Experimental Physiology

Stimulation of intramembranous bone repair in rats by ghrelin

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Researchers in our laboratory have previously shown that ghrelin, a gastric peptide hormone, may regulate mesenchymal cell differentiation into adipocytes and myocytes. Here we show that ghrelin promotes osteogenesis of intramembranous bone and improves the repair of calvarial bone defects in rats. Rats with a 9 mm full-thickness calvarial bone defect received either Bio-Oss® (control group) or Bio-Oss® mixed with 20 μg ghrelin (treatment group), followed by local administration of saline or ghrelin (10 μg), respectively, on days 5, 10 and 15. After 6 and 12 weeks, new bone formation was assessed. Animals treated with ghrelin showed a significant increase in new bone formation as demonstrated by an increment in bone mineral density and fluorescence labelling of tetracycline relative to the control group. At 6 weeks, bone mineral density increased from 54 ± 7 (control group) to 78 ± 9 mg cm^{-2} in the treatment group, while the tetracycline fluorescence labelling increased by 61 ± 15%. A similar increment was observed at 12 weeks. Quantitative reverse transcriptase-polymerase chain reaction showed that expression of alkaline phosphatase (ALP), osteocalcin and collagen type I was elevated. Relative to the control animals, mRNAs for ALP, osteocalcin and collagen type I increased 2.4 ± 0.4-, 4.7 ± 1.9- and 4.0 ± 1.7-fold, respectively, in animals treated with ghrelin for 6 weeks ($P < 0.05$). At 12 weeks, mRNA levels of ALP, osteocalcin and collagen type I showed a decline relative to levels at 6 weeks but still remained significantly higher than in the control group, with fold changes of 2.4 ± 0.8, 2.4 ± 1.2 and 2.1 ± 0.7, respectively ($P < 0.05$). This study demonstrated that ghrelin stimulates intramembranous osteogenesis.

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Ghrelin, a 28 amino acid peptide, is secreted by gastric oxyntic glands and circulates in blood (Kojima *et al.* 1999). Since its discovery in 1999, ghrelin has been the focus of a wide range of studies concerning neuroendocrine control mechanisms, food intake and energy metabolism, cardiovascular function, immunological functions, and growth and development. As the endogenous ligand for the growth hormone secretagogue receptor (GHSR), ghrelin was originally reported to stimulate growth hormone release (Kojima *et al.* 1999; Wren *et al.* 2000; Takaya *et al.* 2000). In rats, ghrelin has been found to stimulate food intake (Wren *et al.* 2000; Nakazato *et al.* 2001), to induce adiposity (Tschop *et al.* 2000) and to increase body weight (Wren *et al.* 2000; Nakazato *et al.* 2001; Tschop *et al.* 2000), suggesting a possible role in regulation of feeding behaviour and energy metabolism. In the gastrointestinal system, ghrelin has recently been reported to regulate secretion of gastric acid (Masuda *et al.* 2000; Date *et al.* 2001), gastric motility (Masuda *et al.* 2000; Trudel *et al.* 2002) and pancreatic protein output (Zhang *et al.* 2001). The detection of GHSR in heart and aorta suggests a role of ghrelin in the regulation of cardiovascular function (Van Der Lely *et al.* 2004). This notion is confirmed by the finding that chronic administration of ghrelin improves cardiac contractility in growth hormone-deficient rats and even in rats with chronic heart failure (Van Der Lely *et al.* 2004).

Several studies have shown that ghrelin may play a role in the regulation of cell proliferation and differentiation. Dependent on the cell type, ghrelin either stimulates or

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inhibits cell proliferation. Mitotic effects of ghrelin have been demonstrated in preosteoblasts (Kim *et al.* 2005; Maccarinelli *et al.* 2005), neuronal precursors (Zhang *et al.* 2004*a*, 2005; Sato *et al.* 2006), preadipocytes (Zhang *et al.* 2004*b*; Kim *et al.* 2004), cardiomyocytes (Pettersson *et al.* 2002) and the rat GH3 pituitary cell line (Nanzer *et al.* 2004). In cell lines derived from carcinomas, including prostate (Cassoni *et al.* 2004), thyroid (Volante *et al.* 2003), mammary gland (Cassoni *et al.* 2001) and lung (Ghe *et al.* 2002), ghrelin acts to inhibit proliferation. *In vitro* studies suggest that ghrelin induces the differentiation of several cell types, including osteoblasts (Kim *et al.* 2005; Maccarinelli *et al.* 2005), adipocytes (Zhang *et al.* 2004*a*; Kim *et al.* 2004) and neurons (Zhang *et al.* 2004*b*, 2005; Sato *et al.* 2006). Investigators (Filigheddu *et al.* 2007; Zhang *et al.* 2007) have reported that ghrelin stimulates proliferating myoblast cells to differentiate and fuse into multinucleated myotubes. Since osteoblasts, adipocytes and myocytes are all derived from a common precursor cell, the mesenchymal stem cell, these reports suggest that ghrelin may play a role in the modulation of mesenchymal cell differentiation.

Emerging evidence has supported the concept that ghrelin is involved in the physiological regulation of bone formation. *In vitro*, ghrelin has been demonstrated to stimulate the proliferation of both primary cultured osteoblast cells and immortalized osteoblast cell lines including UMR106 cells and MC3T3-E1 cells, respectively (Kim *et al.* 2005; Maccarinelli *et al.* 2005). *In vivo*, systemic administration of ghrelin has been reported to increase the bone mineral density of rat femurs (Fukushima *et al.* 2005). However, it is unknown whether ghrelin affects intramembranous bone formation during physiological remodelling and its repair after bone injury. Intramembranous bone ossification accounts for most of the bone growth of the face and calvaria. In contrast to endochondral ossification, intramembranous bone formation is a relatively simple process with no cartilage anlagen and osteoblasts differentiating directly from mesenchymal cells and may be regulated by a mechanism involving different cellular and extracellular processes compared with the processes controlling endochondral bone growth. Craniofacial defects occur as a result of trauma, developmental anomalies, oncological resection, infection and pathology (Goldberg *et al.* 1991). An estimated 250 000 bone transplant procedures are done annually in the USA (Goldberg *et al.* 1991). Despite the significant advance in understanding of the mechanism of bone regeneration in long bone and the development of many bone substitutes and bone growth factors, reconstruction of craniomaxillofacial defects continues to be a challenge for surgeons.

The object of this study was to determine whether ghrelin promotes the repair of calvarial bone defects. We report here that: (1) ghrelin stimulates the new bone formation in a non-healing calvarial bone defect as demonstrated by an increase in bone mineral density and in the tetracycline fluorescence labelling; and (2) ghrelin upregulates the mRNA expression of three common osteoblast differentiation markers: alkaline phosphatase (ALP), osteocalcin and collagen type I.

Methods

Calvarial bone defect model and implant fabrication

Male Sprague–Dawley rats (250 ± 25 g) were purchased and housed at the animal care facility for a week of acclimation. All animals were maintained on standard rat chow and water *ad libitum*. Animal care and surgical procedures for this investigation were approved by the Sun Yat-sen University Animal Care and Handling Committee.

Rats were randomly divided into three groups: unfilled group; control group, in which bone defects were filled with Bio-Oss® implant (Osteohealth, Shirley, NY, USA); and treatment group, in which bone defects were filled with Bio-Oss® implant plus ghrelin. Anaesthesia was induced with an intraperitoneal injection of 150 mg kg^{-1} sodium pentobarbitone. An incision was made through full-thickness skin along the mid-line of the scalp from a point midway between the base of the ears to approximately 2.5 cm anterior. Sharp subperiosteal dissection reflected the pericranium from the outer table of the cranial vault, exposing the parietal bones. A trephine drill under copious saline irrigation was used to create a full-thickness calvarial critical size defect. Care was taken not to damage the dura mater in the procedure. The defects were round, measuring 9 mm in diameter. A critical size defect used by previous workers has been defined as a calvarial defect that does not heal spontaneously, and a 5 mm or greater defect in rats is a non-healing defect (Schmitz & Hollinger, 1986). A 9 mm diameter bone defect in the calvaria in our experiments would therefore create such a non-healing defect. For the unfilled group, four rats with bone defects were left with the defects unfilled and killed at 12 weeks to verify the successful creation of a calvarial non-healing bone defect, and served as the non-healing control group. For the control group, twenty-two rats had bone defects filled with 20 mg Bio-Oss® implant. In the treatment group, twenty-two rats had bone defects filled with 20 mg Bio-Oss® mixed with 20 μg ghrelin (Phoenix Pharmaceutical Co., Burlingame, CA, USA). In all three groups, the pericranium and skin were closed with resorbable sutures.

Rats in the treatment group received a local administration of 10 μg ghrelin in 100 μl saline by pericranial injection at the bone defect site on days 5, 10 and 15 after surgery, while control rats were given 100 μl saline injection on the same days. Fourteen and 4 days before killing the animals, oxytetracycline at a dose of 25 mg (kg body weight)$^{-1}$ was administered intraperitoneally to label the newly formed bone.

Dual-energy X-ray bone absorptiometry measurement

Animals were killed at 6 and 12 weeks after surgery by overdose of CO_2 inhalation. The calvaria, extending to 5 mm beyond the bone defect, were carefully removed from each animal using an electric drill with a 702 bur. The calvarial specimens were placed in 4% paraformaldehyde for 24 h. Bone mineral density (BMD) in the bone defect area was measured by dual-energy X-ray bone absorptiometry.

Histology and histomorphometry

All calvarial specimens were dehydrated in graded dilutions of acetone and embedded in methylmethacrylate. Horizontal sections (30 μm thick) were cut and subsequently stained with Toluidine Blue. The callus area directly in the 9 mm gap was analysed using the Axioskop 40 Zeiss imaging system (Carl Zeiss Shanghai Co. Ltd., Shanghai, China). New bone surface area was measured and calculated as a percentage of total bone surface area using the software AxioVision Rel 4.5 (Carl Zeiss Shanghai Co. Ltd.). Fluorochrome labelling was analysed using unstained sections under fluorescence microscopy to measure the distance of two tetracycline labelling lines. The new bone formation rate was then calculated by dividing the distance between the two labels by the interlabelling period in days. Five sections from each sample and four ×20 microscope fields per section (at the 12, 3, 6 and 9 o'clock positions) were analysed. Results are expressed as means ± S.E.M. Analysis was done using Student's *t* test or ANOVA as appropriate, and a statistical significance was accepted at $P < 0.05$.

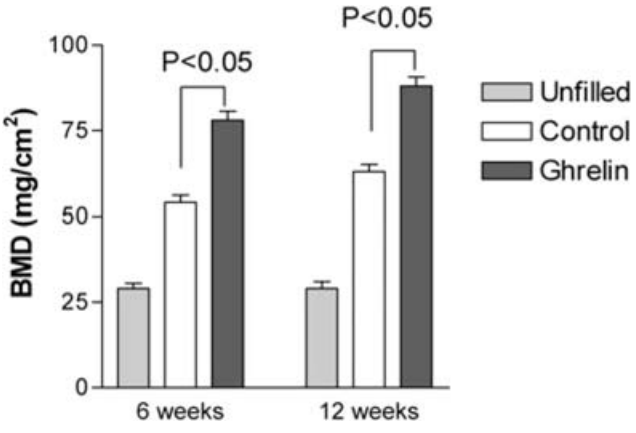

Figure 1. Increase in the bone mineral density of callus in rats treated with ghrelin
Rats had calvarial bone defects filled with either Bio-Oss® (control group) or Bio-Oss® mixed with 20 μg ghrelin (treatment group). On days 5, 10 and 15 after surgery, a local injection at the bone defect site of either 10 μg ghrelin in 100 μl saline or 100 μl saline was administered. Bone mineral density was measured by dual-energy X ray bone absorptiometry. Ghrelin treatment significantly increased the BMD in callus compared with control saline treatment at 6 and 12 weeks ($P < 0.05$). The BMD in calvarial bone defects left unfilled was small even at 12 weeks.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Following killing of the animals, the defect sites were surgically collected and immediately frozen using liquid nitrogen. The extracted tissue was then ground to powder form with a mortar and pestle. Total RNA was extracted using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) and subsequently cleaned using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The quality of the isolated RNA was assessed by gel electrophoresis and samples were stored at −80°C until further use. Single-strand cDNA synthesis was performed as follows: 30 μl of reverse transcription mixture contained 1 μg of Dnase I pretreated total RNA, 0.75 μg of oligo d(T) primer, 6 μl of 5× RT buffer, 10 mM dithiothreitol, 0.5 mM deoxynucleotides, 50 units of RNase inhibitor, and 240 units of reverse transcriptase (Invitrogen). The RT reaction was carried out at 40°C for 70 min followed by heat inactivation at 95°C for 3 min. The PCR was conducted in a total volume of 25 μl, containing 2.5 μl of cDNA, 5 ml $MgCl_2$, 0.2 mM deoxyribonucleoside triphosphates (dNTPs), 0.25 μm of each primer, 1.25 unit Ampli Taq Polymerase, and 1 μl of 800× diluted fluorescent dye SYBR® green I stock (Invitrogen, Carlsbad, CA, USA). The PCR programme was: 95°C for 2 min; and then 45 cyles of 94°C for 15 s, 70°C for 15 s and 72°C for 25 s. Melt curve analysis was from 60 to 95°C at 0.2°C s^{-1} with Optics Ch1 On (Bio-Rad, Hercules, CA, USA). The PCR products were also visualized by 1% agarose gel electrophoresis. The PCR bands were isolated from the gel, then sequenced, and confirmed with known sequences. The mRNA expression was quantified using the comparative cross-threshold (CT, the PCR cycle number that crosses the signal threshold) method (Babu & Nutman, 2003). The CT of the housekeeping gene β-actin was subtracted from the CT of the target gene to obtain ΔCT. The normalized fold-changes of ALP, osteocalcin and collagen type I mRNA expression were expressed as $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = \Delta CT$ sample minus ΔCT control.

Primers used in this study were as follows:

alkaline phosphatase (ALP; 101 bp product), 5′-CGTCTCCATGGTGGATTATGC-3′ (sense), 5′-TGGCAAAGACCGCCACAT (antisense); osteocalcin (OCN; 63 bp product), 5′-GAGCTAGCGGACCACATTGG-3′ (sense), 5′-CCTAAACGGTGGTGCCATAGA-3′ (antisense); collagen type I (COL I; 65 bp product), 5′-TTCACCTACAGCACGCTTGTG-3′ (sense), 5′-GATGACTGTCTTGCCCCAAGTT-3′ (antisense); Runx2 (119 bp product), 5′-CCACCACTCACTACCACACG (sense), 5′-GGACGCTGACGAAGTACCAT (antisense); Osterix

(137 bp product), 5′-GCTGCCTACTTACCCGTCTG (sense), 5′-GTTGCCCACTATTGCCAACT (antisense); and β-actin (67 bp product), 5′-TTCAACACCCCAG-CCATGT-3′ (sense), 5′-GTGGTACGACCAGAGGCAT-ACA-3′ (antisense).

Results

Increase in bone mineral density in callus from bone defects treated with ghrelin

As shown in Fig. 1, unfilled bone defects demonstrated little mineralization with a small value of bone mineral density in the callus, demonstrating the successful creation of a calvarial critical size non-healing defect. Bone defects treated with ghrelin showed a significant increase in the bone mineral density value compared with the control group at 6 and 12 weeks, indicating an increment in the new bone formation in animals treated with ghrelin.

Stimulation of new bone formation by ghrelin

To confirm the above observation that ghrelin acts to enhance the new bone formation in calvarial bone defects, we next analysed the new bone surface area. Histological observation revealed a large amount of mineralizing osteoid both in bone defects filled with

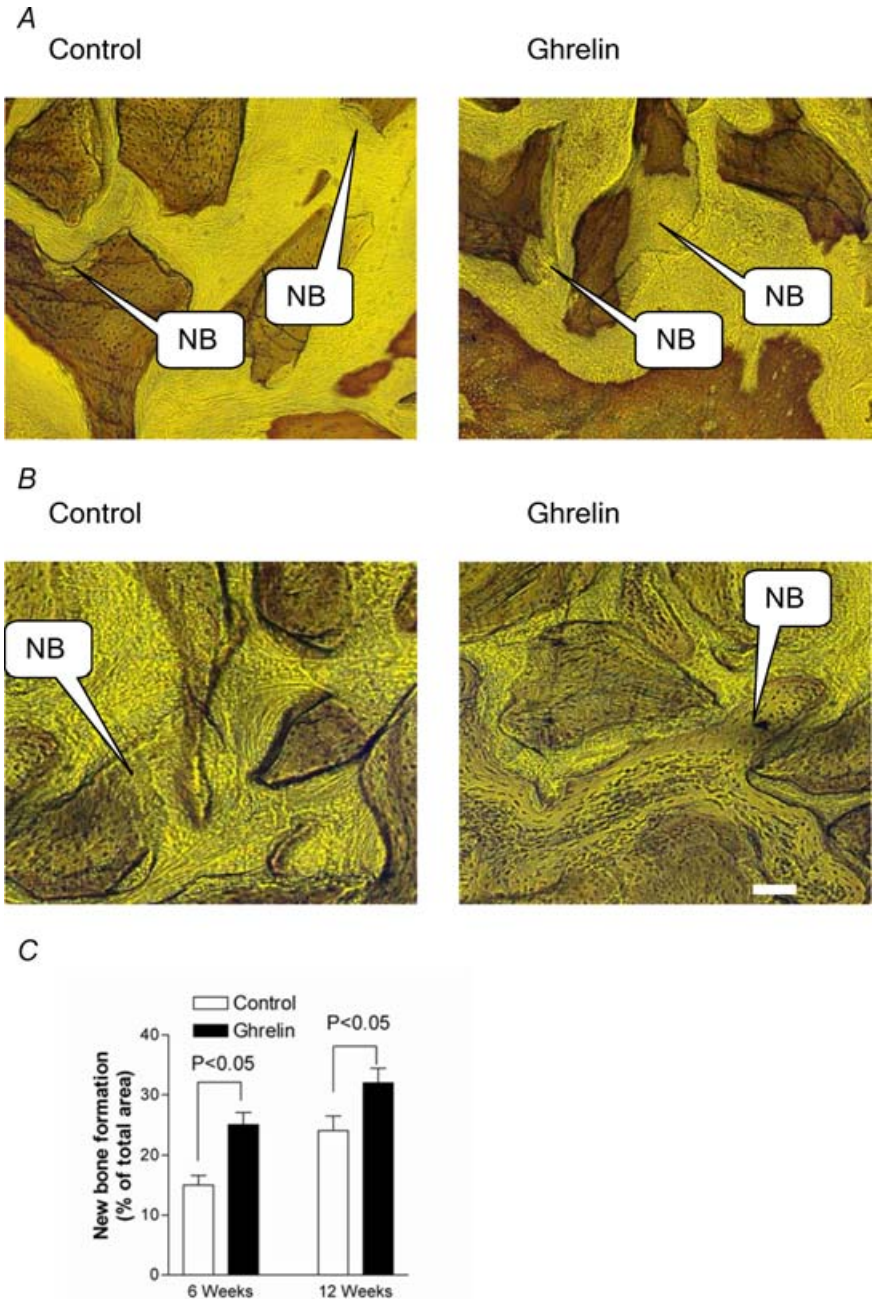

Figure 2. Stimulation of new bone formation by ghrelin
A, 6 weeks after implantation with Bio-Oss®, new bone formation was observed around the Bio-Oss® particles. The Bio-Oss® plus ghrelin-treated group showed a marked increase in new bone formation compared with the control group. Representative histological sections of new bone (NB) formation (Toluidine Blue stained) are shown. *B*, at 12 weeks, a large amount of new bone was observed around the Bio-Oss® implants. Bone maturation became obvious at this time point. In addition to the increment in the amount of new bone, mature bone with trabecular bone structure was present in animals treated with ghrelin, indicating the formation of functional bone structure (arrow). *C*, the area of new bone was measured and expressed as the percentage relative to the total area in tissue sections as described in the Methods. Data are expressed as means ± S.E.M. A significant difference was observed between ghrelin and control groups ($P < 0.05$). Scale bar represents 100 μm.

Bio-Oss® and in those treated with Bio-Oss® plus ghrelin, indicating an active intramembranous bone repair, while those bone defects left unfilled demonstrated little new bone formation. Animals treated with ghrelin showed a significant increase in mean new bone surface area relative to the control group at 6 (25 ± 5.2 *versus* $15 \pm 4.1\%$, $P < 0.05$) and 12 weeks (32 ± 6.9 *versus* $24 \pm 6.6\%$, $P < 0.05$; Fig. 2).

Consistent with the histological observation, tetracycline labelling was obvious in both the control group and ghrelin-treated animals. Rats treated with ghrelin demonstrated a significant increase in new bone formation rate by 14 ± 6 and $26 \pm 4\%$, respectively, relative to the control group at 6 and 12 weeks (Fig. 3).

Increase in the mRNA expression of ALP, osteocalcin and collagen type I

To further confirm the stimulatory effect of ghrelin on calvarial bone repair, we examined the mRNA expression of three osteoblast differentiation markers: ALP, osteocalcin and collagen type I. At 6 weeks, mRNA levels of all three genes examined were significantly elevated in

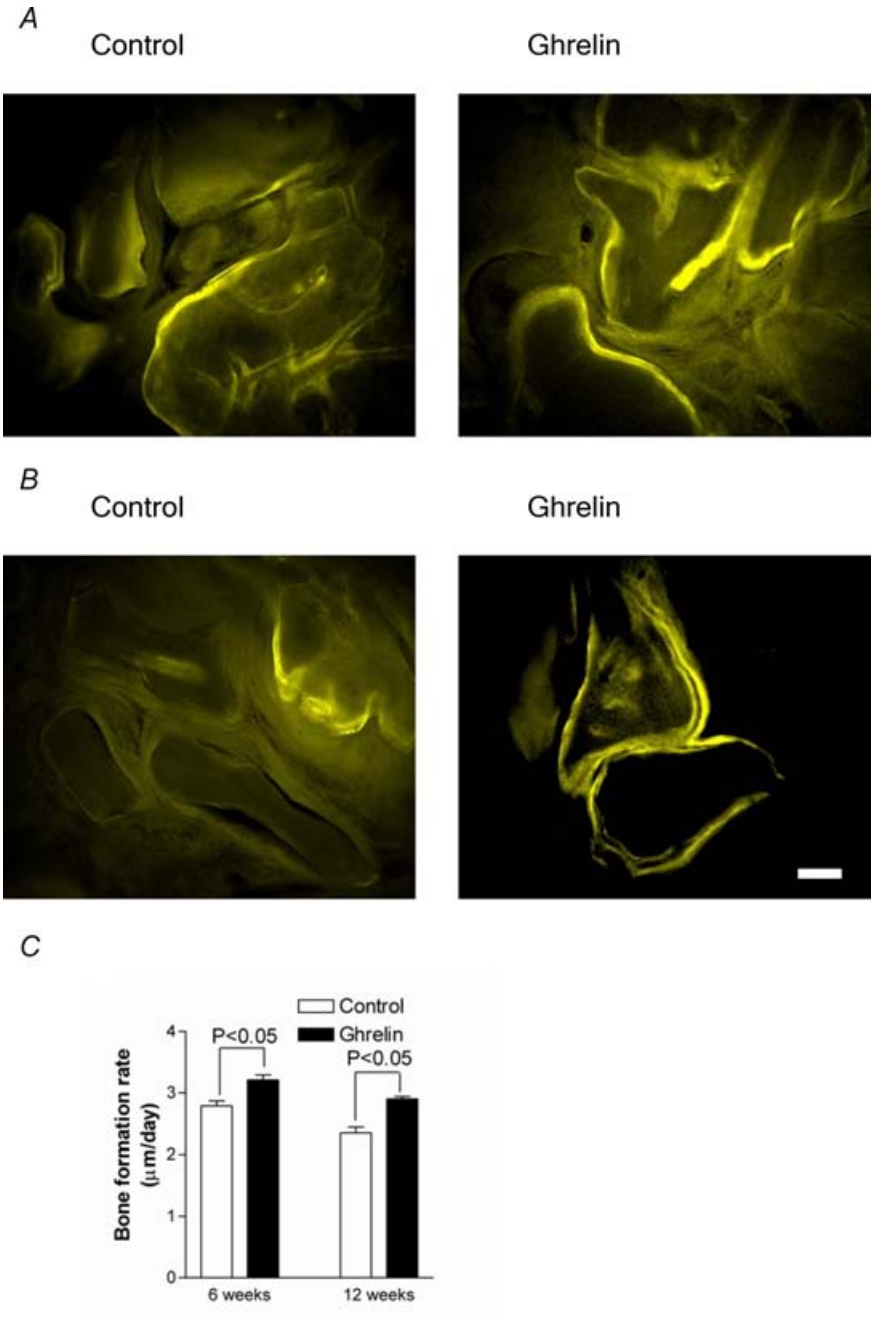

Figure 3. Increase in tetracycline labelling by ghrelin
Oxytetracycline at a dose of 25 mg (kg body weight)$^{-1}$ was administered intraperitoneally to label the newly formed bone days 14 and 4 before killing of the animals. An increase in tetracycline labelling was observed in animals treated with ghrelin compared with the control group at 6 (*A*) and 12 weeks (*B*). The new bone formation rate was calculated as the average distance (in μm) between two tetracycline labelling lines divided by the days, and data are expressed as means ± S.E.M. *C*, the difference in new bone formation rate between ghrelin-treated and control groups was significant ($P < 0.05$). Scale bar represents 100 μm.

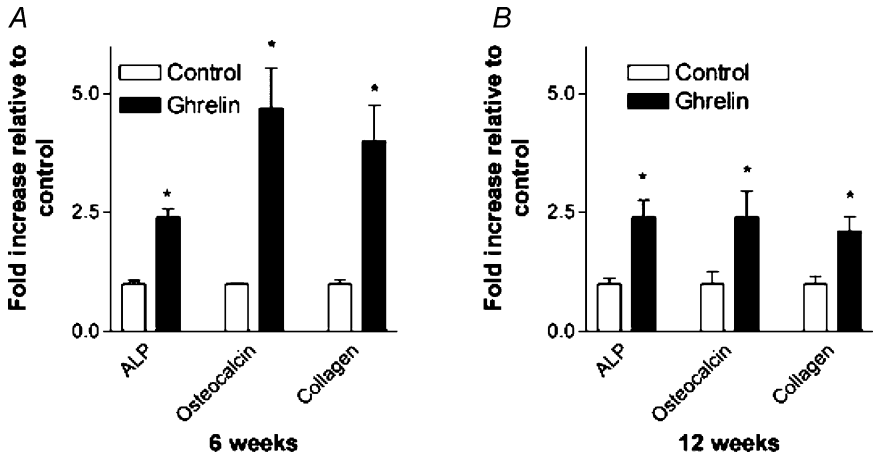

Figure 4. Increase in mRNA levels of ALP, osteocalcin and collagen type I
Animals treated with ghrelin showed a significant increase in mRNA levels of ALP, osteocalcin and collagen type I relative to control animals at 6 (*A*) and 12 weeks (*B*). $^{*}P < 0.05$ compared with the control group.

animals treated with ghrelin compared with the control group (Fig. 4*A*). Relative to the control group, mRNAs for ALP, osteocalcin and collagen type I increased 2.4 ± 0.4-, 4.7 ± 1.9- and 4.0 ± 1.7-fold, respectively, in animals treated with ghrelin ($P < 0.05$ for all comparisons). At 12 weeks, mRNA levels of ALP, osteocalcin and collagen type I showed a decline relative to levels at 6 weeks but still remained significantly higher than in the control group, with fold changes of 2.4 ± 0.8, 2.4 ± 1.2 and 2.1 ± 0.7, respectively ($P < 0.05$; (;Fig. 4*B*).

Upregulation of osterix mRNA

In order to determine the transcriptional factors by which ghrelin stimulates bone repair, we next analysed the expression of transcriptional factors Runx2 and osterix. These two transcriptional factors have been reported to play an important role in the determination and differentiation of osteoblasts (Franceschi *et al.* 2007). As shown in Fig. 5, ghrelin treatment significantly increased the expression of osterix mRNA at 6 weeks relative to the control group ($P < 0.05$), while Runx2 demonstrated no significant change. At 12 weeks, mRNA levels of both osterix and Runx2 were unchanged compared with the control group.

Discussion

The major finding of the present study is that ghrelin stimulates intramembranous bone repair. This conclusion is supported by two distinct observations: (1) local administration of ghrelin enhances new bone formation in calvarial bone defects, as demonstrated by an increase in the bone mineral density, new bone surface area and in the tetracycline labelling; and (2) callus from the bone defects treated with ghrelin showed a significant increase in the expression of ALP, osteocalcin and collagen type I, three common markers of osteogenesis.

Emerging evidence supports the concept that ghrelin is actively involved in bone remodelling. First, ghrelin receptor has been demonstrated at the mRNA and protein levels both in primary cultured osteoblasts and in immortalized osteoblast cell lines, indicating that ghrelin may have a physiological function on osteoblast cells (Kim *et al.* 2005; Fukushima *et al.* 2005). Second, osteoblastic cells have been reported to respond to exogenous ghrelin by an increase in cell proliferation and differentiation (Kim *et al.* 2005; Maccarinelli *et al.* 2005; Fukushima *et al.* 2005). Third, *in vivo* administration of ghrelin increases the bone mineral density in femur, characterized by endochondral bone formation (Fukushima *et al.* 2005). This effect is not dependent on growth hormone because a similar

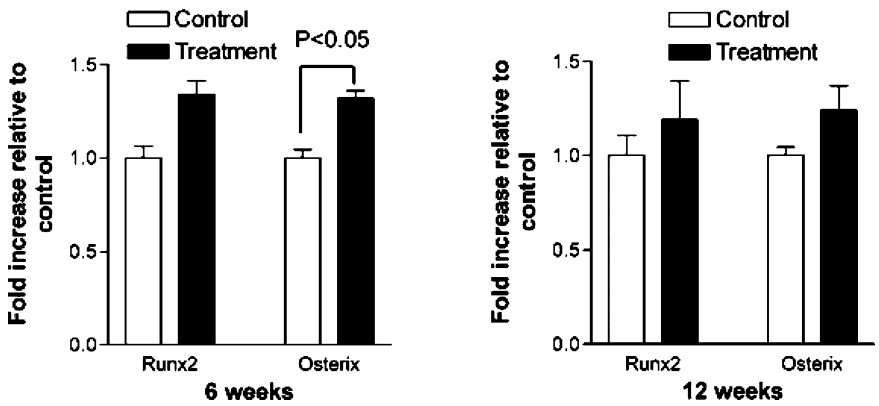

Figure 5. Stimulation of osterix mRNA expression
Rats treated with ghrelin demonstrated a significant increase in mRNA levels of osterix relative to control animals at 6 weeks, while the change in Runx2 mRNA expression was insignificant ($P = 0.12$). At 12 weeks, no significant change in osterix or Runx2 mRNA was observed.

stimulatory effect was observed in growth hormone-deficient dwarf rats (Fukushima *et al.* 2005). Last, *in vitro* experiments showed that ghrelin may stimulate ossification by osteoblasts (Kim *et al.* 2005; Maccarinelli *et al.* 2005). All these studies suggest that ghrelin may be involved in bone remodelling in physiological conditions. Our results are consistent with these reports and extend these observations by disclosing a stimulatory effect of ghrelin in the repair of an intramembranous bone defect.

Two distinct features distinguish intramembranous bone ossification from endochondral bone formation. First, intramembranous bone calcification is simple and does not require a cartilage anlagen. Second, osteoblasts directly differentiate from the mesenchymal stem cells and form the new bone during physiological growth and remodelling of the face and calvaria and during pathological bone repair after injury. Any factor affecting the proliferation and differentiation of osteoblasts is therefore a potential target for the therapy of bone defects in the face and calvaria. Our finding provides clear evidence that ghrelin may induce bone regeneration during intramembranous bone repair. This effect may occur by stimulating the differentiation of osteoblasts from the mesenchymal stem cells around the bone defect, given the facts that: (1) ghrelin is a potent stimulator of the proliferation and differentiation of osteoblasts; and (2) expression of the osteoblast differentiation markers ALP, osteocalcin and collagen type I is augmented by ghrelin. Together with other studies (Kim *et al.* 2005; Maccarinelli *et al.* 2005; Fukushima *et al.* 2005), our findings suggest that ghrelin may be used to improve the healing of bone defects in the face and calvaria.

Workers in our laboratory have previously reported that ghrelin may function to regulate the differentiation of adipocytes and myocytes. Since osteoblasts, adipcytes and myocytes are derived from common precursor cells, the mesenchymal stem cells, our findings and the observations of others that ghrelin stimulates the differentiation of osteoblast cells (Kim *et al.* 2005; Maccarinelli *et al.* 2005) and myocytes (Filigheddu *et al.* 2007; Zhang *et al.* 2007) suggest that ghrelin may serve as an important regulator in the lineage determination of mesenchymal stem cells. This conclusion is in line with recent findings revealing the role of ghrelin in the regulation of growth and development. The significance of ghrelin in growth and development is demonstrated by studies showing that ghrelin regulates the proliferation and differentiation of a variety of cell types derived from endodermic, mesodermic and ectodermic tissues (Chanoine, 2005). In particular, ghrelin has been demonstrated to be a key controller in pancreatic β-cell development (Prado *et al.* 2004; Wierup & Sundler, 2005).

In conclusion, our study provides evidence supporting the concept that ghrelin may function to stimulate bone repair in intramembranous bone defects.

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