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Systemic administration of low-dose naltrexone increases bone mass due to blockade of opioid growth factor receptor signaling in mice osteoblasts

Kenjiro Tanaka*, Hisataka Kondo, Kazunori Hamamura, Akifumi Togari

Department of Pharmacology, School of Dentistry, Aichi-Gakuin University, Nagoya 464-8650, Japan

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ABSTRACT

Aims: Opioid receptor blockers such as naloxone and naltrexone have been suggested to have a bone mass-increasing effect. However, the mechanisms at play have not been clarified. We examined the effects of naltrexone on osteoblasts and determined the expression of opioid growth factor receptor (OGFR) in osteoblasts. Naltrexone blocks the OGFR and other canonical opioid receptors. Thus, we designed experiments to clarify the effects of naltrexone on bone tissue by examining the physiological role of OGFR signaling in osteoblasts and the changes in bone structure after naltrexone systemic administration in mice.

Main methods: We used mouse osteoblast-like cell line MC3T3-E1 for *in vitro* experiments. We cultured MC3T3-E1 cells in the presence of the OGFR agonist met-enkephalin (met-enk). Then, we measured cell proliferation activity and analyzed the expression levels of cell proliferation-related genes. For our *in vivo* experiments, we administered naltrexone intraperitoneally to mice daily for 28 days and administered the animals in the control group equivalent volumes of saline. After sacrificing the mice, we performed micro-computed tomography and bone morphology analyses.

Key findings: Met-enk suppressed cell proliferation in MC3T3-E1 cells. Moreover, Low dose naltrexone administration significantly increased their femoral bone mass, bone formation ratio, and osteoblast number/bone surface values when comparing the values for the same variables in the control group.

Significance: Our results suggest that naltrexone increases bone mass due to osteoblast number increments caused by the OGFR signaling block. Opioid receptor blockers have potential as therapeutic agents for osteoporosis as well as opioid antagonists.

1. Introduction

Osteoporosis is one of the most prevalent chronic conditions in the elderly population, and its consequent fractures affect human health, quality of life, and societal burden [1]. Bone tissue is reconstructed continuously in a dynamic balance between osteoblastic bone formation and osteoclastic bone resorption [2]. Several drugs used in clinical practice influence the bone remodeling process and may induce osteoporosis [3]. Thus, drug prescriptions for patients with a high risk of osteoporosis or patients already suffering from osteoporosis should be formulated responsibly.

Opioid is a generic term for endogenous or synthetic peptides exhibiting alkaloid and morphine-like activities, such as those of narcotic analgesics and related synthetic analgesics. Population-based clinical studies have shown that the chronic administration of opioids increases the risk of fractures and reduces bone mineral density [4–8]. The adverse effects of opioids on bone tissue are usually attributed to inhibitory effects on the endocrine system (hypogonadism) [8–10]. However, other studies have suggested that opioids also act directly on osteoblasts [5,11,12].

Furthermore, opioid receptor blockers such as naloxone and naltrexone may affect bone tissue and bone cells [10,13–15]. Parenteral administration of low-dose naloxone has been shown to promote bone healing in an ovine “drill hole” [14], and low-dose naloxone administration into mouse bone marrow has been shown to increase the cancellous bone mass [15]. However, a controversial report concluded that intraperitoneal administration of naloxone does not change the bone mass of mice [16]. Several unclear points remain, and no unified conclusion exists on the action of naloxone on bone tissue.

Opioid growth factor receptor (OGFR) is a non-canonical opioid receptor present around the cellular nucleus that has no structural or functional homology with canonical opioid receptors such as μ-, κ-, and

* Corresponding author at: Department of Pharmacology, School of Dentistry, Aichi-Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya, Aichi 464-8650, Japan.
E-mail address: kenjiro1@dpc.agu.ac.jp (K. Tanaka).

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δ-opioid receptors [17]. The opioid growth factor (OGF or methionine-enkephalin: met-enk) is the native ligand for the OGFR [15,17,18]. Met-enk is encoded by the proenkephalin gene (*Penk*) [19]. Naloxone and naltrexone are also known to bind the OGFR with greater affinity than they bind the canonical opioid receptor [15]. Studies have identified *Penk* and *Ogfr* expression in osteoblast precursor cells and in osteoblasts [15,20–22]. However, the detailed function of OGF–OGFR signaling in osteoblasts and its physiological role in osteoblasts are not well understood.

The influence of naltrexone on bone tissue is also not well understood. Hence, we decided to first examine the effects of naltrexone on osteoblasts and found the expression of *Penk* and *Ogfr* in osteoblasts, with the *Ogfr* expression being higher than that of the canonical opioid receptor gene. Therefore, for this study, we aimed to clarify the effect of naltrexone on bone tissue by examining the physiological role of OGF–OGFR signaling in osteoblasts and to assess bone structure changes after systemic naltrexone administration in mice.

2. Materials and methods

2.1. Cell culture

We purchased the mouse osteoblast-like cell line MC3T3-E1 from the RIKEN Cell Bank (RIKEN BRC, Ibaragi, Japan) and cultured them in α-MEM (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing 10% FBS and 1% penicillin/streptomycin at 37 °C in a 5% CO_2 atmosphere. To induce differentiation, we replaced the culture medium with α-MEM containing 50 μg/mL ascorbic acid and 5 mM β-glycerophosphate. We changed the culture medium every 2–3 days.

2.2. Cell proliferation assays

We used a Cell Counting Kit-8 (CCK-8) (DOJINDO, Kumamoto, Japan) to perform cell proliferation assays. We plated the MC3T3-E1 cells onto 24-well plates (1×10^{-4} cells per well, cultured in a 5% CO_2 environment at 37 °C). After cell seeding, we added met-enk to the culture medium and cultured the cells for 72 more hours, changing the culture medium every 2 days. We added 100 μL of CCK-8 solution to each well 72 h after the addition of met-enk and incubated the plates for 2 h at 37 °C according to the manufacturer's protocol. Next, we measured the absorbance of each solution at 450 nm.

2.3. RNA extraction and quantitative real-time PCR

For *in vivo* experiments, we extracted total RNA from the right femurs. For *in vitro* experiments, total RNA samples were extracted from the cells. In both cases, we used NucleoSpin® RNA Plus (Takara Bio, Shiga, Japan) to isolate the total RNA samples following the manufacturer's protocol. Next, we used 500 ng of RNA in a reverse transcription to obtain cDNA using the PrimeScript™ RT Master Mix (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. We analyzed gene expression using the 7900HT Real-Time PCR system with its SDS system software (Applied Biosystems, Waltham, Massachusetts, USA). Reaction mixtures were prepared in 20 μL volumes using a THUNDERBIRD® SYBR qPCR Mix (TOYOBO, Osaka, Japan). The cycling conditions were 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data are presented as relative mRNA levels calculated using $2^{-\Delta\Delta Ct}$ (Ct = Ct of the target gene minus Ct of GAPDH). Table 1 shows a list of the primers used.

2.4. Mice

We originally obtained 7-week-old ICR male mice (Japan SLC, Shizuoka, Japan). We treated the mice in accordance with the Guidelines for Animal Experiments at the School of Dentistry, Aichi-Gakuin University. All experiments concerning animals were performed in compliance with the ARRIVE guidelines and the National Institutes of Health guide for the care and use of laboratory animals. We used a total of 21 mice in this study. Food and water were available *ad libitum*. Animals were housed together in automatically controlled temperature conditions (23 °C ± 1 °C) and humidity (50% ± 10%) under a 12-h light:12-h dark cycle.

2.5. Drugs and treatment

For *in vivo* experiments, we dissolved naltrexone (Tocris Bioscience, Bristol, UK) in saline. We randomized 7-week-old ICR male mice by weight and assigned them to three groups. The mice were treated with either naltrexone (0.1 mg/kg/day, n = 7 or 1 mg/kg/day, n = 7) or saline (n = 7) intraperitoneally every evening for 4 weeks. Because many animal experiments using opioids and opioid blockers have selected 4 weeks as the administration period, this period was also selected in this experiment [14,16]. In previous experiments with naltrexone systemic administration, 1 mg/kg was used as the therapeutic dose and 0.1 mg/kg as the low dose; we used a similar dose in this experiment [14–16,23,24]. We measured their body weights weekly. After the mice were sacrificed, we dissected bone tissue samples and maintained them at −80 °C for total RNA extractions later [25].

For *in vitro* experiments, we dissolved met-enk (Sigma, St. Louis, Missouri, USA) and naltrexone in sterile water.

2.6. Bone densitometry and body composition analyses

We subjected the distal region of the left femurs to three-dimensional micro-computed tomography (μCT) analyses using an R-mCT μCT scanner (RIGAKU, Tokyo, Japan). Scanning was initiated 0.5 mm above the distal femoral growth plates, and we analyzed a total of 75 consecutive 20-μm-thick sections, encompassing a length of 1.5 mm of the secondary spongiosa. We obtained the measured volume of interest in the femur by selecting the cancellous bone (separate from the cortical shaft) using contour areas that were drawn semiautomatically [26]. We used the TRI/3D-BON software (Ratoc, Tokyo, Japan) to analyze the cancellous parameters: bone volume/total volume (BV/TV, %), trabecular number (Tb.N, 1/mm), trabecular thickness (Tb.Th, μm), and trabecular Spacing (Tb.Spac, μm) [26,27].

2.7. Bone histomorphometry

For the dynamic histomorphometric analysis, we injected all mice subcutaneously with calcein (20 μg/g) at 1 and 3 days before death. At the end of the experiments, we dissected the femurs of each mouse and fixed them in 70% ethanol. We made 5-μm-thick sagittal sections as undecalcified sections. To assess mineralizing surface/bone surface (MS/BS, %), mineral apposition rate (MAR, μm/day), and bone formation rate (BFR, $\mu m^3/\mu m^2$/day), we used the metaphyseal cancellous bone in the femur to obtain the bone fraction in a rectangular area of 0.34 mm^2 (0.5 mm × 0.67 mm), with its closest and furthest edges being 0.5 and 1.0 mm medial to the growth plate, respectively [25]. To obtain decalcified sections, we dissected the left tibiae of mice, fixed them in 4% paraformaldehyde, and then decalcified them in 20% ethylenediaminetetraacetic acid (EDTA) for 3 weeks. We made sagittal sections (5 μm thick) as decalcified sections and stained them with hematoxylin for osteoblast analysis and tartrate-resistant acid phosphatase (TRAP) for osteoclast analysis. We carried out measurements within an area of 0.8 mm^2 (1.0 mm × 0.8 mm), with the closest and furthest edges being 2.0 and 3.0 mm distal to the growth plate of the proximal ends of the tibia, respectively. We evaluated osteoblast number/bone surface (Ob.N/BS) by scoring osteoblast cells attached to the bone surface. We evaluated osteoclast number/bone surface (Oc.N/BS) and osteoclast surface/bone surface (Oc.S/BS) by scoring TRAP-positive multinucleated cells attached to the bone surface [25].

Table 1
List of primers used in this study.

	Forward	Reverse
Gapdh	5′-AGGTCGGTGTGAACGGATTTTG-3′	5′-TGTAGACCATGTAGTTGAGGTCA-3′
Ogfr	5′-GAGTATGGACGACCCGGAAT-3′	5′-CGCCAGTTTCGGTACCTTGT-3′
Penk	5′-AAATGCAGCTACCGCCTGG-3′	5′-GAAGCCTCCGTACCGTTTCAT-3′
p21	5′-GCAAAGTGTGCCGTTGTCTC-3′	5′-CGTCTCCGTGACGAAGTCAA-3′
p53	5′-ATATCAGCCTCGAGCTCCCT-3′	5′-GCAACAGATCGTCCATGCAG-3′
p27	5′-GTTTCAGACGGTTCCCCGAA-3′	5′-TTACGTCTGGCGTCGAAGG-3′
p16	5′-CGAACTCTTTCGGTCGTACCC-3′	5′-TTGAGCAGAAGAGCTGCTACG-3′
Alp	5′-CTGATCATTCCCACGTTTT-3′	5′-ACTGGGCCTGGTAGTTGTTG-3′
Col1a1	5′-GAGAGGTGAACAAGGTCCCG-3′	5′-AAACCTCTCTCGCCTCTTGC-3′
Oc	5′-TTCTGCTCACTCTGCTGACC-3′	5′-GGGACTGAGGCTCCAAGGTA-3′

Ogfr: opioid growth factor receptor, Penk: proenkephalin, *Alp*: alkaline phosphatase, *Col1a1*: collagen type I alpha 1 chain, *Oc*: osteocalcin.

2.8. Statistical analysis

All data are expressed as mean ± SD. We used Dunnett's test following one-way ANOVA for multiple comparisons, and Student's *t*-test for comparison between the two groups. Differences with $p < 0.05$ were considered statistically significant. Analyses were done with Mac statistical analysis Ver. 3.0 (ESUMI Co., Ltd. Tokyo, Japan).

3. Results

3.1. Met-enk suppressed cell proliferation in MC3T3-E1 cells

To investigate the effect of naltrexone on osteoblasts, we analyzed the expression of various opioid receptors in osteoblast-like cell line MC3T3-E1 cells. Another study identified the expression of μ-, κ-, and δ-opioid receptor genes (*OPRM*, *OPRK*, and *OPRD*, respectively) in osteoblasts [11]. We also found that the expression of *Oprm*, *Oprk*, and *Oprd*. However, these expressions were very low, and the expression of *Ogfr* was approximately 500 times that of *Oprm* (Fig. 1A). These results were obtained in the early stage of differentiation of MC3T3-E1 cells, and similar results were obtained in the middle stage and the late stage of differentiation (data not shown). Moreover, other studies confirmed the expression of *Penk*, a precursor gene of met-enk, in osteoblasts, suggesting met-enk production by the osteoblasts themselves [15]. Our results of real-time PCR analysis indicated that *Ogfr* expression in MC3T3-E1 cells and *Penk* expression were significantly increased during osteoblast differentiation (Fig. 1B). Met-enk has been shown to suppress the proliferation of various cancer cells and human osteoblast cells [15,28–31]. Therefore, we examined the effects of met-enk on MC3T3-E1 cells. As shown in Fig. 1C, sustained exposure of met-enk to MC3T3-E1 cells significantly reduced cell proliferation activity. Moreover, the inhibitory effect on osteoblast proliferation by met-enk disappeared in combination with naltrexone (Fig. 1D). To better characterize these mechanisms, we examined the expression of cell cycle related genes in response to met-enk on MC3T3-E1 cells. We extracted total RNA from MC3T3-E1 cells exposed to met-enk for 2, 4, and 8 h and analyzed the resulting gene expression by real-time PCR. The expression of *p21* and *p53* was significantly increased after exposure to met-enk for 4 h (Fig. 1E). Although, increased expression of *p27* and *p16* in cancer cells was observed by met-enk stimulation [29,31], the expression was not changed in MC3T3-E1 cells (Fig. 1E). These results suggest that met-enk suppresses the proliferation of osteoblasts through the increased expression of *p21* and *p53*.

3.2. Naltrexone suppressed the mRNA expression of p21 in MC3T3-E1 cells

Next, we examined the effect of naltrexone on osteoblasts. We cultured MC3T3-E1 cells for 10 days in the sustained presence of naltrexone at 0, 0.1, or 10 μM and then extracted the total RNA samples. The results of our real-time PCR analysis indicated that *p21* was significantly lower in osteoblasts cultured for 10 days in the presence of 10 μM naltrexone compared with the expression in cells cultured in the absence of naltrexone (Fig. 2A). On the contrary, naltrexone had no effect on *p53* expression (Fig. 2B). Moreover, we examined the effect of naltrexone on osteoblast differentiation to investigate OGF–OGFR signaling in detail. We cultured MC3T3-E1 cells in differentiation media with the sustained presence of naltrexone at 10 μM and then extracted total RNA. We investigated the expression change of *Osteocalcin* (*Oc*), *Alkaline phosphatase* (*Alp*), and *Collagen type I alpha 1 chain* (*cola1a*), which are osteoblast differentiation genes. We observed a partial significant change in *Oc*, but no significant changes in *Alp* and *cola1a*, in MC3T3-E1 cells cultured with naltrexone (Fig. 2C). These findings suggest that naltrexone regulates osteoblast proliferation, but has little or no effect on osteoblast differentiation.

3.3. Systemic administration of low-dose naltrexone in mice increased bone mass by increasing bone formation

To evaluate the effect of systemic administration of naltrexone on bone mass, we performed μCT-based bone densitometry on bones after intraperitoneal administration of 0, 0.1, or 1 mg/kg of naltrexone in mice daily for 28 days. As shown in Fig. 3A and B, the BV/TV of the distal end of the femur in 12-week-old males was significantly higher in naltrexone-treated mice at 0.1 mg/kg than in saline-treated mice. Moreover, the Tb.N of the femur was significantly higher in naltrexone-treated mice at 0.1 mg/kg than in saline-treated mice (Fig. 3C). We found no significant differences in Tb.Th or Tb.Spac between mice injected with naltrexone at 0.1 or 1 mg/kg and those injected with saline (Fig. 3D and E). The administration dose of naltrexone used in this study did not induce adverse effects.

Next, to examine the mechanisms responsible for bone mass-increasing effects after naltrexone treatment, we performed a bone histomorphometrical analysis on bone formation parameters by assessing calcein double labeling in the distal femur after systemic administration of naltrexone for 28 days. Our analysis provided an *in vivo* estimate of osteoblastic activity with respect to the accumulation of bone mass. Bone formation parameters such as MAR and BFR were significantly higher in naltrexone-treated mice (0.1 mg/kg) than in saline-treated mice (Fig. 4A, B, and C). We also measured changes in the number of osteoblasts by hematoxylin staining in the proximal tibia after the systemic administration of naltrexone for 28 days. Our results indicated that Ob.N/BS of the tibia was significantly higher in low dose naltrexone-treated mice than in saline-treated mice (Fig. 4D).

In addition, to confirm the effects of naltrexone on bone resorption, we performed a bone histomorphometric analysis on bone resorption parameters such as Oc.S/BS and Oc.N/BS with TRAP-positive osteoclasts. Our results showed that Oc.N/BS of the tibia was significantly higher in low dose naltrexone-treated mice than in saline-treated mice (Fig. 4E). We found no significant differences in Oc.S/BS between mice administered naltrexone and those administered saline (Fig. 4F). These

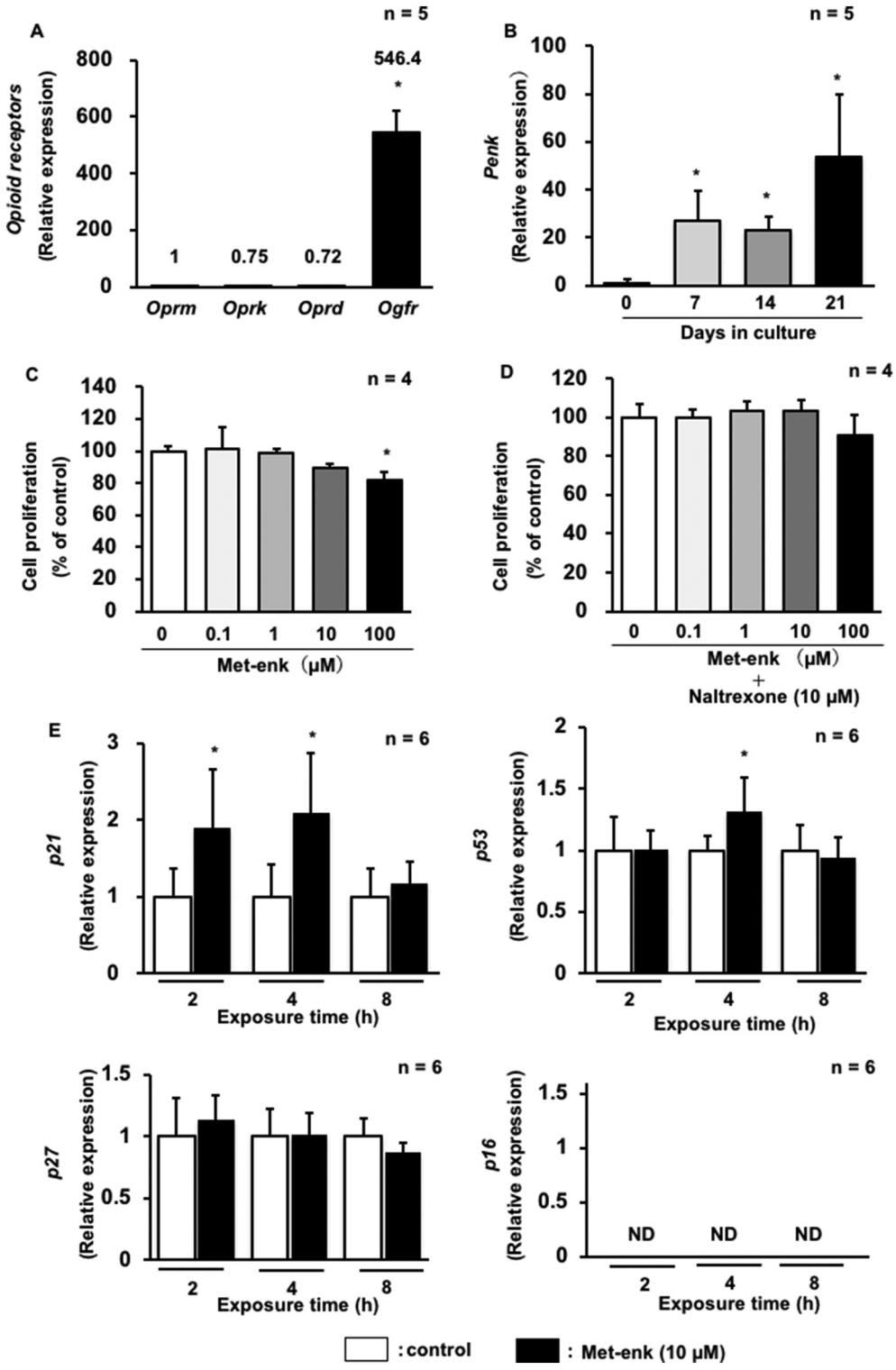

Fig. 1. Expression of *Ogfr* and *Penk*, and effects of met-enk on the cell proliferation of MC3T3E1 cells.

A: Expression of canonical opioid receptors and opioid growth factor receptor. We isolated total RNA from MC3T3-E1 cells and analyzed gene expression by real-time PCR. Values are expressed as means ± SD from independent cultures of five wells. *p < 0.05, significantly different from the value of *Oprm*. B: Expression of *Penk* during osteoblast differentiation. We cultured MC3T-E1 cells in differentiation media for set time periods; we then isolated total RNA and analyzed gene expression by real-time PCR. Values are expressed as means ± SD from independent cultures of five wells. *p < 0.05, significantly different from the value of day 0. C, D: We treated cells with 0, 0.1, 1, 10, or 100 μM met-enk or with met-enk and 10 μM naltrexone for 72 h and then performed the WST assay. Similar results were obtained in three independent experiments. Values are expressed as means ± SD from independent cultures of four wells. *p < 0.05, significantly different from the control value obtained in MC3T3-E1 cells cultured in the absence of met-enk. E: We treated cells with 10 μM met-enk for 2, 4, and 8 h, harvested them, and then performed real-time PCR. Each value represents the mean ± SD from independent cultures of six wells. *p < 0.05, significantly different from each control value obtained in MC3T3-E1 cells cultured in the absence of met-enk. These experiments were performed at least three times with similar results.

results suggest that low-dose administration of naltrexone to mice increases bone mass by promoting bone formation *via* an increase in the number of osteoblasts. Finally, we extracted total RNA from the distal end of the femurs of mice injected intraperitoneally with naltrexone or saline for 28 days and analyzed the expression of *p21* and *p53* by real-time PCR. Consistent with our *in vitro* results, the expression of *p21* in bone was significantly decreased in low dose naltrexone-treated mice than in saline-treated mice (Fig. 4G). In contrast, there was no change in the expression of p53 in bone (data not shown).

4. Discussion

In this study, we first examined the effects of naltrexone on osteoblasts. Expression of the canonical opioid receptor genes (*Oprm*, *Oprk*, and *Oprd*) has been observed in osteoblasts [5,11,14]. Hence, we examined the expression of opioid receptor genes in osteoblasts using real-time PCR. However, the expression level of *Oprm*, *Oprk*, and *Oprd* was very low. Remarkably, we found that *Ogfr* expression was very high (approximately 500-fold) compared with the expression of canonical opioid receptor genes. Other studies have also confirmed the expression

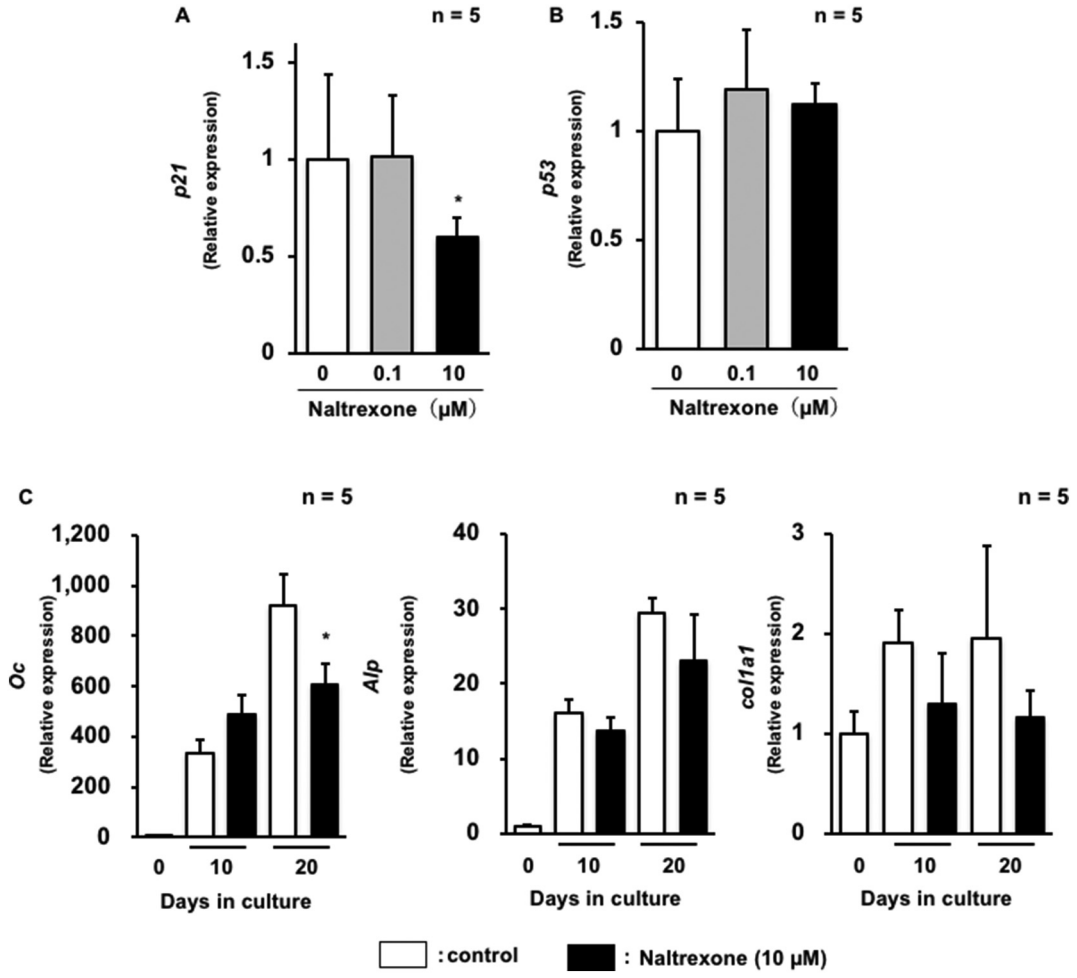

Fig. 2. Effects of naltrexone on proliferation- and differentiation-related genes.
A, B: We cultured MC3T-E1 cells in differentiation media for 10 days in the presence of 0.1 or 10 μM naltrexone. We isolated total RNA and analyzed gene expression by real-time PCR. Each value represents the mean ± SD from independent cultures of five wells. *p < 0.05, significantly different from each control value obtained in MC3T3-E1 cells cultured in the absence of naltrexone. C, D, E: After seeding the cells, at the time of confluence (day 0), we replaced the culture medium with differentiation medium and cultured the cells in the presence of 10 μM naltrexone. Cells were harvested at days 10 and 20. We isolated total RNA and performed real-time PCR. Each value represents the mean ± SD from independent cultures of five wells. *p < 0.05, significantly different from the control value at the same time. These experiments were performed at least three times with similar results.

of *Penk* (the precursor gene of met-enk) and *Ogfr* in mesenchymal stem cells and osteoblasts [15,22,32,33]. In addition, the presence of OGF–OGFR signaling in osteoblasts has also been suggested [15], but detailed investigations have not been carried out. Consistent with other reports, we confirmed the expression of *Penk* and *Ogfr* in MC3T3-E1 cells and demonstrated that *Penk* expression increases significantly during osteoblast differentiation (Fig. 1A and B). These results suggest that met-enk is produced in osteoblasts and serves as an autocrine or paracrine substance. Naltrexone is an OGFR antagonist, and its affinity to OGFR is higher than that of the canonical opioid receptor [15]. Therefore, we focused on studying the OGF–OGFR signaling in osteoblasts in detail.

Our results demonstrated that met-enk significantly decreased the cell proliferation in MC3T3-E1 cells in a concentration-dependent manner. Elhassan et al. [33] showed that the addition of met-enk to the medium reduced the osteoblast number, whereas the addition of naltrexone increased it. We observed that naltrexone tended to promote the proliferation of MC3T3-E1 cells, but we did not find a significant difference (data not shown). The effects of naloxone and naltrexone on osteoblast proliferation are controversial. Donahue et al. [31] found that cell proliferation decreased by 25% 72 h following the addition of a pulse dose (< 6 h) of 1 mM naloxone. However, they also found that continuous dosing with naltrexone resulted in an increase in tumor cell proliferation, which is consistent with the findings obtained by Cheng et al. [28]. The effect of naloxone and naltrexone on cell proliferation *in vitro* may change with exposure time, concentration, timing analysis, and other variables [28,31,34]. A more detailed study is needed to elucidate this issue.

The cyclin-dependent kinase inhibitors *p16* and *p21* have been shown to be required for the OGF–OGFR axis to inhibit cancer cell proliferation [28,31]. We examined the target gene of OGF–OGFR signaling by real-time PCR and demonstrated the presence of *p21* in MC3T3-E1 cells. Additionally, we found that *p53*, a tumor suppressor gene, is active in the OGF–OGFR signaling pathway of MC3T3-E1 cells. However, our gene expression analysis of the femur confirmed *p21* expression but not *p53* expression change. Next, to investigate the OGF–OGFR signaling of osteoblasts in more detail, we examined the effect of naltrexone on osteoblast differentiation. Naltrexone has been shown to promote the differentiation of mesenchymal stem cells into osteoblasts [15], but no report has investigated its effects on osteoblast differentiation. We analyzed the expression change of osteoblast differentiation genes by real-time PCR when MC3T3-E1 cells were cultured in the presence of naltrexone. We found no significant expression changes in *Alp* and *col1a1*, although we observed a general decreasing

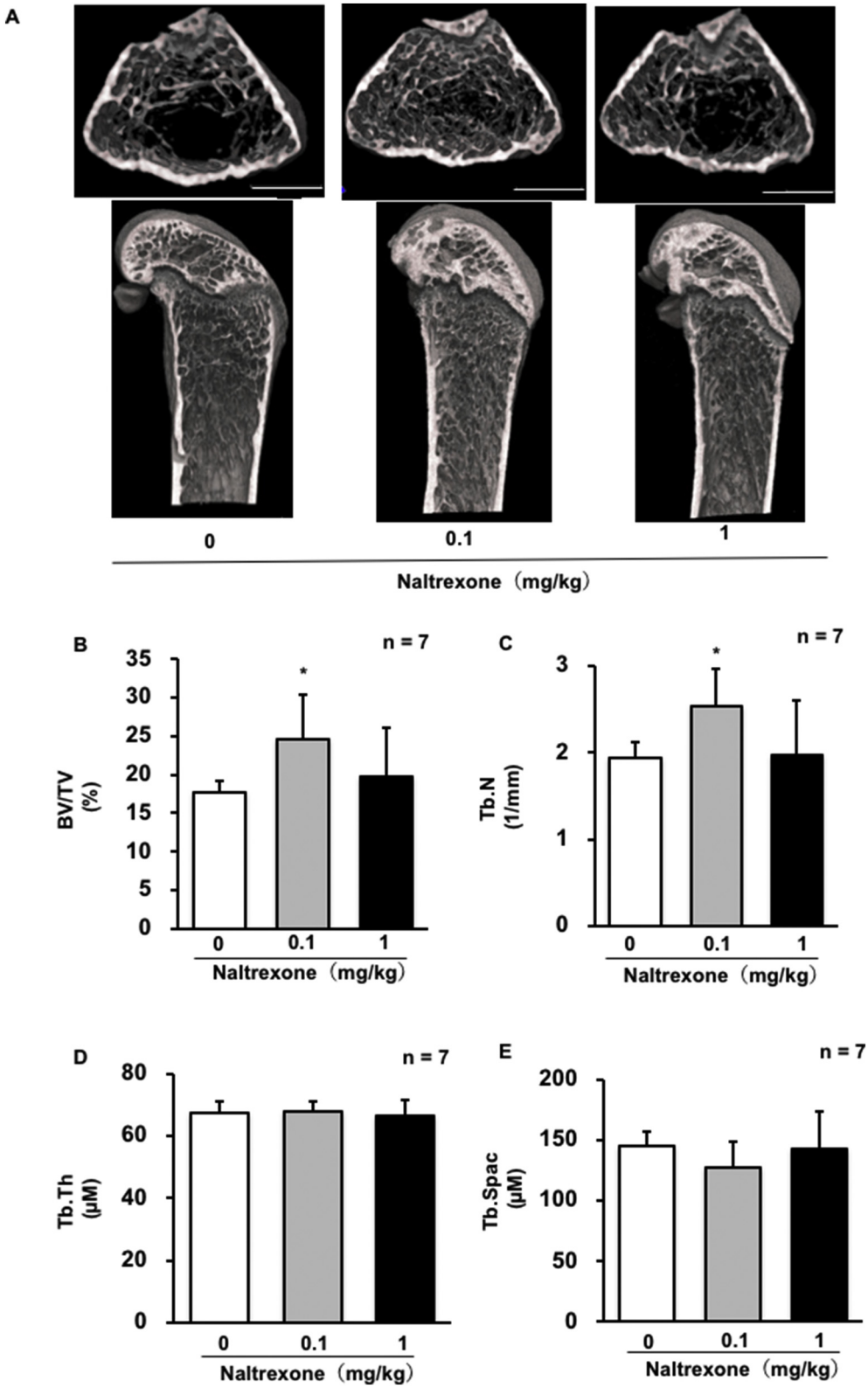

Fig. 3. Effect of naltrexone administration on femoral bone mass.
μCT-based bone densitometry of the distal region of the left femur in 12-week-old male mice administered saline or naltrexone at 0.1 or 1 μg/kg body weight/day for 28 days. n = 7 mice per group. A: Representative μCT images in each group. Scale bars = 1 mm. B: Bone volume/tissue volume (BV/TV). C: Trabecular number (Tb.N). D: Trabecular thickness (Tb.Th). E: Trabecular separation (Tb.Spac). Values are expressed as means ± SD. *$p < 0.05$, significantly different from the values in control mice.

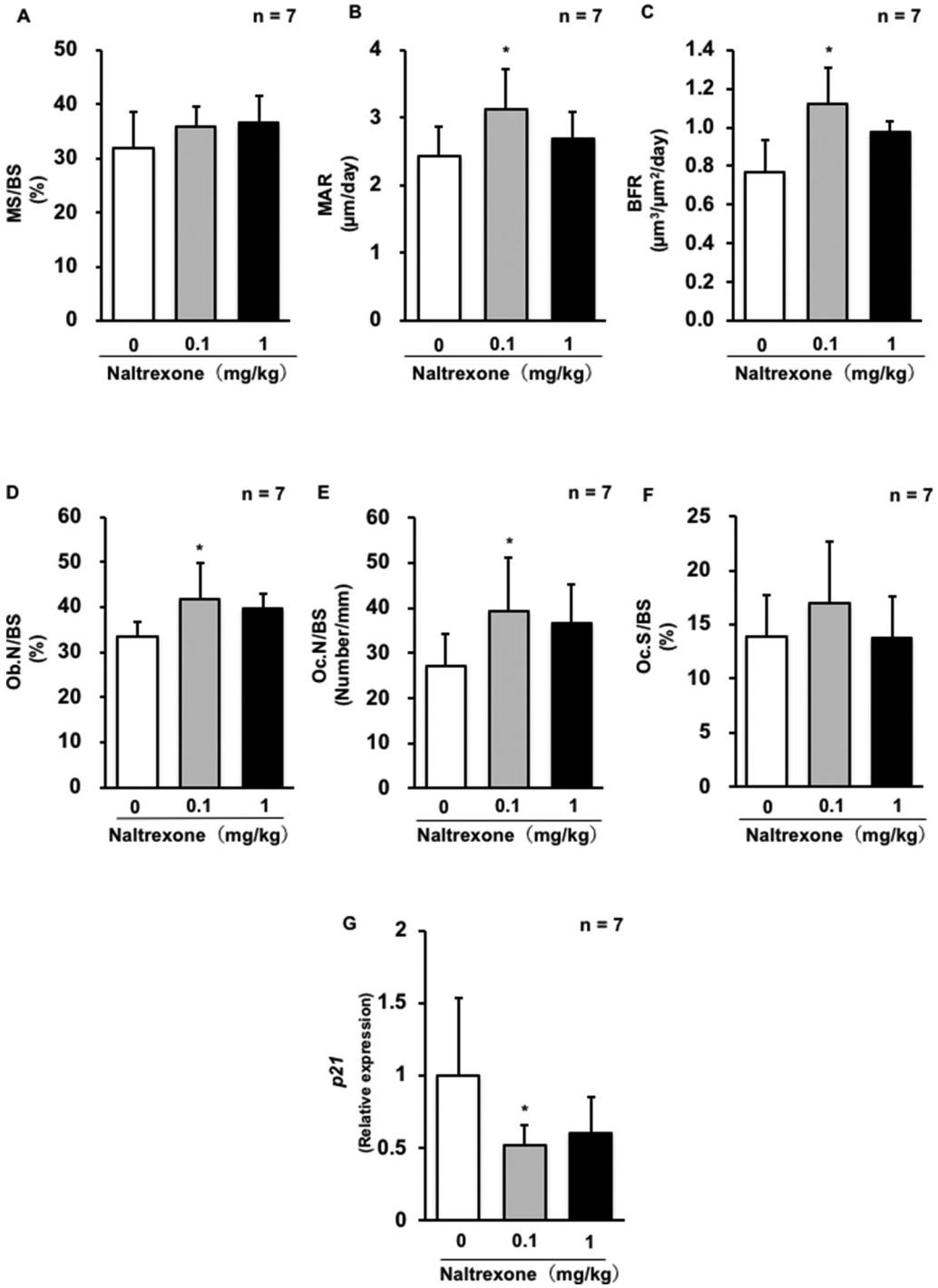

Fig. 4. Effects of naltrexone administration in mice on bone formation and bone resorption.
Dynamic bone morphometry of bone formation in the distal femurs of mice administered naltrexone. A: Analysis of mineral surface/bone surface (MS/BS) and B: mineral apposition rates (MAR) and C: bone formation rate (BFR). n = 7 mice per group. Values are expressed as means ± SD. *p < 0.05, significantly different from the values in control mice. D: Static bone morphometry by hematoxylin staining in the proximal tibia. Analysis of osteoblast number/bone surface (Ob.N/BS) of mice administered naltrexone. n = 7 mice per group. Values are expressed as means ± SD. *p < 0.05, significantly different from the values in control mice. Static bone morphometry of bone proliferation by TRAP staining of the proximal tibia. E: Analysis of osteoclast number/bone surface (OC.N/BS). F: Osteoclast surface/bone surface (Oc.S/BS). n = 7 mice per group. Values are expressed as means ± SD. *p < 0.05, significantly different from the values in control mice. G: We isolated total RNA from the right distal region of the right femur of 12-week-old male mice administered saline or naltrexone and then performed real-time PCR. n = 7 mice per group. Values are expressed as means ± SD. *p < 0.05, significantly different from control mice.

trend for the differentiation-related genes, such as a partial decrease in *Oc* (Fig. 2C). Our results suggest that naltrexone's blockade of OGF–OGFR signaling in osteoblasts does not affect differentiation and that naltrexone is primarily regulates cell proliferation.

In this study, we also investigated the detailed bone structure changes of systemic naltrexone administration to mice. We examined the change in femoral bone mass and the balance of bone formation and bone resorption of mice by systemic administration of a therapeutic dose (1 mg/kg) and a low dose (0.1 mg/kg) of the OGFR antagonist naltrexone. Few studies on the effects of naloxone and naltrexone on bone tissue have been reported. For example, Thakur et al. [15] reported that the topical administration of 1 mM naloxone into the bone marrow causes an increase in bone mass. Petrizzi et al. [14] reported that subcutaneous administration of low-dose naloxone (0.02 mg/kg) aids mineralization and enhances earlier remodeling of the cortical bone in sheep. The results of these studies are consistent with our results using low doses of naloxone. However, these studies focused on the change in bone mass due to local administration to the bone marrow and the healing effects on cortical bone defects. Therefore, to the best of our knowledge, other than our study, no studies have examined changes in bone tissue by the systemic administration of low-dose naloxone or naltrexone. In contrast, Janas and Folwarczna [16] reported that systemic administration of low-dose naloxone in rats had no effect on bone mass or bone strength. Their study considered only the therapeutic dose, and their results, similar to ours, revealed no change in bone mass at the therapeutic dose. We demonstrated that

systemic administration of low-dose naltrexone (0.1 mg/kg) in mice causes an increase in bone mass. Moreover, we found that the bone mass increase results from the promotion of bone formation accompanying the increases in osteoblast numbers. The results of previous animal experiments and ours can be summarized as follows: 1. Naloxone and naltrexone have a bone mass-increasing effect when administered topically or systemically. 2. The effective doses are lower than the therapeutic doses. 3. The bone mass-increasing effect by administration of low-dose naloxone or naltrexone seems to be caused by osteoblast proliferation.

In this study, we discovered that systemic administration of low-dose naltrexone causes an increase in mouse femoral bone mass. On the other hand, the lack of effect of the therapeutic dose of naltrexone was also observed. It has been reported that low-dose opioid antagonist administration is effective for cancer and autoimmune diseases [35–37]. Elhassan et al. [33] reported that the short time inhibition of OGF–OGFR signaling by administration of a low-dose opioid antagonist is compensated by increased met-enk secretions. Thus, increased met-enk secretions inhibit cancer cell proliferation and autoimmune activation. In this study, we also analyzed whether the low-dose naltrexone administration promotes met-enk production in bone tissues. We harvested the femurs of mice administered with naltrexone systemically for 28 days and performed real-time PCR analysis using extracted RNA samples. However, we found no significant increase in *Penk* expression in the bone tissues (data not shown). It is unknown why the increase in bone mass seen in low doses of naltrexone disappeared in therapeutic amount. Naltrexone acts throughout the body and affects the endocrine system [38–40]. In this experiment only bone tissue was analyzed, and changes of the whole body including the brain, changes of hormones and serum cytokines were not examined. It is also possible that a therapeutic amount of naltrexone affected the endocrine system and as a result no change in bone mass was observed. Experimental studies on animal models and human patients have confirmed the pain-relieving effect of low-dose naloxone (lower than 1 mg/kg) [41–45]. It is possible that such a pain-relieving effect is associated with bone mass changes.

In addition, we revealed that naltrexone does not influence the differentiation and mainly regulates cell proliferation *in vitro*, but MAR and BFR increase was observed *in vivo*. It is possible that such discrepancy may have an effect on the endocrine system by systemic administration of naltrexone. Future studies should also consider the tracking of potential biomarkers such as hormones, serum cytokines, or serum met-enk levels. Alternatively, the effect of naloxone and naltrexone on cell proliferation *in vitro* may change under experimental conditions [28,31,34]. Therefore, the effect of naltrexone on osteoblast differentiation may also change under experimental conditions including exposure time. A more detailed study is needed to elucidate this issue.

Although our study focused on the blockade of OGF–OGFR signaling by naltrexone, the effect of blocking the canonical opioid receptor signaling must also be carefully considered. For example, dynorphin knockout mice had increased bone formation markers, suggesting that dynorphin inhibits bone formation [46]. Furthermore, orally active dual μ-/δ-opioid receptor antagonist H-Dmt-Tic-Lys-NH-CH 2-Ph (MZ-2) causes an increase in bone mass [47]. Thus, blocking of the canonical opioid receptors by naloxone and naltrexone may lead to the improvement of certain parameters associated with bone formation. In this study, we cannot rule out the effects of naltrexone blocking the canonical opioid receptors. Further work will be necessary to elucidate the changes in bone mass by administration of selective opioid agonists and antagonists. Moreover, in this study we examined the effect of naltrexone on osteogenesis by osteoblastic cells. However, in *in vivo* experiments, the change in Oc.N/BS values have been confirmed by administration of naltrexone to mice, so the influence of naltrexone on osteoclasts should also be considered. No studies on the expression and function of OGFR in osteoclasts have been conducted. Therefore, future studies investigating OGF-OGFR signaling in bone tissue in detail are required to analyze the expression and function of OGFR in osteoclasts.

5. Conclusion

We showed that the systemic administration of low doses of naltrexone causes increased bone mass *via* the proliferation of osteoblasts. In addition, our results suggest that the blocking of OGF–OGFR signaling in osteoblasts regulates the proliferation of osteoblasts through the suppression of *p21* expression. We found that opioid receptor blockers (widely used in the clinic especially during the perioperative period) have potential as bone mass increasing agents. Further studies are needed to explore their use as bone mass promoters.

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Conflict of interest statement

The authors declare no conflicts of interest associated with this manuscript.

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