

Loss of chemerin triggers bone remodeling in vivo and in vitro



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ABSTRACT

Objective: It was reported that chemerin as an adipocyte-secreted protein could regulate bone resorption and bone formation. However, the specific molecular and gene mechanism of the chemerin role is unclear. The aim of this study is to evaluate the role of chemerin in bone metabolism.

Methods: In the present study, we investigated the effects of chemerin on bone remodeling in *rarres2* knockout (*Rarres2*^{-/-}) mice and examined the role of chemerin as a determinant of osteoblast and osteoclast differentiation in Mc3t3-E1 and Raw264.7 cell lines.

Results: The results showed that the bone mineral density and volume score, trabecular thickness, weight and bone formation marker BALP increased, but Tb.Sp and bone resorption marker TRACP-5b decreased in *Rarres2*^{-/-} mice. Furthermore, the mRNA and protein expression of biomarkers of osteoblasts (β -catenin, RANKL and OPG) significantly increased, but those of osteoclasts (CTSK and RANK) decreased in *Rarres2*^{-/-} mice. In vitro, chemerin markedly suppressed β -catenin and OPG, but increased RANKL, CTSK and RANK expression. Moreover, knockdown of chemerin using RNA interference enhanced osteoblastogenesis genes and inhibited osteoclastogenesis genes in Mc3t3-E1 and Raw264.7 cells.

Conclusions: Taken together, these data suggest an inhibitive effect of chemerin on osteoblast differentiation and proliferation through inhibition of Wnt/ β -catenin signaling, as well as a stimulative effect of chemerin on osteoclast differentiation and proliferation via activation of RANK signaling. The maintenance of a low chemerin level may be a strategy for the prevention and treatment of osteoporosis.

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Keywords Bone metabolism; Chemerin; Metabolic dysfunction

1. INTRODUCTION

It is well known that healthy bone maintains a well-orchestrated ongoing balance between the osteoblast-induced bone formation and osteoclast-induced bone resorption [6,15]. Enhanced bone marrow adipogenesis is accompanied by an inhibition of osteoblastogenesis and bone formation [10]. Thereby, overweight and obesity may disrupt the steady state of bone equilibrium, leading to metabolic bone dysfunction and osteoporosis [13]. The increase in body fat and secretion of adipokines, such as chemerin, indicates a high risk of developing osteoporosis in patients with obesity [6,13,15]. Chemerin, encoded by the gene *Rarres2*, is a novel adipocyte-derived signaling molecule highly expressed in adipose tissue, liver, placenta, intestine, pancreas, adrenal gland and ovary [4]. It performs a variety of

biological functions, such as modulation of inflammation, adipogenesis and energy homeostasis [2,4,11].

Clinical studies have indicated that higher plasma chemerin levels were found in patients with metabolic syndrome, obesity and osteoporosis [3,7,16]. The chemerin concentrations were positively correlated with a risk of osteoporotic fracture and negatively correlated with the bone mineral density (BMD) and stiffness index in obese women [3,7,16]. The experimental evidence demonstrated that chemerin regulated osteoblast differentiation without affecting osteoclasts [10,12]. Additionally, chemerin/chemokine-like receptor 1 (CMKLR1) balanced adipogenic and osteoblastogenic differentiation of bone marrow stromal cells (BMSCs) through inhibition of Wnt/ β -catenin signaling [10]. In contrast, chemerin induced bone loss in vivo by regulating osteoclastic bone resorption through extracellular-regulated kinase 5 (ERK5)

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Abbreviations

BALP	Bone alkaline phosphatase
BMD	Bone mineral density
BMSCs	Bone marrow stromal cells
BV/TV	Bone volume/tissue volume
CMKLR1	Chemokine-like receptor 1
CTSK	Cathepsin K
DMEM	Dulbecco's modified eagle medium
ERK5	Extracellular regulated kinase 5
FBS	Fetal bovine serum
OPG	Osteoprotegerin
PVDF	Polyvinylidene fluoride
Rarres2	Retinoic acid receptor responder protein 2
RANK	Receptor activator of nuclear factor-kappa B
RANKL	Receptor activator of nuclear factor-kappa B ligand
Runx2	Runt-related transcription factor 2
Tb.Sp	Trabecular separation
TBST	Tris-buffered saline with 0.1% Tween-20
Tb.Th	Trabecular thickness
TRACP-5b	Tartrate-resistant acid phosphatase 5b
WT	Wild type

phosphorylation without affecting osteoblastic bone formation [10,12,14]. Although it is known that chemerin affects bone metabolism in vitro and in vivo, the specific molecular and gene mechanisms of chemerin effects on bone remodeling remains largely unknown. In this study, we addressed the molecular basis of chemerin action on bone remodeling in the global *rarres2* knockout mice in vivo as well as Mc3t3-E1 and Raw264.7 cell lines of chemerin knockdown in vitro.

2. MATERIALS AND METHODS

2.1. Drugs and reagents

Trizol reagent (Cat. No. R401-01) was obtained from Vazyme Biological Technology Corporation, Nanjing, China. RIPA lysis buffer (Cat. No. 01408/30450) was purchased from Kangwei Century Corporation, Beijing, China. Antibodies against receptor activator of nuclear factor-kappa B ligand (RANKL) (Cat. No. 23408-1-AP) and β -catenin (Cat. No. 17565-1-AP) were purchased from Proteintech, Wuhan, China. Antibodies against β -actin (Cat. No. bs-0061R), osteoprotegerin (OPG) (Cat. No. bs-20624R), receptor activator of nuclear factor-kappa B (RANK) (Cat. No. bs-2695R) and cathepsin K (CTSK) (Cat. No. bs-1611R) were acquired from Boaoseng, Beijing, China. Bone alkaline phosphatase (BALP) and tartrate-resistant acid phosphatase 5b (TRACP-5b) ELISA kits (Cat. No. 09/2020) were purchased from Meilian, Shanghai, China. The RANKL ELISA kit (Cat. No. YFXEM00536) was purchased from YIFEIXUE BIOTECH, Nanjing, China. Dulbecco's Modified Eagle Medium (DMEM) (Cat. No. RNBH5726) was purchased from Sigma—Aldrich, St. Louis, MO. Fetal bovine serum (FBS) (Cat. No. 42F3495K) and Opti-MEM (Cat. No. 2185849) were purchased from Gibco, Shanghai, China. Recombinant Mouse Chemerin (Cat. No. MQZ0317111) was purchased from R&D Systems, Inc. USA. The siRNA oligos against mouse chemerin were purchased from TranS-heep Bio, Shanghai, China. The TRAP/ALP stain kit (Cat. No. SKK0946) was purchased from Wako, Inc.

2.2. Animals

The *Rarres2*^{-/-} mice were purchased from View Solid Biotech, Beijing, China (No. FW20180629-01ZXC). Twelve-week-old female

C57BL/6J mice (wild type, $n = 8$) and C57BL/6J *Rarres2* knockout (*Rarres2*^{-/-}, $n = 8$) mice were kept in a standard laboratory condition of temperature 23 ± 2 °C, relative humidity $50 \pm 15\%$ and 12-h light—dark cycles with standard water and diet. The animal experiments were approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine (No. 201910A009).

The body weight of all mice was measured at 6:00 pm every Friday for 8 weeks. Then, after fasting for 12 h, 0.7 mL blood was collected from all the 20-week-old female mice under anesthesia. The blood was centrifugated at 3500 RPM for 15 min to isolate the serum. Additionally, the bilateral femurs and tibiae were carefully separated and stripped of peripheral soft tissue. Visceral fats of all mice were weighed. The serum and bone samples were stored at -80 °C for further analysis.

2.3. Cell culture

Mc3t3-E1 cells were purchased from the Cell Bank of Type Culture Collection, Chinese Academy of Science (Shanghai, China) and maintained in α MEM with 10% FBS and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere with 5% CO₂. To induce Mc3t3-E1 cell differentiation, cells were cultured in osteogenic medium (0.1 mg/mL ascorbic acid, 10 mM β -glycerophosphate) for 7 days after they reached confluence. Culture medium was changed every second day. Recombinant chemerin was used to treat normal Mc3t3-E1 cells at 40 ng/mL for evaluation of the effect of chemerin on osteoblastogenesis. The Mc3t3-E1 cells were cultured on 12-well plates at a seeding density of 5×10^4 cells/well. After 24 h, transfection was performed with a 1:1 mixture of siRNA duplexes (with final concentration of 100 nM), using Lipofectamin RNAiMax. The siRNA sequences were 5'-GCCGGAGUGCACAAUCAAATT-3' and 5'-UUUGAUUGUGCA-CUCCGGCTT-3'. The silencing of chemerin was confirmed by qRT-PCR analysis after 24 h transfection. After the silencing of chemerin in Mc3t3-E1 cells, these cells were incubated with recombinant chemerin (40 ng/mL) for 24 h. Cells were then collected for western blot and qRT-PCR analysis.

Raw264.7 cells were purchased from the Cell Bank of Type Culture Collection, Chinese Academy of Science (Shanghai, China). To induce Raw264.7 cell differentiation, cells were stimulated with 50 ng/mL RANKL and maintained in DMEM with 10% FBS and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere with 5% CO₂. Culture medium was changed every second day. Recombinant chemerin was used to treat normal Raw264.7 cells at 10 ng/mL for evaluation of the effects of chemerin on osteoclastogenesis. For Raw264.7 cell transfection experiments, cells were cultured on 12-well plates at a seeding density of 1×10^5 cells/well. After 24 h, Raw264.7 cells were transfected with a 1:1 mixture of siRNA duplexes (with final concentration of 50 nM), using Lipofectamin RNAiMax. The siRNA sequences were 5'-UUGUGAGGUUGGAAUUUAATT-3' and 5'-UUAUUUCCAACCUCACAATT-3'. The silencing of chemerin was confirmed by qRT-PCR analysis after 24 h transfection. After the silencing of chemerin in Raw264.7 cells, these cells were incubated with recombinant chemerin (10 ng/mL) for 24 h. Cells were then collected for western blot and qRT-PCR analysis.

2.4. Cell co-culture

The transwell co-culture system (Cell Culture Inserts, 0.4 μ m, 24-well; Corning, USA) was assembled using 1×10^4 Mc3t3-E1 cells and 1×10^4 Raw264.7 cells. The Mc3t3-E1 cells were seeded in 24-well plates and maintained in α MEM with 1% penicillin-streptomycin and 10% FBS at 37 °C in a humidified atmosphere with 5% CO₂. Upon reaching confluence, differentiation was induced by treating the cells

with osteogenic differentiation inducers (0.1 mg/mL ascorbic acid, 10 mM β -glycerophosphate) for 4 days. The Raw264.7 cells were seeded in transwell plates and maintained in DMEM supplemented with 1% penicillin-streptomycin and 10% FBS at 37 °C in a humidified atmosphere with 5% CO₂. After confluence for 24 h, transwell plates containing Raw264.7 cells were moved to the corresponding 24-well plates containing Mc3t3-E1 cells to create the Raw264.7 cell/Mc3t3-E1 cell co-culture system. Culture medium was changed every second day. After 48 h of co-culture of Mc3t3-E1 cells and Raw264.7 cells, chemerin (40 ng/mL) was added into Mc3t3-E1 cells at the bottom of 24-well plates. The levels of osteoclastogenesis-related protein and mRNA in Raw264.7 cells were detected by western blot and qRT-PCR after Mc3t3-E1 cells were incubated with chemerin (40 ng/mL) for 24 h. In addition, the supernatants were collected after Mc3t3-E1 cell incubation with chemerin (40 ng/mL) for 0 h and 12 h, respectively. The protein levels of RANKL in the supernatants were detected by ELISA.

2.5. Micro-CT detection

Femur samples were cleaned and stored in 4% paraformaldehyde for 24 h. Then, all samples were dehydrated with 70% ethyl alcohol for 48 h. These samples were detected by micro-CT scanner at 10 μ m, 90 kV and 88 μ A resolution (Quantum GX, PerkinElmer, America). The trabecular bone was analyzed from 1 mm to 1 cm below the growth plate. Three dimensional (3D) images and bone mineral density were analyzed by Analyzer (PerkinElmer, America). Bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp) were also analyzed to evaluate bone mass.

2.6. Chemerin assay in the cell supernatant

Mc3t3-E1 cells and Raw264.7 cells were cultured in 24-well plates at a seeding density of 2×10^4 /well and 5×10^4 /well, respectively. The next day, all wells were replaced with fresh medium. After 12, 24, and 48 h, the chemerin of the supernatants from Mc3t3-E1 and Raw264.7 cells were measured with the competitive mouse chemerin ELISA kit. According to the manufacturer's specification, all measurements were performed in duplicate and the mean of the two measurements was considered. As with the presence of chemerin in the FBS of the culture medium, data of 0 h were subtracted from all groups during data statistics.

2.7. ALP and TRAP staining

The ALP and TRAP levels were measured with the TRAP/ALP stain kit (Cat. No. SKK0946). Briefly, the Mc3t3-E1 cells were washed with PBS 3 times and then treated with pre-cold ALP fixative for 10 min. Then Mc3t3-E1 cells were treated with ALP substrate at 37 °C for 30 min. Finally, the blue areas of Mc3t3-E1 cells were observed under a microscope. Image J software was used to quantify the blue areas. Raw264.7 cells were washed with PBS 3 times and treated with pre-cold fixative for 10 min. Cells were then treated with TRAP stain solution at 37 °C for 30 min. The nuclei were then stained with nuclear staining solution (blue) for 5 s and immediately washed with distilled water. Finally, the distribution of osteoclasts was observed under a microscope. Image J software was used to quantify the pink area.

2.8. Total RNA extraction and real-time PCR

Total RNA was extracted with Trizol (Vazyme) from bone tissues or cells according to the manufacturer's instructions. In brief, the femurs were ground with 30 mL liquid nitrogen in mortar until it became a powder. The powdered femurs were placed in 2-mL Eppendorf tubes containing 1 mL Trizol and mechanically disaggregated using the bead-based tissue lyser equipment (Retsch, MM 400, Verder Retsch Shanghai Trading Co., Ltd) to shake for 60 s at 30 Hz. After 5 min

centrifugation to remove tissue debris (12000g at 4 °C), the supernatant was collected for subsequent operation. In addition, the total RNA was isolated from cells with or without siRNA transfection using Trizol reagent. RNA concentration was calculated through spectrophotometric assays of 260/280 nm cDNA was synthesized from 1 μ g RNA using MMLV reverse transcriptase. Real-time quantitative PCR was utilized for mRNA detection via the Applied Biosystems 7500 instrument. The oligos primers were provided in Table 1. The amplification condition consisted of 40 cycles of an initial denaturation at 95 °C for 5 min, 95 °C for 10 s and 60 °C for 35 s. The 2^{- Δ CT} method was used to analyze the PCR data.

2.9. Western blot analysis

The total proteins of bone tissues or cells were extracted using RIPA agents and quantified with the BCA protein assay kit to determine protein levels. Briefly, the femurs were ground with 30 mL liquid nitrogen in mortar until it became a powder. The powder of femurs was placed in 2-mL Eppendorf tubes containing 0.5 mL RIPA lysis buffer and mechanically disaggregated using the bead-based tissue lyser equipment to shake for 60 s at 30 Hz. After the homogenates were centrifuged at 12000 g for 5 min (4 °C), the supernatant was collected for subsequent operation. Protein extracts from cells were prepared in RIPA lysis buffer containing phosphatase and proteinase inhibitors. Protein concentrations were determined with the BCA protein assay kit. The 40 μ g protein sample was separated in 10% SDS-PAGE gel at 30 mA for 90 min. Then, the protein was transferred to PVDF membranes at 300 mA for 90 min. Membranes were blocked with 5% skimmed milk for 1 h at room temperature, then probed overnight at 4 °C with antibodies against β -catenin (1: 2000), RANKL (1:1000), OPG (1:1000), RANK (1:1000), CTSK (1:1000) and β -actin (1:4000), respectively. The next day, the membranes were washed with 1 \times TBST for 10 min and incubated with the corresponding secondary antibody for 1 h at room temperature. Finally, the proteins were developed by ECL chemiluminescent liquid with Image Quant LAS 4000 mini and analyzed by Image J.

2.10. Statistical analysis

Data were analyzed by SPSS 25.0. Comparisons between the means of two groups were analyzed by two-tailed unpaired Student's t-test. Data were presented as mean \pm SEM with P < 0.05 considered statistically significant.

Table 1 — The oligonucleotide primers.

Gene	The oligonucleotide primers
<i>Chemerin</i>	Forward Sequence 5'-TACAGTGGCTCTGGAGGATTC-3' Reverse Sequence 5'-CTTCTCCCGTTGGTTTGATTG-3'
<i>RANKL</i>	Forward Sequence 5'-GCAGCATCGCTCTGTTCCGTGA-3' Reverse Sequence 5'-CCTGCAGGAGTCAGGTAGTGTGTC-3'
<i>OPG</i>	Forward Sequence 5'-CCGAGTGTGTGAGTGTGAGG-3' Reverse Sequence 5'-CCAGCTGCACCACCTCCAA-3'
<i>Runx2</i>	Forward Sequence 5'-CTGTGGTACCGTCATGGCC-3' Reverse Sequence 5'-GGAGCTCGCGGAGTAGTTC-3'
<i>BALP</i>	Forward Sequence 5'-CCTAGACACAGCACTAACA-3' Reverse Sequence 5'-GTCGATCAGGTTGTTCCGATTC-3'
<i>TRAP</i>	Forward Sequence 5'-CACTCCACCTGAGATTTG-3' Reverse Sequence 5'-CCCCAGAGACATGATGAAGTCA-3'
<i>RANK</i>	Forward Sequence 5'-AGAAGCACACCGGGGAGCAAC-3' Reverse Sequence 5'-ACAGAGATGAAGAGGAGCAGAACG-3'
<i>β-catenin</i>	Forward Sequence 5'-CCTAGCTGGTGGACTGCAGAA-3' Reverse Sequence 5'-CACCAGTGGCCAGAAATGATGA-3'
<i>GAPDH</i>	Forward Sequence 5'-GGTTGTCTCTCGCAGCTTCA-3' Reverse Sequence 5'-TGGTCCAGGGTTTCTTACTCC-3'

3. RESULTS

3.1. Bone mass, body weight and serum detection

To address the role of chemerin in bone metabolism, we first examined bone mass, bone mineral density (BMD), BV/TV, Tb.Th and Tb.Sp of femurs (Figure 1). Compared to wild-type (WT) mice, *Rarres2*^{-/-} mice possess a higher bone mass (Figure 1A and B). In *Rarres2*^{-/-} mice, compared with WT mice, the bone volume score, trabecular thickness, body weight, weight of visceral fat and bone formation marker BALP increased by 10.69% ($P < 0.01$), 62.5% ($P < 0.05$), 13.5% ($P < 0.01$), 57.0% ($P < 0.05$) and 14.1% ($P < 0.05$), respectively (Figure 1C, D, F, G and H), while Tb.Sp and the bone resorption marker TRACP-5b of femurs decreased by 12.8% ($P < 0.05$) and 6% ($P < 0.05$) (Figure 1E and I). These results indicate that chemerin is a negative regulator for bone remodeling.

3.2. Bone remodeling—related markers

To confirm the deletion of chemerin in *Rarres2*^{-/-} mice, we measured the protein and mRNA levels of chemerin in femurs. The *Rarres2*^{-/-} mice did not show any chemerin protein or gene expression in femurs

(Figure 2A and I). Next, to address the role of chemerin in bone remodeling, the mRNA and protein expression of biomarkers for osteoblasts (β -catenin, RANKL, OPG) and osteoclasts (CTSK, RANK) were measured in *Rarres2*^{-/-} mice. As shown in Figure 2C, E, F and G, the protein levels of RANKL, CTSK and RANK and the ratio of RANKL/OPG decreased by 24.4% ($P < 0.01$), 54.4% ($P < 0.01$), 48.8% ($P < 0.05$) and 33.3% ($P < 0.05$), while the osteogenesis marker genes β -catenin and OPG increased by 28.2% ($P < 0.01$) and 43.9% ($P < 0.05$) (Figure 2B and D) in *Rarres2*^{-/-} mice compared with WT mice. As shown in Figure 2K and N, the mRNA of TRAP and RANKL/OPG decreased by 43.3% ($P < 0.05$) and 50.1% ($P < 0.01$) while the mRNA of BALP, Runx2 and β -catenin increased by 376.3% ($P < 0.01$), 466.7% ($P < 0.01$) and 109.4% ($P < 0.01$) in *Rarres2*^{-/-} mice compared with WT mice (Figure 2J, L and M). Taken together, results suggest that chemerin not only increases osteoclast activity, but also decreases osteoblast function.

3.3. Effect of chemerin on bone formation in Mc3T3-E1 cells

To examine the inhibitive action of chemerin on osteoblastogenesis, we treated osteoblastic cells with chemerin and knocked down chemerin

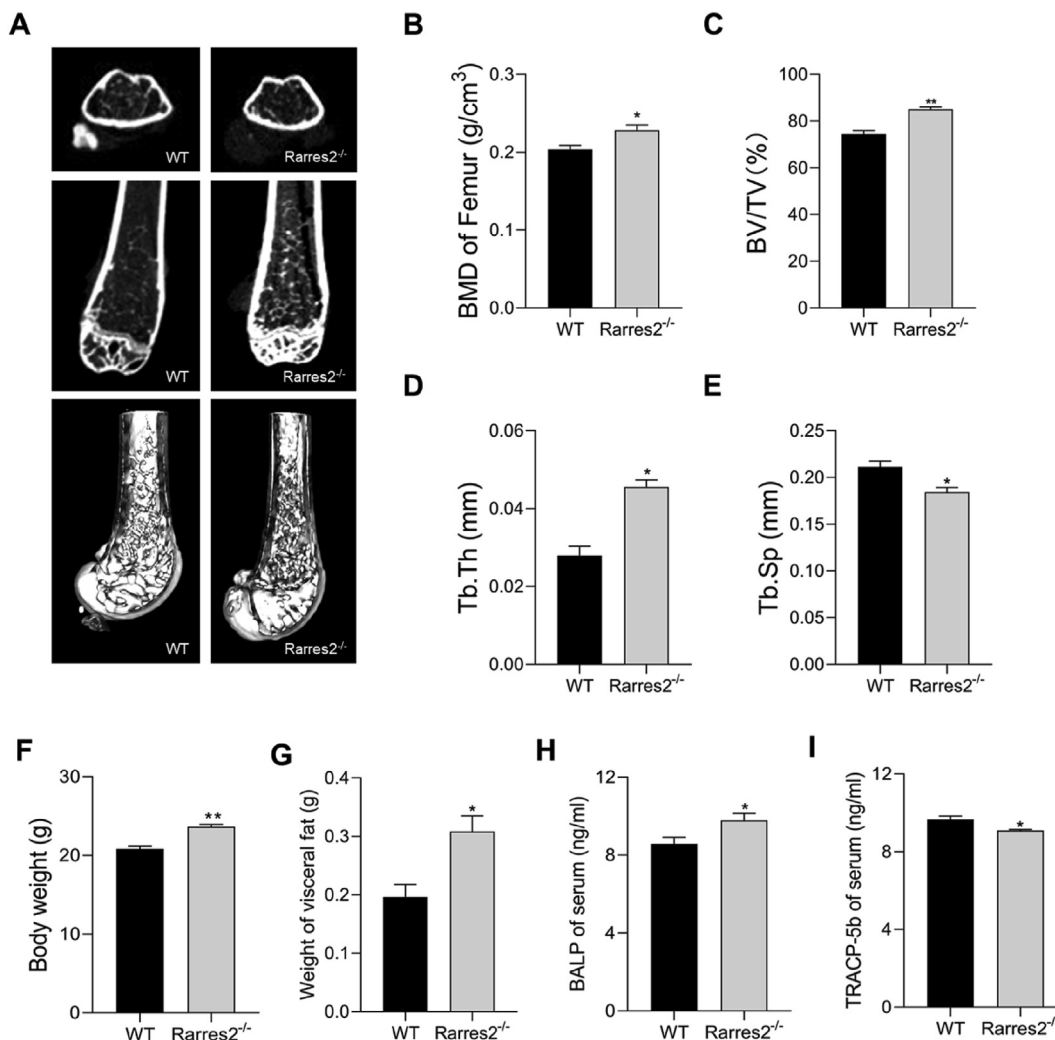


Figure 1: The BMD of femurs, BV/TV, Tb.Th, Tb.Sp, body weight, serum BALP and TRACP-5b in *Rarres2*^{-/-} mice compared to WT mice ($n = 3-7$). (A–B) Micro-ct detection of BMD in femurs of *Rarres2*^{-/-} mice compared to WT mice. (C–E) Analysis of BV/TV, Tb.Th and Tb.Sp in femurs of *Rarres2*^{-/-} mice compared to WT mice. (F–G) Comparisons of body weight and visceral fat between WT and *Rarres2*^{-/-} mice. (H–I) ELISA detection of BALP and TRACP-5b in serum of *Rarres2*^{-/-} mice compared to WT mice. The mice used were 20-week-old female mice. * $P < 0.05$ & ** $P < 0.01$ compared to WT mice.

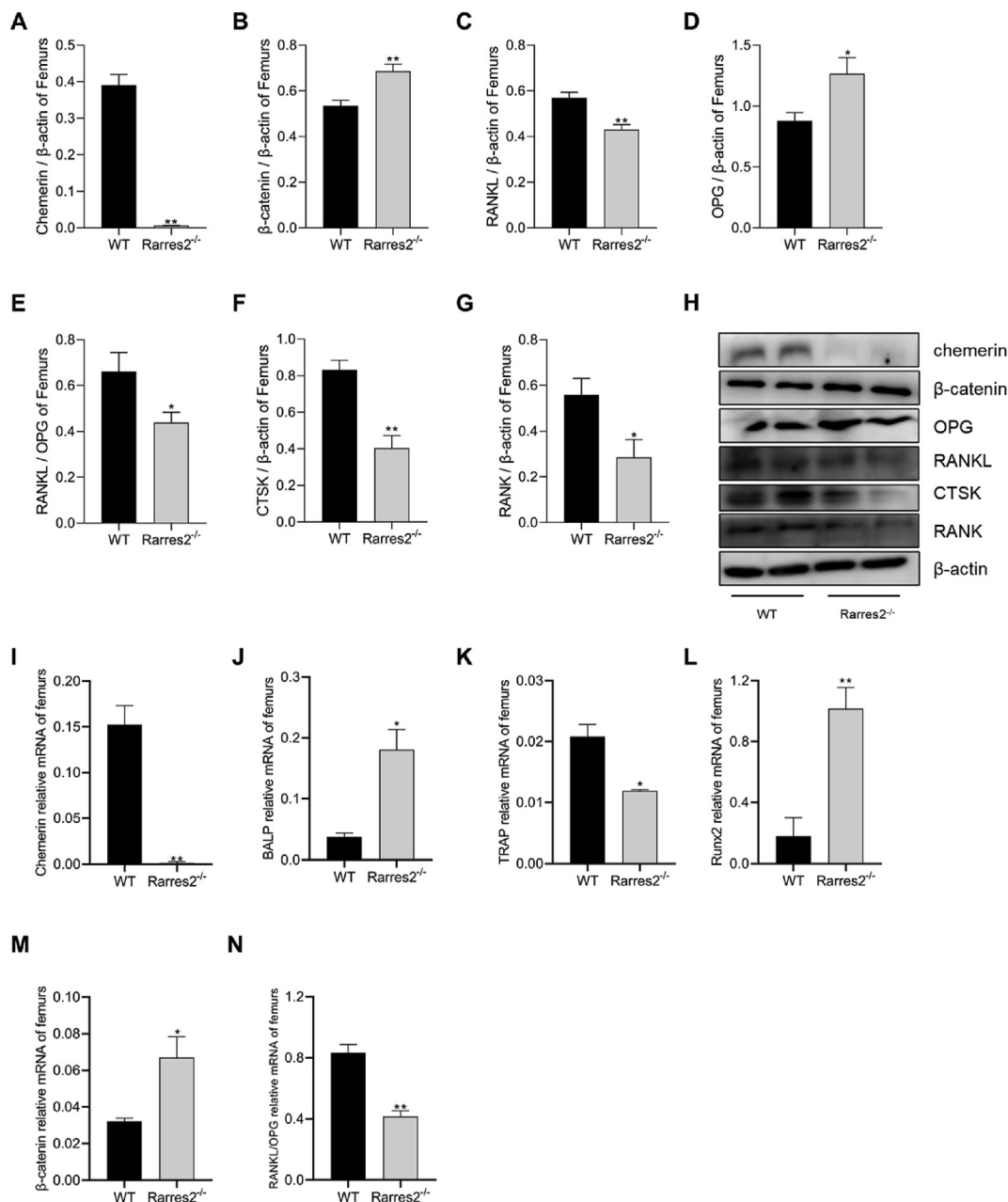


Figure 2: Protein and mRNA expression of bone remodeling markers in *Rarres2*^{-/-} mice compared to WT mice (n = 3–8). (A) Western blot analysis and respective density histograms of chemerin in femurs of *Rarres2*^{-/-} mice compared to WT mice. β -actin was used as the loading control. (B–E) Western blot analysis and respective density histograms of bone formation–related proteins, including β -catenin, RANKL, OPG and RANKL/OPG in femurs of *Rarres2*^{-/-} mice compared to WT mice. β -actin was used as the loading control. (F–G) Western blot analysis and respective density histograms of bone resorption–related proteins, including CTSK and RANK in femurs of *Rarres2*^{-/-} mice compared to WT mice. β -actin was used as the loading control. (H) Western blot analysis of chemerin, β -catenin, RANKL, OPG, CTSK and RANK in femurs of *Rarres2*^{-/-} mice compared to WT mice. β -actin was used as the loading control. (I) Real-time PCR analysis mRNA of chemerin in femurs of *Rarres2*^{-/-} mice compared to WT mice. (J–N) Real-time PCR analysis mRNA of BALP, TRAP, Runx2, β -catenin and RANKL/OPG in femurs of *Rarres2*^{-/-} mice compared to WT mice. * $P < 0.05$ & ** $P < 0.01$ compared to WT mice.

using an siRNA in vitro. First, we investigated the level of chemerin released in the culture supernatant of Mc3t3-E1 cells. As shown in Figure S1A, the levels of chemerin in Mc3t3-E1 cell supernatant were significantly increased at 12 h, 24 h and 48 h ($P < 0.01$) compared with control. Furthermore, in Mc3t3-E1 cells treated with chemerin compared to normal Mc3t3-E1 cells, the bone formative marker gene BALP, Runx2, β -catenin and OPG mRNA levels decreased by 56.7% ($P < 0.05$), 12.1% ($P < 0.05$), 22.4% ($P < 0.01$) and 61.4% ($P < 0.01$)

respectively, but the RANKL mRNA increased by 472.5% ($P < 0.01$) (Figure 3A–E). On the protein level, β -catenin and OPG decreased by 35.9% ($P < 0.01$) and 58.4% ($P < 0.05$) respectively, but RANKL significantly increased by 68.1% ($P < 0.05$) (Figure 3G–I). Moreover, the ratio of RANKL/OPG significantly increased by 558.5% and 220.6% ($P < 0.01$) at mRNA and protein levels, respectively (Figure 3F and J). In Mc3t3-E1 cells with chemerin silencing compared to normal Mc3t3-E1 cells, the efficiency of chemerin silence increased to 84.8%

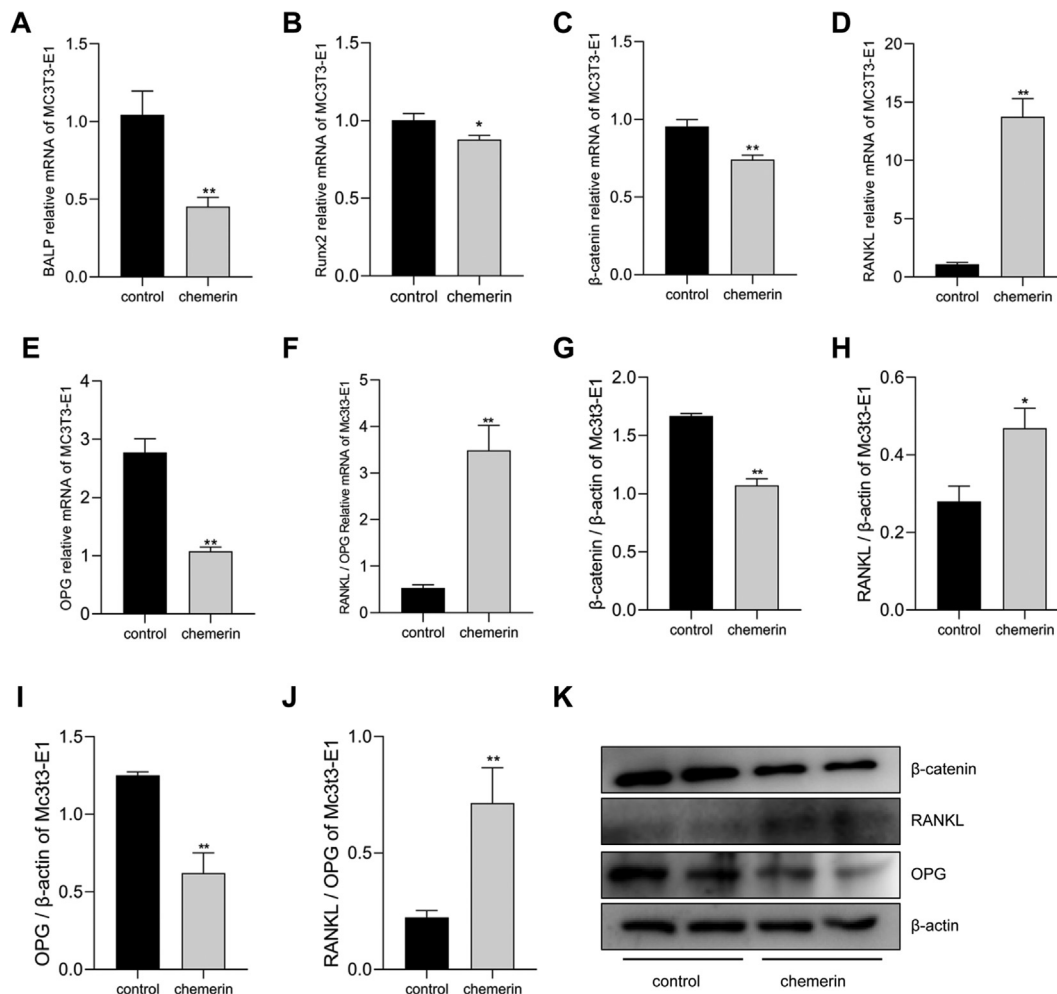


Figure 3: Chemoerin affected the expression of bone formation-related genes, including BALP, Runx2, β -catenin and the activation of the RANKL/OPG pathway in Mc3t3-E1 cells ($n = 3-7$). (A–F) Real-time PCR analysis mRNA of BALP, Runx2 and β -catenin, RANKL, OPG and RANKL/OPG in Mc3t3-E1 cells treated with chemoerin compared to normal Mc3t3-E1 cells. (G–J) Western blot analysis and respective density histograms of β -catenin, RANKL, OPG and RANKL/OPG in Mc3t3-E1 cells treated with chemoerin compared to normal Mc3t3-E1 cells. β -actin was used as the loading control. (K) Western blot analysis of β -catenin, RANKL, OPG and RANKL/OPG in Mc3t3-E1 cells treated with chemoerin compared to normal Mc3t3-E1 cells. Density values are expressed in arbitrary units. The data collected are expressed as the mean \pm SEM for the indicated proteins. * $P < 0.05$ & ** $P < 0.01$ compared to normal Mc3t3-E1 cells.

($P < 0.01$) (Figure 4A) and bone formation marker gene BALP, Runx2, β -catenin and OPG mRNA levels increased by 111.4% ($P < 0.01$), 68.0% ($P < 0.05$), 57.2% ($P < 0.05$) and 182.1% ($P < 0.05$), respectively, but RANKL mRNA decreased by 56.2% ($P < 0.05$) (Figure 4B–F). On the protein level, β -catenin and OPG increased by 32.7% ($P < 0.05$) and 150.0% ($P < 0.01$), respectively, but RANKL decreased by 35.8% ($P < 0.01$) (Figure 4H–J). Furthermore, the ratio of RANKL/OPG significantly decreased by 84.4% ($P < 0.01$) at the mRNA level and 69.9% ($P < 0.05$) at the protein level (Figure 4G and K).

To further investigate the inhibitive action of chemoerin on osteoblastogenesis, we treated chemoerin-silenced Mc3t3-E1 cells with chemoerin. In chemoerin-silenced Mc3t3-E1 cells treated with chemoerin (compared to chemoerin-silenced control cells), the bone formative marker gene BALP, Runx2 and β -catenin mRNA levels decreased by 78.8% ($P < 0.01$), 28.6% ($P < 0.05$) and 37.0% ($P < 0.05$), respectively, but RANKL/OPG mRNA increased by 304.4% ($P < 0.01$) (Figure S2A–D). Furthermore, the ALP staining was performed to analyze the early osteogenesis of Mc3t3-E1 cells in the absence or

presence of chemoerin, respectively. As shown in Figure 5A–C, the level of ALP increased by 29.6% ($P < 0.01$) in chemoerin-silenced Mc3t3-E1 cells compared with the normal control group, whereas it decreased by 22.8% ($P < 0.01$) in chemoerin-silenced Mc3t3-E1 cells treated with chemoerin compared with chemoerin-silenced control cells. Taken together, these results suggest that chemoerin affects bone formation through inhibition of Wnt/ β -catenin signaling and elevation of the RANKL/OPG ratio in osteoblasts.

3.4. Effect of chemoerin on bone resorption in Raw264.7 cells

To examine the stimulative actions of chemoerin on osteoclastogenesis, we further knocked down chemoerin using siRNA and treated osteoclastic cells with chemoerin in Raw264.7 cells. First, we investigated the level of chemoerin released in the culture supernatant of Raw264.7 cells. As shown in Figure S1B, the levels of chemoerin in Raw264.7 cell supernatant were significantly increased at 12, 24, and 48 h ($P < 0.01$) compared with the control. Furthermore, in Raw264.7 cells treated with chemoerin compared to normal Raw264.7 cells, the bone resorptive marker gene TRAP, CTSK and RANK mRNA levels

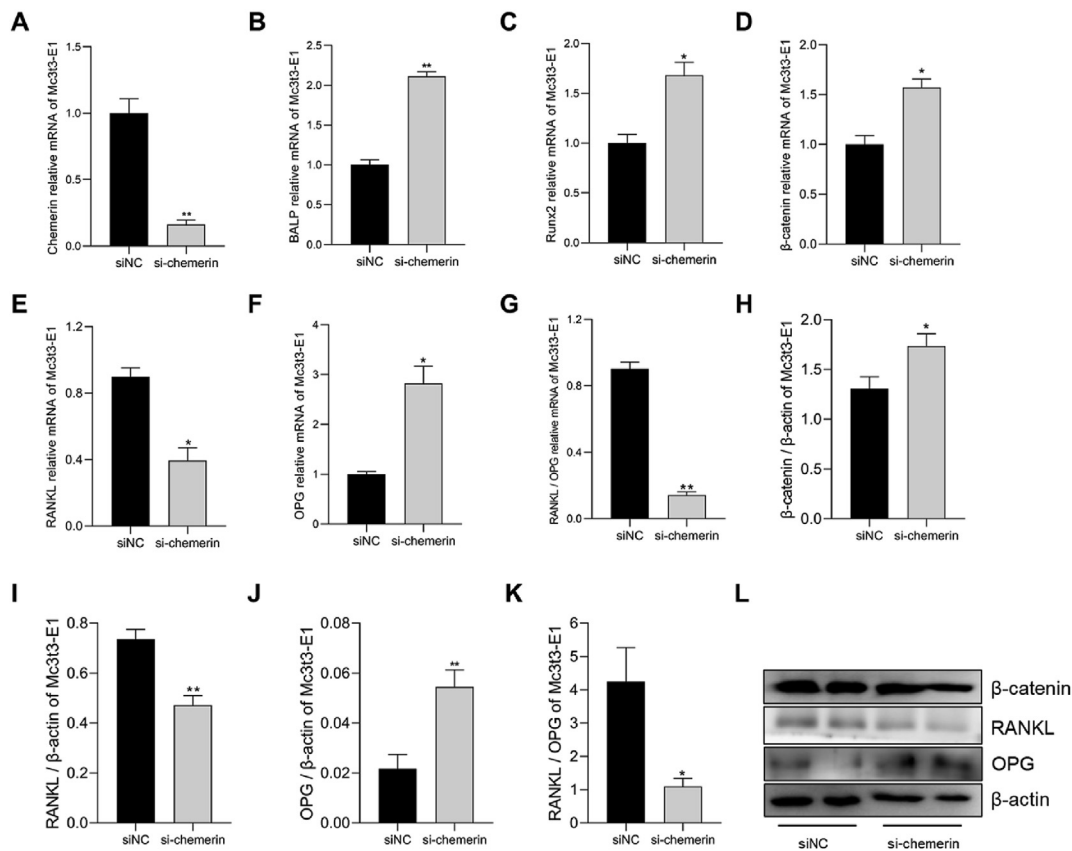


Figure 4: Chimerin silence affected the expression of bone formation-related genes, including BALP, Runx2, β -catenin and the activation of the RANKL/OPG pathway in Mc3t3-E1 cells ($n = 3-8$). (A–G) Real-time PCR analysis mRNA of BALP, Runx2 and β -catenin, RANKL, OPG and RANKL/OPG in si-chemerin Mc3t3-E1 cells compared to normal Mc3t3-E1 cells. (H–K) Western blot analysis and respective density histograms of β -catenin, RANKL, OPG and RANKL/OPG in si-chemerin Mc3t3-E1 cells compared to normal Mc3t3-E1 cells. β -actin was used as the loading control. (L) Western blot analysis of β -catenin, RANKL and OPG in si-chemerin Mc3t3-E1 cells compared to normal Mc3t3-E1 cells. β -actin was used as the loading control. Density values are expressed in arbitrary units. The data collected are expressed as the mean \pm SEM for the indicated proteins. * $P < 0.05$ & ** $P < 0.01$ compared to normal Mc3t3-E1 cells.

significantly increased by 150.8% ($P < 0.05$), 28.2% ($P < 0.01$) and 89.8% ($P < 0.01$), respectively (Figure 6A–C), and the CTSK and RANK protein levels significantly increased by 60% ($P < 0.05$) and 167.1% ($P < 0.01$), respectively (Figure 6D and E). In Raw264.7 cells with si-chemerin compared with normal Raw264.7 cells, the chimerin mRNA decreased by 74.4% ($P < 0.01$) (Figure 7A). The osteoclastogenesis marker gene TRAP, CTSK and RANK levels decreased by 44.3% ($P < 0.05$), 19.1% ($P < 0.05$) and 35.8% ($P < 0.05$), respectively (Figure 7B–D), and the CTSK and RANK protein levels decreased by 40.9% ($P < 0.01$) and 33.6% ($P < 0.05$), respectively (Figure 7E and F).

To further investigate the stimulative actions of chimerin on osteoclastogenesis, we treated chimerin-silenced Raw264.7 cells with chimerin. In chimerin-silenced Raw264.7 cells treated with chimerin compared to chimerin-silenced control cells, the bone resorptive marker gene TRAP, RANK and CTSK mRNA levels increased by 21.4% ($P < 0.05$), 55.1% ($P < 0.01$) and 24.1% ($P < 0.05$), respectively (Figure S2E–G). Furthermore, TRAP staining was performed to analyze the number and distribution of osteoclasts in the absence or presence of chimerin, respectively. As shown in Figure 8, the level of TRAP decreased by 16.2% ($P < 0.01$) in chimerin-silenced Raw264.7 cells compared with the control group, whereas it increased by 17.0% ($P < 0.05$) in chimerin-silenced Raw264.7 cells treated with chimerin compared with chimerin-silenced control cells.

To address the molecular basis of the chimerin action on bone remodeling, we further treated Mc3t3-E1 cells with chimerin at different times. First, we investigated the level of RANKL released in the supernatants of the co-culture system after Mc3t3-E1 cell incubation with chimerin for 12 h. As shown in Figure 9B, the levels of RANKL in Mc3t3-E1 cell supernatant increased by 184.1% ($P < 0.05$) in the chimerin-treated group compared to the control group. Furthermore, we investigated osteoclastogenesis of Raw264.7 cells in the co-culture system after Mc3t3-E1 cell incubation with chimerin for 24 h. As shown in Figure 9C–F, bone resorption gene RANK and CTSK mRNA levels increased by 88.2% ($P < 0.05$) and 39.0% ($P < 0.05$) in the chimerin-treated group compared to the control group. Additionally, the protein levels of RANK and CTSK increased by 34.2% ($P < 0.01$) and 21.0% ($P < 0.05$) in the chimerin-treated group compared to the control group. These results suggest that treatment with chimerin in Mc3t3-E1 cells may enhance osteoclastogenesis of Raw264.7 cells in the co-culture system. Altogether, these results suggest that chimerin affects bone resorption through activation of RANKL/RANK signaling in osteoclasts.

4. DISCUSSION

Bone remodeling is a dynamic process requiring coordination between formative (osteoblast) and resorptive (osteoclast) cells. If the balance is

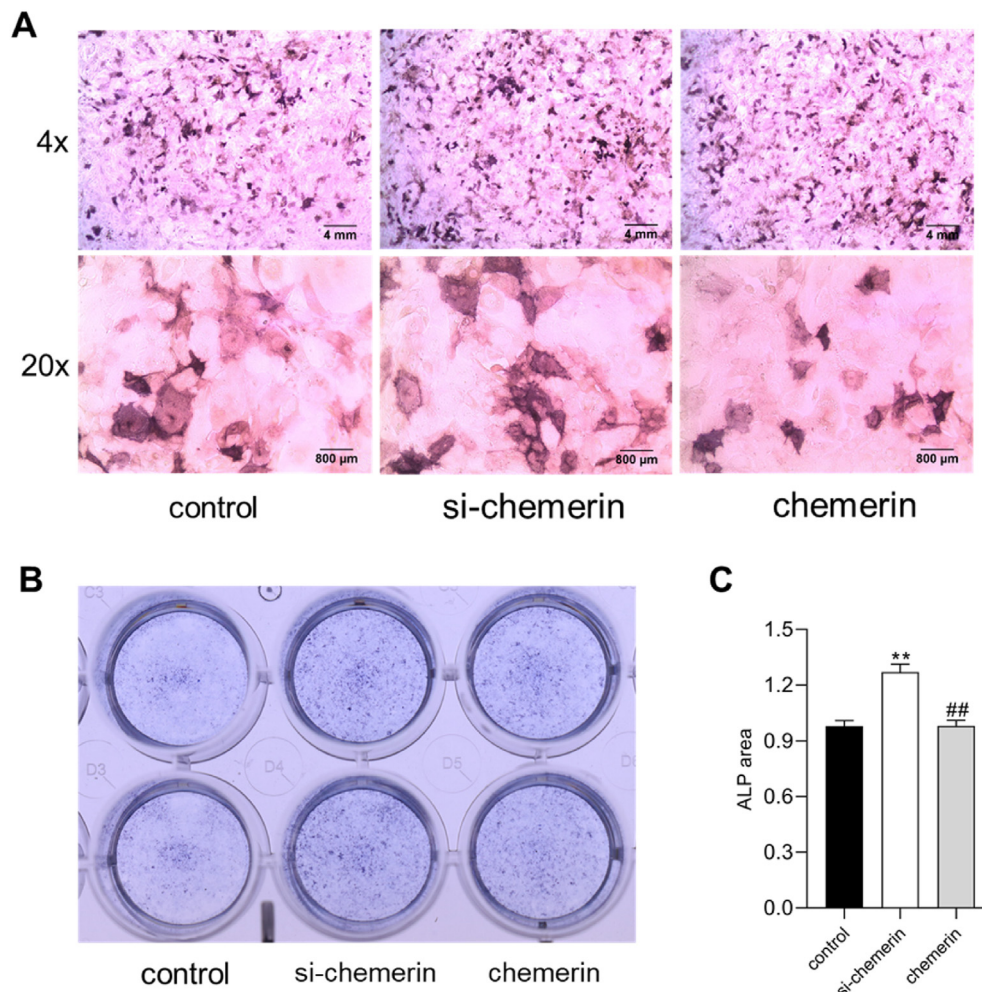


Figure 5: Levels of ALP staining in Mc3t3-E1 cells (n = 5). (A) ALP staining was performed to analyze the early osteogenesis of chemerin-silenced Mc3t3-E1 cells treated with chemerin. Photo capture was performed by a Nikon TE2000 microscope (4x and 20x). (B) ALP staining in 24-well plates. Photo capture was performed by a Canon 80D camera. (C) Histogram of ALP staining area. **P < 0.01 compared to normal Mc3t3-E1 cells. ##P < 0.01 compared to chemerin-silenced Mc3t3-E1 cells.

disrupted, it will lead to metabolic bone diseases such as osteoporosis [17]. Chemerin plays an important role in the pathogenesis of metabolic bone dysfunction via modulation of osteoblast and osteoclast differentiation and function [13]. In the present study, we investigated the role of chemerin in regulating bone metabolism in *rarres2* knockout (*Rarres2*^{-/-}) mice and examined the role of chemerin as a determinant of osteoblast and osteoclast differentiation in Mc3t3-E1 and Raw264.7 cell lines.

Previous studies showed that there were inverse relations between serum chemerin levels and lumbar and femoral BMD in obese postmenopausal women and elderly men and women [3,7,16]. Additionally, abrogation or knockdown of CMKLR1 increased the trabecular bone mass and expression of plasma osteoblast markers, including osterix, alkaline phosphatase, and type I collagen [8]. In accordance with these, we found that plasma BALP levels, bone mass, bone volume score, femur BMD and trabecular thickness increased, but plasma TRACP-5b levels and femur Tb.Sp were reduced in *Rarres2*^{-/-} mice compared to WT mice, suggesting that chemerin increases bone loss rather than bone mass. In addition, we found that body weight increased in *Rarres2*^{-/-} mice in comparison to WT mice. It is possible that the increased body weight of *Rarres2*^{-/-} mice is attributed to

increased visceral fat and bone mass. Our data corroborated previous reports that *Rarres2*^{-/-} mice gained weight and increased fat distribution in subcutaneous adipose tissue after a chronic high-fat diet [5]. Therefore, elevated visceral fat and bone mass could sufficiently explain the observed increase in body weight of *Rarres2*^{-/-} mice. The osteoblast-induced bone-forming activity is important in determining bone mass [18]. The β -catenin derives osteoblast precursor cells (BMSCs) to promote osteoblastogenesis of bone marrow and osteoblast differentiation via stimulation of osteoblastogenic transcription factors, including runx2 and osterix [1]. Chemerin promoted adipogenic differentiation and inhibited BMSCs and osteoblast differentiation [10,12,13]. Therefore, knockdown of chemerin or a decrease in CMKLR1 expression increased osteoblastogenesis of BMSCs in vitro [11,12]. Consistent with this, our study revealed that chemerin inhibited mRNA and protein expression of β -catenin and runx2 in mouse Mc3t3-E1 osteoblasts. We also investigated the effects of abrogation or knockdown of chemerin on the osteoblastogenesis of Mc3t3-E1 osteoblasts and mouse bone tissues. Our study revealed that abrogation or knockdown of chemerin led to increased mRNA and protein expression of β -catenin, runx2 and BALP in Mc3t3-E1 cells and mouse bone tissues. Furthermore, we found that the elevated

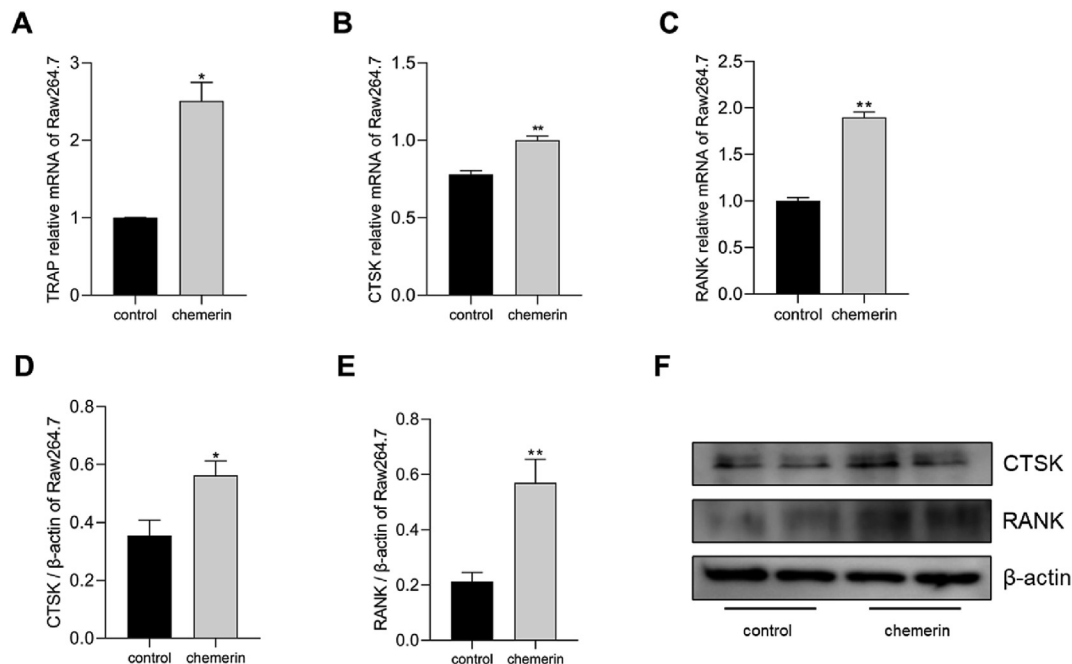


Figure 6: Chemerin affected the expression of bone resorption-related genes, including TRAP, CTSK and RANK in Raw264.7 cells ($n = 3-6$). (A–C) Real-time PCR analysis mRNA of TRAP, CTSK and RANK in Raw264.7 cells treated with chemerin compared to normal Raw264.7 cells. (D–E) Western blot analysis and respective density histograms of CTSK and RANK in Raw264.7 cells treated with chemerin compared to normal Raw264.7 cells. β -actin was used as the loading control. (F) Western blot analysis of CTSK and RANK in Raw264.7 cells treated with chemerin compared to normal Raw264.7 cells. β -actin was used as the loading control. Density values are expressed in arbitrary units. The data collected are expressed as the mean \pm SEM for the indicated proteins. * $P < 0.05$ & ** $P < 0.01$ compared to normal Raw264.7 cells.

expression of β -catenin, runx2 and BALP in chemerin-silenced Mc3t3-E1 cells could be reversed via treatment with chemerin *in vitro*. These results suggest that chemerin not only promotes adipogenesis, but also suppresses osteoblastogenesis and osteoblast differentiation by inhibition of Wnt/ β -catenin signaling.

It is well established that osteoblasts regulate osteoclast activity through stimulation of the expression of receptor activators, such as that of nuclear factor- κ B (RANKL) and osteoprotegerin (OPG) [13,17]. RANKL and OPG play opposite roles in osteoblasts [13]. RANKL is expressed on the osteoblast/stromal cell surface, binding to its receptor to upregulate the receptor activator of nuclear factor- κ B (RANK) expression, resulting in a stimulation of the differentiation/maturation of osteoclasts as well as bone resorption and bone loss [13,17]. Meanwhile, OPG, a soluble decoy receptor secreted by osteoblasts, binds RANKL to block its interaction with RANK, thus resulting in the prevention of osteoclast differentiation and activation [13,17]. As such, the ratio of RANKL/OPG is an indicator of the net stimulus for osteoclastogenesis. Previous studies have reported that treatment with chemerin promoted osteoclastogenesis of hematopoietic stem cells (HSCs) *in vitro* [13,20], and increased bone resorption activity in mature osteoclasts [14]. Other investigators using chemerin or CMKLR1 knockout mice revealed that chemerin or CMKLR1 deficiency enhanced osteoclastic bone resorption and bone loss [9,19]. The cause for this discrepancy has not yet been identified. Herein, our results revealed that treatment with chemerin enhanced CTSK, RANK and TRAP expression in Raw264.7 osteoclasts and elevated RANKL expression and secretion as well as the ratio of RANKL/OPG. However, it decreased OPG expression in Mc3t3-E1 cells, suggesting that chemerin may increase osteoclast activity partly by regulating the ratio

of RANKL/OPG in osteoblasts. In addition, we observed the effect of the knockdown of chemerin on osteoclastogenesis *in vitro* and, more importantly, bone resorption abnormalities in chemerin-null mice. We observed that the abrogation or knockdown of chemerin decreased CTSK, RANK, TRAP mRNA and protein expression in osteoclasts as well as the ratio of RANKL/OPG in osteoblasts and mouse bone tissues. Furthermore, we found that the decreased expression of CTSK, RANK and TRAP in chemerin-silenced Raw264.7 cells as well as the ratio of RANKL/OPG in chemerin-silenced Mc3t3-E1 cells could be reversed by treatment with chemerin *in vitro*. We also observed that treatment with chemerin in osteoblasts elevated the RANKL levels in the supernatants of Mc3t3-E1 cells of the co-culture system and promoted differentiation/maturation of osteoclasts in the co-culture system of osteoblasts and osteoclasts. These results suggested that chemerin could promote osteoclast differentiation and osteoclastogenesis via upregulation of the ratio of RANKL/OPG in osteoblasts and activation of RANK-mediated signaling pathways in osteoclasts. Taken together, our findings provided evidence that chemerin suppressed osteoblastogenesis and osteoblast differentiation via inhibition of Wnt/ β -catenin signaling and enhanced osteoclastogenesis and osteoclast differentiation via activation of RANK signaling.

While this paper was in revision, another report was published indicating that chemerin deficiency decreased osteogenesis and bone mass [9]. Although both studies highlight the effects of chemerin on bone metabolism, the two reports differ in the proposed effects of chemerin. Specifically, the other recent report focused on chemerin's ability to increase bone mass by affecting osteogenic differentiation, while our paper emphasizes the effect of chemerin on osteoblast and osteoclast differentiation through inhibition of Wnt/ β -catenin signaling and

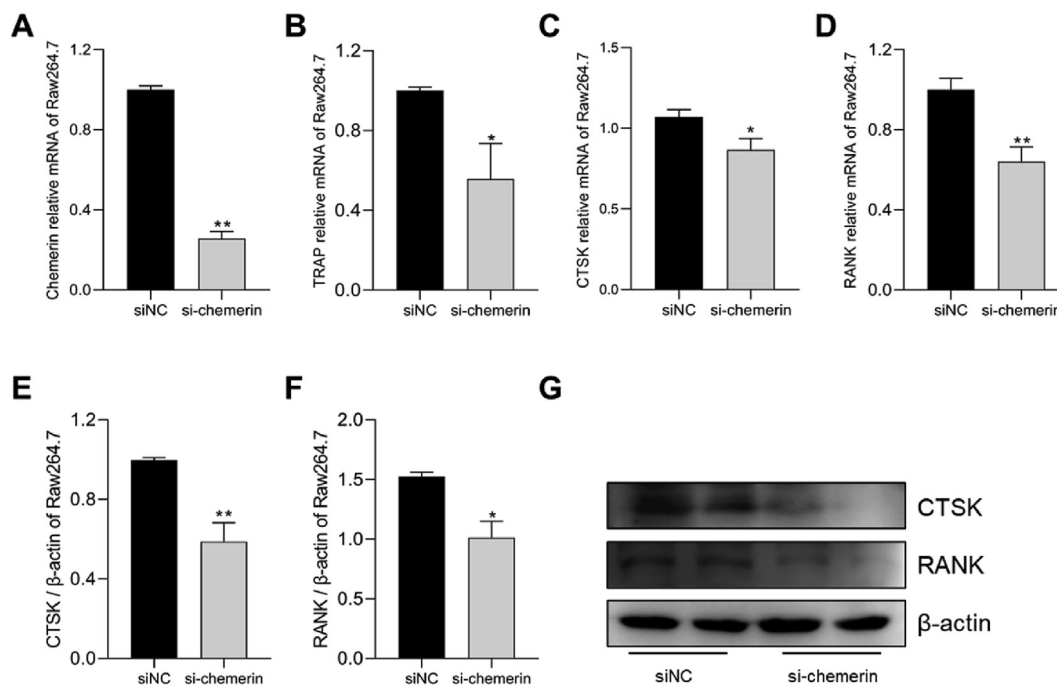


Figure 7: Chemerin silence affected the expression of bone resorption-related genes, including TRAP, CTSK and RANK in Raw264.7 cells ($n = 4-6$). (A–D) Real-time PCR analysis mRNA of TRAP, CTSK and RANK in si-chemerin Raw264.7 cells compared to normal Raw264.7 cells. (E–F) Western blot analysis and respective density histograms of CTSK and RANK in si-chemerin Raw264.7 cells compared to normal Raw264.7 cells. β -actin was used as the loading control. (G) Western blot analysis of CTSK and RANK in si-chemerin Raw264.7 cells compared to normal Raw264.7 cells. β -actin was used as the loading control. Density values are expressed in arbitrary units. The data collected are expressed as the mean \pm SEM for the indicated proteins. * $P < 0.05$ & ** $P < 0.01$ compared to normal Raw264.7 cells.

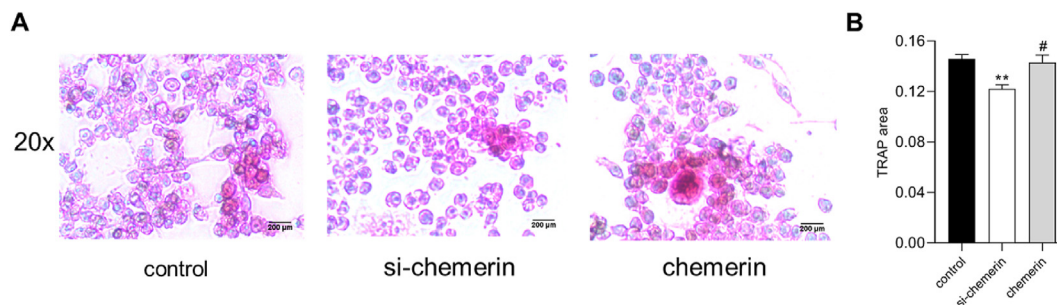


Figure 8: The levels of TRAP staining in Raw264.7 cells ($n = 5$). (A) TRAP staining was performed to analyze the distribution of osteoclasts in chemerin-silenced Raw264.7 cells treated with chemerin. Photo capture was performed by a Nikon TE2000 microscope (20x). (B) Histogram of TRAP staining area. ** $P < 0.01$ compared to normal Raw264.7 cells. # $P < 0.05$ compared to si-chemerin Raw264.7 cells.

activation of RANK signaling. However, the other study did not detect the effect of chemerin on osteoclastogenesis and osteoclast differentiation in vitro. In addition, it should be noted that the two studies differed on the sex of models analyzed. Our studies involved female mice fed a chow diet, while the other manuscript analyzed the effects of chemerin on male mice. Interestingly, in their analysis, they did observe that chemerin enhanced osteogenic differentiation in C3H10T1/2 cells and BMSCs through the Akt/Gsk3 β / β -catenin axis [9]. Unfortunately, these pathways were not detected in our research. Future studies will focus on providing more experimental evidence to clarify effects of chemerin on osteoblasts and osteoclasts.

In conclusion, our experimental results showed that chemerin was a negative regulator for bone microarchitecture, increasing osteoclast

activity and reducing osteoblast function. The levels of β -catenin increased, but CTSK, RANK, Tb.Sp, TRACP-5b and RANKL/OPG decreased in the femurs of *Rarres2*^{-/-} mice. Treatment with chemerin enhanced CTSK, RANK and TRAP expression in Raw264.7 osteoclasts and elevated the ratio of RANKL/OPG, but decreased OPG expression in Mc3t3-E1 cells, suggesting that chemerin suppresses osteoblast differentiation through inhibition of Wnt/ β -catenin signaling and enhances osteoclast differentiation via activation of RANK signaling. These findings are consistent with the speculation that chemerin is involved in the pathogenesis of osteoporosis. These findings deepen our understanding of the physiological functions of chemerin in bone remodeling via its effects on osteoblasts and osteoclasts and provide new insights into potential tactics for prevention and remedy of osteoporosis.

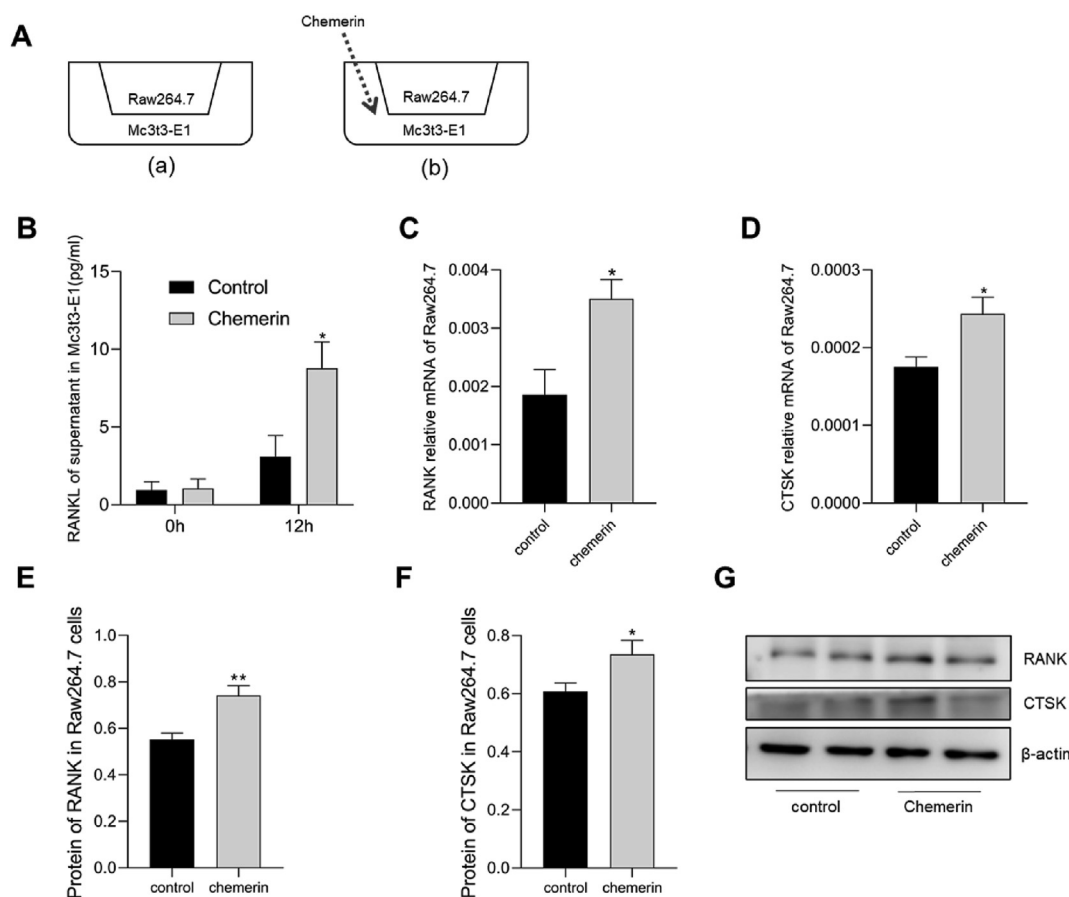


Figure 9: The enhanced osteoclastogenesis of Raw264.7 cells in the co-culture system of osteoblasts and osteoclasts after Mc3t3-E1 cells were incubated with chemerin (n = 3–6). (A) Schematic diagram of the co-culture system of osteoblasts and osteoclasts. (a) Control group; (b) Chemerin group. (B) RANKL in the supernatants of Mc3t3-E1 cells of the co-culture system detected by ELISA. The changes of RANKL protein levels in supernatant after Mc3t3-E1 cell incubation with chemerin for 0 h and 12 h (n = 4). (C–D) The mRNA levels of RANK and CTSK in Raw264.7 cells of the co-culture system after Mc3t3-E1 cell incubation with chemerin for 24 h (n = 3–5). (E–G) Western blot analysis and respective density histograms of RANK and CTSK in Raw264.7 cells of the co-culture system after Mc3t3-E1 cell incubation with chemerin for 24 h (n = 6). β -actin was used as the loading control. Density values are expressed in arbitrary units. The data collected are expressed as the mean \pm SEM for the indicated proteins. *P < 0.05 & **P < 0.01 compared to the control group.

AUTHOR CONTRIBUTIONS

Long Han, Penghua Fang and Wen Min conceived and designed the experiments. Long Han and Yu Zhang performed the experiments. Long Han, Shiwei Wan, Qingbo Wei and Yu Zhang analyzed the data. Guichen Huang, Wenbing Shang, Penghua Fang and Wen Min contributed reagents/materials/analysis tools. Penghua Fang and Wen Min wrote the manuscript.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molmet.2021.101322>.

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