Experimental Cell Research 359 (2017) 112–119

Contents lists available at ScienceDirect

Experimental Cell Research

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

# Neuromedin B and its receptor silencing suppresses osteoclast generation by modulating precursor proliferation via M-CSF/c-Fms/D-type cyclins

Chae-Eun Yeo, Woo Youl Kang, Sook Jin Seong, Seungil Cho, Hae Won Lee, Young-Ran Yoon*, Hyun-Ju Kim*

*Department of Biomedical Science, Cell and Matrix Research Institute, BK21 Plus KNU Biomedical Convergence Program, Clinical Trial Center, School of Medicine, Kyungpook National University and Hospital, Daegu 41944, Republic of Korea*

ARTICLE INFO

*Keywords:*
NMB
NMBR
Osteoclast
M-CSF
c-Fms

ABSTRACT

Neuromedin B (NMB), a mammalian bombesin-like peptide, regulates diverse physiological processes, such as energy metabolism, memory and fear behavior, and cellular growth, through its cognate receptor, NMBR. In this study, we report that NMB expression was upregulated during osteoclast development and that silencing NMB or NMBR attenuated osteoclast generation mediated by macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL). We found that knockdown of NMB or NMBR using a small hairpin RNA suppressed M-CSF-induced proliferation of osteoclast precursor cells without altering osteoclast differentiation. Interestingly, NMB or NMBR knockdown reduced the expression of the M-CSF receptor, c-Fms, which is an important modulator of osteoclast development. Consequently, NMB or NMBR silencing inhibited M-CSF/c-Fms-mediated downstream signaling pathways like activation of ERK and Akt and induction of D-type cyclins, cyclin D1 and D2. Moreover, knockdown of NMB or NMBR accelerated apoptosis in osteoclast lineage cells by inducing caspase-3, caspase-9, and Bim expression. In summary, our study demonstrates that the NMB/NMBR axis plays a pivotal role in osteoclast generation by modulating the proliferation and survival of osteoclast lineage cells.

## 1. Introduction

Osteoclasts are multinucleated cells responsible for bone-resorption [1–3]. These polykaryons are derived from hematopoietic precursors of the monocyte/macrophage lineage from the bone marrow. Their development pathway includes proliferation, differentiation, and activation of cells of the osteoclast lineage and depends on the presence of two key cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) [4].

M-CSF is an essential molecule that regulates the proliferation, differentiation, and survival of monocyte/macrophage precursor cells [5,6]. The critical role of M-CSF in osteoclast development has been well demonstrated in both naturally occurring *op*/*op* (osteopetrotic) mice [7,8] and in *tl*/*tl* (toothless) rats [9]. These animals, which have a point mutation in M-CSF gene (*csf1*), produce non-functional M-CSF and exhibit a severe osteopetrotic phenotype due to the complete absence of mature osteoclasts. Furthermore, administration of exogenous M-CSF to these mutant animals corrects their skeletal defect, namely osteopetrosis [7,9,10].

M-CSF induces biological responses through its unique receptor c-Fms. Binding of M-CSF induces dimerization and activation of the transmembrane receptor tyrosine kinase. This process leads to c-Fms auto-phosphorylation and triggers downstream signaling cascades, including MEK/ERK and PI3K/Akt [5,6]. Although the proliferation and survival of osteoclast lineage cells are affected by M-CSF, osteoclast differentiation is mainly regulated by RANKL. Interaction of RANKL with its receptor RANK is necessary for osteoclastogenesis. It activates NF-κB and mitogen-activated kinases (MAPKs), including JNK, ERK, and p38. These signaling pathways promote the induction and/or activation of transcription factors that are crucial for osteoclastogenesis, such as nuclear factor of activated T cells c1 (NFATc1) [11].

Bombesin-like peptides are a family of neuropeptides that have physiological and pathophysiological roles in the regulation of energy balance and in central nervous system processes [12–14]. These peptides include two members, neuromedin B (NMB) and gastrin-releasing peptide (GRP). NMB was originally isolated from pig spinal

*Abbreviations:* BMM, bone marrow macrophage; M-CSF, macrophage colony-stimulating factor; NFATc1, nuclear factor of activated T cells c1; NMB, neuromedin B; NMBR, neuromedin B receptor; RANKL, receptor activator of NF-κB ligand; TRAP, tartrate-resistant acid phosphatase

* Corresponding authors.
*E-mail addresses:* yry@knu.ac.kr (Y.-R. Yoon), biohjk@knu.ac.kr (H.-J. Kim).

http://dx.doi.org/10.1016/j.yexcr.2017.08.003
Received 13 June 2017; Received in revised form 1 August 2017; Accepted 2 August 2017
Available online 03 August 2017

cord and exerts a number of physiological and pathological effects by binding to its cell surface receptor, NMB receptor (NMBR). NMB expression has been detected in various tissues including the gastrointestinal tract, spinal cord, pancreas, pituitary gland, and several areas of the brain [14–17]. In the central nervous system, NMB and NMBR regulate satiety, body temperature, and behaviors such as anxiety, fear, and memory responses [13]. NMB also controls smooth muscle contraction and mediates the exocrine and endocrine secretions [18–20]. In addition, NMB is known to have a growth effect on various types of tumor cells, osteoblasts, and chondrogenic cells [21–25], suggesting that NMB and NMBR play a critical role in cell proliferation.

In this study, we conducted microarray analysis to discover novel genes that regulate osteoclast development and found that NMB was strongly upregulated in mature osteoclasts. Therefore, we hypothesized that NMB/NMBR may be functionally implicated in osteoclast development. To address this issue, we downregulated NMB/NMBR expression using a small hairpin RNA. We show that NMB or NMBR silencing inhibits osteoclast formation via modulation of precursor proliferation and apoptosis in osteoclast lineage cells. Thus, we identify NMB and NMBR as important modulators in M-CSF and RANKL-induced osteoclast generation.

## 2. Materials and methods

### 2.1. Mice

All experimental procedures were performed in strict accordance with the appropriate institutional guidelines for animal research. The protocol was approved by the Committee on the Ethics of Animal Experiments at Kyungpook National University.

### 2.2. Reagents

RANKL and M-CSF were purchased from R & D Systems (Minneapolis, MN). Antibodies against phospho-c-Fms, phospho-ERK, phospho-Akt, ERK, Akt, cleaved caspase-3, and cleaved caspase-9 were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against NMB, c-Fms, and Bim were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), Abcam (Cambridge, UK), and BD Biosciences (San Jose, CA), respectively. The NMBR antagonist, BIM 23127, was purchased from Tocris Bioscience (Ellisville, MO).

### 2.3. Osteoclast generation

Osteoclasts were prepared from mouse bone marrow cells as previously described [26]. Briefly, bone marrow was isolated from the femora and tibiae of 6- to 8-week-old C57/BL6 mice and incubated in red blood cell lysis buffer (150 mM $NH_4Cl$, 10 mM $KNCO_3$, 0.1 mM EDTA, pH 7.4) for 3 min. The cells were then cultured on bacterial plastic in α-minimal essential medium (α-MEM) containing 10% fetal bovine serum with M-CSF (30 ng/ml) for 3 days. The adherent cells were used as osteoclast precursors (bone marrow-derived cells, BMMs). To generate osteoclasts, BMMs were cultured with M-CSF (10 ng/ml) and RANKL (20 ng/ml) for 4 days and the media were changed every 2 days.

### 2.4. TRAP staining

Cultured cells were fixed in 4% paraformaldehyde for 20 min, and stained for tartrate-resistant acid phosphatase (TRAP) with a 0.1 M acetate solution (pH 5.0) containing 6.76 mM sodium tartrate, 0.1 mg/ml naphthol AS-MX phosphate, and 0.5 mg/ml Fast Red Violet. TRAP-positive multinucleated cells containing more than three nuclei were identified as osteoclasts.

### 2.5. Lentiviral transduction of NMB or NMBR shRNA

The shRNA targeting NMB (NMB-sh), NMBR (NMBR-sh), and the nonspecific scrambled control shRNA (Con-sh) were purchased from Sigma Aldrich (St. Louis, MO). To generate lentiviral particles, 293T cells were transfected with the expression constructs and the virus packaging vectors, ΔH8.2 and VSVG, using the FuGENE HD transfection reagent (Promega, Madison, WI). The viral supernatant was collected 24–48 h after transfection. BMMs were then transduced with the viral supernatant plus protamine sulfate (10 μg/ml) for 24 h and the cells were then selected in the presence of puromycin (4 μg/ml) for 3 days.

### 2.6. RT-PCR

Total RNA was harvested from cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA), and cDNA synthesis was carried out from 1 μg of RNA using the SuperScript synthesis system (Invitrogen). Primers were synthesized on the basis of reported mouse cDNA sequences. PCR reactions were performed for 22–34 cycles each of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and the resulting PCR products were separated by 1.5% agarose gel electrophoresis. The following primers were used: NMB, 5′-ATGACCCG-GCAAGCAGGGAGCTCTT-3′ and 5′-TCACTTCTGCAGTAGTGGCTCCAGC-3′; Cathepsin K, 5′-GGAAGAA-GACTCACCAGAAGC-3′ and 5′-GTCATATAGCCGCCTCCACAG-3′; and GAPDH, 5′-ACTTTGTCAAGCTCATTTCC-3′ and 5′-TGCAGCGAACTTT-ATTGATG-3′.

### 2.7. Real-time PCR

Real time PCR was performed using the SYBR Green dye and an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The following primers were used: NMB, 5′-GTCTCCTGCTCTTCGCATTGT-3′ and 5′-CCGGGAGATCCCAGTTGAA-3′; NMBR, 5′-CCTTCTCTCGG-GCACATGA-3′ and 5′-TTGACACAGGAATTGCTGAAACTC-3′; NFA Tc1, 5′-ACCACCTTTCCGCAACCA-3′ and 5′-TTCCGTTTCCCGTTGCA-3′; Cathepsin K, 5′-GGCTGTGGAGGCGGCTAT-3′ and 5′-AGAGTCAA-TGCCTCCGTTCTG-3′; and GAPDH, 5′-TGTGTCCGTCGTGGATCTGA-3′ and 5′-CCTGCTTCACCACCTTCTTGA-3′.

### 2.8. Western blotting

Cells were harvested after washing with chilled PBS and were then lysed in RIPA lysis buffer containing protease and phosphatase inhibitors. The cell lysates were clarified by centrifugation at 13,000 rpm for 10 min at 4 °C to remove the cellular fraction. The extracted proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. After blocking with 5% skim milk for 1 h, the membranes were incubated overnight with primary antibodies at 4 °C followed by probing with secondary antibodies. Signals were visualized using enhanced chemiluminescence reagents (ECL Plus kit; Amersham Pharmacia Biotech) and analyzed with the LAS3000 luminescent image analyzer (GE Healthcare, Piscataway, NJ).

### 2.9. Proliferation assay

To evaluate cell proliferation, we used the BrdU incorporation assay. BMMs were cultured with M-CSF (30 ng/ml), and after 3 days, BrdU was added to the culture medium for 4 h before analysis. BrdU ELISAs were then performed using the cell-proliferation Biotrak ELISA system (Amersham, GE Healthcare Life Sciences).

### 2.10. Apoptosis assay

Apoptosis assay was performed using a Cell Death Detection ELISA Kit (Roche, Mannheim, Germany), which detects cytoplasmic histone-