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Necrostatin-7 suppresses RANK-NFATc1 signaling and attenuates macrophage to osteoclast differentiation

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ABSTRACT

Osteoclasts play a crucial role in osteolytic bone diseases, such as osteoporosis, rheumatoid arthritis, periodontitis, Paget's disease of bone and bone metastatic tumors. Therefore, controlling osteoclast differentiation and function has been considered a promising therapeutic strategy. Here, we show that necrostatin (Nec)-7, an inhibitor of programmed necrosis, strongly suppressed receptor activator of nuclear factor (NF)- κ B ligand (RANKL)-induced osteoclastogenesis and bone resorption, without compromising macrophage colony-stimulating factor (M-CSF)-supported survival and growth of osteoclast precursor cells. Accordingly, Nec-7 significantly decreased the levels of RANKL-induced osteoclastogenic marker genes, such as cathepsin K. Mechanistically, Nec-7 neither affected MAPK nor NF- κ B activation; however, it strongly inhibited the RANKL receptor (RANK) to nuclear factor of activated T cells c1 (NFATc1) signaling. Lentiviral expression of RANK in bone marrow-derived macrophages significantly restored osteoclastogenesis and NFATc1 amplification in Nec-7-treated cells. In this study, we revealed that Nec-7-sensitive pathways are crucially involved in osteoclast formation and function. Investigation of the molecular mechanism(s) through which Nec-7 inhibits RANK-NFATc1 signaling axis may lead to the development of new therapeutic strategies for bone disease.

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Abbreviations: RANK, receptor activator of nuclear factor-kappa B; RANKL, RANK ligand; M-CSF, macrophage colony-stimulating factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; NF- κ B, nuclear factor-kappa B; IkB α , s; NFATc1, nuclear factor of activated T cells c1; Acp5, acid phosphatase, 5 tartrate resistant; TRAP, tartrate-resistant acid phosphatase; Atp6v0d2, ATPase H⁺ transporting, lysosomal V0 subunit D2; Ctsk, cathepsin K; Dcstamp, dendrocyte expressed seven transmembrane protein; Nec, necrostatin; BMM, bone marrow-derived macrophage.

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1. Introduction

Throughout life, the bone is constantly renewed, and its strength and integrity are maintained by the coordination of osteoclast-mediated bone resorption and osteoblast-mediated bone formation [1,2]. Osteoclasts are multi-nucleated giant cells formed by the fusion and differentiation of the hematopoietic monocyte/macrophage lineage, and when activated, they degrade bone matrix by secreting protons and lysosomal proteases [2,3]. Excessive bone resorption has been shown to be involved in a variety of bone disorders such as osteoporosis, rheumatoid arthritis, Paget's disease of bone, and cancer metastasis to bone [2,4,5], and patients affected by these conditions suffer from fracture, pain and impaired mobility [6–8]. Therefore, osteoclastic bone resorption has been considered a therapeutic target, and in fact, some drugs, such as bisphosphonates and denosumab (a monoclonal antibody against receptor activator of nuclear factor- κ B ligand [RANKL]), which target osteoclasts, have been already clinically used as antiresorptive agents [9,10].

Macrophage colony-stimulating factor (M-CSF) and RANKL are two essential cytokines regulating the development and function of osteoclasts [2,11]. M-CSF mainly contributes to the survival and proliferation of osteoclast precursor cells, while RANKL enables the dynamic differentiation processes including cell-cell fusion through its receptor (RANK) [2,11–13]. Upon RANKL binding to its receptor RANK, a variety of downstream signaling pathways responsible for osteoclast differentiation are activated. Among them, especially in the early phase of osteoclastogenesis (within minutes to an hour after RANKL-RANK engagement), the activation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) [1,14–16], and the activation of nuclear factor- κ B (NF- κ B) molecules [17,18], whose liberation and activation require degradation of inhibitor of κ B (I κ B) protein [19], are strongly induced by RANK-mediated signaling [2,20].

The early phase osteoclastogenic signaling, governed by factors such as MAPKs and NF- κ B, culminates in the activation of nuclear factor of activated T cells c1 (NFATc1), a master regulator for osteoclastogenesis [2,20–22]. In the late phase of osteoclastogenesis (within hours to days after RANKL-RANK engagement), NFATc1 has been shown to bind to its own promoter and activate an autoregulatory transcriptional loop (thus, called the autoamplification of NFATc1) [2,22,23]. Then, NFATc1 transcriptionally controls osteoclast terminal differentiation by activating osteoclastogenic genes, such as *Acp5* (encoding TRAP: a marker for osteoclastogenesis), *Atp6v0d2* (encoding vacuolar-type ATPase, H⁺ transporting V0 subunit D2: a component of a proton pump which contributes to the extracellular acidification of osteoclasts), *Ctsk* (encoding cathepsin K: a key cysteine protease expressed in osteoclasts), and *Dcstamp* (encoding DC-STAMP: a molecule involved in cell-cell fusion during osteoclastogenesis) over time [2,20,24,25].

Necrostatin-7 (5-[[3-(4-Fluorophenyl)-1H-pyrazol-4-yl]methylene]-2-imino-3-(2-thiazolyl)-4-thiazolidinone; CAS registry number: 351062-08-3) (Nec-7) is a chemical compound originally identified as an inhibitor of programmed necrosis (called necroptosis; which is caused by a regulated cell death mechanism that results in morphological features resembling necrosis [26,27]) in *Fas-associated protein with death domain (FADD)*-deficient Jurkat T cells [28]. However, the mechanism of action of how Nec-7 inhibits necroptosis remains totally unknown [28], whereas it has been previously reported that Nec-1, another type of necroptosis inhibitor, prevents necroptosis by inhibiting kinase activity of receptor-interacting serine/threonine-protein kinase 1 (RIPK1) [29].

In this study, we investigated the pharmacological effects of Nec-7, other than necroptosis inhibition, and found that Nec-7

potently inhibited osteoclast differentiation and function. Elucidation of the mechanism underlying its action will provide new insights into bone and osteoclast biology.

2. Materials and methods

For detailed materials and methods, see [Appendix A](#). Supplementary Information.

3. Results

3.1. Nec-7 attenuates osteoclast differentiation and osteoclastic bone resorption *in vitro*

To determine the effect of Nec-7 on osteoclast differentiation, primary bone marrow-derived macrophages (BMMs) were treated with various concentrations of Nec-7 (0.1–3 μ M) in the presence of M-CSF and RANKL for three days. As shown in [Fig. 1A](#) and [B](#), Nec-7 reduced the number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts in a dose-dependent manner, whereas vehicle-treated BMMs efficiently underwent osteoclastogenesis. The attenuated osteoclast differentiation could be caused by inhibitory effects of Nec-7 on the proliferation and/or survival of osteoclast precursor cells. To test this possibility, BMMs were cultured with Nec-7 for three days, and we found that Nec-7 did not show significant cytotoxicity in BMMs at concentrations up to 3 μ M ([Supplementary Fig. S1](#)). In agreement with the significant attenuation of osteoclast differentiation, Nec-7 dose-dependently suppressed osteoclastic bone resorption *in vitro* ([Fig. 1C](#) and [D](#)).

Nec-7 was originally identified as an inhibitor of necroptosis [28]. To address whether the necroptosis mechanisms are generally involved in macrophage to osteoclast differentiation, we tested the effect of another type of necroptosis inhibitor, Nec-1 [29] on osteoclastogenesis. As shown in [Fig. 1E](#), Nec-1 (up to 10 μ M) did not influence osteoclast differentiation, whereas even 2 μ M of Nec-1 significantly suppressed necroptosis in BMMs ([Supplementary Fig. S2](#)), indicating that the Nec-7-sensitive pathways responsible for osteoclastogenesis are, at least, distinct from the Nec-1-sensitive necroptosis pathway(s).

3.2. Nec-7 suppresses RANKL-stimulated osteoclastogenic gene induction

M-CSF and RANKL are essential cytokines for osteoclastogenesis, and M-CSF has been shown to support the survival and proliferation of osteoclast precursor cells [2,23]. The fact that Nec-7 did not affect survival and proliferation of BMMs ([Supplementary Fig. S1](#)) strongly suggested that Nec-7 abrogates RANKL-induced signaling rather than M-CSF-activated signaling. In fact, Nec-7 suppressed osteoclastogenesis in the murine monocyte/macrophage cell line RAW264 ([Supplementary Fig. S3](#)), which only requires RANKL but not M-CSF supplementation for osteoclastogenesis in culture [30], further supporting the notion that Nec-7 inhibits RANKL-RANK signals. We then examined the effects of Nec-7 on the RANKL-stimulated gene induction profiles in BMMs. As expected, Nec-7 dose-dependently suppressed the expression levels of *Acp5*, *Atp6v0d2*, *Ctsk* and *Dcstamp* ([Fig. 2](#)). Collectively, these data clearly demonstrate that RANK downstream signaling contains Nec-7-sensitive elements crucial for osteoclast terminal differentiation.

3.3. Nec-7 affects the RANKL-stimulated autoamplification of NFATc1 and sustained expression of RANK during osteoclastogenesis rather than the early activation of MAPKs and NF- κ B

As important cellular events leading to osteoclastogenesis, the

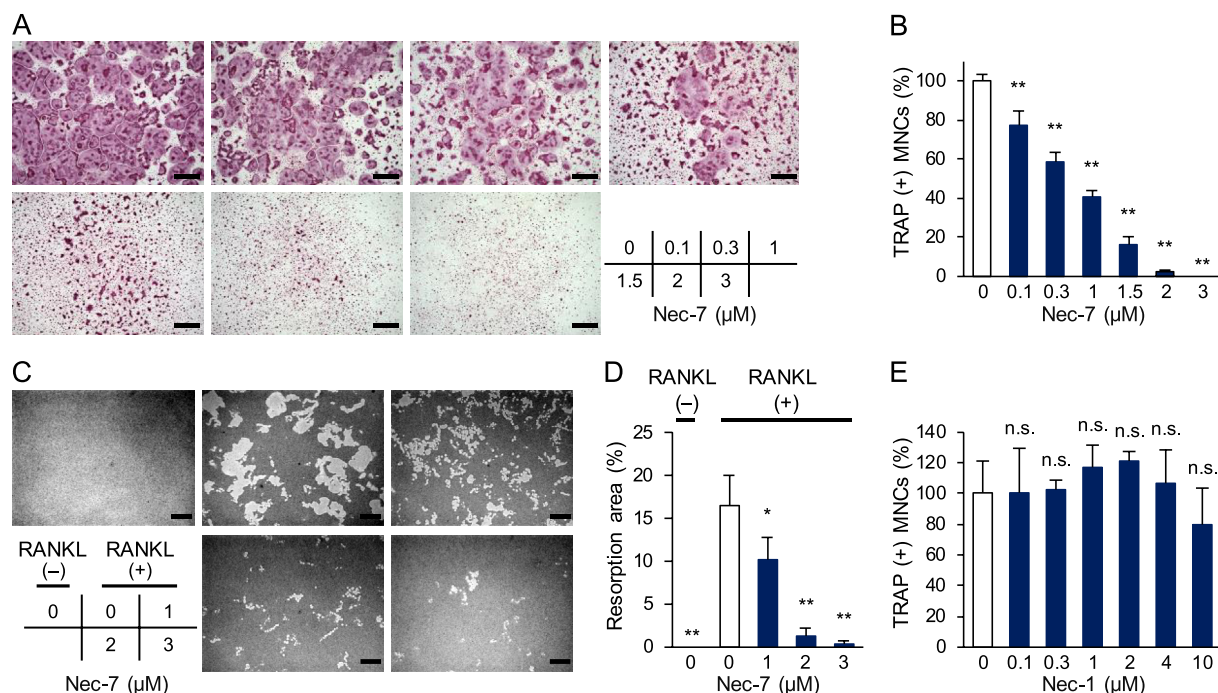


Fig. 1. Nec-7 attenuates osteoclast differentiation and osteoclastic bone resorption *in vitro*. (A, B) Effects of Nec-7 on osteoclast differentiation stimulated by M-CSF (10 ng/ml) and RANKL (50 ng/ml). (A) Representative microscopic images and (B) TRAP-positive multinucleated cell (MNC) number relative to vehicle-treated cells (white bar) are shown. Scale bars, 500 μ m. (C, D) Effects of Nec-7 on osteoclastic bone resorption. BMMs were cultured on inorganic bone biomaterial surface with M-CSF (10 ng/ml) and RANKL (50 ng/ml) in the presence or absence of Nec-7 for five days. (C) Representative microscopic images of resorption pits and (D) quantification of resorption area per view area are shown. Scale bars, 100 μ m. Vehicle-treated cells stimulated with RANKL served as control (white bar). (E) Effects of Nec-1 on osteoclast differentiation stimulated by M-CSF (10 ng/ml) and RANKL (50 ng/ml). BMMs were cultured with the indicated concentrations of Nec-1 and TRAP-positive MNC number relative to vehicle-treated cells (white bar) is shown. Data are presented as mean $\pm$ SD (n = 3). Results are representative of at least three independent experiments.

*p < 0.05; **p < 0.01; n. s., not significant.

activation of MAPKs and NF- κ B take place within minutes to an hour after RANKL-RANK engagement [2,20]. Nevertheless, Nec-7 neither affected the activation of ERK, p38 and JNK (Fig. 3A) nor that of NF- κ B (assessed by I κ B α degradation, a prerequisite of NF- κ B activation [19]) (Fig. 3B), indicating that RANKL-RANK binding itself and the early phase signaling pathways, at least MAPK and NF- κ B pathways, are Nec-7 insensitive. However, intriguingly, Nec-7 significantly suppressed the induction of *NFATc1*, one of the most essential events for osteoclastogenesis [2,20–22] (Fig. 3C). To elucidate the selective inhibitory mechanism of Nec-7 on the late phase of osteoclastogenesis, that is, the autoamplification of NFATc1, we focused on the expression of RANK in osteoclast

precursor cells, since sustained RANKL signaling via RANK is crucial for the maintenance of NFATc1 autoamplification (manuscript in preparation). During osteoclastogenesis, the precursor cells maintained RANK mRNA levels (data not shown) and slightly increased the protein levels of RANK over time (Fig. 3D, vehicle-treated control), while Nec-7-treated cells showed impaired RANK protein expression (Fig. 3D). Of note, Nec-7 also suppressed RANK mRNA levels (Fig. 3E), suggesting that the Nec-7-sensitive pathway is vital for the transcriptional and/or post-transcriptional control of the sustained RANK expression during the course of osteoclast differentiation.

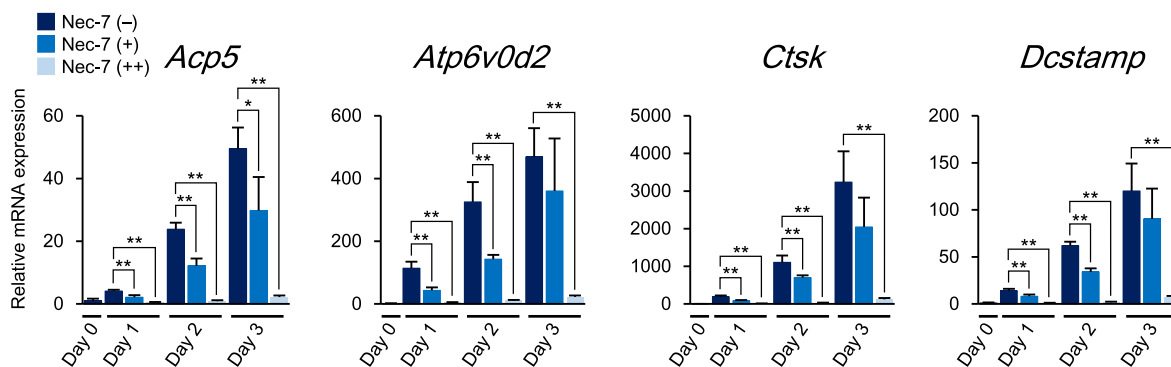


Fig. 2. Nec-7 suppresses RANKL-stimulated osteoclastogenic gene induction. BMMs were cultured with M-CSF (10 ng/ml) and RANKL (50 ng/ml) in the presence or absence of Nec-7 (-, vehicle; +, 1 μ M; ++, 3 μ M). The expression of osteoclastogenic markers (*Acp5*, *Atp6v0d2*, *Ctsk*, *Dcstamp*) during osteoclast differentiation was evaluated by qRT-PCR. Data are presented as mean $\pm$ SD (n = 3). Results are representative of at least three independent experiments. *p < 0.05; **p < 0.01.

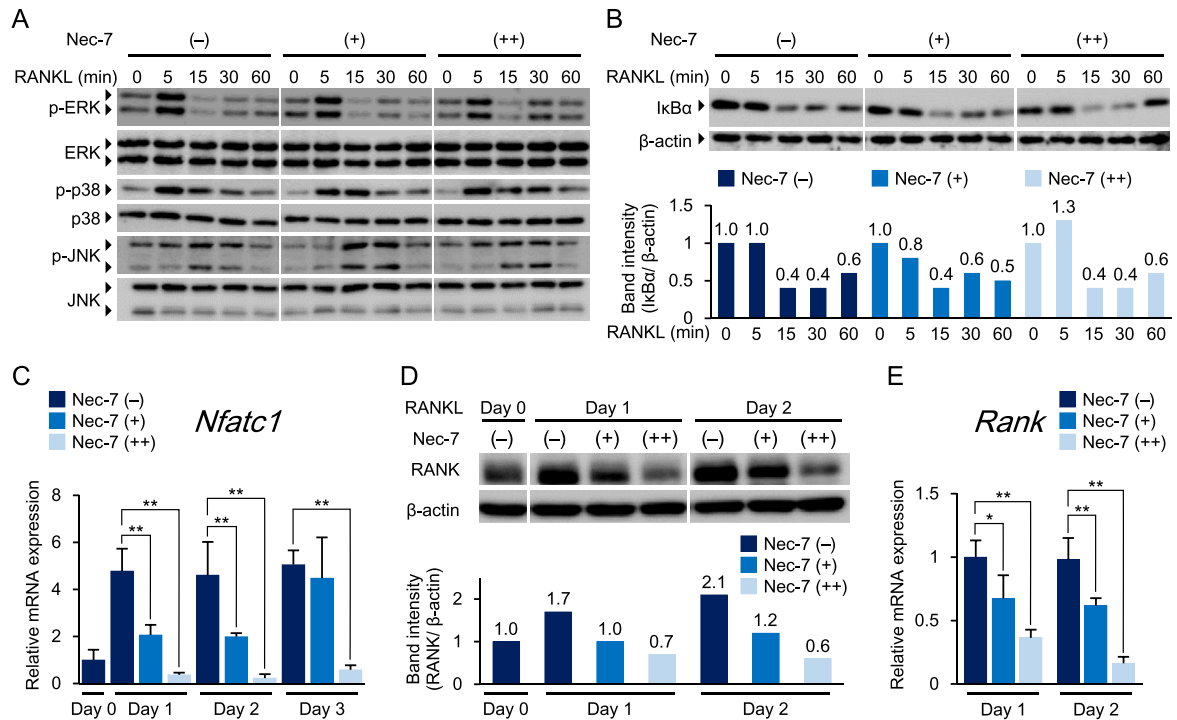


Fig. 3. Nec-7 affects the RANKL-stimulated autoamplification of NFATc1 and sustained expression of RANK during osteoclastogenesis rather than the early activation of MAPKs and NF-κB. (A) Effects of Nec-7 (-, vehicle; +, 1 μM; ++, 2 μM) on the phosphorylation of ERK, p38 and JNK. Total ERK, p38 or JNK was used as a loading control. (B) Effects of Nec-7 (-, vehicle; +, 1 μM; ++, 2 μM) on NF-κB activation were assessed by IκBα degradation (upper panel). The intensities of the IκBα bands were quantified by densitometry, and the relative band density was calculated with respect to β-actin band and expressed as relative to RANKL unstimulated controls (lower panel). Serum-starved BMMs, treated with Nec-7, were stimulated with RANKL (50 ng/ml). Cell lysates were harvested at the indicated time points and subjected to immunoblot analysis. (C) Nec-7 attenuates autoamplification of NFATc1 expression. BMMs were cultured with M-CSF (10 ng/ml), RANKL (50 ng/ml) and various concentrations of Nec-7 (-, vehicle; +, 1 μM; ++, 3 μM), and the effect of Nec-7 on NFATc1 gene expression was analyzed by qRT-PCR, and the data are presented as mean ± SD (n = 3). (D) Impairment of RANK expression in Nec-7 treated cells. The effect of Nec-7 (-, vehicle; +, 1 μM; ++, 2 μM) on RANK protein expression during osteoclastogenesis was analyzed by immunoblotting (upper panel). The intensities of the RANK bands were quantified by densitometry and the relative band density was calculated with respect to β-actin band and expressed as relative to RANKL unstimulated controls (lower panel). (E) Nec-7 suppresses mRNA expression levels of *Rank*. BMMs were cultured with M-CSF (10 ng/ml), RANKL (50 ng/ml) and various concentrations of Nec-7 (-, vehicle; +, 1 μM; ++, 3 μM). The effect of Nec-7 on the mRNA expression of *Rank* during osteoclastogenesis was analyzed by qRT-PCR, and the data are presented as mean ± SD (n = 3). All results are representative of at least three independent experiments. *p < 0.05; **p < 0.01.

3.4. Overexpression of RANK restores osteoclast differentiation and NFATc1 expression in Nec-7-treated BMMs

A line of evidence supports the notion that the attenuated RANK-NFATc1 signaling is a key determinant of the Nec-7 inhibition of osteoclastogenesis. To evaluate this, we investigated the effect of exogenously expressed RANK on the Nec-7-induced impairment of osteoclastogenesis using lentiviral gene transfer. BMMs were infected with lentivirus carrying RANK gene (RANK vector) or with control lentivirus (Empty vector), and infection efficiency was monitored by bicistronically expressed Venus fluorescent protein and was usually around 60% (data not shown). BMMs infected with RANK vector overexpressed RANK both in mRNA and protein levels (Fig. 4A and B) and restored osteoclastogenesis even in the presence of Nec-7 (Fig. 4C and D). Of note, the restored osteoclastogenesis in RANK-overexpressing BMMs was accompanied by recovery of NFATc1 mRNA expression (Fig. 4E), confirming that RANK-NFATc1 signaling is one of the main targets of Nec-7.

4. Discussion

This is the first report demonstrating a novel pharmacological property of Nec-7, other than its action as an inhibitor of programmed cell death. Our results showed that Nec-7 (at

concentrations ranging from 0.1 to 3 μM) dose-dependently inhibited osteoclastogenesis and bone resorption *in vitro* without affecting the cell viability of osteoclast precursor cells. We found that the M-CSF-supported BMM survival and proliferation were not affected by Nec-7, whereas the RANKL-stimulated gene expression profile was strongly abrogated, thus highlighting the Nec-7-sensitive components (molecules, pathways, cellular structures, and so on) in RANK downstream signaling cascades. Indeed, we found that Nec-7 suppressed osteoclastogenesis in RAW cells, which only require exogenous RANKL stimulation for their differentiation.

MAPK and NF-κB activation are important early signaling events induced by RANKL, and numerous reports have shown that perturbations of these pathways result in impaired osteoclastogenesis [2,14–18]. The fact that Nec-7 did not affect MAPK and NF-κB signaling, which unambiguously shows RANK downstream signaling is composed of both Nec-7-sensitive and -insensitive pathways, prompted us to investigate the late phase of osteoclastogenesis, and we found that the RANK expression and NFATc1 autoamplification, the latter of which has been shown to be an essential transcriptional event for osteoclast differentiation and function [22], were particularly abrogated by Nec-7. These results agree with previous studies showing that the genetic disruption of *Rank* or *Nfatc1* results in the failure of osteoclast differentiation *in vitro* and *in vivo*, and thus causes severe osteopetrosis in mice

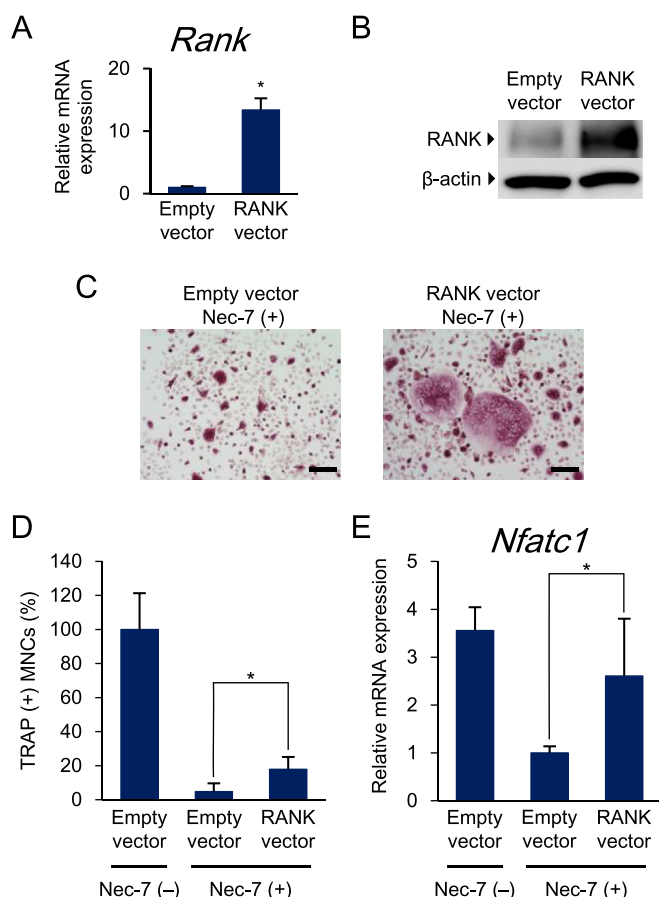


Fig. 4. Overexpression of RANK restores osteoclast differentiation and NFATc1 expression in Nec-7-treated BMMs. (A, B) Lentiviral expression of RANK in BMMs was confirmed at (A) mRNA level and (B) protein level. (C, D, E) Overexpression of RANK rescued the impaired osteoclastogenesis and NFATc1 expression caused by Nec-7. BMMs infected with either Empty vector or RANK vector were cultured with M-CSF (10 ng/ml) and RANKL (100 ng/ml) in the presence or absence of Nec-7 (-, vehicle; +, 3 μ M). (C) Representative microscopic images (scale bars, 100 μ m) and (D) TRAP-positive multinuclear cell (MNC) number relative to Nec-7-untreated BMMs infected with Empty vector are shown. (E) The *NFATc1* mRNA expression was analyzed by qRT-PCR. Cells were harvested 24 h after the RANKL stimulation. The bar graphs are presented as mean $\pm$ SD (n = 4). Results are representative of at least three independent experiments. *p < 0.05.

[22,31]. It is noteworthy that the expression levels of M-CSF receptor (also known as, colony stimulating factor 1 receptor [Csf1r]) were not affected by Nec-7 (data not shown).

The forced expression of RANK restored osteoclastogenesis and autoamplification of NFATc1 in Nec-7-treated BMMs. Therefore, the impaired osteoclast differentiation by Nec-7 is, at least in part, attributable to the decreased RANK-NFATc1 signaling. However, albeit significant, the efficiency of recovered osteoclastogenesis was lower compared to Nec-7-untreated control cells (Fig. 4D). This can be due to the low efficiency of lentiviral gene transfer (around 60%) or may indicate a complex mechanism of the inhibitory action of Nec-7. The M-CSF-M-CSF receptor signaling has been shown to play crucial roles for inducing RANK, a key cell surface molecule that can initiate intracellular machinery that regulates osteoclastogenesis [2,32]. On the other hand, a RANK-mediated positive feedback mechanism has been suggested as a possible regulator of sustained RANK expression during osteoclastogenesis [33]. However, it remains to be seen how exactly RANK-mediated signaling is down-regulated by Nec-7. Chemical biology approaches may be required

to elucidate the precise mode of action of Nec-7.

Necroptosis, one of the programmed necrosis pathways [34], is involved in a variety of disease states such as ischemia-reperfusion injury, drug-induced liver failure and inflammatory bowel diseases [35,36]. We first postulated that the necroptosis-related mechanisms are generally involved in osteoclastogenesis. However, Nec-1, a widely used necroptosis inhibitor which is structurally distinct from Nec-7 [28], did not inhibit osteoclastogenesis in concentrations sufficient to halt necroptosis in BMMs. Thus, we concluded that the necroptosis-related mechanisms, especially Nec-1-sensitive Ripk1-related pathways [29], are not commonly involved in the differentiation of osteoclasts.

Excessive osteoclastic bone resorption is a primary cause of bone loss in many diseases. Therefore, suppressing osteoclast formation is considered one of the most efficient and direct ways to prevent and treat pathological bone destruction [3,5,9]. In fact, bisphosphonates, which suppress the function and cell survival of osteoclasts, had been the first-line drugs for osteoclast-mediated bone loss in osteoporosis, Paget disease of bone, bone metastatic tumors (such as multiple myeloma and breast cancer) and hypercalcemia of malignancy [37,38]. Recently, in efforts to develop potent therapeutics, denosumab (an anti-RANKL monoclonal antibody), which has been reported to potentially inhibit RANK-NFATc1 signaling, has begun to be clinically used [10,39,40]. Hence, molecular understanding of how Nec-7 impacts the RANK-NFATc1 signaling may not only provide physiological and pathophysiological insights into osteoclastic bone resorption but also pave the way for developing new therapeutic approaches for the treatment of bone disorders.

Disclosure

The authors declare no conflict of interest.

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Author contributions

H.F., S.O., N.N. and M. Takeiri performed experiments and analyzed data. H.Y., K. Izumi, M.I., S.I., M. Toyomoto and T.K. provided technical assistance. S.S., K.T., S.H., M. Aoyama, Y.I., M.H., N.T., E.H. contributed to study design and/or manuscript preparation. S.S., K.T., E.H., K. Iwaisako and S.U. helped shape the research. K. Iwaisako and M. Asagiri supervised the study. H.F. and M. Asagiri wrote the manuscript with input from all authors.

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Transparency document

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Appendix A. Supplementary data

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