Am J Transl Res 2015;7(12):2694-2705
www.ajtr.org /ISSN:1943-8141/AJTR0013310

Original Article

Adipocytes exert lipotoxic effects on osteoblast through activating hypoxia signaling pathway *in vitro*

Qi Zhu, Lin Wang, Yue Zuo, Chang Shan, Jing Yu, Lige Song, Keqin Zhang

Department of Endocrinology, Shanghai Tongji Hospital, Tongji University School of Medicine, Shanghai 200065, China

Received July 23, 2015; Accepted November 7, 2015; Epub December 15, 2015; Published December 30, 2015

Abstract: This study aimed to investigate the effect of adipocytes on osteoblastic bone formation *in vitro.* The differentiated and undifferentiated 3T3-L1 cells were co-cultured with primary calvarial osteoblasts. At 48 h, proliferated osteoblasts decreased significantly after co-culture with differentiated preadipocytes as compared to those co-cultured with undifferentiated preadipocytes; at 7 days, the expressions of bone formation-related genes decreased in osteoblasts co-cultured with differentiated preadipocytes; at 14 days, osteoblasts mineralization also decreased significantly after co-culture with differentiated preadipocytes. To further determine whether the decreased proliferation and mineralization were related to the hypoxia signaling pathway, the expressions of hypoxia related-genes were detected. Results showed the expressions of these genes significantly increased after co-culture with differentiated preadipocytes. NF-κB and IL-6 expressions were also up-regulated in osteoblasts co-cultured with differentiated preadipocytes. Osteoblasts from $Hif1\alpha^{f/f}$ mice showed increased proliferation and mineralization after co-culture with adipocytes transfected with adenoviral-cre, accompanied by up-regulated expressions of bone formation-related genes and down-regulated expressions of NF-κB and IL-6. These results demonstrated that adipocytes exert a negative effect on the proliferation and mineralization of osteoblasts via up-regulating the expressions of hypoxia-related genes, and NF-κB and IL-6 may impair the osteoblastic bone formation.

Keywords: Co-culture, adipocytes, osteoblasts, 3T3L1 cells, hypoxia signaling pathway

Introduction

With the acceleration of population aging, osteoporosis has become one of the most prevalent diseases worldwide. Body weight has been reported to be directly associated with bone mineral density (BMD). Low body mass index (BMI) is a risk factor for lower BMD and predicts a greater bone loss in patients with osteoporosis [1]. The higher BMD in obese patients is partly due to the mechanical loading. In addition, obese patients usually have a high level of adipose-derived aromatase which may increase the serum estrogen in obese postmenopausal women as compared to leaner individuals [2]. However, increasing studies revealed that excess adipocytes in obese individuals may negatively affect the bone metabolism and lead to bone fracture [3]. It remains unclear that how adipocytes directly and/or indirectly affect the osteoblasts and what the potential mechanism is. Both adipocytes and osteoblasts are derived from the bone marrow stromal cells, adipogenesis in the bone marrow increases while osteogenesis decreases in osteoporotic patients, and thus the imbalance between adipogenesis and osteogenesis at least partially contributes to the pathogenesis of osteoporosis. *In vitro* studies have shown that co-culture of adipocytes with osteoblasts may inhibit the osteoblasts proliferation [4]. Cawthorn et al [5] found that bone marrow adipose tissue was also a major source of circulating adiponectin by which bone marrow adipose tissue could act beyond the skeleton to exert systemic effects. Based on the above findings, this study was undertaken to investigate the lipotoxic effect of adipocyte on osteoblasts and its potential mechanism.

Trayhurn and Wood for the first time found that the hypoxia of adipose tissues in obese subjects was a key underlying mechanism triggering tissue dysfunction [6]. The adipose tissues

of obese subjects become inflammatory and numerous immune cells including B cells, T cells, and macrophages have been identified in the adipose tissues, which have emerged as an active immunological organ capable of regulating the systemic metabolism [7]. The major cause of obesity-related adipose tissue dysfunction is closely related to oxygen deficiency or "hypoxia", which pushes the adipose tissues toward a pro-inflammatory environment [8, 9]. Hypoxia-inducible factor 1α (HIF-1α) is a key regulator of cellular responses to hypoxia, and may enhance the endochondral ossification through activating vascular endothelial growth factor (VEGF) [10]. However, the up-regulated HIF-1α expression in osteoblasts may inhibit the Wnt signaling pathway and decrease the osteoblastic bone formation [11]. Thus, HIF-1α plays different roles in the osteoblastic bone formation and endochondral bone formation. Furthermore, hypoxia can enhance the apoptosis of osteoblasts in glucocorticoid-induced osteonecrosis leading to increased adipogenesis and decreased osteogenesis in the bone marrow [12]. The adipose tissue volume of the bone marrow increased over age and in patients with osteoporosis [13, 14]. It remains unclear whether accumulated adipocytes can stimulate HIF-1α expression to inhibit the osteoblastic bone formation as osteoblasts are able to interact with adipocytes in the bone marrow.

This study was undertaken to investigate the lipotoxic effect of adipocytes in the bone marrow, and the potential mechanism was explored. Using a co-culture system [15], mouse osteoblasts were exposed to either differentiated or undifferentiated pre-adipocytes. The HIF-1α expression was measured in the adipocytes and its influence on the proliferation and differentiation of osteoblasts was investigated. In this study, results showed adipocytes could affect the proliferation and differentiation of osteoblasts through inhibiting the expressions of osteoblastic bone formation related genes: collagen I (Colla1), osteocalcin (Ocn) and Runx2/Cbfα-1 (Runx2/Cbfa-1). These inhibitions were alleviated when the HIF-1α expression was down regulated in osteoblasts. Findings in this study may provide evidence for the interaction between fat and bone within the bone marrow microenvironment and the potential regulation of hypoxia on bone metabolism.

Materials and methods

Animals

The Hif1α$^{f/f}$ transgenic mice provided by the laboratory of Lianfu Deng (Shanghai Jiaotong University, Shanghai, China) were used in present study [16]. Animals were housed in the Experimental Animal Center of Shanghai Tongji Hospital (Shanghai Tongji University, Shanghai, China). The whole study protocol was approved by the Ethic Committee of Shanghai Tongji Hospital and all the procedures were conducted according to the Guide for the Care and Use of Laboratory Animals.

3T3-L1 cell culture and induced differentiation

3T3-L1 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences in Shanghai, China. 3T3-L1 cells were seeded in 6-well plates (Corning 3506) and grown in preadipocyte growth medium consisting of Dulbecco's Modified Eagle's Medium (DMEM GibcoBRL11965-084) with fetal bovine serum (FBS; 10% v/v GibcoBRL10437-028), penicillin (200 U/ml)/streptomycin (200 mg/ml) (GibcoBRL10378-016). Medium was refreshed once every two days. Two days post-confluence, differentiation was induced using a standard adipogenic cocktail containing 1 mg/ml insulin (Sigma I-5500), 0.5 mM isobutylmethylxanthine (Sigma I-7018), and 1 mM dexamethasone (Sigma D-4902) in DMEM with 10% FBS and penicillin/streptomycin. After 48 h, the differentiation medium was refreshed with DMEM containing 1 mg/ml insulin. After 48 h, the insulin medium was replaced with the adipocyte basal medium which was refreshed once every two days. Oil red staining was performed according to manufacturer's instructions (KeyGEN KGA329). Adipocytes were counted in 10 randomly selected fields.

Primary calvarial osteoblasts culture and alkaline phosphatase staining

Primary calvarial osteoblasts were prepared from 1-week-old *C57Bl/6* mice. First, calvariae were resected and placed into ice-cold PBS to remove surrounding tissues. Then, the calvariae was repeatedly digested with collagenase type II (Invitrogen 17101-015) in Hank's for 6×20 min, and the supernatant was collected since the third digestion, pooled, plated in com-

Table 1. Primers used for quantitative real-time PCR

Gene	Forward	Reverse
Col-1	TCTCCACTCTTCTAGTTCCT	TTGGGTCATTTCCACATGC
OCN	CAAGTCCCACACAGCAGCTT	AAAGCCGAGCTGCCAGAGTT
Cbfα-1	CCGCACGACAACCGCACCAT	CGCTCCGGCCCACAAATCTC
HIF-1α	TCCATGTGACCATGAGGAAA	CTTCCACGTTGCTGACTTGA
HIF-1β	TCACGAAGGTCGTTCATCTG	GATGTAGCCTGTGCAGTGGA
HIF-2α	AGTAGCCTCTGTGGCTCCAA	TCCAGGGCATGGTAGAACTC
GAPDH	GGTCGGTGTGAACGGATTTG	ATGAGCCCTTCCACAATG

plete medium with α-MEM (GibcoBRL 12571-063) and 10% FBS and incubated at 37°C with 5% CO_2. The activity of osteoblasts was evaluated by osteoblast alkaline phosphatase (ALP) staining. ALP activity was assessed according to the manufacturer's instructions (Vector Blue SK-5300).

In vitro differentiation and adenoviral-cre infection of osteoblasts

The isolated osteoblasts from Hif1α$^{f/f}$ transgenic mice were maintained in the basal compartment of 6-well plates of transwell plates with α-MEM medium containing 10% FBS for 48 h, and then transfected with adenoviral-cre or adenoviral-enhanced green fluorescent protein (EGFP) (2×10^7 plaque-forming units [PFU] adenovirus per well) for 48 h, followed by incubation in osteoblast differentiation medium containing 10 mM/L β-glycerophosphate, 50 mg/L vitamin C and 0.1 μmol/L dexamethasone, or in complete medium containing α-MEM and 10% FBS. The medium was refreshed once every 2 days.

3T3-L1 cells co-culture with primary calvarial osteoblasts

Differentiated 3T3-L1 cells were cultured in the upper chambers (pore size: 0.4 μm, area: 4.2 cm^2; Corning Incorporated, NY, USA) with primary calvarial osteoblasts grown in the lower chambers of 6-well plates (area: 9.6 cm^2). There was a unidirectional medium flow from upper to lower chambers while direct intercellular contact was avoided. 3T3-L1 cells were grown at 50,000/cm^2 for 48 h until 100% confluence was achieved. Differentiation medium was then replenished until cells were grown in adipocyte basal medium. Adipocytes in the upper chambers were then co-cultured with monolayer primary calvarial osteoblasts at 40,000/cm^2 in the lower chambers. Fresh culture medium was added to both upper and lower chambers and the co-culture was maintained for 48 h for the examination of cell proliferation and 2 weeks for the examination of mineralization. Cells were divided into 4 groups as follows: (1) differentiated 3T3-L1 cells were plated in the upper chambers with osteoblasts in the lower chambers, and there was a unidirectional medium flow from the adipocytes to the osteoblasts; (2) undifferentiated 3T3-L1 cells were placed in the upper chambers with osteoblasts in the lower chambers; (3) differentiated 3T3-L1 cells were plated in the upper chambers with osteoblasts from Hif1α$^{f/f}$ transgenic mice in the lower chambers and infected with adenoviral-EGFP; (4) differentiated 3T3-L1 cells were plated in the upper chambers with osteoblasts from Hif1α$^{f/f}$ transgenic mice in the lower chambers and infected with adenoviral-cre. Contact between the medium in the bottom and the membrane was carefully avoided during cell culture. Medium in both upper and lower chambers was refreshed once every 2 days. The supernatant was collected and stored at -20°C for further measurements. After 7 days, cells were collected for mRNA and protein extraction; after 14 days, cells were subjected to alizarin red staining.

Cell proliferation and mineralization assays

Cell proliferation was assessed by using the Cell-Light EdU DNA cell proliferation kit (RiboBio, Guangzhou, China), according to the manufacturer's instructions. For the mineralization assay, confluent primary calvarial osteoblasts were grown in osteoblast differentiation medium containing 10 mM/L β-glycerophosphate (Sigma G-6251), 50 mg/L vitamin C (Sigma A-7506) and 0.1 μmol/L dexamethasone (Sigma D-1756) for 14 d. Alizarin red staining was employed for the mineralization assay of osteoblasts according to the manufacturer's instructions (GMS80046; Genmed Scientifics Inc. USA).

Quantitative real-time PCR

RNA was isolated from mouse bone tissues using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed using the PrimeScript™

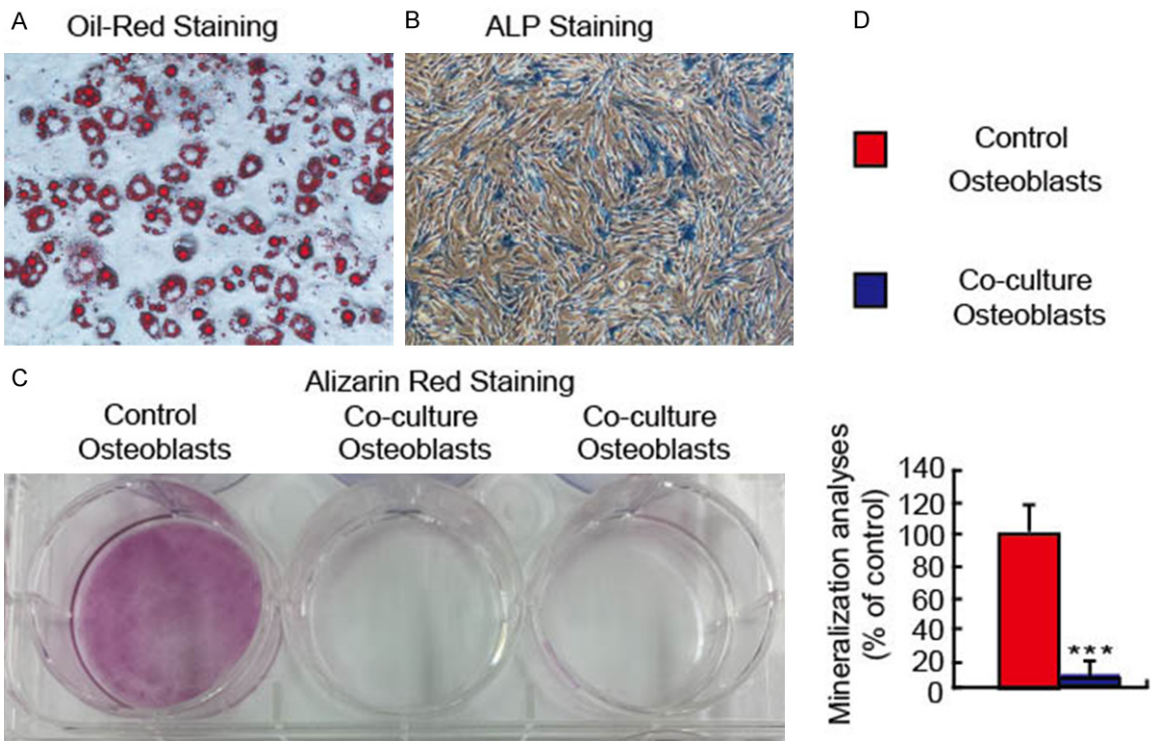

Figure 1. Inhibitory effect on the mineralization of osteoblasts co-cultured with adipocytes. A. Oil-Red staining for differentiated preadipocytes. B. ALP staining for primary calvarial osteoblasts. C. Differentiated osteoblasts were co-cultured with differentiated or undifferentiated adipocytes. Adipocytes were treated with adipogenic cocktail or left untreated (controls). Mineralization (alizarin red staining) assay was performed at 14 days after co-culture. D. Mineralization dramatically decreased in osteoblasts co-cultured with differentiated adipocytes than in control group. *P<0.05; **P<0.01; ***P<0.001. Data are from 3 independent experiments (3 wells/experiment).

RT reagent Kit (Takara 037A) as previously described, and cDNA after reverse transcription was amplified by real-time PCR [17] using a Light Cycler system (Roche Molecular Biochemicals, Indianapolis, IN, USA) with SYBR Premix Ex Taq™ Kit (Takara RR420A). Samples were denatured at 95°C for 10 s. Amplification was carried out as follows: denaturation at 95°C for 1 s, annealing for 5 s and extension at 72°C for 20 s for a total of 40 cycles. Then, the SYBR Green fluorescence was detected to reflect the amount of double-stranded DNA after incubation at 86°C for 3 s. A melting curve was obtained at the end of each run to discriminate specific cDNA from nonspecific cDNA products. Products were denatured at 95°C for 3 s, and then incubated at 58°C for 15 sec and at 95°C. To determine the copies of targeted DNA in the samples, purified PCR fragments of known concentrations were serially diluted and served as external standards that were measured in each experiment. The mRNA expression of a target gene was normalized to that of GAPDH in the same sample. The primers used for real-time PCR were the same to those used for routine PCR. All the primers were designed and synthesized in Sangon Biotech (Shanghai, China; Tab). Primers are listed in **Table 1**.

Western blot assay

Cells were washed with PBS and total protein was extracted in ice-cold buffer (keyGEN whole cell lysis assay KGP 250-2100). Protein concentration was determined with a protein assay kit (KeyGEN BCA Protein Quantitation Assay). Then, total protein was subjected to separation by SDS-PAGE and transferred onto nitrocellulose membranes which were then blocked in non-fat milk in Tris-buffered saline supplemented with 0.1% Tween-20. Proteins were detected with antibodies against collagen I (1:1000; abcam138492), HIF-1α (1:200; abcamH1alpha67), NF-κB (1:1000; abcam131485), IL-6 (1:1000; abcam6672). GAPDH (1:1000 Beyotime AG019) was used as a loading control. Bands were visualized by using enhanced chemiluminescence (KeyGENE ECL Detection Kit KGP1123-KGP1121) [18].

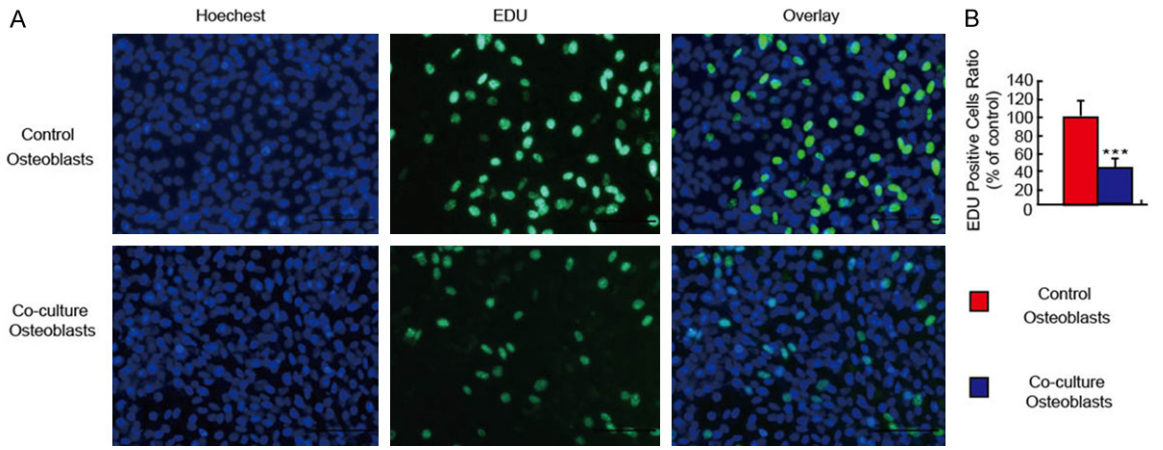

Figure 2. Inhibitory effect on the proliferation of osteoblasts co-cultured with adipocytes. A. Differentiated osteoblasts were co-cultured with differentiated or undifferentiated adipocytes. Adipocytes were treated with adipogenic cocktail or left untreated (controls). Proliferation (EDU staining) assay was performed at 48 h after co-culture. B. Proliferation dramatically decreased in osteoblasts co-cultured with differentiated adipocytes than in control group. *P<0.05; **P<0.01; ***P<0.001. Data are from 3 independent experiments (3 wells/experiment).

Computer-assisted image examination

After histochemical examination, or Edu staining, images were captured with a Nikon digital camera, processed and analyzed using Image-Pro Plus6.0 analysis software. All the measurements were performed in a blinded manner. The region of interest (ROI) was outlined manually using the software.

Statistical analysis

Data for image analysis are presented as means ± standard error (SEM). Comparisons were performed using a two-way analysis of variance (ANOVA), and a value of $P<0.05$ was considered statistically significant. Statistical analysis was done with SPSS version 10.0.

Results

Inhibited mineralization of osteoblasts co-cultured with adipocytes

To assess whether the adipocytes had an inhibitory effect on the osteoblastic mineralization, osteoblastic bone formation was evaluated by Alizarin red staining at 14 days after co-culture with or without differentiated preadipocytes. Oil-Red staining showed preadipocytes differentiated into adipocytes (**Figure 1A**). ALP staining was employed to assess the primary calvarial osteoblasts (**Figure 1B**). Alizarin red staining showed mineralization at 14 days in osteoblasts co-cultured with undifferentiated preadipocytes and in those co-cultured with differentiated preadipocytes. However, the mineralization showed a decreased trend in osteoblasts co-cultured with differentiated preadipocytes (**Figure 1C**). The mineralization quantification showed the mineralization in osteoblasts co-cultured with adipocytes was significantly lower than osteoblasts co-cultured with undifferentiated adipocytes at 14 days (**Figure 1D**).

Inhibited proliferation of osteoblasts co-cultured with adipocytes

To assess whether the adipocytes had an inhibitory effect on the osteoblastic proliferation, osteoblastic proliferation was determined by EDU staining at 48 h after co-culture with or without differentiated preadipocytes. Hoechest stained the nucleus of all the cells while EDU staining showed all the osteoblasts entered S1 phase at 48 h after co-culture. However, the proliferation decreased in osteoblasts co-cultured with differentiated preadipocytes as compared to those co-cultured with undifferentiated preadipocytes (**Figure 2A** and **2B**).

Effects of co-culture on the expressions of genes related to osteoblastic bone formation, hypoxia as well as IL-6 and NF-κB in osteoblasts

To assess whether adipocytes had an inhibitory effect on the osteoblastic bone formation, the expressions of genes related to osteoblastic bone formation were detected by real-time PCR

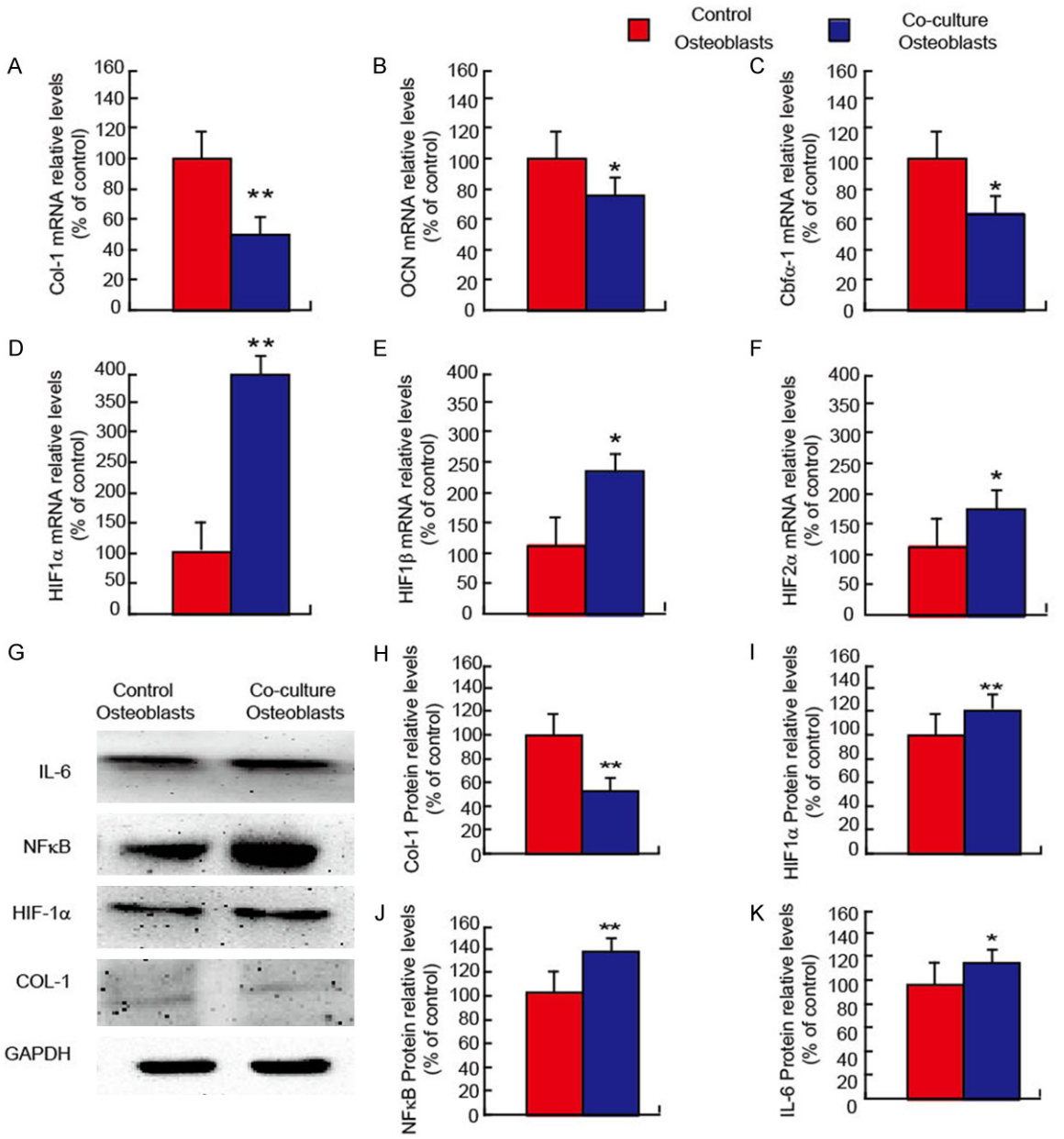

Figure 3. Effects of co-culture on the expressions of genes related to osteoblastic bone formation and hypoxia as well as IL-6 and NF-κB in osteoblasts. Real-time RT-PCR was performed on primary calvarial osteoblasts co-cultured with undifferentiated adipocytes and those co-cultured with differentiated adipocytes at 7 days for the detection of expressions of Col1a1 (A), OCN (B) and Cbfα-1 (C), as well as HIF-1α (D), HIF-1β (E) and HIF-2α (F). GAPDH served as a loading control. mRNA expressions of *Cbfα1*, Col1a1 and *OCN* were down-regulated significantly in osteoblasts co-cultured with differentiated adipocytes. mRNA expressions of HIF-1α, HIF-1β and HIF-2α were up-regulated significantly in osteoblasts co-cultured with differentiated adipocytes. Western blot assay of osteoblasts co-cultured with differentiated and undifferentiated adipocytes for the detection of expressions of col I, HIF-1α, NF-κB and IL-6. GAPDH was used as loading control (G). Col I (H), HIF-1α (I), NF-κB (J) and IL-6 (K) protein expression relative to GAPDH protein expression were normalized to those in osteoblasts without co-culture. Data are expressed as mean ± SEM from 3 independent experiments. *P<0.05; **P<0.01; ***P<0.001 vs control group.

at 7 days after co-culture with or without differentiated preadipocytes. Real-time PCR showed that the mRNA expressions of genes related to osteoblastic bone formation: Col-1 (**Figure 3A**), OCN (**Figure 3B**) and Cbfα-1 (**Figure 3C**) significantly decreased in osteoblasts co-cul-

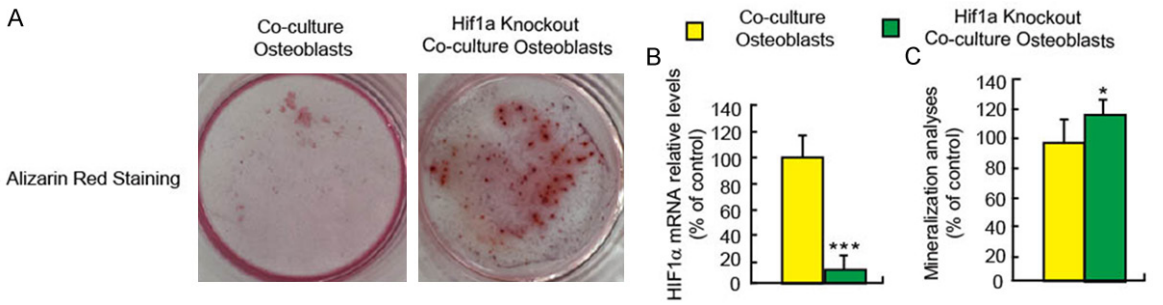

Figure 4. Effects of Hif1α deletion on the mineralization of osteoblasts co-cultured with adipocytes. A. Mineralization (alizarin red staining) assay was performed in osteoblasts at 14 days after co-culture with adipocytes transfected with adenovirus. B. Realtime PCR showed HIF-1α expression was down-regulated in Hif1α$^{f/f}$ osteoblasts co-cultured with adipocytes transfected with adenoviral-cre. C. Mineralization increased in Hif1α$^{f/f}$ osteoblasts co-cultured with adipocytes transfected with adenoviral-cre than in those co-cultured with adipocytes transfected with adenoviral-EGFP. *P<0.05; **P<0.01; ***P<0.001. Data are from 3 independent experiments (3 wells/experiment).

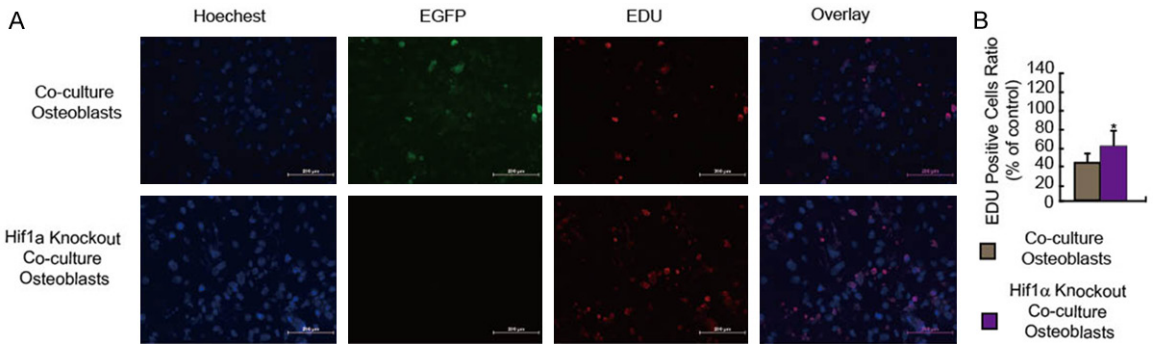

Figure 5. Effects of HIF-1α deletion on the proliferation of osteoblasts co-cultured with adipocytes. A. Osteoblasts from Hif1α$^{f/f}$ mice co-cultured with adipocytes transfected with adenoviral-cre or adenoviral-EGFP. Proliferation (EDU staining) assay was performed at 48 h after transfection with adenovirus. B. Proliferating osteoblasts significantly decreased in both groups, while they increased in Hif1α$^{f/f}$ osteoblasts co-cultured with adipocytes transfected with adenoviral-cre than in Hif1α$^{f/f}$ osteoblasts co-cultured with adipocytes transfected with adenoviral-EGFP. *P<0.05; **P<0.01; ***P<0.001. Data are from 3 independent experiments (3 wells/experiment).

tured with differentiated preadipocytes as compared to those co-cultured with undifferentiated preadipocytes at 7 days.

To further assess whether adipocytes exerted an inhibitory effect on the osteoblastic bone formation through the hypoxia signaling pathway, the expressions of hypoxia related genes were detected by real-time PCR at 7 days after co-culture with or without differentiated preadipocytes. Real-time PCR showed that the expressions of hypoxia related genes: HIF-1α (**Figure 3D**), HIF-1β (**Figure 3E**) and HIF-2α (**Figure 3F**) significantly increased in osteoblasts co-cultured with differentiated preadipocytes as compared to those co-cultured with undifferentiated preadipocytes at 7 days.

The protein expressions of several cytokines were also detected in osteoblasts co-cultured with or without differentiated preadipocytes at 7 days by western blot assay. Western blot assay showed the protein expressions of HIF-1α, IL-6 and NF-κB significantly increased in osteoblasts co-cultured with differentiated preadipocytes as compared to those co-cultured with undifferentiated preadipocytes at 7 days, but the col-1 protein and mRNA expressions were down-regulated significantly osteoblasts co-cultured with differentiated preadipocytes (**Figure 3G-K**).

Effects of HIF-1a deletion on the mineralization of osteoblasts co-cultured with adipocytes

To assess whether the increased HIF-1α had a negative effect on the bone formation and mineralization of osteoblasts co-cultured, osteoblasts were collected from Hif1α$^{f/f}$ mice, transfected with adenoviral-cre or adenoviral-EGFP,

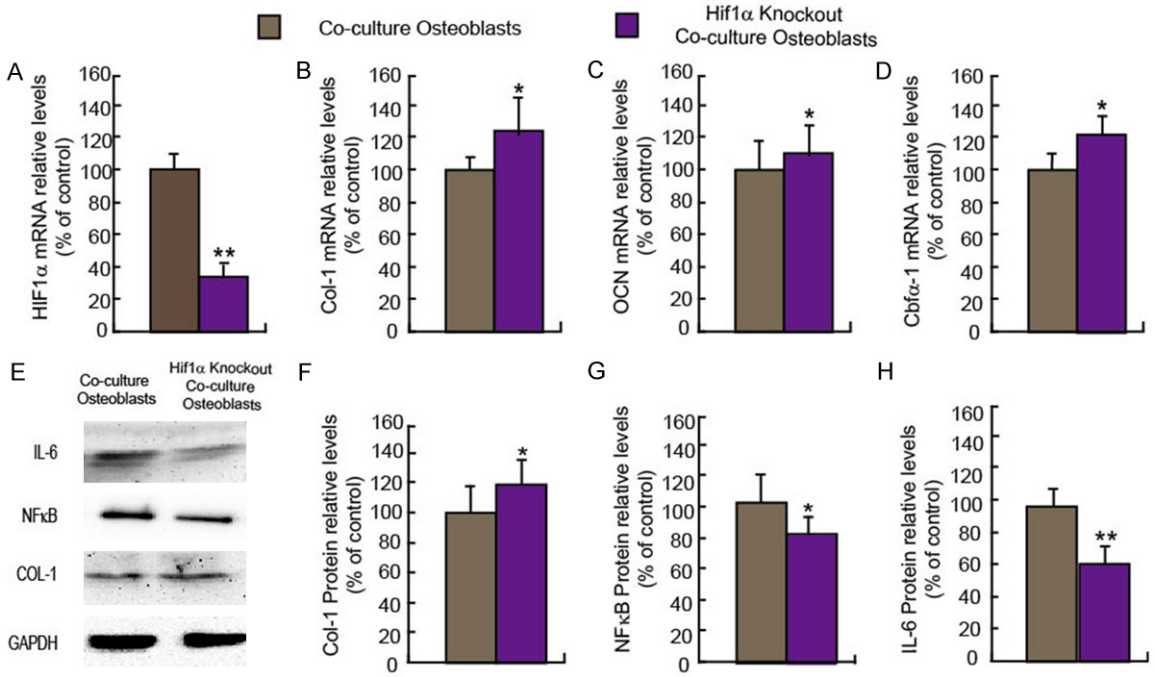

Figure 6. Effects of HIF-1α deletion on the expressions of genes related to bone formation as well as IL-6 and NF-κB in osteoblasts. Real-time RT–PCR was performed for the detection of expressions of HIF-1α (A), and Col I (B), OCN (C) and Cbfα-1 (D) on primary calvarial osteoblasts from c Hif1α$^{f/f}$ mice co-cultured with adipocytes transfected with adenoviral-cre or adenoviral-EGFP at 7 days. mRNA expression relative to GAPDH expression was normalized to that in Hif1α$^{f/f}$ osteoblasts co-cultured with adipocytes transfected with adenoviral-EGFP. HIF-1α expression was down-regulated significantly in osteoblasts co-cultured with adipocytes transfected with adenoviral-cre. The expressions of Runx2/Cbfa1, Col1a1, and Ocn were up-regulated significantly in osteoblasts co-cultured with adipocytes transfected with adenoviral-cr. Western blot assay for the expressions of col I, NF-κB and IL-6 in osteoblasts from Hif1α$^{f/f}$ mice transfected with adenoviral-cre or adenoviral-EGFP and co-cultured with adipocytes at 7 days. GAPDH was used as loading control (E). Protein expressions relative to GAPDH were normalized to those in Hif1α$^{f/f}$ osteoblasts co-cultured with adipocytes transfected with adenoviral-EGFP (F-H). Data are expressed as mean ± SEM from 3 independent experiments. *P<0.05; **P<0.01; ***P<0.001 vs control group.

and co-cultured with differentiated preadipocytes. Real-time PCR showed HIF-1α expression significantly decreased at 48 h after transfection with adenoviral-cre as compared to Hif1α$^{f/f}$ osteoblasts transfected with adenoviral-EGFP in the co-culture system (**Figure 4B**). Osteoblastic mineralization was evaluated by Alizarin red staining at 14 days after transfection with adenoviral-cre or adenoviral-EGFP (**Figure 4A**). Results showed the bone mineralization increased after transfection with adenoviral-cre as compared to Hif1α$^{f/f}$ osteoblasts transfected with adenoviral-EGFP in the co-culture system (**Figure 4C**).

Effects of HIF-1α deletion on the proliferation of osteoblasts co-cultured with adipocytes

To assess whether increased HIF-1α had an inhibitory effect on the proliferation of osteoblasts co-cultured, the proliferation of osteoblasts was determined at 48 h by EDU staining. Hoechest stained cell nucleus. After co-culture with differentiated preadipocytes, the proliferation increased in Hif1α$^{f/f}$ osteoblasts transfected with adenoviral-cre as compared to Hif1α$^{f/f}$ osteoblasts transfected with adenoviral-EGFP in the co-culture system (**Figure 5A** and **5B**).

Effects of HIF-1α deletion on the expressions of genes related to osteoblastic bone formation as well as IL-6 and NF-κB in osteoblasts

To assess whether adipocytes had an inhibitory effect on the osteoblastic bone via the hypoxia signaling pathway, the expressions of osteoblastic bone formation related genes were detected by real-time PCR in Hif1α$^{f/f}$ osteoblasts transfected with adenoviral-EGFP or adenoviral-cre at 7 days after co-culture with differentiated preadipocytes. HIF-1α expression significantly decreased in Hif1α$^{f/f}$ osteoblasts at 48 h after transfection with adenoviral-cre when compared with those transfected with

adenoviral-EGFP (**Figure 6A**). Real-time PCR showed the expressions of osteoblastic bone formation related genes: Col-1 (**Figure 6B**), OCN (**Figure 6C**) and Cbfα-1 (**Figure 6D**) significantly increased in Hif1α$^{f/f}$ osteoblasts transfected with adenoviral-cre when compared with those transfected with adenoviral-EGFP at 7 days after co-culture.

Western blot assay was also employed to determine the protein expressions of several genes in Hif1α$^{f/f}$ osteoblasts at 7 days after co-culture with differentiated preadipocytes. Results showed that the expressions of IL-6 and NF-κB significantly decreased in Hif1α$^{f/f}$ osteoblasts transfected with adenoviral-cre when compared with those transfected with adenoviral-EGFP at 7 days after co-culture with differentiated preadipocytes. The col-1 protein and mRNA expressions were up regulated significantly in Hif1α$^{f/f}$ osteoblasts transfected with adenoviral-cre following co-culture (**Figure 6E-H**).

Discussion

In the present study, co-culture system was employed to simulate the intercellular interaction in the aging bone marrow where the functions of mature osteoblast decline [19] while the adipocytes become predominant and might have a toxic effect on the osteoblastic bone formation [20]. The mechanisms of inhibitory effects of adipocytes on the osteoblastic differentiation remain unclear, and TNF-α and free fatty acid have been reported to be associated with these inhibitory effects [4, 21]. Results of this study demonstrated that adipocytes could negatively regulate the osteoblastic bone formation *in vitro* through inhibiting the proliferation and differentiation of osteoblasts. Additionally, this inhibitory effect could be reversed by inhibiting HIF-1α expression on osteoblasts. Moreover, up-regulated expressions of NF-κB and IL-6 were also found to be associated with the lipotoxicity of adipocytes in models of this study.

Osteoblasts are susceptible to the influence of bone marrow components and adjacent vasculatures. The oxygen tension in the bone marrow is about 5-9%. Osteoblasts have functional mitochondria where oxidative phosphorylation occurs [22]. The reduction in oxygen tension may cause a hypoxic or anoxic environment where the osteoblast phenotype may be inhibited [23-25]. Hypoxia/HIF-1α may cooperate with Osx to inhibit Wnt signaling pathway in osteoblasts [26]. However, it was suggested that low oxygen tension could promote the differentiation of osteoblasts into osteocytes [16, 27]. Wang et al [10] demonstrated that HIF-1α over-expression in osteoblast increased the bone formation, which was associated with the increased angiogenesis and VEGF expression. However, HIF-1α deficiency may delay the progression from condensed mesenchymal stromal cells to proliferating chondrocytes and then to hypertrophic chondrocytes, leading to impaired chondrogenesis and compromised endochondral bone formation. Thus, HIF-1α is required for the normal bone development [28]. HIF-1α may act as a survival factor by activating VEGF expression, which is required for the chondrocyte survival [29]. Hypoxia can activate Sox9 expression and inhibit Runx2 expression. Runx2 is expressed in the condensed mesenchymal stromal cells and prehypertrophic chondrocytes and has a high expression in the osteoblast lineage [28]. Results showed the proliferation and mineralization of osteoblasts decreased without statistical significance after transfected with adenoviral-cre in contrast to Hif1α$^{f/f}$ osteoblasts transfected with adenoviral-EGFP (Data not shown). These results showed that HIF-1α plays different roles in the endochondral bone formation and osteoblastic bone formation. The high HIF expression under normoxic condition is accompanied by increased expression of a large number of glycolytic HIF target genes and increased glycolysis [30] which might be a potential mechanism for the inhibitory effect of HIF-1α on osteoblastic bone formation in vitro.

Furthermore, whether HIF-1α played a pivotal role in the lipotoxic effect of adipocytes was investigated. Recent findings show that HIF-1α is a key participant in the metabolism and function of immune cells [31]. Hypoxia in the adipose tissues has been postulated as a possible contributor to the obesity-related chronic inflammation, insulin resistance, and metabolic dysfunction [4]. HIF-1α stabilization in immune cells may occur in an oxygen-independent manner [32, 33]. NF-κB, which plays a central role in regulating the immune response to infection, is required for the bacteria-induced HIF-1α mRNA expression in macrophages [32]. IL-6 could enhance HIF-1α mRNA expression via signal

transduction and activator of transcription (STAT3) signaling pathway [34]. Oxidative stress has been found to increase in osteoporosis and may activate p53 and $p66^{shc}$ in the bone. On the other hand, ROS stimulate the FoxO family of transcription factors and sirtuins to combat oxidative stress and maintain the skeletal homeostasis [35]. Results in this study showed that adipocytes inhibited the proliferation and mineralization of osteoblasts through up-regulating the expressions of NF-κB and IL-6 in osteoblasts and then increasing the HIF-1α expression, which demonstrated the lipotoxic effect of adipocytes in age related bone disease was related to the chronic inflammation and immunity. Further study was still needed to confirm it *in vivo*.

The novelty of findings in this study was to assess the potential mechanism of age related bone loss as well as the microenvironment of the bone marrow. Results of this study implied that the adipocytes had a negative effect on the nuclear binding of Runx2, and the adipocyte induced chronic inflammation stimulated the HIF-1α expression in differentiating adipocytes to affect the nuclear binding of Runx2 in mature osteoblasts and then influence the expression of downstream osteogenic genes, such as OSX and OCN, which might be another mechanism of age-related bone loss.

In summary, results in this study demonstrate that the lipotoxic effect of adipocytes on osteoblasts is related to the HIF-1α expression in osteoblasts as well as IL-6 and NF-κB expressions. Although this toxic effect might be also associated with either the induction of PPAR-γ pathway or free fatty acid-induced lipotoxicity in osteoblasts, further studies are required to confirm the specific pathways involved in this effect and to investigate whether HIF-1α inhibitor or anti-inflammatory drugs may serve as a potential therapeutic approach for senile osteoporosis.

Acknowledgments

We thank Lianfu Deng (Shanghai Ruijin Hospital, Shanghai Jiaotong University) for generously providing $HIF\text{-}1^{f/f}$ mice. This work was supported by the National Basic Research Program of China (No. 81170802) (to K Zhang), National Natural Science Foundation of China to L Song (No. 81300712), Shanghai Science and Technology Foundation to L Song (No. 13ZR1438000), Research Fund for the Doctoral Program of Higher Education of China to L Song (No. 20130072120014).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Keqin Zhang, Department of Endocrinology, Shanghai Tongji Hospital, Tongji University School of Medicine, Shanghai 200065, China. Tel: 011-86-21-6611-2032; Fax: 011-86-21-6611-2032; E-mail: keqzhang2007@126.com

References

[1] De Laet C, Kanis JA, Oden A, Johanson H, Johnell O, Delmas P, Eisman JA, Kroger H, Fujiwara S, Garnero P, McCloskey EV, Mellstrom D, Melton LJ 3rd, Meunier PJ, Pols HA, Reeve J, Silman A and Tenenhouse A. Body mass index as a predictor of fracture risk: a meta-analysis. Osteoporos Int 2005; 16: 1330-1338.

[2] Dytfeld J, Ignaszak-Szczepaniak M, Gowin E, Michalak M and Horst-Sikorska W. Influence of lean and fat mass on bone mineral density (BMD) in postmenopausal women with osteoporosis. Arch Gerontol Geriatr 2011; 53: e237-242.

[3] Shapses SA and Sukumar D. Bone metabolism in obesity and weight loss. Annu Rev Nutr 2012; 32: 287-309.

[4] Elbaz A, Wu X, Rivas D, Gimble JM and Duque G. Inhibition of fatty acid biosynthesis prevents adipocyte lipotoxicity on human osteoblasts in vitro. J Cell Mol Med 2010; 14: 982-991.

[5] Cawthorn WP, Scheller EL, Learman BS, Parlee SD, Simon BR, Mori H, Ning X, Bree AJ, Schell B, Broome DT, Soliman SS, DelProposto JL, Lumeng CN, Mitra A, Pandit SV, Gallagher KA, Miller JD, Krishnan V, Hui SK, Bredella MA, Fazeli PK, Klibanski A, Horowitz MC, Rosen CJ and MacDougald OA. Bone marrow adipose tissue is an endocrine organ that contributes to increased circulating adiponectin during caloric restriction. Cell Metab 2014; 20: 368-375.

[6] Trayhurn P and Wood IS. Adipokines: inflammation and the pleiotropic role of white adipose tissue. Br J Nutr 2004; 92: 347-355.

[7] Grant RW and Dixit VD. Adipose tissue as an immunological organ. Obesity (Silver Spring) 2015; 23: 512-518.

[8] Wellen KE and Hotamisligil GS. Obesity-induced inflammatory changes in adipose tissue. J Clin Invest 2003; 112: 1785-1788.

[9] Hosogai N, Fukuhara A, Oshima K, Miyata Y, Tanaka S, Segawa K, Furukawa S, Tochino Y, Komuro R, Matsuda M and Shimomura I. Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. Diabetes 2007; 56: 901-911.

[10] Wang Y, Wan C, Deng L, Liu X, Cao X, Gilbert SR, Bouxsein ML, Faugere MC, Guldberg RE, Gerstenfeld LC, Haase VH, Johnson RS, Schipani E and Clemens TL. The hypoxia-inducible factor alpha pathway couples angiogenesis to osteogenesis during skeletal development. J Clin Invest 2007; 117: 1616-1626.

[11] Chen D, Li Y, Zhou Z, Wu C, Xing Y, Zou X, Tian W and Zhang C. HIF-1alpha inhibits Wnt signaling pathway by activating Sost expression in osteoblasts. PLoS One 2013; 8: e65940.

[12] Zou W, Yang S, Zhang T, Sun H, Wang Y, Xue H and Zhou D. Hypoxia enhances glucocorticoid-induced apoptosis and cell cycle arrest via the PI3K/Akt signaling pathway in osteoblastic cells. J Bone Miner Metab 2015; 33: 615-24.

[13] Nuttall ME and Gimble JM. Controlling the balance between osteoblastogenesis and adipogenesis and the consequent therapeutic implications. Curr Opin Pharmacol 2004; 4: 290-294.

[14] Justesen J, Stenderup K, Ebbesen EN, Mosekilde L, Steiniche T and Kassem M. Adipocyte tissue volume in bone marrow is increased with aging and in patients with osteoporosis. Biogerontology 2001; 2: 165-171.

[15] Maurin AC, Chavassieux PM, Frappart L, Delmas PD, Serre CM and Meunier PJ. Influence of mature adipocytes on osteoblast proliferation in human primary cocultures. Bone 2000; 26: 485-489.

[16] Wan C, Gilbert SR, Wang Y, Cao X, Shen X, Ramaswamy G, Jacobsen KA, Alaql ZS, Eberhardt AW, Gerstenfeld LC, Einhorn TA, Deng L and Clemens TL. Activation of the hypoxia-inducible factor-1alpha pathway accelerates bone regeneration. Proc Natl Acad Sci U S A 2008; 105: 686-691.

[17] Zhang Q, Xiao XH, Li M, Li WH, Yu M, Zhang HB, Ping F, Wang ZX and Zheng J. Chromium-containing traditional Chinese medicine, Tianmai Xiaoke Tablet improves blood glucose through activating insulin-signaling pathway and inhibiting PTP1B and PCK2 in diabetic rats. J Integr Med 2014; 12: 162-170.

[18] Wang LN, Wang Y, Lu Y, Yin ZF, Zhang YH, Aslanidi GV, Srivastava A, Ling CQ and Ling C. Pristimerin enhances recombinant adeno-associated virus vector-mediated transgene expression in human cell lines in vitro and murine hepatocytes in vivo. J Integr Med 2014; 12: 20-34.

[19] Rosen CJ and Bouxsein ML. Mechanisms of disease: is osteoporosis the obesity of bone? Nat Clin Pract Rheumatol 2006; 2: 35-43.

[20] Cartwright MJ, Tchkonia T and Kirkland JL. Aging in adipocytes: potential impact of inherent, depot-specific mechanisms. Exp Gerontol 2007; 42: 463-471.

[21] Abuna RP, De Oliveira FS, Santos Tde S, Guerra TR, Rosa AL, Beloti MM. Participation of TNF-alpha in Inhibitory Effects of Adipocytes on Osteoblast Differentiation. J Cell Physiol 2016; 231: 204-14.

[22] Gay C and Schraer H. Frozen thin-sections of rapidly forming bone: bone cell ultrastructure. Calcif Tissue Res 1975; 19: 39-49.

[23] Matsuda N, Morita N, Matsuda K and Watanabe M. Proliferation and differentiation of human osteoblastic cells associated with differential activation of MAP kinases in response to epidermal growth factor, hypoxia, and mechanical stress in vitro. Biochem Biophys Res Commun 1998; 249: 350-354.

[24] Utting JC, Robins SP, Brandao-Burch A, Orriss IR, Behar J and Arnett TR. Hypoxia inhibits the growth, differentiation and bone-forming capacity of rat osteoblasts. Exp Cell Res 2006; 312: 1693-1702.

[25] Park JH, Park BH, Kim HK, Park TS and Baek HS. Hypoxia decreases Runx2/Cbfa1 expression in human osteoblast-like cells. Mol Cell Endocrinol 2002; 192: 197-203.

[26] Chen D, Li Y, Zhou Z, Xing Y, Zhong Y, Zou X, Tian W and Zhang C. Synergistic inhibition of Wnt pathway by HIF-1alpha and osteoblast-specific transcription factor osterix (Osx) in osteoblasts. PLoS One 2012; 7: e52948.

[27] Hirao M, Hashimoto J, Yamasaki N, Ando W, Tsuboi H, Myoui A and Yoshikawa H. Oxygen tension is an important mediator of the transformation of osteoblasts to osteocytes. J Bone Miner Metab 2007; 25: 266-276.

[28] Dunwoodie SL. The role of hypoxia in development of the Mammalian embryo. Dev Cell 2009; 17: 755-773.

[29] Dai J and Rabie AB. VEGF: an essential mediator of both angiogenesis and endochondral ossification. J Dent Res 2007; 86: 937-950.

[30] Palomaki S, Pietila M, Laitinen S, Pesala J, Sormunen R, Lehenkari P and Koivunen P. HIF-1alpha is upregulated in human mesenchymal stem cells. Stem Cells 2013; 31: 1902-1909.

[31] Palazon A, Goldrath AW, Nizet V and Johnson RS. HIF transcription factors, inflammation, and immunity. Immunity 2014; 41: 518-528.

[32] Rius J, Guma M, Schachtrup C, Akassoglou K, Zinkernagel AS, Nizet V, Johnson RS, Haddad GG and Karin M. NF-kappaB links innate immunity to the hypoxic response through tran-

scriptional regulation of HIF-1alpha. Nature 2008; 453: 807-811.
[33] Peyssonnaux C, Datta V, Cramer T, Doedens A, Theodorakis EA, Gallo RL, Hurtado-Ziola N, Nizet V and Johnson RS. HIF-1alpha expression regulates the bactericidal capacity of phagocytes. J Clin Invest 2005; 115: 1806-1815.
[34] Shi X, Jin L, Dang E, Chang T, Feng Z, Liu Y and Wang G. IL-17A upregulates keratin 17 expression in keratinocytes through STAT1- and STAT3-dependent mechanisms. J Invest Dermatol 2011; 131: 2401-2408.
[35] Almeida M. Aging mechanisms in bone. Bonekey Rep 2012; 1: pii: 102.