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Liraglutide regulates proliferation, differentiation, and apoptosis of preosteoblasts through a signaling network of Notch/Wnt/Hedgehog signaling pathways

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Abstract. – OBJECTIVE: This study aims to investigate whether liraglutide can affect proliferation, osteogenic differentiation and serum deprivation-induced apoptosis of preosteoblast cell line MC3T3-E1 through the Notch, Wnt/β-catenin, and Hedgehog (Hh) signaling pathways.

MATERIALS AND METHODS: MC3T3-E1 cells were exposed to different treatments (via Notch inhibitor DAPT, an Hh inhibitor cyclopamine, or serum deprivation) or transfections of different siRNAs (targeting glucagon-like peptide-1 receptor (GLP-1R), β-catenin, or Gli1) in the presence or absence of 100 nM liraglutide. Cell proliferation, mRNA levels of osteogenic differentiation-related genes, mRNA and protein levels of the Notch and Hh signaling pathway proteins, and apoptosis-related proteins were assessed.

RESULTS: Liraglutide significantly increased proliferation of MC3T3-E1 cells, expression levels of the Notch and Hh signaling pathway proteins and β-catenin, and mRNA levels of osteogenic differentiation-related genes and TCF7L2. Moreover, liraglutide promoted a translocation of β-catenin, increased a ratio of Bcl-2/Bax proteins, reduced serum deprivation-induced apoptosis of MC3T3-E1 cells, and a ratio of caspase-3/procaspase-3. However, a cotreatment with liraglutide and DAPT reversed the alterations. A cyclopamine treatment and knockdowns of GLP-1R, Gli1, and β-catenin significantly reduced the expression of Notch proteins. Furthermore, the knockdown of GLP-1R, β-catenin, or Gli1 significantly increased apoptosis, which could be inhibited by liraglutide.

CONCLUSIONS: In summary, liraglutide can promote proliferation and differentiation of MC3T3-E1 cells, and inhibit their serum deprivation-induced apoptosis by activating both the Notch and Hh signaling pathways involving β-catenin and Gli1. These results provide a therapeutic foundation that patients with diabetes and osteoporosis may be cured with treatments of liraglutide.

Key Words:

Glucagon-like peptide-1, Liraglutide, Osteogenic differentiation, Apoptosis, Notch signaling pathway, Hedgehog signaling pathway..

Abbreviations

T1DM: type 1 diabetes mellitus; GLP-1 RA: glucagon-like peptide-1 receptor agonist; GLP-1R: glucagon-like peptide-1 receptor; GLP-1: glucagon-like peptide-1; Sox9: SRY box-containing gene 9; Runx2: Runt-related transcription factor 2; BMP: bone morphogenetic proteins; TGF: tumor growth factor; MAPK: mitogen-activated protein kinase; PDGF: platelet-derived growth factor; IGF: insulin-like growth factor; FGF: fibroblast growth factor-2; NICD: Notch's intracellular domain; TGFβ: transforming growth factor β; BMD: bone mineral density; OPG: osteoprotegerin; Col I: collagen type I; ERK: extracellular signal regulated kinase; Dlk1: δ-like homolog 1; H: heterodimerization domain; NRR: negative regulatory region; RAM: Rbpjκ association module; BMSC: bone mesenchymal stem cells; RANKL: receptor activator of nuclear factor κB ligand; OC: osteocalcin; Sufu: suppressor of fused; Kif7: kinesin family protein 7; IGF-1: insulin-like growth factor-1; PPARγ: peroxisome proliferator activated receptor gamma; TZD: thiazolidinedione; CSF: colony-stimulating factor; PI3K: phosphoinositide 3-kinase; Hh: Hedgehog; GLI1: glioma-associated oncogene 1; Ptch1: patched 1 gene; FITC: fluorescein isothiocyanate; PI: propidium iodide; CO_2: carbon dioxide.

Introduction

According to the International Diabetes Federation Diabetes Atlas (9th Edition, 2019), approximately 463 million adults (20-79 years) worldwide have suffered from diabetes, among whom 54.5 million are Chinese[1]. The complica-

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tions of diabetes include cardiovascular events, nephropathy, retinopathy, peripheral neuropathy, and osteoporosis, which severely reduce the quality of life of patients[1]. Diabetes can increase bone resorption and reduce bone formation, leading to osteoporosis[2]. Osteoporosis, one of the most common diseases that seriously threaten the health of middle-aged and elderly people, is characterized by systemic damages to bone mass, strength, and microstructures, therefore, increasing the risk of fragile fractures[3]. Diabetes, especially type 2 diabetes, and osteoporosis are two common metabolic diseases associated with morbidity and mortality of elderly patients; incidentally, these two diseases share many similar characteristics, including genetic susceptibility, molecular mechanisms, and pathophysiology[3].

Glucagon-like peptide-1 (GLP-1) is a 30-amino-acid peptide hormone produced by intestinal epithelial endocrine L cells. The GLP-1 receptor (GLP-1R) is a membrane-bound cell surface protein that is coupled with stimulatory G proteins and adenylate cyclase[4]. GLP-1 may affect the adipose-bone axis by promoting osteogenic differentiation and inhibiting adipogenic differentiation of bone mesenchymal precursor cells expressing GLP-1R. Several GLP-1R agonists (GLP-1 RAs) are currently used to treat type 2 diabetes and obesity[5]. GLP-1 RAs may also affect a balance between osteoclasts and osteoblasts, leading to more bone formation and less bone resorption[6].

Osteoblasts, the key cells involved in bone formation, are differentiated from preosteoblasts. Mature osteoblasts can be distinguished between the lining cells of stationary bones and the embedded cells in the bone matrix. Embedded cells are characterized by a dendritic appearance and play a key role in mechanical force transduction and bone reconstruction[7]. Mature osteoblasts may die from apoptosis[8]. Studies have shown that such apoptosis is involved in various osteogenic differentiation stages of many signaling pathways, such as Wnt/β-catenin, Notch, bone morphogenetic protein (BMP)/transforming growth factor (TGF)-β/phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR), mitogen-activated protein kinase (MAPK), Hedgehog (Hh), platelet-derived growth factor, insulin-like growth factors, fibroblast growth factor-2, and Ca^{2+} pathways[9]. These signaling pathways are presumed to interact during osteogenic differentiation.

The Wnt signaling pathway regulates various cellular events, such as proliferation, migration, polarization, and gene expression. Wnt is a key regulator of mouse and human osteoblast differentiation and activity[10]. The classic Wnt pathway leads to stabilization of β-catenin and its transfer to the nucleus. β-catenin is an important transcriptional coactivator that regulates the transcription of target genes for Wnt signaling. The importance of Wnt signaling during bone formation has been well studied[11]. β-catenin is necessary for osteogenesis and works in multiple stages of osteoblast differentiation to regulate osteoblasts and osteoclasts[12-19].

Notch signaling is a highly conserved pathway regulating cell proliferation and differentiation. It participates in cellular events in embryos and tissues, including development and regeneration of bone tissues[20]. The Notch signaling pathway consists of following four parts: ligands, receptors, DNA-binding proteins, and downstream-regulated genes. Four Notch receptors (Notch1, Notch2, Notch3, and Notch4) exist in humans and mice. These receptors initiate signaling by directly binding to Jagged ligands (Jag1 or Jag2) or delta-like ligands (Dll1, Dll2, and Dll3) in neighboring cells[21]. The interactions result in the cleaving of Notch receptors through the γ-secretase complex, releasing a Notch's intracellular domain (NICD) into the cytoplasm. NICD then activates classical and nonclassical Notch signaling mechanisms, resulting in the transcription of its downstream target genes, such as hairy enhancers of split family genes (Hes1, Hes5, and Hes7) and Hes-related YRPF motif family genes (Hey1, Hey2, and HeyL)[22]. The role of the Notch signaling pathway in skeletal development and homeostasis has been demonstrated in transgenic mouse, and Notch mutations were found to be related to certain genetic skeletal diseases[20]. Notch signaling is abnormal in osteosarcoma and osteoarthritis, and animal model experiments have indicated that Notch signaling plays an important role in skeletal development and functions[23]. Notch1 can induce osteoprotegerin (OPG) in bone cells, inhibit Wnt antagonists sclerostin and Dkk1, and activate Wnt/β-catenin signaling[24]. Moreover, Notch can directly inhibit Wnt signaling in stromal cells and osteoblasts, thus may impair osteoblast maturation[25,26]. Phenotypes overlap between Notch1- and β-catenin-activated osteocytes. Furthermore, Wnt signaling not only

enhances osteoblast formation, but also directly inhibits osteoclast formation by inducing OPG to indirectly affect osteoclast precursors[27].

The Hh signaling pathway is an evolutionarily conserved pathway. It plays an important role in the normal embryonic development of invertebrates and vertebrates[28]. Three Drosophila Hh homologs [Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh)] exist in mammals[29,30]. The activation of Hh signaling involves following three proteins: Hh ligand, Ptch protein, and Smo protein. Ptch is a receptor of the Hh protein. The main target genes of the Hh signaling pathway include Patched 1 gene (Ptch1), Ptch2, and Gli1. An increased mRNA expression level of Ptch1, Ptch2, and Gli1 is an exact sign of activation of the Hh signaling pathway, which regulates Hh signals through negative (Ptch1) and positive (Gli1) feedback mechanisms[31]. Hh proteins are produced by differentiated mesenchymal cells during bone formation. The existence of Hh proteins drive or maintain cell differentiation in these cells, thus regulating cartilage and bone formation, which are particularly important for osteoblast maturation[32,33]. Hh signaling activation can increase osteoblast production and osteoblast activity in rodent system cell lines[34]. For example, an activation of Hh signaling in osteoblasts has resulted in an increase in matrix deposition at the fracture site in mice, and an *in vitro* osteoblast culture system has confirmed that the activation of this pathway corresponds to enhanced osteoblast activity[35].

Our previous study has found that liraglutide, a long-acting GLP-1RA that continuously activates GLP-1R, promotes proliferation and osteogenic differentiation of preosteoblast MC3T3-E1 cells by activating the PI3K/Akt signaling pathway through GLP-1R and β-catenin[36]. We also have demonstrated that liraglutide inhibits serum deprivation-induced apoptosis of MC3T3-E1 cells through the cAMP/protein kinase A (PKA)/β-catenin and PI3K/Akt/glycogen synthase kinase-3β (GSK3β) pathways[37]. As mentioned above, many signaling pathways participate in bone differentiation, and a signaling network may be formed between signaling pathways. Therefore, we wonder whether the Notch and Hh signaling pathways can regulate proliferation, osteogenic differentiation, and apoptosis of the preosteoblast cell line MC3T3-E1 by liraglutide. The present study aims to explore whether the Notch, Wnt/β-catenin, and Hh signaling pathways affect the regulation of MC3T3-E1 cells by liraglutide.

Materials and Methods

Cell Culture and Treatments

MC3T3-E1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) (CRL-2594) and cultured in an alpha minimal essential medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco, Life Technologies, Rockville, MA, USA) and 1% penicillin-streptomycin at 37°C with 5% carbon dioxide (CO_2). The cells were exposed to different treatments (e.g., *via* a Notch inhibitor DAPT, an Hh inhibitor cyclopamine, siRNA transfections, or serum deprivation) in the presence or absence of 100 nM liraglutide at different growth stages , as indicated in the previous work[36,37].

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cells by using the MiniBEST Universal RNA Extraction Kit (TaKaRa, Otsu, Shiga, Japan) and then reverse-transcribed with the TIANScript RT Kit (Tiangen, Beijing, China) following the manufacturers' instructions. PCR was performed at a total volume of 20 µL containing 10 µL of GoTaq® qPCR Master Mix (Promega, Madison, WI, USA), by using the BIO-RAD CFX96 Touch q-PCR system (Hercules, CA, USA) programmed as follows: 95°C for 10 min; 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 15 s, and extension at 72°C for 20 s, then, finalized with a gradient increase in temperature from 65°C to 95°C at 0.5°C/5 s. The relative expression of each gene was determined using a $2^{-\Delta\Delta}$Ct method and normalized to GAPDH mRNA. The primers used in this study are shown in Table I.

Western Blot

The collected cells were lysed in radioimmunoprecipitation assay lysis buffer containing a proteinase inhibitor cocktail (Beyotime, Shanghai, China), and the supernatants were collected. The protein concentration was determined using a bicinchoninic acid protein assay kit (Beyotime, Shanghai, China) following the manufacturer's instructions. Equal amounts of proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then transferred to polyvinylidene difluoride membranes by using a transfer apparatus (Tanon, Shanghai, China), and blocked with 5% nonfat milk for 1 h at room temperature. The membrane was incubated with specific primary antibodies (β-catenin, Protein-

Table I. Primers in this study.

Gene name		5′-3′ Sequence	Size
GAPDH	Forward	TGTTTCCTCGTCCCGTAGA	161 bp
	Reverse	GATGGCAACAATCTCCACTTTG	
GLP-1R	Forward	TCTGGCTACATAAGGACAACT	178 bp
	Reverse	CCAACAAGGATGGCTGAA	
β-catenin	Forward	AAGAAGCCCCGTGTTTTGACA	172 bp
	Reverse	TGCCCCACATCTCTCAGGG	
Gli1	Forward	GACTTTCTGGTCTGCCCTTTT	155 bp
	Reverse	AGCCCGCTTCTTTGTTAATTTGA	
Col1	Forward	CGCCATCAAGGTCTACTGC	148 bp
	Reverse	GAATCCATCGGTCATGCTCT	
OC	Forward	GAGGGCAATAAGGTAGTGAA	160 bp
	Reverse	CATAGATGCGTTTGTAGGC	
RUNX2	Forward	AGTCCCAACTTCCTGTGCT	243 bp
	Reverse	GGTGAAACTCTTGCCTCGTC	
OPG	Forward	AGGGCGTTACCTGGAGAT	152 bp
	Reverse	AGGGTGCTTTCGATGAAG	
GLP-1R	Forward	TCTGGCTACATAAGGACAACT	178 bp
	Reverse	CCAACAAGGATGGCTGAA	
TCF7L2	Forward	AAGCCTCCAGAGCAGACAAA	191 bp
	Reverse	TTTTGGGGTCTACGTCAGCT	
Notch1	Forward	GATCACATGGACCGATTGCC	175 bp
	Reverse	GATTGCCCAGGTAGCCATTG	
Jag1	Forward	GTCCCACTGGTTTCTCTGGA	244 bp
	Reverse	ATATACCGCACCCCTTCAGG	
Hes1	Forward	CGAGCGTGTTGGGGAAATAC	156 bp
	Reverse	GGTAGGTCATGGCGTTGATC	
Shh	Forward	GCTGACCCCTTTAGCCTACA	168 bp
	Reverse	GCTCCCGTGTTTTCCTCATC	
OCN	Forward	AAGCCTTCATGTCCAAGCAG	173 bp
	Reverse	GCGTTTGTAGGCGGTCTTC	

tech, Wuhan, China: 1:1000; GLP-1R, Proteintech, 1:1000; GAPDH, Proteintech, 1:5000; Notch1, Proteintech, 1:1000; Jag1, Affinity, 1:1000; Hes1, Affinity, 1:1000; Gli1, Proteintech, 1:2000; Shh, Proteintech, 1:600; Bax, Proteintech, 1:1000; Bcl-2, Proteintech, 1:1000; caspase3, Proteintech, 1:1000) overnight at 4°C. Subsequently, the membrane was incubated with horseradish peroxidase-labeled secondary antibodies (1:5000 dilution) for 60 min at room temperature. The bands were visualized using an enhanced chemiluminescence kit (Santa Cruz Biotechnology, USA) and photographed with the Tanon1600 Imaging system (Tianneng Company, Shanghai, China). The housekeeping protein GAPDH was used as an internal control.

Cell Viability Assays

The MC3T3-E1 cells were seeded at a rate of 1 × 10^5 cells/well in 96-well plates. The cells were incubated with the Notch signaling pathway and γ-secretase inhibitor DAPT (50 μM) in the presence or absence of 100 nM liraglutide for 72 h. After each treatment, 20 μL of freshly prepared 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/mL in phosphate-buffered saline) was added to the plates and incubated at 37°C for 4 h. Then, the supernatants were discarded, and 150 μL of dimethyl sulfoxide was added to dissolve the crystals. Finally, an optical density value of each well at 490 nm was measured using a microplate spectrophotometer (BioTek Instruments, San Jose, CA, USA).

Knockdown of GLP-1R, β-Catenin, and Gli1 by Using Small Interfering RNA (siRNA)

Table II presents a total of nine siRNAs that target GLP-1R, β-catenin, and Gli1 (three siRNAs per gene) and a negative control (NC) siRNA. All siRNAs were synthesized by GenePharma Biotechnology (Shanghai, China). The MC3T3-E1 cells were seeded in a 6-well plate and cultured for 24 h without antibiotics, then transfected with siRNA (50 pmol/well) by using a Lipofectamine 2000 regent (Invitrogen, Life Technologies, Grand Island, NY, USA) following

Table II. SiRNAs in this study.

Name	Sequence (5′-3′)
NC siRNA	UUCUCCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT
Mus GLP-1R siRNA1	GGAACUACAUCCACCUGAATT UUCAGGUGGAUGUAGUUCCTT
Mus GLP-1R siRNA2	GUGCAAGACUGACAUCAAATT UUUGAUGUCAGUCUUGCACTT
Mus GLP-1R siRNA3	GACUGGUGUUCCUGCUCAUTT AUGAGCAGGAACACCAGUCTT
Mus Gli1 siRNA1	CGGAGUUCAGUCAAAUUAATT UUAAUUUGACUGAACUCCGTT
Mus Gli1 siRNA2	CCACAAGUCAAUAGCUAUATT UAUAGCUAUUGACUUGUGGTT
Mus Gli1 siRNA3	GUUCAGUCAAAUUAACAAATT UUUGUUAAUUUGACUGAACTT
Mus β-catenin siRNA1	GGUUGCUUUGCUCAACAAATT UUUGUUGAGCAAAGCAACCTT
Mus β-catenin siRNA2	GGGUGCUAUUCCACGACUATT UAGUCGUGGAAUAGCACCCTT
Mus β-catenin siRNA3	GCAGAAUACAAAUGAUGUATT UACAUCAUUUGUAUUCUGCTT

the manufacturer's instructions. The cells were cultured for 48 h. The siRNA silencing efficiency was determined by qRT-PCR and Western blot for further experiments.

Immunofluorescence Assays

The treated MC3T3-E1 cells were fixed in 4% paraformaldehyde, washed with 0.1% bovine serum albumin in phosphate-buffered saline, and permeabilized with 0.5% Triton X-100. After blocking with goat serum for 1 h. The cells were incubated with anti-β-catenin antibody (Proteintech, 1:100) overnight at 4°C, followed by Alexa Fluor®594-conjugated goat anti-rabbit IgG(H+L) (Proteintech, Wuhan, China) for 1 h. Then, the cells were incubated with DAPI for 5 min. The images were observed under an UltraVIEW VoX–IX81 confocal laser scanning microscope (Olympus, Tokyo, Japan).

Cell Apoptosis Based on Flow Cytometry

Cell apoptosis was determined using an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (Beyotime, Shanghai, China) following the manufacturer's protocol. Briefly, the cells were collected and suspended with 195 µL annexin V-FITC binding buffer at a concentration of 50,000–100,000 cells. Then, the cells were incubated with 5 µL annexin V-FITC and 10 µL PI for 15 min at room temperature and analyzed using a CytExpert flow cytometer (Beckmann Coulter, Miami, FL, USA).

Statistical Analysis

Data were analyzed using the Graphpad Prism software (Version 6.0; San Diego, CA, USA) and expressed as mean ± standard deviation. All experiments were repeated three times. The groups were compared using one-way analysis of variance with Tukey's post-hoc analysis. In the analysis, a *p*-value less than 0.05 was considered statistically significant.

Results

Liraglutide Promoted Proliferation and Osteogenic Differentiation Through the Notch Signaling Pathway Via GLP-1R

The expression of the Notch signaling proteins Notch1, Jag1, and Hes1 was initially detected in cells treated with liraglutide for 60 min. Liraglutide significantly increased the protein expression levels of Notch1, Jag1, and Hes1, which reached a peak at 20 min after the treatment and then gradually decreased (Figure 1A). However, their protein expression levels at 60 min were significantly higher than those of the control group. The data suggest that liraglutide can activate the Notch signaling pathway.

Then, the Notch signaling inhibitor DAPT was used to further investigate the probable involvement of the Notch signaling pathway. Liraglutide significantly increased proliferation of the MC3T3-E1 cells and the mRNA levels of colla-

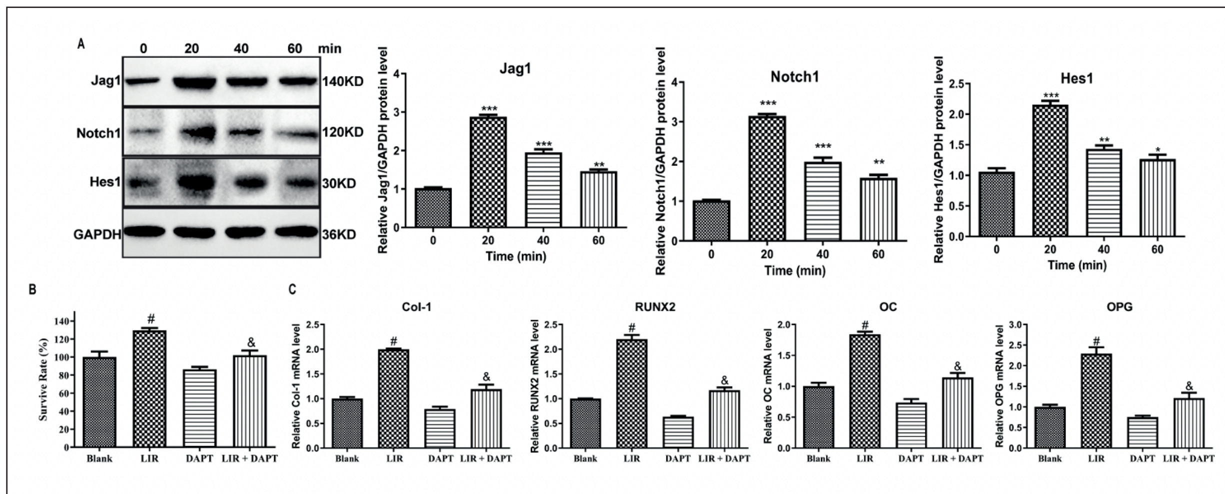

Figure 1. Liraglutide promoted proliferation and osteogenic differentiation of MC3T3-E1 cells through the Notch signaling pathway. **A,** MC3T3-E1 cells were treated with 100 nM liraglutide. The expression of Notch-related proteins (Notch1, Jag1, and Hes1) was determined by Western blot at the indicated time points. **B, C,** MC3T3-E1 cells were treated with the Notch inhibitor DAPT (50 μM) in the presence or absence of 100 nM liraglutide. The cell viability (**B**) and mRNA levels of osteogenesis-related genes (**C**) were assessed at 48 h after treatment. Col-1, collagen-1; RUNX2, runt-related transcription factor 2; OC, osteocalcin; OPG, osteoprotegerin. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared with the control group (0 nM); $^{\#}p < 0.05$ compared with the blank group; $^{\&}p < 0.05$ compared with the LIR group.

gen 1 (Col-1), osteocalcin (OC), runt-related transcription factor 2 (RUNX2), and OPG, whereas a cotreatment with DAPT and liraglutide reduced cell proliferation and mRNA levels of these four genes (Figures 1B and 1C). These results indicate that DAPT can reduce the increased proliferation and osteogenic differentiation of MC3T3-E1 cells by liraglutide.

Finally, we investigated whether GLP-1R was involved in the activation of the Notch signaling pathway by liraglutide *via* a siRNA knockdown. The knockdown efficiency of three siRNAs that targeted GLP-1R was evaluated, and siRNA1 was selected for further experiments (**Supplementary Figure 1**). As opposed to the NC siRNA combined with liraglutide, a knockdown of GLP-1R significantly decreased the expression of Notch1, Jag1, and Hes1 (Figure 2). This indicates that the activation of the Notch signaling pathway by liraglutide depends on GLP-1R.

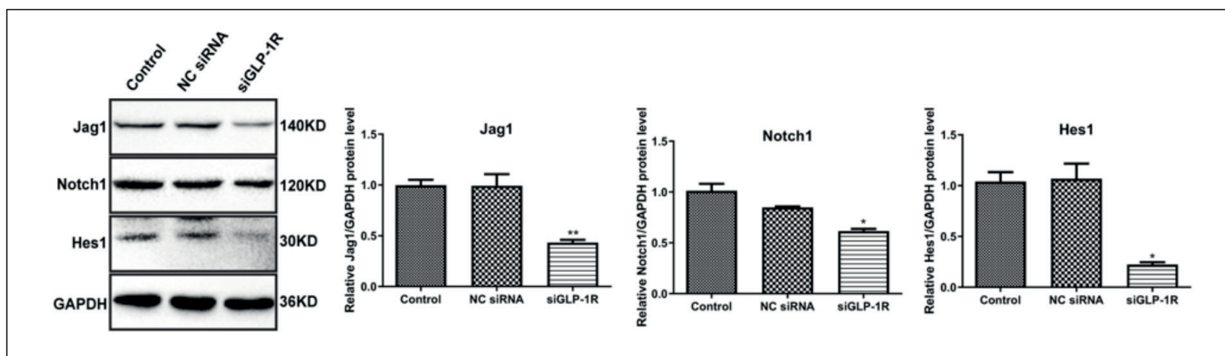

Figure 2. Knockdown of GLP-1R inhibited the activation of the Notch signaling pathway by liraglutide in MC3T3-E1 cells. MC3T3-E1 cells were transfected with NC siRNA or GLP-1R siRNA in the presence of 100 nM liraglutide. The expression of Notch-related proteins (Notch1, Jag1, and Hes1) was showed using Western blot at the indicated time points. Representative Western blot images and quantification of the protein expression from three independent experiments were presented. $*p < 0.05$ and $**p < 0.01$ compared with the NC siRNA group.

Wnt and Notch Signaling Pathways Mediated the Anabolic Effects of Liraglutide Via β-Catenin

Previous studies[24-26] have reported that the Notch signaling pathway affects the Wnt signaling pathway in bone cells and stromal cells. Therefore, we wonder if this interaction is involved in the regulation of MC3T3-E1 cells by liraglutide. The cells were treated with the Notch inhibitor DAPT in the presence or absence of liraglutide. The mRNA level of the Wnt signaling factor TCF7L2, and the expression and localization of β-catenin were all assessed. Liraglutide significantly increased the mRNA level of TCF7L2 and the protein expression of β-catenin, and promoted a translocation of β-catenin from cytoplasm to nucleus (Figure 3). However, a cotreatment with DAPT and liraglutide significantly reduced the mRNA level of TCF7L2 and the expression of β-catenin, which remained in cytoplasm.

Subsequently, the effect of β-catenin siRNA on the Notch signaling pathways activated by liraglutide was investigated. The knockdown efficiency of three siRNAs that targeted β-catenin was evaluated, and siRNA1 was selected for further experiments (Supplementary Figure 2). As opposed to the NC siRNA combined with liraglutide, the knockdown of β-catenin significantly decreased the expression of Notch1, Jag1, and Hes1 (Figure 4). This result indicates that the activation of the Notch signaling pathway by liraglutide depends on β-catenin.

In summary, our data described above suggest that the Wnt and Notch signaling pathways can mediate the effects of liraglutide on proliferation and osteogenic differentiation of MC3T3-E1 cells *via* β-catenin.

Hh and Notch Signaling Pathways Mediated the Anabolic Effects of Liraglutide Via Gli1

A further investigation was focused on whether the Hh signaling pathway was involved in the anabolic effects of liraglutide, and the process of an interaction with the Notch signaling pathway was determined. Liraglutide significantly increased the mRNA and protein expression levels of the Hh signaling pathway proteins Shh and Gli1, whereas a cotreatment of liraglutide and the Hh signaling inhibitor cyclopamine inhibited the activation of the Hh pathway by liraglutide and significantly reduced the mRNA and protein expression levels of Shh and Gli1 (Figure 5). The cotreatment with liraglutide and the Hh signaling

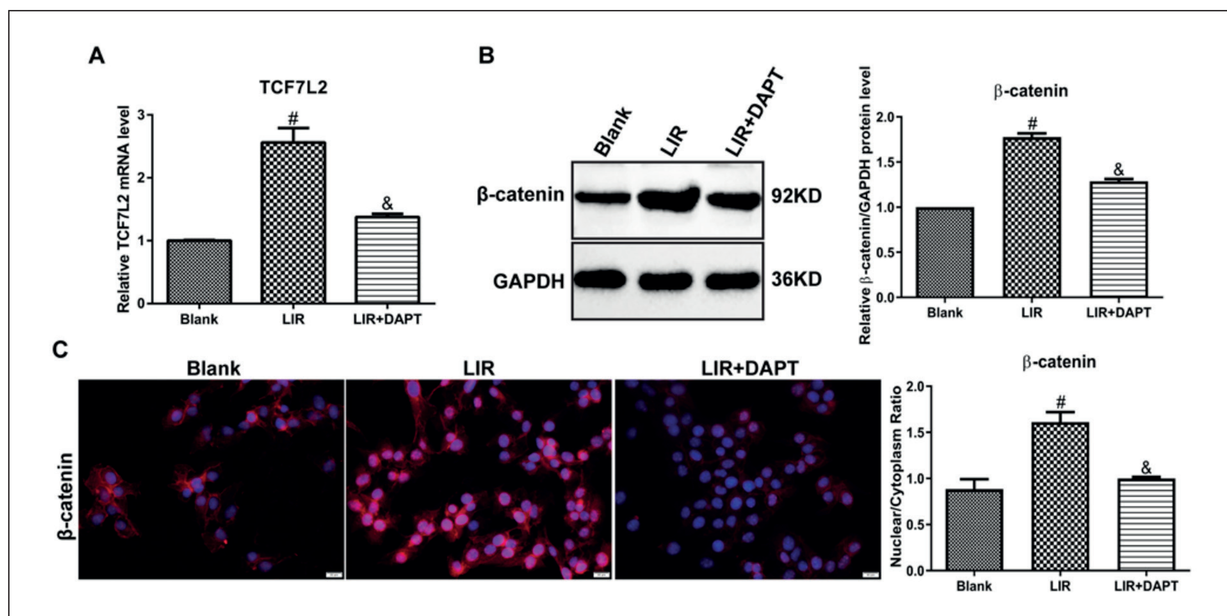

Figure 3. DAPT inhibited the upregulated mRNA expression of TCF7L2 and the increased expression and nuclear localization of β-catenin induced by liraglutide. MC3T3-E1 cells were treated with the Notch inhibitor DAPT (50 μM) in the presence or absence of 100 nM liraglutide for 48 h. **A,** mRNA levels of TCF7L2 were detected using qRT-PCR. **B,** The protein expression of β-catenin was determined using Western blot. **C,** Nuclear localization of β-catenin was observed using an immunofluorescence assay. $^{\#}p < 0.05$ compared with the blank group; $^{\&}p < 0.05$ compared with the liraglutide group. Magnification ×300.

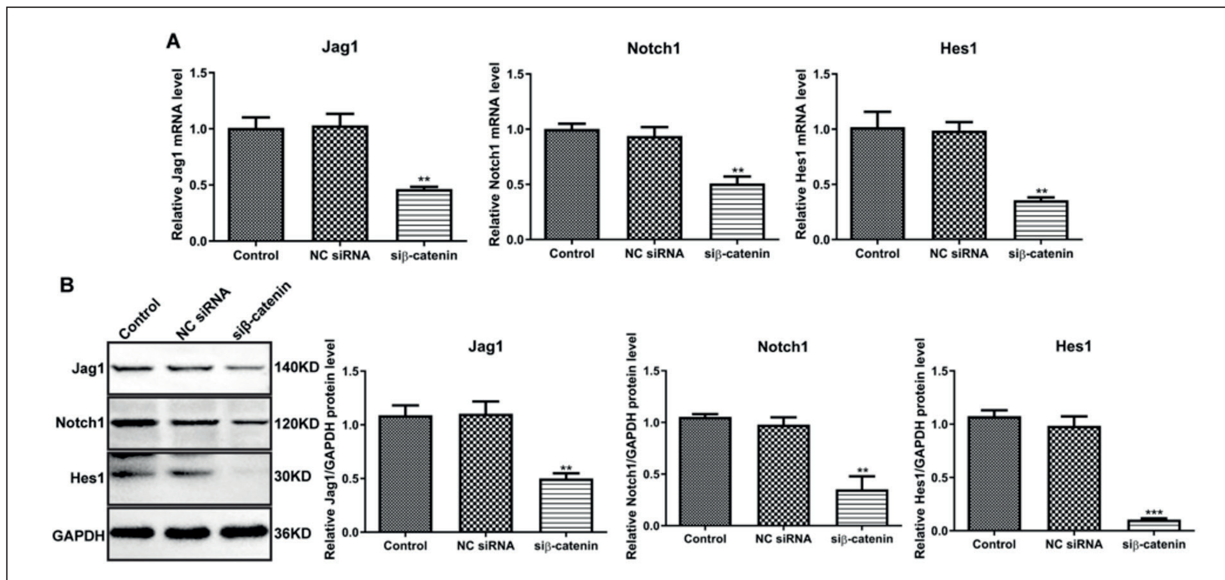

Figure 4. Knockdown of β-catenin inhibited the activation of the Notch signaling pathway by liraglutide in MC3T3-E1 cells. MC3T3-E1 cells were transfected with NC siRNA or β-catenin siRNA in the presence of 100 nM liraglutide for 48 h. **A,** mRNA levels of Notch-related proteins (Notch1, Jag1, and Hes1) were detected using qRT-PCR. **B,** The protein expression of Notch-related proteins (Notch1, Jag1, and Hes1) was determined using Western blot. Representative Western blot images and quantification of the protein expression from three independent experiments were presented. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the NC siRNA group.

inhibitor cyclopamine reduced the mRNA and protein expression levels of Gli1, but the correlation was not statistically significant (Figure 6A). The cotreatment significantly reduced the mRNA and protein expression levels of Gli1, Notch1, Jag1, and Hes1, and the activation of the Hh and Notch pathways was inhibited by liraglutide (Figure 6B).

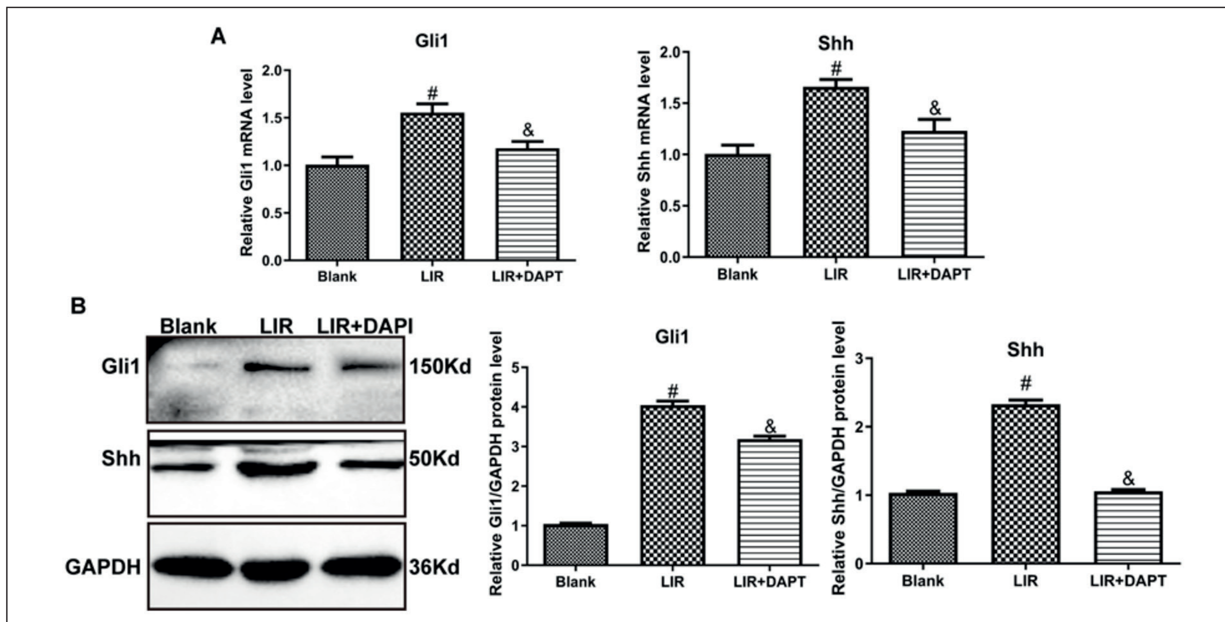

Figure 5. Liraglutide activated the Hedgehog signaling pathway that could be inhibited by DAPT. MC3T3-E1 cells were treated with the Notch inhibitor DAPT in the presence or absence of 100 nM liraglutide for 48 h. The mRNA (**A**) and protein (**B**) expression levels of Hedgehog-related proteins (Shh and Gli1) were determined using qRT-PCR and Western blot, respectively. $^{\#}p < 0.05$ compared with the blank group; $^{\&}p < 0.05$ compared with the LIR group.

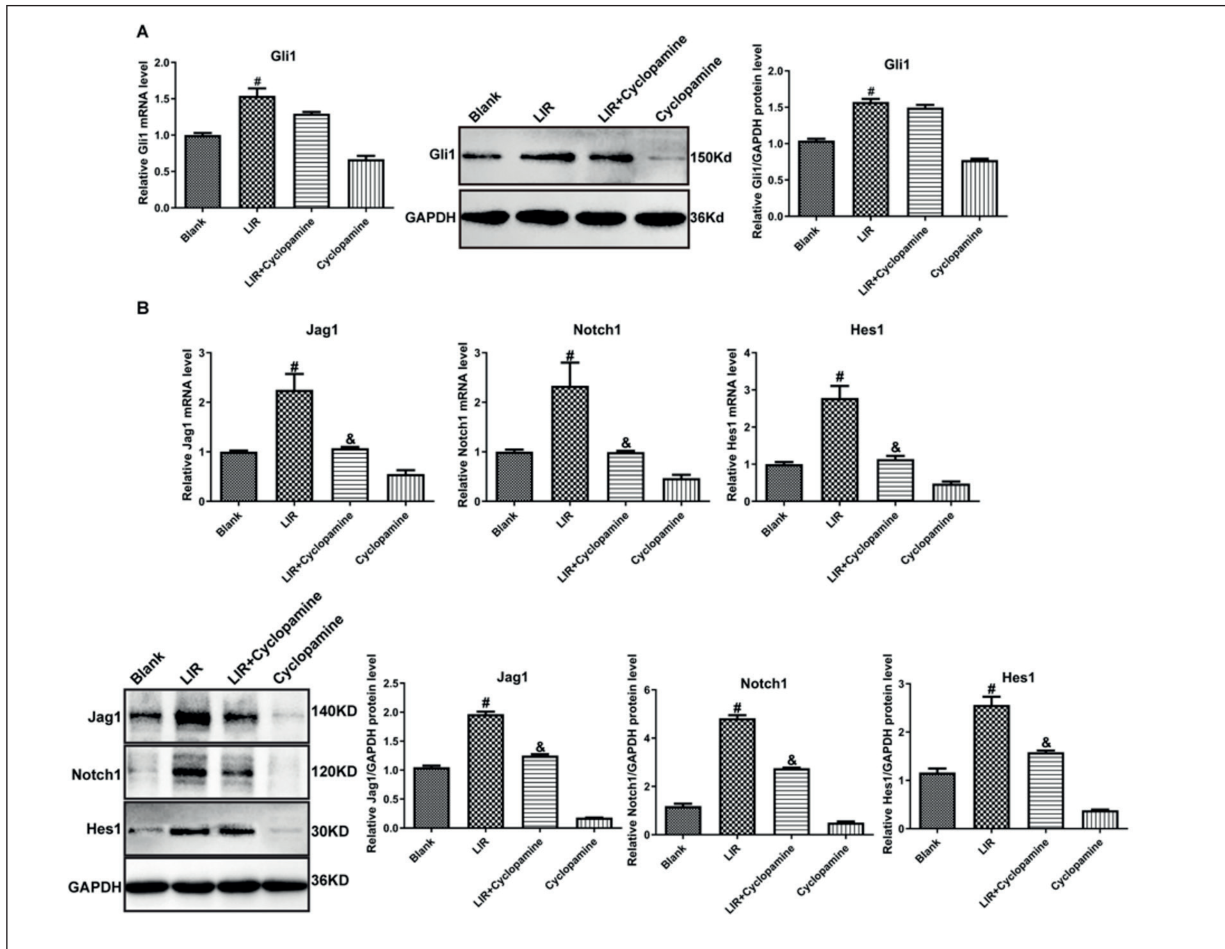

Figure 6. The Hedgehog inhibitor cyclopamine inhibited the activation of the Notch signaling pathway by liraglutide in MC3T3-E1 cells. MC3T3-E1 cells were treated with the Hedgehog inhibitor cyclopamine (10 μM) in the presence or absence of 100 nM liraglutide for 48 h. The mRNA and protein expression levels of Gli1 (**A**) and Notch-related proteins (Notch1, Jag1, and Hes1) (**B**) were determined using qRT-PCR and Western blot, respectively. $^{\#}p < 0.05$ compared with the blank group; $^{\&}p < 0.05$ compared with the LIR group.

The effects of Gli1 knockdown on osteogenic differentiation and the Notch signaling pathway were also investigated. The knockdown efficiency of three siRNAs that targeted Gli1 was first evaluated, and siRNA1 was selected for further experiments (Supplementary Figure 3). The Gli1 knockdown significantly reduced the mRNA expression levels of the osteogenic differentiation proteins RUNX2 and OCN, and the promotion of osteoblast differentiation was inhibited by liraglutide (Figure 7). The interference by Gli1 significantly downregulated the mRNA and protein expression levels of Notch1, Jag1, and Hes1. These results indicate that liraglutide can also activate the Notch signaling pathway by activating the Hh pathway. The data suggest that liraglutide can activate the Hh signaling pathway, and both Hh and Notch signaling pathways can mediate the anabolic effects of liraglutide via Gli1.

Liraglutide Inhibited Serum Deprivation-Induced Apoptosis of MC3T3-E1 Cells Via a GLP-1R/Notch/β-Catenin/Gli1 Pathway Network

Our previous work has shown that liraglutide can inhibit serum deprivation-induced apoptosis of the preosteoblast cell line MC3T3-E1 through the cAMP/PKA/β-catenin and PI3K/Akt/GSK3β pathways[37]. Therefore, we wonder whether the Notch and Gli1 pathways could regulate apoptosis by liraglutide. Liraglutide significantly reduced serum deprivation-induced apoptosis of the MC3T3-E1 cells, increased a ratio of Bcl-2/

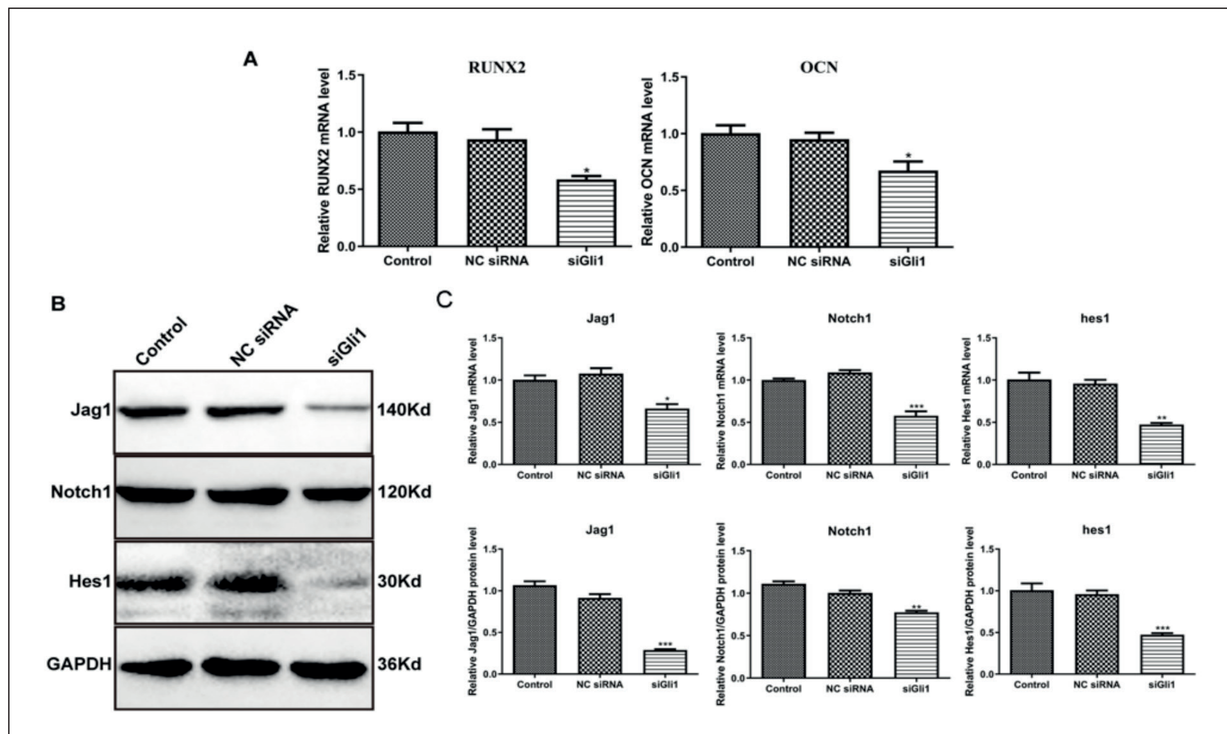

Figure 7. Knockdown of Gli1 inhibited osteogenic differentiation of MC3T3-E1 cells activated by the Notch signaling pathway by liraglutide. MC3T3-E1 cells were transfected with NC siRNA or GLI1 siRNA in the presence of 100 nM liraglutide for 48 h. **A,** mRNA levels of osteogenic differentiation-related genes (RUNX2 and OCN) were detected using qRT-PCR. **B,** mRNA levels of Notch-related proteins (Notch1, Jag1, and Hes1) were detected using qRT-PCR. **C,** The protein expression levels of Notch-related proteins (Notch1, Jag1, and Hes1) were determined using Western blot (n = 3). Representative Western blot images and quantification of the protein expression from three independent experiments were presented. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with the NC siRNA group.

Bax proteins, but reduced a ratio of caspase-3/procaspase-3 (Figures 8A and B). By contrast, a cotreatment with DAPT and liraglutide increased apoptosis, reduced the ratio of Bcl-2/Bax protein, and increased the ratio of caspase-3/procaspase-3 ratio, which subsequently relieved the inhibitory effect of liraglutide on the serum deprivation-induced apoptosis of the MC3T3-E1 cells. Moreover, the knockdown of GLP-1R, β-catenin, or Gli1 significantly increased the apoptosis, which could be inhibited by liraglutide (Figure 8C).

Discussion

Our previous studies have found that liraglutide can promote proliferation and osteogenic differentiation of the preosteoblast cell line MC3T3-E1 by activating the PI3K/Akt signaling pathway through GLP-1R involving β-catenin, and inhibit serum deprivation-induced apoptosis of the MC3T3-E1 cells through the cAMP/PKA/β-catenin and PI3K/Akt/GSK3β pathways[36,37]. In the present study, we further investigate whether other osteogenic differentiation-related signaling pathways, including Notch and Hh, are involved in these processes. Our data suggest that liraglutide promotes proliferation and osteogenic differentiation and inhibits serum deprivation-induced apoptosis of the MC3T3-E1 cells. The promotion or inhibition depends on the activation of the Notch signaling pathway through GLP-1R involving β-catenin or the activation of both the Notch and Hh signaling pathways through Gli1.

The relationship between intestines and bones has increasingly been recognized as a part of the overall integrated physiology of organisms. GLP-1 is secreted from the intestine due to the stimulation of nutrient intake. This process exhibits a variety of physiological functions, including the regulation of islet hormone secretion and glucose levels[38]. GLP-1 may affect the adipose-bone axis by promoting osteogenic differentiation and inhibiting the adipogenic differentiation of bone

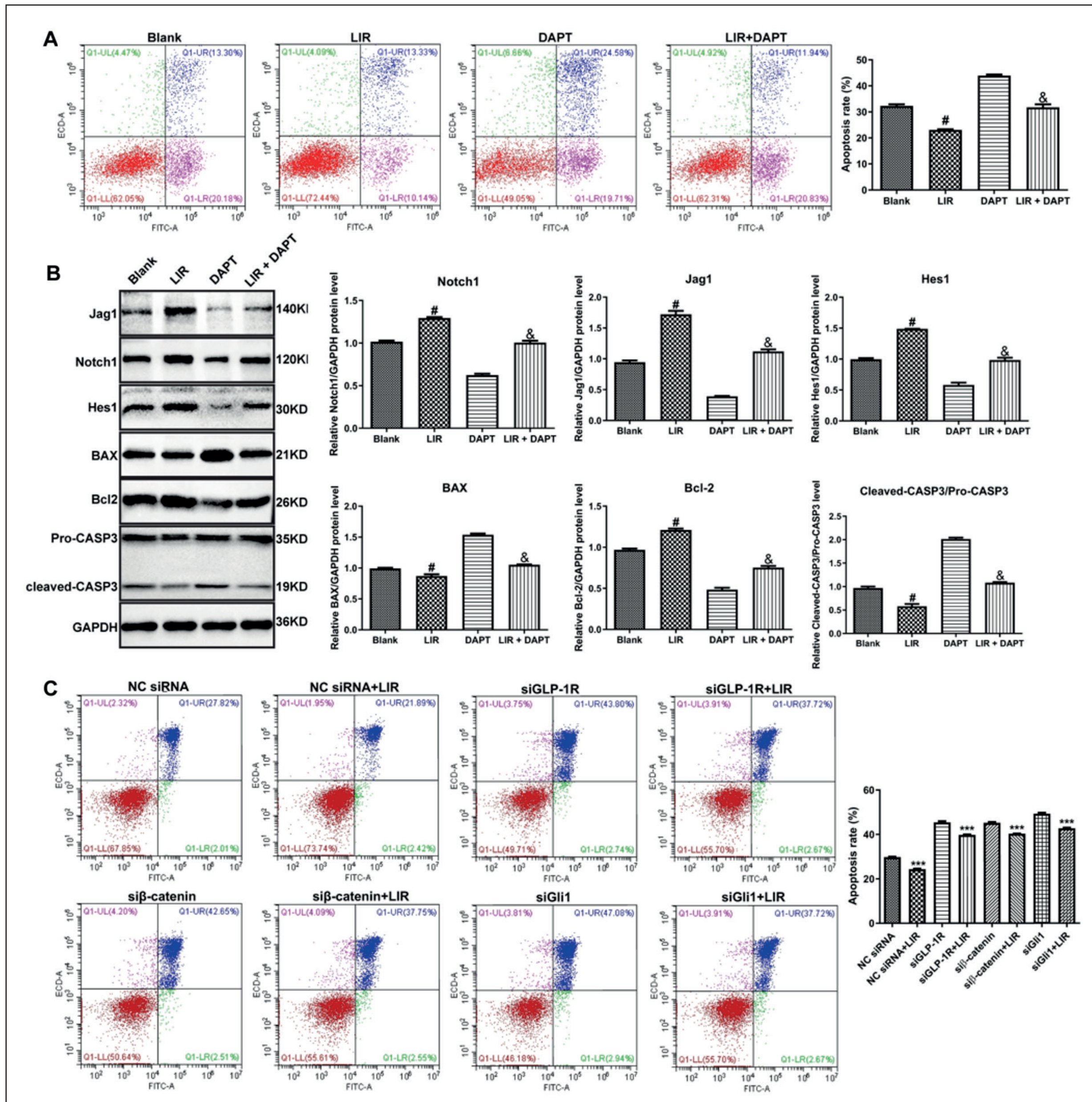

Figure 8. Involvement of the Notch signaling pathway, GLP-1R, β-catenin, and Gli1 in the regulation of serum deprivation-induced apoptosis of MC3T3-E1 cells by liraglutide. **A, B,** Apoptosis of MC3T3-E1 cells was induced using serum deprivation for 24 h, then the cells were treated with the Notch inhibitor DAPT in the presence or absence of liraglutide for 48 h. **A,** Cell apoptosis was evaluated by flow cytometry. **B,** The expression levels of Notch-related proteins (Notch1, Jag1, and Hes1) and apoptosis-related proteins (Bax, Bcl-2, and caspase3) were detected using Western blot. **C,** Apoptosis of MC3T3-E1 cells was induced by serum deprivation for 24 h, then the cells were transfected with siRNAs in the presence or absence of liraglutide for 48 h. Cell apoptosis was evaluated by flow cytometry. Q1-LL, live; Q1-LR, apoptotic cells; Q1-UR, dead cells; Q1-UL, slate apoptotic cells. $^{\#}p < 0.05$ compared with the blank group; $^{\&}p < 0.05$ compared with the LIR group; $^{***}p < 0.001$ compared with the corresponding siRNA treatment group.

mesenchymal precursor cells expressing GLP-1R. Verma et al[39] have demonstrated that the bone marrow adipocytes in osteoporosis patients can be significantly increased, indicating that a decrease in osteoblasts is usually accompanied by an increase in adipocytes. Similarly, in a mouse model of aging, bone formation can be significantly reduced when adipocytes in the bone marrow are increased[40]. Moreover, GLP-1 was found to directly interact with osteoblasts to affect its

function[41]. Consequently, GLP-1 RAs have been approved for the treatment of obesity, especially in patients with osteoporosis[42].

Osteoblast differentiation is regulated by a variety of signaling proteins and transcription factors. Wnt/β-catenin, Notch, BMP/TGF-β, PI3K/Akt/mTOR, MAPK, Hh, and other signaling pathways are involved in osteoblast differentiation[9]. In addition, the role of the Notch signaling pathway in skeletal development and homeostasis has been demonstrated in transgenic mouse studies. Notch mutations are also related to certain genetic skeletal diseases. Moreover, Notch signaling is abnormal in osteosarcoma and osteoarthritis. Animal model experiments have confirmed that Notch signaling plays an important role in skeletal development and functions[23]. Consequently, our research has attempted to determine whether Notch is involved in the regulation of osteogenic differentiation by liraglutide. The results of this study showed that a liraglutide treatment can significantly increase the protein expression levels of the Notch-related proteins Notch1, Jag1, and Hes1. The Notch signaling pathway consists of following four parts: ligand, receptor, DNA-binding protein, and downstream transcriptional genes[20]. Notch1, Jag1, and Hes1 correspond to ligands, receptors, and downstream target genes, respectively. Our findings indicate that liraglutide can activate the Notch signaling pathway in MC3T3-E1 cells. By contrast, a cotreatment with the Notch pathway inhibitor DAPT and liraglutide can significantly impair the effect of liraglutide alone on MC3T3-E1 cells. These data suggest that liraglutide can regulate proliferation and osteogenic differentiation of osteoblasts by activating the Notch signaling pathway.

GLP-1 functions by binding to GLP-1R. Animal experiments with different GLP-1R deletions have reported that GLP-1 may affect bone metabolism. Yamada et al[43] have found that GLP-1R-deficient mice showed reduced cortical bone, increased bone fragility, and increased osteoclast and bone resorption activity. Mabilleau et al[44] have demonstrated that ultimate load, yield load, stiffness, total absorption, post-yield energy, cortical thickness, and bone outer diameter were significantly reduced in GLP-1R gene knockout mice, but quantity and quality of minerals did not manifest differences. Moreover, animal experiments have revealed that GLP-1 improved bone metabolism and presented antiosteoporosis effects, mainly in the osteoblastic lineage. These results are consistent with our present finding that liraglutide can regulate osteogenic differentiation by activating GLP-1R.

The classic Wnt signaling pathway regulates differentiation and maturation of osteoblasts[10]. GLP-1 was found to directly activate the Wnt pathway. GLP-1 can also stabilize β-catenin by binding to GLP-1R, thus increasing intracellular cAMP levels[45]. β-catenin, an important part of the Wnt signaling pathway, is essential for skeletal development. β-catenin-deficient mouse embryos failed to develop normally, even on day 7 after mating[46], but various conditional or null allelic β-catenin mutations indicate that β-catenin is important for the cell fate of endothelial, epidermal, and neural crest cells[47-49]. β-catenin is widely expressed in osteoblasts. It promotes survival and differentiation of osteoblasts through Wnt-dependent and Wnt-independent pathways. Our results suggest that the Notch signaling inhibitor DAPT can significantly reduce the mRNA expression level of TCF7L2 and inhibit the nuclear transfer of β-catenin.

We have further demonstrated that interfering with β-catenin can significantly reduce the mRNA and protein expression levels of Notch1, Jag1, and Hes1. Both the Notch and Wnt/β-catenin pathways are involved in osteoblast differentiation, and cross-regulatory networks may exist between signaling pathways. Shao et al[50] have found that Notch regulates osteoblast differentiation through interactions with the typical Wnt pathways during the transformation of osteoblasts. Our research has also confirmed that liraglutide regulates osteogenic differentiation through the Notch signaling pathway involving β-catenin.

We have also revealed that a liraglutide treatment can significantly upregulate the mRNA and protein expression levels of the Hh signaling molecules Hh and Gli1. Increased mRNA expression of Gli1 is an exact sign of activation of the Hh signaling pathway[31]. Therefore, the liraglutide treatment has activated the Hh signaling pathway. Subsequent experiments have shown that the Notch signaling inhibitor DAPT can significantly inhibit the activation effect of liraglutide on the Hh signaling pathway. Moreover, the mRNA and protein expression levels of Notch1, Jag1, and Hes1 have been significantly reduced by the Hh signaling pathway inhibitor cyclopamine or the knockdown of Gli1. These results indicate that both the Notch and Hh pathways affect each other in the regulation of osteoblastic differentiation by liraglutide through Gli1.

Osteoblasts, the key cells involved in bone formation, are differentiated from preosteoblasts. Osteoporosis is accompanied by a large number of osteoblastic apoptosis, resulting in significantly reduced bone formation[3]. Therefore, an inhibition of preosteoblastic apoptosis helps to increase the number of osteoblasts and promote bone formation[20,51,52]. Osteoporosis is strongly related to osteoblast apoptosis. Advanced glycation end products can accumulate in bones[53], accelerate osteoblast apoptosis, and inhibit bone formation[54]. Meanwhile, an oxidative stress can inhibit osteoblast differentiation and induce osteoblast apoptosis[55]. Our previous study[37] also have shown that liraglutide can inhibit serum deprivation-induced apoptosis of the preosteoblast cell line MC3T3-E1. Under an external stimulation, the regulation of apoptosis and the fate of cells ultimately depend on a ratio of two regulatory factors, namely, the antiapoptotic protein Bcl-2 and the proapoptotic protein Bax[56]. Caspase, a cysteine protease, plays a vital role in an execution of apoptosis, which is a cascade of caspase activation and apoptotic proteases. Caspase-3 is considered as a key protease in the caspase family, and activated by various apoptosis-stimulating factors. Activated caspase-3 can act on some other caspases and degrade certain proteins in apoptotic cells[57]. Our results showed that liraglutide can significantly inhibit serum deprivation-induced apoptosis, increase the ratio of Bcl-2/Bax, and reduce the ratio of caspase-3/procaspase-3. Meanwhile, the Notch pathway inhibitor DAPT can significantly impair the inhibition of apoptosis by liraglutide. These findings indicate that the Notch pathway is also involved in the regulation of osteoblast apoptosis by liraglutide. The subsequent experiments found that the knockdown of GLP-1R, β-catenin, or Gli1 can weaken the inhibitory effect of liraglutide on osteoblast apoptosis. The data suggest that the GLP-1R, Wnt/β-catenin, and Hh pathways may be involved in the regulation of osteoblast apoptosis by liraglutide. This study is the first research to report the pharmacological mechanism of liraglutide on osteoblast differentiation. The series of experiments confirm that liraglutide promotes osteoblastic proliferation and osteogenic differentiation by regulating the Notch, Wnt/β-catenin and Hh signaling pathways. The subsequent apoptotic tests also confirm that liraglutide inhibits apoptosis of the preosteogenic cell line MC3T3-E1 induced by serum deprivation.

Conclusions

On the basis of our previous work, the present study further confirmed that liraglutide promotes proliferation and differentiation of MC3T3-E1 cells and inhibits their serum deprivation-induced apoptosis by activating both the Notch and Hh signaling pathways involving β-catenin and Gli1. All information from our studies indicate that liraglutide may function through a signaling network of the PI3K/Akt, Notch, Wnt, and Hh pathways, which play an important role in bone metabolism and homeostasis. This study explained a pharmacological mechanism of liraglutide in detail and expanded its understanding. At the same time, an important pathway in the differentiation of osteoblasts was found. The theoretical molecular basis provides us with new therapeutic targets for the treatment of patients with diabetes and osteoporosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Availability of Materials

The datasets generated and analyzed in the present scan be availed from the corresponding author upon request.

Authors' Contribution

Hao-wei Hou designed/performed most of the investigation and data analysis and wrote the manuscript. Yu-kun Li provided pathological assistance. Peng Xue and Yan Wang contributed to the interpretation and analyses of the data. All of the authors have read and approved the manuscript.

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