

# Expression of glucagon-like peptide-1 receptor and glucose-dependent insulinotropic polypeptide receptor is regulated by the glucose concentration in mouse osteoblastic MC3T3-E1 cells

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**Abstract.** Glucose-dependent insulinotropic polypeptide receptor (GIPR) and glucagon-like peptide-1 receptor (GLP-1R) are incretin receptors that play important roles in regulating insulin secretion from pancreatic  $\beta$  cells. Incretin receptors are also thought to play a potential role in bone metabolism. Osteoblasts in animals and humans express GIPR; however, the presence of GLP-1R in these cells has not been reported to date. Thus, the aim of this study was to determine whether GLP-1R and GIPR are expressed in osteoblastic cells, and whether their expression levels are regulated by the extracellular glucose concentration. Mouse osteoblastic MC3T3-E1 cells were cultured in medium containing normal (5.6 mM) or high (10, 20 or 30 mM) glucose concentrations, with or without bone morphogenetic protein-2 (BMP-2). RT-PCR, western blot analysis and immunofluorescence were carried out to determine GIPR and GLP-1R mRNA and protein expression levels. Cell proliferation was also assessed. The GLP-1R and GIPR mRNA expression levels were higher in the MC3T3-E1 cells cultured in medium containing high glucose concentrations with BMP-2 compared with the cells cultured in medium containing normal glucose concentrations with or without BMP-2. GLP-1R protein expression increased following culture in high-glucose medium with BMP-2 compared with culture under normal glucose conditions. However, the cellular localization of GLP-1R was not affected by either glucose or

BMP-2. In conclusion, our data demonstrate that the expression of GLP-1R and GIPR is regulated by glucose concentrations in MC3T3-E1 cells undergoing differentiation induced by BMP-2. Our results reveal the potential role of incretins in bone metabolism.

## Introduction

Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are incretin hormones that regulate insulin secretion from pancreatic  $\beta$  cells. GLP-1 and GIP are secreted by the small intestine in response to meal ingestion. GLP-1 is secreted from L cells in the lower small intestine, while GIP is secreted from K cells in the upper small intestine. These incretins stimulate insulin secretion from  $\beta$  cells by binding to the GLP-1 receptor (GLP-1R) and GIP receptor (GIPR). Incretins also have extra-pancreatic functions in a variety of tissues. For example, GLP-1 affects the central nervous system, resulting in neuroprotection against amyloid  $\beta$  (1) and the suppression of appetite (2). It has also been reported to increase glucose uptake in the cardiovascular system (3) and prolong gastric acid secretion (4). GIP affects the central nervous system by stimulating memory recognition and neuroprotection (5). Several studies have also demonstrated that GIP mediates lipogenesis in adipocytes (6) and bone remodeling (7).

The incretin receptors, GLP-1R and GIPR, play important roles in glucose metabolism. GLP-1R and GIPR are members of the G protein-coupled receptor (GPCR) family. GLP-1R and GIPR are expressed in a variety of organs, such as the pancreas, stomach, intestine, heart, lungs, pituitary gland and brain (8,9). However, GLP-1R, but not GIPR, is expressed in the kidneys. In addition, GIPR, but not GLP-1R, is expressed in the adrenal cortex, adipose tissue and bone (9,10). The binding of incretins to their receptors activates adenylate cyclase, which increases intracellular cyclic adenosine monophosphate (cAMP) and is followed by changes in the intracellular  $Ca^{2+}$  concentration, ATP production in the mitochondria and insulin secretion (11).

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There is a correlation between glucose metabolism and bone metabolism. For example, insulin signals in osteoblasts activate osteocalcin production, which enhances glucose metabolism. However, this activation does not occur under conditions of impaired bone resorption (12). The expression of insulin and adiponectin receptors increases during osteoblastic differentiation (13). Adiponectin, which regulates glucose and lipid metabolism, stimulates bone morphogenetic protein-2 (BMP-2) expression in osteoblasts (14).

Incretins act in an insulin-dependent or -independent manner. The GLP-1-mediated suppression of hepatic glucose production is one of the insulin-independent effects (15). However, the insulin-dependent effects of GLP-1 are still controversial. For example, it is unclear whether the effects of GLP-1 on adipocytes are dependent on or independent of insulin since the direct role of GLP-1R on adipocytes has not been experimentally confirmed (16-18). It has been suggested that incretins have a direct effect on bone as the genetic disruption of pancreatic GLP-1R increases bone resorption in mice (19,20). Although GIPR is expressed in osteoblasts (21), it is unclear whether GLP-1R is also expressed in osteoblasts. GIP has been reported to inhibit bone resorption and enhance bone formation by stimulating osteoblast proliferation and inhibiting osteoblast apoptosis (22). Notably, GIPR-knockout mice exhibit reduced bone strength and quality (23,24).

The objective of this study was to clarify the presence of GLP-1R and GIPR in mouse osteoblastic MC3T3-E1 cells and examine the association between incretin receptors in osteoblasts and glucose metabolism.

## Materials and methods

**Materials.** MC3T3-E1 cells, a mouse osteoblastic cell line, were obtained from the RIKEN Cell Bank (Tsukuba, Japan). The reagent used for total RNA isolation (RNA STAT-60™) was purchased from Amsbio (Abingdon, UK). Rabbit polyclonal anti-GLP-1R and GIPR antibodies, which cross-react with mouse antigens, were obtained from Abcam (Cambridge, UK). Recombinant human BMP-2 (rhBMP-2) was purchased from R&D Systems (Minneapolis, MN, USA). The other reagents used were of the highest grade commercially available.

**Cell culture.** MC3T3-E1, mouse osteoblast-like cells, were maintained at 37°C under 95% air/5% CO<sub>2</sub> in Minimum Essential Medium Eagle  $\alpha$  modification ( $\alpha$ -MEM; Sigma, St. Louis, MO, USA) containing 5.6 mM glucose, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 10% fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA). For glucose and BMP-2 treatment, 3.75x10<sup>4</sup> cells were seeded onto 35-mm dishes and incubated overnight at 37°C. The dishes were then randomly divided into 8 groups and were incubated in medium containing normal (5.6 mM) or high (10, 20 or 30 mM) glucose concentrations without or with 100 ng/ml rhBMP-2.

**Reverse transcription-polymerase chain reaction (RT-PCR).** The MC3T3-E1 cells were cultured in  $\alpha$ -MEM supplemented with 10% FBS and 5.6 mM glucose for 72 h. Total RNA was then isolated using RNA STAT-60™ reagent according to the instructions provided by the manufacturer. cDNA was synthesized from 1.0  $\mu$ g of total RNA with oligo(dT) primers

using SuperScript™ III Reverse Transcriptase (Invitrogen, San Diego, CA, USA) under the conditions suggested by the manufacturer. GLP-1R, GIPR and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression was detected by RT-PCR using the following primers (forward and reverse), as previously described: GLP-1R, 5'-GGAGTGTGAAGAGTC TAAGC-3' and 5'-GCAGTACTGCATGAGCAGGA-3' (25); GIPR, 5'-CTGCCTGCCGCACGGCCAGAT-3' and 5'-GCG AGCCAGCTCAGCCGGTAA-3' (22); GAPDH, 5'-ACCACA GTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCT GTA-3'. For GLP-1R, the PCR conditions comprised an initial activation of polymerase at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 30 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. For GIPR, the PCR conditions comprised an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The quality of the template was examined by amplifying GAPDH cDNA in each PCR condition. The amplified PCR products were analyzed by electrophoresis on a 2% agarose gel in Tris-acetate-EDTA buffer, stained with ethidium bromide and photographed under ultraviolet transillumination.

**Quantitative PCR.** The cells were seeded at a density of 3.75x10<sup>4</sup> cells in 35-mm dishes and maintained at 37°C. The following day, the medium was substituted with medium containing various concentrations of glucose, as described above. Total RNA was isolated from the cells following culture for 24, 48 and 72 h, and was converted to cDNA. Four samples were prepared for each treatment. Quantitative PCR assays were carried out in triplicate for each sample using a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). PCR analyses were conducted with gene-specific primers and fluorescently labeled TaqMan GLP-1R-specific probes (Mm00445292\_m1), GIPR (Mm01316344\_m1), Runx2 (Mm00501580\_m1), alkaline phosphatase (ALP, Mm01285814\_g1) and GAPDH (Mm99999915\_g1). The PCR conditions comprised activation at 95°C for 20 sec, followed by 40 cycles of 95°C for 1 sec and 60°C for 20 sec. The  $\Delta\Delta$ Ct method of relative quantification was used to determine the fold change in the expression of each gene. First, the threshold cycle (Ct) of the target mRNAs was normalized to the Ct values of the internal control (GAPDH) in the same sample ( $\Delta$ Ct = Ct<sub>target</sub> - Ct<sub>GAPDH</sub>), which was followed by normalization to the control ( $\Delta\Delta$ Ct =  $\Delta$ Ct<sub>sample</sub> -  $\Delta$ Ct<sub>control</sub>). The fold change in expression was calculated as the relative quantification value (RQ; 2<sup>- $\Delta\Delta$ Ct</sup>).

**Western blot analysis.** Total protein was extracted from confluent cells cultured for 72 h on 60-mm dishes in normal- (5.6 mM) or high-glucose (30 mM) medium with or without BMP-2. Briefly, the cells were washed twice with phosphate-buffered saline (PBS), scraped from the dish and collected by centrifugation. The cells were then resuspended in 100  $\mu$ l of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 10 mM iodoacetamide, 5 mM NaF, 5 mM sodium pyrophosphate, 10 mM EDTA, 10 mM  $\beta$ -glycerophosphate, pH 7.5, containing 2% Triton X-100 and protease inhibitor cocktail), incubated on ice for 15 min and centrifuged at 19,000 x g for 15 min at

4°C. The supernatant was mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (26) and heated at 100°C for 5 min. Proteins (10 µg of total protein/well) were then separated on 5-20% polyacrylamide gels (SuperSep Ace; Wako, Osaka, Japan) and transferred onto a polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA, USA). The membrane was blocked with 1% bovine serum albumin (BSA) in PBS containing 0.1% Tween-20 for 40 min at room temperature and then incubated for 120 min with primary antibody (anti-GLP-1R, 1:5,000 dilution, ab39072; anti-α-tubulin, 1:10,000 dilution, ab4074). The membranes were then washed 6 times for 5 min each in buffer containing PBS and 0.1% Tween-20, followed by incubation with a peroxidase-coupled secondary antibody (1:2,000 dilution; Pierce, Rockford, IL, USA). ECL Prime (GE Healthcare Life Sciences, Piscataway, NJ, USA) was used to detect peroxidase activity. The optical densities of the bands were quantified using ImageJ 1.47 software (NIH, Bethesda, MD, USA). GLP-1R expression was normalized to α-tubulin expression, which was used as an internal loading control.

**Immunofluorescence.** The MC3T3-E1 cells ( $3.2 \times 10^4$  cells/well) were grown on a glass coverslip in 24-well plates for 72 h in normal- (5.6 mM) or high-glucose (30 mM) medium with or without BMP-2. Shortly before fixation, fresh medium containing 500 ng/ml Hoechst 33342 (Cell Signaling Technology, Danvers, MA, USA) was added followed by incubation for 1 h at 37°C. The cells were then fixed with freshly prepared 4% paraformaldehyde (Sigma). The fixed cells were permeabilized with 0.1% Triton X-100 in 10 mM glycine-PBS, blocked with 3% BSA in PBS at room temperature for 30 min and incubated with rabbit anti-GLP-1R antibody (1:1,000 dilution, ab39072) for 1 h. After several washes with 0.1% BSA in PBS, the cells were incubated with anti-rabbit secondary antibody conjugated with AlexaFluor 488 (Molecular Probes, Eugene, OR, USA) for 1 h, washed again with 0.1% BSA in PBS and mounted in ProLong Gold Antifade reagent (Life Technologies, Eugene, OR, USA). The samples were observed under an All-in-One Fluorescence Microscope (BZ-9000; Keyence, Osaka, Japan).

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.** The MC3T3-E1 cells were seeded in 96-well plates at a density of  $5 \times 10^3$  cells/100 µl/well. Following incubation for 24, 48 or 72 h in normal- (5.6 mM) or high-glucose (10, 20 or 30 mM) medium containing BMP-2, cell proliferation was assessed using the MTT Cell Counting kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. MTT solution (10 µl) was added to each well and to the control wells without cells. Following incubation at 37°C for 1 h, the amount of the MTT formazan product was analyzed spectrophotometrically at a wavelength of 450 nm. The absorbance was normalized to that of cell-free medium.

**Statistical analysis.** Statistical analysis was performed using SPSS II for Windows (SPSS Inc., Chicago, IL, USA). The statistical significance of differences among groups was calculated using analysis of variance. Data are presented as the means ± standard error of the mean (SEM).  $P < 0.05$  was regarded as statistically significant and  $P < 0.01$  as highly significant.

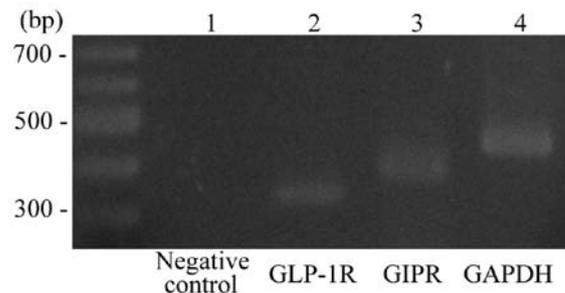


Figure 1. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of glucagon-like peptide-1 receptor (GLP-1R) and glucose-dependent insulinotropic polypeptide receptor (GIPR) mRNA expression in MC3T3-E1 cells. Lane 1, negative control; lane 2, GLP-1R (337 bp); lane 3, GIPR (382 bp); lane 4, GAPDH (452 bp).

## Results

**Expression of incretin receptors in MC3T3-E1 cells.** The MC3T3-E1 cell line, the most widely studied mouse osteoblastic cell line, was used in this study. We first examined the mRNA expression of GLP-1R and GIPR in these cells by RT-PCR with specific primers. Bands at 337 and 382 bp, corresponding to GLP-1R and GIPR, respectively, were detected (Fig. 1), indicating that these osteoblasts expressed both types of incretin receptors.

**Quantitative PCR.** The MC3T3-E1 cells were cultured in medium containing various glucose concentrations. The expression of GLP-1R mRNA following 24 h of culture was unaffected by changes in glucose concentrations either in the presence or absence of BMP-2. After 48 h of culture, GLP-1R mRNA expression increased in the presence of BMP-2 compared with the absence of BMP-2 by 3-fold in the cells cultured in 5.6 mM (normal) glucose, 4-fold in the cells cultured in 10 mM glucose, and 6-fold in the cells cultured in 20 and 30 mM glucose. After 72 h of culture, GLP-1R mRNA expression increased in the presence of BMP-2 by 3-fold in the cells cultured in 5.6 mM glucose, 4-fold in the cells cultured in 10 mM glucose, 11-fold in the cells cultured in 20 mM glucose and 16-fold in the cells cultured in 30 mM glucose (Fig. 2A and Table I). Notably, in the presence of BMP-2, GLP-1R mRNA expression increased in response to higher glucose concentrations. After 48 h of culture, GLP-1R mRNA expression in the presence of BMP-2 in the cells cultured in 30 mM glucose was 2-fold higher than that in the cells cultured in 5.6 mM glucose. After 72 h of culture, GLP-1R mRNA expression in the cells cultured in 30 mM glucose was 4-fold higher than that in the cells cultured in 5.6 mM glucose (Fig. 2A and Table I). GLP-1R mRNA expression increased in a time-dependent manner, since its expression after 72 h of culture in the cells cultured in 30 mM glucose in the presence of BMP-2 was 5-fold higher than that after 24 h of culture (Table I).

GIPR mRNA expression was stimulated by BMP-2 at each glucose concentration (Fig. 2B and Table II). GIPR mRNA expression increased in response to higher glucose concentrations in the presence of BMP-2, being 4-fold higher in the cells cultured in 30 mM glucose than in the cells cultured in 5.6 mM glucose after 24 h of culture (Fig. 2B and Table II).

Table I. Effects of glucose, BMP-2 and incubation time on GLP-1R mRNA expression.

Glucose concentration (mM)	24 h		48 h		72 h	
	BMP-2 (-)	BMP-2 (+)	BMP-2 (-)	BMP-2 (+)	BMP-2 (-)	BMP-2 (+)
5.6	0.82±0.08	2.25±0.18	1.16±0.17	3.40±0.05	1.62±0.15	4.17±0.36
10	1.14±0.15	2.21±0.15	0.90±0.09	3.67±0.25	1.90±0.23	8.23±0.56
20	1.52±0.25	3.45±0.28	1.29±0.09	8.46±0.51	1.11±0.07	11.79±0.84
30	2.16±0.27	3.38±0.16	1.31±0.13	8.22±0.49	1.10±0.04	17.49±0.55

Data are presented as the means ± SEM of RQ values (n=4/group). BMP-2, bone morphogenetic protein-2; GLP-1R, glucagon-like peptide-1 receptor; SEM, standard error of the mean; RQ, relative quantification.

Table II. Effects of glucose, BMP-2 and incubation time on GIPR mRNA expression.

Glucose concentration (mM)	24 h		48 h		72 h	
	BMP-2 (-)	BMP-2 (+)	BMP-2 (-)	BMP-2 (+)	BMP-2 (-)	BMP-2 (+)
5.6	0.91±0.05	3.29±0.12	0.28±0.03	1.50±0.14	0.96±0.11	1.09±0.03
10	0.97±0.05	3.95±0.20	0.43±0.04	2.94±0.26	0.68±0.06	2.98±0.40
20	1.09±0.10	8.76±0.26	1.01±0.03	5.78±0.32	0.94±0.09	5.94±0.50
30	1.00±0.10	11.54±0.28	0.86±0.05	8.04±0.35	1.00±0.05	9.33±0.74

Data are presented as the means ± SEM of RQ values (n=4/group). BMP-2, bone morphogenetic protein-2; GIPR, glucose-dependent insulinotropic polypeptide receptor; SEM, standard error of the mean; RQ, relative quantification.

Table III. Effects of glucose, BMP-2 and incubation time on Runx2 mRNA expression.

Glucose concentration (mM)	24 h		48 h		72 h	
	BMP-2 (-)	BMP-2 (+)	BMP-2 (-)	BMP-2 (+)	BMP-2 (-)	BMP-2 (+)
5.6	1.60±0.04	2.09±0.02	1.53±0.07	3.99±0.04	4.73±0.15	4.23±0.06
10	1.40±0.05	2.15±0.05	1.96±0.06	2.63±0.05	3.66±0.21	5.12±0.10
20	1.37±0.04	2.11±0.03	1.91±0.06	3.08±0.07	2.82±0.12	7.23±0.08
30	1.21±0.07	2.27±0.02	2.03±0.05	3.68±0.11	3.46±0.23	6.18±0.06

Data are presented as the means ± SEM of RQ values (n=4/group). BMP-2, bone morphogenetic protein-2; SEM, standard error of the mean; RQ, relative quantification.

After 48 and 72 h of culture, GIPR mRNA expression in the cells cultured in 30 mM glucose increased by 5- and 9-fold, respectively, compared with the cells cultured in 5.6 mM glucose in the presence of BMP-2. However, GIPR mRNA expression was not affected by the incubation time (Table II).

The expression of Runx2 mRNA increased in a time-dependent manner in the presence of BMP-2. The glucose concentration affected Runx2 mRNA expression after 48 and 72 h of culture in the presence of BMP-2 (Fig. 3 and Table III). The expression of ALP mRNA was below the limit of detection at all culture duration tests (data not shown).

**Western blot analysis.** Western blot analysis was performed to determine the protein expression levels of incretin receptors in MC3T3-E1 cells. GLP-1R expression was determined in the total cell lysates using an anti-GLP-1R antibody. GLP-1R expression appeared to be greater in the cells cultured in 30 mM glucose with BMP-2 than in the cells cultured in 5.6 mM glucose with or without BMP-2 (Fig. 4).

**Immunofluorescence.** Immunocytochemical analysis revealed a vesicular staining pattern in the cytoplasm and cell surface, suggesting that some cell compartments contained GLP-1R.

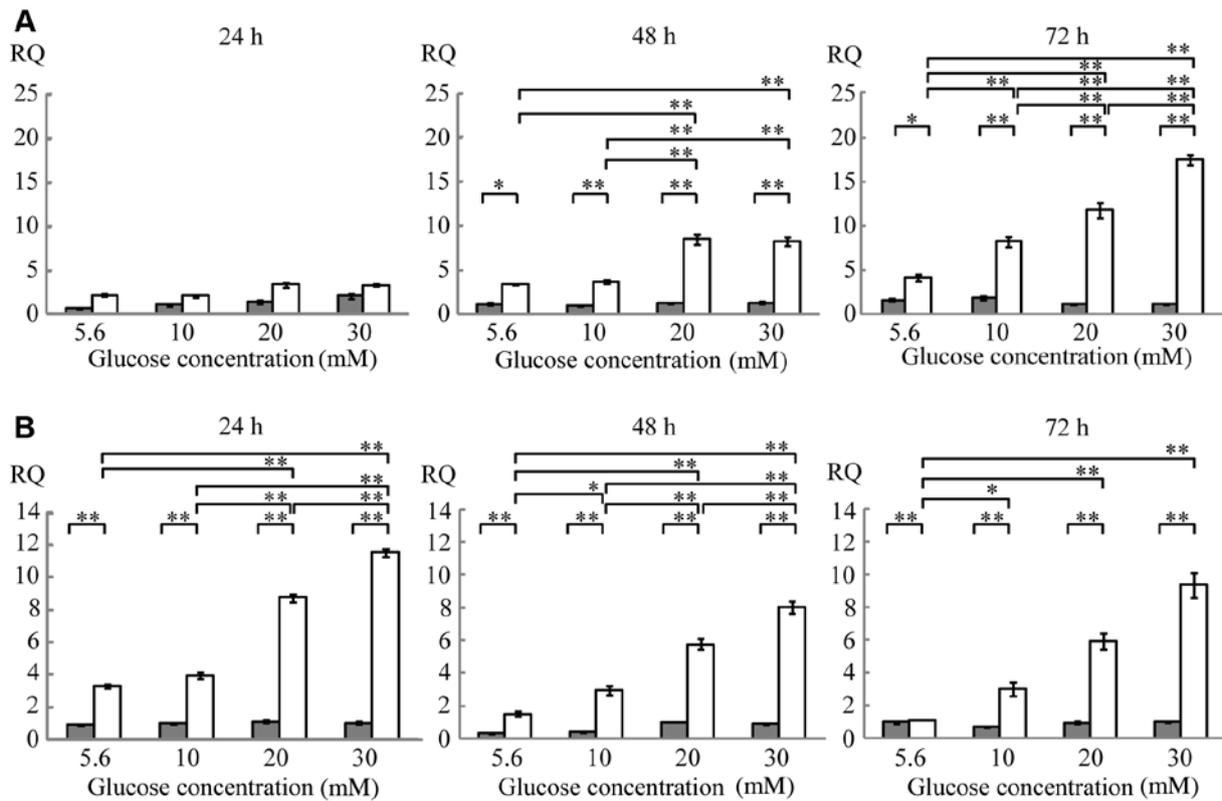


Figure 2. Effects of the glucose concentration on glucagon-like peptide-1 receptor (GLP-1R) and glucose-dependent insulintropic polypeptide receptor (GIPR) mRNA expression. MC3T3-E1 cells were cultured in medium containing 5.6 (normal), or 10, 20 and 30 mM (high) concentrations of glucose in the absence (gray bar) or presence (white bar) of bone morphogenetic protein-2 (BMP-2). (A) GLP-1R mRNA expression after 24, 48 and 72 h of culture. (B) GIPR mRNA expression after 24, 48 and 72 h of culture. Values are the means  $\pm$  standard error of the mean (SEM) (n=4/group). \*P<0.05 and \*\*P<0.01.

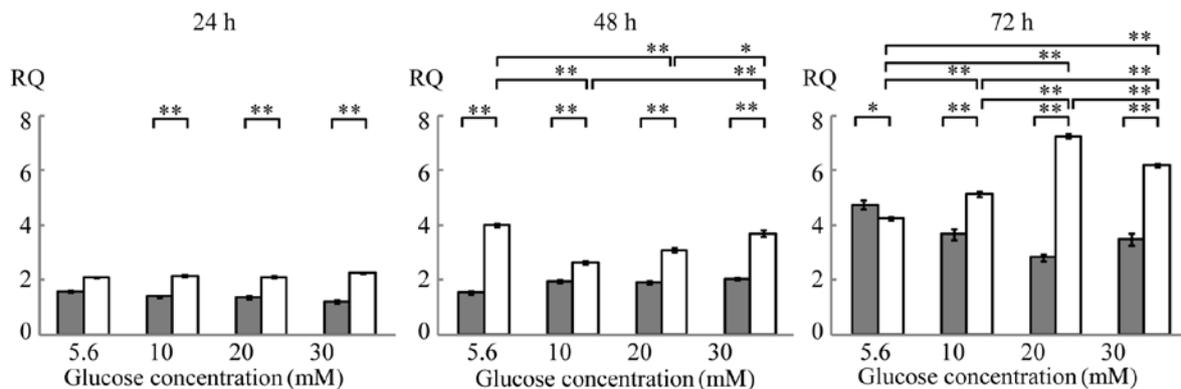


Figure 3. Effects of the glucose concentration on Runx2 mRNA expression. MC3T3-E1 cells were cultured in medium containing 5.6 (normal), or 10, 20 and 30 mM (high) concentrations of glucose in the absence (gray bar) or presence (white bar) of bone morphogenetic protein-2 (BMP-2). Runx2 mRNA expression was determined after 24, 48 and 72 h of culture. Values are the means  $\pm$  standard error of the mean (SEM) (n=4/group). \*P<0.05 and \*\*P<0.01.

The staining intensity was slightly higher in the cells treated with 30 mM glucose in the presence of BMP-2; however, the cellular distribution of GLP-1R was not apparently affected by either glucose or BMP-2 (Fig. 5).

**MTT assay.** MTT assay revealed a time-dependent increase in the number of cells in all groups analyzed. However, cell proliferation was not affected by treatment with high glucose or BMP-2 (Fig. 6).

## Discussion

The aim of this study was to determine whether incretin receptors are expressed in osteoblasts and reveal the correlation between glucose metabolism and bone metabolism.

Previous studies have demonstrated that GIPR is expressed in osteoblastic cells (21); however, the presence of GLP-1R in these cells still remains uncertain. Although osteoblastic cells are thought to express a functional receptor for GLP-1 (27),

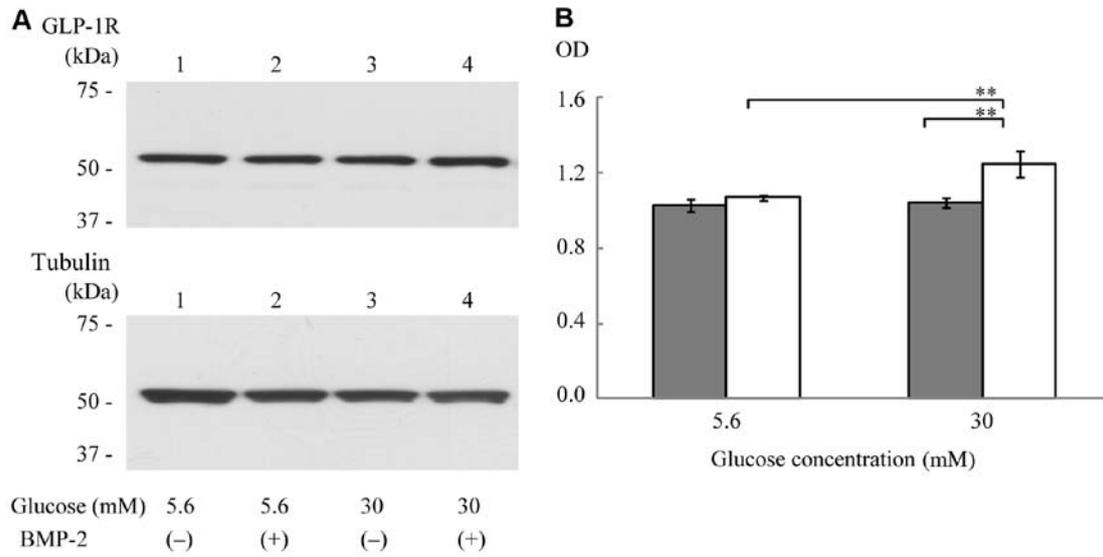


Figure 4. Western blot analysis of glucagon-like peptide-1 receptor (GLP-1R) protein expression. Confluent MC3T3-E1 cells were cultured in medium containing normal (5.6 mM) or high (30 mM) concentration of glucose in the presence or absence of bone morphogenetic protein-2 (BMP-2). (A) Samples were subjected to SDS-PAGE and immunoblotted with anti-GLP-1R (53 kDa) and anti- $\alpha$ -tubulin (50 kDa) antibodies. Lane 1, 5.6 mM glucose without BMP-2; lane 2, 5.6 mM glucose with BMP-2; lane 3, 30 mM glucose without BMP-2; lane 4, 30 mM with BMP-2. (B) Effects of glucose and BMP-2 on relative GLP-1R protein expression. The optical densities (OD) of the bands corresponding to GLP-1R and  $\alpha$ -tubulin were quantified using ImageJ software. Values are the means  $\pm$  standard error of the mean (SEM) (n=5/group). \*\*P<0.01.

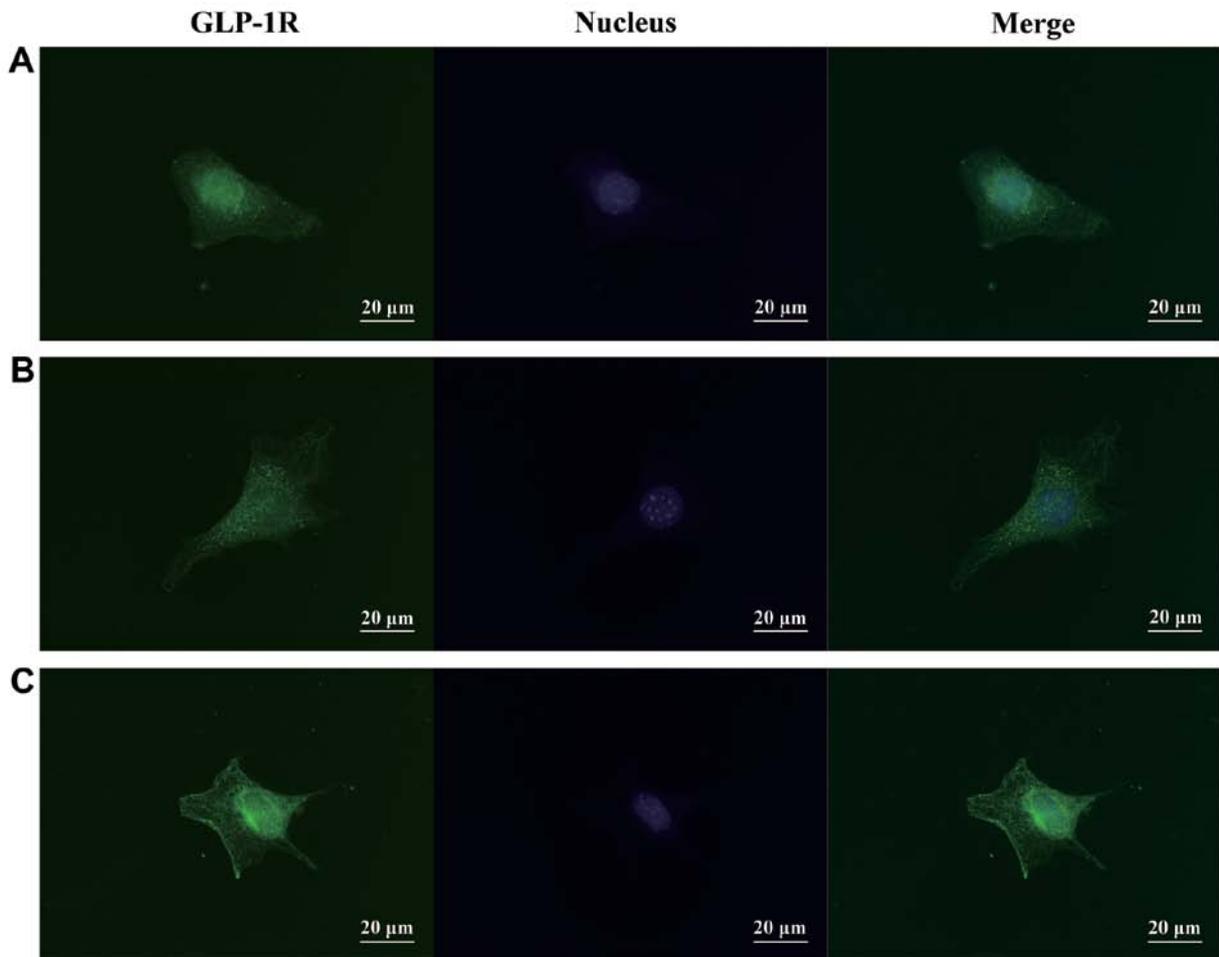


Figure 5. Immunofluorescence staining of glucagon-like peptide-1 receptor (GLP-1R) in MC3T3-E1 cells. Cells were cultured in medium containing normal (5.6 mM) or high (30 mM) concentrations of glucose in the absence or presence of bone morphogenetic protein-2 (BMP-2). (A) Glucose of 5.6 mM without BMP-2; (B) glucose of 30 mM without BMP-2; (C) glucose of 30 mM with BMP-2. Scale bar, 20  $\mu$ m.

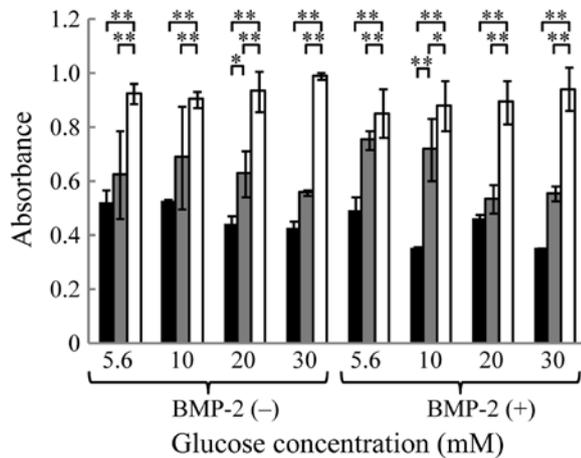


Figure 6. Effects of the glucose concentration on proliferation of MC3T3-E1 cells. MC3T3-E1 cells were cultured in medium containing 5.6 (normal), or high 10, 20 and 30 mM concentrations of glucose in the absence (-) or presence (+) of bone morphogenetic protein-2 (BMP-2). Cell proliferation was determined by MTT assay after 24 (black bar), 48 (gray bar) and 72 h (white bar) of culture. Values are shown as the mean absorbance  $\pm$  standard error of the mean (SEM) (n=3/group). \*P<0.05 and \*\*P<0.01.

there is no direct evidence to confirm the mRNA and protein expression of GLP-1R in these cells. It has been reported that GIP exerts direct effects on bone, whereas the effects of GLP-1 on bone metabolism are mediated by the thyroid hormone (20,28). Our RT-PCR analysis revealed that the MC3T3-E1 cells expressed GLP-1R and GIPR, suggesting that GLP-1 may directly affect bone metabolism, similar to GIP.

In our study, at normal glucose concentrations (5.6 mM), the expression of both incretin receptors was very low and was close to the lower limit of detection. However, treatment with 30 mM glucose increased the expression levels of GLP-1R and GIPR mRNA by 4- and 9-fold, respectively. GLP-1R and GIPR knockout mice have a higher risk of osteoporosis, suggesting that both receptors are involved in bone metabolism (29). On the other hand, another study revealed that treatment with a GLP-1R agonist may induce polyarthritis in some patients with type 2 diabetes mellitus (30). Thus, it is possible that the optimal effects of incretins may be achieved at moderate expression levels.

The glucose concentration is known to regulate many cellular events, including cell apoptosis, proliferation and differentiation. For example, it has been reported that incubation in high glucose concentrations stimulates the apoptosis of neurons, retinal pericytes and endothelial cells (31-33). However, high glucose does not affect the proliferation of human mesenchymal stem cells (34). MTT assays in this study revealed that treatment with high glucose did not affect the proliferation of MC3T3-E1 cells, which suggests either that the proliferation of osteoblasts is not affected by high glucose or that a longer duration of exposure is necessary to influence proliferation (35).

In our study, the expression of GLP-1R, but not GIPR, increased in a time-dependent manner. Although both GLP-1R and GIPR mediate insulin secretion from pancreatic  $\beta$  cells in response to ligand binding, these receptors have the opposite effects in other cell types. For example, GLP-1 has been shown to inhibit glucagon secretion in pancreatic  $\alpha$  cells in contrast to GIP (36). Therefore, while GLP-1R and GIPR are co-expressed

in the same tissues, such as the pancreas, they have different roles. Moreover, some tissues (e.g., kidneys and adipose tissue) express either GLP-1R or GIPR. Thus, the different reactions of GLP-1R and GIPR to culture duration may reflect the different roles of these receptors. Notably, glucose metabolism seems to be altered after the loss of L cells, but is not affected by the loss of K cells (37), which suggests that GLP-1 has a greater effect on glucose metabolism than GIP.

BMP-2 stimulates bone formation and osteoblastic differentiation. Runx2, an osteogenic master transcription factor, is an important mediator of BMP-2 expression (38). Runx2 protein has been detected in pre-osteoblasts, and its expression is upregulated in immature osteoblasts, but downregulated in mature osteoblasts. Thus, the MC3T3-E1 cells in this study were undergoing differentiation induced by BMP-2.

GLP-1R and GIPR belong to the GPCR family, the members of which are expressed in a variety of tissues and are potential targets for drug discovery (39). It has been reported that the administration of insulin and thiazolidinediones increases fracture risk (40-42), whereas inhibitors of dipeptidyl peptidase-4 (DPP-4) are associated with reduced fracture risk (43). DPP-4 inactivates GLP-1 and its inhibitors improve glycemic control in patients with type 2 diabetes by preventing incretin degradation (44).

GLP-1 and GIP also reduce the expression of the receptor of advanced glycation end products (AGE) in endothelial and mesangial cells by increasing cAMP levels (45,46). Chronic hyperglycemia induces non-enzymatic glycosylation and the transformation of various proteins, particularly type I collagen, into AGE, leading to a deterioration of bone mineralization and impaired biomechanical properties of the skeleton. AGE can also affect bone metabolism by inducing the expression of pro-inflammatory cytokines, such as tumor necrosis factor, which promote bone resorption and inhibit osteoblastic activity and maturation (8).

In conclusion, in the present study, we confirmed that MC3T3-E1 cells express both GLP-1R and GIPR. To the best of our knowledge, this is the first study to demonstrate that both incretin receptors are expressed in the osteoblastic cell line. Our results also suggest that GLP-1R expression is regulated by glucose concentration, particularly in the presence of BMP-2, suggesting that GLP-1R links bone metabolism and glucose metabolism in osteoblasts, and that GLP-1 may have potential therapeutic applications in bone diseases.

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