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C/EBP α and PU.1 Exhibit Different Responses to RANK Signaling for Osteoclastogenesis

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Abstract

The transcription factors C/EBP α and PU.1 are upregulated by RANKL through activation of its receptor RANK during osteoclastogenesis and are critical for osteoclast differentiation. Herein we investigated the mechanisms underlying how C/EBP α and PU.1 regulate osteoclast differentiation in response to RANK signaling. We showed that C/EBP α or PU.1 overexpression could initiate osteoclastogenesis and upregulate the expressions of the osteoclast genes encoding the nuclear factor of activated T-cells, C1, cathepsin K, and tartrate-resistant acid phosphatase independently of RANKL. However, while PU.1 upregulated C/EBP α , C/EBP α could not upregulate PU.1. RANK has a unique cytoplasmic domain, 535IVVY538 motif, which is crucial for osteoclast differentiation. We demonstrated that mutational inactivation of RANK IVVY motif blocked osteoclast differentiation and significantly attenuated C/EBP α , but not PU.1, expression, indicating that RANK-IVVY-induced signaling is dispensable to PU.1 upregulation during osteoclastogenesis. However, C/EBP α or PU.1 overexpression failed to promote osteoclastogenesis in cells expressing mutated RANK IVVY motif. We noted that RANK-IVVY-motif inactivation significantly repressed osteoclast genes as compared with a vector control, suggesting that IVVY motif might also negatively regulate osteoclast inhibitors during osteoclastogenesis. Consistently, IVVY-motif inactivation triggered upregulation of *RBP-J*, a potent osteoclast inhibitor, during osteoclastogenesis. Notably, C/EBP α or PU.1 overexpression in cells expressing mutated RANK IVVY motif failed to control the deregulated *RBP-J* expression, resulting in repression of osteoclast genes. Accordingly, *RBP-J* silencing in the mutant cells rescued osteoclastogenesis with C/EBP α or PU.1 overexpression. In conclusion, we revealed that while PU.1 and C/EBP α are critical for osteoclastogenesis, they respond differently to RANKL-induced activation of RANK IVVY motif.

Keywords

C/EBP α ; differentiation; IVVY motif; osteoclast; PU.1; RANK; RBP-J

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1. Introduction

Bone is continuously remodeled through the balance activities of the osteoblasts, the bone-forming cells, and the osteoclasts, the bone resorbing cells (1,2). During skeletal remodeling and healthy states, bone resorption is synchronized by bone formation. However, in many bone diseases, the rate of bone resorption exceeds that of bone formation (3,4). As such, the osteoclast has been regarded as a key player in the bone loss stemming from various bone diseases (5). Osteoclasts are polykaryons that are originated from the macrophages upon stimulation by the macrophage colony-stimulating factor (M-CSF) and the receptor activator of NF- κ B (RANK) ligand (RANKL) (6). M-CSF promotes the proliferation and survival of the bone marrow macrophages (BMMs), and RANKL, through its receptor RANK, mediates the differentiation of BMMs into osteoclasts. Specifically, activation of RANK by RANKL strongly upregulates the expressions of many crucial transcription factors, such as CCAAT/enhancer binding protein α (C/EBP α) (7), a member of the C/EBP family of transcription factors, and the spleen focus-forming virus proviral integration 1 (PU.1 also called Spi-1) (8), a member of the ETS family transcription factor. C/EBP α and PU.1 are both critical for osteoclastogenesis through induction or upregulation of osteoclast genes (6–8). Importantly, RANK has a specific motif within its cytoplasmic domain, 535IVVY538, which is essential for osteoclast formation by regulating gene expression (9–12).

C/EBP α is critical for hematopoiesis through its ability to induce the expressions of genes responsible for myeloid cell differentiation, including macrophages (13,14). Mice deficient in the *C/EBP α* gene die shortly after birth and exhibit defective granulocyte development as well as impaired homeostasis (15,16). We have recently demonstrated that newborn *C/EBP α* -deficient mice also display osteopetrosis due to impaired osteoclast development (7). Consistently, C/EBP α can induce the expressions of the osteoclast genes encoding nuclear factor of activated T-cells, C1 (*NFATc1*), cathepsin K (*Ctsk*), and tartrate-resistant acid phosphatase (*TRAP*) during osteoclast differentiation (17). Similarly, PU.1 is also important for the development of cells of the hematopoietic lineage, including macrophages (18–21). Mice deficient in the *PU.1* gene die during embryonic development or shortly after birth (8). The *PU.1*-deficient mice also develop osteopetrosis from impaired osteoclast development (8). Furthermore, PU.1 is critical for the induction of *NFATc1*, *Ctsk*, and *TRAP* during osteoclast differentiation (22–24).

Although C/EBP α and PU.1 are both upregulated by RANKL and are also crucial for osteoclast differentiation by inducing gene expression (6–8,17), the mechanisms underlying how C/EBP α and PU.1 regulate osteoclast differentiation in response to RANK activation are unknown. Moreover, while the RANK IVVY motif is essential for induction of osteoclast genes including *Ctsk* and *TRAP* which are known to be regulated by C/EBP α and PU.1 (7,8,17), the roles of RANK IVVY motif in regulating the expressions of C/EBP α and PU.1 have not been investigated. The current study was aimed at investigating the roles of C/EBP α and PU.1 in mediating osteoclast differentiation in response to RANKL/RANK signaling by using a gain-of-function strategy in a RANK-IVVY motif dependent manner. The results provide an important insight into the roles of C/EBP α , PU.1, and RANK signaling in osteoclast differentiation.

2. Materials and Methods

2.1. Reagents

The chemicals were purchased from Sigma. Recombinant mouse RANKL (catalog no. 462-TEC) and M-CSF (catalog no. 416-ML) were obtained from R&D Systems. Anti-Human FAS activating antibody (α -FAS, catalog no. 05-201) was obtained from Millipore. Anti-FLAG antibody (catalog no. F1804) was from Sigma. Anti- β actin (catalog no. SC-81178) and anti-RBP-J (catalog no. SC-271128) antibodies were from Santa Cruz Biotechnology. Recombinant recognition sequence binding protein at the J_κ site (*RBP-J*) and Scramble shRNA lentiviral constructs were purchased from Sigma.

2.2. Plasmid Generation and Viral Transduction

The pMX-puro-3xFLAG-C/EBP α (FLAG-C/EBP α) and pMX-puro-3xFLAG constructs were generated in a previous study (17). The pMX-puro-3xFLAG-PU.1 (FLAG-PU.1) construct was generated by first amplifying the mouse PU.1 cDNA from the pSport6-PU.1 vector (Addgene). We then subcloned the amplified PU.1 cDNA in-frame with the 3xFLAG sequence into the pMX-puro-3xFLAG vector. The resulted construct was confirmed by sequencing. The pMX-puro-GFP (GFP), pMX-puro-FAS-RANK (FAS-RANK), and pMX-puro-FAS-mIVVY (FAS-mIVVY) vectors were generated and kindly provided by Dr. Xu Feng (University of Alabama at Birmingham) (9,25). The 293GPG retroviral packaging cell line was used for retrovirus generation as described previously (26). In brief, 293GPG cells were cultured in Dulbecco's Modified Eagle Medium with 10% heat-inactivated fetal bovine serum, G418, tetracycline, penicillin/streptomycin, and puromycin before being transfected with pMX retroviral constructs using the calcium phosphate precipitation method. Retroviral supernatant was harvested at 2, 3, and 4 days post transfection. For the lentivirus generation, the RBP-J lentiviral vector or a Scramble shRNA lentiviral construct along with packaging vectors were co-transfected into HEK-293 cells using the calcium phosphate precipitation method. The lentiviral supernatant was collected at 60 hours post transfection. The viral supernatant was used to infect BMMs for osteoclastogenesis assays.

2.3. In Vitro Osteoclastogenesis Assays

BMMs were isolated from long bones of 4-to 6-week old C57BL/6 mice, and 5×10^4 cells/well in 24-well culture dishes were cultured in α -Minimal Essential Medium with 10% heat-inactivated fetal bovine serum and M-CSF (20 ng/ml) for 24 hours. Some cells were then directly differentiated into osteoclasts as indicated in individual experiments, and other cells were infected with a virus before being submitted to osteoclastogenesis assays as indicated in the related experiments (27,28). At the end of the assays, the cultures were stained for TRAP activity using a leukocyte acid phosphatase kit (catalog no. 387-A, Sigma) according to the instruction of the manufacturer to examine osteoclast formation. The assays were quantified by counting and/or accessing the size of the multinucleated TRAP-positive cells (more than three nuclei) in representative areas. The experiments involving mice were approved by The University of Alabama at Birmingham Institutional Animal Care and Use Committee. The osteoclastogenesis assays were carried in duplicate and repeated independently at least three times.

2.4. Western Blotting Analysis

Western blotting was carried out as described in a previous study (29). In brief, cells were cultured as indicated in the individual experiments before protein collection for gel electrophoresis. Membranes were washed, and enhanced chemiluminescence detection was carried using Luminata Forte HRP Substrate from Millipore. Membranes were visualized using a C-DiGit® Blot Scanner and Image Studio Software from Li-Cor. The Western blotting analysis was repeated independently at least three times using β actin as a loading control.

2.5. Quantitative Real-time PCR (qPCR) Analysis

qPCR analysis was performed as described in a previous study (30). In brief, cells were cultured as indicated in the individual experiments, and total RNA was collected using TRIzol reagent (Life Technologies). 1 μ g of total RNA was transcribed into cDNA using the ProtoScript® First Strand cDNA Synthesis Kit (New England BioLabs) according to the instruction of the manufacturer. qPCR reactions were carried by utilizing the Fast SYBR® Green Master Mix reagent (Life Technologies) using hypoxanthine-guanine phosphoribosyl-transferase as an endogenous control for normalization. The qPCR analysis was repeated independently three times.

2.6. Reverse Transcription PCR (RT-PCR) Analysis

BMMs were cultured as indicated in the individual experiments, and total RNA was collected for cDNA synthesis as indicated above in 2.5. Gene amplification was carried using *Taq* DNA polymerase (catalog no E001, Novo Protein) (25). RT-PCR primers to detect the chimeric receptors (FAS-RANK and FAS-mIVVY) are 5' - ATGCTGGGCATCTGGACCCTCCTA - 3' for the Human FAS extracellular domain (Forward) and 5' - GAAGTCACAGCCCTCAGAATC - 3' for the mouse RANK intracellular domain (reverse). Primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), used as a loading control, are 5' - TCATTGAGAGCAATGCCAGC - 3' (Forward) and 5' - ACATCATCCCTGCATCCACTG - 3' (Reverse). The RT-PCR reaction was loaded on 2% agarose gel for electrophoretic analysis. The RT-PCR analysis was repeated independently three times.

2.7. Statistical Analysis

Data are reported as averages \pm SD. Statistical significance was assessed using the Student's t test. p values less than 0.05 were considered significant.

3. Results

3.1. C/EBP α or PU.1 can initiate osteoclastogenesis independently of RANKL.

C/EBP α and PU.1 are critical for osteoclast formation both *in vitro* and *in vivo* (7,8,17,31). In order to examine the influence of C/EBP α and PU.1 on osteoclastogenesis, we first examined their roles in osteoclast lineage commitment. We stimulated BMMs, widely used as primary osteoclast precursors, with M-CSF plus RANKL for 0 or 3 hours and then accessed the expressions of C/EBP α and PU.1 by qPCR. The data showed that the combined

stimulation of BMMs with M-CSF/RANKL could significantly upregulate *C/EBP α* and *PU.1* (Fig. 1A), confirming previous reports that RANKL can upregulate *C/EBP α* and *PU.1* very early during osteoclastogenesis (7,8). We then investigated the effects of *C/EBP α* or *PU.1* overexpression in mediating osteoclast lineage commitment without RANKL stimulation (Fig. 1B-D). We have recently reported that *C/EBP α* overexpression could initiate osteoclastogenesis independently of RANKL (7,17). We confirmed this finding and showed that *C/EBP α* overexpression in BMMs, as confirmed by Western blotting (Fig. 1B), could generate TRAP-positive mononucleated cells independently of RANKL (Fig. 1C,D). Notably, we confirmed that, similarly to *C/EBP α* , *PU.1* overexpression could also generate TRAP-positive mononucleated cells independently of RANKL (Fig. 1 B-D) (8,32). In confirming the abilities of *C/EBP α* and *PU.1* to induce lineage commitment, we showed that *C/EBP α* or *PU.1* overexpression could significantly induce the expressions of *c-Fos* (Fig. 1E), an early osteoclast transcription factor (33), and *NFATc1* (Fig. 1F), a master transcriptional regulator of osteoclast differentiation, as compared to a GFP control (34). Importantly, we demonstrated that *c-Fos* or *NFATc1* overexpression could not upregulate *C/EBP α* or *PU.1* (Suppl. Fig. 1), confirming the previous studies that *c-Fos* and *NFATc1* are target genes of *C/EBP α* and *PU.1* during osteoclastogenesis (6–8). Moreover, *C/EBP α* or *PU.1* overexpression could significantly induce the expressions of the osteoclast genes encoding *Ctsk* (Fig. 1G) and *TRAP* (Fig. 1H) as compared to a GFP control under the stimulation by M-CSF alone.

Given that RANKL could transiently upregulate *C/EBP α* and *PU.1* both of which could induce osteoclast lineage priming (Fig. 1), we examined the abilities of *C/EBP α* and *PU.1* to upregulate each other independently of RANKL (Fig. 2). *PU.1* overexpression significantly upregulated *C/EBP α* under stimulation by M-CSF alone as compared to a GFP control (Fig. 2A). However, *C/EBP α* overexpression failed to upregulate *PU.1* in the absence of RANKL as compared to the GFP control (Fig. 2B). These results indicated that while overexpression of *C/EBP α* or *PU.1* could similarly induce the lineage commitment, *C/EBP α* was a target gene of *PU.1* during osteoclastogenesis.

3.2. *PU.1* generates more osteoclasts than *C/EBP α* from pre-committed BMMs.

Next, we compared the roles of *C/EBP α* and *PU.1* in mediating osteoclast differentiation which follows the lineage commitment by using the gain-of-function strategy (Fig. 3). Treatment of BMMs with low amount of RANKL was shown to be sufficient to promote lineage commitment but was unable to sustain osteoclast differentiation (35–37). Using this strategy, we overexpressed *C/EBP α* or *PU.1* using a retrovirus and then treated the infected cells with M-CSF plus low amount (1 ng/ml) or optimum amount (10 ng/ml) of RANKL as determined in our previous assay to stimulate osteoclast differentiation with RANKL-evoked lineage priming (Fig. 3A,B) (17). Whereas *C/EBP α* or *PU.1* overexpression could promote osteoclast differentiation with low RANKL doses, the *PU.1* overexpressers generated significantly more osteoclasts than the *C/EBP α* overexpressers (Fig. 3B,C). Consistently, *PU.1* could also generate more osteoclasts than *C/EBP α* in BMMs treated with optimum RANKL doses (Fig. 3B,C). However, we found that *PU.1* overexpression did not influence the osteoclast size as compared with BMMs overexpressing *C/EBP α* or expressing the GFP control (Fig. 3D,E). The results indicated that *PU.1* exhibited a stronger influence on

osteoclast differentiation than *C/EBP α* , and showed *PU.1* and *C/EBP α* displayed similar effects on OC size.

3.3. *C/EBP α* and *PU.1* respond differently to RANKL-induced activation of the RANK IVVY motif.

RANK contains an unique cytoplasmic domain, the IVVY motif, at the amino acids 535–538 that is essential for osteoclastogenesis by inducing gene expression (9–11). To gain more insight into the role of the RANK IVVY motif in osteoclastogenesis, we compared the influence of *C/EBP α* and *PU.1* in osteoclast differentiation through RANKL-induced activation of the RANK IVVY motif (Fig. 4). RANK and FAS are both members of the tumor necrosis factor receptor superfamily, which are activated by ligand-induced receptor trimerization (38). Upon binding to RANK, RANKL triggers RANK trimerization and subsequently transduces intracellular signaling to drive gene expression and ultimately osteoclastogenesis (Fig. 4A). In order to delineate specific motifs within the RANK cytoplasmic domain that mediate osteoclastogenesis, Xu and colleagues developed a chimeric receptor system which consists of the Human FAS external domain linked to the transmembrane and cytoplasmic domains of mouse RANK (39). This chimeric receptor system can be specifically activated by a Human FAS activating antibody (α -FAS) which can only activate the Human FAS, but not the mouse FAS, external domain (9). The authors showed that treatment of BMMs expressing the chimeric receptor system with M-CSF and α -FAS could induce gene expression and promote osteoclastogenesis in a similar fashion as RANKL (Fig. 4B) (9,28,40). In investigating whether the RANK IVVY motif could regulate the expressions of *C/EBP α* and *PU.1* during osteoclastogenesis, we used two chimeras (FAS-RANK and FAS-mIVVY) that were previously developed and validated by Xu and colleagues (9). FAS-RANK contains the Human FAS external domain linked to the transmembrane and cytoplasmic domains of normal mouse RANK, and FAS-mIVVY has the Human FAS external domain linked to the transmembrane and cytoplasmic domains of mouse RANK bearing an inactivating mutation in the IVVY motif (Fig. 4C). BMMs were infected with a retrovirus encoding FAS-RANK, FAS-mIVVY, or a GFP control before being submitted to gene expression analysis (Fig. 4D,E) and osteoclastogenesis assays (Fig. 4F). To confirm the expressions of FAS-RANK and FAS-mIVVY in the infected cells, we utilized both a RT-PCR strategy by designing a forward primer against the Human FAS extracellular domain and a reverse primer against the mouse RANK cytoplasmic domain (Fig. 4D) and Western blotting using an anti-Human FAS antibody (Fig. 4E). We confirmed that FAS-RANK and FAS-mIVVY were highly expressed in the BMMs expressing the chimeras but not in cells expressing the GFP control. Whereas treatment of the FAS-RANK expressers with M-CSF and α -FAS generated numerous osteoclasts, the FAS-mIVVY expressers failed to form osteoclast (Fig. 4F). Accordingly, the FAS-RANK, but not the FAS-mIVVY, expressers, could induce the expressions of *NFATc1*, *Ctsk*, and *TRAP* during osteoclast differentiation (Fig. 4G). These results confirmed the previous reports that the RANK IVVY motif is critical for osteoclastogenesis through induction of osteoclast genes (9,27). However, we found that the mutational inactivation of the RANK IVVY motif significantly repressed *C/EBP α* expression but exerted no significant effect on *PU.1* expression (Fig. 4H). These results indicated that *C/EBP α* and *PU.1* responded differently to RANK-IVVY signaling during osteoclastogenesis upon RANK activation.

3.4. C/EBP α or PU.1 fails to mediate osteoclast differentiation with inactivation of the RANK IVVY motif.

Next, we investigated whether *PU.1* overexpression could rescue osteoclastogenesis in cells expressing the mutated RANK IVVY motif. Toward this end, BMMs were first infected with a retrovirus encoding FAS-RANK or FAS-mIVVY before being infected with another retrovirus encoding a GFP control, C/EBP α , or PU.1 (Fig. 5A). BMMs doubly expressing FAS-RANK or FAS-mIVVY plus the GFP control, C/EBP α , or PU.1, as confirmed by RT-PCR (Fig. 5B) and Western blotting (Fig. 5C), were stimulated with M-CSF plus α -FAS to promote osteoclast differentiation (Fig. 5D). These osteoclastogenic assays utilized 10 ng/ml and 100 ng/ml of α -FAS which were validated in previous studies to promote osteoclast differentiation with permissive and optimum activation of the chimeric receptor system, respectively (9,25,27,28,40). As expected, treatment of BMMs doubly expressing FAS-RANK plus the GFP control, C/EBP α , or PU.1 with 10 ng/ml or 100 ng/ml of α -FAS in the presence of M-CSF generated numerous osteoclasts (Fig. 5D). Consistently, we noted that the cells doubly expressing the FAS-RANK and PU.1 generated significantly more osteoclasts than the FAS-RANK and C/EBP α double expressers under both the permissive and optimum α -FAS stimulation (Fig. 5E). However, treatment of BMMs doubly expressing FAS-mIVVY plus the GFP control, C/EBP α , or PU.1 with the permissive and optimum doses of α -FAS in the presence of M-CSF formed no osteoclast (Fig. 5D,E), indicating that C/EBP α or PU.1 overexpression could not rescue osteoclastogenesis from the mutational inactivation of RANK IVVY motif. Moreover, similarly to the RANKL-induced osteoclast differentiation (Fig. 3), PU.1 overexpression did not influence the osteoclast size as compared with C/EBP α or the GFP control under stimulation by optimum α -FAS levels (Fig. 5F,G), further confirming that PU.1 and C/EBP α might not regulate osteoclast size. Collectively, the results indicated that mutational inactivation of the RANK IVVY motif blocked osteoclast differentiation, and overexpression of C/EBP α or PU.1 could not rescue osteoclast differentiation from the mutational inactivation of the RANK IVVY motif.

3.5. Inactivation of the RANK cytoplasmic IVVY motif triggers RBP-J upregulation but exerts no significant effect on the expression of interferon regulatory factor 8 (IRF-8).

While C/EBP α or *PU.1* overexpression could initiate osteoclastogenesis independently of RANKL (Fig. 1) and promote osteoclast differentiation from pre-committed BMMs (Fig. 3), C/EBP α or PU.1 overexpression failed to mediate osteoclast differentiation with inactivation of the RANK IVVY motif (Fig. 5). In our attempt to understand this discrepancy, we found that IVVY-motif inactivation led to significantly lower expressions of *Ctsk* and *TRAP* as compared with a GFP control (Fig. 4G). This observation suggested that the RANK IVVY motif, besides positively regulating the expressions of osteoclast genes, might also negatively regulate the expressions of osteoclast inhibitors during osteoclastogenesis. This is consistent with the notion that deregulation of potent negative regulators of osteoclastogenesis from the inactivation of the IVVY motif might negatively affect the ability of C/EBP α or *PU.1* to promote osteoclast differentiation in cells expressing FAS-mIVVY.

Among the factors that can potentially inhibit osteoclastogenesis, IRF-8 and RBP-J have been the most studied (41–47). Hence, we examined the role of RANK IVVY motif in regulating

the expressions of *IRF-8* and *RBP-J* during osteoclast differentiation. Mutational inactivation of RANK IVVY motif showed no significant effect on *IRF-8* expression (Fig. 6A), but led to a significant increase in *RBP-J* expression (Fig. 6B), indicating that this RANK motif could negatively regulate *RBP-J* expression during osteoclastogenesis. It was recently shown that RBP-J inhibits osteoclastogenesis by suppressing the immunoreceptor tyrosine-based activation motif (ITAM)-associated receptor costimulatory signaling, which is critical for induction of osteoclast genes during osteoclast differentiation (48,49). We showed that overexpression of *C/EBP α* or *PU.1* was unable to control the deregulated *RBP-J* expression from the inactivation of RANK IVVY motif as compared with normal RANK (Fig. 6C,D). To exclude the contribution of RANK signaling in regulating *RBP-J* expression upon the activation of the chimeric receptor system, we demonstrated that *C/EBP α* or *PU.1* overexpression in BMMs not expressing the chimeric receptors exhibited no over effect on *RBP-J* expression (Suppl. Fig. 2). In addressing the molecular basis of the failure of *C/EBP α* or *PU.1* overexpression to mediate osteoclast differentiation in the mutant cells, we revealed that *C/EBP α* or *PU.1* overexpression in the FAS-mIVVY expressers significantly repressed *NFATc1* (Fig. 6C,E), *Ctsk* (Fig. 6C,F), and *TRAP* (Fig. 6C,G) during osteoclast differentiation. Our data mimicked the reported role of RBP-J in repressing osteoclast genes during osteoclastogenesis (41). The results indicated that the inability of *C/EBP α* or *PU.1* overexpression to rescue osteoclast differentiation in the context of RANK-IVVY-motif inactivation was due in part to the deregulated *RBP-J* expression, which negatively affected osteoclast gene expression.

3.6. RBP-J silencing rescues osteoclastogenesis in cells expressing the mutated RANK IVVY motif.

Finally, we investigated whether *RBP-J* silencing in BMMs expressing the mutated RANK IVVY motif could mediate osteoclastogenesis with *C/EBP α* or *PU.1* overexpression (Fig. 7). We were able to knockdown the *RBP-J* gene efficiently in BMMs using a shRNA construct that was purchased from Sigma as accessed by qPCR (Fig. 7A) and Western blotting (Fig. 7B). *RBP-J* silencing significantly enhanced RANKL-induced osteoclastogenesis (Fig. 7C,D), confirming the established role of RBP-J as a strong inhibitor of osteoclast differentiation (50) (41). Next, in investigating whether *RBP-J* silencing could rescue osteoclastogenesis in cells doubly expressing the mutated RANK IVVY motif plus *C/EBP α* or *PU.1*, BMMs were first infected with a lentivirus encoding the *RBP-J* shRNA construct or a Scramble control to silence the *RBP-J* gene before being infected with a mixture of retrovirus encoding FAS-RANK or FAS-mIVVY plus FLAG-*C/EBP α* , FLAG-*PU.1*, or a GFP control (Fig. 7E,F). The data showed that *RBP-J* silencing in BMMs doubly expressing FAS-RANK plus *C/EBP α* , *PU.1*, or the GFP control significantly enhanced osteoclast differentiation as compared with cells triply expressing the Scramble control along with FAS-RANK plus FLAG-*C/EBP α* , FLAG-*PU.1*, or the GFP control (Fig. 7G,H). Notably, *RBP-J* silencing in the cells doubly expressing the mutated RANK IVVY motif plus the GFP control generated a few small TRAP-positive multinucleated cells as compared to the Scramble control cells doubly expressing the mutated RANK IVVY motif plus the GFP control (Fig. 7G,H). Notably, forced expression of *C/EBP α* or *PU.1* in the cells doubly expressing the *RBP-J* shRNA construct and the mutated RANK IVVY motif generated more osteoclasts than cells triply expressing the *RBP-J* shRNA construct, the Fas-

mIVVY, and the GFP control, but formed less osteoclasts than the cells triply expressing the RBP-J shRNA construct, normal RANK, and the transcription factors (Fig. 7G,H). Consistently, we noted that *RBPJ*-depleted BMMs doubly expressing Fas-RANK or Fas-mIVVY plus *PU.1* generated more osteoclasts than the *RBPJ*-depleted BMMs doubly Fas-RANK or Fas-mIVVY plus *C/EBPα* or the *GFP* control. (Fig. 7H). In examining the effects of *RBPJ*-silencing on the basal levels of *C/EBPα* and *PU.1*, we found that *RBPJ*-silencing showed no significant effect on *C/EBPα* or *PU.1* expression (Suppl. Fig. 3). Collectively, the results indicated that *RBPJ*-silencing alone was sufficient to initiate osteoclastogenesis in the cells expressing the mutated RANK IVVY motif, but *C/EBPα* or *PU.1* overexpression further enhanced osteoclast differentiation.

4. Discussion

RANK signaling triggers upregulation of the transcription factors PU.1, C/EBPα, c-Fos, and NFATc1, which are essential for osteoclast formation (6). Whereas PU.1 is long known to be essential for osteoclastogenesis (8) (22), the role of C/EBPα in osteoclastogenesis has only recently been documented (7,17). Importantly, PU.1 or C/EBPα can upregulate *c-Fos* and *NFATc1* during osteoclastogenesis, establishing C/EBPα and PU.1 as the earliest known osteoclast transcription factors (51). However, the mechanisms through which C/EBPα and PU.1 regulate osteoclast differentiation in response to RANK activation remain unknown. The current study sought to address this issue *in vitro*.

We confirmed that PU.1 and C/EBPα can mediate osteoclast lineage priming by inducing gene expression in a RANKL-independent manner (7). Notably, PU.1 can upregulate *C/EBPα*, but *C/EBPα* is unable to upregulate PU.1, establishing *C/EBPα* as a target gene of *PU.1* during osteoclastogenesis. Our finding agrees with another study identifying *C/EBPα* as a target gene of *PU.1* in granulocyte (52), but disagrees with other reports that *C/EBPα* can upregulate *PU.1* during early myeloid cell fate decision (53,54). Nonetheless, we found that while C/EBPα and PU.1 could similarly initiate osteoclastogenesis independently of RANKL, *PU.1* exhibited a stronger ability in promoting osteoclast differentiation than *C/EBPα* in pre-committed BMMs. Given that *PU.1* and *C/EBPα* show similar abilities in inducing osteoclast genes, the stronger ability of *PU.1* in mediating osteoclast differentiation than C/EBPα may not stem from its ability to upregulate *C/EBPα*. We believe that this may instead result from the ability of PU.1 or C/EBPα to induce different sets of genes, besides a common set of genes, during osteoclastogenesis. Our notion is underscored by a recent study demonstrating that C/EBPα and PU.1 exhibit distinct responses in the human acute leukemia HL-60 and NB4 cell lines (55).

In comparing the roles of C/EBPα and PU.1 in osteoclast differentiation, we examined their response to RANK signaling from activation of the IVVY motif which is essential for osteoclastogenesis (9–11) (56). We showed that RANK IVVY motif upregulates *C/EBPα* but is dispensable to *PU.1* induction, indicating that C/EBPα and PU.1 respond differently to RANK-IVVY-induced signaling during osteoclastogenesis. The fact that *C/EBPα* is repressed with the inactivation of RANK IVVY motif despite normal *PU.1* expression indicated that PU.1 may function with other unknown factors that are regulated by the RANK IVVY motif to upregulate C/EBPα during osteoclast differentiation. Nonetheless,

we speculate that a different region within the RANK cytoplasmic domain is responsible for PU.1 upregulation during osteoclastogenesis. Studies have shown that RANK transduces two types of signaling pathways emanating from the IVVY motif, a TNF receptor associated factor (TRAF) independent site, and its TRAF-binding sites (9,27,57). RANK has three functional TRAF-binding motifs (369PFQEP373, 559PVQEET564, and 604PVQEQQ609) that are as essential as the IVVY motif for osteoclastogenesis (57). Importantly, it was reported that the TRAF-binding sites and IVVY motif of RANK do not function independently but cooperate in mediating osteoclastogenesis (27). We believe that the TRAF-binding sites of RANK are likely to regulate PU.1 during osteoclastogenesis. Notably, the fact that RANK IVVY-motif inactivation fails to mediate osteoclastogenesis despite normal *PU.1* expression indicates that the convergence of the IVVY and TRAF signaling pathways to induce a common set of genes (e.g *Ctsk* and *TRAP*) as well as the unique *C/EBP α* and PU.1 target genes is essential for osteoclast differentiation.

In further characterizing the influence of RANK-IVVY signaling on osteoclast differentiation, we hypothesized that *C/EBP α* , unlike PU.1, overexpression might rescue osteoclastogenesis from the IVVY-motif inactivation. We reasoned that *C/EBP α* overexpression in the context of RANK IVVY-motif inactivation should rescue the *C/EBP α* target genes in the presence of the PU.1 target genes to promote osteoclastogenesis. Consistently, PU.1 overexpression in cells expressing the mutated IVVY motif formed no osteoclast, indicating that repression of the *C/EBP α* target genes from IVVY-motif inactivation which caused *C/EBP α* downregulation impeded osteoclastogenesis despite *PU.1* overexpression. However, *C/EBP α* overexpression in the mutant cells also formed no osteoclast despite normal *PU.1* expression. In elucidating this discrepancy, we showed that *PU.1* or *C/EBP α* overexpression failed to induce osteoclast genes in the context of RANK-IVVY-motif inactivation. We noted that RANK IVVY motif inactivation caused significantly lower *Ctsk* and *TRAP* expressions as compared with a vector control, suggesting that the RANK IVVY motif might also promote osteoclast differentiation by down-regulating osteoclast inhibitors. This assumption goes with the idea that RANK IVVY motif inactivation can also trigger upregulation of potent osteoclast inhibitors which affects the ability of *C/EBP α* or PU.1 to promote osteoclastogenesis.

We later revealed that RANK-IVVY-motif inactivation triggered *RBP-J* upregulation, a potent osteoclast inhibitor. Mechanistically, we demonstrated that *C/EBP α* or *PU.1* overexpression failed to control the deregulated *RBP-J* expression from the IVVY-motif inactivation, leading to repression of osteoclast genes and inhibition of osteoclast differentiation. Osteoclastogenesis requires two essential cross-talk signaling induced by RANK activation and the ITAM-associated receptors (*DAP12* and *FcR γ*) (48,49,58). Our finding is consistent with a recent study indicating that *RBP-J* suppresses the ITAM-mediated costimulatory signaling and limits the cross-talk between the ITAM and RANK signaling during osteoclastogenesis. Notably, *RBP-J* silencing partially rescues osteoclastogenesis from the inactivation of the IVVY motif. Whereas the IVVY motif can upregulate *C/EBP α* , but not PU.1, and downregulates *RBP-J*, *C/EBP α* or PU.1 overexpression only partially rescues osteoclast differentiation in *RBP-J* depleted cells expressing the mutated IVVY motif. This finding indicates that our understanding of the

mechanism by which the IVVY motif mediates osteoclastogenesis remains incomplete (9–11).

On the basis of our findings, we propose a working model to, in conjunction with other studies (8,9,27,33), summarize the roles of transcription factors and RANK signaling in osteoclast differentiation (Fig. 8). The model expands on the findings of a recent study that has demonstrated that the IVVY motif and TRAF-binding sites of RANK are equally important for osteoclastogenesis (27). Hence, RANKL-induced activation of the TRAF-independent IVVY signaling pathway upregulates C/EBP α , which subsequently activates transcription factors (e.g. c-Fos and NFATc1) for induction of osteoclast genes (e. g. *Ctsk* and *TRAP*) and osteoclast differentiation (33,34). Unlike C/EBP α , PU.1 is likely to be regulated by the RANK TRAF-binding sites during osteoclastogenesis. PU.1 can then upregulate C/EBP α , c-Fos, and NFATc1 expressions to induce osteoclast genes and thereby promote osteoclastogenesis. However, we believe that C/EBP α is mainly regulated by the RANK IVVY motif during osteoclast differentiation. In the light of various studies reporting that transcription factors can function in complex with other factors to regulate gene expression for osteoclastogenesis (22,23,59), we anticipate that PU.1 and C/EBP α may induce a common set of osteoclast genes, including *Ctsk*, *TRAP*, and *NFATc1*. Moreover, C/EBP α and PU.1 may also induce a different set of genes that are also critical for osteoclastogenesis. Finally, the IVVY motif can negatively regulate the osteoclast inhibitor RBP-J in mediating osteoclast differentiation.

In conclusion, whereas PU.1 can upregulate C/EBP α during the lineage commitment and exhibits a stronger osteoclastogenic potential than C/EBP α , the ability of PU.1 to upregulate C/EBP α may not be the primary factor responsible for its stronger ability in mediating osteoclast differentiation than C/EBP α . C/EBP α and PU.1 display different responses to RANKL/RANK signaling through activation of the IVVY motif. The IVVY motif mediates osteoclastogenesis by positively regulating osteoclast activators and negatively regulating osteoclast inhibitors. Our study provides an important insight into the mechanism underlying the responses of transcription factors to RANK signaling during osteoclastogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

BMMs	bone marrow macrophages
C/EBPα	CCAAT/enhancer binding protein-alpha
Ctsk	cathepsin K

FAS-RANK	a chimeric receptor with the Human FAS external domain linked to the normal mouse RANK transmembrane and cytoplasmic domains
FAS-mIVVY	a chimeric receptor with the Human FAS external domain linked to the mouse RANK transmembrane and cytoplasmic domains bearing an inactivating mutation in the IVVY motif
ITAM	immunoreceptor tyrosine-based activation motif
IRF-8	interferon regulatory factor 8
M-CSF	macrophage colony-stimulating factor
NFATc1	nuclear factor of activated T-cells, C1
RANK	receptor activator of NF- κ B
RANKL	receptor activator of NF- κ B ligand
RBP-J	recombinant recognition sequence binding protein at the J κ site
TRAP	tartrate-resistant acid phosphatase 5
TRAF	TNF receptor associated factor

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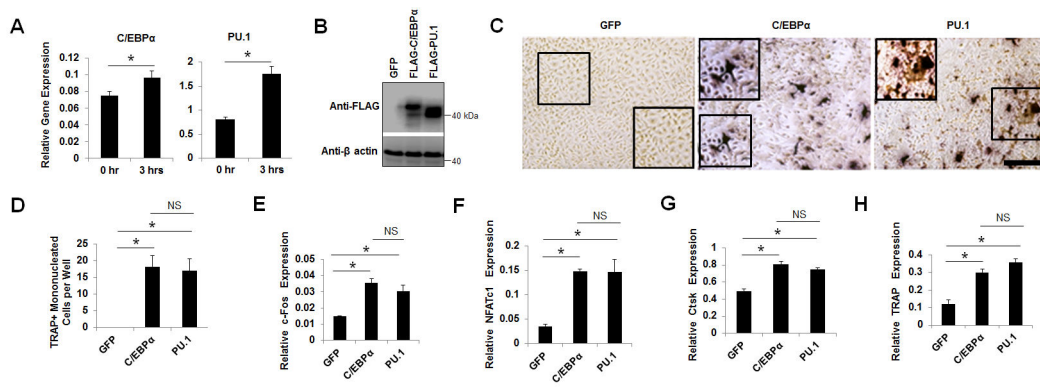


Fig. 1. C/EBPα or PU.1 overexpression can initiate osteoclastogenesis independently of RANKL. A, analysis of *C/EBPα* and *PU.1* expression in BMMs stimulated by M-CSF and RANKL for 0 hour or 3 hours by qPCR. B, analysis of gene overexpression in BMMs expressing a GFP control, FLAG-C/EBPα, or FLAG-PU.1 cultured with M-CSF for 4 days by Western blotting. C-H, BMMs expressing GFP, FLAG-C/EBPα (C/EBPα), or FLAG-PU.1 (PU.1) were cultured with M-CSF for 4 days. Some cells were submitted to TRAP staining to examine osteoclastogenesis (C) and then quantified for the number of TRAP-positive mononucleated cells (D). Scale bar = 250 μm. The remaining cells were subjected to qPCR analysis for *c-Fos* (E), *NFATc1* (F), *Ctsk* (G), or *TRAP* expression (H). Error bars show averages ± S.D. *, $p < 0.05$; NS, not significant..

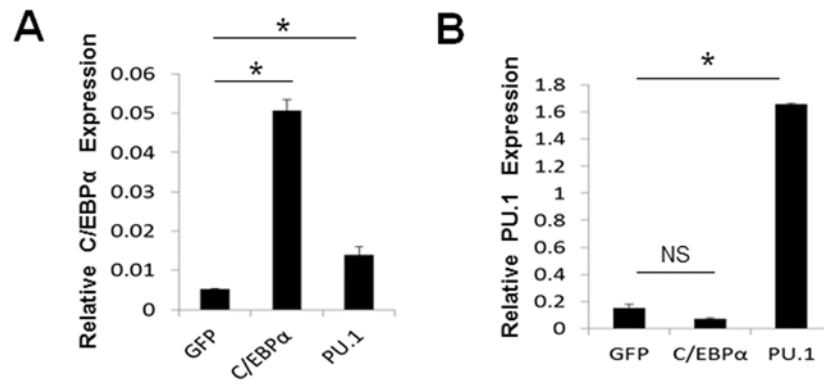


Fig. 2. Analysis of the effects of C/EBPα or PU.1 in inducing the expressions of each other. A, analysis of *C/EBPα* expression in BMMs expressing a GFP control (GFP), FLAG-C/EBPα (C/EBPα), or FLAG-PU.1 (PU.1) cultured with M-CSF for 4 days by qPCR. B, analysis of *PU.1* expression in BMMs expressing GFP, C/EBPα, or PU.1 cultured with M-CSF for 4 days. Error bars show averages \pm S.D. *, $p < 0.05$. NS, not significant.

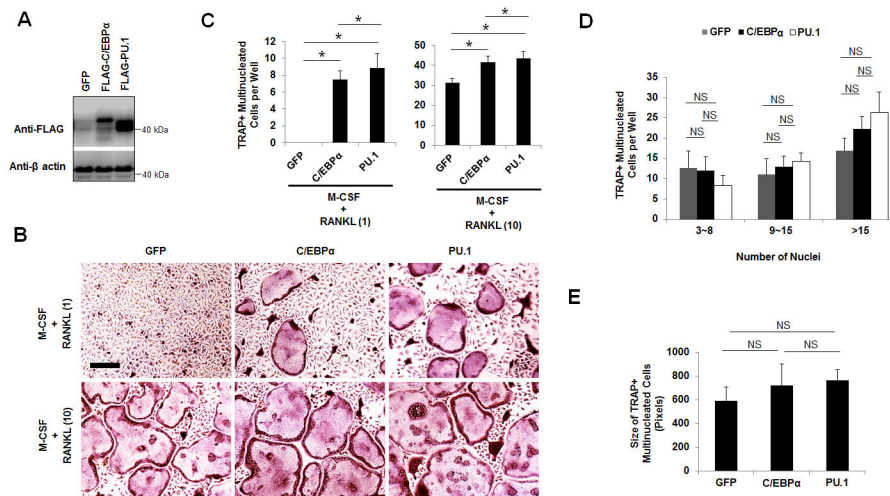


Fig. 3. PU.1 overexpression generates more osteoclasts than C/EBPα overexpression from pre-committed BMMs. *A*, gene expression analysis from BMMs expressing a GFP control (GFP), FLAG-C/EBPα, or FLAG-PU.1 stimulated by M-CSF for 4 days by Western blotting. *B*, TRAP staining for osteoclast differentiation from BMMs expressing GFP, FLAG-C/EBPα (C/EBPα), or FLAG-PU.1 (PU.1) stimulated by M-CSF plus RANKL for 4 days. Scale bars = 200 μm. *C*, quantifications for *B* are shown. *D* and *E*, quantifications of the osteoclast size for *B* via the number of nuclei (*D*) and area (*E*) of TRAP-positive multinucleated cells. The numbers in parentheses show concentrations in nanograms per milliliter. Error bars show averages ± S.D. *, $p < 0.05$; NS, not significant.

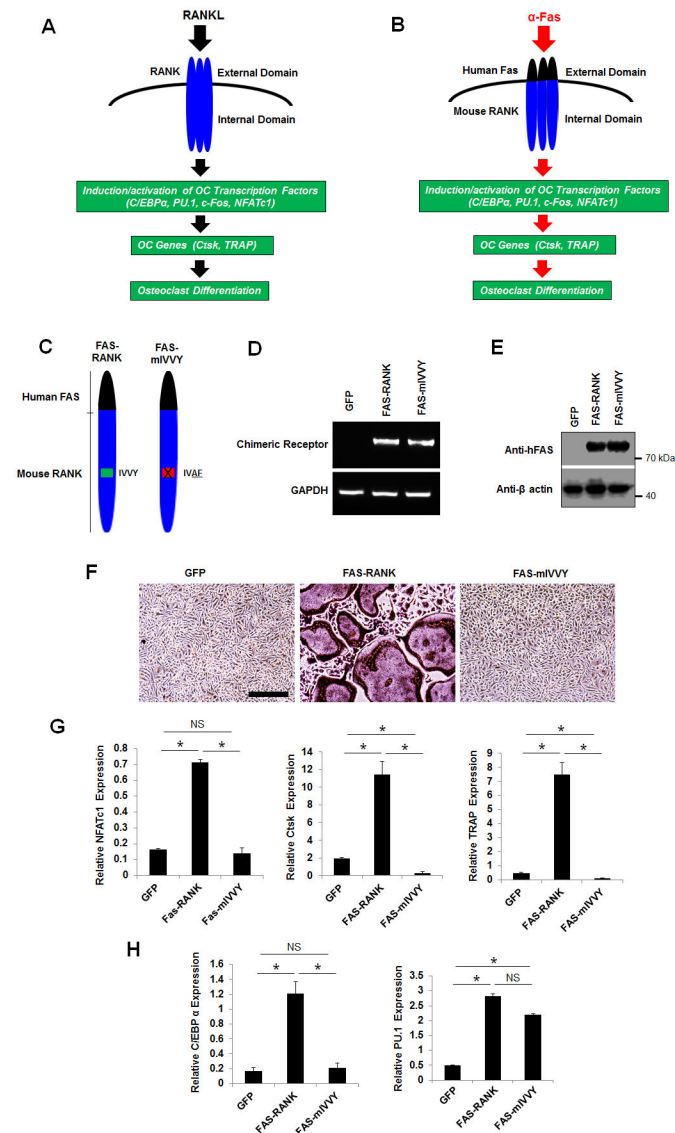


Fig. 4. Mutational inactivation of the RANK cytoplasmic 535IVVY538 motif blocks osteoclast differentiation and attenuates *C/EBP α* , but not *PU.1*, expression. **A**, a schematic of RANK activation by RANKL to induce osteoclast differentiation. **B**, a schematic of the chimeric receptor system which can be activated by α -FAS to promote osteoclast differentiation. **C**, schematics of FAS-RANK and FAS-mIVVY. **D** and **E**, analysis of the expressions of the chimeras in BMMs expressing a GFP control (GFP), FAS-RANK, or FAS-mIVVY cultured with M-CSF for 4 days by RT-PCR (**D**) or Western blotting (**E**). **F**, TRAP staining for osteoclast differentiation from BMMs expressing GFP, FAS-RANK, or FAS-mIVVY treated with M-CSF plus α -FAS (100 ng/ml) for 4 days. Scale bar = 250 μ m. **G** and **H**, analysis of osteoclast genes from BMMs expressing GFP, FAS-RANK or FAS-mIVVY treated with M-CSF plus α -FAS (100 ng/ml) for 2 days by qPCR. Error bars show averages \pm S.D. *, p < 0.05; NS, not significant.

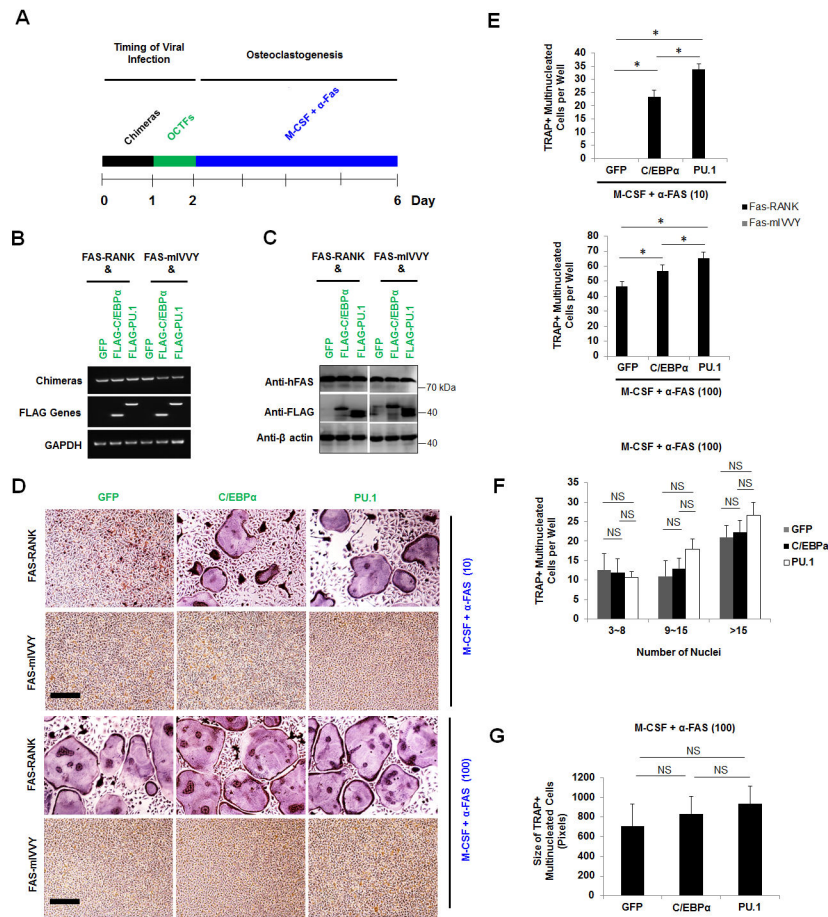


Fig. 5. C/EBP α or PU.1 overexpression fails to mediate osteoclast differentiation with the mutational inactivation of the RANK cytoplasmic 535IVVY538 motif. **A**, a schematic of the experimental strategy. **B** and **C**, gene expression analysis from BMMs doubly expressing FAS-RANK or FAS-mIVVY plus a GFP control (GFP), FLAG-C/EBP α (C/EBP α), or FLAG-PU.1 (PU.1) cultured with M-CSF for 4 days by RT-PCR (**B**) and Western blotting (**C**). **D**, TRAP staining for osteoclast differentiation from BMMs doubly expressing FAS-RANK or FAS-mIVVY plus GFP, C/EBP α , or PU.1 treated with M-CSF and α -FAS for 4 days. Scale bar = 200 μ m. **E**, quantifications for **D** are shown. **F** and **G**, quantifications of the osteoclast size for **D** via the number of nuclei (**F**) and area (**G**) of TRAP-positive multinucleated osteoclasts from FAS-RANK expressers treated with M-CSF and α -FAS (100 ng/ml). The numbers in parentheses show concentrations in nanograms per milliliter. Error bars show averages \pm S.D. *, $p < 0.05$; NS, not significant.

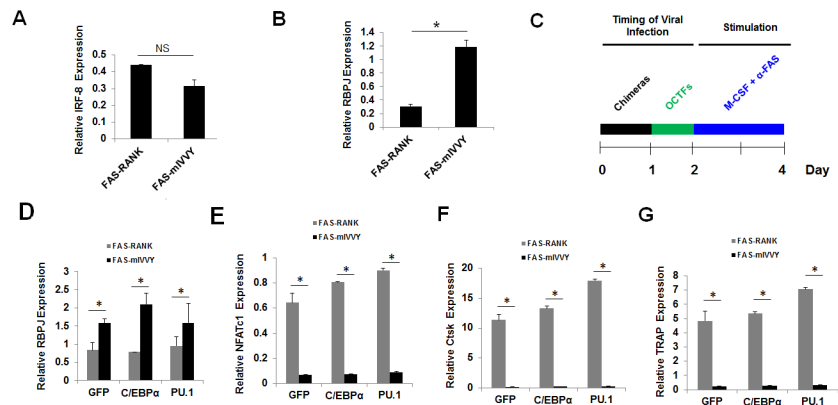


Fig. 6. Mutational Inactivation of the RANK cytoplasmic 535IVVY538 motif triggers *RBP-J* upregulation but represses osteoclast genes. **A** and **B**, analysis of the expressions of *IRF-8* (**A**) and *RBP-J* (**B**) from BMMs expressing FAS-RANK or FAS-mIVVY stimulated by M-CSF plus α-FAS (100 ng/ml) for 2 days by qPCR. **C**, a schematic of the experimental strategy for **D-G**. **D-G**, BMMs doubly expressing FAS-RANK or FAS-mIVVY plus a GFP control (GFP), FLAG-C/EBPα (C/EBPα), or FLAG-PU.1 (PU.1) were cultured with M-CSF and α-FAS (100 ng/ml) for analyses of the expressions of the osteoclast inhibitor *RBP-J* (**D**) and the osteoclast genes *NFATc1* (**E**), *Ctsk* (**F**) and *TRAP* (**G**) by qPCR. Error bars show averages ± S.D. *, p < 0.05; NS, not significant.

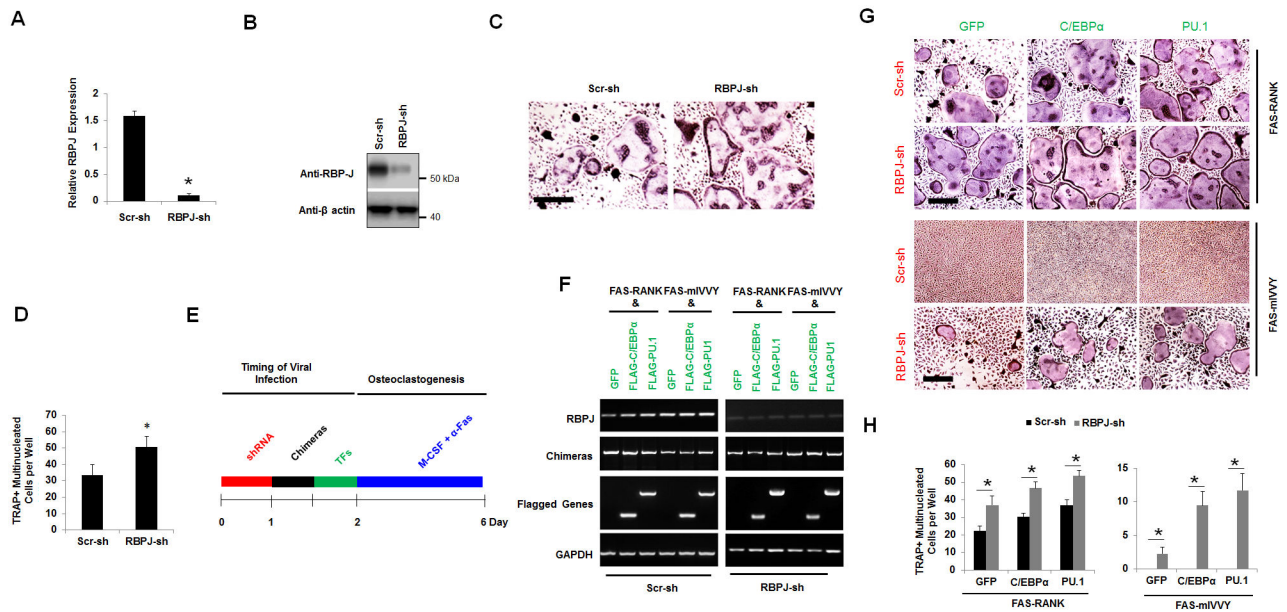


Fig. 7. *RBP-J* silencing rescues osteoclastogenesis from inactivation of the RANK cytoplasmic 535IVVY538 motif. **A** and **B**, analysis of *RBP-J* knockdown in BMMs expressing a Scramble shRNA control (Scr-sh) or *RBP-J* shRNA (RBPJ-sh) construct cultured with M-CSF for 4 days by qPCR (**A**) and Western blotting (**B**). **C**, TRAP staining for osteoclast differentiation from BMMs expressing Scr-sh or RBPJ-sh treated with M-CSF and RANKL (10 ng/ml) for 4 days. Scale bar = 250 μm. **D**, quantification for **C** is shown. **E**, a schematic for the experimental strategy for **F-H**. **F**, analysis of gene expression from BMMs triply expressing the shRNA constructs (*RBPJ*-shRNA or Scr-sh), the chimeric receptor system (FAS-RANK or FAS-miVYV), and the GFP control (GFP), FLAG-C/EBPα (C/EBPα), or FLAG-PU.1 (PU.1) treated with M-CSF for 4 days by RT-PCR. **G**, TRAP staining for osteoclast differentiation from BMMs triply expressers as in **F** treated with M-CSF and α-FAS (100 ng/ml) for 4 days. Scale bar = 200 μm. **H**, quantifications for **G** are shown. Error bars show averages ± S.D. *, $p < 0.05$.

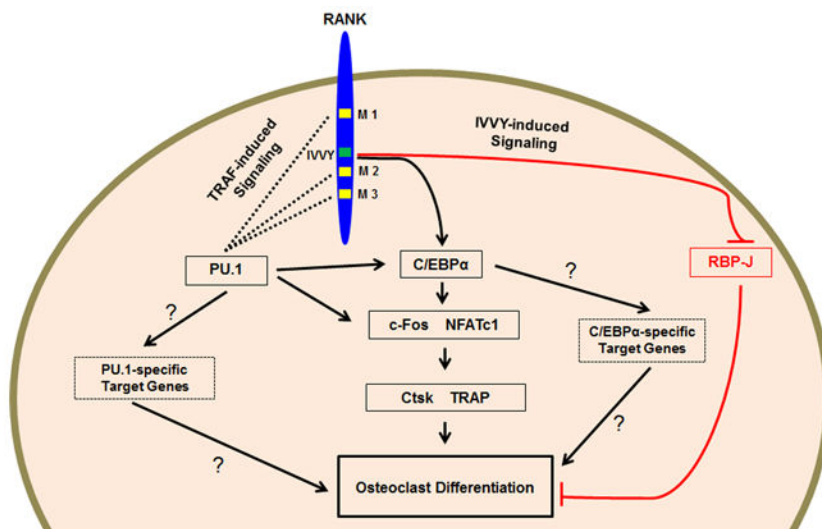


Fig. 8. Proposed working model. RANK emits two types of signaling pathways from the IVVY motif and TRAF-binding sites that are equally important for osteoclastogenesis through activation of transcription factors for induction of osteoclast genes. RANK has three functional sites, 369PFQEP373 (M1), 559PVQEET564 (M2), and 604PVQEQG609 (M3), that can recruit TRAFs to transduce osteoclastogenic signaling. Moreover, RANK has a TRAF-independent 535IVVY538 motif (IVVY) that can also transduce signaling for osteoclastogenesis. Furthermore, osteoclastogenesis is likely to require two different but unique sets of genes that are specifically regulated by C/EBPα and PU.1, respectively. The question marks indicate unknown mechanisms.