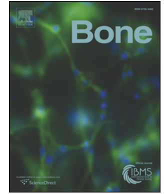
Bone 110 (2018) 221–229

Contents lists available at ScienceDirect

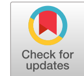
Bone

journal homepage: www.elsevier.com/locate/bone



Full Length Article

Secreted Clusterin protein inhibits osteoblast differentiation of bone marrow mesenchymal stem cells by suppressing ERK1/2 signaling pathway



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ARTICLE INFO

Article history:
Received 15 November 2017
Revised 18 February 2018
Accepted 19 February 2018
Available online 21 February 2018

Keywords:
Mesenchymal stem cells
BMSCs
Clusterin
sCLU
Osteoblast
Adipocyte
Osteoblast differentiation

ABSTRACT

Secreted Clusterin (sCLU, also known as Apolipoprotein J) is an anti-apoptotic glycoprotein involved in the regulation of cell proliferation, lipid transport, extracellular tissue remodeling and apoptosis. sCLU is expressed and secreted by mouse bone marrow-derived skeletal (stromal or mesenchymal) stem cells (mBMSCs), but its functional role in MSC biology is not known. In this study, we demonstrated that Clusterin mRNA expression and protein secretion in conditioned medium increased during adipocyte differentiation and decreased during osteoblast differentiation of mBMSCs. Treatment of mBMSC cultures with recombinant sCLU protein increased cell proliferation and exerted an inhibitory effect on the osteoblast differentiation while stimulated adipocyte differentiation in a dose-dependent manner. siRNA-mediated silencing of *Clu* expression in mBMSCs reduced adipocyte differentiation and stimulated osteoblast differentiation of mBMSCs. Furthermore, the inhibitory effect of sCLU on the osteoblast differentiation of mBMSCs was mediated by the suppression of extracellular signal-regulated kinase (ERK1/2) phosphorylation. In conclusion, we identified sCLU as a regulator of mBMSCs lineage commitment to osteoblasts versus adipocytes through a mechanism mediated by ERK1/2 signaling. Inhibiting sCLU is a possible therapeutic approach for enhancing osteoblast differentiation and consequently bone formation.

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1. Introduction

Bone marrow skeletal (also known as stromal or mesenchymal) stem cells (BMSCs) are a subpopulation of adult stem cells that reside in the bone marrow within a specific perivascular niche and are characterized by their ability for self-renewal and multipotent differentiation into mesodermal cells, including osteoblast, adipocytes, and chondrocytes [1–4]. Several pre-clinical and clinical studies have suggested the possible use of BMSC-based therapy for enhancing bone regeneration in a number of conditions, such as non-union fracture, bone reconstruction and augmentation in cranial, oral, maxillo-facial and long bone defects [5]. Thus, understanding the regulatory mechanisms underlying the differentiation of BMSCs into bone-forming osteoblastic cell lineage is important to provide novel therapeutic targets that can be used to direct the differentiation of BMSCs into the osteoblastic lineage to enhance bone formation.

In this context, we and others have demonstrated that the regulation of BMSCs differentiation into osteoblasts is mediated by the secreted factors produced by BMSCs [3,6]. These osteogenic secreted factors include the secreted Frizzled-related protein 1 (sFRP-1) [7], Delta like-1/Fetal antigen 1 (Dlk1/FA1) [8,9], Leukemia inhibitor factor (LIF) [10], Vascular endothelial growth factor A (VEGF) [11], WNT1-induced Secreted Protein-1 (WISP1) [12], Semaphorin 3A (Sema3A) [13] and Nel-Related Protein 1, NELL-1 [14]. We have also previously employed global, hypothesis-generating methods of transcriptomics or proteomics to identify novel factors important for BMSCs commitment to osteoblastic cells and to bone formation [15,16]. By comparing the transcriptome and secretome of BMSC-derived osteoprogenitor cells versus adipoprogenitor cells [17], we identified Clusterin and found that its expression was significantly upregulated in BMSCs-derived adipocytes (Abdallah BM and Kassem M, unpublished data).

Clusterin (CLU, also known as Apolipoprotein J), is a heterodimeric protein, that found in two forms: nuclear form (nCLU) and soluble form (sCLU). sCLU is ubiquitously expressed in many tissues including

Abbreviations: ALP, alkaline phosphatase; Runx2, runt-related transcription factor 2; Col1a1, collagen type 1; OCN, osteocalcin; OPN, osteopontin; PPARγ2, peroxisome proliferator-activated receptor gamma 2; C/EBP-α, CCAAT/enhancer-binding protein alpha; aP2, adipocyte lipid-binding protein 2; LPL, lipoprotein lipase; APM1, adiponectin; MSX2, msh homeo box 2; DLX5, distal-less Homeobox 5.

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https://doi.org/10.1016/j.bone.2018.02.018
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brain, liver, testis, ovary, and heart and is present in the circulation and in all biological fluids as a component of high density lipoprotein (HDL) complex with Apolipoprotein A-1 (ApoA1) [18–20]. sCLU is a pro-cell survival factor, that is involved in the regulation of cell proliferation, apoptosis, tissue remodeling, complement inhibition, lipid transport, and carcinogenesis [18,21,22]. sCLU has been reported to be protective against oxidative stress-induced apoptotic cell death in a variety of cells including BMSCs [23], [24] [25]. Furthermore, increased expression of CLU was shown to be associated with oxidative stress and inflammation in many diseases including neurodegenerative diseases, cancers and inflammatory diseases [26]. The function of sCLU as an anti-apoptotic factor is mediated by the modulation of NF-κB, PI3K/AKT and ERK1/2 signaling pathways [27–29]. Regarding bone metabolism, sCLU was reported to inhibit osteoclast bone resorption by suppressing macrophage colony-stimulating factor, M-CSF-mediated ERK activation [30]. However, the role of sCLU in osteoblast differentiation from BMSCs and in bone formation has not been reported. In this study, we demonstrated that *sClu* is expressed by BMSCs and that its steady-state gene expression is increased during adipocyte differentiation and decreased during osteoblast differentiation. Functional analysis revealed that sCLU stimulates cell proliferation and the early commitment of BMSCs toward the adipocytic lineage at the expense of the osteoblastic lineage, an effect mediated via ERK1/2 phosphorylation.

2. Materials and methods

2.1. Animals

C57BL/6 mice were originally purchased from Charles River. Mice were bred and housed under standard conditions (21 °C, 55% relative humidity) on a 12-h light/12-h dark cycle at the animal housing unit and the Physiology Laboratory, College of Science, King Faisal University, Saudi Arabia, in accordance with the protocol approved by the Standing Research Ethics Committee. Ad libitum food (Altromin® Spezialfutter GmbH & Co. KG, Lage, Germany) and water were provided. Sera were collected from young female mice (2 months old) and old female mice (18 months old).

2.2. Isolation and cultivation of BMSCs

Mouse BMSCs were isolated from the bone marrow of wild-type 8-weeks-old male C57BL/6 J mice as described previously [31]. In brief, The femur and tibia were dissected from mice and bone marrow was flushed out with a 21-gauge syringe containing, complete isolation media (CIM), which consists of RPMI-1640 (Roswell Park Memorial Institute) supplemented with 10% fetal bovine serum (FBS; GIBCO), 100 U/mL penicillin (GIBCO, Thermo Fisher Scientific, Darmstadt, Germany) and 100 μg/mL streptomycin (GIBCO). Cells were filtered, washed with PBS and cultured in 40 mL CIM in a 175-cm2 flask in 5% CO2 incubator at 37 °C. Non-adherent cells were removed after 24 h by washing with PBS, and adding 30 mL of fresh CIM. Cells were passaged every 1 week with using 0.25% trypsin/1 mM EDTA [32]. BMSCs cultures were used between passages 2 to 4 only.

Recombinant mouse Clusterin Protein was purchased from R&D Systems GmbH (Wiesbaden, Germany).

2.3. Cell proliferation study

Short-term in vitro cell growth was determined by culturing the cells at 2000 cells/well in 4 well plates. Cells were trypsinized and counted by the hemocytometer. We measured 4–6 biological replicates for each time point.

2.4. Osteoblast differentiation

Cells were cultured at 15,000 cells/cm^2 in CIM medium. At 70% cell confluence, cultured media were changed to osteogenic-induction medium (OIM) consists of: α-minimum essential medium (α-MEM; Gibco) containing 10% FBS, 100 U/mL of penicillin, 100 mg/mL of streptomycin, 50 μg/mL of vitamin C (Sigma-Aldrich), 10 nM dexamethasone and 10 mM β-glycerol-phosphate (Sigma-Aldrich). Cells were cultured in OIM for 12 days (or as indicated). The media were changed every 2–3 days during the time course of osteoblast differentiation.

2.5. Adipocyte differentiation

Cells were cultured at 15,000 cells/cm^2 in CIM medium. At 100% cell confluence, cultured media were replaced by adipogenic-induction medium (AIM) consists of: DMEM supplemented with 9% horse serum, 450 μM 1-methyl-3-isobutylxanthine (IBMX), 250 nM dexamethasone, 5 μg/mL insulin (Sigma-Aldrich) and 1 μM rosiglitazone (BRL 49653, Cayman Chemical). Cells were cultured in AIM for 12 days (or as indicated). The media were changed every 2–3 days during the time course of adipocyte differentiation.

2.6. Alkaline phosphatase (ALP) activity assay and number of viable cells measurement

Number of viable cells was determined using the Cell Titer-Blue cell viability assay according to the manufacturer's instructions (Promega, USA) at OD 579. ALP activity was determined following the manual instructions of ALP assay kit (Abcam plc, Cambridge, UK). The color of the reaction was measured at 405 nm. ALP activity was normalized to cell number (measured by number of viable cells) and then represented as fold change over control non-induced cells [33].

2.7. Alizarin red staining for mineralized matrix

Cells were fixed with 70% ice-cold ethanol for 1 h at −20 °C, and stained with 40 mM Alizarin red S (AR-S; Sigma-Aldrich), pH 4.2 for 10 min at room temperature. For the quantification of mineralized matrix in culture, Alizarin red stain was eluted using 10% (*w*/*v*) cetylpyridinium chloride solution (Sigma-Aldrich) with shaking for 20 min and the absorbance of the eluted dye was measured at 570 nm.

2.8. Oil Red O staining and quantification

Cells were fixed in 4% paraformaldehyde for 10 min at room temperature, then stained with Oil Red O (0.5 g in 60% isopropanol) (Sigma-Aldrich) for 1 h at room temperature to stain the fat droplets. Lipids were quantified by elution of Oil Red O in isopropanol for 10 min at room temperature. The absorbance of the extracted dye was detected at 490 nm. Oil Red O measurements were normalized to cell umber (measured by number of viable cells) and then represented as fold change over control non-induced cells.

2.9. RNA extraction and real-time PCR analysis

Total RNA was extracted from tissues and cells using a single-step method of TRIzol (Thermo Fisher Scientific). cDNA was synthesized from 1 μg of total RNA using revertAid H minus first strand cDNA synthesis kit (Fermentas). Quantitative real time PCR was performed with Applied Biosystems 7500 Real-Time system using Fast SYBR® Green Master Mix (Applied Biosystems, California, USA) with specific primers (Supplementary Table 1). The expression of each target gene was normalization to β-Actin and *Hprt* mRNA expression as reference genes, using a comparative CT method [(1/(2delta-CT)) formula, where delta-CT is the difference between CT-target and CT-reference] with Microsoft Excel 2007® as described [34].

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