# Cholesterol loading affects osteoblastic differentiation in mouse mesenchymal stem cells

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## ARTICLE INFO

## ABSTRACT

Effects of cholesterol on osteoblastic differentiation was evaluated in mouse bone marrow derived mesenchymal stem cells (MSCs). Cholesterol-treated MSCs showed a stimulated differentiation process with induced mRNA and protein levels of osteogenic lineage markers, increased alkaline phosphatase (AKP) activity and more mineralized nodules. However, the stimulation extent was reduced when incubating the cells with cholesterol plus the ACAT (acyl-CoA:cholesterol acyltransferase) inhibitor Sandoz58035 or SiRNA-ACAT1 [which blocks the esterification of free cholesterol (FC) to cholesteryl ester (CE)], indicating the osteogenic potency of cholesterol was mostly due to CE levels. The key role of BMP2 and Runx2 in the effects of cholesterol on MSC osteogenesis was elucidated. These results point to cholesterol as a modulator of osteoblastic differentiation, which separate cholesterol itself from other components of modified lipoproteins.

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## 1. Introduction

Cholesterol plays an indispensable role in regulating the properties of cell membranes in mammalian cells [1,2]. Cellular cholesterol levels are precisely controlled by several pathways, including the esterification of free cholesterol (FC) to cholesteryl ester (CE) by the enzyme acyl-CoA:cholesterol acyltransferase (ACAT) and the efflux through high-density lipoprotein (HDL) [2,3]. However, excess cholesterol in serum or cells increases the risk of developing atherosclerosis, cardiovascular diseases, osteoporosis and other morbidities [4–6]. The regulation of cholesterol homeostasis is now receiving a new focus, and this may throw light on diseases caused by cholesterol excess.

The bone is a living tissue that is continuously being remodeled through the interplay of two functions: bone resorption by osteoclasts and bone formation by osteoblasts [7]. The loss of equilibrium between osteoblastic and osteoclastic functions results in osteoporosis that is characterized by reduced bone mass and higher bone fragility [8]. Accumulating evidence suggests that bone metabolism is associated with serum lipid conditions [9–13]. Individuals with lower bone density and osteoporosis also present higher lipid levels [6,9]. Some *in vitro* and *in vivo* studies indicate that cholesterol and its metabolites inhibit the functional activity of osteoblasts and thereby induce reduced bone mineralization [10–13].

In contrast to increased understanding on the association of lipid-related factors with bone metabolism, little attention has been paid on the role of free or esterified cholesterol in the remodeling of bone. In the present study, mouse bone marrow derived mesenchymal stem cells (MSCs) were loaded with cholesterol complexed to methyl-β-cyclodextrin (Chol:MβCD), which could deliver cell-permeable cholesterol into cells. After exposition to Chol:MβCD, the osteoblastic differentiation potency of MSCs was determined by the expression of specific osteogenic markers, alkaline phosphatase (AKP) activity assay and alizarin red S (ARS) staining. And the involvement of BMP2 and Runx2 in the effects of cholesterol was clarified. Additionally, to elucidate whether the changes were regulated by the cellular content of FC or CE, in some cases the cells were pre-incubated with the ACAT inhibitor Sandoz58035 (S58035) or ACAT1 specific small interfering RNAs (SiRNA-ACAT1) (which blocks the formation of CE), and then incubated with Chol:MβCD in the presence of S58035 or SiRNA-ACAT1.

## 2. Experimental

### 2.1. Cell culture

Female Kunming mice (specific pathogen free grade) were purchased from Center for New Drugs Evaluation of Shandong University. Animals were kept in an environmentally controlled breeding room. They were fed standard laboratory chow with water and fasted for 12 h before the experiments. The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Animal Care and Use

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**Table 1**
Sequences of the primers used in this study. The sizes of the PCR products are given in base-pairs (bp).

| Genes | NCBI number | Forward primers (5′–3′) | Reverse primers (5′–3′) | Sizes |
|---|---|---|---|---|
| BMP2 | NM_007553 | TGGCCCATTTAGAGGAGAACC | AGGCATGATAGCCCGGAGG | 279 bp |
| Runx2 | NM_009820 | CCGCACGACAACCGCACCAT | CGCTCCGGCCCACAAATCTC | 289 bp |
| AKP | NM_007431 | GGTAGATTACGCTCACAACAA | AGGCATACGCCATCACAT | 164 bp |
| OCN | L24431 | GAGCCCTTAGCCTTCCAT | GCGGTCTTCAAGCCATAC | 297 bp |
| GAPDH | NM_008084 | GACTTCAACAGCAACTCCCAC | TCCACCACCCTGTTGCTGTA | 125 bp |

Committee of Shandong province, China. Six-week old mice were killed by cervical dislocation under sodium pentobarbital anesthesia. Mouse MSCs were isolated from femur and tibia bone marrow as previously described [14]. The isolated cells were cultured and passaged as described by Li et al. [14]. All the following experiments were performed using the third passage cells. MSC lineage was confirmed by the presence of immunoreactivity for CD29 and CD44 in >95% of the cells. Osteoblast differentiation of MSCs was assessed in the complete medium supplemented with 0.01 μM dexamethasone, 50 μg/ml ʟ-ascorbic acid and 10 mM β-glycerophosphate as osteogenic stimuli (OS).

### 2.2. Cholesterol loading

All treatment concentrations involving Chol:MβCD ("water-soluble cholesterol" containing 30 mg of cholesterol/g solid) were based on cholesterol weight. Increasing concentrations of Chol:MβCD were loaded into MSCs in the absence of OS for 48 h (MTT assay) or 72 h (cellular cholesterol determination). Subconfluent MSCs were incubated with Chol:MβCD (5, 10 and 15 μg/ml) in the presence of OS for 4 days (AKP activity measurement and qRT-PCR), 7 days (Western blotting) or 14 days (ARS staining). In some conditions, before cholesterol loading, the cells were pre-incubated 4 h with the ACAT inhibitor S58035 or pre-incubated 24 h with SiRNA-ACAT1.

### 2.3. Cellular cholesterol determination

Cellular lipids were extracted by using a hexane/isopropyl alcohol (3:2) mixture, followed by cellular protein extraction with 0.2 mol/l NaOH as described [15]. Total cholesterol and FC were determined by using kits from Applygen Technologies Inc. (Beijing, China). We added known amounts of cholesterol to the assay material to generate standard curves. And the amounts of total cholesterol and FC in our cell extracts were calculated according to the standard curves. CE content was determined by subtracting FC from total cholesterol. Cellular protein content was determined based on a BCA assay by using a Byotime (Shanghai, China) kit.

### 2.4. Cell growth assay

MTT assay was performed as previously described [16]. Subconfluent MSCs were treated for 48 h with 5, 10 and 15 μg/ml Chol:MβCD in the complete culture medium. MTT reagent was then added and incubated with the cells for 4 h. The reagent was converted by metabolically active cells into a colored formazan product measurable at 570 nm after DMSO dissolution. The number of active cells is related to the relative metabolic activity (i.e., formazan production).

Trypan blue exclusion method was performed to count the cell number and to see cell viability, as described [17]. MSCs were treated 48 h with Chol:MβCD, harvested by centrifugation at 1000 rpm and incubated in 0.2% trypan blue for 10 min at 37 °C. Cells were then washed in PBS and counted using a haemocytometer. In this assay, blue cells were concluded to have lost membrane integrity and were scored as nonviable. All counts were performed in triplicate.

### 2.5. Quantitative real-time RT-PCR (qRT-PCR)

After 4 days treatment with test agents, cellular total RNA was extracted by using a kit from Invitrogen (Carlsbad, CA) and reverse transcription was performed. The mRNA levels for specific genes were determined by qRT-PCR as described [14], using 20 ng of total RNA. The primer sequences for BMP2, Runx2, AKP, OCN and GAPDH are listed in Table 1. QRT-PCR was performed by using a Bio-rad real-time PCR system according to the manufacturer's protocol. Melt-curve analysis was performed to validate the used primer sets. The mRNA expression of specific genes was calculated relative to GAPDH levels by the 2-ΔΔCt method and expressed as fold change over the controls.

### 2.6. AKP activity determination

Reagent treated cells were washed twice with ice-cold PBS and lysed by two cycles of freezing and thaw. AKP activity in the cell layer was measured colorimetrically by monitoring the release of $p$-nitrophenol from $p$-nitrophenyl phosphate at 37 °C using an AKP assay kit (Nanjing Jiancheng Biotech., China). Production of 1 mg $p$-nitrophenol at 37 °C in 15 min was described as 1 unit (U). AKP activity was normalized to total protein determined using a BCA assay kit (Beyotime, China), and expressed as U/g protein.

### 2.7. ARS staining

After 14 days incubation with different agents, ARS staining was performed to detect the mineralized nodules in treated and control MSCs, as previously described [18]. Additionally, the bound dye was eluted with 10% cetylpyridinium chloride, and ARS in samples was quantified by measuring absorbance at 550 nm. Parallel wells served for DNA isolation by using a standard kit (BioVision, CA). The osteoblastic extent was normalized to total DNA content determined using a DNA quantification kit (Sigma, MO), and expressed as μmol ARS/μg total DNA.

### 2.8. Western blot analysis

Western blotting was performed to detect the protein expression levels of BMP2 and Runx2 in treated and control MSCs, as previously described [16]. Thirty micrograms of protein from each sample served to measure the expression of target proteins using GAPDH as internal control. All primary antibodies and the appropriate horseradish peroxidase conjugated second antibodies were from Santa cruz biotechnology (CA, USA). Protein was visualized by chemiluminescence method using an ECL kit (Amersham, Germany).

### 2.9. RNA interference

The RNA interference technique was used for down-regulating Runx2 or ACAT1 gene expression. SiRNAs were obtained from Shanghai GenePharma Corporation (Shanghai, China). The siRNA sequence targeting Runx2 (GenBank No. NM_009820) was 5′-CCA-TAACAGTCTTCACAAA-3′ (corresponding to 994–1012 nt of ORF), and that of ACAT1 (GenBank No. NM_144784) was 5′-GGTGAAA-