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TRIM38 regulates NF-κB activation through TAB2 degradation in osteoclast and osteoblast differentiation

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ABSTRACT

The tripartite motif protein 38 (TRIM38), a member of the TRIM family, is involved in various cellular processes such as cell proliferation, differentiation, apoptosis, and antiviral defense. However, the role of TRIM38 in osteoclast and osteoblast differentiation is not yet known. In this study, we report the involvement of TRIM38 in osteoclast and osteoblast differentiation. Overexpression of TRIM38, in osteoclast precursor cells, attenuated receptor activator of nuclear factor kappa B ligand (RANKL)-induced osteoclast formation, RANKL-triggered NF-κB activation, and expression of osteoclast marker genes, such as NFATc1, osteoclast-associated receptor (OSCAR), and tartrate-resistant acid phosphatase (TRAP); and down-regulation of TRIM38 expression showed the opposite effects. Ectopic expression of TRIM38 in osteoblast precursors induced increased osteoblast differentiation and function. Elevated expression of alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteocalcin was also observed due to blockade of NF-κB activation. Conversely, knockdown of TRIM38 showed the opposite effects. TRIM38 also induced degradation of lysosome-dependent transforming growth factor beta-activated kinase 1 and MAP3K7-binding protein 2 (TAB2), further blocking NF-κB activation. Taken together, our data suggest that TRIM38 plays a critical role in bone remodeling as a negative regulator of NF-κB in both osteoclast and osteoblast differentiation.

1. Introduction

Bone homeostasis is maintained by the balance between bone resorption and bone formation carried out by osteoclasts and osteoblasts, respectively. Imbalance between the activities of osteoclasts and osteoblasts can cause various bone diseases, such as osteoporosis.

Osteoclast formation requires the presence of macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) [1,2]. Upon RANKL stimulation, osteoclast precursor cells undergo complete differentiation to form mature osteoclasts via TNF receptor-associated factor 6 (TRAF6) recruitment. This activates the NF-κB and MAPK signaling pathways, including that of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, to induce expression of osteoclast marker genes, such as nuclear factor of activated T cells c1 (NFATc1), osteoclast-associated receptor (OSCAR), and tartrate-resistant acid phosphatase (TRAP) [1,3].

Osteoblasts, which are responsible for bone formation, are differentiated from mesenchymal stem cells; and the process of differentiation is under the control of several transcription factors and signaling cascades. It has been shown that bone morphogenic factor 2 (BMP2) is sufficient to commit pluripotent mesenchymal cells to the osteoblast lineage by regulating signaling molecules that stimulate specific transcriptional programs required for bone formation [4]. BMP2 activates ERK, JNK, and p38 kinase signaling pathways to promote the expression and activation of an osteogenic-specific transcription factor, runt-related transcription factor 2 (*Runx2*) [5]. Runx2 is a master transcription factor that regulates the expression of several osteogenic genes, including alkaline phosphatase (ALP) and osteocalcin, which are key regulators of osteoblast differentiation and function [6].

NF-κB plays important roles in bone formation via regulation of both osteoclasts and osteoblasts [7]. NF-κB has recently been identified as a critical factor involved in inhibition of bone formation in osteoporosis [8]. Therefore, targeting of NF-κB could provide a novel and

Abbreviations: TRIM38, tripartite motif 38; BMM, bone marrow-derived macrophage-like cell; NFATc1, nuclear factor of activated T-cells c1; TRAP, tartrate-resistant acid phosphatase; RANKL, receptor activator of nuclear factor kappa B ligand; M-CSF, macrophage colony stimulating factor; TRAF6, TNF receptor associated factor 6; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; OSCAR, osteoclast-associated receptor; BMP2, bone morphogenic factor 2; RUNX2, runt-related transcription factor 2; NF-κB, nuclear factor kappa B; ALP, alkaline phosphatase; TAK1, transforming growth factor beta activated kinase 1; TAB2, transforming growth factor beta activated kinase 1 binding protein 2

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efficacious treatment strategy for osteoporosis and other inflammatory bone diseases. Inhibition of NF-κB may lead to increased bone formation and decreased bone resorption.

It has been reported that transforming growth factor beta-activated kinase 1 and MAP3K7-binding protein 2 (TAB2) functions as an adaptor protein that binds to TRAF6 and transforming growth factor beta-activated kinase 1 (TAK1), thereby inducing TAK1 activation, and promotes the subsequent activation of inhibitory kappa B kinases (IKK) [9].

The tripartite motif-containing (TRIM) proteins, also known as E3 ubiquitin-protein ligases, are characterized by the presence of RING finger, one or two zinc-binding motifs called B-boxes, and an associated coiled-coil region (RBCC); there are currently 70 known TRIM proteins in humans [10]. The TRIM family proteins are involved in a broad range of cellular processes, including apoptosis, intracellular trafficking, and modulation of innate immune responses during various pathological conditions [11]. Recently, numerous reports have suggested a role for TRIMs in regulation of the NF-κB pathway. It has been suggested that TRIM 4, 8, 20, 22, 23, 25, and 38 positively regulate NF-κB, whereas TRIM 9, 13, 19, 21, 22, 30a, 38, 40, 45, and 59 negatively regulate NF-κB [12–17].

TRIM38 is a typical TRIM protein, and contains a RING, two B-Boxes, a CCD, and a PRY-SPRY domain [13]. TRIM38 has been reported to have controversial roles in NF-κB signaling. Large scale genomic analysis in humans showed that TRIM38 activates NF-κB and MAPK kinases [18,19]. However, another study demonstrated that TRIM38 acts as a negative regulator in TLR3/4-mediated and TNF/IL-1-triggered inflammatory signaling. These data suggest that regulation of NF-κB by TRIM38 may have important implications in biological processes. Although TIM38 regulates NF-κB signaling pathway in various cells, the role of TRIM38 in bone homeostasis has not yet been elucidated.

In this study, we identified TRIM38 as a novel factor that regulates both osteoclasts and osteoblasts via the NF-κB pathway. Overexpression of TRIM38 inhibited RANKL-induced osteoclast differentiation and RANKL-triggered NF-κB activation, whereas TRIM38 overexpression enhanced osteoblast differentiation by inhibiting NF-κB activation. Furthermore, knockdown of TRIM38 induced the enhancement of osteoclastogenesis and attenuation of osteoblastogenesis. We found that TRIM38 promoted TAB2 degradation through a lysosomal dependent pathway. This subsequently resulted in the inhibition of the NF-κB pathway. Our findings revealed a new role of TRIM38 in bone remodeling, and provided the molecular basis for a therapeutic target in bone diseases.

2. Materials and methods

2.1. Reagents

Cell culture media and supplements were obtained from Hyclone (HyClone Laboratories, Lagan, UT, USA). Recombinant human M-CSF and RANKL were purified from bacteria. Recombinant human BMP2 was purchased from Cowellmedi (Busan, Korea). Alizarin Red, β-glycerophosphate, and *p*-nitrophenyl phosphate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ascorbic acid was purchased from Junsei Chemical (Tokyo, Japan).

2.2. Osteoclast differentiation and TRAP staining

Murine osteoclasts were prepared from bone marrow cells, which were obtained by flushing the femurs and tibiae from male 6-week-old ICR (Institute for Cancer Research) mice. All animal experiments were approved by the Chonnam National University Medical School Research Institutional Animal Care and Use Committee and were carried out in accordance with the approved guidelines.

Bone marrow cells were cultured in α-MEM containing 10% fetal bovine serum with M-CSF (30 ng/ml) for 3 days, and bone marrow-derived macrophage-like cells (BMMs) were used as the osteoclast precursor. To generate osteoclasts, BMMs were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 3 days. All cells were cultured at 37 °C and 5% CO_2. Cultured cells were fixed and stained for TRAP. TRAP-positive multinuclear cells that contained more than three nuclei were denoted as osteoclast. Cells were observed using the Leica DMIRB microscope equipped with an N plan 10 × 0.25 numerical aperture objective lens (Leica Microsystems, Wetzlar, Germany). Images were obtained with a ProgRes CFscan (Jenoptik, Jena, Germany) camera, and the ProgRes Capture Pro software was used (Jenoptik).

2.3. Pit formation assay

BMMs were cultured on Osteo assay plates (Corning, Corning, NY, USA) in the presence of M-CSF (30 ng/ml) with or without RANKL for 4 days. Resorption lacunae were visualized under bright-field, using a Leica DMIRB microscope with a ProgRes CFscan (Jenoptik) camera and the ProgRes Capture Pro software (Jenoptik).

2.4. Osteoblast differentiation

Primary osteoblast precursor cells were isolated from the calvarial bone of neonatal mice via enzymatic digestion with α-MEM containing 0.1% collagenase (Life technologies, Carlsbad, CA, USA) and 0.2% dispase II (Roche Diagnostics GmbH, Mannheim, Germany). All mice handling and experiments were performed as per guidelines of the National Institutes of Health (Guide for the Care and Use of Laboratory Animals). The experimental protocol was approved by the Chonnam National University Medical School Research Institutional Animal Care and Use Committee. The Ethics Committee of Chonnam National University Hospital also approved our experimental protocols. Following enzyme removal, the collected cells were cultured in α-MEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Osteoblast differentiation was induced by incubating cells in osteogenic medium containing 100 ng/ml BMP2, 50 μg/ml ascorbic acid, and 100 μM β-glycerophosphate for 4 to 9 days; culture medium was replaced every 3 days for the ALP activity assay. Osteoblast precursor cells were lysed with osteoblast lysis buffer (50 mM NaCl, pH 7.6, 150 mM NaCl, and 0.1% Triton X-100 and 1 mM EDTA). The cell lysates were incubated with *p*-nitrophenyl phosphate substrate (Sigma-Aldrich), and ALP activity was measured with a spectrophotometer at 405 nm. For alizarin red staining, cells were cultured for 9 days, and were fixed with 70% ethanol and stained with 40 mM alizarin red (pH 4.2). Nonspecific staining was removed with PBS wash, and alizarin red staining was visualized with a CanoScan 4400F (Canon inc., Japan). Alizarin red was then dissolved with 10% cetylpyridium (Sigma-Aldrich) for 15 min at room temperature, and alizarin red activity was measured with a spectrophotometer at 562 nm.

2.5. Quantitative polymerase chain reaction (PCR)

Cells were lysed in Qiazol (Qiagen GmbH, Hilden, Germany), and total RNA was isolated according to manufacturer's protocol. Purified RNA was reverse transcribed with Goscript™ Reverse Transcriptase (Promega, Madison, WI, USA), and the resultant cDNA was used for SYBR-based real-time PCR. Assays were performed in triplicates with a Rotor-Gene6 instrument (Qiagen). The thermal cycling conditions were as follows: 15 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. Total mRNA was normalized to the endogenous housekeeping gene *Gapdh*. The relative quantitation value for each target gene was expressed as $2^{-(Ct-Cc)}$ (Ct and Cc are the mean threshold cycle differences after normalizing to *Gapdh*). The relative expression levels of samples were presented by a semi-log plot. The GenBank (http://www.ncbi.nlm.nih.gov/Genbank) accession numbers for the genes and the primer sequences discussed in this paper are: *Nfatc1* (NM_001164109.1, NP_001157581.1) 5′-CTC GAA AGA CAG CAC TGG AGC AT-3′ and 5′-CGG CTG CCT TCC GTC TCA TAG-3′; *Oscar*