Lentiviral-mediated Shh reverses the adverse effects of high glucose on osteoblast function and promotes bone formation via Sonic hedgehog signaling

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Abstract. Patients with diabetes tend to have an increased incidence of osteoporosis, which may be associated with hyperglycemia; however, the pathogenic mechanisms governing this interaction remain unknown. The present study sought to investigate whether elevated extracellular glucose levels of bone mesenchymal stem cells (BMSCs) could influence osteoblastic differentiation and whether the intracellular Sonic hedgehog (Shh) pathway could adjust the effects. Furthermore, to verify the results *in vivo*, a rat tooth extraction model was constructed. BMSCs were incubated in eight types of culture medium, including low glucose (LG), LG + lentivirus (Lenti), LG + Lenti-small interfering RNA (Lenti-siRNA), LG + Lenti-Shh, high glucose (HG), HG + Lenti, HG + Lenti-siRNA and HG + Lenti-Shh. The lentiviral transfection efficiency was observed using a fluorescence microscope; protein and mRNA expression was detected by western blotting and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The matrix mineralization and alkaline phosphatase (ALP) activity of BMSCs were examined by Alizarin red staining and ALP activity assays, respectively. The expression of osteogenesis-related genes in BMSCs were quantified by RT-qPCR. The alveolar ridge reduction was measured and histological sections were used to evaluate new bone formation in the tooth socket. With high concentrations of glucose, Shh expression, matrix mineralization nodules formation, ALP activity and the levels of bone morphogenic protein 4 (BMP4), bone sialoprotein (BSP) and osteopontin (OPN) expression were greatly reduced compared with LG and corresponding control groups. Whereas activated Shh signaling via Lenti-Shh could increase the number of matrix mineralization nodules, ALP activity, and the expression levels of BMP4, BSP and OPN in BMSCs. Additionally, *in vivo* assays demonstrated that Lenti-Shh induced additional bone formation. Collectively, the results of the present study indicated that HG inhibited the Shh pathway in osteoblasts and resulted in patterning defects during osteoblastic differentiation and bone formation, while the activation of Shh signaling could suppress these deleterious effects.

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Introduction

Diabetes mellitus (DM) is a metabolic disease that is characterized by hyperglycemia (1). DM has multiple possible long-term complications, including vascular lesions, neuropathy, retinopathy, nephropathy and bone osteoporosis. DM was determined to affects the skeletal system (2). Females with DM have a three-fold higher risk of fracture when compared with non-diabetic females (3). In addition, fracture patients with diabetes require longer hospitalization times than those without diabetes (4); however, the underlying mechanisms associated with fracture risk in diabetes are yet to be completely elucidated. Evidence has also demonstrated that DM accelerates alveolar bone resorption (5). In our previous study, rats subjected to experimental DM exhibited more serious alveolar bone destruction when compared with the normal group (6). This also suggested that type 2 DM (T2DM) elevated the levels of tumor necrosis factor-α, interleukin-1β and lipopolysaccharide in the gingival crevicular fluid of rats (6). The enhanced levels of inflammatory mediators contribute to an increase in osteoblast apoptosis, decreasing their abundance (7). Decreased activity of osteoblasts under diabetic conditions has been discussed in numerous studies in both humans and animal models (8,9); however, despite numerous studies that have addressed this problem, the precise molecular mechanism underling the effects of glucose in altering bone formation and osteoblastic differentiation is yet to be elucidated.

Lentiviral vector technology has now become an effective tool for therapeutic gene delivery (10-12). Effectively, lentiviral vectors can infect dividing and non-dividing cells then integrate exogenous genes or exogenous short hairpin RNAs into a host chromosome, which culminates in the stable and long-term expression of the target genes (13).

Hedgehog (Hh) signals serve an important role in evolution and numerous biological events (14). The Hh gene family comprises at least three members, including Sonic hedgehog (Shh), Desert hedgehog and Indian hedgehog. Shh is involved in the formation of organs and tissues; Shh proteins are involved in the same signaling pathway. The activated Shh ligand binds to the transmembrane protein receptor protein patched homolog 1 (PTCH), which results in the release of the downstream protein smoothened (SMO). SMO subsequently transduces signaling downstream which in turn activates the transcription factors glioma-associated homologs. Phosphorylated Gli enters the nucleus and activates target genes (14). Without the Shh ligand, PTCH binds to SMO and suppresses the activation of SMO, as well as the associated genes (15). The Shh pathway is involved in multiple developmental processes by regulating cell differentiation (16). A previous study reported that Shh induces osteoblastic differentiation and bone formation as evidenced by an *in vitro* culture system and *in vivo* transplantation experiments (17). Additionally, it was demonstrated that by adding Shh to bone mesenchymal stem cells (BMSCs), the differentiation of uncommitted BMSCs to the osteoblast lineage was promoted (18). A previous study revealed that the altered expression of Shh and bone morphogenic protein 4 (BMP4), and their downstream genes are associated with variations of the telencephalon in the embryos of diabetic mice (19). In addition, the study of Dunaeva *et al* (20) demonstrated that diabetes is associated with a functional inhibition of the Shh signaling pathway.

These studies indicated that the Shh signaling pathway was associated with osteoblastic differentiation and DM, while DM could be associated with the skeletal system and osteoblastic activity. We hypothesized that high glucose (HG) levels altered the Shh signaling pathway in osteoblasts and affected osteoblast functions. Therefore, in the present study, the Shh pathway was induced and suppressed vial entiviral vector-mediated Shh/small interfering (si)RNA gene transfer *in vitro*; we also investigated whether in an *in vivo* animal model, whether alterations in Shh expression may alter the effects of HG on osteoblastic differentiation of BMSCs and new bone formation.

Materials and methods

Statement of ethics. All animal experiments were conducted in strict accordance with the principles of medical ethics and were approved by Animal Care and Use Committee of The Second Affiliated Hospital of Harbin Medical University (Harbin, China). All necessary permits were obtained for the described field studies.

Isolation and culture of BMSCs. BMSCs were isolated from 3-week-old male Sprague-Dawley rats (n=30, average weight of 80-100 g), which were purchased from the Department of the Animal Experiment Center of The Second Affiliated Hospital of Harbin Medical University. Their housing conditions were as follows: 18-26˚C, humidity 40-70%, fresh air, 12 h light/dark cycle, and free access to food and water. Briefly, rats were sacrificed, soaked in 75% ethanol for 10 min, and the femurs and tibias were isolated from the soft tissues. The proximal and distal ends of the femur and tibia were removed. The exposed bone marrow cavity was flushed with 30 ml of Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences) containing 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, Merck KGaA). The cell suspension was obtained by repeated rinsing through a 5-gauge needle, and was placed in a centrifuge tube. The supernatant was discarded after centrifugation at 1,000 x g for 10 min at room temperature. Cells were then seeded at a density of $2x10^6$ cells/ml in a 25 cm^2 plastic culture flask in the low glucose (LG)-DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and incubated in 5% CO_2 at 37˚C. Following a 72-h incubation, the unattached cells were removed by replacing the medium and the remaining cells were considered to be BMSCs. When the cells reached 90% confluence, they were trypsinized by 0.25% trypsin and 0.02% ethylenediamine tetraacetic acid (Gibco; Thermo Fisher Scientific, Inc.) for 2 min, and then passaged at a density of 10^2 per cm^2. The first-passage BMSCs were cultured in 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin with DMEM containing different concentrations of glucose, 5% CO_2 at 37˚C (LG-DMEM, 1,000 mg/l glucose; HG-DMEM, 4,500 mg/l glucose). The third passage generation cells were used for further studies.

Transduction with lentiviral vectors. Lentivirus gene transfer vectors fused with a green fluorescent protein (GFP) sequence were constructed by Shanghai GenePharma Co., Ltd. Lentiviral vectors can infect BMSCs then integrate RNAs into a host chromosome. The targeting sequences for *Rattus norvegicus* Shh (NM-017221) were 5'-GGTGCCAAGAAGGTCTTC TAC-3', and the titer of Lenti-Shh (activate Shh signaling), Lenti-siRNA (inhibit Shh signaling) and Lenti (negative control) were all $1x10^8$ UT/ml. The third passage of BMSCs was plated in 24-well plates at a density of $0.5x10^5$ cells/well in 0.5 ml medium per well prior to transduction. Following a 24-h incubation the cells had grown to 30-50% confluence; 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin with the differing DMEM were removed and transductions were performed at a multiplicity of infection (MOI) of 10 according to a preliminary experiment. BMSCs were incubated in 10% FBS-DMEM under eight different conditions: LG + Lenti-Shh, LG + Lenti-siRNA, LG, LG + Lenti, HG + Lenti-Shh, HG + Lenti-siRNA, HG and HG + Lenti. The volume of fresh medium was 0.5 ml per well. On the third day, the culture medium was replaced with 10% FBS LG-DMEM or 10% FBS HG-DMEM (1 ml/well), respectively. Every other day medium was replenished, and it was suitable to proceed experiments until day 6. The transduction efficiency and the morphology of the BMSCs was evaluated by two researchers independently via fluorescence and phase contrast microscopes (Olympus Corporation) in 5-7 fields per view (magnification, x100).

Western blot analysis. Following transduction, cells were cultured in the eight different types of media for 6 days, and were then lysed in ice-cold radioimmunoprecipitation assay (Santa Cruz Biotechnology, Inc.) and phenylmethyl sulfonyl fluoride lysis buffer (Santa Cruz Biotechnology, Inc.) for 2 sec. The cells were subsequently scraped from the plate with a cell scraper, and transferred to a 1.5 ml microcentrifuge tube. The lysates were separated by centrifugation at 12,000 x g for 5 min at 4˚C. The supernatants were collected and stored at -80˚C until western blotting was performed. The protein concentration was quantified with a bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology). The samples were separated by 12% SDS-PAGE based on the molecular weight of the target proteins (Shh, 19.45), and were then transferred to a polyvinylidene difluoride membrane (EMD Millipore). After blocking with 5% non-fat milk in TBST and 1X Tris-buffered saline with 0.1% Tween-20, the membranes were incubated with the following antibodies: Shh (C9C5) rabbit monoclonal antibody (dilution 1:1,000; cat. no. 2207, Cell Signaling Technology, Inc.) and anti-β-actin (dilution 1:1,000; cat. no. 03-102, Sigma-Aldrich; Merck KGaA) at 4˚C overnight. The membranes were then incubated with horseradish peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L), according to the origin of the primary antibodies, (dilution 1:5,000; cat. no. ZB-2305, OriGene Technologies, Inc.) for 1 h at room temperature and were detected by using enhanced chemiluminescence plus reagents (GE Healthcare Life Sciences) according to the manufacturer's protocols; the emitted light was captured on an X-ray film.

Alizarin red staining. The mineralization of the third passage BMSCs was determined using Alizarin red staining (Sigma-Aldrich, Merck KGaA). BMSCs were seeded in 24-well plates at a density of $5.0x10^4$ cells/well. Following transduction, the eight different media types were removed and replaced with osteogenic medium containing 50 μg/ml ascorbic acid (Sigma-Aldrich; Merck KGaA), 10 mmol/l β-glycerophosphate sodium (Sigma-Aldrich; Merck KGaA), and 10% FBS LG-DMEM or 10% FBS HG-DMEM. The culture medium was replaced every other day and staining was performed after three weeks (21 days later, osteogenic-inducing medium can promote the formation of mineralized nodules). Cells were fixed with 70% ethanol for 1 h at room temperature, washed with PBS twice per well and stained with 40 mM Alizarin red (pH 4.2, Sigma-Aldrich; Merck KGaA) for 15 min at room temperature. The stained cells were rinsed with dH_2O four times to eliminate nonspecific staining, and then observed using a light microscope (Nikon Corporation). We randomly selected 5 fields of vision per sample for analysis (magnification, x100).

Alkaline phosphatase (ALP) activity assay. Following transduction, the third passage cells in 24-well plates were treated with the osteogenic medium as described above. The culture medium was changed every 2 days. Following 2 weeks of incubation in 5% CO_2 at 37˚C, ALP activity of the BMSCs was detected using an ALP assay kit (Nanjing KeyGen Biotech Co., Ltd.) according to the manufacturer's protocol. The absorbance was recorded using a microplate absorbance reader (Bio-Rad Laboratories, Inc.; Model-680) at 405 nm. The protein concentration was measured with a BCA protein assay kit (Beyotime Institute of Biotechnology). The ALP activity was normalized to the total protein content.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA samples for eight groups were analyzed to measure the effects of high glucose on the expression of osteogenesis-related genes in BMSCs. RT-qPCR was also used to quantify the expression levels of the seven-transmembrane protein SMO. On day 6 following transduction, total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and the concentration of total RNA was measured with an ultraviolet spectrophotometer (Thermo Fisher Scientific, Inc.). Total RNA (300 ng) was reverse transcribed into cDNA using a Prime Script™ RT Reagent kit (Takara Bio, Inc.) and RT was performed at 44˚C for 60 min. Then, the mixture was incubated at 95˚C for 5 min. All of the primers for PCR are listed in Table I. qPCR was performed on a CFX-96 machine (Bio-Rad Laboratories, Inc.) using a SYBR PrimeScript EX Taq PCR (Takara Bio, Inc.) according to the manufacturer's protocols. All reactions were conducted in a 20 μl mixture containing cDNA preparation (1 μl), 10 μl SYBR® Premix Ex Taq™ (Tli RNaseH Plus), 1 μl of each primer and 7 μl diethylpyrocarbonate water. The thermocycling conditions were 95˚C for 10 min, and then 40 cycles of 95˚C for 20 sec and 60˚C for 30 sec. A melting curve was acquired using 95˚C for 15 sec, 60˚C for 30 sec, and 95˚C for 15 sec. The expression levels were normalized to the expression of the housekeeping gene β-actin. Data were analyzed using the $2^{-\Delta\Delta Cq}$ method (21).

Animal experiments. A total of 60, 3-month-old male Sprague-Dawley rats with average weight of 200-220 g, were obtained from the Department of the Animal Experiment Center of The Second Affiliated Hospital of Harbin Medical University. Their housing conditions were as follows: 18-26˚C, humidity 40-70%, fresh air, 12 h light/dark cycle with free access to food and water. All animals were acclimatized for at least 1 week. A total of 30 rats were fed an adequate high fat (HF) diet (22) (34.5% fat, 17.5% protein, 48% carbohydrate; Beijing HFK Bio-Technology), while the remaining animals (n=30) received normal chow for 4 weeks. Then, the 30 rats on the HF diet were intraperitoneally injected with streptozotocin (STZ; 30 mg/kg, Sigma-Aldrich; Merck KGaA) dissolved in 0.1 mol/l citrate buffer, pH 4.5, to induce models of diabetes. A diagnosis of diabetes was confirmed by measurement of blood glucose randomly on the third and seventh days following STZ administration. Rats were considered diabetic when the random blood glucose levels were >11.1 mmol/l (23).

Rat tooth extraction model. The left incisors of the mandibular of 60 rats were cut at the gingival level with a small diamond bur under anesthesia (10% chloral hydrate, 300 mg/kg animal bodyweight, intraperitoneal injection) at days 10, 8, 6, 4 or 2 following the induction of diabetes; before this, mandibular incisor was fully extracted. On day 0, the left incisors of the 60 animals were carefully extracted (24,25). The animals were divided into four groups: A normal group as a control

Table I. Primer sequences employed reverse transcription-quantitative polymerase chain reaction.

Genes	Sequences (5'-3')	Size (bp)	Accession no.
β-actin	F: CCCATCTATGAGGGTTACGC	207	NM_031144
	R: TTTAATGTCACGCACGATTTC		
BSP	F: GATGAAAATGAGCAGGTCGTC	95	NM_012587
	R: GCTTCTTCTCCGTTGTCTCCT		
OPN	F: AGGACAGCAACGGGAAGAC	241	M99252
	R: TGAAACTCGTGGCTCTGATGT		
BMP4	F: GCTCTGCTTTTCGTTTCTTCTT	199	NM_012827
	R: TCCAGTAGTCGTGTGATGAGGT		
SMO	F: TGATGGCTGGAGTAGTGTGGT	117	NM_012807
	R: TGAGCAGGTGGAAATAGGATG		

F, forward; R, reverse; BMP4, bone morphogenic protein 4; BSP, bone sialoprotein; OPN, osteopontin; SMO, smoothened.

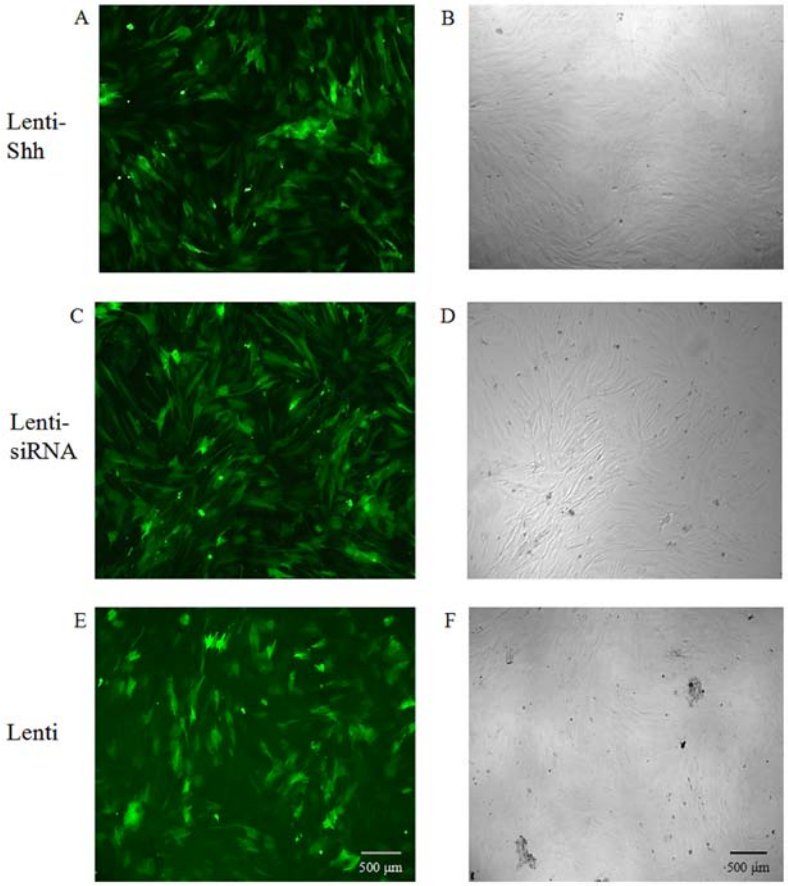

Figure 1. Microscopy analysis of transduced cells. Rat BMSCs were transduced with (A and B) Lenti-Shh, (C and D) Lenti-small interfering RNA and (E and F) Lenti. Fluorescence and phase contrast microscopy analyses to investigate transduction and the morphology of the BMSCs. Scale bar, 500 μm. BMSCs, bone marrow stromal stem cells; Lenti, lentivirus; Shh, Sonic hedgehog.

(untreated post incisor extraction, n=15), a normal group with MOI=10, 50 μl Lenti-Shh into the tooth socket (n=15), a DM group with MOI=10, 50 μl Lenti-Shh into the tooth socket (n=15), and a DM group (untreated post incisor extraction, n=15). Microliter syringes (Shanghai GAOGE Industrial and Trading Co., Ltd.) were used to inject Lenti-Shh into the tooth socket. The extraction sockets of the teeth were closed with periodontal dressing paste.

The rats were sacrificed at 8 weeks following tooth extraction; the mandible was dissected and the samples were fixed in 10% neutral buffered formalin at room temperature for 2 days. Soft X-ray images of the mandibles were obtained via cabinet X-ray systems (Faxitron, MC-20). To determine the alveolar ridge reduction after tooth extraction, the distances between the highest point of the mesial at the first molar to the lingual alveolar ridge margin of the incisor were measured

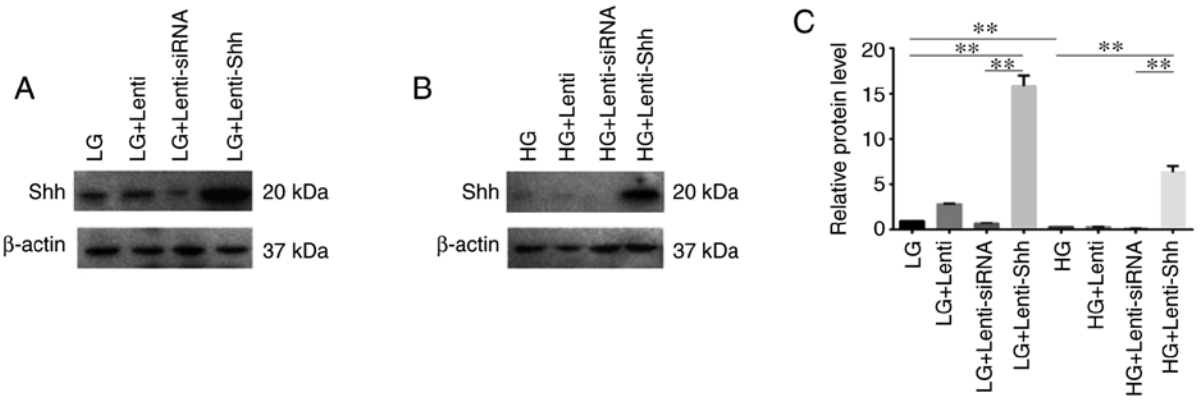

Figure 2. Efficiency of lentiviral-mediated suppression or overexpression of Shh in BMSCs with LG-DMEM or HG-DMEM. (A and B) Expression of Shh protein and (C) relative protein level of Shh in bone marrow stromal stem cells cultured in eight different media conditions: LG, LG + Lenti, LG + Lenti-siRNA, LG + Lenti-Shh, HG, HG + Lenti, HG + Lenti-siRNA and HG + Lenti-Shh. **P<0.01. HG, high glucose; LG, low glucose; Lenti, lentivirus; Shh, Sonic hedgehog; siRNA, small interfering RNA.

by an image analyzing software (ImageJ; v2.1.4.7, National Institutes of Health) as described previously (26). The reduction rate was calculated according to the following formula: (1-a/b) x100% (a, the length after incisor extraction; b, the length before incisor extraction). Subsequently, the left half of the mandible was decalcified, specimens were decalcified in 10% ethylene diamine tetraacetic acid for 4 weeks, dehydrated and then embedded in paraffin. The paraffin serial sections in 6 μm were obtained in the buccal and lingual direction and stained with hematoxylin and eosin (HE) staining at room temperature for 1 h. The histological sections were used to evaluate new bone formation of the tooth socket in five randomly selected fields per view. Histologic evaluation was performed at 100 magnification using a light microscope (BX50, Olympus Corporation).

Statistical analysis. Statistical analyses were performed using Microsoft Excel and SPSS 23.0 software (IBM Corp.). All of the data are presented as the mean ± standard deviation. Statistical analysis for differences between multiple groups was conducted using one-way analysis of variance followed by a Tukey's post hoc test for multiple comparisons. The experiments were conducted in triplicate. P<0.05 was considered to indicate a statistically significant difference.

Results

Transfection efficiency. As the lentiviral constructs were tagged with the GFP reporter, the infection efficiency was examined using fluorescence microscopy and phase contrast microscopy. At 72 h following transduction, the BMSCs expressed GFP, suggesting that the lentivirus had integrated into the genome of the BMSCs and the infection efficiency was ~80% when the MOI was 10 (Fig. 1).

Efficiency of lentiviral-mediated suppression or overexpression of Shh in BMSCs with LG-DMEM or HG-DMEM. The present study performed western blotting in order to determine the expression of Shh under LG-DMEM and HG-DMEM treatment following transduction with Lenti-Shh (activate Shh signaling), Lenti-siRNA (inhibit Shh signaling) and Lenti

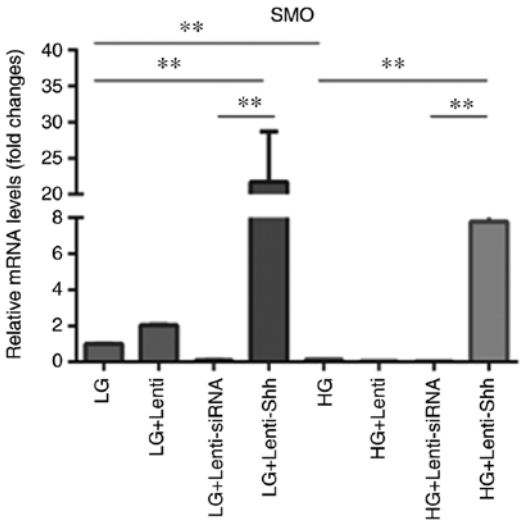

Figure 3. Expression levels of SMO in bone marrow stromal stem cells cultured under eight media conditions: LG, LG + Lenti, LG + Lenti-siRNA, LG + Lenti-Shh, HG, HG + Lenti, HG + Lenti-siRNA and HG + Lenti-Shh. **P<0.01. HG, high glucose; LG, low glucose; Lenti, lentivirus; Shh, Sonic hedgehog; siRNA, small interfering RNA; SMO, smoothened.

(negative control), respectively. As presented in Fig. 2A-C, Shh protein expression was markedly decreased in BMSCs when cultured in HG medium compared with LG medium, which indicated that HG inhibited Shh protein expression in BMSCs. In addition, Lenti-Shh treatment significantly upregulated Shh expression in LG and HG medium compared with the corresponding controls. These results suggested that following transduction with Lenti-Shh, the decreased expression of Shh in BMSCs under HG conditions could be reversed.

Effect of HG on the expression levels of SMO in BMSCs. Hh signaling in vertebrates is initiated by the binding of secreted Hh proteins to the twelve-transmembrane protein PTCH, which typically suppresses the activity of the seven-transmembrane protein SMO (14). SMO in turn promotes the expression of Hh target genes by the Gli family of transcription factors and activates Hh signaling (15). The present study performed RT-qPCR in order to quantify the expression levels of SMO (Fig. 3),

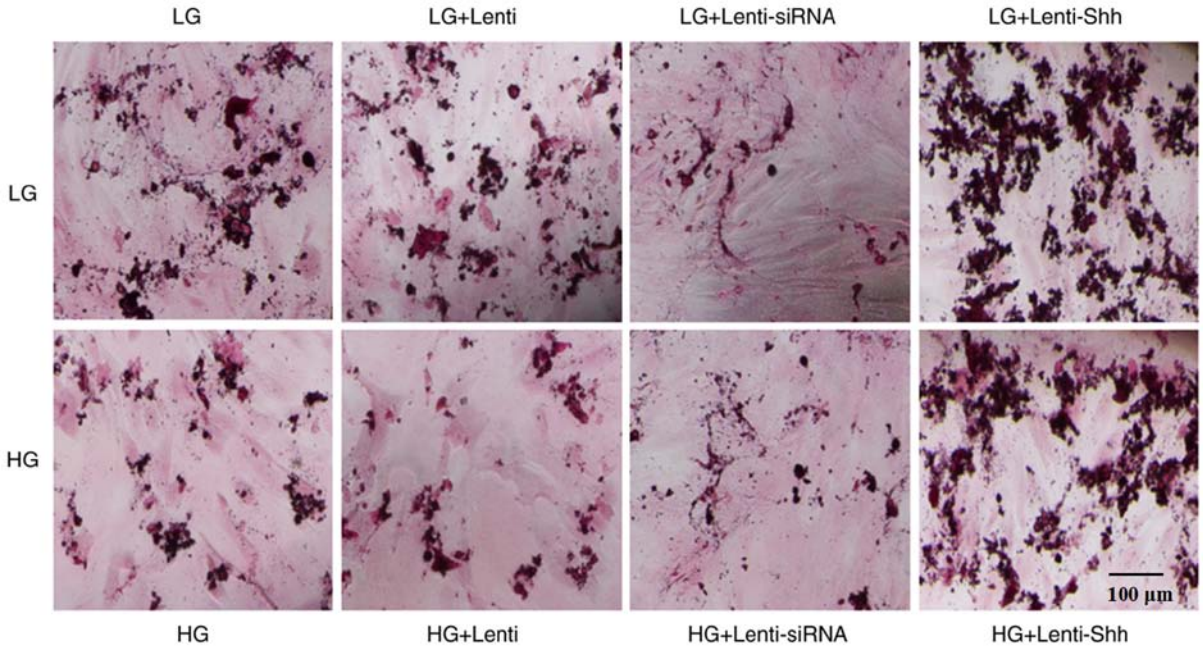

Figure 4. Extent of matrix mineralization in bone marrow stromal stem cells as determined by Alizarin red S staining cultured under eight different media conditions: LG, LG + Lenti, LG + Lenti-siRNA, LG + Lenti-Shh, HG, HG + Lenti, HG + Lenti-siRNA and HG + Lenti-Shh. Scale bar, 100 μm. HG, high glucose; LG, low glucose; Lenti, lentivirus; Shh, Sonic hedgehog; siRNA, small interfering RNA.

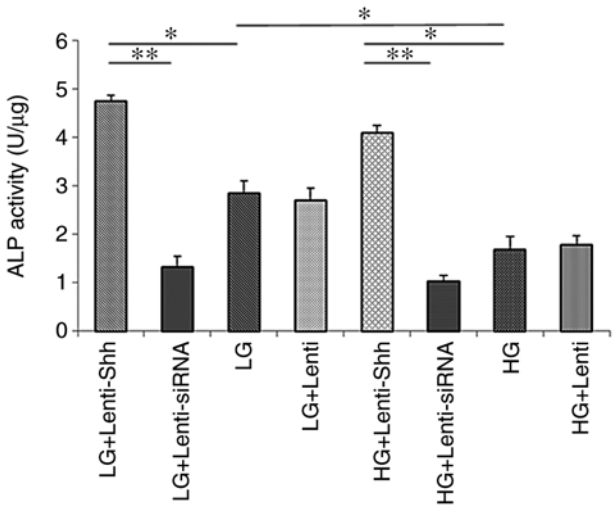

Figure 5. ALP activity of bone marrow stromal stem cellscultured in eight media conditions: LG + Lenti-Shh, LG + Lenti-siRNA, LG, LG + Lenti, HG + Lenti-Shh, HG + Lenti-siRNA, HG and HG + Lenti was analyzed on day 14. *P<0.05, **P<0.01. ALP, alkaline phosphatase activity; HG, high glucose; LG, low glucose; Lenti, lentivirus; Shh, Sonic hedgehog; siRNA, small interfering RNA.

and the results indicated that SMO expression was significantly decreased in the HG group compared with LG group. Following transduction with Lenti-Shh, the LG + Lenti-Shh and HG + Lenti-Shh groups exhibited significantly upregulated SMO expression compared with the corresponding controls. However, the levels of SMO were decreased in the LG + Lenti-siRNA and HG + Lenti-siRNA groups; the levels SMO expression in the LG + Lenti and HG + Lenti control groups were similar to that of the LG and HG groups, respectively. These results suggested that HG levels inhibited the expression of SMO; however, following transduction with Lenti-Shh, the decreased expression of SMO in BMSCs under HG conditions could be reversed.

Effect of high glucose and Shh pathway on matrix mineralization of BMSCs. Osteoblast-secreted matrix is mineralized into nodules (27). In the present study immunohistochemical staining of mineralized nodules with Alizarin red S after 21 days of culture was performed in 24-well plates. The results presented in Fig. 4 demonstrated that matrix mineralization in the HG group was decreased compared with the LG group. Following infection with Lenti-Shh, a notable increase in mineralized nodules was observed under LG and HG conditions, compared with LG and HG alone, respectively. However, the LG + Lenti-siRNA and HG + Lenti-siRNA groups exhibited markedly decreased matrix mineralization compared with the other groups; the extent of staining in the LG + Lenti and HG + Lenti control groups was similar to that of the LG and HG groups, respectively. Taken together, these results indicated that HG levels inhibited the matrix mineralization of BMSCs and this negative effect could be reversed by activating the Shh pathway via transduction with Lenti-Shh.

Effects of HG and Shh pathway on ALP activity in BMSCs. The ALP activity levels of BMSCs cultured in eight different media conditions were detected and are presented in Fig. 5. The results demonstrated that the ALP activity in the HG group was significantly reduced compared with the LG group. Following transduction with Lenti-Shh, the significantly increased ALP activity levels were detected under both glucose conditions compared with the LG and HG groups, respectively (P<0.05). Conversely, the LG + Lenti-siRNA and HG + Lenti-siRNA groups exhibited notably decreased ALP

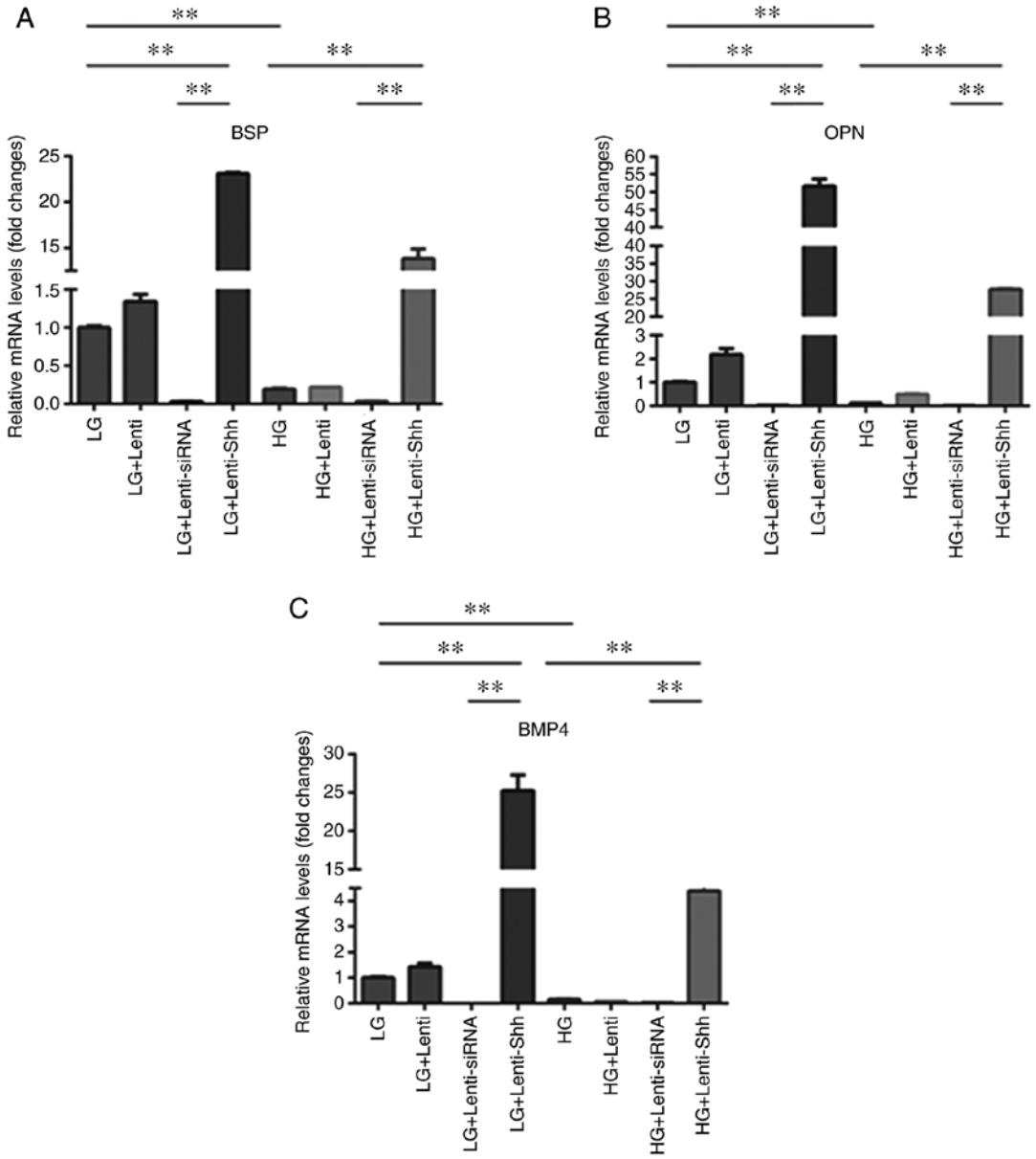

Figure 6. Gene expression of bone marrow stromal stem cells cultured in eight media conditions: LG, LG + Lenti, LG + Lenti-siRNA, LG + Lenti-Shh, HG, HG + Lenti, HG + Lenti-siRNA and HG + Lenti-Shh. The expression of (A) BSP, (B) OPN and (C) BMP4 was quantified by reverse transcription-quantitative polymerase chain reaction. **P<0.01. BMP4, bone morphogenic protein 4; BSP, bone sialoprotein; OPN, osteopontin; HG, high glucose; LG, low glucose; Lenti, lentivirus; Shh, Sonic hedgehog; siRNA, small interfering RNA.

activity levels compared with the other groups compared with the corresponding groups under the same glucose conditions. In addition, the ALP activity levels of the LG + Lenti and HG + Lenti control groups similar to the LG and HG groups, respectively. These results indicated that HG concentrations inhibited the ALP activity of BMSCs and this negative effect could be reversed by activating the Shh pathway via transduction with Lenti-Shh.

Effects of HG on the expression of osteogenesis-related genes in BMSCs and the role of Shh protein. To provide further evidence as to whether a HG inhibits the expression of osteogenesis-related genes in BMSCs and to determine whether Lenti-Shh/siRNA could adjust gene expression, the present study performed RT-qPCR to quantify their relative expression levels. As presented in Fig. 6A-C, the expression levels of bone sialoprotein (BSP), osteopontin (OPN) and BMP4 were significantly downregulated in the HG group compared with the LG group, which indicated that HG inhibited osteogenesis-related gene expression in BMSCs. Following transduction with Lenti-Shh, the LG + Lenti-Shh and HG + Lenti-Shh groups exhibited significantly increased BSP, OPN and BMP4 expression levels compared with the LG and HG groups respectively ($P<0.05$). Additionally, the LG + Lenti-siRNA and HG + Lenti-siRNA groups exhibited markedly decreased expression levels compared with the other groups under the same glucose conditions. The expression levels of the aforementioned genes in the LG + Lenti and HG + Lenti control groups, were similar to those of the LG and HG groups, respectively. These results suggested that the decreased expression of BSP, OPN and BMP4 in BMSCs under HG conditions could be reversed following transduction

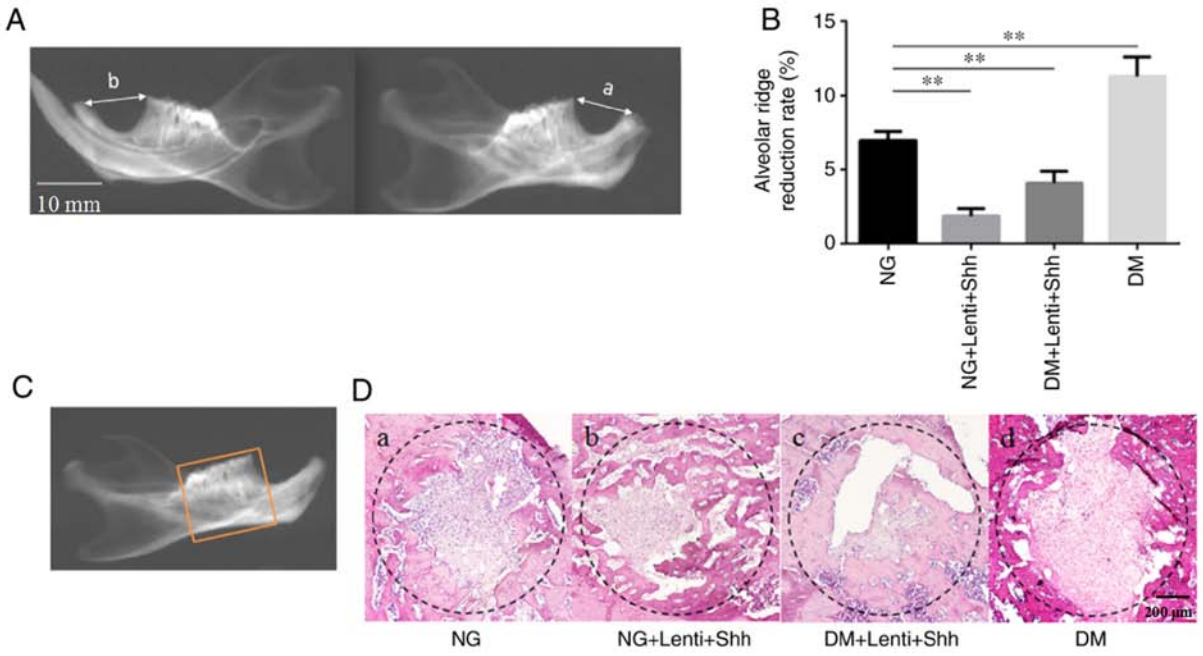

Figure 7. (A) Soft X-ray and histological analyses.The distances between the highest point of the mesial at the first molar to the lingual alveolar ridge margin of the incisor were determined: (a) The length after incisor extraction and (b) the length before incisor extraction. Scale bar, 10 mm. The reduction rate was calculated according to the following formula: (1-a/b) x100%. (B) The reduction rate results. (C) The orange border area was selected for histological analysis. (D) Hematoxylin and eosin staining: (a) Normal group, (b) normal group with Lenti+Shh, (c) DM group with Lenti+Shh and (d) DM group. Scale bar, 200 μm. **P<0.01. DM, diabetes mellitus; NG, normal group; Lenti, lentivirus; Shh, Sonic hedgehog.

with Lenti-Shh. The *in vitro* experiments were performed in triplicate.

Histological analyses suggests that Lenti-Shh enhances bone formation and reverses the adverse effect of diabetes on tooth sockets in a rat model. The present study sought to confirm whether DM alters the alveolar ridge absorption and the formation of new bones in the tooth socket. The rate of alveolar ridge reduction was measured among the four groups at 8 week (Fig. 7A and B). The results demonstrated that the rate of alveolar ridge reduction was significantly decreased in the normal group compared with the DM group. The reduction rates of the normal group with Lenti-Shh and DM + Lenti-Shh were significantly lower than the normal group. In addition, the histological analysis of the socket revealed that more fibrous connective tissue and reduced new bone formation were detected in the DM group when compared with the normal group (Fig. 7D-a and -d). Importantly, more newly formed bone was detected in the normal group with Lenti-Shh and DM +Lenti-Shh, than in the normal group (Fig. 7D-b and -c). New bone formation from the edge of sockets towards the center and an increased abundance of osteoclasts were evident in the Lenti-Shh treated groups. These data suggest that diabetes inhibited the formation of new bones and that Lenti-Shh may protect alveolar bone, while enhancing new bone formation; thus, increasing the expression of Shh may reverse the negative effect of diabetes.

Discussion

The present study investigated the effects of HG and the Shh pathway on BMSC differentiation, and the expression of osteogenesis-related genes. From cells transducing with Lenti-Shh and Lenti-siRNA vectors, the results indicated that: i) Shh signaling in osteoblasts was triggered by Lenti-Shh; ii) activation of Shh signaling enhanced the expression of BMP4, BSP and OPN; iii) HGmayimpair osteogenic differentiation; and iv) the inhibitory effects of HG on the differentiation of BMSCs were reversed via transduction with Shh lentiviral vectors. To verify these observations *in vivo*, the present study used a rat tooth extraction model, and found that: i) Lenti-Shh treatment resulted in a revealed reductions of alveolar bone to a lesser extent; and ii) significant bone formation inside the extraction socket when compared with the normal and DM groups. Taken together, the results of the present study demonstrated that osteogenic differentiation was affected by the Shh pathway under HG conditions. Furthermore, these effects could be controlled by way of activating Shh signaling.

The Hh signaling pathway serves a key role in numerous processes during embryonic development, as well as the proliferation and differentiation of stem cells, and induces bone formation (17,28,29). Oliveira *et al* (30) reported that activation of the Hh pathway increased the expression of a panel of genes associated with the osteoblast phenotype development in human mesenchymal stem cells. In addition, the Hh pathway exerts a pivotal function in driving undifferentiated cells to the osteoblast lineage (30). In addition, several lines of evidence support the notion that activation of the Hh pathway by purmorphamine increases the transcription of numerous genes, including Gli1, PTCH and ALP; however, ALP activity was induced in mouse embryonic mesoderm fibroblasts and pre-osteoblast cell lines (31,32). The results of the present study consistently demonstrated that activation of Shh signaling via

transduction with Lenti-Shh vectors upregulated the expression of BMP4, BSP and OPN. The present study also observed that the ALP activity and matrix mineralization of BMSCs were increased by triggering Shh signaling. BMP4, BSP and OPN are early transcription factors that are associated with osteoblast differentiation (33). In addition, ALP is involved in the formation and reconstruction of bones (34-36). Therefore the aforementioned bone related genes should be investigated further, in an attempt to provide additional insight into the relationship between the Shh pathway and HG levels.

The incidence of diabetes-associated bone metabolic disorders is increasing (37). If poorly controlled, the quality of life of patients could be severely affected. A previous study demonstrated that patients with T2DM had a 50% increased mortality and a 21% increased incidence of hip fracture when compared with individuals without T2DM (38). To a certain extent, our previous findings confirmed that the alveolar bone resorption was more severe in rats with diabetes compared with non-diabetic rats (4). Glucose in the microenvironment markedly affects the gene regulation of cells. A study reported that high levels of glucose inhibits the mRNA expression of ALP and RUNX family transcription factor 2 in periodontal ligament stem cells (39). The results of the present study consistently demonstrated fewer mineralized nodules in the HG groups, as well as ALP activity and the reduced mRNA expression of osteogenic related genes when compared with LG groups. This is in accordance with the findings that in the presence of HG concentrations, osteoblasts undergo differentiation; however, with a significant delay compared with control- or mannitol-treated cells (40). Additionally, SMO significantly impairs Shh-induced chemotaxis and is accompanied by elevated expression levels of PTCH, which is a protein that inhibits SMO activity (20). In the present study, Shh protein and SMO gene expression were decreased under HG conditions when compared with LG conditions. This may be due to HG-induced downregulation of genes associated with the Shh pathway. In addition the present study observed that, following transduction with Lenti-Shh, the osteoblastic differentiation of BMSCs was promoted, as were the expression of Shh and SMO under HG and LG conditions. As hypothesized, the activation of Shh signaling could prevent HG-mediated BMSCs dysfunction thereby upregulating osteoblast differentiation; however, this beneficial effect was attenuated following transduction with Lenti-siRNA, which was accompanied with a decrease in the expression of bone-related genes, ALP activity and matrix mineralization deposition. As the results of the present study demonstrated the high efficiency of lentiviral-mediated overexpression and downregulation of Shh on osteoblastic differentiation under HG conditions in BMSCs *in vitro*, future studies should investigate and verify these effects in animal models prior to use in clinical settings. The alveolar ridge reduction rate was significantly higher in DM rats than the NG rats, which indicated reduced bone formation in DM group. Following treatment with Lenti-Shh, a significant loss in alveolar bone was prevented in the NG + Lenti-Shh and DM + Lenti-Shh groups. These results indicated that the activation of Shh signaling could promote bone formation.

Due to its widespread role in embryonic and postnatal development, Shh pathway activators have attracted a great deal of attention as potential therapies (14). The study of Wang *et al* (41) used an adenovirus that expresses a secreted form of ShhN peptide to activate Hh signaling in periosteal mesenchymal progenitors and observed robust bone formation. Furthermore, in an additional study, a >90% transduction efficiency at 1 week was recorded, and continued to demonstrate stable expression for 8 weeks as identified in rat BMSCs transduced with Lenti-CMV-EGFP (42). In the present study, we transduced BMSCs with lentiviral vectors fused with GFP at an MOI of 10 and >80% of the cells were GFP positive. Lentiviral vectors were selected as gene delivery vehicles for research due to their capacity to efficiently transduce non-dividing cells, shuttle large genetic payloads and maintain stable long-term transgene expression. It has been reported that Hh signaling could be regulated by the presence of the Shh protein and GANT61 (43). However, proteins and chemicals usually have other side effects on cells. The strong cytotoxicity of GANT61 has a significant effect on cell survival (44,45), which limits its ease of application in animal experiments. An increasing amount of studies investigating bone tissue engineering have investigated the application of cytokines, specific growth factors and artificial scaffolds for promoting bone formation *in vivo* via transducing signals to modulate cellular activities (46,47). In the present study, the area of new bone formation in the NG + Lenti-Shh and DM + Lenti-Shh groups was larger than that of the NG and DM groups, respectively. This indicated that Lenti-Shh transgene expression contributed to *in vivo* bone mineralization and osteogenesis. Thus, lentiviral transgene expression may be considered as an effective therapeutic strategy for overcoming local bone defects, particularly for those with diabetes.

In conclusion, the results of the present study are in agreement with the hypothesis that HG alters the Shh pathway in osteoblasts, which results in defects during osteoblastic differentiation; the activation of Shh signaling could reverse these deleterious effects and promote bone formation. Understanding the mechanism underlying the effects of HG on osteoblastic differentiation and matrix mineralization deposition may provide novel insight into the possible therapeutic and prevention methods of osteopenia associated with diabetes. Of note, further investigation is warranted to validate the use of Lenti-Shh transgene expression to ensure the safety and efficiency for human use.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZLJ designed the study, performed the research, analyzed data, and wrote the paper. BZ designed the *in vitro* experiments and edited the manuscript. YL designed the *in vivo* experiments and edited the manuscript. ZLJ, HJ, ZSL, MYL, XFC, YYJ and HDB made substantial contributions to the analysis and interpretation of data, and drafted the manuscript. All authors reviewed the manuscript.

Ethics approval and consent to participate

All animal experiments were conducted in strict accordance with the principles of medical ethics and were approved by Animal Care and Use Committee of the Second Affiliated Hospital of Harbin Medical University (Harbin, China). All necessary permits were obtained for the described field studies.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that there are no competing interests.

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