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Original Article

Exendin-4 promotes the osteogenic differentiation of osteoblasts via the Hedgehog/Gli1 signaling pathway

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Abstract: This study aimed to investigate the effect and mechanisms of Exendin-4 mediated-Hedgehog/Gli1 signaling pathway on the differentiation of osteoblasts in mouse. The alkaline phosphate activity, alizarin red staining and expression of Gli1, GLP-1R, Hedgehog, Runx2 and osteocalcin were analyzed using PCR and Western blot analysis after treating the osteoblastic cell line MC3T3-E1 with Exendin-4. Osteoblasts were treated with Gli1-siRNA and Hedgehog receptor antagonist Cyclopamine (Cy) and analyzed for their impact on the Hedgehog/Gli1 signaling pathway. Our results showed that optimal treatment of Exendin-4 was 7 days at 10^{-7} mol/L. Exendin-4 significantly promoted osteoblast formation in the cell line in a dose-dependent manner and up-regulated the expression of GLP-1R, Hedgehog and Gli1. Gli1-siRNA significantly down regulated the expression of Gli1 and Runx2, and offset Exendin-4-induced osteoblast differentiation. Similarly, Cy offset Exendin-4-induced Gli1 up-regulation. It is clear that Exendin-4 can promote the osteogenic differentiation of osteoblasts through Hedgehog/Gli1 signaling pathway.

Keywords: Exendin-4, Hedgehog signaling, Gli1, osteoblast, differentiation

Introduction

Osteoporosis is characterized by microstructure change, density reduction and strength reduction in bone. Clinically, it often results in bone pain and brittle fracture. This is mainly due to imbalance in bone formation and resorption, where generally bone resorption is far more than bone formation, which ultimately leads to reduced bone strength, bone pain, and even bone fracture. Glucagon like peptide-1 (GLP-1) is a polypeptide secreted by intestinal L cells. It binds GLP-1 receptor (GLP-1R) to play the roles [1, 2]. Exendin-4 is a natural analogue of GLP-1 secreted in the saliva of the Gila monster in the Southwestern United States. This polypeptide contains 39 amino acids with 53% amino acid sequence identity to mammalian GLP-1. It is a highly effective activator of the pancreatic GLP-1 receptor and has high affinity with and similar biological functions to GLP-1R [3-5]. Study has shown that Exendin-4 can significantly improve osteoporosis in rats [6]. Hedgehog/Gli1 is a very important signal pathway for cell differentiation and organ formation [7]. Hedgehog (Hh) is a secreted glycoprotein and can bind the Ptch receptor on the cell membrane to change the conformation of the receptor to activate the effector Gli1. Once translocated into the nucleus, Hh can regulate the transcription of a number of genes to impact the growth, proliferation and differentiation of cell [8]. Hh signal directs the differentiation of bone marrow mesenchymal stem cells (BMMSCs) toward osteoblasts (OB), not to fat [9]. In vitro studies showed that Ihh signaling promotes the differentiation of osteoblast progenitor cells toward cartilage osteoblasts [10, 11]. In this study, we investigated the effects and mechanism of Exendin-4 mediated Hedgehog/Gli1 signaling pathway on the differentiation of osteoblasts in vitro and the findings would provide insights into the bone metabolism following Exendin-4 treatment of diabetes.

Materials and methods

Cell line and culture

The osteoblastic cell line MC3T3-E1 was purchased from Yingniurui Biotechnology, Wuxi,

Table 1. Primers for qRT-PCR

Gene	Primer
Hedgehog	F: CCGCTACTCTACAAGCAATT
	R: GTCGGCTCCACTGTTCTC
GAPDH	F: GCAAGTTCAACGGCACAG
	R: CGCCAGTAGACTCCACGAC
Runx2	F: GGACTGGGTATGGTTTGTAT
	R: GCTGAAGAGGCTGTTTGA
OCN	F: ACCACATCGGCTTTCAGG
	R: CATAGGGCTGGGAGGTCA
GLP-1R	F: TCTCCAAACTGAAGGCTAAT
	R: ATTCTATCGCCACCTTCC
Gli1	F: TCCCACATCCTCAGTCCC
	R: GTCCATCACAAAGGCAAA

China. The cells were maintained in MEM-α medium supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA) and 1% streptomycin at 37°C in 5% CO_2. To investigate the effect of Exendin-4, the cells were either cultured in the above medium, or osteogenic media (the above medium with 50 ug/mL ascorbic acid and 10 m mol/L β-sodium glycerol phosphate) containing various concentration of Exendin-4 (0 to 10^{-7} M) as previously described [12].

Alkaline phosphate (ALP) activity assay

Cell homogenates were assayed for ALP activity using an ALP assay kit (Jiancheng Biotech Institute, Nanjing, China) following the supplier's instructions. Briefly, $1x10^5$ cells were harvested, washed twice with cold PBS and lysed with RIPA buffer that contains protease and phosphotase inhibitors cocktail (Roche, UK). The supernatants were collected after centrifugation at 12000 rpm for 20 min. The ALP activities in the cell supernatants were quantified after centrifugation, and the wavelength of spectrophotometer was 520 nm. The values were normalized to total protein concentration.

Alizarin red staining

Cells were washed with PBS (pH7.2, calcium- and magnesium-free), fixed in 70% ethanol for 60 min at room temperature. The fixed slides were washed twice with PBS (pH7.2, calcium- and magnesium-free) and stained with Alizarin red staining solution at 37°C for 60 min in the dark. After gently rinsed in double distilled water for 3-5 min, the slides were examined under microscope. Then, Alizarin red was destained with 10% cetylpyridinium chloride (Sigma, USA) for 30 min at room temperature. The OD value was read using an ELISA plate reader at 562 nm wavelength.

Transfection

Cells were grown to 80% confluency in MEM-α (Hyclone, USA) supplemented with 10% FCS at 37°C in 5% CO_2 cell culture incubator. The cells were transfected with siRNA and scramble control (GenePharma, Suzhou, China) at a final concentration of 100 nM using Lipofectamine 3000 reagent (Thermofisher, USA) according to the supplier's instructions. After 4 to 6 h, the cells were cultured in fresh complete medium. Since the calcium deposition in the MC3T3-E1 cells lasted for 21 days, the cells were subjected to three rounds of transfection, prior to performing experiments. The sequences of Gli1-siRNA and scramble control were Gli1-siRNA-F: 5'-GATCC CAT CCA TCA CAG ATC GCA TTT CTC GAG AAA TGC GAT CTG TGA TGG ATG TTTTT G, Gli1-siRNA-R 5'-AATTC AAA AAC ATC CAT CAC AGA TGG CAT TTC TCG AGA AAT GCGATCTGTGATGGATGG, scramble control-F: 5'-GGACGAGAATTCATCTATGTCCTGCTCCTAGGTAGATACA, scramble control-R: 5'-ACATAGATGAATTCGCTAGTATTGAGGATCCTCGCC.

Hedgehog receptor antagonist (Cy) treatment

Cells were seeded in the wells of 6-well plates at 10^4 cells/ml. After induced into osteoblasts as described above, 10U mol/L Cy (Sigma, USA) was added to each well and cultured for 3 days.

Fluorescent quantitative PCR

Total RNA was extracted using Zon Reagent TRIzon kit (CWBIO, Beijing, China) according to the manufacturer's instructions. The cDNA was synthesized using a reverse transcription system (Promega Corporation, USA) according to the manufacturer's recommendations. The qPCR Master Mix (Promega Corporation, USA) were used for real-time RT-PCR to quantify the expression of cDNA. PCR was performed on an Agilent Mx3000P QPCR Systems (Agilent, CA) using the primers listed in **Table 1**. The cycling conditions were: 95°C for 1 min, followed by 40 cycles of 95°C for 10 s, 52°C for 30 s and 72°C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as an internal con-

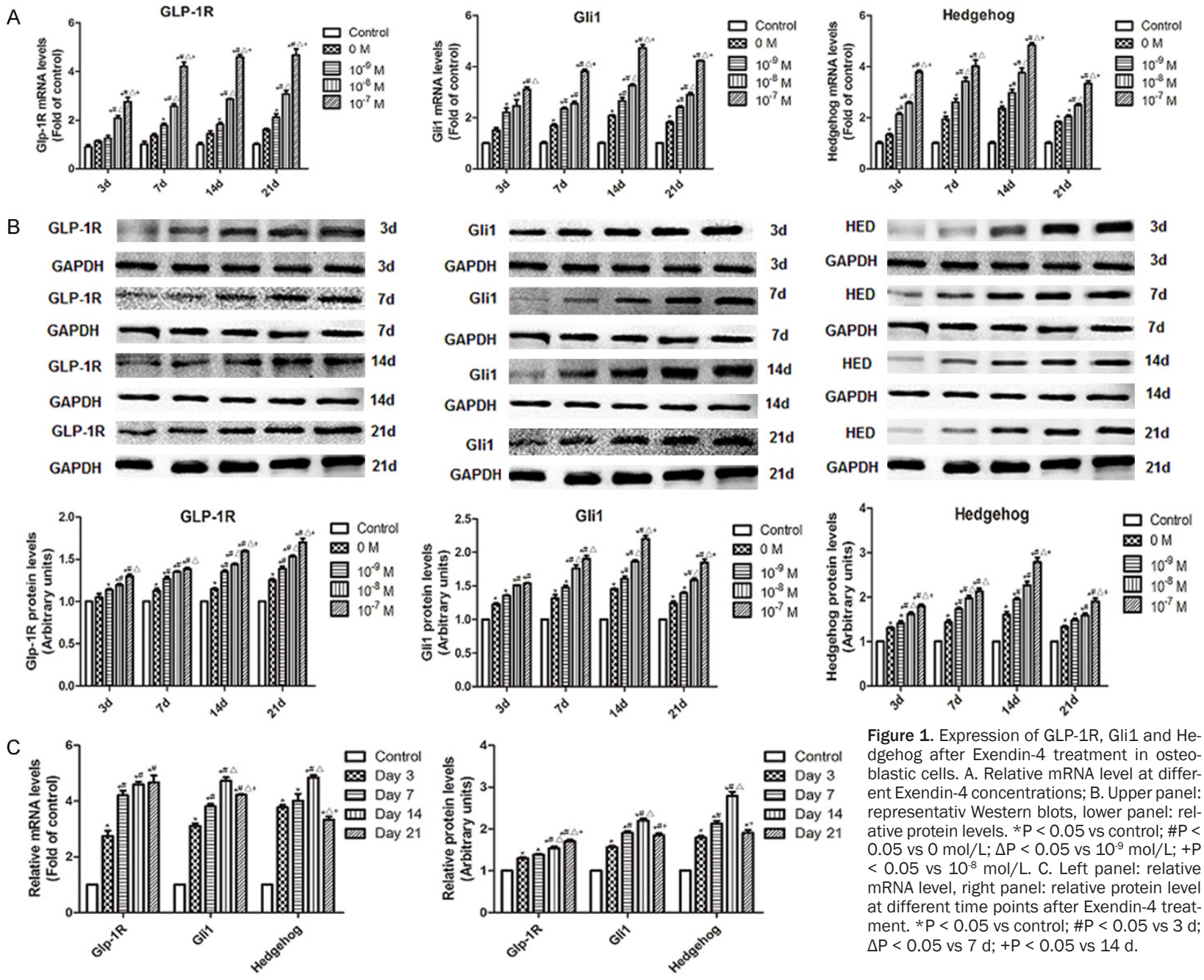

Figure 1. Expression of GLP-1R, Gli1 and Hedgehog after Exendin-4 treatment in osteoblastic cells. A. Relative mRNA level at different Exendin-4 concentrations; B. Upper panel: representativ Western blots, lower panel: relative protein levels. *P < 0.05 vs control; #P < 0.05 vs 0 mol/L; ΔP < 0.05 vs 10^{-9} mol/L; +P < 0.05 vs 10^{-8} mol/L. C. Left panel: relative mRNA level, right panel: relative protein level at different time points after Exendin-4 treatment. *P < 0.05 vs control; #P < 0.05 vs 3 d; ΔP < 0.05 vs 7 d; +P < 0.05 vs 14 d.

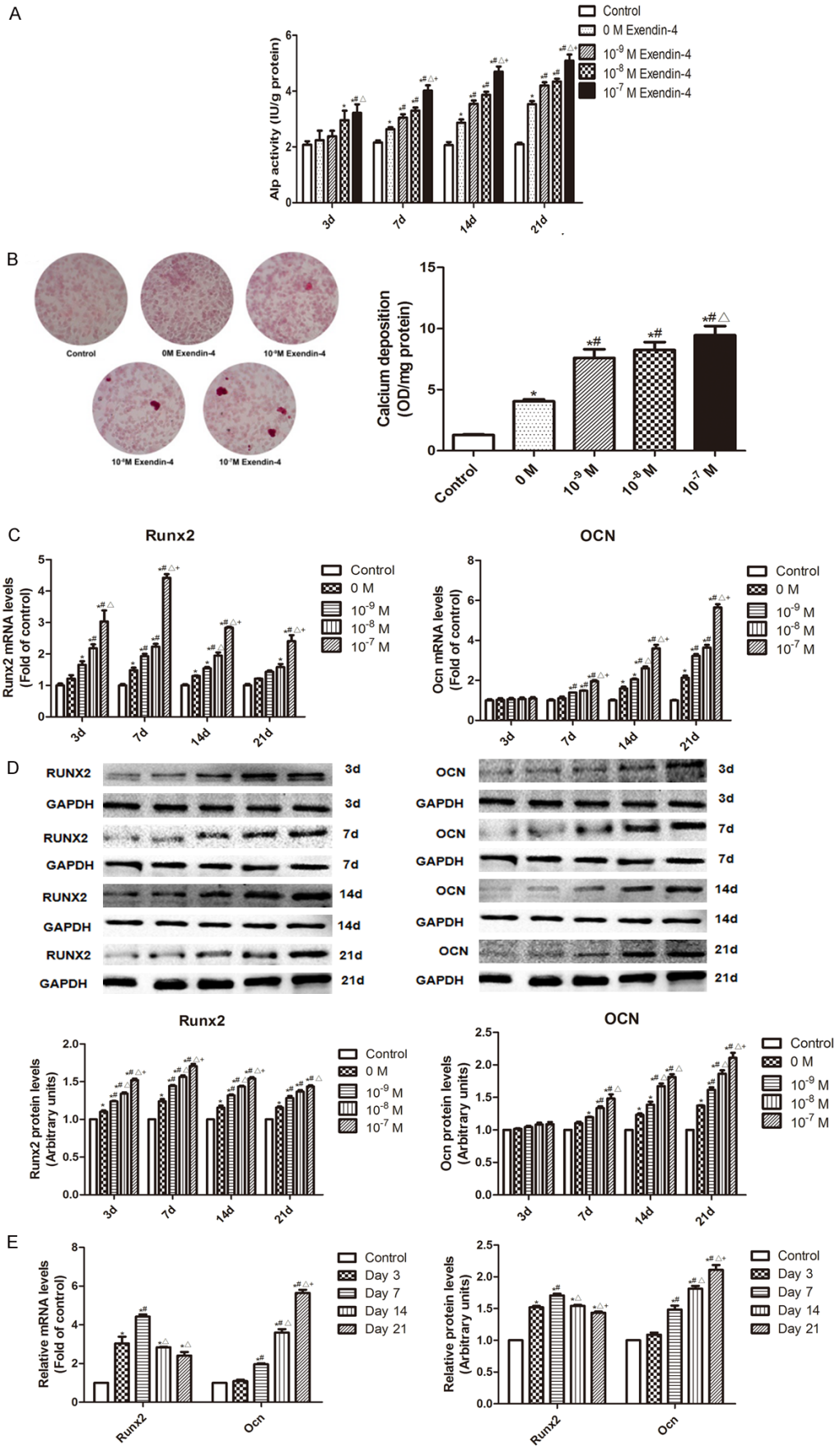
A
Control
0 M Exendin-4
10-9 M Exendin-4
10-8 M Exendin-4
10-7 M Exendin-4
Alp activity (IU/g protein)
3d
7d
14d
21d
B
Control
0M Exendin-4
10-9M Exendin-4
10-8M Exendin-4
10-7M Exendin-4
Calcium deposition (OD/mg protein)
Control
0 M
10-9 M
10-8 M
10-7 M
C
Runx2
Runx2 mRNA levels (Fold of control)
OCN
Ocn mRNA levels (Fold of control)
D
RUNX2
GAPDH
OCN
3d
7d
14d
21d
Runx2
Runx2 protein levels (Arbitrary units)
OCN
Ocn protein levels (Arbitrary units)
E
Relative mRNA levels (Fold of control)
Relative protein levels (Arbitrary units)
Day 3
Day 7
Day 14
Day 21
Runx2
Ocn

Figure 2. ALP activities, calcium deposition, expression of Runx2 and OCN in MC3T3-E1 cells after treatment of exendin-4. A: ALP activity, B: Left panel, Alizarin red staining, right panel: relative calcium deposition. C. Relative mRNA levels. D. Upper panel: representative Western blots, lower panel: relative protein level. *P < 0.05 vs control; #P < 0.05 vs 0 mol/L; ΔP < 0.05 vs 10^{-9} mol/L; +P < 0.05 vs 10^{-8} mol/L. E. Left panel: relative mRNA level, right panel: relative protein level at different time points after Exendin-4 treatment. *P < 0.05 vs control; #P < 0.05 vs 3 d; ΔP < 0.05 vs 7 d; +P < 0.05 vs 14 d.

trol. Relative expression was calculated by using comparative Ct method and obtaining the fold change value ($2^{-\Delta\Delta}$Ct) according to previously described protocol [13].

Western blot analysis

Cells were harvested, washed twice with cold PBS and lysed with RIPA buffer that containing protease and phosphotase inhibitors cocktail (Roche, UK). The supernatants were collected after centrifugation at 12000 rpm for 20 min. The protein was applied to polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane, and then incubated with the proper primary antibodies (rabbit anti-GLP-1R, anti-rat RUNX2, OCN, Gli1 and Hedgehog polyclonal antibodies, ABclonal, USA). Primary antibodies were detected using goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000, Santa Cruz Biotechnology, USA) and immunoreactive bands were visualized using Western Lighting Chemiluminescence Reagent Plus (PerkinElmer, USA) according to the manufacturer's instructions. The intensity of blot signals was quantitated using Quatity One 4.62 software (Bio-Rad, USA).

Statistical analysis

All data were expressed as means ± standard error of the mean (SEM) obtained from at least three independent experiments. Statistical comparisons between experimental and control groups were assessed by using the one way analysis of variance (ANOVA) followed by Student Newman Keuls (S-N-K). $P < 0.05$ was considered statistically significant.

Results

Exendin-4 up-regulated the expression of GLP-1R, Hedgehog and Gli1

The expression of GLP-1R, Hedgehog and Gli1 increased with the increasing concentration of Exendin-4 ($P < 0.05$) at mRNA and protein levels (**Figures 1A**, **1B**, S1). For GLP-1R, the expression increased over the experimental period, and for Gli1 and Hedgehog, the expression reached the highest on day 14 and then declined (**Figure 1C**).

Exendin-4 promoted osteoblast differentiation

Analysis showed that ALP activity began to increase after 3 day treatment in the osteoblastic cells and was significantly higher in the cells treated with 10^{-8} mol/L and 10^{-7} mol/L Exendin-4 than in control ($P < 0.05$). After induction for 7 days and longer, the activity was higher in Exendin-4-treated cells than control or untreated cells. In addition, the activity increased as the Exendin-4 concentration increased (**Figure 2A**). Alizarin red staining showed that the calcium deposition was significantly higher in Exendin-4-treated cells ($P < 0.05$) (**Figure 2B**). Mineralized nodule of osteoblast appeared after 3 day of Exendin-4 treatment at concentrations of 10^{-8} mol/L and 10^{-7} mol/L (**Figure 2B**). PCR and Western blot analyses showed that compared with control, the expression of Runx2 and osteocalcin (OCN) was up-regulated significantly ($P < 0.05$) in Exendin-4-treated cells and the up-regulation was higher at higher Exendin-4 concentrations (**Figures 2C**, **2D**, S2). At Exendin-4 concentration of 10^{-7} mol/L, the mRNA and protein levels of Runx2 reached the highest on day 7 and then decreased, while the levels of OCN increased significantly with treatment times ($P < 0.05$) (**Figure 2E**).

Gli1 knockdown offset Exendin-4-induced osteoblast differentiation

When the osteogenic cells were transfected with Gli1-siRNA, both Gli1 mRNA and proteins were significantly reduced ($P < 0.01$) as compared with scramble control and untreated control (**Figures 3A**, **3B**, S3). After the knockdown, the ALP activity was decreased in both scramble control and exendin-4-treated cells (**Figure 3C**), but not different between the control and scramble control. Similar changes were obse-

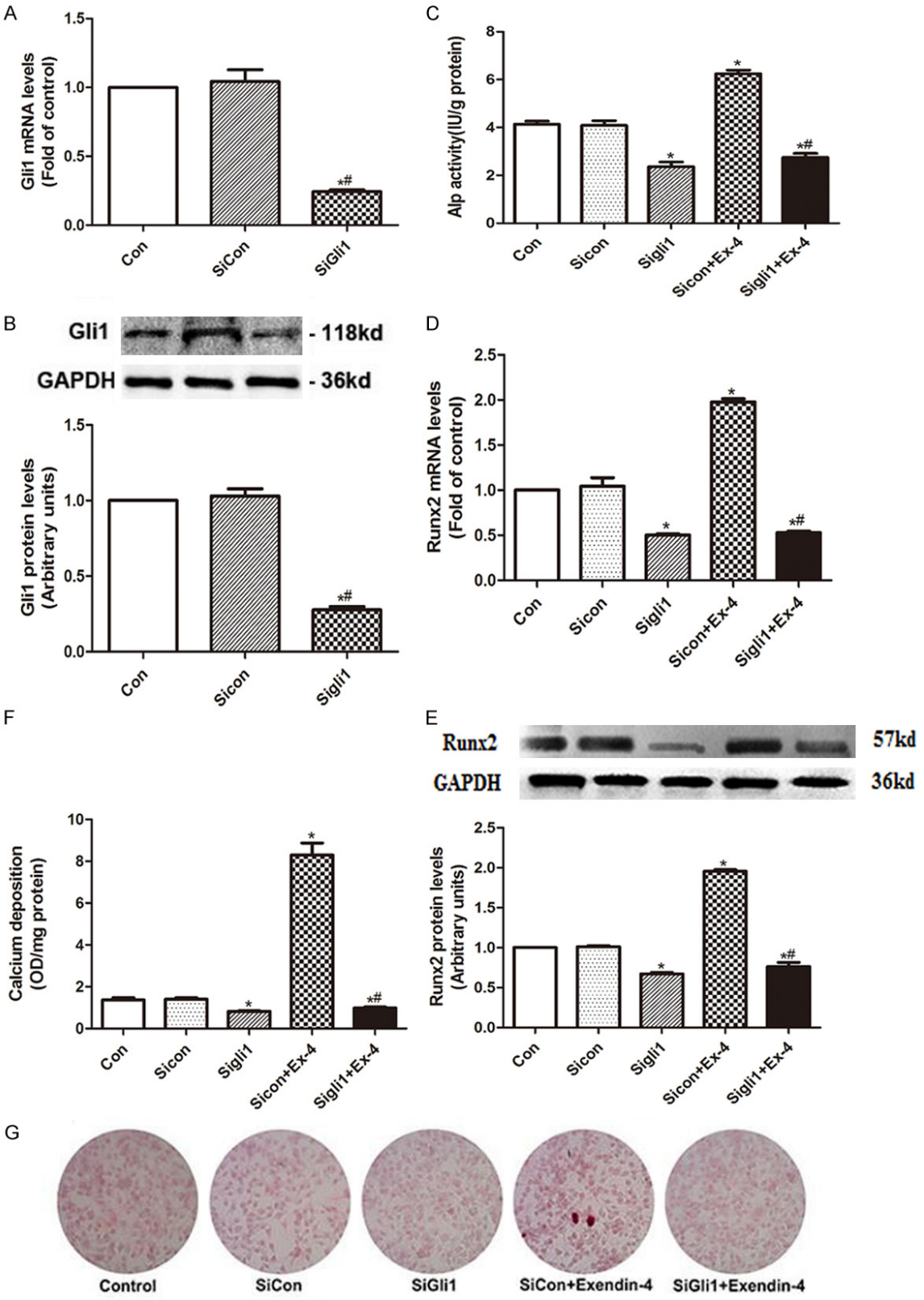

Figure 3. Expression of Gli1, Runx2 and calcium deposition in MC3T3-E1 cells following Gli1 knockdown. A. Relative mRNA level; B. Upper panel: representative Western blots, low panel: protein level; C. ALP activity; D. Relative mRNA level of Runx2; E. Upper panel: representative Western blots, low panel: protein level; F. Calcium deposition; G. Alizarin red staining. *P < 0.05 vs SiCon; #P < 0.05 vs SiCon+Exendin-4.

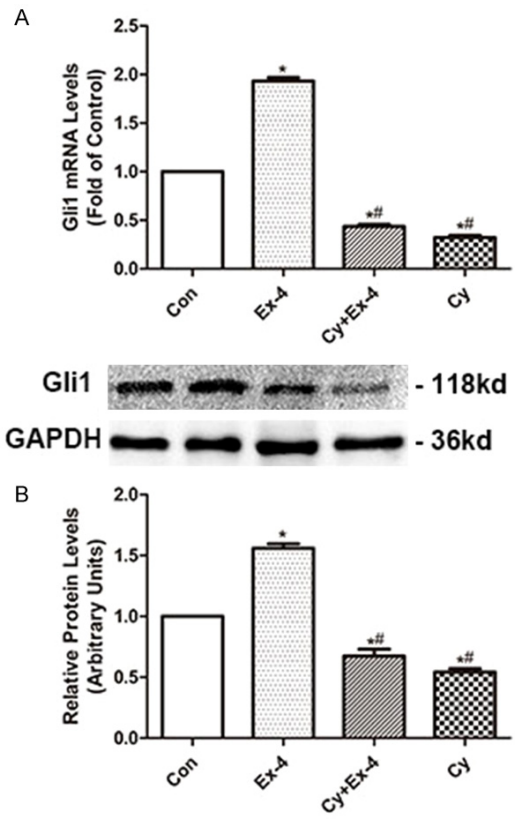

Figure 4. Expression of Gli1 in MC3T3-E1 cells following Cy treatment. A. Relative mRNA level, B. Upper panel: representative Western blots, low panel: protein level. *$P < 0.05$ vs control; #$P < 0.05$ vs Exendin-4 treatment.

rved for Runx2 expression at mRNA and protein level (**Figures 3D**, **3E** and S3) and calcium deposition measured by Alizarin red staining (**Figure 3F** and **3G**).

Cy offset Exendin-4-induced Gli1 up-regulation

Compared with Exendin-4-treated cells, Cy treatment down-regulated the expression of Gli1 at mRNA and protein level ($P < 0.05$, **Figures 4** and S4).

Discussion

Osteoporosis is characterized by reduced bone mass, thinning and fracture of trabecular bone and decreased quantity of bone. GLP-1 binds GLP-1R to glucagon-like peptide 1 receptor GLP-1R to exert its biological function. GLP-1R belongs to glucagon receptor subfamily in the 7 transmembrane receptor family, which is widely distributed in various human organs [14]. Recent studies have shown that GLP-1 bind GLP-1R to improve bone mass, microstructure and quality [15]. Yamada et al found that in GLP-1R knockout mice, the bone mass was reduced and brittleness and number of osteoclast were increased, while Exendin-4 was able to reverse the effect [16]. These studies indicate that GLP-1 is involved in the regulation of bone metabolism as well as the expression of GLP-1R. In order to investigate whether GLP-1R is expressed in osteoblast-like cells and the effect of Exendin-4 on the expression of GLP-1R, we determined the expression level of GLP-1R in MC3T3-E1 cells. We found that the expression of GLP-1R increased with the increasing concentrations and treatment time of Exendin-4, indicating that Exendin-4 up-regulates the expression of GLP-1R in a concentration and time-dependent manner.

When GLP-1 was infused subcutaneously into type 2 diabetic rat models for 3 days, the volume of trabecular bone, trabecular bone pattern factor and structure model index were markedly increased and bone formation was significantly increased, indicating that GLP-1 promotes bone formation [17]. Sun et al found that BMSCs from non-diabetic ovariectomized (OVX) rats differentiated toward osteoblast and lipocyte after Exendin-4 treatments with increased mRNA levels of Runx2, ALP and Coll-1 and decreased mRNA levels of PPARc and c/EBPA, suggesting that Exendin-4 preserves bone in the OVX rats via increasing osteogenic differentiation and inhibiting adipogenic differentiation [18]. Meng et al measured the mRNA and protein levels of abiogenic and osteogenic markers after treating BMSCs with Exendin-4 and found that Exendin-4 could promote the differentiation of BMSCs into osteoblasts and inhibit their adipogenic differentiation [19]. However, Hu et al found that GLP-1R agonists could reduce the osteogenic differentiation of MC3T3-E1 cells via the AMPK/mTOR signaling pathway [20]. Due to these inconsistencies, we investigated the expression of several classic osteogenic markers following exposure of MC3T3-E1 cells to Exendin-4 to verify whether the peptide can promote the osteogenic differentiation of MC3T3-E1 cells. It is generally believed that bone ALP is associated with calcification of bone, and in osteoblasts ALP produces phosphate, which deposits in the bone as calcium phosphate. Studies have shown

that the activity of ALP is elevated during osteogenic differentiation, which can serve as an indication of preosteoblast differentiation into osteoblast [18, 21]. Alizarin red forms chelation complexes with calcium in the calcium nodules on the surface of differentiated osteoblasts as orange-colored deposition, which can be semi-quantitatively determined spectrometrically as an indication of calcium salt in the cell matrix to measure the extend of osteoblast differentiation [22]. Our results showed that Exendin-4 increased the ALP activity in the cells in a time- and concentration-dependent manner. Seven days after Exendin-4 treatment at 10^{-7} mol/L, mineralized nodules were observed in the cells and calcium deposition was higher with increasing Exendin-4 concentration 21 days after Exendin-4 treatment. These findings indicate that Exendin-4 promotes the differentiation of preosteoblast to osteoblast and calcium deposition in a time and concentration-dependent manner.

In addition, Exendin-4 up-regulated the expression of Runx2 and OCN, two key osteogenic biomarkers. Runx2 is a transcription factor of Runt-related gene family [23]. It promotes the early osteogenic differentiation and is the earliest and most specific marker of osteoblast differentiation. Runx2 is also necessary for the differentiation of BMMSCs into bone. In RUNX2 deficient mouse or human, there is a lack of osteoblasts and cleidocranial dysplasia due to bone defect [24, 25]. OCN is a γ-carboxyglutamic protein, mainly synthesized and secreted by non-proliferating osteoblast. It is accumulated after mineralization peak period in the bone and regulated by Runx2. Therefore, it is also a specific marker for bone differentiation [26]. Meng et al found that the expression of Runx2 and OCN was significantly up-regulated 7 and 28 days after treatment with Exendin-4, respectively [19]. Similarly, we found that the expression of the two genes increased with increasing concentration and time of Exendin-4 treatment. The Runx2 reached the highest level 7 days after Exendin-4 treatment and OCN reached the peak in 21 days. Therefore, it is likely that Exendin-4 may up-regulate RUNX2 in early stage, OCN in later state of osteoblast differentiation of MC3T3-E1 cells, suggesting that Exendin-4 functions in various stage of osteoblasts differentiation to promote osteogenic differentiation in a concentration-dependent manner.

Hedgehog/Gli1 signal pathway is closely related to osteogenesis. It not only plays an important role during the development of bone tissue, but also promotes differentiation of osteoblast-related cells, regulates the formation of osteoblast and angiogenesis in the bone [27]. It has been shown that as a conserved and important pathway, the Hedgehog signaling pathway plays an important role in bone formation and metabolism [28] and transient inhibition of the pathway would result in permanent deficiency of in the bone structure [24]. Wang et al found that Hedgehog signaling pathway promoted the differentiation of MSCs to osteoblasts and chondrocytes [29]. Chávez et al used Hedgehog pathway inhibitor Cy in cultured bone cells isolated from the parietal bone of neonatal rats and found that the proliferation and differentiation of the bone cells were inhibited [30], indicating that the Hedgehog pathway is involved in the regulation of proliferation and differentiation of osteoblasts. Our results showed that the expression of Hedgehog and Gli1 increased with the increasing concentration of Exendin-4 and reached peaks 14 days after Exendin-4 treatment. Therefore, Exendin-4 may regulate bone metabolism via up-regualting Hedgehog and Gli1.

Activation of the Hedgehog/Gli1 signaling pathway has been shown to promote bone formation by upregulating the expression of osteotropic cytokines such as Runx2 that increases bone metabolism and mass [31]. In Gli1 knockout mouse, Runx2 was not expressed in the chondrocyte, resulting in obvious bone defects [32]. In addition, if there is a mutation in the Gli1 binding site of RUNX2 promoter region that prevents Runx2 from binding Gli1, the transcription level of RUNX2 was significantly decreased [33]. These results suggest that Gli1 indirectly influence the regulation of Runx2-regulated genes during osteogenic differentiation. In order to further clarify whether Exendin-4 impacts the differentiation of pre osteoblast to osteoblast via Gli1, we knockdown the expression of Gli1 using siRNA. It was found that after the knockdown of Gli1, Exendin-4 was no longer able to up-regulation the expression of RUNX2, suggesting that the up-regulation is mediated by Gli1. Furthermore, after the knockdown, ALP activity and alizarin red staining were no longer increased after Exendin-4 treatment in the MC3T3-E1 cells. Previous studies have shown that the

Hedgehog/Gli, TGFβ-Smads and RTK-PI3K-AKT signaling pathways can up-regulate the expression of Gli1 to regulate cell signaling [34]. Therefore, in order to determine whether Exendin-4-induced up-regulation of Gli1 is mediated by the Hedgehog signaling pathway, the Hedgehog signal pathway blocker Cy was used block the pathway. Analysis showed that after the block, Exendin-4-induced up-regulation of Gli1 disappeared, demonstrating that the up-regulation is mediated by the Hedgehog signal pathway. Taken together, our findings have demonstrated that Exendin-4 promotes the differentiation of MC3T3-E1 cells to osteoblasts by up-regulating the expression of Gli1 via the Hedgehog signaling pathway.

In conclusion, we have shown that Exendin-4 can increase the ALP activity and mineralized nodule in osteoblast, and up-regulate the expression of GLP-1R, Hedgehog, Gli1, RUNX2 and OCN to promote the differentiation of preosteoblast towards osteoblast via the Hedgehog/Gli1 pathway.

Disclosure of conflict of interest

None.

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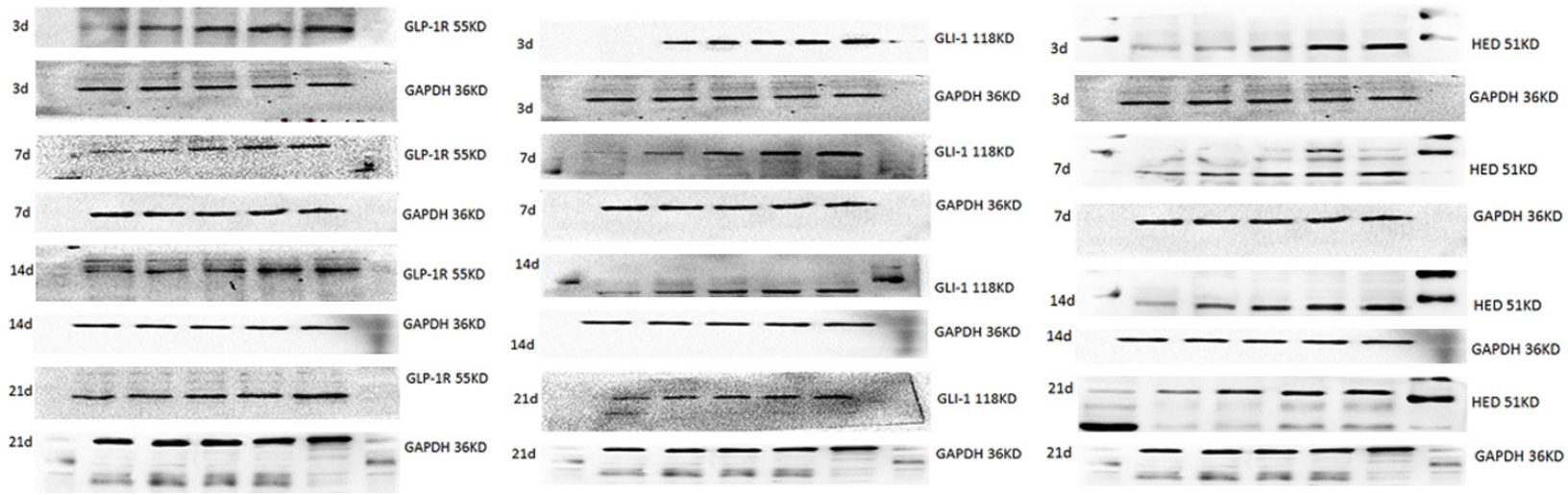

Figure S1. Original Western blots of GLP-1R, Gli-1 and HED after Exendin-4 treatment in osteoblastic cells.

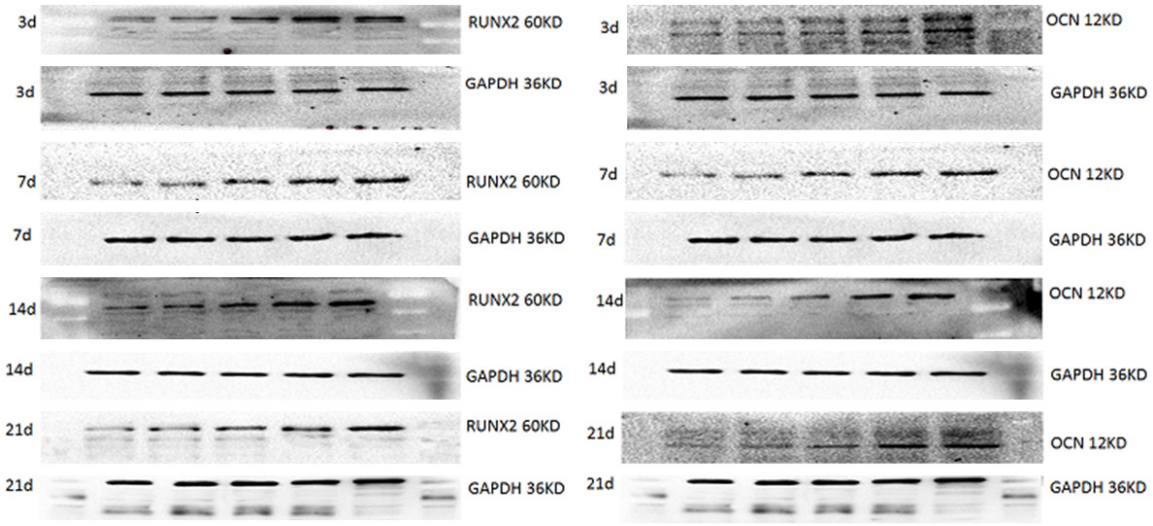

Figure S2. Original Western blots of RUNX2, OCN after Exendin-4 treatment in osteoblastic cells.

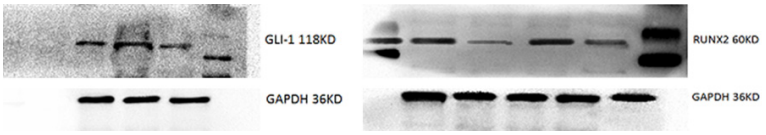

Figure S3. Original Western blots of Gli1 and Runx2 in MC3T3-E1 cells following Gli1 knockdown.

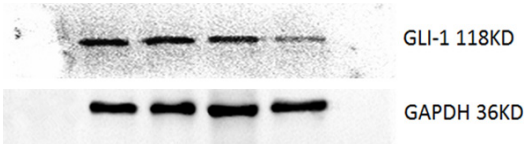

Figure S4. Original Western blots of Gli1 in MC3T3-E1 cells following Cy treatment.