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ORIGINAL PAPER

Ghrelin stimulates proliferation of human osteoblastic TE85 cells via NO/cGMP signaling pathway

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Abstract Ghrelin regulates bone formation and osteoblast proliferation, but the detailed signaling pathway for its action on osteoblasts remains unclear. In human osteoblastic TE85 cells, we observed the effects and intracellular signaling pathway of ghrelin on cell proliferation using BrdU incorporation method. Ghrelin, at 10^{-10}–10^{-8} M concentration, significantly increased BrdU incorporation into TE85 cells. The action of ghrelin was inhibited by D-Lys3-GHRP-6, a selective antagonist of GHS-R. Nitric oxide (NO) scavenger hemoglobin and the NO synthase inhibitor NAME eliminated the stimulatory action of ghrelin on proliferation, while NO donor SNAP and NO synthase substrate L-AME stimulated proliferation of osteoblastic TE85 cells. The cGMP analogue, 8-Br-cGMP, stimulated TE85 cell proliferation, and ghrelin did not enhance proliferation in the presence of 8-Br-cGMP. Inhibition of cGMP production by the guanylate cyclase inhibitor prevented ghrelin-induced osteoblastic TE85 cell proliferation. In conclusion, ghrelin stimulates proliferation of human osteoblastic TE85 cells via intracellular NO/cGMP signaling pathway.

Keywords Ghrelin · Osteoblast · Nitric oxide · cGMP · Proliferation

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Ghrelin is a 28-amino acid hormone released from the stomach [1]. It was first identified as an endogenous growth hormone secretagogue (GHS) [2]. Now, it has been confirmed that ghrelin exert multiple functions such as regulation of energy metabolism, preservation of cardiovascular function, and stimulation of cell proliferation [3–5]. The stimulatory effects of ghrelin on cell proliferation were firstly described in HepG2 hepatoma cells and further confirmed in many other kinds of cells [6–8]. Recent studies indicate that ghrelin is also involved in the regulation of bone growth and osteoblast proliferation [9]. For example, synthetic growth hormone secretagogues (GHSs) increased bone mineral content in rodents [10, 11]. Ghrelin stimulated proliferation and differentiation, but inhibited apoptosis in mouse osteoblastic MC3T3-E1 cells [12].

Ghrelin activates the G-protein-coupled growth hormone secretagogue receptor (GHS-R) to achieve multiple functions [13]. Although the signaling mechanism of GHS-R has attracted much research interest, some discrepancies exist in the signal transduction pathway of GHS-R. For example, it was suggested that GHS-R is linked to phospholipase C (PLC) activation and resulted in increased inositol triphosphate (IP3) production and calcium mobilization in rat somatotrophs [14]. On the other hand, GHS-R was found to be related to cyclic AMP (cAMP) signaling

pathway in ovine somatotrophs [15]. Moreover, Nitric oxide (NO) and cyclic GMP (cGMP) signaling pathways were reported to mediate the inhibitory effect of ghrelin on voltage-gated potassium channels in somatotrophs and the stimulatory effect of ghrelin on growth hormone secretion via GHS-R on somatotrophs [16, 17].

Primary osteoblasts as well as osteoblastic cell lines express ghrelin receptor, GHS-R. The signaling mechanism of GHS-R on osteoblasts remains unclear. In the present study, we showed that ghrelin stimulates osteoblast proliferation via GHS-R and demonstrate that intracellular NO/cGMP signaling pathway mediate the action of ghrelin on stimulating osteoblast proliferation.

Results

Ghrelin stimulated proliferation of osteoblastic TE85 cells

Firstly, we showed that ghrelin dose-dependently stimulated the proliferation of human osteoblastic TE85 cells. After incubated with ghrelin for 24 h, the cell number of osteoblastic cells was significantly increased as indicated by MTT assay. Ghrelin from 10^{-10} M exhibited significant stimulatory effects of proliferation and reached the maximal effects at 10^{-8} M concentration (Fig. 1a). BrdU incorporation is an accurate method to measure the proliferation of cells, and we used this method to confirm the proliferation of osteoblastic TE85 cells. After incubation for 24 h with the cells, ghrelin increased the incorporation of BrdU into TE85 cells dose-dependently with significant responses from 10^{-10} M to 10^{-8} M (Fig. 1b).

GHS-R receptor mediated the effects of ghrelin

Ghrelin achieves the bioactive effects by activating the GHS-R. To test the involvement of GHS-R, we observed the action of the selective antagonist of GHS-R receptor on ghrelin-induced proliferation of human osteoblastic TE85 cells. Pretreatment of the cells with the selective antagonist of GHS-R receptor, D-Lys3-GHRP-6 (10^{-3} M), totally blocked ghrelin-induced proliferation of human osteoblastic TE85 cells as measured by BrdU ELISA (Fig. 2). This result suggested that the effect of ghrelin is mediated by GHS-R.

NO signaling pathway was involved in the effects of ghrelin

In the next step of clarifying the signaling pathways involved in the action of ghrelin on osteoblastic TE85 cell proliferation, we found that nitric oxide (NO) contributed to the action of ghrelin on osteoblastic TE85 cell

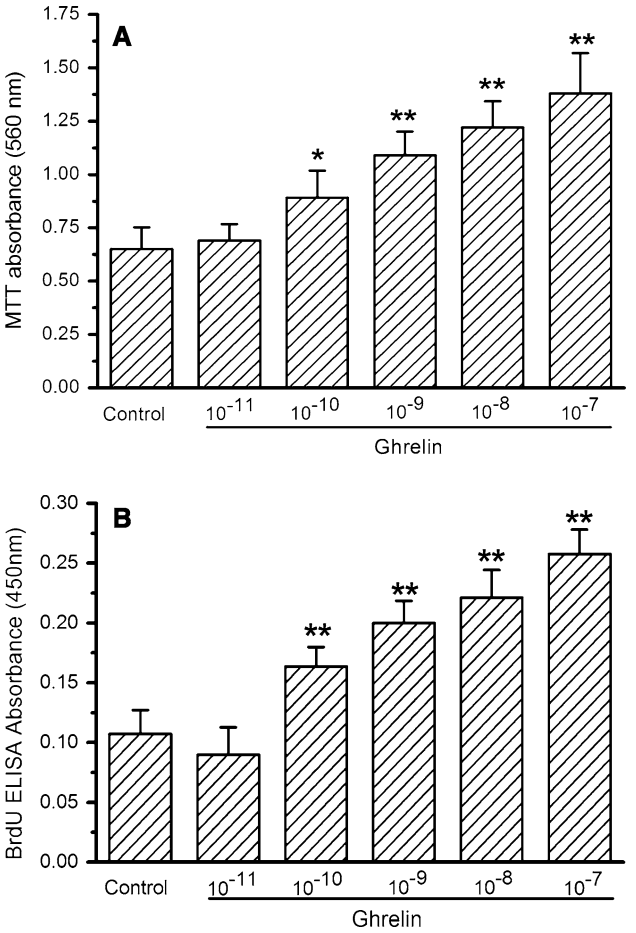

Fig. 1 The effects of ghrelin on proliferation of human osteoblastic TE85 cells. **a** Ghrelin dose-dependently increased the formation of MTT crystals in TE85 cells after 24 h incubation. **b** The BrdU incorporation assay using ELISA showed that ghrelin stimulated proliferation of TE85 cells. The data were presented as mean ± SEM, $n = 12$. $^{*}P < 0.05$ and $P < 0.01$ vs control. $^{**}P < 0.01$ vs control

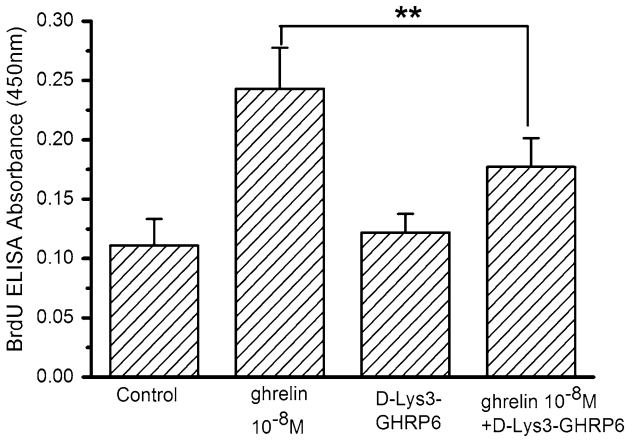

Fig. 2 The effects of GHS-R antagonist on ghrelin-induced proliferation of human osteoblastic TE85 cells. D-lys3-GHRP6, the antagonist of GHS-R, blocked the effects of ghrelin on stimulating proliferation of TE85 cells as measured by BrdU ELISA. The data were presented as mean ± SEM, $n = 8$. $^{**}P < 0.01$

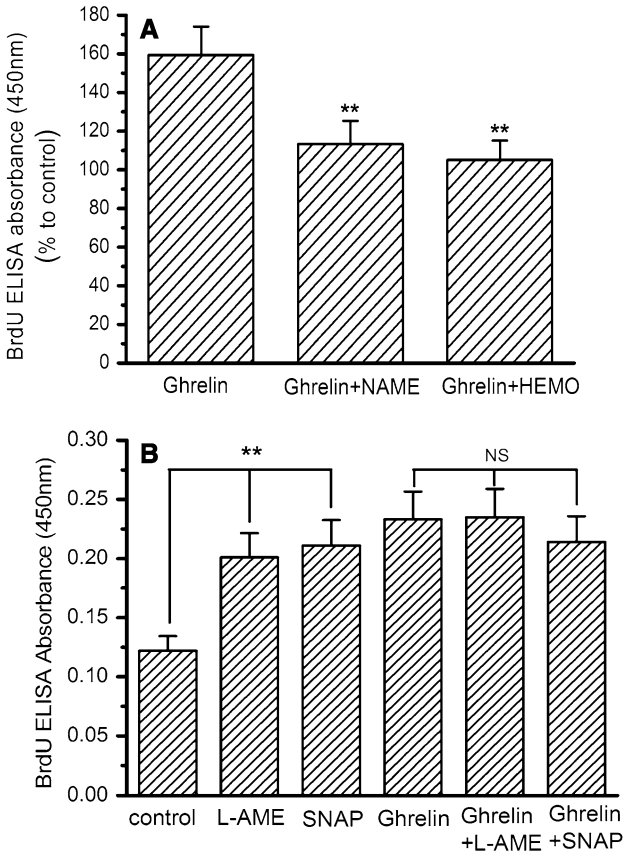

Fig. 3 The effects of inhibitors and activators for nitric oxide production on ghrelin-induced proliferation of human osteoblastic TE85 cells. **a** NO scavenger hemoglobin and the NO synthase inhibitor NAME abolished the stimulatory action of ghrelin on proliferation of TE85 cells. The data were presented as mean ± SEM, $n = 12$. $^{*}P < 0.05$ vs. ghrelin group. **b** NO donor SNAP and NO synthase substrate L-AME stimulated proliferation of osteoblastic TE85 cells. In the presence of SNAP and L-AME, ghrelin could not enhance further the proliferation of TE85 cells. The data were presented as mean ± SEM, $n = 12$. $^{**}P < 0.01$ vs. control group. NS means no significant difference

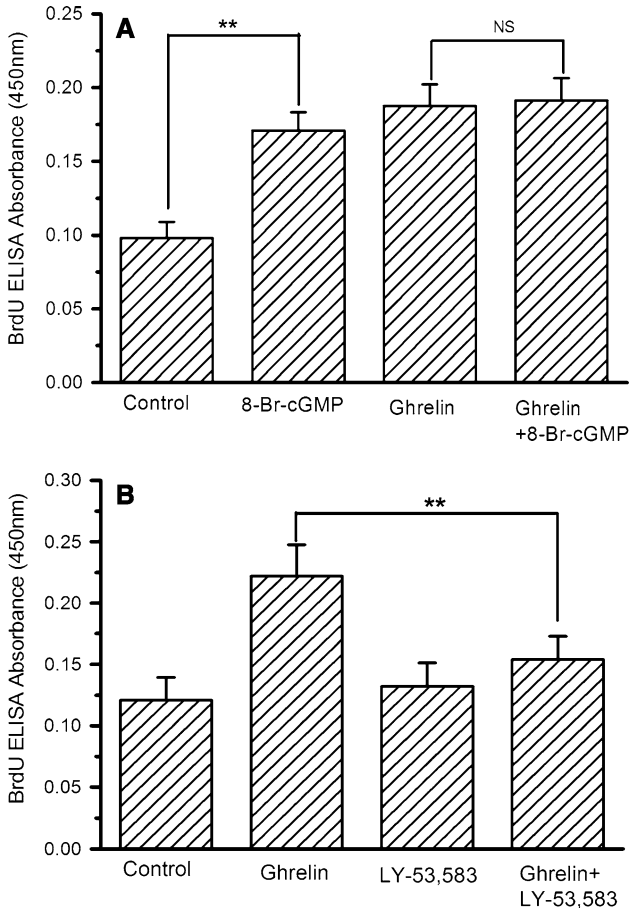

Fig. 4 The effects of activator and inhibitor of cGMP signaling on ghrelin-induced proliferation of human osteoblastic TE85 cells. **a** cGMP analogue, 8-Br-cGMP, stimulated proliferation of TE85 cells. **b** Guanylate cyclase inhibitor, LY-83,583, completely blocked ghrelin-induced osteoblastic cell proliferation. The data were presented as mean ± SEM, $n = 12$. $^{**}P < 0.01$ vs. control group. NS means no significant difference

proliferation. Two compounds that block NO action, the NO scavenger hemoglobin and the NOS inhibitor NAME, both abolished the stimulatory action of ghrelin on proliferation of osteoblastic TE85 cells as measured by BrdU ELISA (Fig. 3a). On the other hand, we examined the effects of NO donor SNAP and NOS substrate L-AME on osteoblast proliferation. As shown in Fig. 3b, SNAP and L-AME stimulated proliferation of osteoblastic TE85 cells. Ghrelin could not enhance proliferation in the presence of SNAP and L-AME, which indicates the same signaling targets of ghrelin to SNAP and L-AME.

cGMP signaling pathway mediated the effects of ghrelin

To test the role of cGMP in NO-mediated signaling pathway in osteoblastic TE85 cell proliferation, we first observed the effects of cell-permeable analogue of cGMP, 8-Br-cGMP, on basal and ghrelin-stimulated osteoblastic TE85 cell proliferation. 8-Br-cGMP (10^{-8} M) stimulated osteoblastic TE85 cell proliferation in a manner similar to ghrelin as measured by BrdU ELISA. Ghrelin did not cause further proliferation on the top of 8-Br-cGMP, indicating ghrelin uses cGMP signaling pathway to stimulate osteoblastic TE85 cell proliferation (Fig. 4a). Furthermore, we observed that guanylate cyclase (GC) blocker, LY-83,583 (10 μM), completely blocked ghrelin-induced osteoblastic TE85 cell proliferation (Fig. 4b).

Nitric oxide activated the GC/cGMP signaling pathway in the action of ghrelin

To clarify the role of NO in the activation of GC/cGMP in the route of ghrelin action, we provided NO by adding

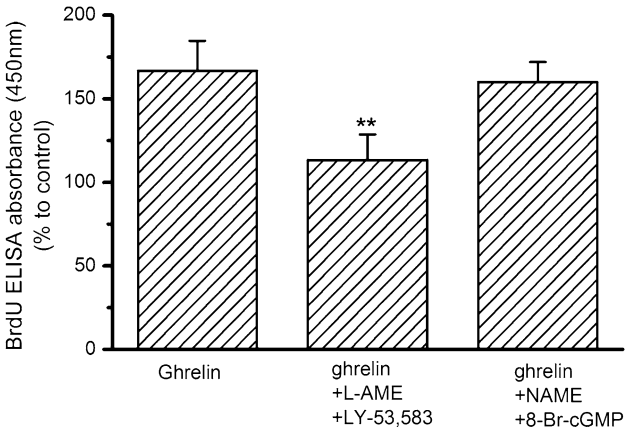

Fig. 5 The relationship between nitric oxide and cGMP in ghrelin-induced proliferation of human osteoblastic TE85 cells. Ghrelin could not exert stimulatory effects on TE85 cell proliferation by adding in L-AME and simultaneously inhibited GC by LY-83,583. NAME combined with 8-Br-cGMP could not inhibit ghrelin-induced proliferation of TE85 cells. The data were presented as mean ± SEM, $n = 6$. $^{**}P < 0.01$ vs. ghrelin group

in L-AME and simultaneously inhibited GC by LY-83,583 when treating the cells with ghrelin. Under this condition, ghrelin could not exert stimulatory effects on osteoblastic TE85 cell proliferation. On the other hand, inhibition of NO production by NAME combined with activation of cGMP by 8-Br-cGMP could not inhibit cell proliferation (Fig. 5). It demonstrated that cGMP is the downstream molecule following NO in the action route of ghrelin.

Discussion

The present study provides novel evidence for the stimulatory effects of ghrelin on osteoblast proliferation. It has shown that ghrelin both stimulates and inhibits cell growth depending on the cell types. For example, ghrelin enhances proliferation in H9c3 cardiomyocytes, HepG2 human hepatoma cells, PC3 prostate cancer cells, and PAC1 pancreatic cancer cells [6, 18–20]. On the other hand, ghrelin suppresses proliferation of breast cancers, lung tumor cells, and thyroid adenocarcinoma [21–23]. For osteoblasts, ghrelin stimulates proliferation of mouse osteoblasts [12, 24]. Our present results obtained in human TE85 osteoblastic cells support that ghrelin stimulates osteoblast proliferation. It strengthens the hypothesis that ghrelin is a hormonal regulator of bone growth.

Ghrelin exerts its actions by binding to GHS-R. There are two types of GHS-R named as GHS-R1a and GHS-R1b [25]. GHS-R1a has an essential biological function in mediating the effects of GHS, while the functional role of GHS-R1b remains to be defined. The GHS-R1a was distributed widely in peripheral tissues, and it has been reported that GHS-R1a is expressed by osteoblasts [12]. The block of ghrelin-induced proliferation by GHS-R antagonist D-Lys3-GHRP-6 strongly suggests that ghrelin stimulates osteoblastic TE85 cell proliferation via its active GHS-R.

The intracellular signaling pathways for GHS-R remain unclear. Ghrelin stimulates IP3 production and calcium mobilization from intracellular calcium stores in rat somatotrophs [14]. cAMP/PKA signaling pathway was suggested to be involved in the action of ghrelin on ovine somatotrophs [15]. Interestingly, NO/cGMP signaling pathway was considered to mediate stimulatory effects of ghrelin on growth hormone secretion via GHS-R on pig somatotrophs and to mediate the inhibitory effects of ghrelin on voltage-gated potassium channels on rat GH3 cells [16, 17]. In this study, we provide the evidence for the involvement of NO/cGMP signaling pathway in ghrelin-induced proliferation on osteoblastic TE85 cells.

We first demonstrated that NO mediates the effects of ghrelin on osteoblastic TE85 cell proliferation. NO is a very small lipophilic molecule defusing rapidly in the cytoplasm and results in the activation of diverse biological function [26]. NO undertakes important role in bone metabolism [27, 28]. It has shown that NO mediates osteoblast proliferation. Lin et al. reported that NO stimulates proliferation of fetal calvarial osteoblastic cells [28]. It was reported that NO mediates 17 beta-estradiol-stimulated osteoblast proliferation and the osteoblast proliferation stimulated by pulsed electromagnetic field [29, 30]. Moreover, NO protects osteoblasts from oxidative stress-induced apoptotic insults [31]. In our study, NO production by the donor SNAP and NOS substrate L-AME increased the proliferation of human osteoblastic TE85 cells, which is accordant with previous reports. Ghrelin could not enhance proliferation of human osteoblastic TE85 cells in the presence of SNAP and L-AME, and inhibition of NO action by NO scavenger hemoglobin and the NOS inhibitor NAME abolished the stimulatory action of ghrelin on proliferation of osteoblastic TE85 cells. It strongly supports that NO mediates the effects of ghrelin on stimulation of human osteoblastic TE85 cell proliferation.

The downstream molecule of NO induced by ghrelin is cGMP. GC and its product cGMP constitute the main subsequent molecule of the NO signaling system in most cellular model [32]. Our results demonstrated that the ghrelin treatment and cGMP activation both stimulated osteoblast proliferation, and no further stimulatory action of ghrelin was observed under the condition of cGMP activation. It suggests that ghrelin acts on osteoblastic

TE85 cells through the intracellular mechanism that is same to cGMP. The block of ghrelin-stimulated osteoblast proliferation by GC inhibitor further confirmed that ghrelin's action on osteoblastic cell proliferation is mediated by cGMP.

Taken as a whole, our results reveal that NO/cGMP signaling pathway plays an important role in osteoblast proliferation. This finding is supported by the report that NO regulates osteoblast growth and this effect is mediated by cGMP [33]. Moreover, activation of GC and production of cGMP in osteoblasts stimulated by natriuretic peptides induces osteoblast proliferation and bone growth [34]. However, the involvement of mitogen-activated protein kinase (MAPK) signaling pathway in ghrelin-induced osteoblast proliferation was suggested [12]. The activation of MAPK by cGMP-dependent protein kinase was reported in human and mouse platelet [35], suggesting the interaction of cGMP signaling pathway with MAPK signaling pathway. We hypothesize that there may be possible relationship between these two signaling pathways in osteoblast proliferation. The major findings of our study are illustrated in Fig. 6.

Ghrelin is a critical hormone connecting physiological processes regulating nutrition, body composition, and growth [36]. It is speculated that ghrelin ensures sufficient amounts of energy to be available for growth hormone to stimulate growth and repair [37]. Ghrelin stimulates growth hormone secretion and enhances activation of GH/IGF-1 system. GH/IGF-1 system is an important regulator in bone growth and metabolism [38, 39]. The direct action of ghrelin on osteoblasts may cooperate with GH/IGF-1 system to facilitate bone growth.

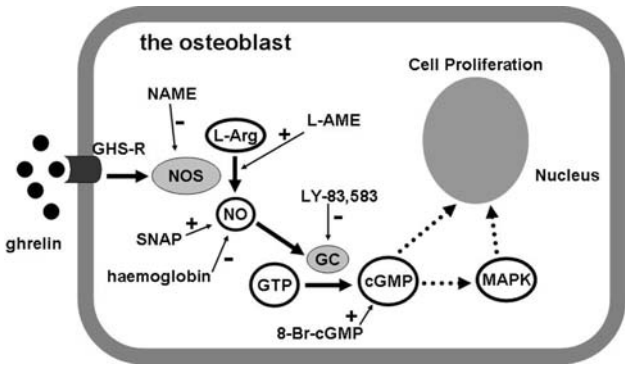

Fig. 6 The illustration of the signaling pathway in mediating ghrelin-induced osteoblast proliferation. Ghrelin binds to GHS-R and induced NO production. NO stimulates cGMP production via activation of GC. cGMP induces osteoblast proliferation via possibly activating MAPK or through other signaling molecules. Solid line means the pathway supported by the results and the dashed line means the signaling pathway that has not confirmed. + and − mean stimulation and inhibition, respectively

Materials and methods

Materials

Ghrelin, D-lys3-GHRP6, S-nitroso-*N*-acetylpenicillamine (SNAP), L-arginine methyl ester hydrochloride (L-AME), hemoglobin, Nw-nitro-L-arginine-methyl ester hydrochloride (NAME), 8-Bromo-cyclic GMP (8-Br-cGMP), and Ly-53,583 were purchased from Sigma (St. Louis, MO, USA). Mccoy's 5A medium, fetal bovine serum (FBS), and penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). 5-Bromo-2′-deoxyuridine (BrdU) ELISA kits were purchased from Roche (Sandhofer, Mannheim, Germany).

Cell culture

Human TE85 osteoblastic cells were cultured in Mccoy's 5A medium with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in humidified atmosphere of 5% CO_2 and 95% air. The medium was changed every 2–3 days.

MTT assay

Human TE85 osteoblastic cells were planted into 96-well plates in the density of 10^4 per well. Ghrelin was added into the medium on the next day after plantation. After 24 h incubation, cell numbers were assayed by quantifying the ability of viable cells to convert soluble MTT dye into insoluble dark blue crystals. MTT stock solution was added (1 part to 10 parts medium) to each well of a 96-well tissue culture plate and incubated at 37°C for 4 h. Acid isopropanol (400 μl of 10 M HCl in 100 ml of isopropanol) was added to each well and mixed thoroughly to ensure that all the crystals had dissolved. The absorbances of dissolved crystals were measured by an ELISA reader at a wavelength of 570 nm (BioRad).

BrdU ELISA assay

BrdU ELISA is based on the fixation and denaturation of cells within the plates in which they were grown, followed by the detection of the immobilized antigen with the peroxidase-coupled antibody and the reaction with soluble peroxidase substrate TMB (3,3′,5,5′-tetramethylbenzidine). The cells were planted into 96-well plates in the density of 10^4 per well. Ghrelin and BrdU were added into the medium in the next day after plantation. The cells were fixed and denatured by incubation with 100 μl FixDenat for 30 min at room temperature. After aspiration of FixDenat, the cells were washed with 1 ml blocking buffer (1% BSA in PBS) and then incubated for 90 min with 100 μl

anti-BrdU peroxidase-conjugated antibody. The incubation with anti-BrdU peroxidase-conjugated antibody was terminated by washes with blocking buffer for three times, and then 100 μl TMB substrates were added into the wells. After 5 min incubation with TMB, 25 μl 1 N H_2SO_4 was added to stop the reaction. The absorbances of reaction products were measured by an ELISA reader at wavelength of 450 nm (BioRad). BrdU, antibodies, FIxDenat, and TMB were provided by the BrdU ELISA kit.

Statistic analysis

All data were presented as mean ± SEM. The data were analyzed by one-way ANOVA. $P < 0.05$ was considered as significant difference for all statistical analysis.

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