Inflammation, Vol. 42, No. 5, October 2019 (© 2019)
DOI: 10.1007/s10753-019-01026-w

ORIGINAL ARTICLE

Ghrelin Fights Against Titanium Particle-Induced Inflammatory Osteolysis Through Activation of β-Catenin Signaling Pathway

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Abstract—Aseptic loosening is a major complication of prosthetic joint surgery, in which exaggerated inflammation and impaired osteoblastogenesis are detected. Ghrelin is a recently discovered neuropeptide that is closely associated with inflammatory conditions and bone regeneration. Here, we report that titanium particles inhibited ghrelin expression in MC3T3-E1 cells. Furthermore, exogenous ghrelin effectively inhibited titanium particle-induced inflammation *in vitro* by interacting with its receptor GHSR1a; as an inhibitor of GHSR1a, Dlys repressed the function of ghrelin. Moreover, ghrelin attenuated the impairment of osteoblastogenesis and the exaggeration of osteolysis induced by titanium particles. Furthermore, the protective role of ghrelin in aseptic loosening might be associated with the Wnt/β-catenin signaling pathway. Collectively, these findings suggest that ghrelin might be a potential therapeutic target for wear-debris-induced inflammation and osteolysis.

KEY WORDS: ghrelin; wear debris; osteolysis; β-catenin; osteoblastogenesis.

Ruize Qu, Xiaomin Chen and Yongjian Yuan contributed equally to this work.

Key Messages

- Aseptic loosening is now a well-applied joint surgery, while exaggerated inflammation and impaired osteoblastogenesis are detected, which are mainly caused by wear-debris-induced osteolysis.
- Ghrelin is a recently discovered neuropeptide that is closely associated with inflammatory conditions and bone regeneration.
- In this study, we found ghrelin might be a potential therapeutic target for wear-debris-induced inflammation and osteolysis, and the activation of β-catenin signaling may be involved.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10753-019-01026-w) contains supplementary material, which is available to authorized users.

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INTRODUCTION

Terminal rheumatic arthritis and severe osteoarthritis are devastating diseases that affect individuals worldwide [1]. Total joint replacement is a type of widely used treatment strategy for these diseases, with millions of patients undergoing this surgery each year [2]. However, researchers have found that total joint replacement surgery can also lead to increased rates of surgical error and implant fracture, as well as wear-debris-induced osteolysis caused by loosening of the prosthesis, which may affect the longevity of the implant and patient quality of life [3, 4]. Among these side effects, wear-debris-induced osteolysis remains the most usual-seen side effect after total joint replacement [5, 6]. A chronic inflammatory response to wear debris is now considered to be a main reason of wear-debris-induced osteolysis, which is caused by polyethylene, poly(methyl methacrylate), cobalt, chromium, and titanium (Ti) emerged at the surface of prostheses [7, 8]. As previously reported, wear debris can induce the accumulation of macrophages, fibroblasts, osteoblasts, and osteoclasts and increases the secretion of proinflammatory cytokines, including IL-6, IL-1β, and TNF-α [9]. Increased secretion of these cytokines can lead to more severe bone resorption and excessive osteoclast formation [10]. Moreover, ideal treatment methods for wear-debris-induced osteolysis remain unknown [11].

Ghrelin is a novel peptide mainly produced by X/A-like cells of the stomach [12, 13]. However, it is also detected in a number of additional tissues [14]. It has been reported that ghrelin acts as a critical factor in various physiological and disease processes, including neurogenesis, tumorigenesis, hypertension, and tissue regeneration [15–18]. Moreover, the anti-inflammatory function of ghrelin has been extensively studied [19, 20]. Its mechanism might be mediated through the inhibition of inflammatory cytokines by binding to its receptor, GHSR1a, thus protecting the body from inflammation [21–23], while the effect of ghrelin to wear-debris-induced osteolysis remains unknown.

Wnt/β-catenin signaling is one of the key signaling pathways in various inflammation reactions [24]. It has been reported to regulate many life processes, including growth, development, disease, aging, and death [25, 26]. Furthermore, Wnt proteins can bind to receptors located on the cell membrane through an autocrine or paracrine action, which activates a number of intracellular signaling pathways, including β-catenin, and thus regulates the differentiation and maturation of osteoblasts [27]. Reports have shown that the Wnt/β-catenin signaling pathway can exert an effect in wear-debris-induced osteolysis [5], and ghrelin can protect against many inflammatory diseases by activating the Wnt/β-catenin signaling pathway [28].

In this study, we established a murine calvarial model of Ti particle-induced osteolysis and an MC3T3-E1 osteoblast cell culture to determine whether treatment with ghrelin would prevent wear-debris-induced osteolysis by intervening in osteoclastogenesis and inflammatory reactions and, more importantly, its mechanism in osteolysis.

RESULTS

Titanium Particles Decreased Ghrelin Expression *In Vitro* and *In Vivo*

To determine whether Ti particles affect ghrelin expression, MC3T3-E1 osteoblast cells were cultured with and treated by micron-size pure titanium particles (5×10^6 particles/mL), and the expression of ghrelin was subsequently measured. The results in Fig. 1b show that Ti stimulation substantially decreased the ghrelin expression in cells. Moreover, the protein levels and mRNA levels of ghrelin expression were apparently downregulated in both the mouse and cell models with the stimulation of Ti particles, as shown in Fig. 1c–f. To determine Ti's effects on the expression of ghrelin *in vivo*, immunohistochemistry staining was further performed. As indicated in Fig. 1g, the ghrelin expression in the Ti-stimulated mouse models was elevated compared with the non-Ti-stimulated mice. Collectively, ghrelin expression was inhibited with Ti stimulation both *in vitro* and *in vivo*.

Ghrelin Suppresses Severity of Ti-Induced Inflammatory Osteolysis Both *In Vitro* and *In Vivo* by Combining with GHSR1a

Given that the loss of ghrelin may lead to severe inflammation in many inflammatory diseases, including OA, we subsequently sought to determine whether additional treatment of ghrelin could prevent the Ti-induced inflammation *ex vivo* and *in vivo*. The ALP activity was initially measured in the cell models. As shown in Fig. 2a, Ti stimulation attenuated the activity of ALP in the MC3T3-E1 cells, while the additional treatment of ghrelin substantially enhanced the ALP activity. Moreover, osteoblast-related factors, including ALP, Runx-2, osteorix, osteocalcin, Col 1, and Axin-2, were examined through real-time PCR. The results showed that Ti stimulation suppressed the secretion of these cytokines, while the additional application of ghrelin substantially recovered their secretion, indicating a lower severity of inflammatory osteolysis

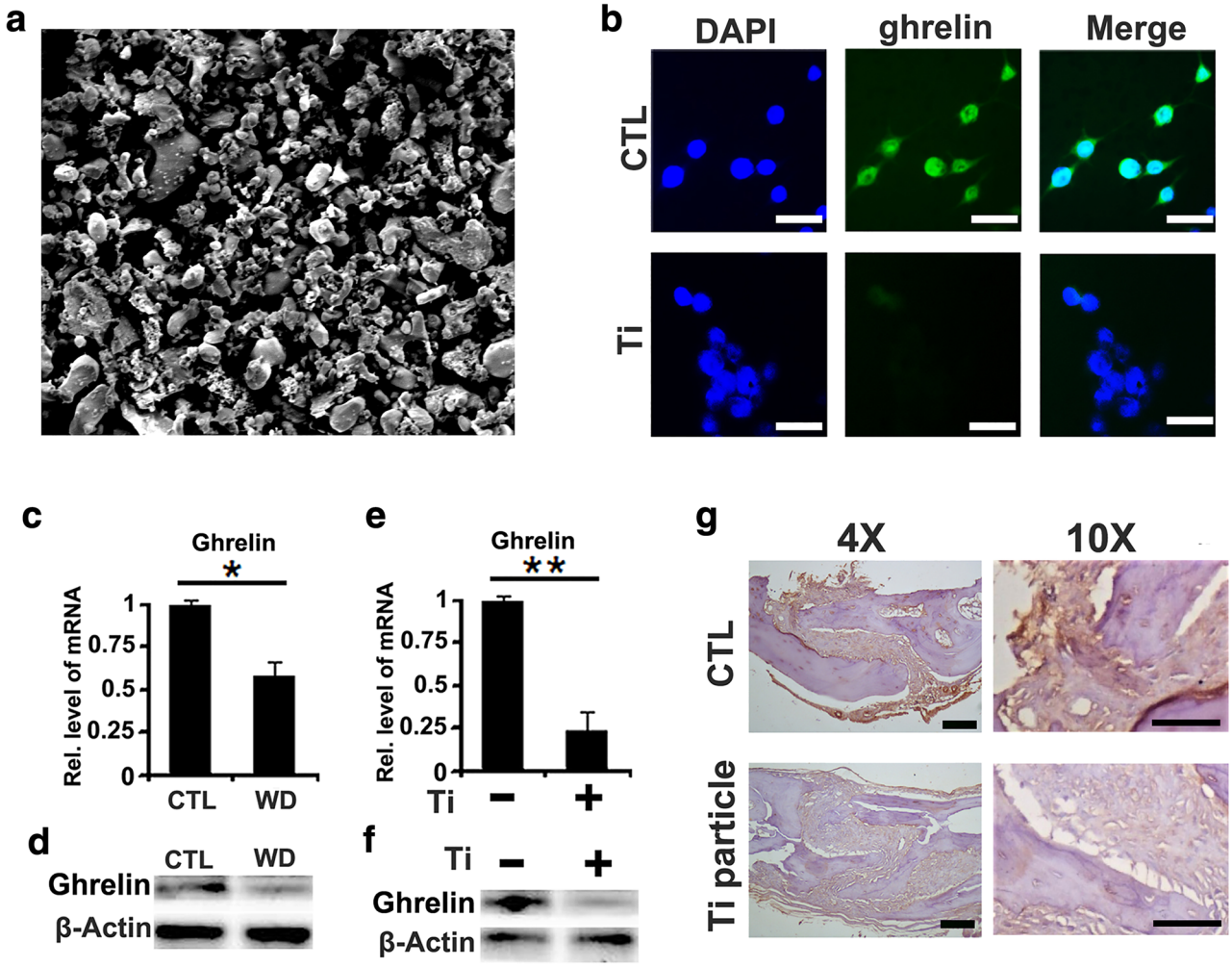

Fig. 1. Stimulation of Ti particles diminished ghrelin expression *in vivo* and *ex vivo*. **a** Scanning election microscopy (SEM) appearance of titanium particles (magnification × 5000). **b** Stimulation of Ti apparently decreased ghrelin expression in cells, as measured by immunofluorescence. **c** mRNA was extracted from mouse models, and real-time PCR was conducted. Expression of ghrelin decreased with the stimulation of wear debris. **d** Total protein was obtained from mouse models, and western blot showed that ghrelin expression was inhibited with the stimulation of wear debris. **e** Expression of ghrelin diminished with the stimulation of Ti particles, as indicated by real-time PCR. **f** Ghrelin expression was downregulated with Ti particle stimulation, as assessed *via* western blot. **g** Ghrelin secretion was diminished with the stimulation of Ti particles *in vivo*, as indicated by immunohistochemistry. $*p < 0.05$, $**p < 0.01$ *vs* the control group. Scale bar, 150 μm.

ex vivo (Fig. 2b–g). Micro-CT was conducted in each experimental group, and the results in Fig. 2h indicated that ghrelin treatment apparently improved the degree of bone destruction *in vivo*. Moreover, TRAP staining, HE staining, and immunohistochemistry of osteorix were examined in the calcaria tissues of the mouse wear debris models of each group. As shown in Fig. 2i, the TRAP expression, which indicates the osteoclast activity, decreased with ghrelin application. HE staining showed a lower bone destruction with ghrelin treatment, and immunohistochemistry showed a higher osteorix secretion with the treatment of ghrelin, which together indicated a lower inflammatory osteolysis severity with ghrelin application *in vivo*.

Dlys, a GHSR1a inhibitor, was subsequently applied in both the cell and mouse models to clarify the acting mechanism of ghrelin. Osteoblast-related factors, including ALP, osteorix, Runx-2, Col 1, osteocalcin, and Axin-2, were initially examined through real-time PCR and western blot. The results showed that ghrelin treatment increased the expression of these cytokines, while Dlys

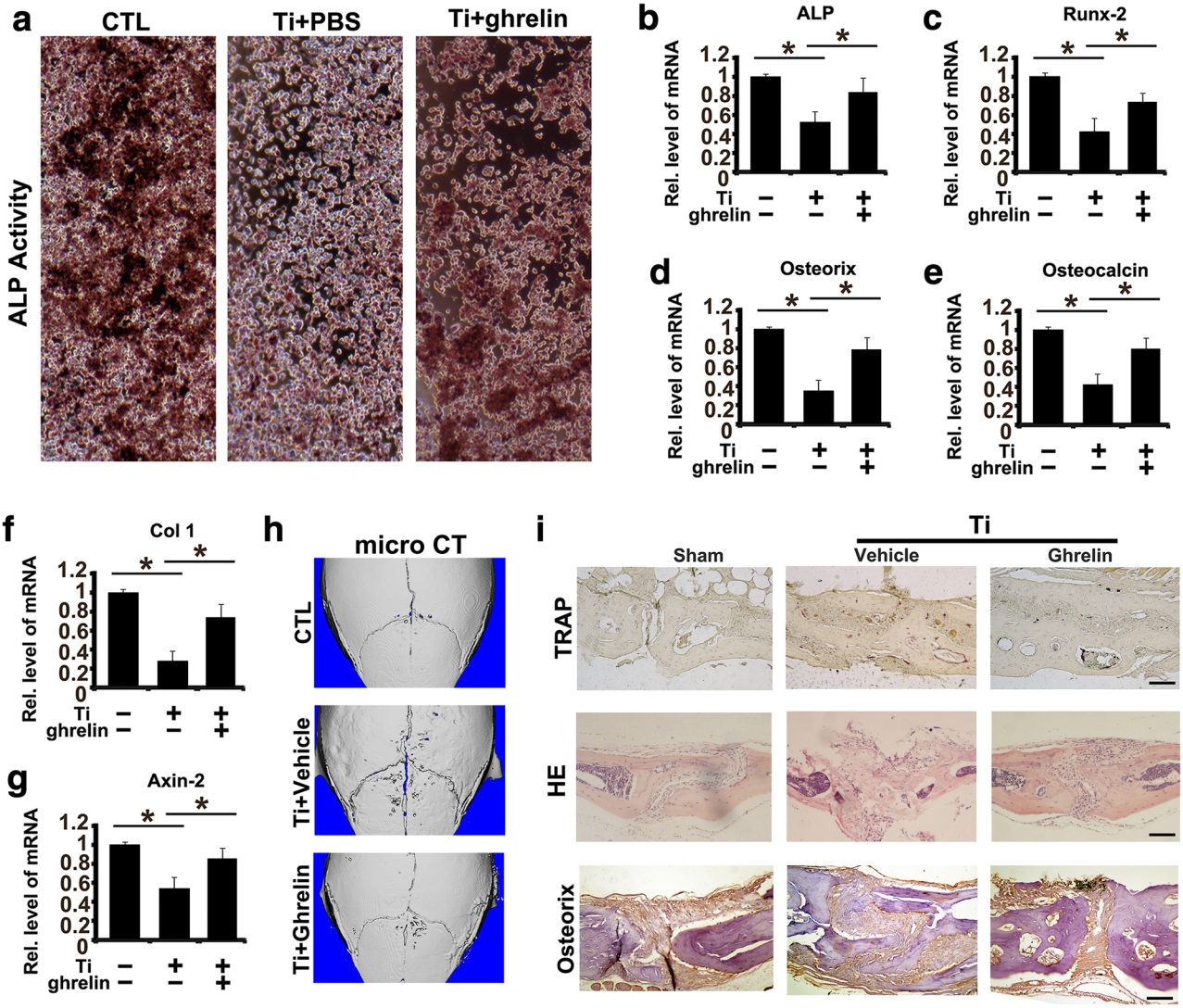

Fig. 2. Ghrelin improves severity of Ti-induced inflammatory osteolysis in mouse and cell models. **a** ALP activity was diminished with Ti stimulation *in vitro*, and additional ghrelin application apparently recovered it. **b**–**g** Osteoblast-related factors, including ALP, Runx-2, osteorix, osteocalcin, Col 1, and Axin-2, increased with ghrelin treatment in MC3T3-E1 stimulated with Ti particles, as indicated by real-time PCR. **h** Ghrelin treatment apparently improved the degree of bone destruction detected by micro-CT. **i** TRAP expression decreased with ghrelin application. HE staining showed lower bone destruction with ghrelin treatment, and immunohistochemistry showed higher osteorix secretion with ghrelin treatment. $*p < 0.01$ *vs* the control group. Scale bar, 100 μm.

apparently inhibited their secretion *ex vivo* (Fig. 3a, b). These findings indicate that the inhibition of GHSR1a can antagonize ghrelin's effect in osteolysis. Furthermore, micro-CT and additional analyses were performed. As shown in Fig. 3c–g, the healing effect of ghrelin substantially decreased with Dlys application *in vivo*. In summary, ghrelin can suppress Ti-induced inflammatory osteolysis *in vitro* and *in vivo*, which occurred by combining with GHSR1a.

Ghrelin Inhibits Inflammation and Apoptosis Stimulated by Ti Particles in MC3T3-E1 Cells

It is well-known that osteoblasts play a critical role in inflammatory osteolysis, and the inhibition of the inflammation extent in osteoblasts can exert a positive effect on osteolysis treatment. To determine whether ghrelin exerts an effect on osteoblasts in inflammatory osteolysis, mRNA was extracted, and real-time PCR was performed to examine

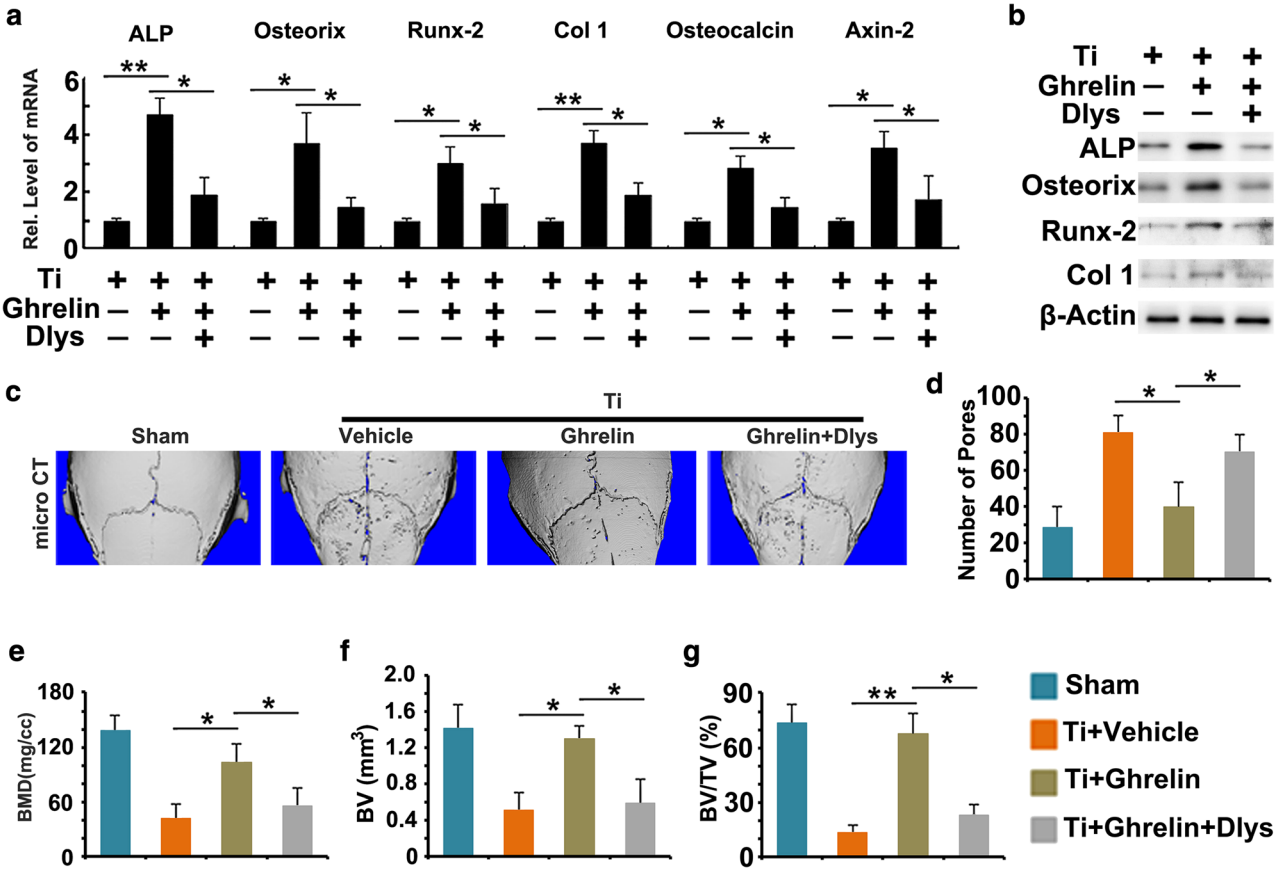

Fig. 3. Ghrelin mainly exerts its effects in wear-debris-induced osteolysis by combining with GHSR1a. **a** Expressions of osteoblast-related factors, including ALP, osteorix, Runx-2, Col 1, osteocalcin, and Axin-2, were apparently inhibited with Dlys stimulation *in vivo*, as measured by real-time PCR. **b** Secretion of ALP, osteorix, Runx-2, and Col 1 were substantially diminished with Dlys stimulation in cell models through western blot examination. **c**–**g** Healing effect of ghrelin substantially decreased with Dlys application, which was examined by micro-CT and further analysis. $*p < 0.005$, $**p < 0.05$ *vs* the control group.

the expression of the inflammatory and apoptosis cytokines mainly involved in osteolysis, including TNF-α, IL-6, iNOS, COX-2, caspase-3, caspase-7, and caspase-9. The results showed that the expressions of these cytokines were enhanced with Ti particle stimulation, while the additional application of ghrelin apparently downregulated the secretion of these cytokines *in vitro* (Fig. 4a). Furthermore, the expressions of IL-1β and IL-6 were examined through enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 4b, c, the expressions of these two cytokines enhanced by Ti stimulation were substantially diminished with additional ghrelin treatment, which indicated a lower inflammation extent *ex vivo*. Total protein was subsequently extracted from each experimental group, and western blot was conducted to determine the expression of cytokines, including iNOS, COX-2, caspase-3, caspase-7, and caspase-9. Figure 4d, e shows that these expressions increased with Ti stimulation and apparently decreased with the treatment of ghrelin *ex vivo*. Based on these results, it can be concluded that ghrelin may exert an effect in osteolysis by suppressing inflammation and the apoptosis of osteoblasts.

Ghrelin Exerts an Effect in Inflammatory Osteolysis Caused by Ti Particles Through β-Catenin Signaling Pathway

The protein and mRNA of MC3T3-E1 stimulated with Ti particles with or without the treatment of ghrelin were obtained for the analysis of the β-catenin expression. As shown in Fig. 5a, b, the stimulation of Ti particles substantially diminished the β-catenin expression, while additional ghrelin application apparently increased its level *ex vivo*,

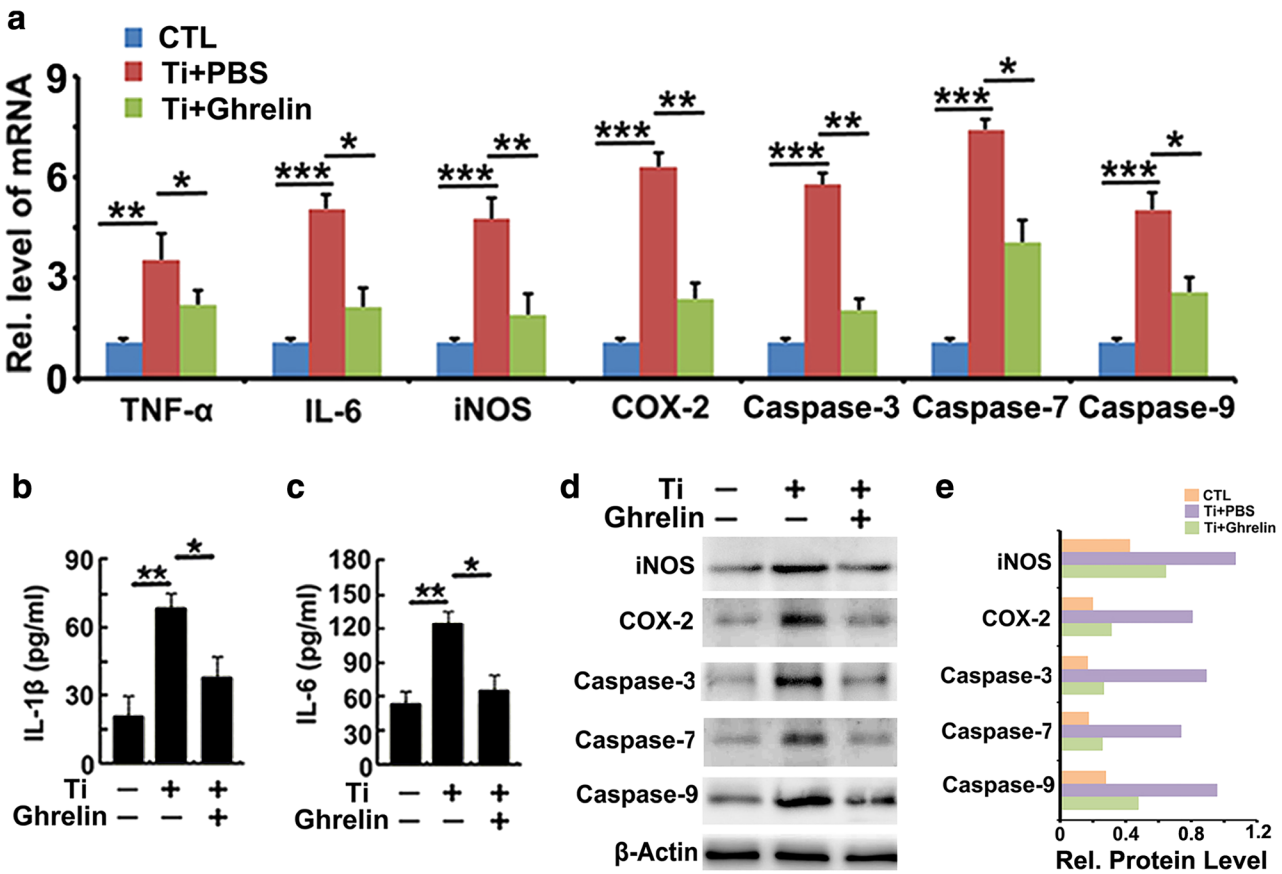

Fig. 4. Ghrelin inhibits inflammation and apoptosis caused by Ti particles *in vitro*. **a** Inflammatory and apoptosis cytokines, including TNF-α, IL-6, iNOS, COX-2, caspase-3, caspase-7, and caspase-9, were enhanced with Ti particle stimulation, while additional application of ghrelin apparently downregulated their expression, as measured by real-time PCR. **b**, **c** Expressions of IL-1β and IL-6 enhanced by Ti stimulation were substantially diminished with additional ghrelin treatment, as detected by ELISA. **d**, **e** Ghrelin apparently inhibited expression of inflammatory and apoptosis cytokines, such as iNOS, COX-2, caspase-3, caspase-7, and caspase-9, which were enhanced with Ti particle stimulation, as indicated by western blot. $^{*}p < 0.005$, $^{**}p < 0.05$, $^{***}p < 0.01$ *vs* the control group.

which signaled activation of β-catenin signaling. Active and non-active β-catenin were also detected through western blot. As was shown in Fig. 5c, d, expression of both active and non-active β-catenin got decreased with Ti stimulation, while got apparently enhanced with the treatment of ghrelin *in vitro*. In addition, nuclear translocation of β-catenin was enhanced with ghrelin application; however, the use of its inhibitor Dlys dramatically antagonized such translocation *ex vivo* (Fig. 5e), which further illustrated the relationship and under mechanism between ghrelin and β-catenin signaling in wear-debris-induced osteolysis. Moreover, ICG001, a widely applied β-catenin inhibitor, was applied in an *in vitro* experiment with MC3T3-E1 cells stimulated with Ti and treated with ghrelin. Osteoblast-related factors, including ALP, Col 1, osteorix, Runx-2, and osteocalcin, were subsequently examined. The results showed that the application of ghrelin substantially enhanced the secretion of these factors, while the addition of ICG001 substantially inhibited this effect *ex vivo* (Fig. 5f). The ALP activity was also measured in *ex vivo* models. The results showed that the application of ICG001 apparently suppressed the activity of ALP compared with cells simply treated with ghrelin *ex vivo* (Fig. 5g). ICG001 was subsequently applied in the mouse model. Micro-CT and further analysis were performed. As shown in Fig. 5h–l, the healing effect of ghrelin substantially decreased with ICG001 application *in vivo*. In summary, the results indicated that β-catenin may be involved in the development of wear-debris-induced osteolysis caused by Ti particles, with the characteristics of an enhancement of inflammation and an

inhibition of osteoblastogenesis. In addition, ghrelin can exert its effect in wear-debris-induced osteolysis by activating the β-catenin signaling pathway, through combining with GHSR1a (Fig. 5m).

DISCUSSION

As a most effective method for severe joint diseases, TJA has been widely accepted and applied in orthopedic treatment, while implant failure mainly caused by wear-debris-induced osteolysis followed by aseptic loosening typically results in the need for revision surgery. Despite the unclear mechanism, the inflammatory response induced by wear debris and the osteoclastic bone resorption at the bone-implant interface have been proven to be key reasons for wear-debris-induced osteolysis [29, 30]. Treatment in inflammation and osteoclast formation can be an effective method in the prevention and curation of wear-debris-induced bone loss after TJA [31, 32].

Ghrelin is a novel peptide reported to have great efficiency in inflammatory diseases [21, 33–35]. Furthermore, it has been reported that ghrelin not only mediates the inhibition of inflammatory cytokines but also acts as a gastrointestinal peptide to stimulate appetite [36], which makes ghrelin an outstanding potential anti-inflammatory factor in the curation of many diseases [37, 38]. In addition, ghrelin's effects in antagonizing apoptosis have been reported in numerous diseases [39–41]. Inflammation and apoptosis caused by Ti particles have been reported to impel the development of wear-debris-induced osteolysis [42–44]. Inflammatory cytokines, such as TNF-α, IL-6, iNOS, and COX-2, are essential inflammatory biomarkers for examining the severity of inflammation [45, 46]. Moreover, the expression of caspase-3, caspase-7, and caspase-9 can strictly reflect the severity of apoptosis [47, 48]. In this study, we found an enhancement of these inflammatory cytokines, as well as apoptosis-related factors with the stimulation of Ti in both cell and mouse models, indicating that wear debris including Ti particles could be harmful to the development of wear-debris-induced osteolysis by causing inflammatory reactions and apoptosis. However, the application of ghrelin has been found to exert positive effects in both inhibiting the expression of inflammatory cytokines, such as TNF-α, IL-6, iNOS, and COX-2, and downregulating the apoptosis indexes, including caspase-3, caspase-7, and caspase-9, in both cell and mouse models. These findings indicate ghrelin's potential effect in wear-debris-induced osteolysis in inhibiting inflammation and apoptosis.

Reports have shown that dual effects would occur as long as the host bone is in contact with wear debris, which was conducted by inhibiting periprosthetic bone regeneration, with concurrent enhancement of bone erosion [49]. Moreover, the interface of loosening implants and bone has been shown to have osteogenic bone formation [50, 51]. Furthermore, it has been indicated that wear debris could inhibit new bone formation through preventing the differentiation of osteoblast precursors [52]. Therefore, it can be concluded that wear debris as Ti particles can induce the decreation of osteogenic differentiation and mineralization, which was proven to be one of the main causes of wear-debris-induced osteolysis [53, 54]. However, in this study, we found that the induced expression of ALP, osteorix, Runx-2, and Col 1 was significantly improved with the treatment of ghrelin in Ti-stimulated MC3T3-E1 cells, which demonstrated ghrelin's effect in antagonizing the decreation of osteogenic differentiation and mineralization caused by wear debris. To further identify ghrelin's acting mechanism in inflammatory osteolysis, Dlys was applied to identify the link between the combination of ghrelin to GHSR1a and its treating effect to wear-debris-induced osteolysis. The results showed that the inhibition of GHSR1a substantially suppressed ghrelin's effect on osteolysis induced by Ti particles, which indicated that ghrelin mainly exerts its effect in osteolysis by combining with GHSR1a. Moreover, in the *in vivo* experiment of calvarial osteolysis, the mouse model further supported this conclusion. Based on these results, it can be concluded that ghrelin has the ability to increase osteogenic differentiation and mineralization and decrease osteoclastogenesis in wear-debris-induced osteolysis by combining with GHSR1a.

The β-catenin signaling pathway has been reported to have a close relationship with osteoblastic differentiation and mineralization [55–57], and reports have also shown that it can exert effects in osteolytic diseases, including wear-debris-induced osteolysis [58]. Activation of Wnt/β-catenin signaling pathway can impel Wnt protein to bind to its related receptors as frizzled receptor. Following, forming a complex with Axin and Frat-1, therefore inhibits the activity of glycogen synthase kinase-3 (GSK3). The inhibition induces dephosphorylation of substrate β-catenin and promotes its accumulation. Then β-catenin will then enter the cell nucleus, interacting with transcription factor T cytokine (TCF) and lymphoid-enhancing factor (LEF), so as to mediate Wnt-induced gene transcription changes and promote division, differentiation, and maturation of osteoblast [59]. However, wear debris including Ti particles has previously been reported to induce the secretion of DKK1, which can antagonize the Wnt/β-catenin signaling pathway

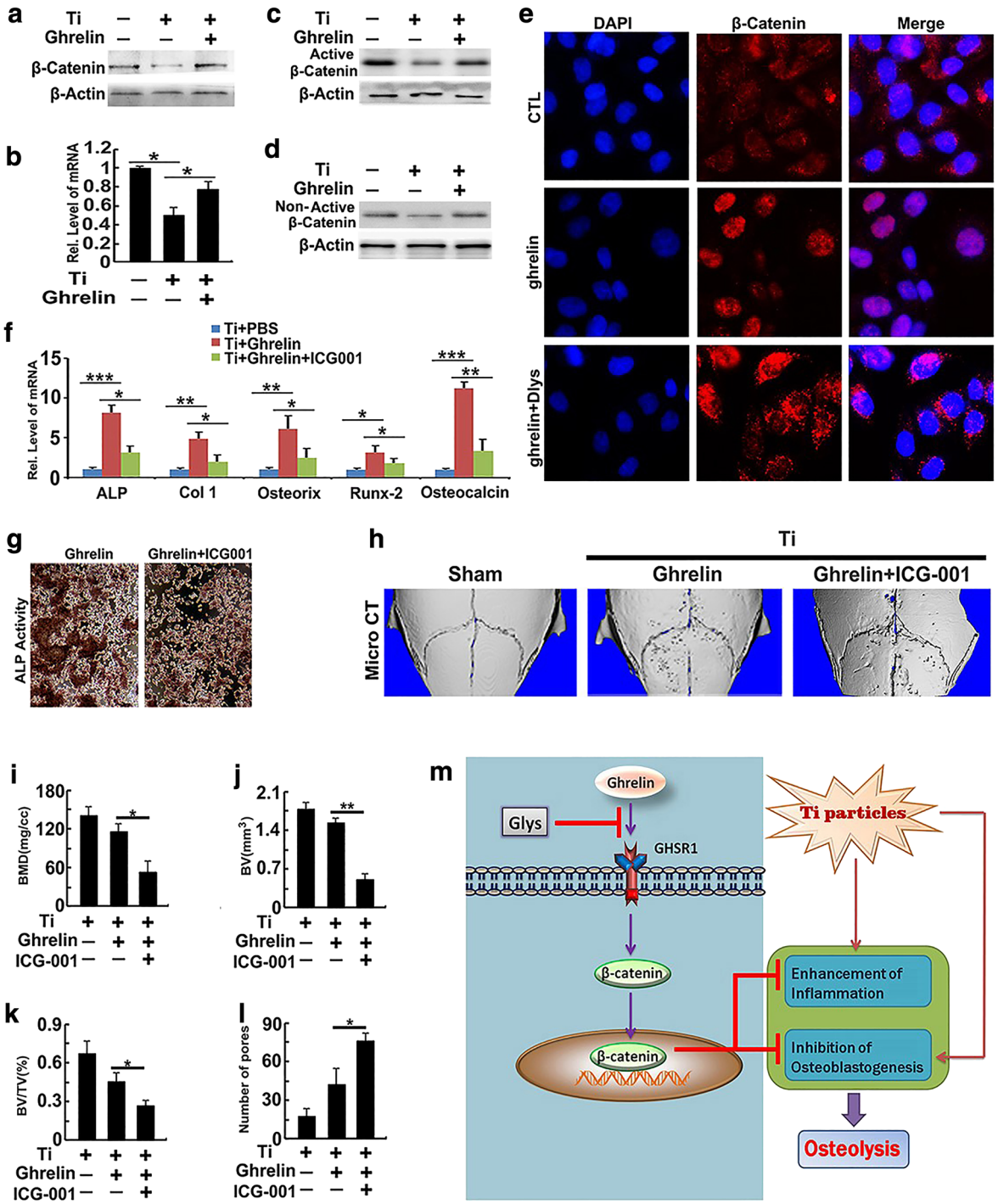

Fig. 5. Ghrelin antagonizes wear-debris-induced osteolysis through activating β-catenin signaling pathway. **a**–**d** Additional ghrelin application apparently increased the level of β-catenin, including active, non-active, and total β-catenin, as indicated by western blot and real-time PCR. **e** Nuclear translocation detection of β-catenin with or without the treatment of ghrelin and its inhibitor Dlys. **f** Ghrelin apparently enhanced, while the β-catenin inhibitor ICG001 substantially suppressed the expression of osteoblast-related factors, including ALP, Col 1, osteorix, Runx-2, and osteocalcin, as detected by real-time PCR. **g** ICG001 apparently suppressed activity of ALP, compared to cells simply treated with ghrelin. **h**–**l** Healing effect of ghrelin substantially decreased with ICG001 application detected by micro-CT and further analysis. **m** Schematic depicting ghrelin's proposed effect on wear-debris-induced osteolysis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* the control group. Scale bar, 150 μm.

and thus decrease β-catenin expression, affecting the processes of osteoblastic differentiation and mineralization [60]. In this study, we observed that Ti particles apparently downregulated the secretion of active, non-active, and total β-catenin, while the treatment of ghrelin substantially activated the β-catenin signaling pathway and attenuated Ti particle-induced bone loss. Nuclear translocation detection of β-catenin also showed the relationship and the under acting mechanism between ghrelin and β-catenin signaling in wear-debris-induced osteolysis. In addition, the protective effects of ghrelin on osteoblasts were inhibited following ICG-001 administration. Among these results, it can be suggested that ghrelin can antagonize the inhibitory effect of bone regeneration caused by wear debris, with the help of β-catenin signaling pathway activation.

Collectively, the findings reported in this study indicated that ghrelin can exert positive effects on the treatment of wear-debris-induced osteolysis through suppressing inflammatory cytokine secretion, reducing apoptosis, antagonizing bone destruction, and promoting bone formation, thereby providing insights into the molecular mechanisms that underlie Ti-induced inflammatory osteolysis. This study supports the concept that the treatment of ghrelin could be developed into an effective and safe method to treat osteolysis-related disease caused by chronic inflammation and excessive osteoclast formation.

MATERIALS AND METHODS

Ti Particles

Commercial pure Ti particles (catalog #IRMM531A) were purchased from Sigma Corporation (Sigma, St. Louis, MO, USA). Their distribution and size were subsequently examined by five continuous measurements with a Coulter counter (Beckman Coulter Inc., USA). The average size of the particles was 3.32 ± 2.39 mm. More than 90% of the particles were < 3.6 mm, 50% were < 1.6 mm, and 10% were < 1.0 mm. Ti particles were baked for 6 h at 180 °C and washed in ethanol to eliminate endotoxins, as previously reported [9]. The levels of endotoxin were examined with a quantitative Limulus Amebocyte Lysate Assay, and the results showed that the threshold of positivity was 0.05 EU/mL. Endotoxin-free particles were selected for the experiment (Fig. 1a).

Animals

C57BL/6 mice at 12 weeks of age were purchased from the Experimental Animal Center of Shandong University. The animals were treated for over 7 days in a standard environment (23 ± 2 °C, 12-h light/dark cycle). All experiments were conducted in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee of Shandong University.

Ti Particle-Induced Calvarial Osteolysis Model

The mouse model of Ti particle-induced osteolysis at calvarial was induced as previously reported [61, 62]. Briefly, mice were randomly assigned to three groups ($n = 7$ per group): the sham control (CTL), Ti particles only (Ti), and Ti particles with ghrelin (ghrelin group). The mice were anesthetized using chloral hydrate *via* intraperitoneal injection. Following, 0.5-cm sagittal incision was made in each mouse and the periosteum remained intact. A 25-gauge needle was used to inject 100 μL of PBS with or without 20 mg Ti particles resuspended in it directly over the calvarial bone and periosteum in sterile technique, mice in ghrelin group were applied with treatment of ghrelin (1 μg/g body weight, sc-364,689, Santa Cruz Biotechnology, U.S.A.) in 0.2 mL sterile phosphate-buffered saline (PBS), and mice in the CTL group and Ti group were treated with PBS, all *via* intraperitoneal injection. Both ghrelin and PBS treatment were conducted every day. All mice were administered an oral antibiotic in the form of enrofloxacin (100 mg/mL) in the drinking water for 3 days postoperation. No adverse effects or death occurred in the period of the experiment. The mice were sacrificed at the 14th day in a CO_2 chamber, and the calvariae tissues were excised and fixed in formalin for further analysis.

Micro-CT Assessment

The gathered calvariae tissues were initially fixed in formalin and subsequently examined by micro-CT with a Sky-scan1176 scanner and associated analysis software (SkyScan, Aartselaar, Belgium). The scanning protocol was set at an isometric resolution of 18 mm, with X-ray energy settings of 80 kV and 100 mA. The reconstruction of three-dimensional (3D) images was conducted using Cone Beam Reconstruction software (SkyScan), followed by further processing with a CT analyzer (CT An, SkyScan) to achieve quantitative analysis. A cylindrical volume of interest (VOI of 3*3*1 mm) was positioned on the calvaria in its center to reduce the bias of the 3D analysis of the calvaria as previously described. In brief, the scanned images from each group were evaluated at the same thresholds to allow 3D structural reconstruction of each sample. The osteolysis in calvaria of each treatment group was examined through structural reconstruction. The

bone mineral density (BMD), bone volume (BV), BV against tissue volume (BV/TV), and number of pores within the VOI were obtained as previously described [9].

Histology Analysis

Calvariae tissues obtained from seven mice per group were fixed in formalin for 48 h and were subsequently decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 3 weeks, followed by embedded in paraffin wax. Cross-sections were cut, and hematoxylin and eosin (H&E) was applied to stain. Moreover, serial sections were stained using tartrate-specific acid phosphatase-positive (TRAP) osteoclasts. Photos were subsequently obtained and examined with light. The histomorphometric analysis was performed on the most central section and on four adjacent sections using image analysis software. The region of interest (ROI) was set around the center of the middle suture. The eroded surface was determined and quantified using the methods established as was previously reported [63]. The inflammatory cell infiltration was detected *via* a periosteum thickness examination. The osteoclast number was examined at the range of the ROI, which is TRAP-positive and located in the resorption lacuna. Moreover, the osteoclast surface per bone surface (OCs/BS, %) was detected with Image Pro-Plus 6.0 software and the manufacturer's instructions.

Immunohistochemistry

Calvariae tissues of the mouse models of each experiment were harvested. Calvariae tissues were fixed in formalin for 7 days, followed by decalcification in 10% EDTA for 3 weeks. The tissues were subsequently dehydrated and embedded in paraffin, and 6-μm sections were cut. Moreover, the sections were deparaffinized through xylene immersion, rehydrated by graded ethanol, and treated with 0.1% trypsin for 45 min at 37 °C. After blocking in 20% goat serum for 60 min at RT, the sections were hatched with ghrelin (1:500 dilution, sc-50297, Santa Cruz, USA) or osteorix (1:1000 dilution, ab22552, Abcam Corporation, USA) at 4 °C overnight, followed by cultivation with horseradish peroxidase-conjugated secondary antibody (Zhongshanjinqiao Biotechnology, P.R., China) for 60 min at RT. The signal was detected using a Vector Elite ABC Kit (Vectastain, Vector).

Cell Culture

The MC3T3-E1, a widely accepted preosteoblast cell line was employed. Briefly, MC3T3-E1 cells were cultured at 37 °C in 5% CO_2 in DMEM (Gibco, USA) with 10% fetal bovine serum (FBS) (Gibco, USA) for 24 h. Non-adherent cells were removed, and the adherent cells were further cultured until the cells became approximately 90% confluent. The cells were seeded in 96-well plates at a density of 1.5×10^4 cells/well in DMEM that contained 10% FBS and 0.1 mg/mL Ti particles with/without treatment of ghrelin for 48 h, and the culture medium and cells were gathered for further analysis.

ALP Staining

After being cultured in osteogenic medium for 1 week, the cells were stained for ALP. In brief, after fixation in 4% paraformaldehyde for 15 min, the mMSCs were rinsed three times with PBS and were subsequently immersed in BCIP/NBT working solution in the dark for 10 min. The staining results were then microscopically analyzed.

Immunofluorescence

MC3T3-E1 cells were selected to verify the expression of ghrelin or β-catenin with immunofluorescence staining. According to our previously reported study [64], with minor modifications, MC3T3-E1 cells were stimulated with Ti particles in the presence or absence of ghrelin for 1 h. Immunofluorescence staining of ghrelin (1:500 dilution, sc-50297, Santa Cruz, USA) or β-catenin (1:1000 dilution, 17565-1-AP, Proteintech Corporation, USA) was subsequently performed on these cells and examined using a confocal fluorescence microscope system.

Western Blot Analysis

Total protein was collected from cells with P0013 cell lysis buffer (Beyotime Corporation, Shanghai, China) and then centrifuged at 12,000 rpm for 6 min at 4 °C. Cytoplasmic and nuclear proteins were isolated using a commercially available Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Corporation, Shanghai, China) according to the manufacturer's instructions. The protein concentrations in the supernatants were examined with a BCA protein assay kit (Beyotime Corporation, Shanghai, China). Equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membranes. After blocking with 5% BSA for 1 h at room temperature, the membranes were incubated with primary antibodies against β-actin (1:1500 dilution, #2148, Cell Signaling Technology, USA), iNOS (1:1000 dilution, ab15323, Abcam Corporation, USA), ghrelin (1:500 dilution, sc-50297, Santa Cruz, USA), ALP (1:1000 dilution, 11400-1-AP, Proteintech Corporation,

Table 1. Primers for Real-Time PCR

Source	Name	Forward	Reverse
Mouse	ALP	5′-CGGAGCAAGCAACATCTCAG-3′	5′-GGGTCGTTTTCCAGGACAGT-3′
	Osteorix	5′-TGAGCTGGAACGTCACGTGC-3′	5′-AAGAGGAGGCCAGCCAGACA-3′
	Runx-2	5′-TTGACCTTTGTCCCAATGC-3′	5′-AGGTTGGAGGCACACATAGG-3′
	Col-1	5′-CGGACCTAAAGGGGAGATGG-3′	5′-CCAGCCGCACTGAATTGAGT-3′
	Osteocalcin	5′-CAGGGTGTTGCCCAAACTG-3′	5′-GGCTGCGTTCTTCTTTGCT-3′
	Axin-2	5′-GTCTCTACCTCATTTCCCGAGAAC-3′	5′-CGAGATCAGCTCAGCTGCAA-3′
	TNF-α	5’-CCAACATGCTGATTGATGACACC-3′	5′-GAGAATGCCAATTTTGATTGCCA-3′
	IL-6	5′-CTGCAAGAGACTTCCATCCAG-3′	5′-AGTGGTATAGACAGGTCTGTTGG-3′
	iNOS	5′-CTCTTCGACGACCCAGAAAAC-3′	5′-CAAGGCCATGAAGTGAGGCTT-3′
	Caspase-3	5′-CTCGCTCTGGTACGGATGTG-3′	5′-TCCCATAAATGACCCCTTCATCA-3′
	Caspase-7	5′-AAGACGGAGTTGACGCCAAG-3′	5′-CCGCAGAGGCATTTCTCTTC-3′
	Caspase-9	5′-GGCTGTTAAACCCCTAGACCA-3′	5′-TGACGGGTCCAGCTTCACTA-3′
	Ghrelin	5′-TGTTTGCAGAGCACTACTTGAA-3′	5′-CAGTCACCTCTAAGCCAAAGAAA-3′
	β-Catenin	5′-ACGGTGCCGCGCCGCTTATA-3′	5′-TAGCCATTGTCCACGCAGCGG-3′
	GAPDH	5′-AGCAGTCCCGTACACTGGCAAAC-3′	5′-TCTGTGGTGATGTAAATGTCCTCT-3′

USA), osteorix (1:1000 dilution, ab22552, Abcam Corporation, USA), Runx-2 (1:1000 dilution, ab76956, Abcam Corporation, USA), Col 1 (1:1000 dilution, 14695-1-AP, Proteintech Corporation, USA), β-catenin (1:1000 dilution, 17565-1-AP, Proteintech Corporation, USA), phospho-β-catenin (1:1000 dilution, 05-665, Merck Technology, USA), or non-phospho β-catenin (1:1000 dilution, #8814, Cell Signaling Technology, USA) at RT for 1–2 h, followed by the application of appropriate HRP-conjugated secondary antibodies for 1 h at RT. Immunoreactive bands were imaged with a DNR Bio-Imaging system based on the manufacturer's instructions. The expression of cytoplasmic protein was normalized to β-actin using ImageJ software, as was previously reported [65].

Real-Time Quantitative PCR

Total mRNA was extracted from the MC3T3-E1 cells of each experimental group with TRIzol Reagent (Takara Biotechnology, Otsu, Japan). Following extraction, reverse transcription was completed to convert total RNA to cDNA with the PrimeScript RT Reagent Kit (Takara Biotechnology, Otsu, Japan) following the manufacturer's protocol. PCR was performed with an RNA PCR kit (Takara Biotechnology, Otsu, Japan). The $2^{-\Delta\Delta CT}$ method was utilized to quantify the data. The PCR primer sequences are listed in Table 1.

Enzyme-Linked Immunosorbent Assay

The concentrations of IL-1β and IL-6 in the cell culture media from MC3T3-E1 cells were measured *via* ELISA according to the manufacturers' instructions (ELISA kits for IL-1β: eBioscience, Frankfurt, Germany; ELISA kits for IL-6: R&D Systems, Minneapolis, MN, USA). All assays were performed in duplicate, as previously reported [66].

Statistical Analysis

The data are presented as the mean ± standard deviation, and GraphPad Prism v. 6.0 software was used for statistical analyses. Student's *t* test or one-way analysis of variance (ANOVA) was employed to determine the statistical significance of the differences. A value of $p < 0.05$ was regarded as statistically significant.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Yunpeng Zhao, Weiwei Li, and Yuhua Li

Performed the experiments: Ruize Qu, Xiaomin Chen, Wenhan Wang, Cheng Qiu, Long Liu, Peng Li, Liang Liu, Zhaoyang Zhang, and Yongjuan Yuan

Analyzed the data: Krasimir Vasilev, John Hayball, Yunpeng Zhao, and Weiwei Li

Contributed reagents/materials/analysis tools: Yunpeng Zhao and Weiwei Li

FUNDING INFORMATION

This work was supported by the Key Research and Development Projects of Shandong Province (Grant No. 2015GSF118115), the Natural Science Foundation of Shandong Province (Grant Nos. BS2014YY048, BS2015SW028, ZR2014HM101, and ZR2016HM53), the Cross-disciplinary Fund of Shandong University (Grant No. 2018JC007), the

Population and Family Planning Commission of Shandong Province (Grant No. 2016w80333), and the National Natural Science Foundation of China (Grant No. 81501880 to Yunpeng Zhao and 81602761 to Weiwei Li).

COMPLIANCE WITH ETHICAL STANDARDS

All experiments were conducted in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee of Shandong University.

Competing Interests. The authors declare that there are no competing interests.

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