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MHC class II transactivator negatively regulates RANKL-mediated osteoclast differentiation by downregulating NFATc1 and OSCAR

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ABSTRACT

Nuclear factor of activated T cells (NFAT) c1 plays a key role in receptor activator of nuclear factor κB ligand (RANKL)-induced osteoclast differentiation and function via induction of osteoclast-specific target genes including osteoclast-associated receptor (OSCAR), cathepsin K, and tartrate-resistant acid phosphatase. To elucidate which downstream target genes are regulated by NFATc1 during osteoclastogenesis, we used microarray analyses to examine gene expression profiles in the context of bone marrow-derived macrophages overexpressing a constitutively active form of NFATc1. Herein, we demonstrate that MHC class II transactivator (CIITA) is up-regulated downstream of NFATc1. Overexpression of CIITA in osteoclast precursors attenuates RANKL-induced osteoclast formation through down-regulation of NFATc1 and OSCAR. Epigenetic overexpression of CIITA regulates NFATc1 and OSCAR by competing with c-Fos and NFATc1 for CBP/p300 binding sites. Furthermore, silencing of CIITA by RNA interference in osteoclast precursors enhances osteoclast formation as well as NFATc1 and OSCAR expression. Taken together, our data reveal that CIITA can act as a modulator of RANKL-induced osteoclastogenesis.

1. Introduction

Bone is continuously remodeled by osteoclasts and osteoblasts which are responsible for bone resorption and formation, respectively [1,2]. The balance of both processes are important for maintaining proper bone density and excess osteoclast activity can lead to bone diseases such as osteoporosis, periodontal disease, and rheumatoid arthritis. Osteoclasts differentiate from monocyte/macrophage lineage cells upon stimulation with two essential cytokines, macrophage colony-stimulating factor (M-CSF) and RANKL, both of which are expressed on the surface of stromal/osteoblast cells and are requisite components of the bone growth microenvironment [2,3].

RANKL, a TNF family member, directly regulates osteoclast differentiation and function. RANKL activates and induces expression of key transcription factors such as NF-κB, Mitf, PU.1, c-Fos, and NFATc1, which are important for osteoclastogenesis in vitro and in vivo [3,4]. During osteoclastogenesis, RANKL stimulates c-Fos gene expression and c-Fos binding to the promoter region of NFATc1 up regulates NFATc1 gene expression [5]. NFATc1 and c-Fos act synergistically to induce the expression of key osteoclast-regulating genes by binding to the promoter regions of genes such as: cathepsin K, tartrate-resistant acid phosphatase (TRAP), and OSCAR [5–7]. Ectopic expression of NFATc1 can mediate osteoclast differentiation in the absence of RANKL and NFATc1-deficient embryonic stem cells fail to differentiate into osteoclasts in response to RANKL [5,8], suggesting that NFATc1 acts as a key modulator of osteoclastogenesis.

Although osteoblast/stromal cells play an important role in physiologic bone metabolism, it has been shown that T-, B-, and macrophage cells may also play a role in osteoclast differentiation and function in pathological conditions [9,10]. Factors produced during immune responses are capable of profoundly affecting bone metabolism and a series of recent papers have suggested that the skeletal and immune systems share a number of regulatory molecules including cytokines, receptors, signaling molecules, and transcription factors [9,11].

CIITA is a non-DNA-binding coactivator that serves as the master control factor for the expression of MHC class II (MHCII) and other genes required for MHCII-restricted antigen presentation [12–14]. CIITA can also modulate immune responses by repressing the transcription of IL-4 and Fas ligand by competing with NFAT for binding to the general coactivator CREB-binding protein (CBP) [15,16]. Although CIITA regulates immune responses in various cells, the role of CIITA in osteoclast differentiation has yet to be determined.

In this study, we used microarray analyses to elucidate the molecules downstream of NFATc1 during osteoclastogenesis. We report that NFATc1 induces CIITA expression and that CIITA negatively regulates RANKL-induced osteoclast formation by sequestering the p300 form the NFATc1 and OSCAR promoter regions, thereby inhibiting transactivation of both genes. Our data suggest that negative

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Table 1
The nucleotide sequences used for plasmid construction.

Constructs	Nucleotide sequences
CIITA	5′-CGGGATCCACCATGAACCACTTCCAGGCCATCCTG-3′ 5′-CCGCTCGAGTCTCAGACTGATCCTGGCATCCAG-3′

feedback regulation of CIITA plays a role in RANKL-mediated osteoclastogenesis.

2. Materials and methods

2.1. Constructs

CIITA was prepared by RT-PCR using RNA from osteoclasts. Primer sequences are provided in Table 1. The amplified PCR fragments were cloned into the pMX-IRES-EGFP vector or pcDNA3.1. NFATc1 and OSCAR reporter vectors, expression vectors for c-Fos, NFATc1, and p300, and retroviral vectors expressing a constitutively active form of NFATc1 were previously described [6,17–19].

2.2. Osteoclast formation

Murine osteoclasts were prepared from bone marrow cells as previously described [20,21]. In brief, bone marrow cells were cultured in α-MEM containing 10% FBS with M-CSF (30 ng/ml) for 3 days. The attached cells, bone marrow-derived macrophages (BMMs), were used as osteoclast precursors. To generate osteoclasts, BMMs were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 3 days. Cultured cells were fixed and stained for TRAP as previously described.[20] TRAP-positive multinuclear cells [TRAP(+) MNCs], containing more than 3 nuclei, were counted.

2.3. Retroviral infection

To generate retroviral stocks, retroviral vectors were transfected into the packaging cell line Plat E using FuGENE 6 (Roche Applied Sciences, Indianapolis, IN). Viral supernatant was collected from cultured media 24–48 h after transfection. BMMs were incubated with viral supernatant for 8 h in the presence of polybrene (10 μg/ml). After retroviral supernatants were removed, BMMs were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 3 days.

2.4. Semiquantitative RT-PCR

RT-PCR was performed as previously described [22]. Primer sequences are provided in Table 2.

Table 2
The nucleotide sequences used for RT-PCR.

Genes	Nucleotide sequences
CIITA	5′-ACTGGCCTCCAGCCTTCAGAAGTG-3′ (forward) 5′-GTTCTGTCCTGGAGTGTCGAAGAG-3′ (reverse)
c-Fos	5′-ATGGGCTCTCCTGTCAACACACAG-3′ (forward) 5′-TGGCAATCTCAGTCTGCAACGCAG-3′ (reverse)
NFATc1	5′-CTCGAAAGACAGCACTGGAGCAT-3′ (forward) 5′-CGGCTGCCTTCCGTCTCATAG-3′ (reverse)
OSCAR	5′-CTGCTGGTAACGGATCAGCTCCCCAGA-3′ (forward) 5′-CCAAGGAGCCAGAACCTTCGAAACT-3′ (reverse)
TRAP	5′-CTGGAGTGCACGATGCCAGCGACA-3′ (forward) 5′-TCCGTGCTCGGCGATGGACCAGA-3′ (reverse)
HPRT	5′-GTAATGATCAGTCAACGGGGGAC-3′ (forward) 5′-CCAGCAAGCTTGCAACCTTAACCA-3′ (reverse)
GAPDH	5′-TGACCACAGTCCATGCCATCACTG-3′ (forward) 5′-CAGGAGACAACCTGGTCCTCAGTG-3′ (reverse)

Table 3
The nucleotide sequences used for ChIP assay.

Genes	Nucleotide sequences
NFATc1	5′-CCGGGACGCCCATGCAATCTGTTAGTAATT-3′ (forward) 5′-GCGGGTGCCCTGAGAAAGCTACTCTCCCTT-3′ (reverse)
OSCAR	5′-GAACACCAGAGGCTATGACTGTTC-3′ (forward) 5′-CCGTGGAGCTGAGGAAAAGGTTG-3′ (reverse)

2.5. Western blot analysis

For immunoblotting analysis, BMMs, osteoclasts, and 293T cells were transfected or transduced with various constructs as indicated. Cells were washed with ice-cold PBS and lysed in extraction buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and protease inhibitors). Cell lysates or immunoprecipitated samples were subjected to SDS-PAGE and Western blotting. Primary antibodies used include: c-Fos (Calbiochem, La Jolla, CA), NFATc1 (BD Biosciences, Franklin, NJ), OSCAR [6], IκB, p-ERK, ERK, p-JNK, JNK, p-p38, p38 (Cell Signaling Technology, Beverly, MA), actin, Flag (Sigma-Aldrich, St Louis, MO), and hemagglutinin (HA, Roche Applied Sciences). Horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Piscataway, NJ) were probed and developed with ECL solution (Amersham Biosciences).

2.6. Transfection and luciferase assay

For transfection of reporter plasmids, 293T cells were plated on 24-well plates (3×10^4 cells/well) one day prior to transfection. Plasmid DNA was mixed with FuGENE and transfected into the cells according to the manufacturer's protocol. After 48 h of transfection, the cells were washed twice with PBS and then lysed in reporter lysis buffer (Promega, Madison, WI). Luciferase activity was measured using a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

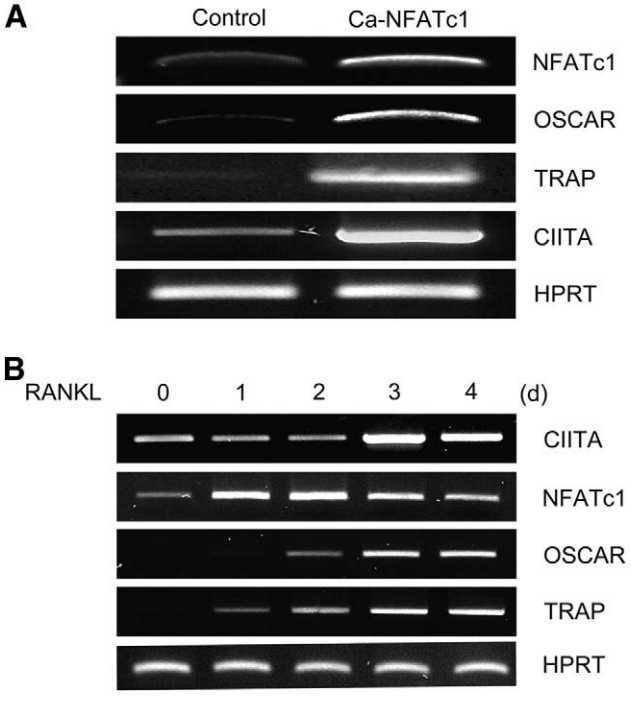

Fig. 1. CIITA expression during osteoclastogenesis. (A) BMMs were transduced with pMX-IRES-EGFP (control) or constitutively active NFATc1 (Ca-NFATc1) retrovirus and cultured for 4 days in the absence of RANKL. (B) BMMs were cultured for the indicated times in the presence of M-CSF and RANKL. (A–B) Total RNA was harvested from cultured cells, and RT-PCR was performed for the expression of NFATc1, OSCAR, TRAP, CIITA, and HPRT.