

Exendin-4 promotes proliferation and differentiation of MC3T3-E1 osteoblasts by MAPKs activation

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Abstract

Glucagon-like peptide-1 (GLP1) and its receptor agonist have been previously reported to play a positive role in bone metabolism in aged ovariectomized rats and insulin-resistant models. However, whether GLP1 has a direct effect on the proliferation and differentiation of osteoblasts or any cellular mechanism for this potential role is unknown. We examined the effects of the GLP1 receptor agonist exendin-4 on the proliferation, differentiation, and mineralization of mouse osteoblastic MC3T3-E1 cells. GLP1 receptor was detected in MC3T3-E1 cells by polymerase chain reaction (PCR) and Western blot assay. Cell proliferation was assessed using MTT assay, revealing that exendin-4 increased cell proliferation at effective concentrations between 10^{-10} and 10^{-5} M. Quantitative PCR analysis showed that exendin-4 increased the mRNA expression of the differentiation markers alkaline phosphatase (ALP), collagen-1 (COL1), osteocalcin (OC), and runt-related transcription factor 2 (RUNX2) under osteogenic conditions. Alizarin red staining confirmed that 10^{-7} M exendin-4 increased osteoblast mineralization by 18.7%. Exendin-4 upregulated the phosphorylation of ERK1/2, p38, and JNK, with the peak effect at 1.5 h in the Western blot analysis. The use of selective MAPK inhibitors, namely PD98059, SB203580, and SP600125, blocked the effects of exendin-4 on kinase activation (ERK1/2, p38, and JNK), as well as cell proliferation and differentiation in MC3T3-E1 cells. These findings demonstrate that exendin-4 promotes both the proliferation and differentiation of preosteoblasts MC3T3-E1 via activation of the MAPK pathway.

Key Words

- ▶ GLP1
- ▶ exendin-4
- ▶ osteoblast
- ▶ MC3T3-E1 cells
- ▶ MAPK

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Introduction

Diabetes mellitus and osteoporosis are both prevailing metabolic disorders and have been recently found to share similar molecular signatures. Multiple molecular factors

that arise as a result of diabetes may lead to secondary osteoporosis (Kurra & Siris 2011). In addition, patients with both diabetes and osteoporosis are increasingly

common in an aging society (Viegas *et al.* 2011). The effects of antidiabetic agents on bone metabolism have thus received increased attention, especially in old diabetic patients with high fracture risk (Montagnani & Gonnelli 2013). It has been reported that the novel incretin-based drugs, such as glucagon-like peptide-1 (GLP1) receptor agonist exenatide, did not affect bone mineral density despite body weight reduction in patients with type 2 diabetes mellitus (Bunck *et al.* 2011). This suggests that GLP1 and its receptor agonist may play a positive role in bone metabolism, but the specific mechanisms are still unclear.

GLP1 is one of the most important incretin hormones and is produced by intestinal L-cells. *In vivo*, GLP1 is degraded rapidly by dipeptidyl peptidase-4 (DPP4) and has a very short half-life of 1–2 min. Exendin-4, a natural GLP1 receptor (GLP1R) agonist, is extracted from the saliva of Gila monster (*Heloderma suspectum*). It shares 53% of its amino acid sequence with the N-terminal region of mammalian GLP1 and has an extra nine amino acid residues at its C terminus. The difference in the second amino acid of GLP1 makes exendin-4 become resistant to DPP-4 cleavage, therefore exendin-4 has a much longer half-life than GLP1, prompting its widespread use in *in vitro* studies (Yamada 2012). Exenatide is the synthetic product of exendin-4 for improving glycemic control.

Beyond its well-known effects on glucose metabolism and the protection of pancreatic beta cells, GLP1 has been reported to have extensive extrapancreatic effects on many other organs (Abu-Hamdah *et al.* 2009), with bone the latest target identified (Dicembrini *et al.* 2012). Animal studies show that GLP1 receptor-knockout mice develop osteoporosis (Yamada *et al.* 2008). GLP1 and exendin-4 can reverse the bone loss observed in insulin-resistant and type 2 diabetic rats, and change their bone turnover markers, most likely through the PTH and Wnt pathways (Nuche-Berenguer *et al.* 2010a). In aged ovariectomized rats, exendin-4 can prevent osteopenia by promoting bone formation and suppressing bone resorption (Ma *et al.* 2013). Recently, specific functional GLP1 receptors have been identified on the cell membranes of mice MC3T3-E1 osteoblasts (Nuche-Berenguer *et al.* 2010b). These results strongly suggest that GLP1 and its receptor agonist may play a positive role in bone metabolism. However, the molecular pathway involved in such beneficial effects has not been elucidated, and how GLP1 and its analog may regulate osteoblast activity is still unknown.

Our study was designed to investigate the function of exendin-4 in mice preosteoblastic MC3T3-E1 cells, and

illustrate the regulative mechanisms that exendin-4 may serve in osteoblasts.

Materials and methods

Reagents

Chemicals including exendin-4, glycerol 2-phosphate disodium salt hydrate (β -GP), ascorbic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-terazolium bromide (MTT), alizarin red, cetylpyridinium chloride, Trizol, and DMSO were purchased from Sigma. For Western blot analysis, rabbit anti-mouse-specific total and phospho (p)-p44/42 (Thr202/Tyr204), total and p-p38 (T180/Y182), total and p-SAPK/JNK (Thr183/Tyr185) antibodies, α -tubulin and GAPDH internal control antibodies, secondary antibody (goat anti-rabbit) and ERK inhibitor PD98059 and JNK inhibitor SP600125 were all purchased from Cell Signaling Technology, p38 inhibitor SB203580 was from Selleckchem (Houston, TX, USA). All other chemicals were of the highest grade available commercially.

Cell culture

MC3T3-E1 cells, a mouse preosteoblastic cell line, were kindly provided by Professor Eryuan Liao, Department of Metabolism and Endocrinology, The Second Xiangya Hospital, Central South University, Changsha, Hunan, People's Republic of China (Liang *et al.* 2013). MC3T3-E1 cells were cultured in DMEM-F12 medium (Gibco by Life Technologies, Shanghai, China) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin in 5% CO₂ at 37°C. The culture media was replaced every 2 days. When the cell density reached approximately 80% confluence, the cells were treated with 0.25% trypsin and transferred to fresh culture flasks at 1:2 ratio.

Differentiation of osteoblastic cells

When the cells reached 80% confluence after 2 or 3 days of culture, they were induced to undergo differentiation using the osteogenic induction medium consisting of DMEM-F12, 10% FBS, 1% penicillin–streptomycin, 50 mg/L ascorbic acid, and 10 mM β -GP. The medium was replaced every 2 days. Exendin-4 (100 nM) was added in the experimental group to study its effect on osteoblasts.

MTT assay for cell viability

MC3T3-E1 cells were incubated in a 96-well plate with a cell density of 1×10^4 cells per well. The cells were then

incubated with exendin-4 (10^{-11} – 10^{-5} M) for 24, 48, or 72 h. The cells in the same volume of culture media were used as control. For inhibition experiments, cells were pretreated with the MAPK inhibitors: PD98059 (ERK1/2 inhibitor, 50 μ M), SB203580 (p38 inhibitor, 10 μ M), or SP600125 (JNK inhibitor, 50 μ M), followed by culture with exendin-4 (100 nM) for 24 h. Following treatments, the culture medium was removed, 10 μ L of freshly prepared MTT (10 μ g/L) was added to each well of the plate, and the plates were placed in a cell incubator at 37°C for 4 h. 150 μ L DMSO was added to each well and mixed thoroughly to lyse the cells and dissolve the dark blue crystals. After 5 min of dissolution, the absorbance was measured at 570 nm using a microplate reader (Bio-Rad iMARK, CA, USA). Cell proliferation was assessed by calculating the relative cell viability, comparing the control and experimental groups.

Real-time PCR for the detection of differentiation markers and GLP1R

Gene expression of osteogenic differentiation markers including alkaline phosphatase (ALP), collagen-1 (COL1), osteocalcin (OC), and runt-related transcription factor-2 (RUNX2) in osteoblastic cells was quantified by fluorogenic quantitative PCR analysis. The MC3T3-E1 cells were plated at 1×10^5 per well in 6-well plates and induced to differentiate in the absence or presence of exendin-4 (100 nM). The cells were also pretreated with or without the MAPK-specific inhibitors PD98059, SB203580, or SP600125 to see whether the effects of exendin-4 on MC3T3-E1 cells differentiation can be blocked. Total RNA from the cells was extracted on day 0, 7, 14, 21, and 28 using Trizol reagent. The integrity of total RNA was assessed by RNA agarose electrophoresis, and the concentration and purity of total RNA were determined with the absorbance at 260/280 nm (NANODROP 2000, Thermo, MA, USA). cDNA was synthesized from 2 μ g total RNA in a standard 20 μ L volume according to the protocol (ReverTra Ace qPCR RT Kit, Toyobo, Osaka, Japan). Real-time PCR amplification was performed with the ABI PRISM 7500 (Applied Biosystems). The 20 μ L PCR system consisted of 1 μ L cDNA, 0.3 μ mol forward primer, 0.3 μ mol reverse primer, 10 μ L SYBR green qPCR mix, and 0.04 μ L ROX (SYBR qPCR Mix, Toyobo, Osaka, Japan). PCR primers (Table 1) were designed using Primer 5.0 software and, primers synthesis was acquired from Invitrogen Co. (Life Technologies). PCR conditions were as follows: 95°C for 10 min, 40 cycles of the three-step PCR (denaturation at 95°C for 1 min, annealing at 56°C for

30 s, and extension at 72°C for 1 min). Expression of the *Ef1 α* (*Eef1 α*) (in differentiation experiments) and β -*actin* genes (in inhibition experiments) was used for normalization. The results were analyzed by the $2^{-\Delta\Delta C_t}$ method.

Glp1r mRNA was synthesized to cDNA and amplified by real-time polymerase chain reaction (RT-PCR) in 50 μ L volumes. The primers used for *Glp1r* (Pereira *et al.* 2015) was given in Table 1. The amplification condition consisted of initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 30s, and extension at 72°C for 1 min. The products were visualized by electrophoresis on a 2% agarose gel. mRNA from mouse pancreas was used as a positive control. A mixture of water and loading buffer in equal proportion was used as a negative control.

Alizarin red staining for mineralization

To examine the mineralization of MC3T3-E1 cells, cells were plated in the differentiation medium at a concentration of 1×10^5 per well in 6-well plates in the presence or absence of 100 nM exendin-4. After 21 days, the medium was removed and stained with alizarin red. The cells were washed with phosphate-buffered saline three times and fixed in 75% ethyl alcohol for 30 min at 4°C. The cells were then washed with distilled water three times and stained in 1% alizarin red solution (pH 4.2) for 30 min at 37°C. Unbound dye was removed by washing several times with distilled water. Mineralized nodules were observed using a low magnification microscope and photographed. To quantify matrix mineralization, 1 mL 100 mM cetylpyridinium chloride was added to each well and incubated for 1 h to dissolve and release the calcium-bound alizarin red. The absorbance of released alizarin red was measured at 570 nm.

Table 1 Primers used for fluorogenic quantitative RT-PCR.

Name	Primer sequence	GenBank number
β - <i>actin</i>	5'-TCTTGGGTATGGAATCCTGTG-3' 5'-AGGTCTTTACGGATGTCAACG-3'	NM_007393.2
<i>Ef1α</i>	5'-CTGAACCATCCAGGCCAAAT-3' 5'-GGCTGTGTGACAATCCAG-3'	NM_010106.2
<i>Alp</i>	5'-AACCCAGACACAAGCATTCC-3' 5'-GAGAGCGAAGGGTCAGTCAG-3'	NM_007431.1
<i>Col1</i>	5'-AGAGCATGACCGATGGATTCC-3' 5'-CCTTCTTGAGGTTGCCAGTC-3'	NM_007742.3
OC (<i>Bglap2</i>)	5'-TGCTTGTGACGAGCTATCAG-3' 5'-GAGGACAGGGAGGATCAAGT-3'	NM_001032298.2
<i>Runx2</i>	5'-AAGTGCGGTGCAAACCTTCT-3' 5'-TCTCGGTGGCTGGTAGTGA-3'	NM_009820.2
<i>Glp1r</i>	5'-TCCTTCGTGAATGTCAGCTG-3' 5'-TGGTGCAAGTGTCTG-3'	(Pereira <i>et al.</i> 2015)

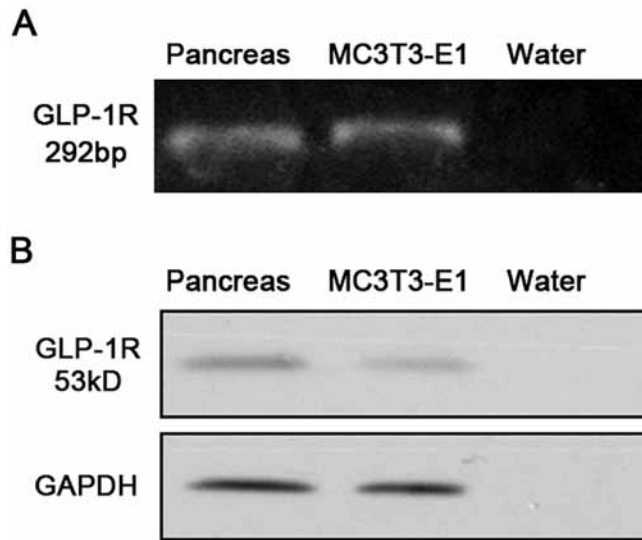


Figure 1
GLP1R was expressed in preosteoblastic MC3T3-E1 cells. (A) Expression of *Glp1r* mRNA in MC3T3-E1 cells and mouse pancreas. (B) GLP1 receptor protein was detectable in MC3T3-E1 cells by Western blot. Expression normalized to that of GAPDH. Equal parts of water and loading buffer were used as a negative control.

Western blots for MAPK assays and GLP1R

Western blot was used to detect the level of key proteins of MAPK assays including ERK1/2, p-ERK1/2, p38, p-p38, JNK, and p-JNK. House-keeping proteins α -tubulin and GAPDH were used as internal controls for normalization. MC3T3-E1 cells were seeded in 30 mm culture dishes at the concentration of 1×10^6 per dish. After 2 days cultivation, the normal medium was replaced with low serum medium (containing 1% FBS) and cultures were maintained for a further 12h. Then, the medium was changed and the cells were treated with 100 nM exendin-4 for 15, 30, 60, 90, 120, 150, and 180 min. For inhibition experiments, cells were pretreated with PD98059 (50 μ M), SB203580 (10 μ M), or SP600125 (50 μ M), followed by incubation with exendin-4 (100 nM) for 1 h. At the harvested time, cells were washed using PBS three times and lysed using the RIPA lysis system with phosphatase inhibitors (Beyotime Biotechnology, Shanghai, China). Ten micrograms of total protein were extracted from each sample, assessed by a BCA protein assay (Beyotime Biotechnology), then separated by 10% SDS-PAGE, and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked with 5% non-fat milk in TBST (containing 50mM Tris-HCl, 150mM NaCl, and 0.05% Tween-20) and incubated overnight at 4°C with the specific primary antibodies: p-ERK1/2, total-ERK1/2, p-p38, total-p38, p-JNK, total-JNK diluted 1:1000. A horseradish peroxidase-conjugated

secondary antibody (Cell Signaling Technology) at 1:2000 dilution was used for immunodetection and incubated for 1 h at room temperature. Lastly, the proteins were visualized by autoradiography using an enhanced chemiluminescence detection system (Thermo, Rockford, IL, USA). Protein bands on the films were quantified by ImageJ software (NIH).

Western blot analysis was also used to detect the specific expression of GLP1R protein in MC3T3-E1 cells, with protein from mouse pancreas used as a positive control. A mixture of water and loading buffer in equal proportion was used as a negative control. The anti-GLP1R antibody used was rabbit polyclonal (53 kDa) (ab39072, Abcam).

Statistical analyses

The data were expressed as means \pm standard deviation and analyzed by SPSS 16.0 software (IBM). Statistical evaluations for the differences between groups were performed by one-way ANOVA followed by the least significant differences test for *post hoc* multiple comparisons. Differences were considered to be significant at $P < 0.05$.

Results

Expression of GLP1R in MC3T3-E1 cells

We investigated whether the GLP1R is present on preosteoblastic MC3T3-E1 cells by PCR and Western blot assay. Expression of the GLP1R in mouse pancreas was used as a positive control. As shown in Fig. 1, the specific GLP1R was expressed in undifferentiated MC3T3-E1 cells.

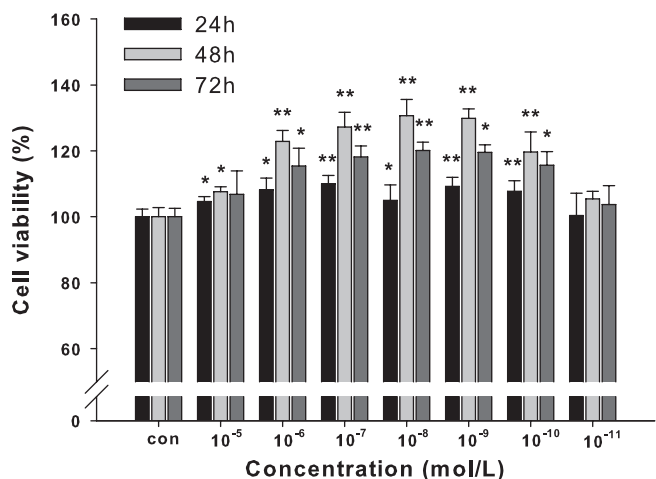
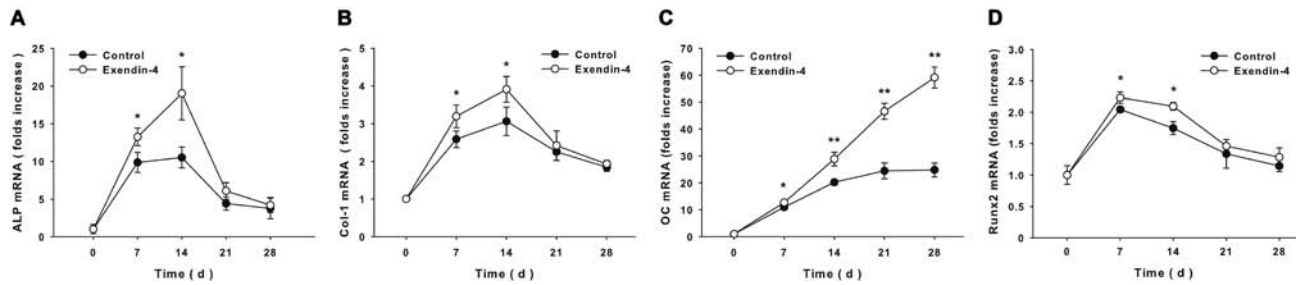


Figure 2
Effects of exendin-4 on cell viability in MC3T3-E1 cells. Cells were incubated with exendin-4 (10^{-11} ~ 10^{-5} M) for 24, 48, or 72 h. Results are represented as cell proliferation relative to the control ($n=3$). * $P < 0.05$, ** $P < 0.01$ compared with the control group.

**Figure 3**

Effects of exendin-4 on the expression of differentiation markers in MC3T3-E1 cells. Cells were induced to differentiate in the presence or absence of exendin-4. Total RNA was collected on day 0, 7, 14, 21, and 28, fluorogenic quantitative PCR was used to evaluate the expressions of mRNA levels of *Alp* (A), *Col1* (B), *Oc* (C), and *Runx2* (D) ($n=3$). * $P<0.05$, ** $P<0.01$ exendin-4 group vs control.

Exendin-4 promoted cell proliferation in MC3T3-E1 cells

The MTT assay showed that exendin-4 at concentrations of 10^{-10} – 10^{-5} M led to a significant increase in cell viability (Fig. 2). The cell proliferation rate calculation showed the maximal effect of 10^{-7} M ($110\pm 2.6\%$ of the basal rate) and 10^{-8} M ($131\pm 4.9\%$ of the basal rate) at 24 and 48 h ($P<0.05$), respectively.

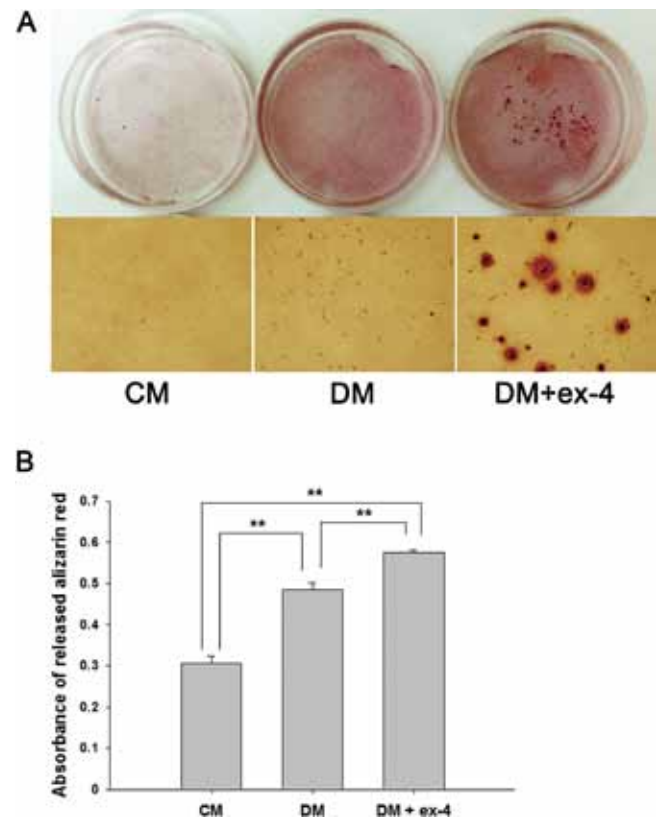
Exendin-4 increased the gene expression of differentiation markers

RT-PCR showed that osteogenesis markers increased rapidly following induction of osteogenesis by culturing in osteogenic media. The expression of mRNA encoding the transcription factor *Runx2* increased from a very early stage, peaking at day 7 before tapering off toward the end of the differentiation period. The expression of *Alp* and *Col1* mRNA levels increased during the early and middle period, peaking at day 14. The late-stage marker *Oc* continued to increase during the whole culture period, peaking at the end of osteogenesis (Fig. 3). These changes indicate that MC3T3-E1 cells differentiated into mature osteoblasts in the presence of osteogenic induction media. Compared with controls, exendin-4 treatment significantly elevated the mRNA levels of *Runx2*, *Alp*, and *Col1* on day 7 and 14 ($P<0.05$). The maximum increases observed for each were 1.20-, 1.81- and 1.28-fold, respectively. *Oc* expression was increased by exendin-4 throughout the entire differentiation period with the maximum increase of 2.38-fold occurring on day 28 ($P<0.05$) (Fig. 3).

Exendin-4 enhanced osteoblastic mineralization

As shown in Fig. 4, mineralized nodules increased in MC3T3-E1 cells following the addition of differentiation

factors into the medium (50 mg/L ascorbic and 10 mM β -GP). Larger nodules were observed by visual inspection and microscopic observation at low magnification in cells incubated with 100 nM exendin-4. Consistent with this,

**Figure 4**

Mineralization of staining by Alizarin red in MC3T3-E1 cells. (A) Cells were cultured for 21 days in the control basal media (CM, left); differentiation media (DM, center); differentiation media with the addition of exendin-4 (right). Upper figures showed mineralized nodules by visual inspection and lower figures showed the photomicrograph (Magnification: $\times 40$). (B) Quantification by cetylpyridinium chloride dissolution and spectrophotometric readings ($n=3$). * $P<0.05$, ** $P<0.01$.

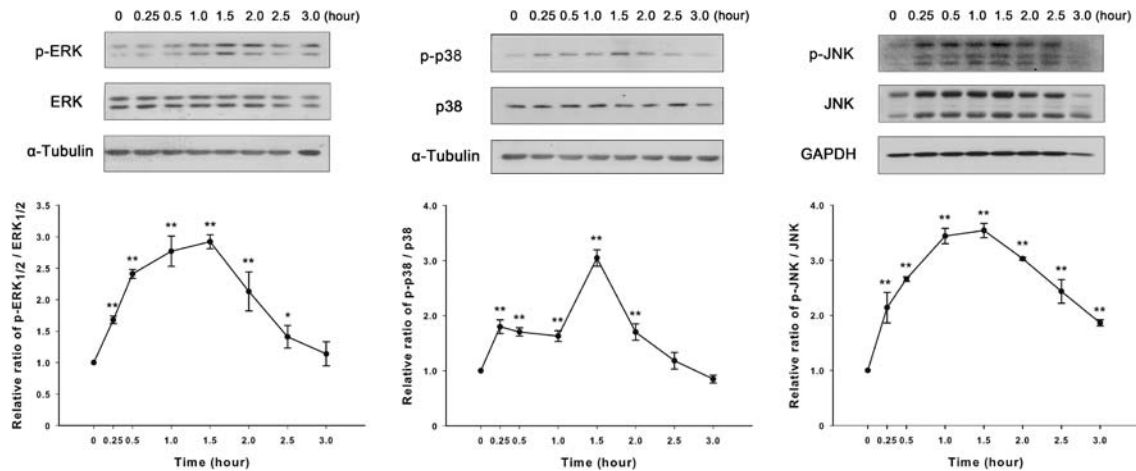


Figure 5 Effects of exendin-4 on MAPK phosphorylation in MC3T3-E1 cells. Cells were treated with exendin-4 and total proteins were extracted at different observed times to detect the phosphorylation of ERK1/2 (A), p38 (B), and JNK (C) ($n=3$). * $P<0.05$, ** $P<0.01$ compared with 0h.

quantification of mineralization by cetylpyridinium chloride dissolution and spectrophotometry showed an 18.7% increase in mineralization in cells treated with exendin-4 compared with cells cultured in differentiation media alone ($P<0.05$).

Exendin-4 induced the phosphorylation of MAPKs

Exendin-4 increased the phosphorylation levels of all three MAPKs between 0.15 and 2.5 h. The peak effects of exendin-4 on ERK1/2, p38, and JNK all appeared at 1.5 h,

which were 2.92 ± 0.11 -, 3.05 ± 0.15 -, and 3.54 ± 0.13 -fold of the baseline level, respectively ($P<0.05$) (Fig. 5).

Exendin-4-induced phosphorylation was abolished by MAPK inhibitors

The upregulation of phosphorylation of MAPK induced by exendin-4 was completely abolished by pretreatment with $50\mu\text{M}$ PD98059 (ERK1/2) inhibitor, $10\mu\text{M}$ SB203580 (p38 inhibitor), or $50\mu\text{M}$ SP600125 (JNK inhibitor). Exendin-4 induced an increase in MAPK phosphorylation of ERK1/2,

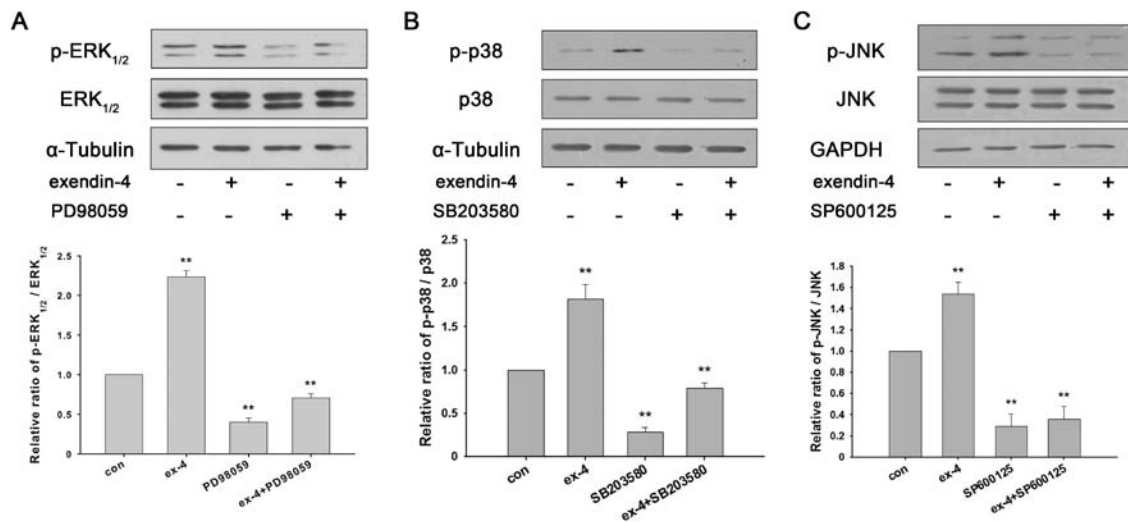
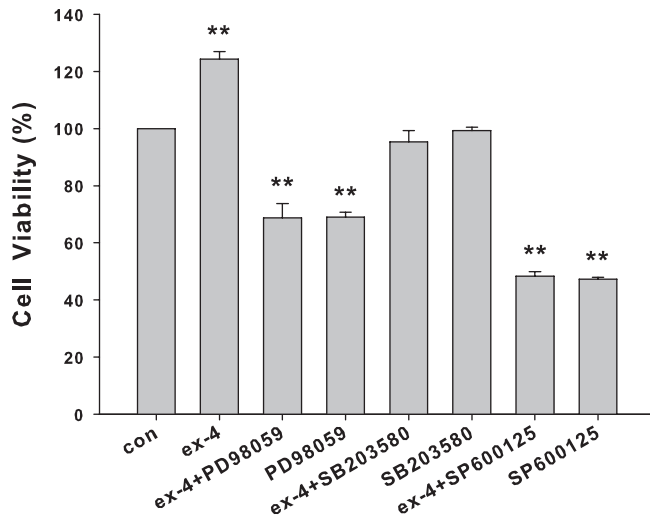


Figure 6 Effects of MAPK-selective inhibitors on exendin-4-induced upregulation of MAPK phosphorylation. Cells were pretreated with $50\mu\text{M}$ PD98059, $10\mu\text{M}$ SB203580, or $50\mu\text{M}$ SP600125 before incubation with 100 nM exendin-4 for 1 h. Total proteins were extracted to detect the phosphorylation of ERK1/2 (A), p38 (B), and JNK (C) ($n=3$). * $P<0.05$, ** $P<0.01$ compared with the control group.

**Figure 7**

Effect of MAPK inhibitors on exendin-4-induced increase of MC3T3-E1 cell proliferation. Cells were pretreated with PD98058, SB203580, or SP600125 before incubation in the presence or absence of 100 nM exendin-4, before performing MTT assays to analyze cell viability ($n=3$). * $P<0.05$, ** $P<0.01$ compared with the control group.

p38, and JNK with 2.24 ± 0.08 -, 1.82 ± 0.17 -, and 1.54 ± 0.11 -fold ($P<0.01$), respectively. Pretreatment with PD98059, SB203580, and SP600125 alone reduced the phosphorylation to $40.0\pm 5.1\%$, $28.6\pm 5.0\%$, and $29.2\pm 11.6\%$ ($P<0.01$), respectively, from control levels. Incubation of exendin-4 and each inhibitor showed a lower activation at $70.5\pm 5.2\%$, $78.9\pm 6.5\%$, and $35.8\pm 11.9\%$, respectively, compared with the control group ($P<0.01$) (Fig. 6).

The effect of exendin-4 on MC3T3-E1 cells can be blocked by MAPK inhibitors

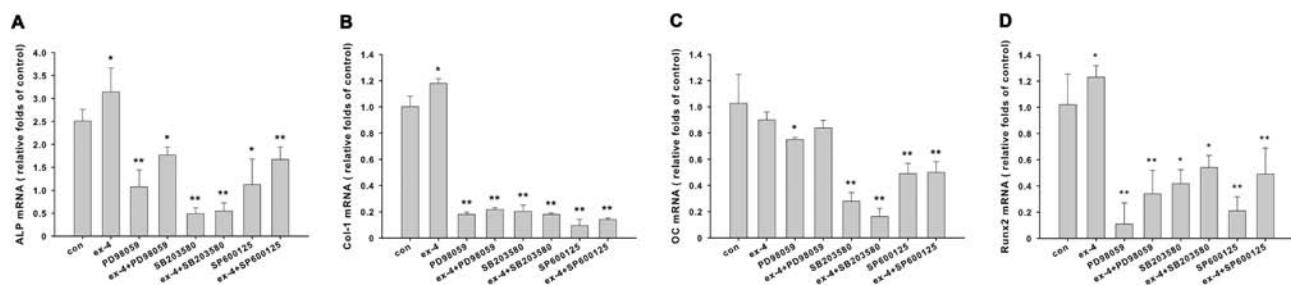
To confirm that the MAPK pathways are involved in the exendin-4-induced osteoblast proliferation and differentiation, we used specific MAPK inhibitors to pretreat the cells.

The MTT assay showed that cells pretreated with PD98059 and SP600125 alone had only $69.0\pm 1.7\%$ and $47.3\pm 0.6\%$ cell viability in comparison with the controls ($P<0.01$). Incubation with the p38 inhibitor SB203580 did not affect cell proliferation. Although incubation for 24 h with 100 nM exendin-4 increased the cell proliferation by $24.3\pm 2.7\%$, intervention using PD98059, SB203580, or SP600125 resulted in a reduction in cell viability of $55.6\pm 5.0\%$, $31.6\pm 2.9\%$, and $76\pm 1.5\%$, respectively, compared with exendin-4 incubation alone ($P<0.05$) (Fig. 7).

The gene expression of osteoblastic differentiation markers in response to MAPK inhibitors was much lower than that in the controls. Exendin-4 increased the mRNA levels of *Alp*, *Col1*, and *Runx2* to 1.26 ± 0.21 -, 1.18 ± 0.03 -, and 1.23 ± 0.09 -fold, respectively, at day 7 of differentiation ($P<0.05$). PD98059 reduced all the mRNA levels of these markers whether treated with exendin-4 or not; this was particularly apparent in the case of *Runx2* expression, which showed only 11–34% of the expression level compared with the control ($P<0.01$). SB203580 also reversed the upregulation effect of exendin-4 on these markers; a most notable effect was seen for *Alp* with only $21.9\pm 7.1\%$ of control expression levels ($P<0.01$). SP600125 likewise downregulated all the mRNA expressions, with the maximal effect of this inhibitor seen for *Col1* expression with only $9.5\pm 4.9\%$ of the control levels when treated alone and $14.2\pm 1.1\%$ of the control level expression when used in conjunction with exendin-4 ($P<0.01$) (Fig. 8).

Discussion

Our results demonstrated that the GLP1 receptor agonist exendin-4 exerted a direct effect on mice preosteoblast MC3T3-E1 cells. Signaling through this receptor resulted in the promotion of cell proliferation and an increase in the gene expression of osteogenic differentiation

**Figure 8**

Effect of MAPK inhibitors on exendin-4-induced increase of MC3T3-E1 cell differentiation. Cells were switched to differentiation media, and pretreated with PD98058, SB203580, or SP600125 before their incubation in the presence or absence of 100 nM exendin-4. Total RNA was extracted at day 7 and quantitative PCR was performed ($n=3$). * $P<0.05$, ** $P<0.01$ compared with the control group.

markers. Exendin-4 also enhanced mineralization in pre-osteoblasts. Exendin-4 upregulated the activity of the MAPK signaling pathway, which is known to play a critical role in osteoblast differentiation and skeletal development. Taken together, these results imply that exendin-4 may play a positive role in bone metabolism by regulating the function of osteoblasts directly.

The gut–brain–bone axis has been recognized since the finding that bone turnover can occur as a response to feeding (Henriksen *et al.* 2003). The relationship between incretin and bone remains unclear. GLP1 has been found to have positive effects on bone metabolism in studies of cell lines (Sanz *et al.* 2010, Pacheco-Pantoja *et al.* 2011), gene-knockout mice (Yamada *et al.* 2008, Mieczkowska *et al.* 2015), and other animal models (Nuche-Berenguer *et al.* 2009, Nuche-Berenguer *et al.* 2010a, Nuche-Berenguer *et al.* 2011, Kim *et al.* 2013, Ma *et al.* 2013). Since GLP1 receptor agonists have been introduced in the pharmacological treatment of type 2 diabetes, their role in bone metabolism is now gaining increased attention.

Although GLP1 and exendin-4 have been proven to benefit the bone in different animal models, whether GLP1 and its receptor agonist can regulate the osteocytes directly has remained controversial. There is much confusion about whether GLP1R is expressed in osteocytes or not. Yamada *et al.* (2008) demonstrated that GLP1R^{-/-} mice have cortical osteopenia and bone fragility as well as increased osteoclastic numbers and bone resorption activity. While their study demonstrates the essential role of the murine GLP1R in control of bone resorption, Yamada and coworkers found that the GLP1R was absent in osteoblasts and GLP1 failed to increase intracellular cAMP levels in osteoblast Saos2 cells (Yamada *et al.* 2008). Furthermore, GLP1 had no effects on either osteoclastic differentiation or osteoblastic apoptosis protection, thus they concluded that GLP1 had no direct effect on osteocytes (Yamada *et al.* 2008). Nevertheless, recent studies have reported that the expression of GLP1R was detected in osteocyte-like MLO-Y4 cells by RT-PCR and Western blot analysis. In addition, GLP1R was present in the osteocytes taken from rat femur as determined by immunohistochemistry and immunofluorescence (Kim *et al.* 2013). These results indicate that the expression of GLP1R varied in different osteocyte cell lines and bone tissue. As in preosteoblast MC3T3-E1 cells, Nuche-Berenguer and co-workers identified the presence of a functional receptor for GLP1 by ¹²⁵I-GLP1 binding studies. This GLP1R in MC3T3-E1 was different from those found previously in the pancreas and liver, as it failed to signal through cAMP. But GLP1R was undetectable in

these cells by RT-PCR (Nuche-Berenguer *et al.* 2010b). In contrast, in our study, the expression of the GLP1R was detected by both RT-PCR and Western blot in preosteoblast MC3T3-E1 cells. In previous studies, Aoyama *et al.* (2014) showed that the expression of GLP1R was influenced by the glucose concentration in MC3T3-E1 cells undergoing differentiation induced by BMP2 (Aoyama *et al.* 2014). Another study found that *GLP1R* mRNA is absent in the pre-osteocytic cell line MLO-A5, but is present in the late osteocytic cell line IDG-SW3 as well as primary osteoblasts and osteoclasts derived from mouse bone marrow. Furthermore, they illustrated that the expression of GLP1R gradually diminishes as osteoblasts differentiate toward maturation (Pereira *et al.* 2015).

Taken together, we conclude that GLP1R is expressed in certain osteocytes including MC3T3-E1 cells, and is affected by several factors such as glucose concentration and different differentiation conditions. In addition, we found that exendin-4 affects osteoblastic cell proliferation and differentiation as well as the kinase activity of MC3T3-E1 cells *in vitro*. This clearly shows that exendin-4 directly and functionally interacts with osteoblastic cells.

Recent reports have suggested that exendin-4 has a direct osteogenic effect. In hyperlipidemic rats, both GLP1 and exendin-4 similarly reversed decreased bone mass in femurs and vertebrae (Nuche-Berenguer *et al.* 2011). In insulin-resistant and type 2 diabetic rats, exendin-4 exerted osteogenic effects and interacted with the Wnt pathway to promote bone formation (Nuche-Berenguer *et al.* 2009, Nuche-Berenguer *et al.* 2010a). Exendin-4 was also found to increase bone mineral density in type 2 diabetic OLETF rats through down-regulation of SOST/sclerostin in osteocytes (Kim *et al.* 2013). More recently, exendin-4 has been reported to prevent osteopenia by promoting bone formation and suppressing bone resorption in aged ovariectomized rats, confirming the effects of GLP1 on bone in an osteoporosis model (Ma *et al.* 2013). Our data indicate that exendin-4 promotes the proliferation of MC3T3-E1 osteoblastic cells at certain concentrations, suggesting that exendin-4 may increase the pool of basic osteocytes that can participate in bone formation. It is interesting that this response exhibits optimums at both intermediate concentrations and intermediate time of treatment. We inferred that these effects are probably owing to the saturability of the receptor. Most studies report that the effective concentrations of GLP1 or exendin-4 for different cell lines are between 10⁻⁹ and 10⁻⁶M (Sanz *et al.* 2010, Heller *et al.* 2011, Xie *et al.* 2014). Our results demonstrate a similar effective

dosage range of exendin-4 for osteoblast proliferation, with a maximum effective dose of 10^{-7} M. The optimum treatment time of 48h may be attributable to a number of factors, including the half-life of exendin-4 and a corresponding decrease in bio-utilization of the drug, as well as increased cell apoptosis given the limited volume of the cell culture plates used in this study.

In addition, we found that exendin-4 treatment increases the expression of the transcriptional regulatory factor *Runx2* and dominant differentiation markers including *Alp*, *Col1*, and *Oc*. Mineralization was also improved at late-stage osteogenesis by exendin-4 treatment, strongly suggesting that the GLP1 receptor agonist exendin-4 possibly enhances the osteogenic capability of osteoblasts to promote bone formation. Thus, we conclude that exendin-4 probably increases bone formation through the following mechanism: exendin-4 first promotes cell proliferation at early time points to increase the number of potential osteoblastic cells for bone formation. Exendin-4 treatment then upregulates the expression of differentiation markers and subsequently promotes osteoblast differentiation. This leads to an increase in the expression of bone-specific enzymes, collagen, and osteocalcin that accelerates cell mineralization and eventually promotes bone matrix formation.

To further explore the mechanisms of exendin-4 on MC3T3-E1 osteoblastic cells, we explored the effect of exendin-4 on kinase activation of the MAPK signaling pathway. MAPKs are classical signal transducers that are well characterized as mediators of cell growth, differentiation, apoptosis, and inflammation. MAPKs exert their effects through the ERK1/2, p38, JNK, and ERK5 pathways. Although the physiological role of the MAPK pathway in osteoblasts remains controversial, it has been determined as one of the most important regulatory signaling pathways in osteoblast proliferation and differentiation (Greenblatt *et al.* 2013). The activation of ERK1/2 has been shown to regulate the proliferation of bone marrow mesenchymal stem cells *in vitro* and accelerate their differentiation toward osteoblasts (Jaiswal *et al.* 2000, Miguel *et al.* 2005). The ERK pathway is associated with multiple key mediators of early- and late-stage osteoblast differentiation, including RUNX2, ATF4, IL1 β , and PTH-related protein (Franceschi *et al.* 2007, Greenblatt *et al.* 2013). Similar to ERK, the p38 pathway also can phosphorylate *Runx2* to promote osteoblast differentiation (Greenblatt *et al.* 2010). Moreover, the p38 pathway has been reported to be necessary in multiple osteoblast differentiation induction studies (Guicheux *et al.* 2003, Zhou *et al.*

2007). The JNK pathway is typically involved in signaling stress response and inflammation, but has also been shown to operate in late-stage osteoblast differentiation (Matsuguchi *et al.* 2009, Hah *et al.* 2013).

In addition, numerous studies have showed that GLP1 and exendin-4 affect the MAPK signaling pathway both *in vivo* and *in vitro*. GLP1 increased ERK1/2- and p38-MAPK levels in pancreatic beta cells (INS 832/13), and the p38 inhibitor SB203580 suppressed GLP1-induced beta cell proliferation (Buteau *et al.* 2001). In human myocytes, GLP1 and exendin-4 both activated ERK1/2 and MAPK, and stimulated glucose uptake, while the ERK1/2 inhibitor PD98059 abolished this effect (Gonzalez *et al.* 2005). Similar effects were observed in normal human adipocytes (Sancho *et al.* 2007). Hindbrain administration of exendin-4 increased MAPK activity that contributed to food intake regulation and body weight suppression in SD rats (Hayes *et al.* 2011). However, in peripheral blood mononuclear cells from type 2 diabetes patients, exendin-4 downregulated ERK and p38 MAPK phosphorylation to reduce oxidative stress and pro-inflammatory responses (He *et al.* 2013). In osteocytes, GLP1 induced a clear increase in the phosphorylation of ERK1/2 MAPKs in MC3T3-E1 cells (Nuche-Berenguer *et al.* 2010b). Therefore, we believe that the activity of MAPKs participates in the mechanism of exendin-4 to promote both proliferation and differentiation in MC3T3-E1 cells and undertook further experiments to test this hypothesis.

Our results show that exendin-4 activates the three MAPKs within a short time period, all with a peak increase of phosphorylation at 1.5 h from the beginning of treatment. However, use of the specific inhibitors PD98059, SB203580, and SP600125 blocked exendin-4 signaling through ERK1/2, p38, and JNK, respectively. This demonstrates that exendin-4 can activate the MAPK pathways in MC3T3-E1 cells.

The use of specific MAPK inhibitors confirmed the necessary role that MAPKs play in osteoblastic cell proliferation and differentiation. The use of the ERK1/2 inhibitor PD98059 resulted in the reduced proliferation of MC3T3-E1 cells and a decrease in the expression of differentiation markers, in particular *Runx2*. This is consistent with previous studies that have shown that ERK-MAPK modulates osteocyte function by regulating the activity of *Runx2* (Ge *et al.* 2007, Ge *et al.* 2009). We have also shown that treatment with SB203580, an inhibitor of p38, did not affect osteoblast proliferation but showed marked inhibitory effects on cellular differentiation, with the greatest decreases seen in *Alp* mRNA expression.

The JNK inhibitor SP600125 used in our experiments also inhibited cell growth and differentiation, as expected.

Finally, the expression profiles of osteoblastic markers and cell viability data suggest that the effects of exendin-4 in promoting osteoblast proliferation and differentiation can be prevented by inhibiting MAPK pathways. In proliferation experiments, exendin-4 incubation in the presence of PD98059 and SP600125 confers much lower cell viability compared with controls, but similar to the group using each inhibitor only. This suggests that the effects of exendin-4 on MC3T3-E1 cells proliferation require ERK- and JNK-MAPK. The upregulation effects of osteoblastic markers induced by exendin-4 were abolished partly or completely by each MAPK-specific inhibitor, suggesting that blocking each MAPK pathway attenuates the effects of exendin-4 on cell differentiation.

Thus, we suggest that the effects of exendin-4 on osteoblast proliferation and differentiation are dependent on MAPK activation. In previous studies performed in the same cell line, Nuche-Berenguer and his team found that 10^{-8} M GLP1 downregulates the gene expression of *Runx2* after 24 h of treatment as well as 5 days of osteogenesis induction (Nuche-Berenguer *et al.* 2010b). Our results show that 10^{-7} M exendin-4 treatment mildly elevated the same gene levels, but during a much longer differentiation period of 7–28 days. As both ERK1/2 and p38 can phosphorylate *Runx2* and regulate its activity, we thought that differential transcription of *Runx2* may be involved in exendin-4-induced osteoblast differentiation. However, the cross-talk among MAPK pathways and their interaction with other critical signaling pathways in osteoblasts require further exploration.

In conclusion, we demonstrate for the first time the effects of the GLP1 receptor agonist exendin-4 on the preosteoblastic MC3T3-E1 cells. Exendin-4 can promote the proliferation and differentiation of MC3T3-E1 cells, partly via the activation of MAPK signaling pathways. This study provides evidence that exendin-4 plays a positive role in bone metabolism by promoting bone formation. With further study, future consideration of GLP1 receptor agonists as a treatment for bone health in diabetes patients, particularly those with osteoporosis, may be an option. Given the large number of patients treated with GLP1 receptor agonists, more studies are needed to understand the precise role that GLP1 plays in bone metabolism.

Declaration of interest

All the authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution

LX and HX designed the study design; YF conducted the study; YF, XZ, and GW involved in data collection and analysis; YF drafted the manuscript; LS and YL revised the manuscript content; YF, LS, XZ, GW, HX, YL, and LX approved the final version of manuscript; And YF takes responsibility for the integrity of the data analysis.

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