# Uncarboxylated osteocalcin reverses the high glucose-induced inhibition of the osteogenic differentiation of MC3T3E1 cells via the GPRC6A/cAMP/PKA/AMPK signaling pathway

LUYAO MA, FANGZI GONG, JIAOJIAO XU and JIANHONG YANG

Medical School, University of Chinese Academy of Sciences, Beijing 100049, P.R. China

Received November 27, 2020; Accepted March 2, 2021

DOI: 10.3892/ijmm.2021.4924

**Abstract.** Diabetic osteoporosis is a serious complication of diabetes affecting human bones. Uncarboxylated osteocalcin (GluOC), a small molecular protein specifically synthesized and secreted from osteoblasts, is of importance in regulating energy metabolism. In previous studies, the authors demonstrated that high glucose inhibited osteoblastic differentiation, but promoted adipocytic differentiation. GluOC promoted osteogenic and inhibited adipogenic differentiation under high glucose conditions. However, the corresponding receptors and signaling pathways through which GluOC exerts its effects on MC3T3E1 cells remain elusive. Thus, in the present study, Cell Counting kit-8 assays and western blot analysis were performed to assess the proliferation of MC3T3E1 cells. Alizarin Red S or Oil Red O staining, as well as reverse transcription-quantitative PCR analysis were performed to examine osteogenic and adipogenic differentiation. The cells were transfected with short interfering RNA or inhibitors to investigate the possible signaling pathways involved. The results revealed that G-protein coupled receptor, class C, group 6, subtype A (GPRC6A) receptor expression was markedly increased following the addition of GluOC to the MC3T3E1 cells. GPRC6A silencing decreased osteogenic gene expression, while it increased adipogenic gene expression. Furthermore, GluOC promoted osteoblast differentiation via the subsequent activation of the cyclic AMP (cAMP)/protein kinase A(PKA)/AMP-activated protein kinase (AMPK) signaling pathway in MC3T3E1 cells. On the whole, the results of the present study suggest that GluOC reverses the high glucose-induced inhibition of osteogenic differentiation via the GPRC6A/cAMP/PKA/AMPK signaling pathway in MC3T3E1 cells, and thus may prove to be beneficial in the treatment of diabetic osteoporosis.

*Correspondence to:* Professor Jianhong Yang, Medical School, University of Chinese Academy of Sciences, 19A Yuquan Road, Beijing 100049, P.R. China
E-mail: yangjh@ucas.edu.cn

*Key words:* diabetic osteoporosis, high glucose, uncarboxylated osteocalcin, osteogenic differentiation, GPCR class C group 6 subtype A receptor

## Introduction

Osteocalcin (OC) is a multipurpose bone-derived hormone (1), which can be γ-carboxylated at one or more of its glutamic acid residues (2). Therefore, OC can be present as one of 2 forms, carboxylated and undercarboxylated OC. Uncarboxylated osteocalcin (GluOC) is the form with no γ-carboxylated glutamic acid residu (3). GluOC is not only an index used to assess the health of bones, but also a biologically active molecule directly secreted into the blood to mediate glucose and energy metabolism (4,5). GluOC can affect the role of glucose by promoting the proliferation of β cells, increasing the secretion of insulin and improving the sensitivity of insulin target tissues, such as muscle and adipogenic tissue (6,7). However, the precise mechanisms through which GluOC improves the effects of glucose on osteoblasts have not yet been fully elucidated, at least to the best of our knowledge.

Osteoporosis is considered a serious skeletal disease, characterized by abnormal bone structure and low bone mineral density (8,9). The imbalance between osteoblast and adipocyte differentiation of bone marrow-derived stromal cells (BMSCs) is a major cause of osteoporosis (10-12). Diabetic osteoporosis is a severe diabetic complication affecting the bones. The number of patients with diabetic osteoporosis is increasing, and the disease is thus gaining increasing attention (13-15). Previous studies have suggested that diabetic patients have a lower bone quality primarily due to hyperglycemia (16,17). In a previous study on primary rat osteoblasts, the authors demonstrated that high glucose induced oxidative stress and resulted in the activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway to induce adipogenic differentiation (18). Moreover, it was demonstrated that high glucose conditions activated the cyclic AMP (cAMP)/protein kinase A(PKA) pathway to stimulate the adipocytic differentiation of MG63 cells (19). GluOC has been shown to inhibit the high glucose-induced inhibition of cellular proliferation, as well as the osteogenic differentiation of MC3T3E1 cells (20). However, the corresponding receptors and signaling pathways through which GluOC exerted its effects on osteoblasts have not yet been fully elucidated.

G-protein coupled receptor, class C, group 6, subtype A (GPRC6A), a relatively recently discovered G-protein-coupled

receptor, has been identified in several tissues and organs in humans, mice and rats, including skeletal muscle, pancreas, kidneys, heart, liver, lungs and brain (21). It has been reported that GPRC6A can bind to a variety of ligands, such as L-α amino acids, calcium, magnesium, testosterone and osteocalcin (22,23). Pi *et al* (24) demonstrated that 293 cells transfected with full-length GPRC6A cDNA exhibited a dose-dependent response to treatment with GluOC; GluOC activated the extracellular signal-regulated kinase (ERK) pathway via the GPRC6A/phospholipase C (PLC)/protein kinase C (PKC) signaling pathway. Otani *et al* (25) found that in 3T3L1 cells, GluOC was assumed to bind to GPRC6A, and this resulted in the increased intracellular accumulation of cAMP, and thus, in the subsequent activation of PKA to promote the synthesis and secretion of adiponectin. Karsenty and Oury (26) also found that GluOC upregulated cAMP levels via GPRC6A to promote the synthesis and secretion of testosterone in testicular mesenchymal cells. In another study, in TC-6 cells, GluOC stimulated PKD1 in a dose-dependent manner via GPRC6A, suggesting the active participation of GPRC6A in the maintenance of glucose homeostasis (27). However, whether GPRC6A acts as a GluOC receptor in MC3T3E1 has not yet been confirmed, at least to the best of our knowledge.

The present study assessed whether GPRC6A functions as a receptor of GluOC in the high glucose-induced inhibition of the osteogenic differentiation of MC3T3E1 cells. To the best of our knowledge, the present study is the first to demonstrate that GluOC activates the GPRC6A/cAMP/PKA/AMP-activated protein kinase (AMPK) signaling pathway to reverse the high glucose-induced inhibition of the osteogenic differentiation of MC3T3E1 cells. Thus, the results of the present study add to a growing body of evidence indicating that in MC3T3E1 cells, GPRC6A plays a significant role in osteogenic differentiation promoted by GluOC. Additionally, these results also highlight a potentially novel means for the prevention or treatment of diabetic osteoporosis.

## Materials and methods

*Materials*. Alizarin Red S and Oil Red O were obtained from Sigma-Aldrich; Merck KGaA. Small interfering (si) RNAs were synthesized by Shanghai Gima Corp. Inhibitors, including SQ22536 [adenylate cyclase (AC) inhibitor], U73122 (PLC inhibitor), H-89 (PKA inhibitor) and BML-275 (AMPK inhibitor) were obtained from TargetMol. Antibodies against PKA (cat. no. 4782), AMPKα (cat. no. 5831), phospho-PKA (cat. no. 5661) and phospho-AMPKα (cat. no. 2535) were purchased from Cell Signaling Technology, Inc. Antibodies against cyclin D1 (cat. no. ab134175), proliferating cell nuclear antigen (PCNA; cat. no. ab29) and β-actin (cat. no. ab8226) were purchased from Abcam.

*Cells and cell culture*. MC3T3E1 cells (cat. no. GNM15) were obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. Initially, the cells were grown in α-MEM (HyClone; Cytiva) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) with 5% $CO_2$ at 37˚C. The concentration of FBS in α-MEM was lowered to 4% when treating the cells, and the medium was replaced every day during the treatments. The control group was exposed to a glucose concentration of 5.5 mM.

*Cell proliferation assay*. A Cell Counting kit-8 (cat. no. CK04; Dojindo Molecular Technologies, Inc.) was used to detect the proliferation of the MC3T3E1 cells. MC3T3E1 cells were plated in 96-well plates ($3x10^3$ cells/well) for 24 h, after which the cells were cultured in 4% FBS α-MEM with various concentrations of GluOC (0, 0.1, 1, 3 or 10 ng/ml) and high glucose (25.5 mM) for 1, 2 or 3 days, as previously described (20). Subsequently, 100 $\mu$l fresh medium and 10 $\mu$l CCK-solution 8 were added to each well, and the cells were cultured for 30 min in a humidified incubator with 5% $CO_2$ at 37˚C. An automated microplate reader (Synergy H1; Biotek Instruments, Inc.) at 450 nm was used to measure the absorbance.

*Mineralization assay*. The MC3T3E1 cells were grown overnight at a density of $1x10^6$ cells/well in 6-well plates, and then cultured in osteoblastic differentiation medium (HyClone; Cytiva) (α-MEM, 4% FBS, 100 nM dexamethasone, 10 mM β-glycerophosphate disodium, 50 mg/l vitamin C) containing high glucose (25.5 mM) or GluOC (3 ng/ml) for 28 days. The cells were fixed at room temperature with 10% formaldehyde for 30 min and calcium nodules were stained using Alizarin Red S. The staining was imaged using a microscope (magnification, x100, DM750; Leica Microsystems, Inc.) and the red areas represented calcified nodules. For quantitative detection, 0.1 M cetylpyridinium chloride was used to dissolve the calcium nodules for 15 min. Following a 20-fold dilution, the absorbance value was measured at 570 nm by an automated microplate reader (Synergy H1; Biotek Instruments, Inc.).

*Assay of lipid droplets*. The MC3T3E1 cells were grown overnight at a density of $1x10^6$ cells/well in 6-well plates and then cultured in adipocytic differentiation medium (α-MEM, 4% FBS, 1 mM dexamethasone, 10 mg/l insulin) containing high glucose (25.5 mM) or GluOC (3 ng/ml) for 16 days. The cells were fixed at room temperature with 10% formaldehyde for 30 min and Oil Red O was used to stain the lipid droplets. The staining was imaged using a microscope (magnification, x500, DM750; Leica Microsystems, Inc.), and the red areas represented lipid droplets. For quantitative detection, isopropanol was used to dissolve lipid droplets for 10 min and the absorbance value was measured at 510 nm by an automated microplate reader (Synergy H1; Biotek Instruments, Inc.).

*Alkaline phosphatase (ALP) assay*. The MC3T3E1 cells were seeded into 6-well plates ($1x10^6$ cells/well) and cultured in 4% FBS α-MEM containing high glucose (25.5 mM), siRNAs (100 pmol), inhibitors (10 $\mu$M) or GluOC (3 ng/ml) for 7 days. A total of 200 $\mu$l pre-cooled PBS was added to collect the cells, and the supernatant was retained to measure ALP activity, using an Alkaline Phosphatase assay kit (cat. no. A059-2; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol.

*Type I collagen (COLI) assay*. The secretion of COLI was performed as described above for the measurement of ALP activity. The secretion of COLI was quantified using a specific

Table I. Primer sequences designed for RT-qPCR.

| Gene | Forward primers (5'→3') | Reverse primers (5'→3') |
|---|---|---|
| Runx2 | GCCTTCAAGGTGGTAGCCC | CGTTACCCGCCATGACAGTA |
| Osx | ACTGGCTAGGTGGTGGTCAG | GGTAGGGAGCTGGGTTAAGG |
| PPARγ | GCATGGTGCCTTCGCTGA | TGGCATCTCTGTGTCAACCATG |
| FAS | GGCTGCAGTGAATGAATTTG | TTCGTACCTCCTTGGCAAAC |
| GPRC6A | TCCGGAGTCAAGCTGGGATA | CCCTTGGCATGTAGCTGGAA |
| β-actin | GCTCTTTTCCAGCCTTCCTT | AGGTCTTTACGGATGTCAACG |

Runx2, runt-related transcription factor 2; Osx, osterix; PPARγ, peroxisome proliferator-activated receptor γ; FAS, fatty acid synthase; GPRC6A, GPCR class C group 6 subtype A receptor.

ELISA kit (cat. no. SEA571Mu; Cloud-Clone Corp.), according to the manufacturer's protocol.

*Reverse transcription-quantitative PCR (RT-qPCR).* The MC3T3E1 cells in 6-well plates ($1x10^6$ cells/well) were treated with or without high glucose (25.5 mM), siRNAs (100 pmol), inhibitors (10 $\mu$M) or GluOC (3 ng/ml). Total RNA extraction from the MC3T3E1 cells was performed using a Total RNA kit (cat. no. DP419; Tiangen Biotech, Co., Ltd.). A total of 2 $\mu$g RNA was reverse transcribed into cDNA using the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (cat. no. A T311-03; TransGen Biotech.) and quantitative PCR (qPCR) was performed in a 20 $\mu$l reaction volume with the TransStart Top Green qPCR SuperMix (+Dye II) (cat. no. AQ132-24; TransGen Biotech.). The following thermocycling conditions were used: 40 cycles at 94˚C for 5 sec, 60˚C for 15 sec and 72˚C for 10 sec. To quantify GPRC6A expression, qPCR was performed after 24 h. The expression of the adipogenic genes, peroxisome proliferator-activated receptor γ (PPARγ) and FAS, was assessed after 3 days. The expression of the osteogenic genes, Runt-related transcription factor 2 (Runx2) and osterix (Osx), was assessed after 5 days. The sequences of the primers used are listed in Table I. Expression data were normalized to β-actin, which was used as the internal standard.

*siRNAs and transfection.* siRNA-1 (siRNA.m1638, CCA ACACAGCTGTTGCTAT) and siRNA-2 (siRNA.m2553, GCAGAAGACTAACACCAAA), which were specific for GPRC6A were used in the present study. GPRC6A (GenBank accession no. NM_153071) was used as a template for synthesizing the siRNAs. Cells at a density of $2x10^5$ cells/well in 24-well plates were cultured overnight in 10% FBS α-MEM, and then transiently transfected with 100 pmol siRNA and 5 $\mu$l Lipofectamine® 2000 (cat. no. 11668-019, Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RT-qPCR analysis was used to quantify GPRC6A expression after 24 h. Subsequently, the medium was changed to 4% FBS α-MEM containing 25.5 mM glucose or 3 ng/ml GluOC. Scrambled siRNA (UUCUCCGAACGUGUCACG UTT) was used as a negative control.

*Radioimmunoassay for cAMP.* MC3T3E1 cells at a density of $2x10^5$ cells/well in 24-well plates were cultured in 4% FBS α-MEM and divided into a control group, high glucose group, GluOC group and a SQ22536 group. The cAMP assay kit (cat. no. ab133051; Abcam) was used to measure the intracellular accumulation of cAMP after 24 h, according to the manufacturer's protocol.

*Western blot analysis.* MC3T3E1 cells in 6-well plates ($1x10^6$ cells/well) were treated either with or without glucose (25.5 mM), siRNAs (100 pmol), inhibitors (10 $\mu$M) and GluOC (3 ng/ml). The cold cell lysates were acquired by ultrasonic disruption with RIPA lysis buffer on ice after two ice-cold rinses with PBS. Cell lysates were centrifuged (10,000 x g) at 4˚C for 20 min, and the protein concentrations of the extracted proteins were measured using a BCA Detection kit (cat. no. B5000; Beijing Lablead Biotech, Co., Ltd.). A total of 10 $\mu$g proteins (for each lane) in cell lysates were loaded and separated by 12% SDS-PAGE, and then transferred to PVDF membranes. The membranes were blocked in TBS-5% Tween-20 (TBST) containing 5% non-fat milk at room temperature for 2 h and incubated overnight at 4˚C with rabbit-derived antibodies, including anti-cyclin D1 (1:10,000), anti-PCNA (1:2,000), anti-β-actin (1:1,000), anti-PKA (1:1,000), anti-phosphorylated (p-)PKA (1:1,000), anti-AMPKα (1:1,000), anti-p-AMPKα (1:1,000). The membranes were then washed 3 times with TBST (10 min each), followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:10,000; cat. no. ab6721; Abcam) at 25˚C for 2 h. The protein bands were visualized using an ECL kit (Biomiga). To quantify the expression of proteins, the intensity of bands was normalized to the respective β-actin control, and the bands of the phospho-proteins were normalized to the total levels of that specific protein. ImageJ version 6 (National Institutes of Health) was used for densitometric analysis.

*Statistical analysis.* Data are presented as the means ± standard deviation of at least 3 independent repeats. Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, Inc.). Differences between the groups were compared using a one-way ANOVA followed by a Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*GluOC promotes the proliferation of MC3T3E1 cells under high glucose conditions.* First, the MC3T3E1 cells

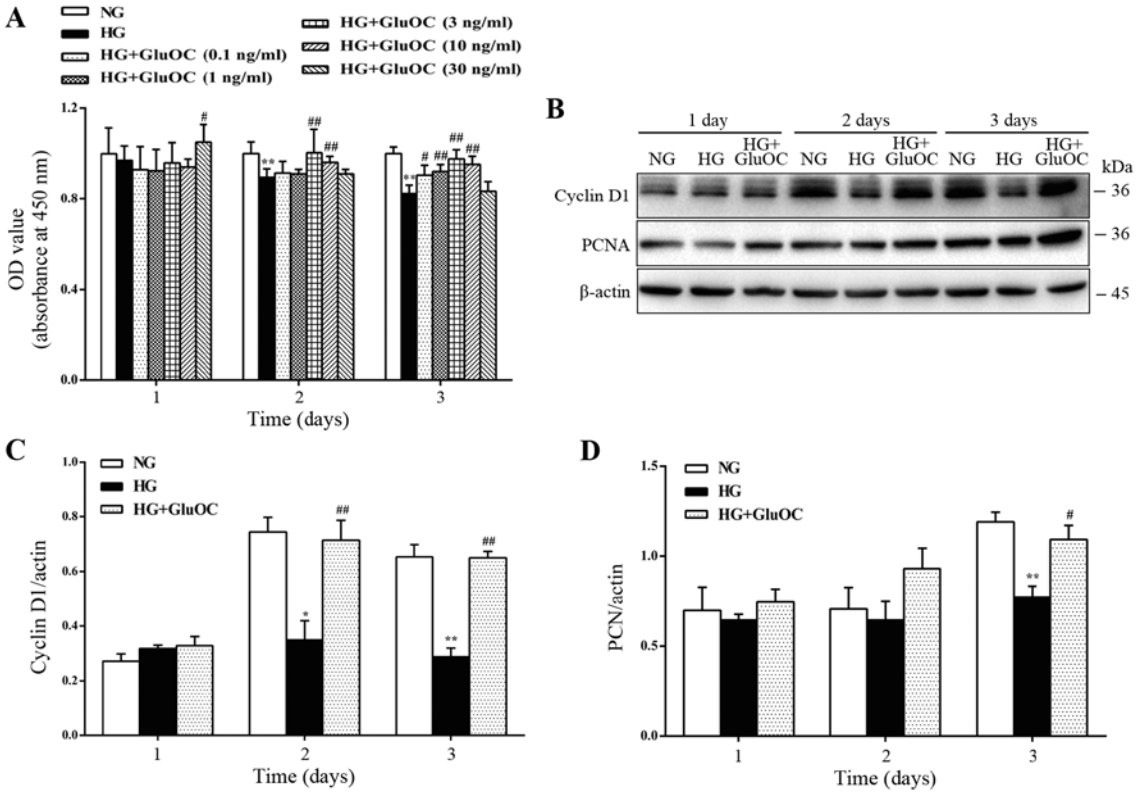


Figure 1. GluOC promotes the growth of MC3T3E1 cells under high glucose conditions. (A) Effects of various concentrations of GluOC treatment for 1, 2 and 3 days on the proliferation of MC3T3E1 cells under high glucose conditions. (B-D) Expression levels of cyclin D1 and PCNA in MC3T3E1 cells treated with 3 ng/ml GluOC for 1, 2 or 3 days under high glucose conditions. $^{*}$P<0.05, $^{**}$P<0.01 vs. NG group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. HG group. GluOC, uncarboxylated osteocalcin; PCNA, proliferating cell nuclear antigen; NG, normal glucose; HG, high glucose.

were treated with high glucose or various concentrations of GluOC (0.1, 1, 3, 10 and 30 ng/ml) to confirm whether GluOC promotes the proliferation of MC3T3E1 cells under high glucose conditions. High glucose inhibited cell proliferation, whereas treatment with 3 ng/ml GluOC significantly alleviated the inhibitory effects of high glucose on the proliferation of the MC3T3E1 cells (Fig. 1A). Thus, in all subsequent experiments, 3 ng/ml GluOC was used as the working dose to treat the MC3T3E1 cells. Similarly, the expression levels of cyclin D1 and PCNA were reduced by high glucose, whereas the addition of GluOC reversed the inhibitory effects of high glucose on cyclin D1 and PCNA expression (Fig. 1B-D). These results suggest that GluOC reversed the high glucose-induced inhibition of the proliferation of MC3T3E1 cells.

*GluOC promotes the osteogenic differentiation and inhibits the adipogenic differentiation of MC3T3E1 cells under high glucose conditions*

*GluOC promotes the mineralization and inhibits the high glucose-induced formation of lipid droplets in MC3T3E1 cells*. It has been reported that MC3T3E1 cells generate calcium nodules during mineralization, and these calcium nodules are considered as an osteoblastic parameter (28). Alizarin Red S staining of calcium nodules indicated that cells treated with GluOC generated significantly more calcium nodules, whereas fewer calcium nodules were observed in the high glucose group (Fig. 2A). Fewer lipid droplets stained by Oil Red O were observed in the GluOC-treated cells (Fig. 2B). GluOC did not affect the cells in the normal glucose group. Both of the images and the results of quantitative analysis indicated that GluOC promoted the mineralization and inhibited the high glucose-induced formation of lipid droplets in MC3T3E1 cells.

*GluOC promotes the expression of COLI and inhibits high glucose-induced ALP activity in MC3T3E1 cells*. Osteoblasts express various phenotypic markers including ALP and collagenous bone matrix protein-COLI (29,30). Thus, ALP activity and the COLI secretion of MC3T3E1 cells cultured with or without GluOC for 7 days under high glucose conditions were examined in the present study. As shown in Fig. 2C, high glucose upregulated ALP activity, whereas treatment with GluOC reversed this effect. Conversely, an increased COLI production was observed in the cells treated with GluOC (Fig. 2D). GluOC did not affect the cells in the normal glucose group. These findings suggested that GluOC increased COLI expression and decreased the high glucose-induced ALP activity in MC3T3E1 cells.

*GluOC promotes the expression of osteogenic genes and inhibits the high glucose-induced expression of adipogenic genes in MC3T3E1 cells*. RT-qPCR was used to further confirm the effects of GluOC on the expression of relevant genes. As shown in Fig. 2E, the gene expression levels of the osteogenic markers (Runx2 and Osx) in the high glucose group were significantly decreased, whereas the expression

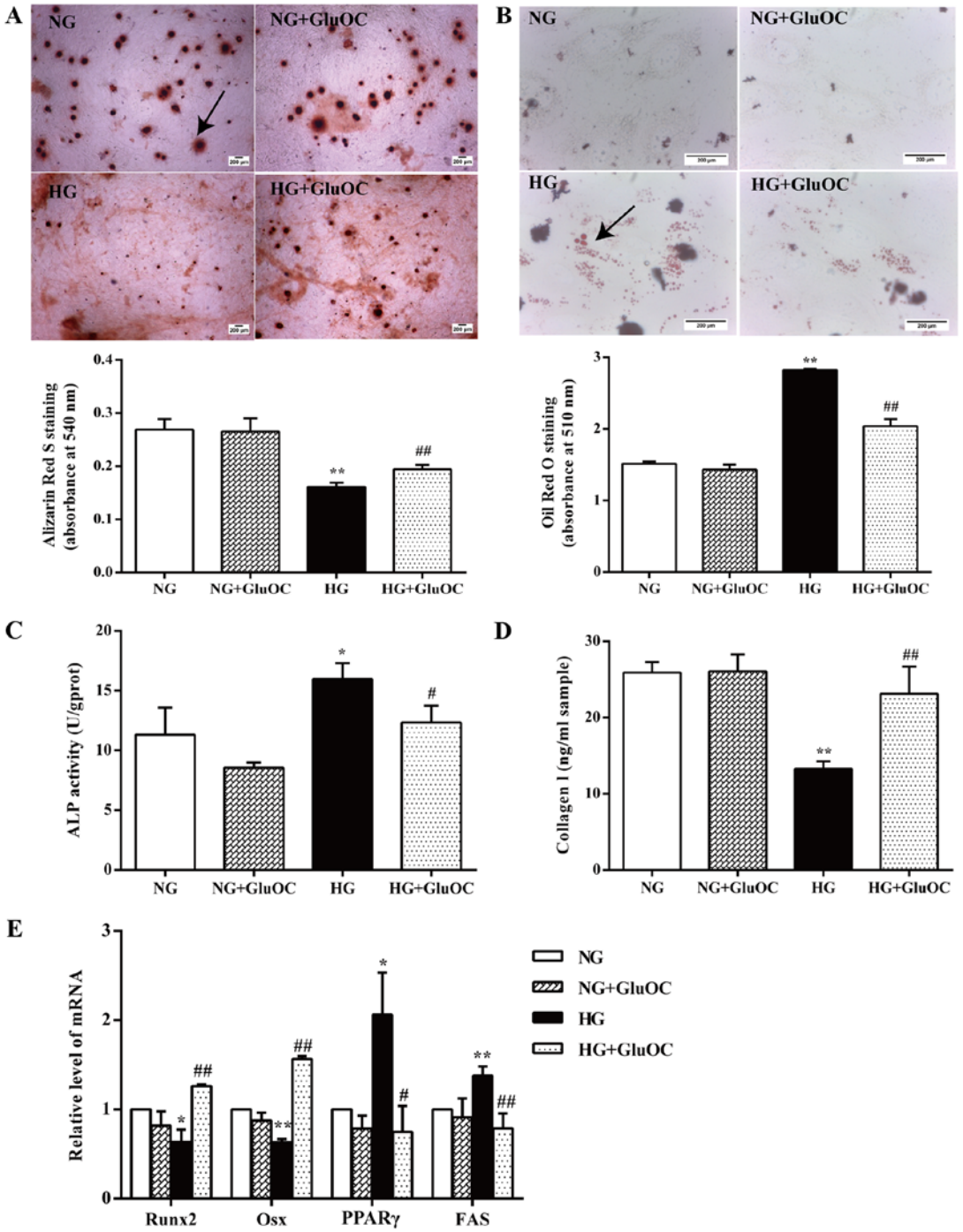


Figure 2. GluOC promotes the osteogenic differentiation and inhibits the adipogenic differentiation of MC3T3E1 cells under high glucose conditions. Cells were cultured in inductive α-MEM containing 5.5 mM glucose (NG group) or 25.5 mM glucose (HG group), with or without 3 ng/ml GluOC (NG + GluOC group and HG + GluOC group, respectively). (A) Representative images of Alizarin Red S staining of calcium nodules and quantitative analysis. Scale bar, 200 $\mu$m. (B) Representative images of Oil Red O staining images of lipid droplets and the quantitative analysis. Scale bar, 200 $\mu$m. (C) ALP activity and (D) COLI expression in the different groups. (E) Expression of the osteogenic-specific genes (Runx2 and Osx), as well as the adipogenic-specific genes (PPARγ and FAS) in MC3T3E1 cells in the different groups. $^{*}$P<0.05, $^{**}$P<0.01 vs. NG group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. HG group. GluOC, uncarboxylated osteocalcin; COLI, type I collagen; Runx2, Runt-related transcription factor 2; Osx, osterix; PPARγ, peroxisome proliferator-activated receptor γ; NG, normal glucose; HG, high glucose.

of adipogenic genes (PPARγ and FAS) was increased. The effects of high glucose were reversed by GluOC; however, GluOC did not affect the cells in the normal glucose group. These results demonstrated that GluOC significantly affected the upregulation of osteogenic gene expression and downregulated adipogenic gene expression in MC3T3E1 cells. Thus, GluOC reversed the inhibitory effects of high glucose on the osteogenic differentiation of MC3T3E1 cells.

*GluOC reverses the high glucose-induced inhibition of osteogenic differentiation via GPRC6A in MC3T3E1 cells*

*GluOC increases the expression of GPRC6A in MC3T3E1 cells.* The expression of GPRC6A, which was hypothesized to be a GluOC receptor in MC3T3E1 cells, was detected to determine whether GPRC6A is the receptor of GluOC. Confluent cells were cultured in 6-well plates with 4% FBS and treated with high glucose or GluOC for 6, 12 or 24 h, respectively. RT-qPCR analysis

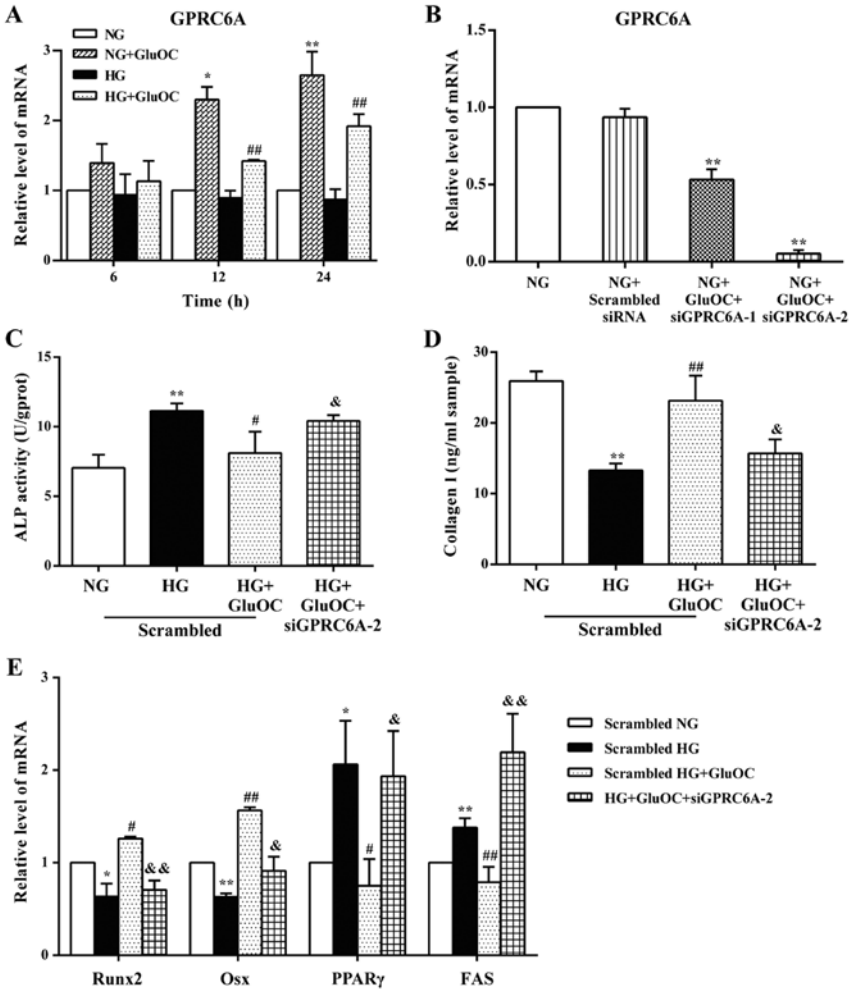


Figure 3. GPRC6A functions as a receptor of GluOC in MC3T3E1 cells. (A) GluOC promoted the expression of GPRC6A. The expression of GPRC6A was detected after treatment with high glucose and GluOC for 6, 12 and 24 h. (B) GPRC6A mRNA levels were analyzed following transfection with GPCR6A targeting siRNAs after 24 h. (C-E) GPRC6A knockdown abrogated the GluOC-mediated increase in osteogenic differentiation, and promoted adipogenic differentiation. (C) ALP activity and (D) COLI expression was determined in the different groups. (E) Expression of the osteogenic-specific genes (Runx2 and Osx) and adipogenic-specific genes (PPARγ and FAS). $^{*}$P<0.05, $^{**}$P<0.01 vs. NG group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. HG group; $^{\&}$P<0.05, $^{\&\&}$P<0.01 vs. HG + GluOC group. GPRC6A, GPCR class C group 6 subtype A receptor; GluOC, uncarboxylated osteocalcin; COLI, type I collagen; Runx2, Runt-related transcription factor 2; Osx, osterix; PPARγ, peroxisome proliferator-activated receptor γ; NG, normal glucose; HG, high glucose.

indicated that treatment with GluOC significantly increased the expression of GPRC6A in MC3T3E1 cells (Fig. 3A).

*GPRC6A functions as a receptor of GluOC in MC3T3E1 cells.* To further confirm whether GluOC stimulates the osteogenic differentiation of MC3T3E1 cells under high glucose conditions via GPRC6A, siRNAs were used to knockdown GPRC6A expression. RT-qPCR analysis indicated that GPR6CA siRNA-2 significantly reduced the expression of GPRC6A by 90% (Fig. 3B). Based on these results, GPR6CA siRNA-2 was selected to knockdown GPRC6A expression in all subsequent experiments. To determine the effects of GPRC6A on the osteoblast differentiation of MC3T3E1 cells, the present study examined the activity of ALP and the expression of COLI, as well as the expression of the aforementioned genes (Runx2, Osx, PPARγ and FAS) following GPRC6A knockdown. The promoting effects of GluOC on the osteogenic differentiation of MC3T3E1 cells were reversed by the knockdown of GPRC6A (the knockdown of GPRC6A increased ALP activity, decreased COLI production, decreased Runx2 and Osx expression, and increased PPARγ and FAS expression), and the results were similar to those in the HG group (Fig. 3C-E). Taken together, these results suggest that GPRC6A functions as a receptor of GluOC in MC3T3E1 cells.

*GluOC reverses the high glucose-induced inhibition of osteogenic differentiation via the GPRC6A/cAMP/PKA/AMPK signaling pathway in MC3T3E1 cells*

*GluOC promotes the osteogenic differentiation of MC3T3E1 cells by activating AC through GPRC6A.* The intracellular signaling pathways activated by the GluOC-mediated activation of GPRC6A in MC3T3E1 cells were determined. The results of western blot analysis revealed that GluOC reversed the high glucose-induced decrease in the phosphorylation of PKA and AMPK, and that this effect was abrogated by the knockdown of GPR6CA or SQ22536, an AC inhibitor. Additionally, it was confirmed that the addition of U73122, a PLC inhibitor, did not affect the effects of GluOC on PKA and AMPK phosphorylation under high glucose conditions (Fig. 4A-F). These findings suggest that GluOC activated downstream PKA and AMPK through GPRC6A-AC rather than GPRC6A-PLC. As shown

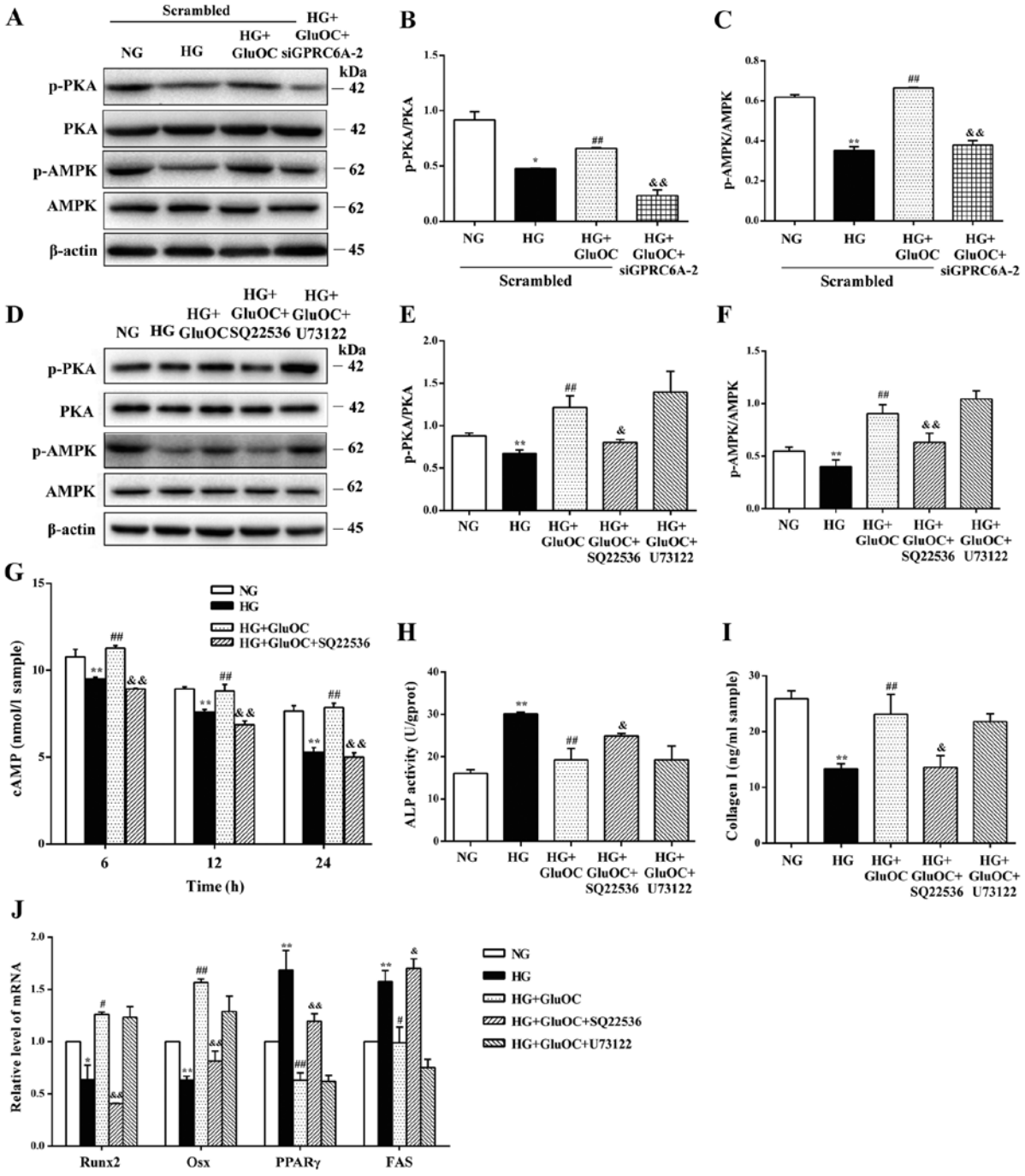


Figure 4. GluOC promotes osteogenic differentiation of MC3T3E1 cells under high glucose conditions via GPRC6A-AC. MC3T3-E1 cells were treated with GluOC (3 ng/ml) and a GPCR6A targeting siRNA, SQ22536 (adenylate cyclase inhibitor), or U73122 (phospholipase C inhibitor). (A-C) Western blot analysis of PKA, p-PKA, AMPK and p-AMPK expression following the siRNA-mediated knockdown of GPCR6A in cells. (D-F) Protein expression levels of PKA, p-PKA, AMPK and p-AMPK treated with or without SQ22536 and U73122. (G) Accumulation of intracellular cAMP following the above treatments. (H-J) GluOC promoted osteogenic differentiation of MC3T3E1 cells via GPRC6A-AC rather than GPRC6A-PLC. (H) ALP activity and (I) the levels of COLI in cells following the above treatments. (J) Expression of osteogenic-specific genes (Runx2 and Osx) and adipogenic-specific genes (PPARγ and FAS). $^{*}$P<0.05, $^{**}$P<0.01 vs. NG group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. HG group; $^{\&}$P<0.05, $^{\&\&}$P<0.01 vs. HG + GluOC group. GPRC6A, GPCR class C group 6 subtype A receptor; GluOC, uncarboxylated osteocalcin; COLI, type I collagen; Runx2, Runt-related transcription factor 2; Osx, osterix; PPARγ, peroxisome proliferator-activated receptor γ; PKA, protein kinase A; AMPK, AMP-activated protein kinase; NG, normal glucose; HG, high glucose.

in Fig. 4G, GluOC increased the intracellular cAMP concentrations, reversing the decrease observed in the HG group; the addition of SQ22536 attenuated this effect, suggesting that GluOC promoted the production of cAMP through AC. Additionally, SQ22536 reversed the increase in the expression of COLI, Runx2 and Osx induced by GluOC, whereas ALP activity, and the expression of PPARγ and FAS were significantly increased under high glucose conditions. U73122, a PLC inhibitor, did not affect the effects of GluOC under high glucose conditions (Fig. 4H-J). These findings suggest that the activation of AC rather than PLC through GPRC6A is the mechanism through which GluOC reverses the inhibitory effects of high glucose on the osteogenic differentiation of MC3T3E1 cells.

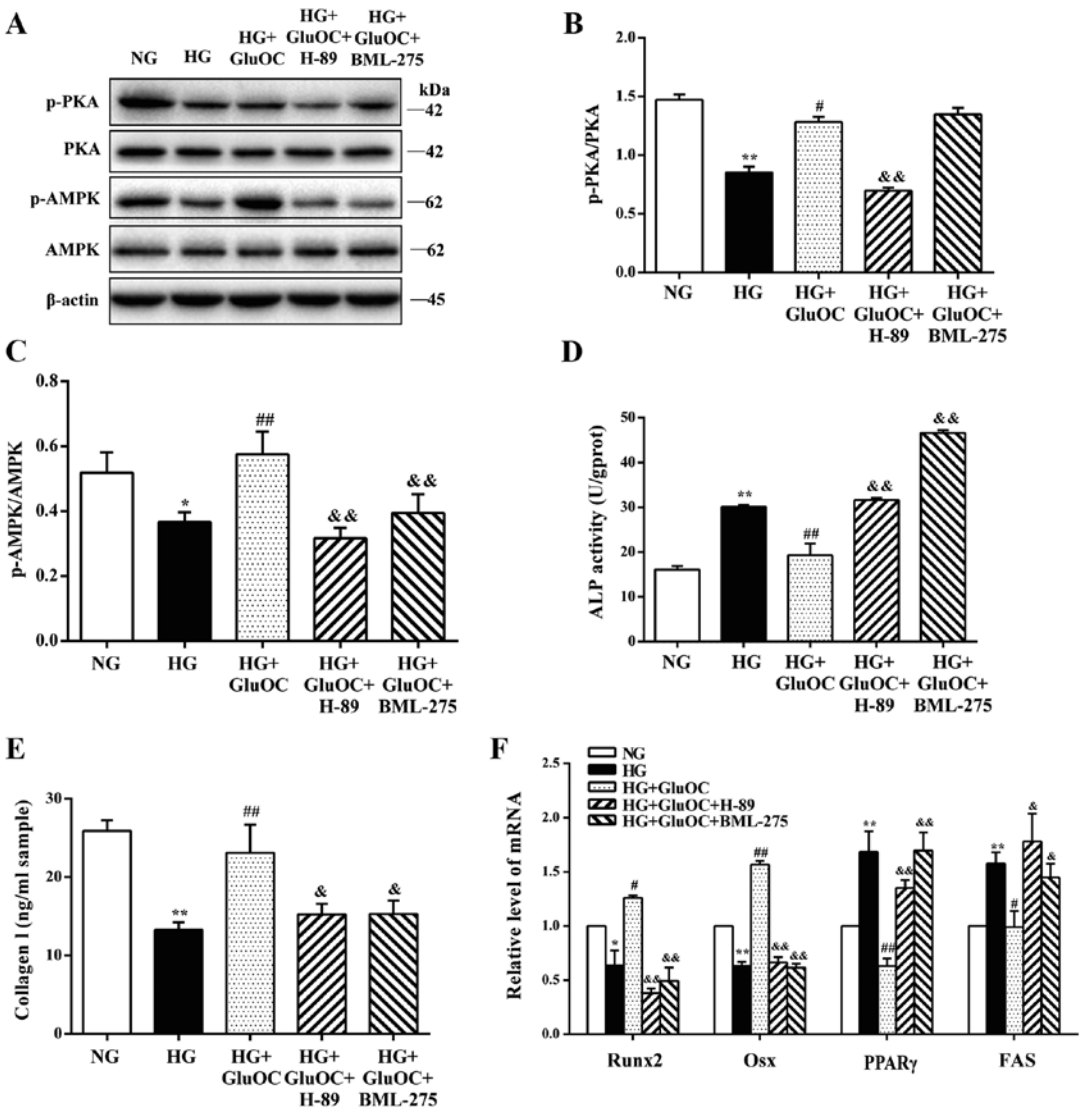


Figure 5. GluOC promotes the osteogenic differentiation of MC3T3E1 cells under high glucose conditions via the PKA/AMPK signaling pathway. MC3T3-E1 cells were treated with GluOC (3 ng/ml) combined with H-89 or BML-275. (A-C) Western blot analysis of PKA, p-PKA, AMPK and p-AMPK expression. (D-F) GluOC promoted the osteogenic differentiation of MC3T3E1 cells via the PKA/AMPK signaling pathway. (D) ALP activity and (E) the levels of COLI in cells following the above treatments. (F) Expression of osteogenic-specific genes (Runx2 and Osx) and adipogenic-specific genes (PPARγ and FAS). $^{*}$P<0.05, $^{**}$P<0.01 vs. NG group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. HG group; $^{\&}$P<0.05, $^{\&\&}$P<0.01 vs. HG + GluOC group. GPRC6A, GPCR class C group 6 subtype A receptor; GluOC, uncarboxylated osteocalcin; COLI, type I collagen; Runx2, Runt-related transcription factor 2; Osx, osterix; PPARγ, peroxisome proliferator-activated receptor γ; PKA, protein kinase A; AMPK, AMP-activated protein kinase; NG, normal glucose; HG, high glucose.

*GluOC phosphorylates PKA and AMPK to promote the osteogenic differentiation of MC3T3E1 cells*. Finally, the crucial functions of PKA and AMPK on the GluOC-mediated promotion of the osteogenic differentiation of MC3T3E1 cells were determined. The addition of H-89 inhibited the phosphorylation of PKA and AMPK, and treatment with BML-275 did not affect the phosphorylation of PKA, but only inhibited the phosphorylation of AMPK, suggesting that AMPK was a downstream factor of GPRC6A/cAMP/PKA (Fig. 5A-C). As shown in Fig. 5D-F, the expression of COLI, Runx2 and Osx induced by GluOC was suppressed by H-89 and BML-275, whereas ALP activity, and the expression of PPARγ and FAS were significantly increased similar to the results in the HG group. These results demonstrated that the sequential activation of PKA and AMPK was the mechanism through which GluOC reversed the inhibitory effects of high glucose on the osteogenic differentiation of MC3T3E1 cells. Taken together, GluOC activated AC and PKA through GPRC6A, and the effects of GluOC were AMPK phosphorylation-dependent, which further promoted the osteogenic differentiation and inhibited the adipogenic differentiation of MC3T3E1 cells under high glucose conditions (Fig. 6).

## Discussion

In the present study, it was found that GluOC reversed high glucose-induced inhibition of osteogenic differentiation of MC3T3E1 cells via the GPRC6A/cAMP/PKA/AMPK signaling pathway, manifested by an increase in COLI protein expression and a decrease in ALP activity, as well as the increased expression of osteoblast-specific genes (Runx2 and Osx), and the decreased expression of adipogenic genes (PPARγ and FAS). The results of the present study provide novel insight into the role of GluOC and the molecular mechanisms underlying the

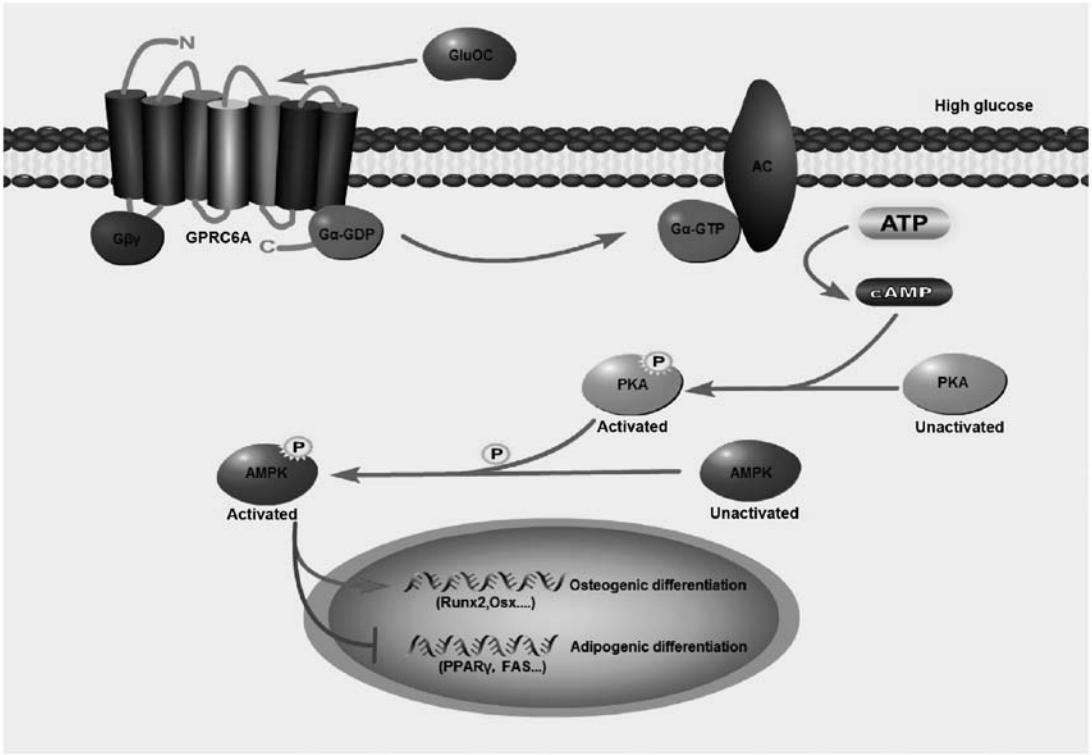


Figure 6. Model for the effects of GluOC on the osteogenic differentiation of MC3T3E1 cells under high glucose conditions. GluOC activates AC and PKA, and the effects of GluOC are AMPK phosphorylation-dependent, which further promotes osteogenic differentiation and inhibits adipogenic differentiation in MC3T3E1 cells under high glucose conditions. GPRC6A, GPCR class C group 6 subtype A receptor; GluOC, uncarboxylated osteocalcin; COLI, type I collagen; Runx2, Runt-related transcription factor 2; Osx, osterix; PPARγ, peroxisome proliferator-activated receptor γ; PKA, protein kinase A; AMPK, AMP-activated protein kinase.

inhibition of the high glucose-induced inhibition of osteogenic differentiation of MC3T3E1 cells.

Diabetic osteoporosis is a severe complication of bones in patients with diabetes, and is becoming an ever-increasing public health concern (31). In a previous study by the authors, it was demonstrated that high glucose, a major cause of diabetic osteoporosis, resulted in abnormal structural changes and a decrease in bone density, specifically of the bone trabecula in KK-Ay mice (32). Additionally, in the present study, it was confirmed that high glucose exerted an inhibitory effect on the osteoblastic differentiation of MC3T3E1 cells. Thus, the identifying factors that inhibit the adipogenic differentiation of osteoblasts induced by high glucose has potentially wide prospects for the prevention of diabetic osteoporosis.

In recent years, bone has been considered as not only a structural organ, but also as an endocrine organ that can secrete protein factors into the circulation (33). GluOC, the active form of OC, is crucial for the regulation of energy metabolism and glucose metabolism (34,35). GluOC relieves diabetes by increasing insulin synthesis and secretion. GluOC can also act on adipocytes and Leydig cells to promote the production of adiponectin and testosterone, respectively (36,37). Studies have demonstrated that GluOC functions by binding to its putative receptor, GPRC6A. GPRC6A is a relatively recently discovered GluOC receptor. A growing number of studies have confirmed that GPRC6A is expressed by β cells, and GluOC directly activates GPRC6A to regulate β-cell proliferation and insulin secretion (38,39). GluOC activates GPRC6A to stimulate mammalian target of rapamycin (mTOR) phosphorylation in myocytes (40) and stimulates Leydig cells to produce vitamin D via the activation of GPRC6A (41). In a previous study, the authors demonstrated that GluOC ameliorated hepatic glucose and lipid metabolism in KK-Ay mice (42). Moreover, GluOC has been shown to exert a positive effect on the osteogenic differentiation of BMSCs (43). The conclusion that GluOC promotes the osteogenic differentiation of MC3T3E1 cells was confirmed in the present study. However, the receptor of GluOC in osteoblasts has not yet been identified. To the best of our knowledge, the present study demonstrated, for the first time, that GPRC6A functioned as a receptor of GluOC in MC3T3E1 cells. GluOC promoted the expression of GPRC6A, irrespective of the glucose concentration. Following transfection with GPR6CA siRNA, the inhibitory effects of GluOC on ALP activity and adipogenic gene expression were abrogated, as well as the effect on the increase in COLI and osteogenic gene expression. These results demonstrated that GPRC6A functioned as a receptor of GluOC in MC3T3E1 cells.

The signaling pathways that are mediated via GPRC6A vary. It has been shown that GluOC activates GPCR6A, thereby increasing the expression of testosterone via the cAMP/CREB pathway (44). GluOC binding to GPRC6A also increases the cAMP concentrations and consequently, PKA phosphorylation in 3T3L1 adipocytes, thereby promoting the production of adiponectin (5). In the present study, it was found that GluOC subsequently activated the GPRC6A/cAMP/PKA/AMPK signaling pathway to reverse the high glucose-induced inhibition of osteoblastic differentiation of MC3T3E1 cells. As shown in Fig. 4, the addition of SQ22536, an inhibitor of AC, reduced the phosphorylation levels of PKA and AMPK compared with the GluOC group, intracellular CAMP

accumulation and the expression of osteogenic-specific markers decreased and the expression of adipogenic-specific markers increased. However, the addition of the PLC inhibitor, U73122, did not affect the results, indicating that GluOC activated AC, rather than PLC after binding to GPRC6A, and that AC mediated the high glucose-induced inhibition of osteoblastic differentiation of MC3T3E1 cells. Blocking the cAMP/PKA/AMPK signal transduction pathway with H-89 or BML-275 also abrogated the effects of GluOC on osteoblastic differentiation, as evidenced by the increased expression of adipogenic gene expression and the increased activity of ALP, as well as the reduced production of COLI and reduced osteogenic gene expression. The above-mentioned results suggest that the GPRC6A/cAMP/PKA/AMPK signaling pathway was responsible for GluOC mediated inhibition of the high glucose-induced inhibition of osteoblastic differentiation of MC3T3E1 cells.

In conclusion, to the best of our knowledge, the present study is the first to demonstrate that GluOC reverses high glucose-induced inhibition of osteogenic differentiation by activating the GPRC6A/cAMP/PKA/AMPK signaling pathway in MC3T3E1 cells (Fig. 6). These findings further clarify the pathogenesis of diabetic osteoporosis at the cellular level, indicating that the GluOC/GPRC6A signaling pathway may potentially serve as a therapeutic target for the prevention or treatment of diabetic osteoporosis.

**Acknowledgements**

Not applicable.

**Funding**

No funding was received.

**Availability of data and materials**

All analyses and results obtained from the current experiments are included in the present study.

**Authors' contributions**

All authors (LM, FG, JX and JY) contributed to the conception and design of the entire study. FG and JX performed an abundant search of relevant literature on osteocalcin. LM performed the experiments and wrote the manuscript. LM and JY were responsible for modifying the manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they no competing interests.

**References**

1. Khrimian L, Obri A, Ramos-Brossier M, Rousseaud A, Moriceau S, Nicot AS, Mera P, Kosmidis S, Karnavas T, Saudou F, *et al*: Gpr158 mediates osteocalcin's regulation of cognition. J Exp Med 214: 2859-2873, 2017.
2. Chen Y, Li J, Liao J, Hu Y, Zhang H, Yang X, Wang Q, Mo Z and Cheng J: Potential protective effect of osteocalcin in middle-aged men with erectile dysfunction: Evidence from the FAMHES project. Sci Rep 8: 6721, 2018.
3. Liang Y, Tan A, Liang D, Yang X, Liao M, Gao Y, Jiang Y, Yao Z, Lin X, Lu Z, *et al*: Low osteocalcin level is a risk factor for impaired glucose metabolism in a Chinese male population. J Diabetes Investig 7: 522-528, 2016.
4. Lin X, Brennan-Speranza TC, Levinger I and Yeap BB: Undercarboxylated osteocalcin: Experimental and human evidence for a role in glucose homeostasis and muscle regulation of insulin sensitivity. Nutrients 10: 847, 2018.
5. Otani T, Matsuda M, Mizokami A, Kitagawa N, Takeuchi H, Jimi E, Inai T and Hirata M: Osteocalcin triggers Fas/FasL-mediated necroptosis in adipocytes via activation of p300. Cell Death Dis 9: 1194, 2018.
6. Fernandes TAP, Gonçalves LML and Brito JAA: Relationships between bone turnover and energy metabolism. J Diabetes Res 2017: 9021314, 2017.
7. Zoch ML, Clemens TL and Riddle RC: New insights into the biology of osteocalcin. Bone 82: 42-49, 2016.
8. Edwards BJ: Osteoporosis risk calculators. J Clin Densitom 20: 379-388, 2017.
9. Golob AL and Laya MB: Osteoporosis: Screening, prevention, and management. Med Clin North Am 99: 587-606, 2015.
10. Ivanova S, Vasileva L, Ivanova S, Peikova L and Obreshkova D: Osteoporosis: Therapeutic options. Folia Med (Plovdiv) 57: 181-190, 2015.
11. Wang C, Meng H, Wang X, Zhao C, Peng J and Wang Y: Differentiation of bone marrow mesenchymal stem cells in osteoblasts and adipocytes and its role in treatment of osteoporosis. Med Sci Monit 22: 226-233, 2016.
12. Guo Q, Chen Y, Guo L, Jiang T and Lin Z: miR-23a/b regulates the balance between osteoblast and adipocyte differentiation in bone marrow mesenchymal stem cells. Bone Res 4: 16022, 2016.
13. Ma R, Zhu R, Wang L, Guo Y, Liu C, Liu H, Liu F, Li H, Li Y, Fu M and Zhang D: Diabetic osteoporosis: A review of its traditional Chinese medicinal use and clinical and preclinical research. Evid Based Complement Alternat Med 2016: 3218313, 2016.
14. Roy B: Biomolecular basis of the role of diabetes mellitus in osteoporosis and bone fractures. World J Diabetes 4: 101-113, 2013.
15. Qi J, Hu KS and Yang HL: Roles of TNF-α, GSK-3β and RANKL in the occurrence and development of diabetic osteoporosis. Int J Clin Exp Pathol 8: 11995-12004, 2015.
16. Bahrambeigi S, Yousefi B, Rahimi M and Shafiei-Irannejad V: Metformin; an old antidiabetic drug with new potentials in bone disorders. Biomed Pharmacother 109: 1593-1601, 2019.
17. Takagi S, Miura T, Yamashita T, Ando N, Nakao H, Ishihara E and Ishida T: Characteristics of diabetic osteopenia in KK-Ay diabetic mice. Biol Pharm Bull 35: 438-443, 2012.
18. Zhang Y and Yang JH: Activation of the PI3K/Akt pathway by oxidative stress mediates high glucose-induced increase of adipogenic differentiation in primary rat osteoblasts. J Cell Biochem 114: 2595-2602, 2013.
19. Wang W, Zhang X, Zheng J and Yang J: High glucose stimulates adipogenic and inhibits osteogenic differentiation in MG-63 cells through cAMP/protein kinase A/extracellular signal-regulated kinase pathway. Mol Cell Biochem 338: 115-122, 2010.
20. Liu J and Yang J: Uncarboxylated osteocalcin inhibits high glucose-induced ROS production and stimulates osteoblastic differentiation by preventing the activation of PI3K/Akt in MC3T3-E1 cells. Int J Mol Med 37: 173-181, 2016.
21. Clemmensen C, Smajilovic S, Wellendorph P and Brauner-Osborne H: The GPCR, class C, group 6, subtype A (GPRC6A) receptor: From cloning to physiological function. Br J Pharmacol 171: 1129-1141, 2014.
22. Pi M, Xu F, Ye R, Nishimoto SK, Kesterson RA, Williams RW, Lu L and Quarles LD: Humanized GPRC6A(KGKY) is a gain-of-function polymorphism in mice. Sci Rep 10: 11143, 2020.
23. Pi M, Nishimoto SK and Quarles LD: GPRC6A: Jack of all metabolism (or master of none). Mol Metab 6: 185-193, 2016.
24. Pi M, Wu Y and Quarles LD: GPRC6A mediates responses to osteocalcin in beta-cells in vitro and pancreas in vivo. J Bone Miner Res 26: 1680-1683, 2011.

25. Otani T, Mizokami A, Hayashi Y, Gao J, Mori Y, Nakamura S, Takeuchi H and Hirata M: Signaling pathway for adiponectin expression in adipocytes by osteocalcin. Cell Signal 27: 532-544, 2015.
26. Karsenty G and Oury F: Regulation of male fertility by the bone-derived hormone osteocalcin. Mol Cell Endocrinol 382: 521-526, 2014.
27. Smajilovic S, Clemmensen C, Johansen LD, Wellendorph P, Holst JJ, Thams PG, Ogo E and Bräuner-Osborne H: The L-α-amino acid receptor GPRC6A is expressed in the islets of Langerhans but is not involved in L-arginine-induced insulin release. Amino Acids 44: 383-390, 2013.
28. Chen JH, Lin X, Bu C and Zhang X: Role of advanced glycation end products in mobility and considerations in possible dietary and nutritional intervention strategies. Nutr Metab (Lond) 15: 72, 2018.
29. Deng A, Zhang H, Hu M, Liu S, Gao Q, Wang Y and Guo C: Knockdown of Indian hedgehog protein induces an inhibition of cell growth and differentiation in osteoblast MC3T3-E1 cells. Mol Med Rep 16: 7987-7992, 2017.
30. Smilic TN, Novakovic TR, Markovic-Jovanovic SR, Smilic LLJ, Mitic JS and Radunovic ML: The relevance of osteoclastic and osteoblastic activity markers follow-up in patients on antiresorptive osteoporosis treatment. J Clin Densitom 21: 322-328, 2018.
31. Khosla S and Hofbauer LC: Osteoporosis treatment: Recent developments and ongoing challenges. Lancet Diabetes Endocrinol 5: 898-907, 2017.
32. Fu C, Zhang X, Ye F and Yang J: High insulin levels in KK-Ay diabetic mice cause increased cortical bone mass and impaired trabecular micro-structure. Int J Mol Sci 16: 8213-8226, 2015.
33. Verma H and Garg R: Comment on 'bone regulates glucose metabolism as an endocrine organ through osteocalcin'. Int J Endocrinol 2016: 9724929, 2016.
34. Wang J, Yan DD, Hou XH, Bao YQ, Hu C, Zhang ZL and Jia WP: Association of bone turnover markers with glucose metabolism in Chinese population. Acta Pharmacol Sin 38: 1611-1617, 2017.
35. Liu JM, Rosen CJ, Ducy P, Kousteni S and Karsenty G: Regulation of glucose handling by the skeleton: Insights from mouse and human studies. Diabetes 65: 3225-3232, 2016.
36. Mizokami A, Kawakubo-Yasukochi T and Hirata M: Osteocalcin and its endocrine functions. Biochem Pharmacol 132: 1-8, 2017.
37. Tao SC and Guo SC: Extracellular vesicles in bone: 'Dogrobbers' in the 'eternal battle field'. Cell Commun Signal 17: 6, 2019.
38. Pi M, Kapoor K, Ye R, Nishimoto SK, Smith JC, Baudry J and Quarles LD: Evidence for osteocalcin binding and activation of GPRC6A in β-cells. Endocrinology 157: 1866-1880, 2016.
39. Dumitru N, Carsote M, Cocolos A, Petrova E, Olaru M, Dumitrache C and Ghemigian A: The link between bone osteocalcin and energy metabolism in a group of postmenopausal women. Curr Health Sci J 45: 47-51, 2019.
40. Ye R, Pi M, Cox JV, Nishimoto SK and Quarles LD: CRISPR/Cas9 targeting of GPRC6A suppresses prostate cancer tumorigenesis in a human xenograft model. J Exp Clin Cancer Res 36: 90, 2017.
41. De Toni L, De Filippis V, Tescari S, Ferigo M, Ferlin A, Scattolini V, Avogaro A, Vettor R and Foresta C: Uncarboxylated osteocalcin stimulates 25-hydroxy vitamin D production in Leydig cell line through a GPRC6a-dependent pathway. Endocrinology 155: 4266-4274, 2014.
42. Zhang XL, Wang YN, Ma LY, Liu ZS, Ye F and Yang JH: Uncarboxylated osteocalcin ameliorates hepatic glucose and lipid metabolism in KKAy mice via activating insulin signaling pathway. Acta Pharmacol Sin 41: 383-393, 2020.
43. Liu Z and Yang J: Uncarboxylated osteocalcin promotes osteogenic differentiation of mouse bone marrow-derived mesenchymal stem cells by activating the Erk-Smad/β-catenin signalling pathways. Cell Biochem Funct 38: 87-96, 2020.
44. Camerino C, Conte E, Cannone M, Caloiero R, Fonzino A and Tricarico D: Nerve growth factor, brain-derived neurotrophic factor and osteocalcin gene relationship in energy regulation, bone homeostasis and reproductive organs analyzed by mRNA quantitative evaluation and linear correlation analysis. Front Physiol 7: 456, 2016.