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25-hydroxycholesterol promotes RANKL-induced osteoclastogenesis through coordinating NFATc1 and Sp1 complex in the transcription of miR-139-5p

Lishan Zhang [a], Yinping Lv [d], Guozhe Xian [c], Yanliang Lin [b, *]

[a] Department of Hand and Foot Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan 250021, China
[b] Center Laboratory, Shandong Provincial Hospital Affiliated to Shandong University, Jinan 250021, China
[c] Department of Hepatobiliary Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan 250021, China
[d] Department of Medical Engineering, Jinan 250021, China

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ABSTRACT

25-hydroxycholesterol (25-HC) is implicated in many processes, including lipid metabolism and the immune response. However, the role of 25-HC in RANKL-induced osteoclastogenesis remains largely unknown. Our results showed that 25-HC inhibited miR-139-5p expression in mouse bone marrow macrophages (BMMs) cultured in receptor activator of NF-κB ligand (RANKL) and monocyte macrophage colony-stimulating factor (M-CSF). Further investigation suggested that 25-HC promoted the expression of nuclear factor of activated T cell cytoplasmic 1 (NFATc1) and Sp1, especially in the presence of RANKL and M-CSF. Meanwhile, 25-HC induced nuclear translocation of NFATc1, resulting in the interaction between NFATc1 and Sp1 that was confirmed by co-immunoprecipitation. Chromatin immunoprecipitation assay indicated that Sp1 could bind to miR-139-5p promoter, but NFATc1 had no binding capacity. Although forming NFATc1/Sp1 complex increased its binding to miR-139-5p promoter, the complex inhibited the transcriptional activity of Sp1. Inhibition of NFATc1 increase the expression of miR-139-5p, which might be due to the release of free Sp1 that could bind to the promoter of miR-139-5p. Enforced expression of miR-139-5p impaired osteoclastogenesis induced by co-treatment with 25-HC and RANKL. These results suggested that 25-HC induced the interaction between NFATc1 and Sp1, reducing the level of free Sp1 to inhibit miR-139-5p expression and promote osteoclastogenesis.

1. Introduction

Skeletal homeostasis is maintained by a bone remodeling process that depends on a delicate balance between osteoblast for bone formation and osteoclast for bone resorption [1]. Therefore, identification of the transcription factors implicating in the osteoclast differentiation would have contributed to improve therapeutic strategies for bone disease.

Osteoclasts are multinucleated giant cells dived from hematopoietic monocyte/macrophage precursors. The osteoclast differentiation requires macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) [1]. RANKL induces osteoclast differentiation by binding to RANK expressed on surfaces of osteoclast precursors in the presence of M-CSF [2]. During RANKL-induced osteoclast differentiation, a cascade of transcriptional factors are activated, including nuclear factor of activated T cells c1 (NFATc1), c-fos, and PU.1 [2]. Activated NFATc1 translocates to the nucleus and then promoted the expression of target genes [3]. PU.1, a hematopoietic transcription factor, plays a key role in RANKL-induced osteoclastogenesis by regulating the transcription of RANK gene [4]. The transcription factor c-fos is also critical for osteoclastogenesis by regulating RANKL-induced NFATc1 expression [5].

The transcription factor Sp1 is constitutively expressed in most cells and serves as a transactivator of target genes. Sp1 is originally recognized as a protein that binds to multiple GGGCGG sequences in HeLa cells [6]. Subsequently, a series of promoters are found to be activated by Sp1 [7–9]. In addition, the recognition sequences of Sp1 are often located near binding sites of other transcription factors, such as CTWNF-I and AP-1 [6], suggesting that Sp1 may

* Corresponding author.
E-mail address: sphyanlianglin@163.com (Y. Lin).

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interact with other transcription factors to regulate gene transcription.

25-hydroxycholesterol (25-HC) is an oxysterol from cholesterol catalyzed by 25-hydroxylase (CH25H). 25-HC mainly functions as an immune regulator. 25-HC regulates transcriptional responses and amplifies inflammatory reactions by mediating recruitment of AP-1 [10]. 25-HC also promotes fibroblast-mediated tissue remodeling through regulation of NF-κB/TGF-β pathway [11]. Our recent results demonstrate that 25-HC inhibits the IL-1β secretion from human macrophages while promotes IL-1β secretion in coculture of macrophage and lung adenocarcinoma cells [12]. Given that IL-1β has been documented to favor the maturation of active osteoclasts [13], we hypothesized that 25-HC participated in osteoclastogenesis. Herein, we set out to explore the role of 25-HC in RANKL-induced osteoclastogenesis.

2. Materials and methods

2.1. Materials

25-hydroxycholesterol (25-HC) and a Leukocyte Acid Phosphatase Kit were purchased from Sigma (St. Louis, MO); α-MEM medium and fetal bovine serum (FBS) were purchased from Invitrogen Life Technologies (Grand Island, NY, USA); Murine M-CSF and RANKL were purchased from R&D Systems (Minneapolis, MN, USA). Anti-NFATc1 (ab2796), anti-Sp1 (ab59257) and anti-beta-actin (ab8227) were purchased from Abcam (Cambridge, MA). Anti-GAPDH (sc25778) was purchased from Santa (Santa Cruz, CA, USA). The rabbit anti-goat secondary antibody (BA1060) was purchased from Boster. The goat anti-rabbit secondary antibody (ZB-2301) was purchased from ZSGB-Bio.

2.2. Animals

Male 8-week-old C57BL/6 mice were obtained from Weitonglihua Animal Center (Beijing, China). All animal experiments were performed according to the protocols approved by the Institutional Laboratory Animal Care and Use Committee at Shandong provincial hospital.

2.3. Osteoclast formation

Murine bone marrow cells (BMCs) derived from C57BL/6 mice were prepared as the source of osteoclasts. In brief, BMCs from murine long bones are washed and cultured in α-MEM containing 10% FBS and 10 ng/ml M-CSF for 72 h. After removing nonadherent cells, the adherent bone marrow-derived macrophages (BMMs) were used as osteoclast precursors. To generate osteoclasts, BMMs were further cultured in α-MEM containing 10% FBS in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 72 h. To determine the effect of 25-HC on RANKL-induced osteoclastogenesis, BMMs were pretreated with 25-HC (100 nM) for 24 h in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 72 h. Subsequently, cells were fixed and stained for tartrate resistant acid phosphatase (TRAP) using a Leukocyte Acid Phosphatase kit (Sigma). TRAP-positive cells containing more than three nuclei were considered as multinucleated osteoclasts. The cell fusion index was calculated according to the formula: Fusion Index (%) = (total number of nuclei in multinucleated cells)/(total number of nuclei) × 100. The fusion indexes were determined in 12–15 microscopic fields containing more than 2000 nuclei from three independent experiments.

2.4. MicroRNA microarray assay

BMMs were cultured with M-CSF (30 ng/ml) in the presence of RANKL (100 ng/ml) and 25-HC (100 nM) for the indicated times, and then total RNA was isolated using TRIzol reagent (Life Technologies, CA, USA) and further purified using RNeasy MinElute Cleanup Kit (Qiagen). 3 μg total RNA from each sample was labeled by Cy3 and Cy5 dye and hybridized onto Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, CA, USA). The miRNA expression levels were estimated using TaqMan Low Density Arrays on a 7500 Real-Time PCR system (Invitrogen, CA). MiRNAs with at least a twofold difference in expression level in treated BMMs compared to control BMMs were selected to determine the expression profile.

2.5. Plasmid constructs, gene transfection and reporter assays

Small interfering RNA duplexes (siRNA) targeting NFATc1 or Sp1 was synthesized based on the targeted 21 nucleotides of NFATc1 (5′- CAAGCATCACGGAGGAGAGCT-3′) and 23 nucleotides of Sp1 (5′GTACAGGACCCCCTTGAGCTCGC-3′). Complete open reading frame (ORF) coding NFATc1 was amplified by PCR, digested and ligated into the *Eco*RI site of flag-tagged pcDNA3 vector (Addgene), and then was sequenced to verify. The ORF coding Sp1 was amplified by PCR, digested and ligated into the region between *Xho*I and *Eco*RI sites of HA-tagged pcDNA3 vector (Addgene). The reporter plasmid, pmiR139-luc, was constructed by inserting a 450 bp fragment of the mmu-miR-139-5p promoter (−410 to +40 region) into the pGL3 basic vector (Promega, Madison, USA).

BMMs cells were seeded at a density of 2×10^6 cells/ml in 6-well microplates overnight. The cells were then transfected with retroviruses carrying siRNAs against NFATc1 or Sp1, miR-139-5p or anti-miR-139-5p (Biosune, Shanghai, China) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) for 6 h, followed by incubation in fresh media for 48 h. The cells are prepared for further experiments.

HEK-293 cells were seeded at a density of 1×10^5 cells/ml in 12-well microplates. Cells were co-transfected with 1 μg pcDNA3-NFATc1 or pcDNA3-Sp1 and 0.5 μg pmiR139-luc using Lipofectamine 3000 (Roche); the pRL-SV40 plasmid (Promega) was used for a normalizing control. After 48 h incubation, cellular lysates were harvested to measure dual luciferase activities according to the manufacturer's instructions.

2.6. Protein extraction and western blot analysis

To obtain total protein, the treated cells were harvested and lyzed using RIPA lysis buffer (Thermo). The cytoplasmic protein and nuclear protein were prepared as previously described [14]. The protein concentration was determined using the bicinchoninic acid method. Equal amounts of proteins were loaded on 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). After blocking for 1 h with 5% nonfat milk, membranes were incubated with primary antibodies including anti-GAPDH (1:1000), anti-beta-actin (1:2000), anti-Sp1 (1: 1000) and anti-NFATc1 (1:500), followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The signals were detected using the enhanced chemiluminescence method and quantified using Scion Image 4.03 software.

2.7. RNA extraction and northern blot analysis

The total RNA was extracted using Trizol reagent (Invitrogen). Equal amount of RNA was subjected to a 15% urea-polyacrylamide gel, and was subsequently transferred to nylon membrane using *Trans*-blot cell (Bio-Rad). The expression of miR-139-5p or U6 was determined using biotin-labeled oligonucleotide probes showed as follows: 5′-ACTCCAACAGGGCCGCGTCTCCA-3′ for mmu-miR-139-

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