Original article

MOLECULAR METABOLISM

# Genetic determination of the cellular basis of the ghrelin-dependent bone remodeling

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## ABSTRACT

**Objective:** Bone mass is maintained through a balance of bone formation and resorption. This homeostatic balance is regulated by various systems involving humoral and local factors. The discovery that the anorexigenic hormone leptin regulates bone mass via neuronal pathways revealed that neurons and neuropeptides are intimately involved in bone homeostasis. Ghrelin is a stomach-derived orexigenic hormone that counteracts leptin's action. However, the physiological role of ghrelin in bone homeostasis remains unknown. In this study, through the global knockout of ghrelin receptor (Ghsr) followed by tissue-specific re-expression, we addressed the molecular basis of the action of ghrelin in bone remodeling *in vivo*.

**Methods:** We performed molecular, genetic and cell biological analyses of Ghsr-null mice and Ghsr-null mice with tissue specific Ghsr restoration. Furthermore, we evaluated the molecular mechanism of ghrelin by molecular and cell-based assays.

**Results:** Ghsr-null mice showed a low bone mass phenotype with poor bone formation. Restoring the expression of Ghsr specifically in osteoblasts, and not in osteoclasts or the central nervous system, ameliorated bone abnormalities in Ghsr-null mice. Cell-based assays revealed ghrelin induced the phosphorylation of CREB and the expression of Runx2, which in turn accelerated osteoblast differentiation.

**Conclusions:** Our data show that ghrelin regulates bone remodeling through Ghsr in osteoblasts by modulating the CREB and Runx2 pathways.



**Keywords** Bone remodeling; Ghrelin; Osteoblast; CREB; RUNX2

## 1. INTRODUCTION

Bone remodeling, the function affected in osteoporosis, comprises two phases: bone formation by matrix-producing osteoblasts and bone resorption by osteoclasts [1,2]. The demonstration that the anorexigenic hormone leptin inhibits bone formation through a hypothalamic relay suggests that other molecule(s) affecting energy metabolism may also modulate bone mass [3–5]. Ghrelin is a peptide of 28 amino acids that was initially isolated from the rat stomach as an endogenous ligand of the growth hormone secretagogue receptor (Ghsr), which affects energy homeostasis by binding ghrelin [6]. Ghrelin has various physiological effects; it stimulates the appetite, increases food intake, and decreases energy metabolism through the Ghsr [7]. Clinically, gastrectomy is known to rapidly cause osteopenia, independent of nutritional defects such as insufficient calcium absorption [8], which indicates that the stomach regulates bone remodeling per se. Previously, we and others have demonstrated that ghrelin regulates osteoblast differentiation without affecting osteoclasts [9–11], and these findings also indicated that ghrelin may play an important role in bone remodeling *in vivo*. In contrast, a recent report using Ghsr-null mice showed that ghrelin regulates bone mass *in vivo* by regulating osteoclastic bone resorption through an unknown mechanism without affecting osteoblastic bone formation [12]. Thus, the physiological role of ghrelin in bone homeostasis remains to be determined.

In this study, through the global knock-down of Ghsr followed by tissue-specific re-expression, we addressed the molecular basis of the action of ghrelin in bone remodeling *in vivo*; these findings demonstrate that ghrelin regulates bone remodeling through osteoblastic Ghsr expression.

## 2. MATERIALS AND METHODS

### 2.1. Animals

Ghsr-null mice (Ghsr-null) [13], Osterix-Cre mice [14], Nestin-Cre mice [15] and Cathepsin K-Cre mice (Ctsk-Cre) [16] were described previously. The Ghsr-null mice were generated by inserting a loxP-flanked transcriptional blocking cassette (TBC) into a putative intron located downstream of the transcriptional start site and upstream of the translational start site of the murine *Ghsr* gene; this construct resulted in the knockout of *Ghsr* expression. Mating these Ghsr-null mice with tissue-specific Cre mice leads to the removal of the loxP-flanked TBC and enables the tissue-specific restoration of *Ghsr* expression in subsequent experiments [13]. We used littermates for all the experiments and we backcrossed at least eight times to ensure that mice are

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on a pure background. We bred Ghsr heterozygous mice with Ghsr heterozygous mice to obtain Ghsr-null and wild-type littermates. We also bred Ghsr-null mice with Ghsr-null/Nes-Cre or Ctsk-Cre or Osx-Cre mice to obtain Ghsr-null/Nes or Ctsk or Osx mice and Ghsr-null littermates. We maintained all the mice in a 12-h light–dark cycle with *ad libitum* access to regular food and water. All of the animal experiments were performed with the approval of the Animal Study Committee of Tokyo Medical and Dental University and conformed to the relevant guidelines and laws.

### 2.2. Cell culture

*In vitro* primary osteoblast and osteoclast-like cell cultures were established as previously described in Ref. [17]. Briefly, calvarial osteoblast cells were isolated from 4-day-old mice by enzymatic digestion in α-minimal essential medium (α-MEM) containing 0.5 mg/ml collagenase-P (Roche) and 0.05% trypsin. To induce osteoblast differentiation, primary osteoblasts or MC3T3-E1 osteoblastic cells were cultured in osteogenic medium (0.1 mg/ml ascorbic acid, 10 mM β-glycerophosphate) for 7 days after they reached confluence. The MEK inhibitor U0126, PKA inhibitor H89 and ghrelin were obtained from Promega, Calbiochem and the Peptide Institute, respectively.

*In vitro* osteoclast differentiation was carried out as previously described in Ref. [17]. Briefly, bone marrow cells from 6- to 8-week-old mouse femurs were cultured in α-MEM supplemented with FBS in the presence of human macrophage colony-stimulating factor (M-CSF, 10 ng/ml; R&D Systems) for 2 days and then differentiated into osteoclasts using human RANKL (50 ng/ml; PeproTech) and M-CSF for 3 days.

The osteoblast proliferation assays were performed using the Cell Counting Kit-8 (DOJINDO) according to the manufacturer's instructions.

All the results are representative of more than three individual experiments.

### 2.3. Quantitative real-time PCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen), and reverse transcription was performed with the ReverTra Ace qPCR RT Kit (TOYOBO) according to the manufacturer's instructions. We performed quantitative analysis of gene expression using an Mx3000P real-time PCR system (Agilent Technologies). The mRNA levels are expressed relative to the housekeeping gene *Gapdh* and were calculated by the comparative threshold cycle (ΔΔCt) method.

### 2.4. Western blot analysis

Western blot analysis was performed according to a previously described standard protocol [17]. Primary antibodies against p38α, phospho-p38α, SAPK/JNK, phospho-SAPK/JNK, p44/42, and phospho-p44/42 (1:1,000; Cell Signaling); β-actin (1:2,000; Sigma); and Runx2 (1:1,000; Santa Cruz) were used.

### 2.5. Dual-luciferase reporter assay

We transfected MC3T3-E1 cells using Lipofectamine 2000 (Invitrogen). The activities of firefly luciferase and Renilla luciferase were determined using the dual-luciferase reporter assay (Promega).

### 2.6. *In vivo* studies

For intracerebroventricular (ICV) injection experiments, ghrelin (10 nmol/kgBW/day) was infused intracerebroventricularlly to 3-month-old female mice for 25 days as previously described in Ref. [5]. Briefly, a hole was made in the skull using a needle at a position 1.0 mm lateral to the central suture and 1.0 mm posterior to the bregma. A cannula was inserted into the third ventricle through the hole and connected to an osmotic pump (ALZET® Osmotic Pumps) placed in the dorsal subcutaneous space of the mouse.

All mice were housed individually in cages. The food intake of the mice was measured by subtracting the amount of uneaten food from the initial amount of premeasured food every morning at 10:00.

We also measured the visceral fat mass weight at 25 days.

### 2.7. Histological and histomorphometric analyses

We injected calcein (25 mg/kg, Sigma) i.p. 5 days and 2 days prior to sacrifice. We then stained the non-decalcified sections of the third and fourth lumbar vertebrae using the von Kossa technique. We performed static and dynamic histomorphometric analyses using the Osteomeasure Analysis System (Osteometrics). We analyzed 8–10 mice in each group.

### 2.8. Micro-CT analyses

We obtained two-dimensional images of the distal femur and femoral diaphysis by micro-CT analysis (Comscan). The following three dimensional morphometric parameters were determined using TRI/3D-BON software (RATOC): trabecular bone fraction (BV/TV), trabecular thickness (Tb. Th), trabecular number (Tb. N), trabecular separation (Tb. Sp), trabecular spacing (Tb. Spac), cortical volume (CtV), cortical thickness (CtTh), cortical perimeter (Ct. Perimeter), and bone mineral density (BMD).

### 2.9. Measurement of growth hormone (GH), insulin-like growth factor 1 (IGF1) and Tartrate-resistant acid phosphatase 5b (TRACP5b)

We measured the serum levels of GH, IGF1 and TRACP5b using a GH ELISA kit (Millipore), an IGF-1 ELISA kit (Alpco Diagnostics) and a TRACP5b ELISA kit (Immunodiagnostic Systems Ltd.) according to the manufacturers' instructions.

### 2.10. Statistical analysis

All data are represented as the mean ± s.d. ($n = 5$ or more), unless specified. We performed statistical analysis using Student's t-tests. Differences were considered to be statistically significant at $P < 0.05$. The results are representative of more than three individual experiments.

## 3. RESULTS

### 3.1. Ghsr signaling is required for proper bone formation and bone mass accrual

To address the role of ghrelin in bone remodeling, we first examined the expression of *ghrelin* (*Ghrl*) and its receptor (*Ghsr*) in bone tissue, osteoblasts and osteoclasts (Figure 1A). Ghrelin was expressed in bone cells at lower levels than in the stomach (Figure 1A). Substantial expression of *Ghsr* was observed in bone (Figure 1A), although the level of *Ghsr* in the bone was lower than that observed in the brain (Figure 1A), where *Ghsr* expression is known to be high [7]. Because ghrelin exerts its actions through the Ghsr, we next took advantage of Ghsr-null mice [13] to study whether ghrelin-Ghsr signaling regulates bone mass *in vivo*. Ghsr-null mice were created by inserting a loxP-flanked transcriptional blocking cassette (TBC) into a putative intron located upstream of the transcriptional start site of the *Ghsr* gene, which hampered Ghsr expression throughout the body.

Indeed, the expression of *Ghsr* was significantly decreased in the bones of Ghsr-null mice (Figure 1B). We analyzed both male and female Ghsr-null mice at 3 months and observed that male and female Ghsr-null mice exhibit low bone mass phenotypes as shown by a decrease in

 

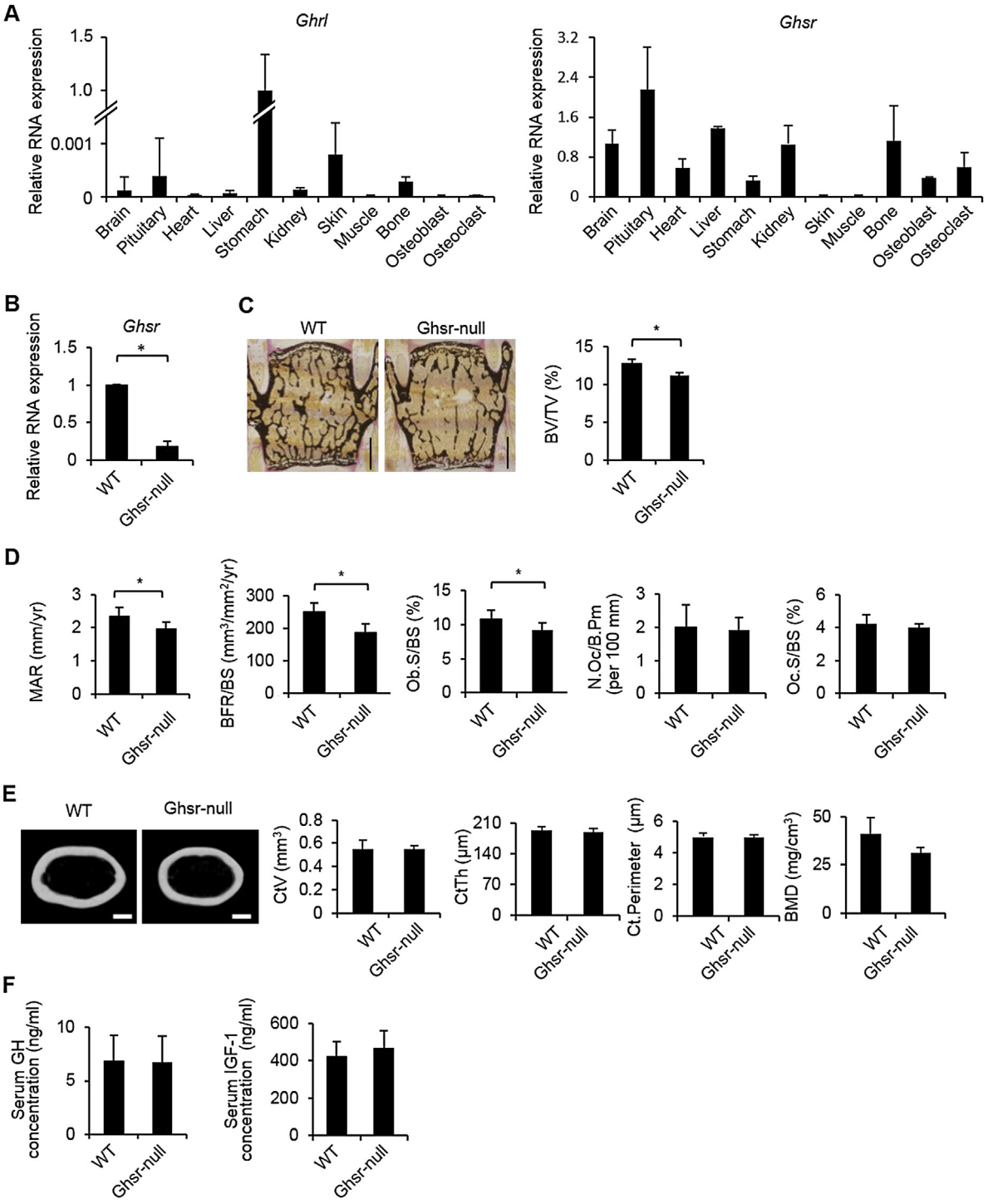


**Figure 1: Ghsr deficiency induces low bone mass**. (A) Gene expression analysis of Ghrl and Ghsr in several tissue types, including brain, bones (femur and tibia), skin, primary osteoblasts, and bone marrow-derived osteoclast-like cells ($n = 3$ or more). (B) Expression of Ghsr in the bones of wild-type (WT) and Ghsr-null male mice. (C, D) Histological (C) and histomorphometric (D) analyses in 3-month-old male mice (B–D, $n = 8$, or more. The L3 and L4 vertebrae were analyzed.). Bone volume/tissue volume (BV/TV; %), mineral apposition rate (MAR; mm/yr), bone formation rate/bone surface (BFR/BS; mm3/mm2/yr), osteoblast surface/bone surface (Ob. S/BS; %), osteoclast number/bone perimeter (No. Oc./B. Pm), and osteoclast surface/bone surface (Oc. S/BS; %). Note the decrease in bone mass in the Ghsr-null mice. (E) Micro-CT analyses of cortical bone in 3-month-old male mice femur. Cortical volume (CtV; $mm^2$), cortical thickness (CtTh; µm), cortical perimeter (Ct.Perimeter; µm), and bone mineral density (BMD; $mg/cm^3$). (F) The serum concentration of GH and IGF-1 in WT and Ghsr-null male mice ($n = 8$). The scale bars represent 500 µm. The error bars indicate the standard deviation (s.d.). *$P < 0.05$.

bone volume/tissue volume (BV/TV), which means percent of total marrow cavity that is occupied by cancellous bone (Figure 1C and Figure S1A). Because female Ghsr-null mice fed a normal diet tend to have lower body weights [13] and because body weight is known to be a strong confounding factor for bone mass, we used only male mice for subsequent experiments. Ghsr-null male mice demonstrated a low-bone-mass phenotype at 6 months (Figure S1B). This phenotype was due to a decrease in bone formation as shown by a decrease in mineral

  

apposition rate (MAR; a linear rate of new bone deposition), bone formation rate/bone surface (BFR/BS; amount of new bone formed in unit time per unit of bone surface), and osteoblast surface/bone surface (Ob.S/BS; percent of bone surface occupied by osteoblasts) (Figure 1D and Figure S1B). However, bone resorption was unaffected as shown by the normal osteoclast number/bone perimeter (No. Oc./B. Pm; the number of osteoclast in hundred millimeter of bone perimeter) and osteoclast surface/bone surface (Oc.S/BS; the percent of bone surface occupied by osteoclasts and TRACP5b, a bone resorption marker) (Figures 1D and 3C, D and Figure S1B). Ghsr-null mice exhibited low trabecular bone mass in vertebrae and long bones (Figure 1C and Figure S1C), whereas cortical bone parameters were unaffected (Figure 1E). Interestingly, the serum levels of growth hormone (GH) and insulin-like growth factor 1 (IGF-1) in Ghsr-null mice were comparable to those of wild-type mice (Figure 1F), which indicated that ghrelin affects bone formation independent of the GH/IGF-1 axis. Collectively, these results indicate that Ghsr signaling is essential for the maintenance of bone mass.

### 3.2. Ghrelin regulates bone remodeling independent of the central nervous system

Leptin regulates bone remodeling through the central nervous system (CNS) [5], which suggests that other peptide(s) regulating food intake and energy metabolism may also play a role in bone remodeling through the CNS. Because ghrelin regulates food intake and energy metabolism through the CNS [6], we evaluated whether it also affects bone metabolism through this pathway. To address this question, we performed an ICV infusion of ghrelin for 25 days. ICV infusion of various hormones or neuropeptide for a relatively short period (less than 1 month) is known to affect bone metabolism [5,18,19]. However, ICV infusion of ghrelin did not affect bone mass. This administration of ghrelin to wild-type mice increased their food intake and visceral fat mass (Figure 2A,B). Remarkably, the ICV infusion of ghrelin did not affect bone mass in either wild-type or Ghsr-null mice (Figure 2C,D). These results indicate that ghrelin does not affect bone remodeling through the CNS. Interestingly, serum level of IGF-1, a downstream hormone that is responsible for the actions of GH [7], was unaffected by the ghrelin ICV infusion (Figure 2E), indicating that the chronic stimulation of Ghsr signaling does not affect the physiological GH/IGF-1 pathway activation *in vivo*.

To confirm the absence of a central effect of ghrelin on bone remodeling, we used a genetic approach to test whether the selective restoration of Ghsr expression in the brain would affect bone metabolism [20—22]. We crossed Ghsr-null mice to Nestin-Cre mice (Ghsr-null/Nes mice) [15], in which the expression of Cre protein leads to the

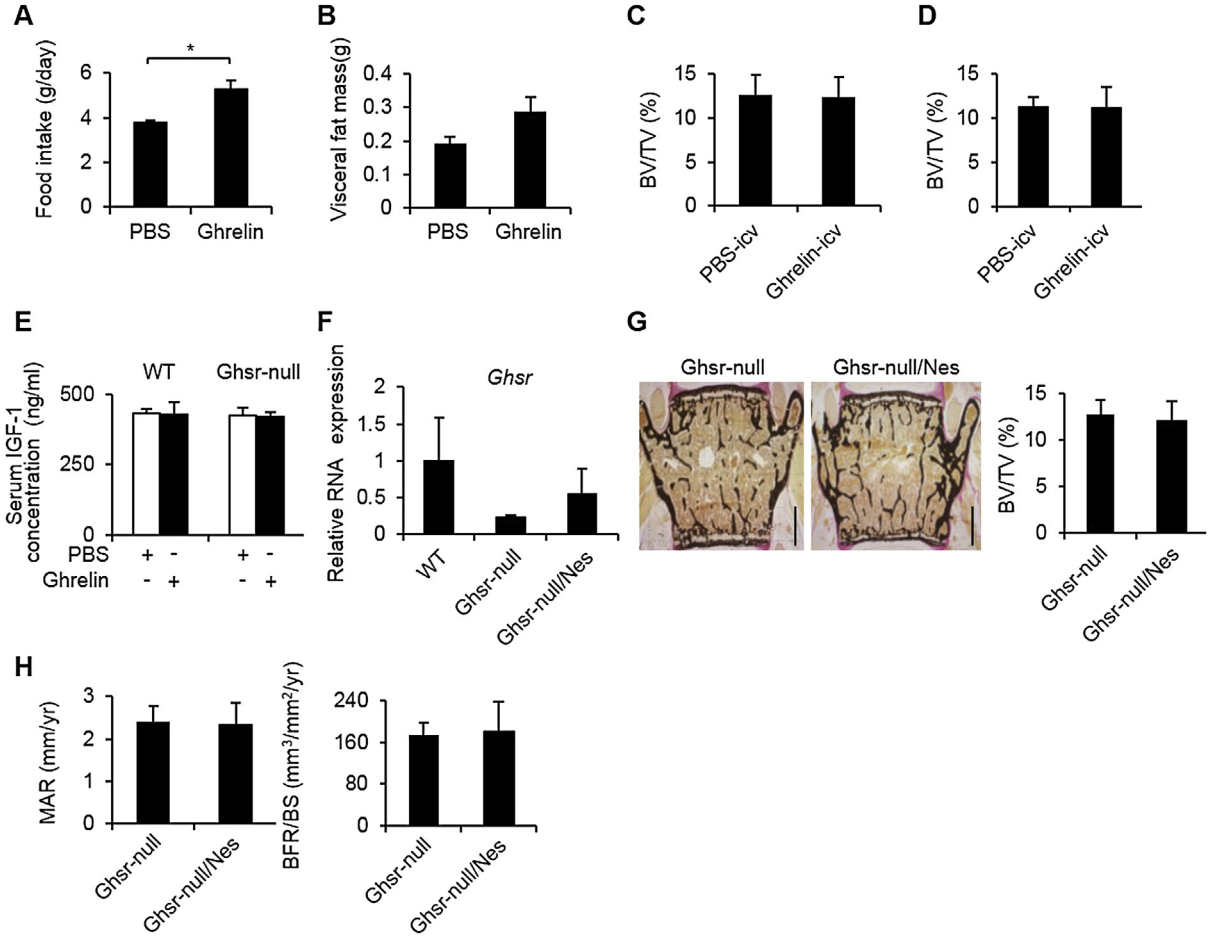


**Figure 2: Ghrelin regulates bone remodeling independent of the central nervous system.** (A—E) Analysis of wild-type (WT) and Ghsr-null male mice with an intracerebroventricular (ICV) infusion of ghrelin (10 nmol/kg body weight/day). Food intake (A), visceral fat mass (B) and histological analysis (C) of wild-type male mice following ghrelin ICV infusion. Histological analysis of Ghsr-null male mice following ghrelin ICV infusion (D). Serum concentration of IGF-1 after ICV ghrelin infusion (E). (F) Expression of *Ghsr* in the brain of Nestin-Cre-Ghsr-null male mice (Ghsr-null/Nes). (G, H) Histological (GF) and histomorphometric (H) analyses of Ghsr-null/Nes male mice. (A—H, $n = 8$, or more. L3 and L4 vertebrae were analyzed.) The scale bars represent 500 μm. The error bars indicate the standard deviation (s.d.). $^*P < 0.05$.

 

removal of the loxP-flanked TBC specifically in the CNS. However, a histologic and histomorphometric analyses of these animals revealed no significant changes in bone remodeling parameters compared with the Ghsr-null mice in the presence of substantial re-expression of Ghsr (Figure 2F,G,H). Taken together, these results excluded the central nervous system as the key mediator of the anabolic actions of ghrelin in bone tissue.

### 3.3. Ghsr in osteoclasts is not responsible for the bone abnormalities of Ghsr-null mice

A previous report showed that the ghrelin-Ghsr pathway regulates bone remodeling by altering osteoclastic bone resorption without any effect on bone formation, although the mechanism underlying this effect was not elucidated [12]. To study whether Ghsr expression in osteoclasts is responsible for the bone abnormalities observed in Ghsr-null mice, we crossed Ghsr-null mice to Cathepsin K Cre mice (Ghsr-null/Ctsk mice) [16]. Bone histology and histomorphometric analyses revealed that the bones of Ctsk-Ghsr-null mice were indistinguishable from those of Ghsr-null mice with respect to bone mass in the presence of substantial re-expression of Ghsr (Figure 3A,B). Moreover, the serum concentration of TRACP5b was not different between Ghsr-null mice and Ghsr-null/Ctsk mice or wild-type mice and Ghsr-null mice (Figure 3B—D). In accordance with these *in vivo* observations, ghrelin treatment did not affect RANKL-induced *in vitro* osteoclast differentiation (Figure 3E). Collectively, these results demonstrate that ghrelin-Ghsr signaling in osteoclasts is dispensable for the anabolic actions of ghrelin in bone tissue.

### 3.4. The restoration of Ghsr in osteoblasts rescues the bone abnormalities of Ghsr-null mice

Ghrelin is known to affect the cellular proliferation and differentiation of osteoblasts *in vitro* [9,11]. Therefore, we next studied whether osteoblastic expression of Ghsr is important for bone metabolism *in vivo*. We crossed Ghsr-null mice to Osx-Cre mice [14] to restore Ghsr expression in osteoblasts (Ghsr-null/Osx mice) and performed histologic and histomorphometric analyses. Recombination of the Ghsr allele was undetectable in all the tissues except bone, as expected, and the small intestine, a crucial organ for calcium and phosphate absorption, albeit faintly (Figure 4A), indicating that Ghsr expression was restored in osteoblasts mainly. Indeed, the expression of *Ghsr* in the bones and primary osteoblasts of Ghsr-null/Osx mice was restored to approximately 60—80% of that observed in control mice (Figure 4B,C). Importantly, serum levels of calcium and phosphate were comparable between Ghsr-null mice and Ghsr-null/Osx mice (Figure 4D), indicating that intestinal Ghsr is dispensable for calcium and phosphate absorption. Consistent with the restored expression of *Ghsr*, the bone volume of Ghsr-null/Osx mice was significantly increased compared to that observed in Ghsr-null mice (Figure 4E). Histomorphometric analysis revealed that the bone formation abnormalities, including decreased bone formation and mineral apposition rates, were also significantly increased compared to those observed in Ghsr-null mice (Figure 4F). In contrast, the osteoclast parameters were unchanged (Figure 4F), indicating that the osteoblastic expression of Ghsr is responsible for the bone abnormalities observed in the Ghsr-null mice. Furthermore, the expression of osteoblast marker genes including *Col1a1*, *Alpl* and *Runx2*, which was decreased in the bones and osteoblasts of Ghsr-null mice, was rescued in Ghsr-null/Osx mice to a level comparable to that observed in wild-type mice (Figure 4G and Figure S2). Taken together, these results demonstrate that the osteoblastic expression of Ghsr is necessary and sufficient for the bone anabolic actions of ghrelin.

### 3.5. Ghrelin regulates osteoblast differentiation and proliferation by modulating the CREB and Runx2 pathways

To further examine the anabolic actions of ghrelin on bone, we next knocked down *Ghsr* expression using a siRNA and treated osteoblastic cells with ghrelin. As expected, ghrelin treatment increased both the differentiation and proliferation of osteoblastic cells (Figure 5A,B), whereas a siRNA against *Ghsr* successfully knocked down the expression of *Ghsr* and abolished osteoblast differentiation

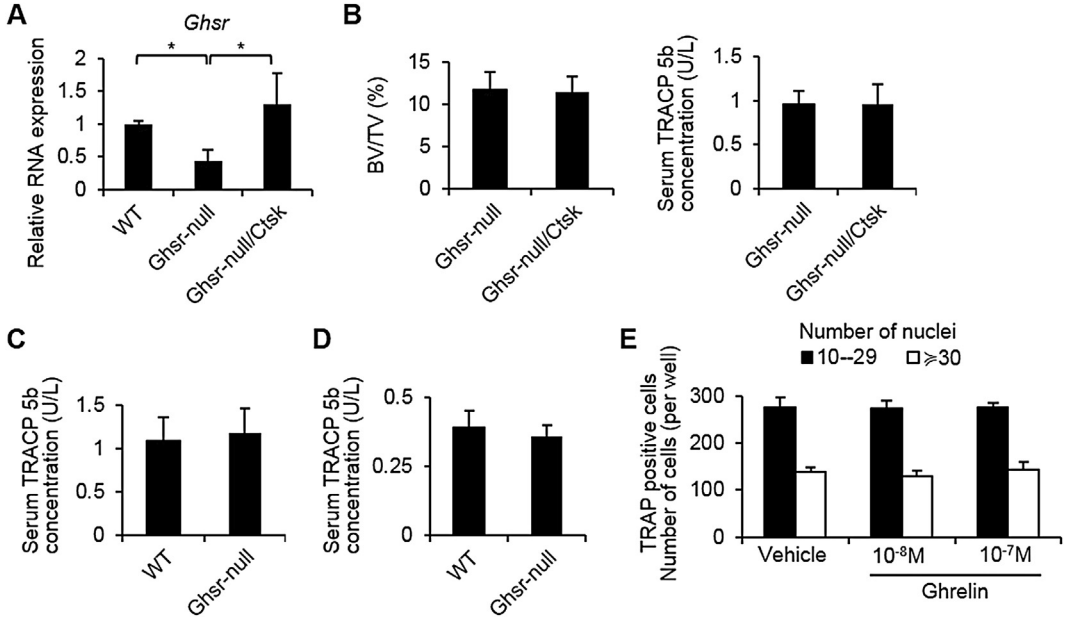


**Figure 3: Ghrelin-dependent bone remodeling does not involve osteoclasts.** (A) Expression of *Ghsr* in the osteoclast of Cathepsin K-Cre-Ghsr-null male mice (Ghsr-null/Ctsk). (B—D) Histological analysis (B) and measurement of a bone resorption marker (B—D) in 3-month-old Ghsr-null/Ctsk male mice (B) and 3-month-old (C) and 6-month-old (D) Ghsr-null male mice. $n = 8$. (E) Number of osteoclasts after ghrelin treatment, $n = 10$. Note the lack of a difference in the bone resorption marker in the Ghsr-null/Ctsk, Ghsr-null, and wild-type mice. The error bars indicate the standard deviation (s.d.). $^*P < 0.05$.

  

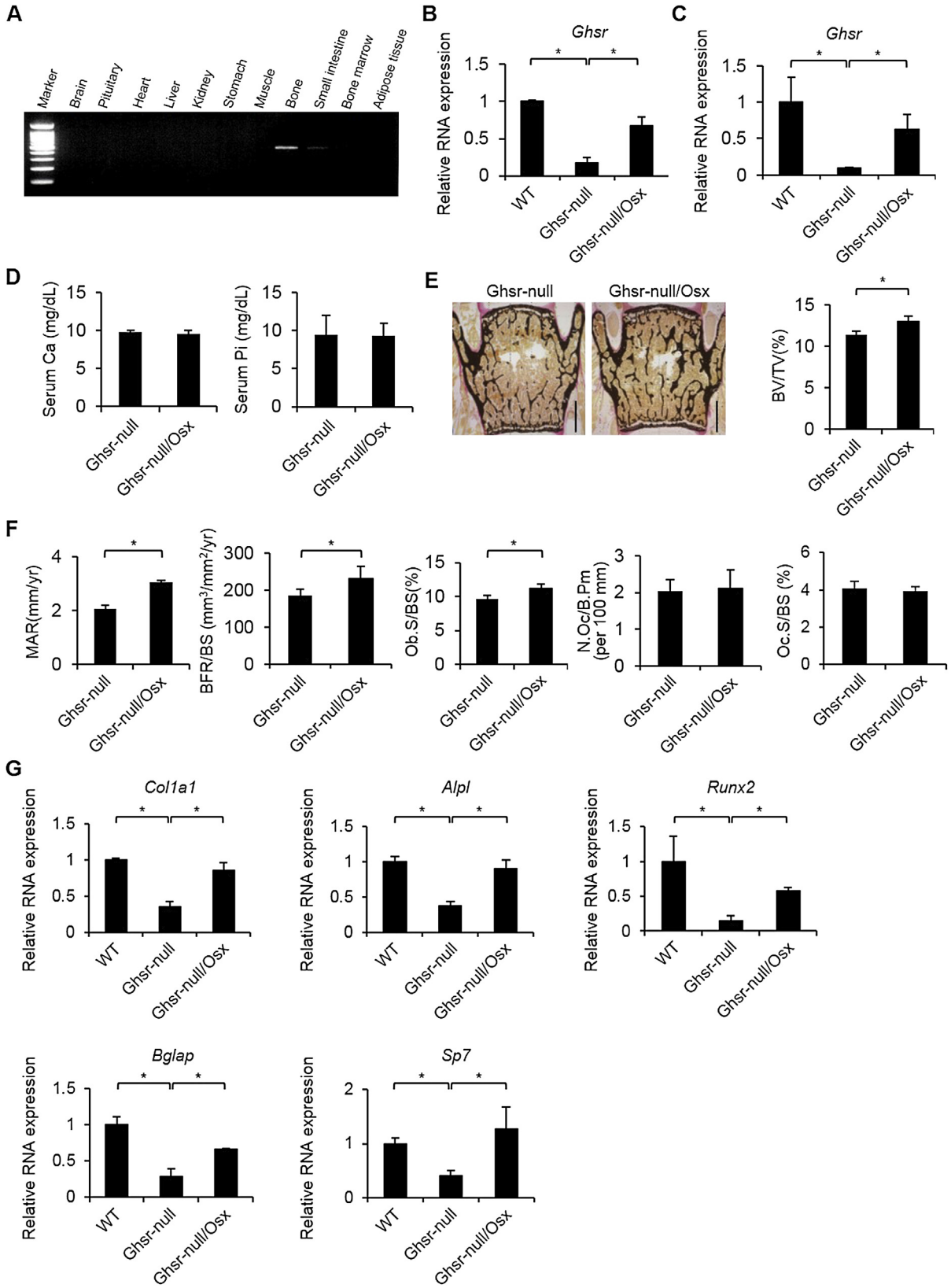


**Figure 4: Rescue of Ghsr expression specifically in osteoblasts ameliorates low bone mass in Ghsr-null mice.** (A) Genomic DNA PCR analysis in the tissues of Ghsr-null/Osx mouse. The PCR can detect the delta allele, which is observed when there has been Cre-mediated removal of transcriptional blocking cassette (TBC) and one of the loxP site. Note the expression of the delta band in the bone and small intestine, albeit faintly. (B and C) Expression of *Ghsr* in the femurs (B), primary osteoblasts (C) of Osx-Cre-Ghsr-null male mice (Ghsr-null/Osx). $n = 3$ or more. (D) Serum calcium and phosphate levels in Ghsr-null and Ghsr-null/Osx mice. Note the lack of difference between Ghsr-null and Ghsr-null/Osx mice. (E and F) Histological and histomorphometric analyses of 3-month-old Ghsr-null/Osx male mice. L3 and L4 vertebrae were analyzed. $n = 8$ or more. (G) Expression of the osteoblast markers in femurs, $n = 5$. Note the rescue of bone abnormalities in Ghsr-null/Osx male mice. The scale bars represent 500 μm. The error bars indicate the standard deviation (s.d.). $^*P < 0.05$.

 

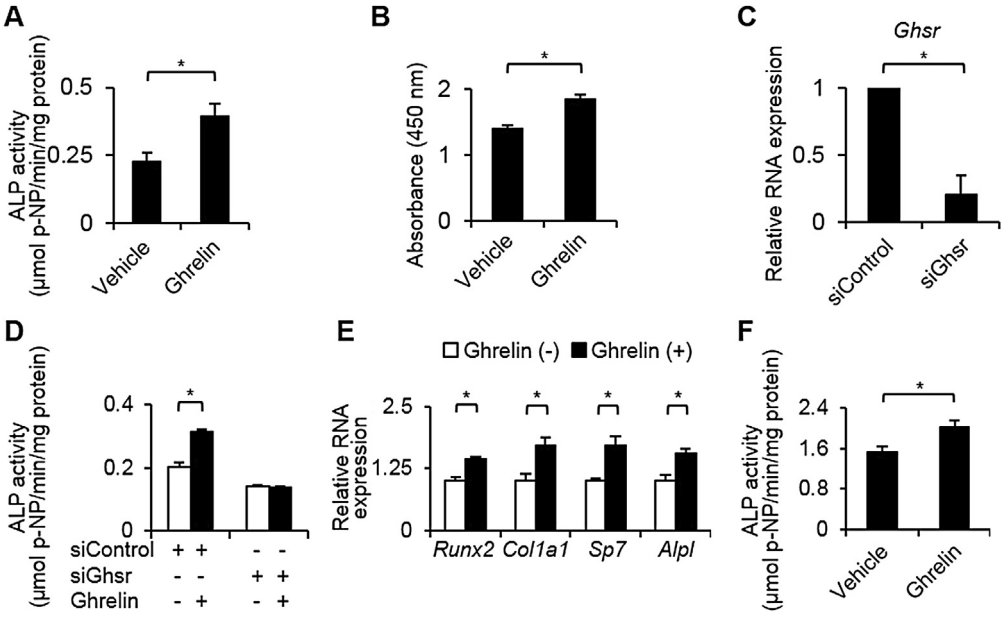


**Figure 5: Ghrelin increases osteoblast differentiation via Ghsr.** (A and B) Effect of ghrelin treatment on ALP activity (A) and proliferation (B) in MC3T3-E1 osteoblastic cells, $n = 10$. (C and D) Effect of si*Ghsr* treatment on gene expression (C) and ALP activity (D) in MC3T3-E1 osteoblastic cells, $n = 10$. (E and F) Effect of ghrelin treatment in primary osteoblasts, $n = 10$. Expression of the osteoblast markers *Runx2*, *Col1a1*, *Osterix* (*Sp7*) and *Alpl* (E) and ALP activity (F). The error bars indicate the standard deviation (s.d.). $^*P < 0.05$.

(Figure 5C,D). We also treated primary osteoblasts with ghrelin and observed that ghrelin accelerated osteoblast differentiation, as demonstrated by an increase in the expression of osteoblast marker genes and alkaline phosphatase (ALP) activity (Figure 5E,F). Taken together, these results further confirmed that ghrelin affects bone formation through Ghsr expression in osteoblasts.

We next studied the molecular mechanism that is responsible for ghrelin/Ghsr-dependent osteoblast differentiation. Because ghrelin is known to exert various physiological functions through MAPK and PKA pathways [23–25], we studied the activation of the MAPK and PKA pathways after ghrelin treatment. Ghrelin induced the phosphorylation of ERK in osteoblastic cells but did not affect the phosphorylation of p38 or JNK (Figure 6A), indicating that ghrelin affects osteoblast differentiation through ERK. Indeed, blocking the ERK pathway, either through siRNA against *ERK* or by U0126, a known inhibitor of ERK, abolished ghrelin-dependent osteoblast differentiation (Figure 6B–D). The addition of a PKA inhibitor attenuated ghrelin-dependent osteoblast differentiation, indicating that PKA signaling is also involved (Figure 6E). CREB is an important transcription factor for osteoblast proliferation and differentiation [26,27]; we observed that ghrelin treatment induced the phosphorylation of CREB (Figure 6F). Moreover, the activation of CREB was required for the optimal induction of osteoblast differentiation by ghrelin, as a siRNA targeting *Creb2* encoding CREB suppressed the ghrelin-mediated increase in ALP activity (Figure 6G,H). Interestingly, ghrelin-dependent CREB phosphorylation was attenuated by treatment with an ERK inhibitor (Figure 6I) or a PKA inhibitor (Figure 6J), indicating that both the ERK and PKA pathways are involved in ghrelin-Ghsr-dependent bone remodeling. Furthermore, we found that ghrelin treatment induced the expression of Runx2, a master regulator of osteoblast differentiation, in osteoblastic cells (Figure 6K). As a result, ghrelin increased the transcriptional activity of the osteocalcin promoter, a well-known target for Runx2 (Figure 6L). Taken together, our results indicate that ghrelin induces osteoblast differentiation through the activation of the osteoblastic ERK–CREB pathway and the induction of Runx2.

## 4. DISCUSSION

In this study, we demonstrated that ghrelin regulates bone remodeling through Ghsr in osteoblasts and not via the central nervous system. Moreover, we demonstrated that ghrelin regulates osteoblast differentiation and proliferation through ERK–CREB and Runx2 (Figure 6M). Ghsr-null mice exhibited a low bone mass phenotype at 3 and 6 months of age, indicating that they did not reach the peak bone mass of the wild-type mice that is usually achieved at 2 months. Therefore, Ghsr regulates bone mass accrual rather than bone loss after reaching peak bone mass.

Because the primary action of ghrelin is exerted through the central nervous system [6,7], we hypothesized that ghrelin also affects bone remodeling through the central nervous system. In addition, because ghrelin is a "bona fide" GH secretagogue [7] and GH is a key regulator of bone remodeling, we were interested in whether bone anabolic action of ghrelin is mediated via the GH/IGF-1 pathway. In this study, we demonstrated that ghrelin does not regulate bone remodeling via receptors expressed in the central nervous system by using cell-specific Ghsr rescue. Moreover, we previously showed that ghrelin increases bone mass in SD rats, natural mutants that lack GH, as effectively as it does in control rats [9]. Recent reports indicate that Ghrl-deficiency leads to altered GH secretion profiles and serum IGF-1 concentrations, which reflect GH signaling [28]. In our Ghsr-null mice, it is also possible that GH secretion is similarly altered, which would affect the bone phenotype. However, in this study, no statistically significant differences in serum IGF-1 levels were observed between wild-type mice and Ghsr-null mice fed standard chow, confirming previous findings, i.e. normal serum IGF-1 levels in Ghsr-null mice fed high fat diet [13]. Thus, the contribution of the GH/IGF-1 axis to the development of the abnormal bone phenotype in Ghsr-null mice would be limited, if any. Taken together, we formally excluded the possibility that ghrelin regulates bone mass via central nervous system or IGF-1 pathway. The observation that disruption of ghrelin-Ghsr axis did not affect serum IGF-1 levels is in line with reports showing unchanged

  

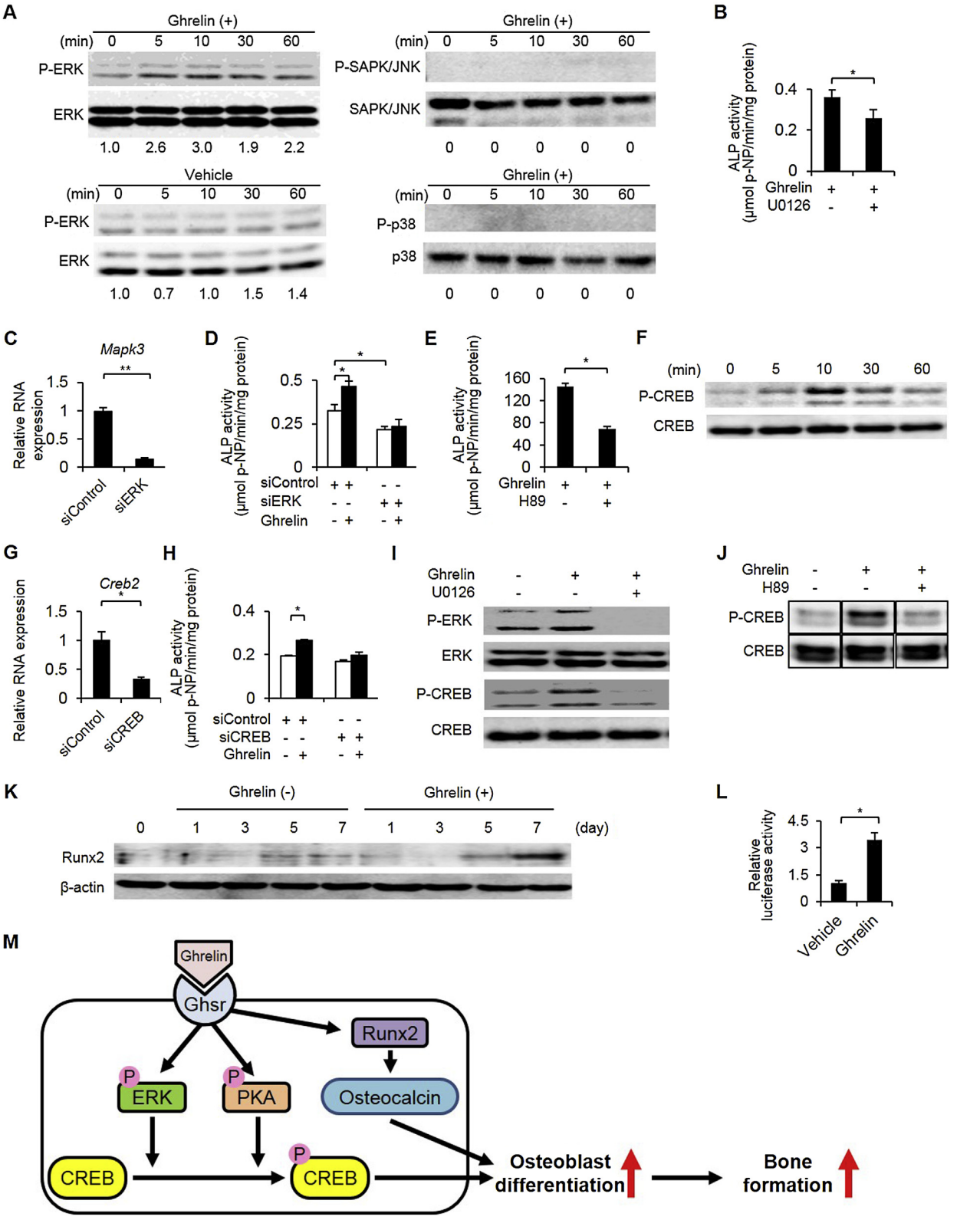


**Figure 6: Ghrelin regulates bone remodeling via the ERK and/or PKA–CREB and Runx2 pathways.** (A, F, I and K) Protein analysis in ghrelin-treated osteoblastic cells. (B–E) Effect of ERK inhibitor (B), siERK (C, D), PKA inhibitor (E) or siCreb2 treatment (G, H) on the expression of ERK (Mapk3) (C) or Creb2 (G) and osteoblast differentiation (B, D, H). (L) The effect of ghrelin on osteocalcin gene promoter activity. (A–L) MC3T3-E1 cells were treated with 10−7 M ghrelin and/or 10 μM MEK inhibitor (U0126) and 10 μM PKA inhibitor (H89). Phosphorylated protein/total protein ratios are indicated (A). P-, phosphorylated; SAPK/JNK, stress-activated protein kinase/Jun-amino-terminal kinase; ERK, Extracellular signal-regulated kinase; CREB, cAMP response element-binding protein, $n = 10$. The error bars indicate the standard deviation (s.d.). $^{*}P < 0.05$, $^{**}P < 0.01$. (M) Schematic representation of ghrelin-dependent osteoblast differentiation.

 

serum IGF-1 levels in two different ghrelin-null mice [28,29]. These results differ from those of Sun and colleagues' Ghsr-knockout mice [30]; however, the cause of this discrepancy is unknown.

A previous report showed that ghrelin directly regulates osteoclast differentiation [12], but other investigators failed to find an effect of ghrelin on osteoclasts [9,10]. The cause for these discrepant observations has not yet been identified. In addition, van der Velde et al. reported that Ghsr affects osteoclastogenesis indirectly via leptin/sympathetic nervous system signaling *in vivo* only at 6 months but not at 3 months, indicating that Ghsr deficiency in osteoclasts, in and of itself, does not directly affect osteoclastogenesis *in vivo* [12]. This finding would explain the absence of the rescue in our Ghsr-null/CtsK mice. In this study, we demonstrated that ghrelin does not affect osteoclastogenesis; we observed no effect of ghrelin on *in vitro* osteoclastogenesis and, more importantly, no bone resorption abnormalities in either Ghsr-null mice or Ghsr-null/CtsK mice. Therefore, the physiological effect of ghrelin on osteoclasts, if any, is limited.

In rats, partial gastrectomy induces a marked and rapid reduction in bone volume [31]. In humans, bone mineral density (BMD) is significantly decreased after gastrectomy also [32]. Indeed, gastrectomy is a well-known cause for secondary osteoporosis in males. The mechanism by which gastrectomy induces bone loss is still unknown. A lack of gastric acid secretion may contribute to bone loss after gastrectomy, but proton pump inhibitors and H2-receptor antagonists are known to affect BMD only modestly [31]. Together with the facts that ghrelin is mainly produced in the fundus of the stomach [6] and that the serum concentration of ghrelin is significantly reduced after gastrectomy [33], our current study revealed that ghrelin is a probable candidate responsible for gastrointestinal system-dependent bone remodeling. Gastrectomy also causes poor dietary intake and rapid gastric emptying and intestinal transit, which may affect bone mass. How these factors affect bone remodeling, in a ghrelin-dependent or ghrelin-independent manner, remains to be elucidated.

Recent findings revealed that whole body homeostasis is maintained by an inter-organ communication network. A neuronal pathway from the liver has been shown to modulate energy expenditure, systemic insulin sensitivity, and more recently, pancreatic beta cell mass [34]. The gut microbiota are also involved in energy metabolism and metabolic diseases, such as obesity [35,36]. Moreover, serotonin, which regulates intestinal movements and is strongly expressed in the gastrointestinal (GI) tract, has been reported to regulate bone mass and bone density both in mice and humans [37,38]. Thus, the digestive organs play major roles in whole-body homeostasis, including bone remodeling. Given that the expression level of ghrelin in bone cells is reduced relative to their expression in the stomach, it is possible that ghrelin secretion in distant organs, especially in the stomach, instead of the local ghrelin produced by bone cells regulate bone remodeling through Ghsr, represents a new example of the inter-organ regulation of bone homeostasis. Together with the control of bone remodeling by the central nervous system, bones and other organs orchestrate an inter-organ communication network to maintain bone homeostasis. In this study we demonstrated that osteoblastic Ghsr is indispensable for the anabolic action of ghrelin in bone; however, this finding does not necessarily indicate that osteoblastic Ghsr by itself is sufficient for this ghrelin-Ghsr-dependent anabolic action. Given the existence of the inter-organ communication system, including bone, it is possible that the expression of Ghsr by other organs may also contribute to bone remodeling indirectly. Osteoblast-specific Ghsr KO mice would address this question. A recent report showed that Osx-driven Cre targets multiple other cell types [39]. Therefore, we checked Ghsr expression in various tissues and found that it is expressed in the intestine of Ghsr-null/Osx mice, albeit faintly. However, serum levels of calcium and phosphate were comparable between Ghsr-null mice and Ghsr-mull/Osx mice. Thus, together with *in vitro* findings using primary osteoblasts, we believe that osteoblastic Ghsr is mainly responsible for the ghrelin-Ghsr dependent bone anabolic action. Here again, osteoblast-specific Ghsr KO mice would address this question. In this study, we demonstrated that ghrelin-Ghsr signaling regulates trabecular bone mass without affecting cortical bone parameters. Leptin has been shown to negatively and positively regulates trabecular bone mass and cortical bone mass, respectively. Thus, the effects of appetite-regulating hormones differ depending on the site of bone. Or, alternatively, it is possible that the expression levels of ghrelin-Ghsr signaling machinery in osteoblasts, such as Ghsr, pErk, pPKA, Runx2, are different depending on the location of the bone, which would affect the severity of the bone phenotype of Ghsr-null mice.

It has been demonstrated that the GH/IGF-1 pathway cooperatively regulates osteoblast differentiation by means of CREB and Runx2 [27]. It is intriguing that ghrelin, a GH secretagogue, and GH/IGF-1 use the same molecular machinery to regulate bone remodeling. To ensure and amplify a signaling pathway, it is reasonable that a hormonal cascade uses the same effector. Indeed, the combined administration of GH and IGF-I was observed to be more anabolic than either IGF-I or GH alone due to a direct action of GH independent of IGF-1 [40].

In conclusion, we demonstrated that ghrelin regulates bone remodeling by affecting osteoblasts. We also clarified that ghrelin does not regulate bone remodeling via the central nervous system. We unambiguously demonstrated that ghrelin plays a physiological role in bone remodeling, consistent with the observation that ghrelin possesses many physiological functions in addition to the regulation of energy homeostasis and appetite, and is implicated in the pathogenesis of ischemic heart disease [41] and various cancers [42]. Given that there are several ongoing clinical studies on the effect of ghrelin on various diseases, such as cancer-induced cachexia and anorexia nervosa [43], our current observation provides evidence that ghrelin can also be used as a potential therapeutic for osteoporosis.

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## CONFLICT OF INTEREST

None declared.

## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2015.01.002.

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