seq analysis

Following RNA isolation, the RNA was quantified (Qubit RNA Assay Kit; Life Technologies, Inc.), and the purity was assessed (RNA6000 Nano Kit and BioAnalyzer 2100; Agilent). Next, 1000 ng RNA was used as the input for the VAHTSTM mRNA-seq v2 Library Prep Kit for Illumina® (Vazyme, Inc., Piscataway, NJ, USA), and sequencing libraries were created according to the manufacturer's protocol. Briefly, poly(A) RNA was purified in two rounds of hybridization to Dynal Oligo (dT) beads. Poly(A)+ RNA was fragmented and then used for first- and second-strand cDNA synthesis with random hexamer primers. The cDNA fragments were treated with the End-It DNA EndRepair Kit to repair the ends, that were then modified with Klenow to add an A base at the 3' end of the DNA fragments, and finally ligated to the adapters. The ligated cDNA products were subjected to PCR amplification. The library quality was determined using a Bioanalyzer 2100 (Agilent). The RNA-seq libraries were sequenced using the Illumina HiSeq™ 2000 platform. Based on the differentially expressed genes, GO functional significance enrichment analysis and KEGG pathway significant enrichment analysis were carried out.

Enzyme-linked immunosorbent assay (ELISA)

Blood samples from mice were centrifuged at 5000 ×g for 15 min and 4°C. The supernatant was stored at -80°C for further separation of Lepr. According to the manufacturer instructions, an ELISA kit (Cusabio, Wuhan, China) was used to measure the protein concentration of Lepr in blood samples from mice.

Western blotting

Western blotting was performed as previously described (Chen, Y. et al., 2020). Briefly, cells were harvested, washed with phosphate-buffered saline, and lysed in RIPA buffer (which contained inhibitors of proteases and phosphatases). Then, the same amount of protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 10% (w/v) gels and transferred (by electrophoretic means) to polyvinylidene fluoride (PVDF) membranes (0.22-μm thickness; Millipore, Billerica, MA, USA). Next, PVDF membranes were incubated at 4°C overnight with antibodies against Lepr (1:2000 dilution; AF497; R&D Systems) and β-actin (1:5000; Proteintech, Rosemont, IL, USA). Immunoblotting with peroxidase-labeled secondary antibody (1:5000; Proteintech, Rosemont, IL, USA) was performed, and an ECL detection kit was used for visualization of chemiluminescence (Imager 600; Amersham, Buckinghamshire, UK). The density of protein bands was measured by ImageJ (US National Institutes of Health, Baltimore, MD, USA).

Cell culture and transfection

BMSCs were harvested by flushing the long bones of mice. Murine osteocyte-like cells (MLO-Y4) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Before intervention, cells were cultured in α-minimum essential medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL). For intervention with a high glucose concentration, Dulbecco's modified Eagle's medium-high glucose (DMEM-

HG; 4.5 g/L) was used. For intervention using a low concentration of glucose, DMEM-LG (1.5 g/L) was used. All cells were stored in a humidified incubator in an atmosphere of 5% CO₂ at 37°C. Liposome 2000 (27623927; Thermo Fisher Scientific, Waltham, MA, USA) was used for cell transfection. Briefly, BMSCs were seeded in six-well plates, and then transfected with a miR-384-5p mimic and its negative control (miR-384-5p mimic NC) and a miR-384-5p inhibitor and its negative control (miR-384-5p inhibitor NC) (RiboBio, Guangzhou, China).

Osteogenic differentiation and adipogenic differentiation

For osteogenic differentiation, 2×10^6 cells/well were seeded in six-well plates in differentiation medium comprising osteo-differentiation medium (dexamethasone [10 nM], β -glycerophosphate [10 μ mol/L], and L-ascorbic acid [50 μ g/mL]) with dexamethasone (100 nM). Treatment was administered for 3 days, and the medium was changed on day 2. At the end of the experiment, cells were washed in PBS and stained with the ALP Assay Kit.

For adipogenic differentiation, 1×10^4 cells/well were seeded in six-well plates and cultured in a complete growth medium. At 100% confluence, the medium was changed to adipo-differentiation medium (3-isobutyl-1-methylxanthine [0.5 mM], indomethacin [100 μ M], insulin [10 μ g/mL], and dexamethasone [1 μ M]). Adipocyte differentiation was identified by the presence of fat globules visualized by the Oil Red O Staining Kit.

Real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Real-time RT-qPCR was carried out according to protocols published previously [24]. RNA extraction from cells was performed using TRIzol[®] Reagent according to manufacturer (Thermo Fisher Scientific) instructions. RNA was reverse-transcribed using a PrimeScript RT Reagent Kit and gDNA Eraser (TaKaRa Biotechnology, Shiga, Japan). Amplification and detection were undertaken with TB Green Premix Ex Taq II (Tli RNaseH Plus) reagent (TaKaRa Biotechnology) and the LightCycler[™] Real-time PCR system (Roche, Basel, Switzerland). The primer sequences (forward and reverse, respectively) were 5'-CAACGAGCGGTTCCGATG-3' and 5'-GCCACAGGATTCCATACCCA-3' for β -actin, and 5'-GAGCAGGCGTGCCATC-3', and 5'-GTACCCGTCAGTTTCACATGATATATTG-3' for Lepr. For miRs, the RNA reverse-transcription reaction was carried out with a PrimeScript miRNA quantitative PCR Starter Kit (TaKaRa Biotechnology) and relative miR expression was normalized to that of U6 (TaKaRa Biotechnology).

Dual luciferase reporter assay

Human embryonic kidney 293T (HEK293T) cells were inoculated on a 24-well plate and grown to 70–80% confluence before transfection. To examine luciferase reporter activity, Lepr-3' untranslated region (UTR) (WT) and Mut-Lepr-3' UTR (MUT) were inserted into psiCHECK-2 (luciferase expression vector). Then, the two vectors were co-transfected into HEK293T cells (NC) or with miR-384-5p mimics, miR-384-5p inhibitor, or NC using Endofectin[™]-MAX Reagent (EF004; GeneCopoeia, Rockville, MD, USA). After 48 h of incubation, HEK293T cells were analyzed for luciferase activity with a dual-luciferase reporter assay system (E1910; Promega, Madison, WI, USA). The relative luciferase signal was represented by the ratio of firefly luciferase activity to that of the sea kidney.

Statistical analyses

Data are represented as the mean $\pm$ SD. Experiments were conducted in triplicate. The significance of differences between groups was analyzed by one-way analysis of variance or the Student's *t*-test. $P < 0.05$ (two-tailed) was considered significant.

3. Results

MDP regulates diet-induced glucose homeostasis and adipogenesis

To examine in detail the effects of MDP on energy metabolism in mice, we used two experimental models of different diets (Figure 1A, B). Model 1 involved feeding a high-fat diet (HFD) and injection with MDP daily (10 days before sacrifice), and Model 2 involved feeding a low-fat diet (LFD) and injection with MDP daily (10 days before sacrifice). In both regimens, the final MDP injection was given 24 h before measurement of metabolic and inflammatory phenotypes. In the HFD model, the bodyweight of mice increased, but the blood glucose level after MDP injection was unaltered (Figure 1C, E). However, in the LFD model, MDP injections could reduce the bodyweight and blood glucose level of mice after 9 weeks on an LFD (Figure 1D, F).

Briefly, H&E staining of interscapular brown adipose tissue (iBAT) revealed that more lipid droplets, smaller parenchyma, and infiltration of more inflammatory cells were observed in the LFD group after MDP injection. However, in the HFD model, MDP injection reduced the number of inflammatory cells, and we noted enlargement of lipid droplets. Then, in the LFD model, the subcutaneous white adipose tissue (scWAT) inflammatory cells were decreased and the lipid droplets became larger after MDP injection. In the HFD model, the number of adipocytes was increased, the number of lipid droplets was increased, and the inflammatory reaction was more robust. Interestingly, in the visceral white adipose tissue (vWAT) group, the mean number of adipocytes in the HFD group was greater than that in the LFD group, and there was no significant difference in the same dietary group (Figure 1G).

Lepr participates in the changes of energy metabolism induced by MDP

We wished to further explore the mechanism of MDP-induced changes in glucose homeostasis and adipogenesis in mice. We identified differentially expressed genes related to energy metabolism using high-throughput PCR arrays.

We discovered that multiple genes related to energy metabolism were affected by MDP intervention. We found significant changes in expression of 12 genes related to metabolism. Among these 12 genes, expression of eight genes was upregulated such as *Acsf6*, *Tbcl1*, and expression of four genes was downregulated such as *Acacb*, among which *lepr* expression was upregulated significantly (>9.0-fold change) (Figure 2A). Analyses of signaling-pathway enrichment using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database revealed enrichment in “Metabolic pathways,” “MAPK signaling pathway,” “TNF signaling pathway,” and “Transcriptional misregulation.” Among them, the change in metabolic pathways was the most obvious (Figure 2B). Analyses of functional enrichment using the Gene Ontology (GO) database showed that these differentially expressed genes were mainly enriched in “cellular process,” “single-organism process,” and “metabolic process”; “cell,” “cell part and organelle”; and “binding, catalytic activity and transporter activity” (Figure 2C).

We further verified the changes of *Lepr* expression in our animal model. The ELISA results for *Lepr* expression in serum are shown in Figure 2D. In the LFD group, *Lepr* expression upon MDP injection was increased compared with that in the control group. In the HFD group, there was no significant difference between the two groups for *Lepr* expression.

Protein expression of *Lepr* in the adipose tissue, alveolar bone, and femur of mice was measured using immunohistochemistry (Figure 2E, F). Among the three types of adipose tissue, *Lepr* expression in LFD mice injected with MDP was increased more than that in the NC group; however, interestingly, *Lepr* expression in HFD mice was decreased more than that in the NC group. In the alveolar bone and femur, compared with that in the NC group, LFD mice and HFD mice injected with MDP had increased *Lepr* expression in osteoblasts, BMSCs, and osteocytes. Expression of *Lepr* protein was augmented in HFD mice upon MDP injection compared with that in the other three groups.

Effect of MDP on the mass and quality of bone in male mice consuming an HFD or LFD

Three-dimensional images of the alveolar bone and femur of mice in the L-NaCl, L-MDP, H-NaCl, and H-MDP groups were constructed using micro-CT (Figure 3A, B). In the mandible and femur, L-MDP mice had decreased bone mass compared with L-NaCl mice. BV/TV and Tb.Th were decreased in the L-MDP compared with L-NaCl mice. In the H-MDP group compared with the H-NaCl group, BV/TV and Tb.N were increased and Tb.Sp was decreased in the mandible and femur. However, in the mandible and femur, we found Tb.Th had no statistical significance, in the H-MDP group compared with the H-NaCl group. In the mandible and femur, we noted Tb.N and Tb.sp had no statistical significance in the L-MDP group compared with the L-NaCl group.

In the L-MDP group, H&E staining of the femur showed that trabeculae became thinner and fewer in number than in the L-NaCl group, and scattered adipocytes could be seen. In the H-MDP group, trabeculae became thicker and more numerous than in the LFD group, and fewer adipocytes could be observed.

miR-384-5p contained the targets of *Lepr* and modulated *Lepr* expression *in vitro*

The binding target sequences of mmu-miR-384-5p and *Lepr*-3'UTR were predicted using a miRNA database (Figure 4A). As described above, *Lepr*-WT and *Lepr*-MUT vectors were constructed. Figure 4B shows the sequences of the two vectors (red rectangle highlights the binding site in WT and core mutation site in MUT). After transfection of HEK293T cells with these constructs, luciferase activity was inhibited by the miR-384-5p mimic in the *Lepr*-WT group but showed no difference in the *Lepr*-MUT group (Figure 4D).

Next, expression of miR-384-5p and *Lepr* was measured at day 0 and day 3 of osteogenic differentiation of BMSCs. During osteogenic differentiation of BMSCs, *Lepr* expression showed an increasing trend, but miR-203 expression was decreased (Figure 4E).

To further validate that miR-384-5p modulated *Lepr* expression, we undertook transfection with the miR-384-5p mimic and miR-384-5p inhibitor and their respective NCs in BMSCs. miR-384-5p expression increased after transfection of the miR-384-5p mimic and decreased by ~80% after induction of miR-384-5p inhibition in BMSCs. Conversely, expression of *Lepr* mRNA decreased after transfection of the miR-384-5p mimic but increased after miR-384-5p inhibition in BMSCs (Figure 4F).

miR-384-5p affected osteogenic differentiation of BMSCs through regulation of Lepr expression

To further explore expression of miR-384-5p and Lepr in bone metabolism induced by MDP, we simulated the HFD environment with a HG medium and the LFD environment with a LG medium. We simulated osteocytes and BMSCs *in vitro*. As described in our previous research [24], we first cultured osteocytes in the two abovementioned culture environments, and the supernatant was extracted after 36 h of intervention with MDP. Next, the extracted supernatants were assigned into four groups: (a) LG medium control (L-NC), (b) LG medium with MDP (L-MDP), (c) HG medium control (H-NC), and (d) HG medium with MDP (H-MDP). Next, we used the supernatants of the four groups stated above to interfere with the osteogenic and adipogenic induction of BMSCs, and carried out related ALP staining or Oil Red O staining in BMSCs (Figure 5A). The ALP content in the L-MDP group was increased compared with that in L-NC group. Likewise, the ALP content in the H-MDP was enhanced compared with that in the H-NC group. ALP staining was strongest in the H-MDP group. Interestingly, the result of Oil Red O staining after adipogenic induction of BMSCs was exactly the opposite to that observed with ALP staining.

We also measured expression of Lepr and miR-384-5p after interventions using extracted supernatants in BMSCs (Figure 5B, C). Expression of Lepr protein was consistent with that of the mRNA expression. Lepr expression increased with MDP induction when compared with that in their respective NC groups. In the H-MDP group, Lepr expression was the highest among the four groups mentioned above. Based on the RT-qPCR results, miR-384-5p expression showed the opposite results compared to Lepr expression. Our results implied that miR-384-5p affected osteogenic differentiation by regulation of Lepr expression.

4. Discussion

We detected changes in the blood glucose level, body weight, and adipose tissue of mice on different diets, with or without MDP, and found an association of energy metabolism with MDP. We also observed drastically increased *Lepr* expression in the alveolar bone and femur in the H-MDP group compared with that in the H-NaCl group and LFD group. However, expression of *Lepr* in adipose tissue was opposite to that in bone tissue. Moreover, the bone mass of mice was decreased after MDP intervention in the LFD group, whereas, in the HFD group, the bone mass of mice increased upon MDP intervention. Finally, we used MDP to interfere with osteocytes cultured under HG and LG conditions, and extracted supernatants to act on BMSCs. We found that miR-384-5p could regulate *Lepr* expression and osteogenic differentiation in BMSCs. This result suggested an important role for miR-384-5p in regulating the energy metabolism and bone formation of mice after MDP intervention.

According to previously described protocols [31], the final MDP injection was administered 24 h before phenotyping of metabolic and inflammatory mediators. In the HFD model, there was no significant change in the blood glucose levels of mice. In the LFD model, MDP injection could reduce the blood glucose level. This result is consistent with observations from studies indicating that inflammation in an animal model is also induced by HFD [32]. In addition, excessive intake of calories usually leads to glucose intolerance and insulin resistance [33].

Mammals have two types of adipose tissue: WAT and BAT [34]. Some commonly studied WATs in rodent models are scWAT and vWAT [35]. WAT stores energy in the form of lipids. BAT uncouples oxidative phosphorylation with ATP production through brown adipocyte-specific uncoupling protein-1, and consumes energy directly in the form of calories [36]. BAT can also improve insulin sensitivity and regulate glucose homeostasis [37].

H&E staining of iBAT showed that the energy supply of adipose tissue increased after MDP intervention in the LFD group, but decreased in the HFD group. However, in the scWAT group, fat droplets became larger and energy storage of adipose tissue increased after MDP intervention in the LFD group, whereas, in the HFD group, “browning” of white fat (which can resist obesity-related harm) was observed. In the WAT group, there was no significant difference between the LFD group and HFD group after MDP intervention, but the LFD group had smaller fat droplets and infiltration of more inflammatory cells, which revealed a healthier state than the HFD group. Owing to the heterogeneity and plasticity of adipocytes, the morphology and function of white adipocytes and brown adipocytes changes if the energy demand changes [38, 39]. Brown adipocytes develop and appear in WAT (browning) [40]. In the LFD group after MDP intervention, the energy supply of BAT decomposition would be greater than that of WAT which, in turn, would promote the energy consumption and weight loss of mice. In contrast, HFD is associated with a state of inflammation, which would cause disorder of the metabolic function of mice. Hence, MDP injection seemed to change the energy metabolism of mice and be more beneficial in the LFD group. Therefore, we attempted to analyze the reason for this effect.

Studies have shown that osteocytes can stimulate the osteogenesis of BMSCs. If BMSCs are co-cultured with osteocytes, the key indicators of osteogenic differentiation—calcium deposition and ALP levels—are increased significantly[22].

Previously, we showed that MDP was beneficial to LFD mice, and we focused on its beneficial mode of action. Hence, we examined the protective effect of MDP intervention on the health of mice under normal conditions. Therefore, we used MDP to interfere with osteocytes and identified differentially expressed genes related to energy metabolism by high-throughput PCR arrays: *Lepr* expression was increased significantly after MDP intervention. We measured *Lepr* expression in serum and found that MDP intervention increased *Lepr* expression in the LFD group, but there was no significant change in the HFD group. Leptin acts as a mediator of inflammation and energy balance by activating *Lepr* [41]. Studies have shown changes in production of leptin and its receptor during acute infection and inflammation [42]. Gregor et al. showed that obese mice on an HFD had chronic low-grade inflammation of adipose tissue and the liver [43]. The binding of *Lepr* and leptin in various peripheral tissues is involved in lipid metabolism, pancreatic-cell function, hematopoiesis, and thermogenesis [44, 45]. Hence, there may have been a dynamic imbalance of *Lepr* expression in the HFD group, which resulted in no significant change of *Lepr* expression in the blood circulation.

In addition to determining *Lepr* expression in the serum of mice treated with MDP under different dietary conditions, we also found that *Lepr* expression in osteocytes increased. Simultaneously, because of regulation of osteoblasts and BMSCs by osteocytes, we also focused on *Lepr* expression in these two types of cells. *Lepr* expression was also increased. Expression of *Lepr* protein in the LFD group treated with MDP showed strong immunohistochemical staining, which was consistent with the results of our high-throughput PCR arrays (Figure 2F). These data suggested that we should pay attention to the beneficial mode of action of MDP, and that a combination of leptin and its receptor is involved in regulation of energy metabolism.

L-MDP mice had a reduction of bone mass compared with that in L-NaCl mice. However, in the HFD group, the bone mass of the MDP-intervention group was higher than that of the control group. This phenomenon may be related to the multi-lineage potential of BMSCs [46]. If the balance between adipocyte differentiation and osteoblast differentiation is disrupted, adipocyte differentiation is increased compared with osteoblast differentiation, which is related to decreased bone mass, increased bone brittleness, and increased susceptibility to fracture [47]. We found that the LFD group was in good health, and that the energy supply after iBAT decomposition increased after MDP intervention (Figure 1G), which led to a decrease in the energy supply of bone tissue, and resulted in the weakening of osteogenesis and a relative decrease in bone mass. In the H-MDP group, mice were unhealthy, and the lipid droplets in iBAT were larger after MDP treatment, suggesting that adipose tissue was reduced to provide energy for decomposition, so bone tissue was needed for additional energy supply.

Bone is an endocrine organ that can regulate metabolism and energy balance during osteogenesis [48, 49]. Hence, an increase in the energy supply of bone tissue resulted in osteogenesis enhancement, which also led to a relative increase in bone mass in the H-MDP group. In the LFD group, more adipocytes were seen in H&E-stained femurs after MDP intervention (indicating that adipogenic differentiation of BMSCs was enhanced), whereas, in the HFD group, adipogenic differentiation was decreased after MDP intervention (indicating that adipogenic differentiation of BMSCs was reduced). These data are consistent with our abovementioned results. Morrison et al. demonstrated that *Lepr* is a sensor for whole-body energy homeostasis, because it

regulates BMSCs, promotes lipogenesis, and inhibits osteogenesis in response to diet and obesity [26]. Therefore, MDP intervention may be through a change in Lepr expression, which then leads to changes in bone metabolism.

Bioinformatics analysis and Dual luciferase reporter assay revealed that miR-384-5p targets Lepr through expression of specific binding sites. miR-384-5p transfection showed that miRNA could inhibit Lepr expression, and that MDP intervention could promote Lepr expression by inhibiting mir-384-5p expression during the osteogenic induction of BMSCs. Consistent with the abovementioned animal-model experiments, Lepr expression in serum and bone tissue increased after MDP intervention. In adipose tissue, expression of Lepr protein was inhibited in the H-MDP group, likely due to the impact of a more inflammatory environment.

Osteocytes can regulate the multi-lineage potential of BMSCs [22]. To further explore how MDP regulates bone formation, we extracted the supernatants of osteocytes after MDP intervention, and interfered with the osteogenic differentiation and adipogenic differentiation of BMSCs. We simulated the HFD environment with an HG medium and the LFD environment with an LG medium in the MLO-Y4 (for osteocytes) cell line. After that, we used the above four groups of supernatants to interfere with the osteogenic and adipogenic induction processes of BMSCs. The ALP content in the LG group intervened with MDP was increased more than that in the NC group, and a similar effect was observed in the HG group. Lepr was expressed on the surface of BMSCs [50] and leptin stimulation of BMSCs *in vitro* was shown to promote mineralization and partly inhibit adipogenic differentiation by binding to Lepr [51]. Therefore, MDP intervention could increase Lepr expression and further promote its ability to enhance the osteogenic differentiation of BMSCs, which also meant that the ability of adipogenic differentiation of BMSCs decreased. Moreover, expression of Lepr protein and mRNA in the four groups of BMSC cells revealed that the inhibitory effect of MDP on miR-384-5p promoted Lepr expression which, in turn, enhanced the osteogenic-differentiation ability of BMSCs.

However, we did not directly evaluate how the supernatants of osteocytes acted on BMSCs after MDP intervention, which may be the result of extracellular-vesicle transduction of miRs on BMSCs [52]. Recent studies have shown that miR-185^{-/-} mice can promote osteogenic differentiation in osteoporosis through the BMP/Smad pathway and inhibit bone loss, thereby revealing the effect of miR inhibition on bone protection [53]. Hence, conditional knockout of miR-384-5p expression may need to be improved further.

5. Conclusions

The supernatants of osteocytes treated with MDP may regulate Lepr expression in BMSCs through miR-384-5p, and then affect the osteogenic differentiation of BMSCs. Our findings indicate a possible mechanism of periodontitis-associated alveolar bone changes caused by the main components of microflora.

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Author contributions Yun-Zhi Feng designed and conducted the study. Shao-Hui Zhang and Yue Guo wrote the manuscript and conducted the animal experiments and cell-culture experiments. Shao-Hui Zhang conducted western blotting and RT-qPCR experiments. Yun Chen and Qiong Liu conducted micro-CT experiments. Li Tan, Zheng-Rong Gao, Jie Zhao and Ya-Qiong Zhao undertook bioinformatics analysis. Yao Feng, Jing Hu and Qin Ye conducted tissue experiments. Ying-Hui Zhou used computer software to record experiments. The first draft of the manuscript was written by Shao-Hui Zhang and Yue Guo and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript

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Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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Figure legends

Figure 1. MDP regulates diet-induced glucose homeostasis and adipogenesis.

(A and B) Experimental design for MDP administration of a HFD (A) and LFD (B).
(C and D) Change in blood glucose level of HFD-fed (C) and LFD-fed (D) male mice treated with MDP for 10 days. $n=3-5$ mice for each group.
(E and F) Bodyweight evaluation of HFD-fed (E) and LFD-fed (F) male mice treated with MDP for 10 days. $n=3-5$ mice for each group.
(G) Representative staining (hematoxylin and eosin) images of iBAT, scWAT, and vWAT from the differently treated mice (Scale bar, 20 μm ; from 3 mice per group). Data are presented as mean $\pm$ SD.

Figure 2. Lepr participates in the changes of energy metabolism induced by MDP.

(A) Heatmap showing differential expression of energy metabolism-related genes in osteocytes after MDP intervention.
(B) and (C) Differential expression of mRNAs in osteocytes based on KEGG and GO databases.
(D) Lepr expression in serum of mice in the four groups.
(E) Representative staining (immunohistochemical) images of iBAT, scWAT, and vWAT from the four groups. Scale bar, 20 μm .
(F) Staining (immunohistochemical) for Lepr in the femur and alveolar bone in mice of the four groups. Scale bar, 20 μm . * $P<0.05$. Data are presented as mean $\pm$ SD from 3 mice per group.

Figure 3. Effect of MDP on the mass and quality of mice in male mice fed a HFD or LFD.

(A and B) Representative sagittal three-dimensional reconstructions of micro-CT images from the mandible (A) and femur (B) of L-NaCl, L-MDP, H-NaCl, and H-MDP groups.
(C) Bone histomorphometry and images of mice from the four groups by micro-CT. BV/TV, bone volume/total tissue volume; BS/BV, bone surface/bone volume; Tb.Sp, trabecular spacing; Tb.Th, trabecular thickness; Tb.N, trabeculae number.
(D) Representative staining (immunohistochemical) images of the femur of mice from the four groups. Scale bar (left), 1000 μm . Scale bar (right), 500 μm . * $P<0.05$. Data are presented as mean $\pm$ SD from 3 mice per group.

Figure 4. miR-384-5p contained Lepr targets and modulated Lepr expression *in vitro*.

(A) Prediction using bioinformatics analysis of the target site of mmu-miR-384-5p with Lepr-3'UTR applied by TargetScan.
(B) Sequencing results for Lepr-3'UTR (WT) and mut-Lepr-3'UTR (MUT). The red rectangle represents the binding site in WT and core mutation site in MUT.
(C) The luciferase expression vector psiCHECK-2 was used to detect the activity of the luciferase reporter gene.
(D) Dual luciferase reporter assay in HEK293T cells. * $P<0.05$.
(E) During the osteogenic differentiation of BMSCs, Lepr expression was upregulated, but miR-384-5p expression was downregulated. * $P<0.05$.
(F) Expression of miR-384-5p and Lepr mRNA in BMSCs after transfection. * $P<0.05$.

Figure 5. miR-384-5p affected osteogenic differentiation of BMSCs through regulation of Lepr expression.

(A) Representative images of alkaline phosphatase (ALP) and Oil Red O staining of BMSCs induced by the supernatants of osteocytes with HG or LG medium after MDP intervention.

(B) Protein expression of Lepr induced by the supernatants of osteocytes with HG or LG medium after MDP intervention. *P<0.05.

(C) Changes in expression of Lepr and miR-384-5p mRNA induced by the supernatants of osteocytes with HG or LG medium after MDP intervention. *P<0.05.

Figures

Fig.1 MDP regulates diet-induced glucose homeostasis and adipogenesis (A and B) Experimental design for MDP administration of HFD (A) and LFD feeding (B). (C and D) Blood glucose change of HFD-fed(C) and LFD-fed(D) male mice treated with MDP for 10 days. n = 3-5 mice for each group. (E and F) Body weight evaluation of HFD-fed(E) and LFD-fed(F) male mice treated with MDP for 10 days. n = 3-5 mice for each group. (G) The representative hematoxylin and eosin staining images of iBAT, scWAT, and vWAT from the differently treated mice (Scale bar,20μm ; from three mice per group). Data are presented as mean±SD.

Figure 1

Please See image above for figure legend.

Fig.2 Lepr participates in the changes of energy metabolism induced by MDP
 (A) The heatmap of differential expression of energy metabolism related genes in osteocytes after MDP intervention.
 (B) and (C) KEGG and GO analyses of differentially expressed mRNAs in osteocytes.
 (D) Serum Lepr level of the four groups
 (E) The representative Immunohistochemical staining images of iBAT, scWAT, and vWAT from four groups. Scale bar, 20 μ m
 (F) Immunohistochemical staining of femur and alveolar bone for Lepr in the four groups. Scale bar, 20 μ m. *P < 0.05, Data are means plus or minus SD from 3 mice per group.

Figure 2

Please See image above for figure legend.

Fig.3. Effect of MDP on bone mass and quality in male mice placed on HFD or LFD
A and B. Representative sagittal three-dimensional reconstructions of micro-CT images from mandible(A) and femur(B) of L-NaCl,L-MDP,H-NaCl and H-MDP groups
C. Bone histomorphometric analyses and images of the four groups by μ CT. Abbreviations: BV/TV, bone volume/total tissue volume; BS/BV, bone surface/bone volume; Tb.Sp, trabecular spacing; Tb.Th, trabecular thickness; Tb.N, trabecular number.
D. The representative immunohistochemical staining images of femur from four groups. Scale bar(left),1000 μ m. Scale bar(right),500 μ m. * $P < 0.05$, Data are means plus or minus SD from 3 mice per group.

Figure 3

Please See image above for figure legend.

Fig.5 miR-384-5p affected osteogenic differentiation of BMSC through regulating Lepr

A. Representative images of alkaline phosphatase(ALP) and oil red o staining of BMSC cells induced by the supernatant of osteocytes with HG or LG medium after MDP intervention

B. Protein level of Lepr induced by the supernatant of osteocytes with HG or LG medium after MDP intervention* $P < 0.05$.

C. Changes in Lepr and miR-384-5p mRNA induced by the supernatant of osteocytes with HG or LG medium after MDP intervention. * $P < 0.05$.

Figure 5

Please See image above for figure legend.