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Supplemental Information

Activation of GLP-1 Receptor Promotes Bone Marrow Stromal Cell Osteogenic Differentiation through β-Catenin

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SUPPLEMENTAL TABLE

Table S1. Oligonucleotide primers used for real-time PCR. Related to Figure 2, 3, 4, 6 and Supplementary Figure S1, S3.

gene	primers	primer sequence(5'-3')	Position (mRNA)	Size (bp)
Runx2	Forward	catggccgggaatgat	729-876	148
	Reverse	tgtgaagaccgttatggtcaaagtg		
Sp7	Forward	atggcgtcctctctgcttg	1-144	144
	Reverse	gtatggcttctttgtgcctcct		
Balp	Forward	catcgcctatcagctaatgcaca	761-910	150
	Reverse	atgaggtccaggccatccag		
Bglap	Forward	gacaagtcccacacagcaactc	7-178	172
	Reverse	caccttactgccctcctgct		
Pparγ	Forward	gtggacctctctgtgatggatg	61-176	116
	Reverse	gctcttgtgaacgggatgtct		
Lpl	Forward	gcccagcaacattatccagtgtc	538-684	147
	Reverse	agcagcatgggctccaaga		
Tcf7l2	Forward	tcaatccggcagcactcattac	862-997	136
	Reverse	ggcatccttgagggcttgtc		
Glp-1r	Forward	catcgcttcagccatccttg	496-641	146
	Reverse	cagccgtgctatacatccacttg		
Gapdh	Forward	ggcacagtcaaggctgagaatg	242-384	143
	Reverse	atggtggtgaagacgccagta		

SUPPLEMENTAL FIGURES AND LEGENDS

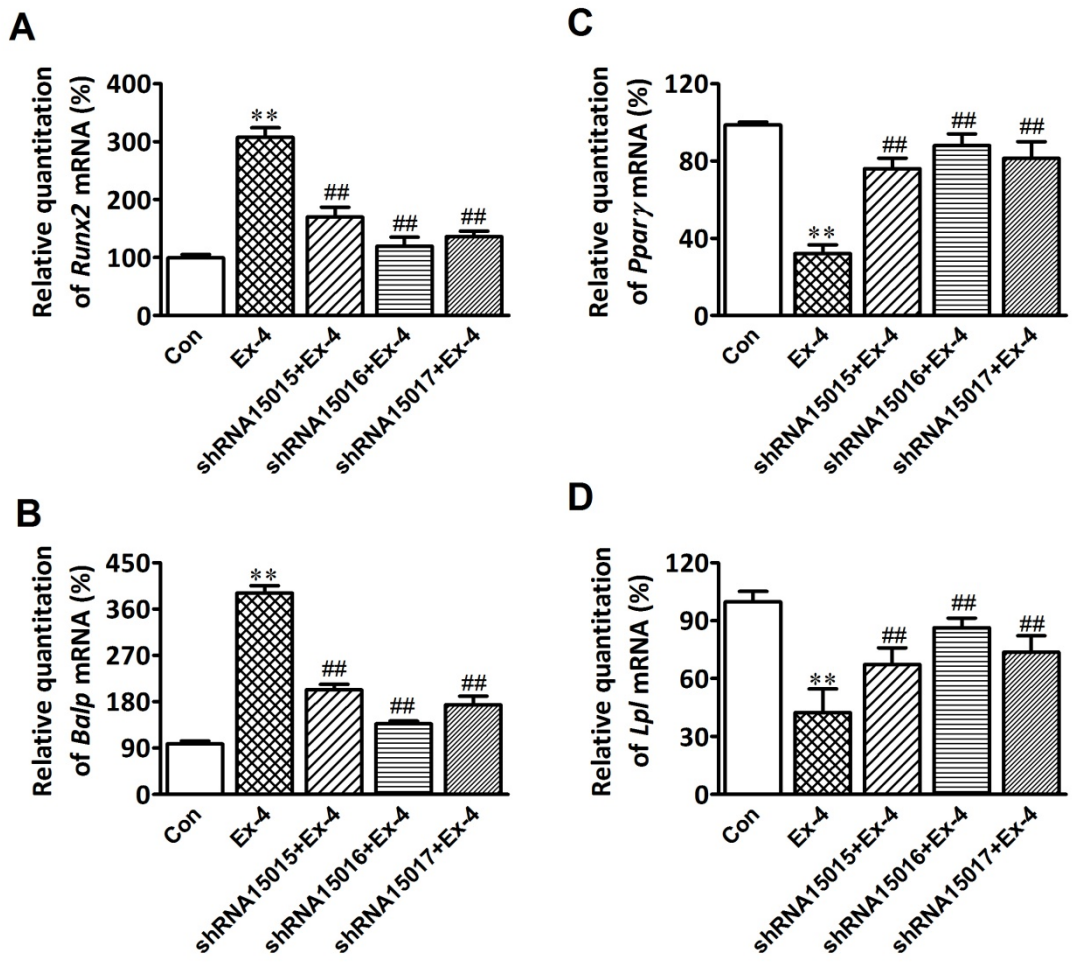

Figure S1

Figure S1 Ex-4 effects on BMSCs differentiation infected with lentiviral shRNAs to down-regulate GLP-1R. Real-time PCR of *Runx2* (A), *Pparγ* (B), *Balp* (C) and *lpl* mRNA in the BMSCs infected with shRNA15015, shRNA15016 and shRNA15017. Data are expressed by mean ± SD from three independent experiments. $^{**}P<0.01$ versus control group (Con) and $^{\#\#}P<0.01$ versus Ex-4 treatment group (Ex-4) by one-way ANOVA followed by a Student-Newman-Keuls *t* test. Related to Figure 3-5.

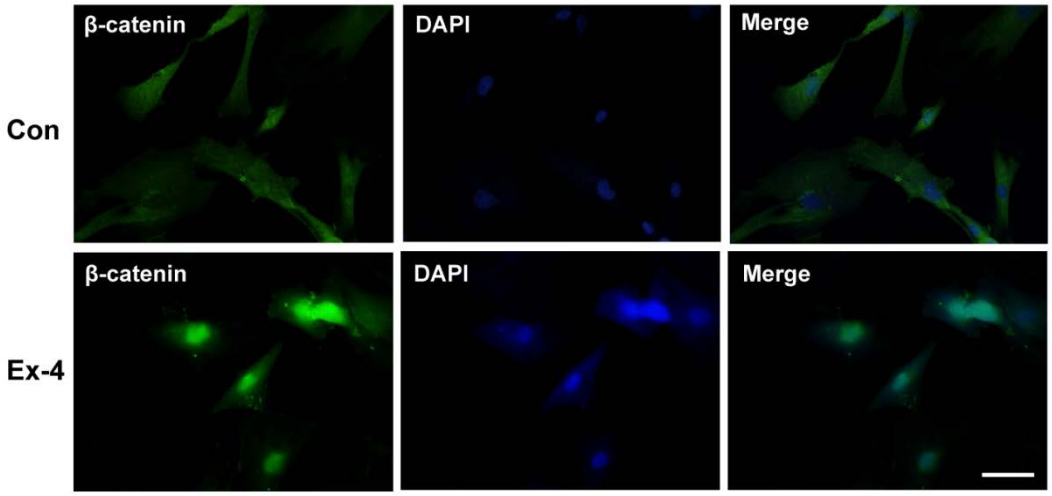

Figure S2

Figure S2 Ex-4 induced β-catenin nuclear translocation in BMSCs by immunofluorescence microscope. The BMSCs were treated with vehicle (Con) or 10 nM Ex-4 (Ex-4) in OIM. Scale bars: 25 μm. Related to Figure 4.

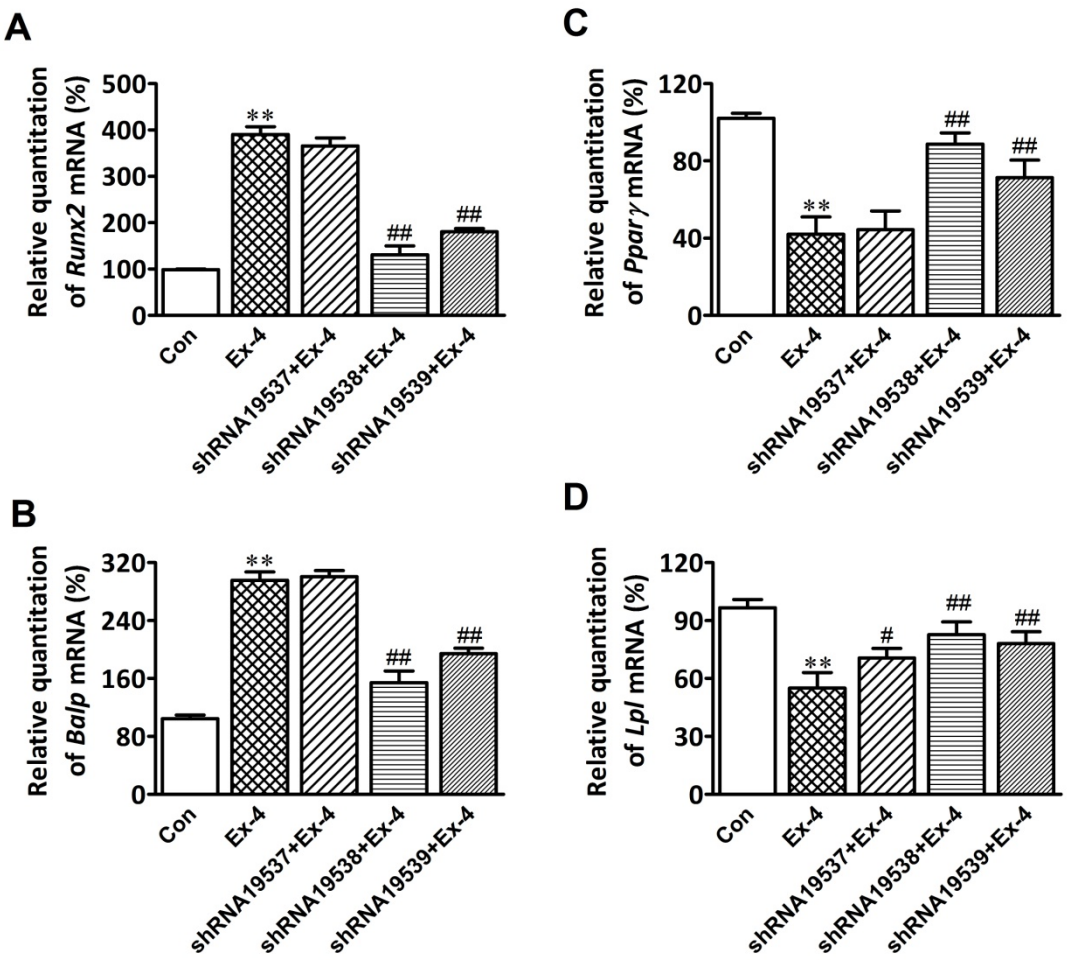

Figure S3

Figure S3 Ex-4 effects on BMSCs differentiation infected with lentiviral shRNAs to down-regulate β-catenin. Real-time PCR of *Runx2* (A), *Pparγ* (B), *Balp* (C) and *lpl* mRNA in the BMSCs infected with shRNA19537, shRNA19538 and shRNA19539. Data are expressed by mean ± SD from three independent experiments. $^{**}P<0.01$ versus control group (Con) and $^{\#\#}P<0.01$ versus Ex-4 treatment group (Ex-4) by one-way ANOVA followed by a Student-Newman-Keuls *t* test. Related to Figure 5, 6.

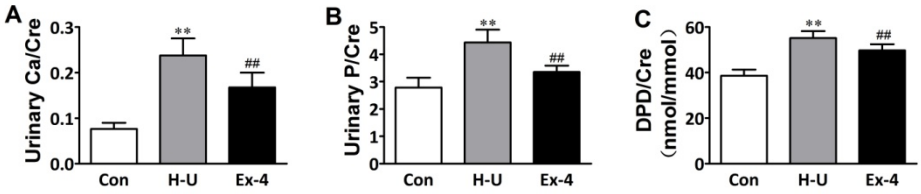

Figure S4

Figure S4 Ex-4 effects on bone resorption markers, Ca/Cre (A), urinary P/Cre (B) and urinary DPD/Cre (C). Bars represent mean and SD, n=12. $^{**}P<0.01$ versus normal control rats (Con) and $^{\#\#}P<0.01$ versus hindlimb-unloaded rats (H-U) by one-way ANOVA followed by a Student-Newman-Keuls *t* test. Related to Figure 2.

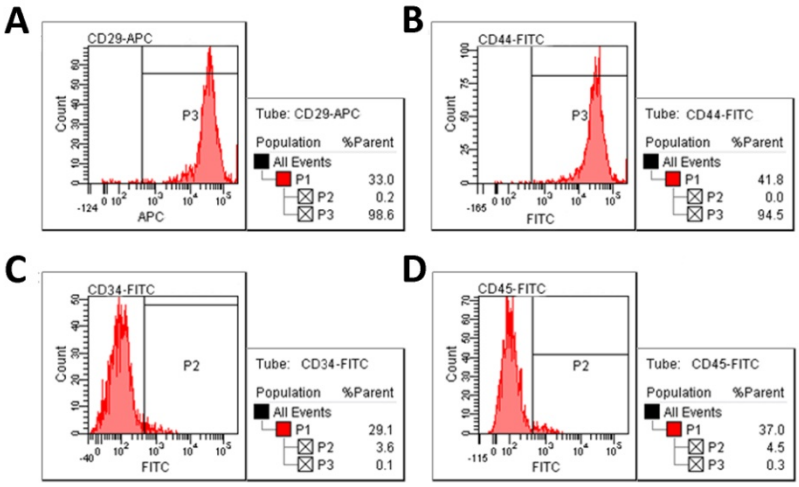

Figure S5

Figure S5 BMSCs phenotype identification. BMSCs surface markers expression. The percentages of BMSCs expressing CD29 (A), CD44 (B), CD34 and CD45 were 98.6%, 94.5%, 3.6% and 4.5%, respectively. Related to Figure 3-6.

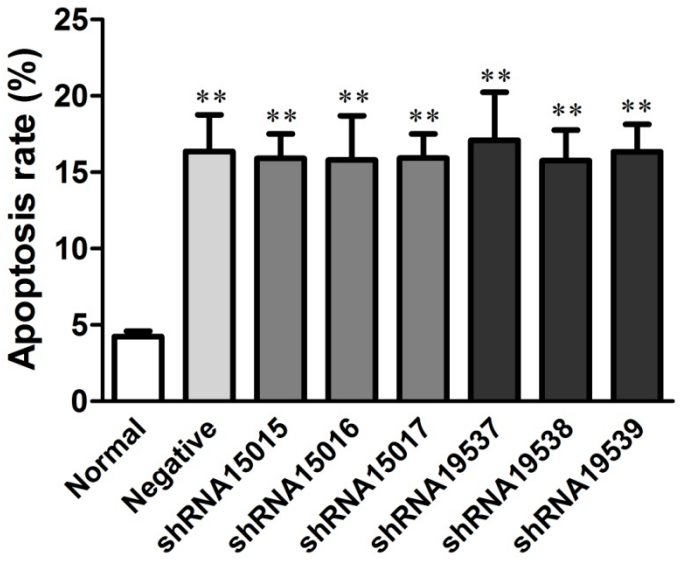

Figure S6

Figure S6 Apoptotic rate of BMSCs infected with lentiviral shRNA by flow cytometry. BMSCs were infected with vehicle (Normal), scrambled-shRNA (Negative), GLP-1R-shRNA (shRNA15015, shRNA15016 and shRNA15017) or β-catenin-shRNA (shRNA19537, shRNA19538 and shRNA19539). Data are expressed by mean ± SD from three independent experiments. ***P*<0.01 versus Normal group by one-way ANOVA followed by a Student-Newman-Keuls *t* test. Related to Figure 3-6.

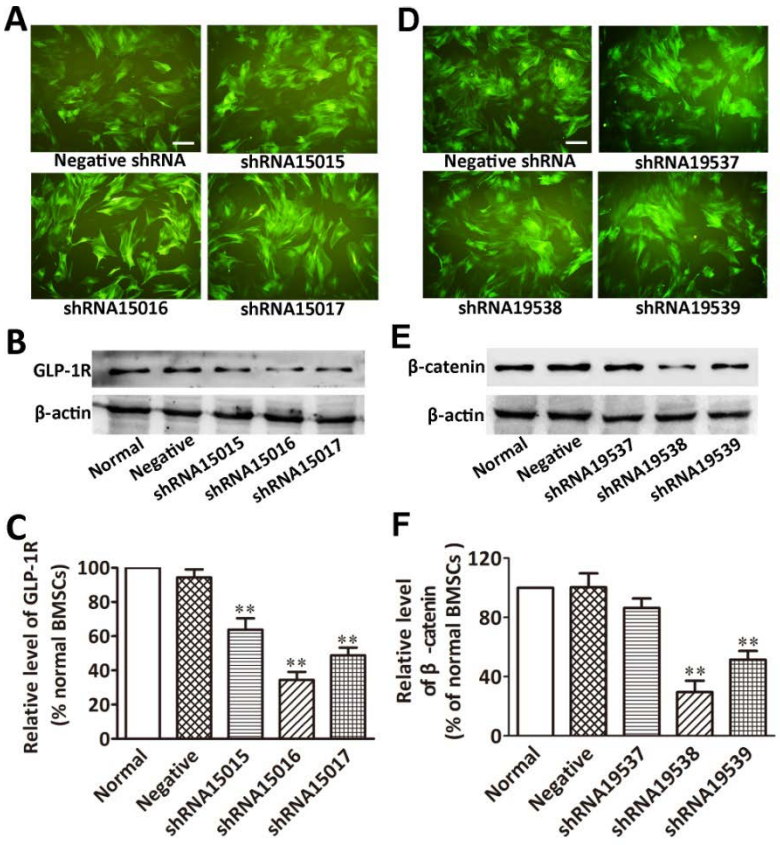

Figure S7

Figure S7 GLP-1R or β-catenin knock-down by lentiviral shRNA in BMSCs. (A) GFP-positive BMSCs indicated the successful infection of GLP-1R shRNAs. Scale bars, 50 μm. (B, C) GLP-1R expression in KO BMSCs by Western blot 72 h after lentivirus infection. GLP-1R expression was successfully inhibited by shRNA15015, shRNA15016 and shRNA15017. (D) GFP-positive BMSCs indicated the successful infection of β-catenin shRNAs. Scale bars, 50 μm. (E, F) β-catenin expression in KO BMSCs detected by Western blot 72 h after lentivirus infection. β-catenin expression was successfully inhibited by shRNA19538 and shRNA19539. n=3 wells from three independent experiments. ***P<0.01* compared with the negative shRNA-treated group. Related to Figure 3-6.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animal experiments

Thirty six 12-weeks-old male Sprague-Dawley rats (body weight 200-250 g) were obtained from the Animal Center of the Fourth Military Medical University (Xi'an, China). After one week of adaptation to the laboratory cages (one rat in each cage), rats were randomly divided into three groups: one normal control group, which consist of rats not subjected to hindlimb-unloading, as well as any other treatment, and two groups of rats subjected to hindlimb-unloading by tail suspension. These two groups were further divided into hindlimb-unloading treatment group, in which the rats were treated with Ex-4, and hindlimb-unloading control group, in which the rats were treated with the vehicle used to dilute Ex-4. The rat tail suspension was performed for 28 days as previously described. Ex-4 4.2 μg/kg/day was intraperitoneally administered to the rats of the hindlimb-unloading treatment group for 28 days, while the rats belonging to the hindlimb-unloading control group received an intraperitoneal injection of the same amount of normal saline for the same number of days. The three groups were subjected to the same nursery/housing conditions, with 12 h dark-light cycles and food and water *ad libitum*.

Bone imaging

Femurs and lumbar vertebrae (L3-L4) were removed and stored at -80℃ for micro-computed tomography (μCT) analysis and biomechanical test. At the day of testing, the femurs and vertebrae were slowly thawed to room temperature and kept wrapped in the saline-soaked gauzes except during measurements. The specimens of femur and lumbar vertebra were scanned using the Explore Locus SP Pre-clinical Specimen Micro-CT (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and the images were reconstructed to an isotropic voxel size of 12 μm. The volume of interest (VOI), which was located 1 mm from the metaphyseal line to the 100 continuous slices above, was selected for data analysis. All 3D image manipulations and analyses were performed by the system software (MicroView, v.2.1, GE Healthcare).

Bone biomechanical test

The femurs and vertebrae were subjected to a three-point bending test and axial compression analysis using a servo-hydraulic materials testing machine (MTS 858 Mini Bionix II, MTS systems Corp, Eden Prairie, MN, USA) to perform a biomechanical evaluation. A force resolution of 0.5 N was applied on the left femur mid-shaft at a speed of 0.1 mm/min as a contact force before the analysis, and then the speed was increased to 2 mm/min when the analysis started. The rats lumbar vertebrae (L3) were compressed until reaching the bone crushing point at a rate of 6 mm/min. The load-deformation curve was generated and analyzed by TestStar II software (MTS systems Corp). The biomechanical parameters were determined by the load-deformation curve.

Biochemical analysis

Serum bone formation markers were measured by commercially available ELISA kits. Serum concentration of osteocalcin (OCN) was analyzed using rat OCN ELISA kit (Biomedical Technologies Inc., Ward Hill, MA, USA), serum levels of bone alkaline phosphatase (BALP) were measured using an ELISA kit (MyBioSource, San Diego, CA, USA) and serum levels of N-terminal propeptide of type 1 procollagen (P1NP) were also measured using an ELISA kit (Immunodiagnostic Systems, Boldon, Tyne & Wear, UK). All measurements were conducted according to the manufacturer's instructions. Urine calcium, urine phosphorus and creatinine concentration were determined by an automated biochemistry analyzer (Cobas Integra 400 Plus; Roche Diagnostics, Basel, Switzerland).

Bone histomorphometry

Rats were treated with an intraperitoneal injection of 25 mg/kg tetracycline (Sigma-Aldrich, St. Louis, MO, USA) and 5 mg/kg calcein (Sigma-Aldrich) was intraperitoneally injected 10 days later for histomorphometry analysis. Static histomorphometry measurements of the trabecular bone were restricted to the secondary spongiosa. Eight-micrometer-thick, double-labeled sections were analyzed for mineral apposition rate (MAR; μm per day), mineralizing surface (MS/BS; %) and bone formation rate (BFR; μm^3 per μm^2 per day).

Bone histology

Tibiae were fixed in 4% paraformaldehyde (Sigma-Aldrich), decalcified in 10% ethylenediaminetetraacetic acid at pH 7.0 (Sigma-Aldrich), and then embedded in paraffin. Longitudinal sections (5-μm thick) were stained with hematoxylin and eosin to count the osteoblasts and with toluidine blue to visualize the bone marrow adipocytes.

BMSCs isolation and cell culture

BMSCs were isolated from the femurs and tibias of 2- to 3-week-old Sprague-Dawley male rats (Animal Center of Fourth Military Medical University). The cells were collected and dissolved in

α-MEM culture medium (Hyclone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Life Technologies, Grand Island, NY, USA), 100 U/ml penicillin (Life Technologies), 100 mg/ml streptomycin (Life Technologies) and incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The BMSCs were a relatively pure population of stromal cells negative for CD34 and CD45 and positive for CD29 and CD44 (Abcam, Cambridge, MA, USA) (Figure S5).

GLP-1R and β-catenin Knockdown

shRNA specific sequences targeting different regions of rat GLP-1R (NM_012728) and β-catenin (NM_053357) mRNA were constructed into the pGV118 lentiviral vector, which possess a green fluorescent protein tag (GeneChem Co, Ltd, Shanghai, China). The pGV118-GFP lentiviral vector with scrambled shRNA (TTCTCCGAACGTGTCACGT) (GeneChem Co, Ltd) was used as a negative control. The lentivirus was packaged and amplified in HEK293T cells. BMSCs were infected for 72 h in a 6-well plate (multiplicity of infection of 30) and were allowed to recover for 48 h in full medium prior to the treatments. Flow cytometry analysis showed that the apoptotic rate of BMSCs without lentivirus shRNA was about 4%, while the apoptotic rates of BMSCs infected with lentivirus shRNA were about 16% (Figure S6). GLP-1R and β-catenin expression were confirmed by Western blot analysis. The BMSCs expressing the green fluorescent protein (GFP) were successfully infected with control-shRNA, GLP-1R-shRNA or β-catenin-shRNA (Figure S7A, D), and the GFP positive BMSCs were approximately 90% after 72h of shRNA infection. Three sequences (si-15015: GGAACTACATCCACCTGAA, si-15016: GGTCTCTTCTGCAACCGAA, and si-15017: GCCATCCTTGTCAGCTTCA) were designed to reduce GLP-1R expression compared with cells infected with lentivirus containing a nonsense control sequence (negative: TTCTCCGAACGTGTCACGT). GLP-1R expression was reduced to 63.8%±6.7%, 34.3%±4.8% and 48.8%±4.5% compared with the control, by the shRNAs 15015, 15016 and 15017, respectively (Figure S7B, S7C). Three sequences (si-19537: AATCAGCTGGCCTGGTTTG, si-19538: CCAGGTGGTCGTTAATAAA, and si-19539: GCTTACGGCAATCAGGAAA) were designed to reduce β-catenin expression in BMSCs. β-catenin expression was reduced to 86.5%±6.3%, 29.5%±7.7% and 51.4%±5.9% compared with the control, by shRNAs 19537, 19538 and 19539, respectively (Figure S7E, F).

Cell Staining Assay

BMSCs were treated with 10 nM Ex-4 alone, pretreated with 100 nM GLP-1R antagonist Ex(9-39) (GL Biochem Ltd) or 0.5 mg/ml DKK1 (R&D Systems, Inc., Minneapolis, MN, USA) for 1 hour, followed by 10 nM Ex-4 treatment; GLP-1R or β-catenin silenced BMSCs by sh-glp-1r or sh-β-catenin were treated with 10 nM Ex-4 alone in OIM or AIM. At the 28th day of culture, cells were fixed in 70% ice-cold ethanol for 1 hour, washed three times in Dulbecco's phosphate-buffered saline (Mediatech, Inc.) and stained with alizarin red S (40 mM) at pH 4.2 (Sigma-Aldrich) for 10 minutes at room temperature to monitor the formation of mineralization nodules. Images of the cells were taken prior to alizarin red stain extraction with 10% cetylpyridinium chloride (CPC, Sigma-Aldrich) in 10 mM sodium phosphate buffer for 20 minutes at room temperature with gentle agitation. Extracted alizarin red stain in CPC was quantified by the absorbance at 570 nm on a microplate reader (BioTek ELX800, Winooski, VT, USA). At the 21st day of culture, cells were stained with Oil Red O (Sigma-Aldrich) to identify the lipid droplets and detect intracellular lipid accumulation. Briefly, cells were washed twice with PBS and fixed for 40 min with 4% paraformaldehyde. The fixed cells were incubated with oil red O for 30 min and then the images were taken. Subsequently, intracellular oil red O was extracted in 60% isopropanol (Tianjin Kemiou Chemical Reagent Co., Ltd, Tianjin, China) and quantified by the absorbance at 570 nm.

Quantitative Real-Time PCR

BMSCs were treated with 10 nM Ex-4 alone, pretreated with 100 nM GLP-1R antagonist Ex(9-39) (GL Biochem Ltd) or 0.5 mg/ml DKK1 (R&D Systems, Inc., Minneapolis, MN, USA) for 1 hour, followed by 10 nM Ex-4 treatment; GLP-1R or β-catenin silenced BMSCs by sh-glp-1r or sh-β-catenin were treated with 10 nM Ex-4 alone in OIM or AIM. Total RNA was extracted from BMSCs pellets using an RNeasy kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. cDNA synthesis was performed using the one step SYBR® PrimeScript™ RT-PCR Kit (TaKaRa Biotech, Dalian, China). Quantitative real-time PCR was performed using the SYBR® Premix Ex Taq™ (TaKaRa Biotech) in Bio-Rad CFX96™ real-time PCR detection system (Bio-Rad, Hercules, CA, USA) with two sets of primers specific for each targeted gene (Table S1). The PCR protocol included a denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s. Detection of the fluorescent product was carried out at the end of the 72 °C extension period. PCR products were subjected to a melting curve analysis, and relative expression was

calculated for each gene by the $2^{-\Delta\Delta CT}$ method, using Gapdh for normalization. *Pparγ, Runx2, Sp7, Balp, Lpl* and *Bglap* mRNA (Takara Biotech) expression were examined at day 3, 7, 10, 14, 14 and 28 after Ex-4 treatment, respectively. Each sample was measured at least in triplicate.

Western Blot Analysis

BMSCs were treated with 10 nM Ex-4 alone, pretreated with 100 nM GLP-1R antagonist Ex(9-39) (GL Biochem Ltd) or 0.5 mg/ml of DKK1 (R&D Systems, Inc., Minneapolis, MN, USA) for 1 hour, followed by 10 nM Ex-4 treatment; GLP-1R or β-catenin silenced BMSCs by sh-glp-1r or sh-β-catenin were treated with 10 nM Ex-4 alone in OIM or AIM. BMSCs were scraped from the culture dish and washed twice with ice-cold PBS. Total protein was extracted from the cell pellet using RIPA lysis buffer (Beyotime), while nuclear protein was extracted using Nuclear/Cytosol Fractionation kit (BioVision, Zurich, Switzerland) according to the manufacturer's protocol. Proteins concentrations were quantified by the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL, USA). The total proteins were separated by 10 % SDS-PAGE and transferred to a 0.45 mm polyvinylidene fluoride blotting membrane (Millipore, Billerica, MA, USA). The membrane was incubated at room temperature in a blocking solution of 5% skimmed milk powder dissolved in TBST containing 0.05% Tween 20, 10 mM Tris, pH 8.0, and 140 mM NaCl (Cell Signaling Technology, Boston, MA, USA) for 1 hour, followed by an incubation with the primary antibodies overnight at 4°C. The membrane was washed three times in TBST (5 minutes each wash), and incubated with horseradish peroxidase-conjugated anti-rabbit (ab136817) or anti-mouse IgG secondary antibody at 1:5000 dilution (Abcam, ab136815) in the blocking solution. The blots were exposed by enhanced chemiluminescence (Pierce, Rockford, IL, USA). The densitometric analysis of Western blots was conducted using a ChemiDoc XRS (Bio-Rad) and the relative band intensities in the scanned images were measured with Quantity One version 4.1.0 (Bio-Rad). The antibodies used in this study included mouse β-actin antibody at 1:10000 dilution (Sigma-Aldrich A5441), rabbit histone H3.1 antibody at 1:500 dilution (Novus Biologicals, Littleton, CO, USA, NB100-81964), rabbit anti-GLP-1R antibody at 1:600 dilution (Abcam, ab39072), mouse RUNX2 antibody at 1:500 dilution (Abcam, ab54868), rabbit osterix antibody 1:500 dilution (Abcam, ab22522), rabbit PPARγ antibody at 1:400 dilution (Abcam, ab19481), rabbit β-catenin antibody at 1:1000 dilution (Cell Signaling Technology, #9562), rabbit phospho-β-catenin (Ser675) antibody at 1:1000 dilution (Cell Signaling Technology, #4176), rabbit PKAc antibody at 1:1000 dilution (Cell Signaling Technology, #4782), rabbit phospho-PI3K p85 (Tyr458)/p55 (Tyr199) antibody at 1:1000 dilution (Cell Signaling Technology, #4228), rabbit phospho-AKT (Ser473) antibody at 1:500 dilution (Cell Signaling Technology, #4060) and rabbit phospho-GSK3β (Ser9) antibody at 1:500 dilution (Cell Signaling Technology, #5558).

Co-immunoprecipitation

BMSCs were treated with vehicle or 10 nM Ex-4. BMSCs total proteins were extracted in NP-40 lysis buffer (Beyotime). The extraction was pre-incubated with protein A/G PLUS-agarose (Santa Cruz Biotechnology, Dallas, Texas, USA) and normal rabbit IgG antibodies for 30 min at 4 °C, and then the mixture was centrifuged at 2500 rpm for 5 minutes at 4°C. Phospho-PKA substrate antibody at 1:50 dilution (Cell Signaling Technology, #5661) was added into the supernatants and incubated for 1 h, followed by the addition of protein A/G PLUS-agarose. The mixture was centrifuged for 5 min, the immunoprecipitates were washed 4 times with PBS and eluted with a protein sample buffer. The immunoprecipitates were subjected to SDS-PAGE separation and immunoblotting analysis using antibodies against PKAc, β-catenin or PI3K (Cell Signaling Technology, #3358).

Immunofluorescence Labeling and Confocal Microscopy Detection

BMSCs were seeded on a 6-chambers slide (2000 cells/chamber) for 24 hour. Next, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 (Worldbio, Xi'an, China). Nonspecific antibody-binding sites were blocked with PBS containing 10% goat serum (Boster, Wuhan, China) at room temperature for 1 h. After the excess of the serum was removed, rabbit anti-GLP-1R antibody (Abcam, ab39072) diluted 1:200 in PBS containing 10% goat serum was applied to the slides and incubated overnight at 4°C. After thorough washes with PBS, the slides were incubated with green FITC-conjugated goat anti-rabbit secondary antibody (Cwbiotech, Beijing, China, CW0114) diluted 1:100 in PBS containing 0.1% saponin and 10% goat serum, for 1 h in the dark. Nuclei were stained using 10 μg/ml Hoechst (Sigma-Aldrich, 33258). Images were taken with a TCS SP2 confocal laser microscopy system (Leica, Wetzlar, Germany) equipped with an inverted DMIRE2 Leica microscope.