BMB
Reports

BMB Rep. 2021; 54(9): 482-487
www.bmbreports.org

IRF2 enhances RANKL-induced osteoclast differentiation via regulating NF-κB/NFATc1 signaling

Inyoung Kim[1], Jung Ha Kim[1], Kabsun Kim[1], Semun Seong[1], Keun-Bae Lee[2] & Nacksung Kim[1,]*

[1]Department of Pharmacology, Chonnam National University Medical School, Gwangju 61469, [2]Department of Orthopedic Surgery, Chonnam National University Medical School and Hospital, Gwangju 61469, Korea

Interferon regulatory factors (IRFs) play roles in various biological processes including cytokine signaling, cell growth regulation and hematopoietic development. Although it has been reported that several IRFs are involved in bone metabolism, the role of IRF2 in bone cells has not been elucidated. Here, we investigated the involvement of IRF2 in RANKL-induced osteoclast differentiation. IRF2 overexpression in osteoclast precursor cells enhanced osteoclast differentiation by regulating the expression of NFATc1, a master regulator of osteoclastogenesis. Conversely, IRF2 knockdown inhibited osteoclast differentiation and decreased the NFATc1 expression. Moreover, IRF2 increased the translocation of NF-κB subunit p65 to the nucleus in response to RANKL and subsequently induced the expression of NFATc1. IRF2 plays an important role in RANKL-induced osteoclast differentiation by regulating NF-κB/NFATc1 signaling pathway. Taken together, we demonstrated the molecular mechanism of IRF2 in osteoclast differentiation, and provide a molecular basis for potential therapeutic targets for the treatment of bone diseases characterized by excessive bone resorption. [BMB Reports 2021; 54(9): 482-487]

INTRODUCTION

Differentiation of osteoclasts is induced by macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL). In bone, RANKL binds to its receptor RANK and activates downstream signaling pathways such as NF-κB, p38, c-Jun N-terminal kinase (JNK), and extracellular signal-related kinase (ERK) (1). Diverse transcription factors are activated by RANKL, including c-Fos and nuclear factor-activated T cells c1 (NFATc1), which play major roles in osteoclastogenesis (2). Activated NFATc1-dependent transcription process acts as a master switch to regulate the downstream target genes such as osteoclast-associated receptor (OSCAR), cathepsin K, and tatrate-resistant acid phosphatase (TRAP) (3). Embryonic stem cells deficient with NFATc1 cannot be differentiated into osteoclasts upon RANKL stimulation (4). However, overexpression of NFATc1 in osteoclast precursors efficiently induces differentiation into osteoclasts even without RANKL signaling (2). As clearly demonstrated *in vitro* and *in vivo*, NFATc1 is an essential element for osteoclast differentiation.

NF-κB and c-Fos regulate expression of NFATc1 through binding to the NFATc1 promoter during osteoclast differentiation (5). NF-κB is a group of transcription factors consisting of RelA (p65), NF-κB1 (p50), NF-κB2 (p52), RelB, and c-Rel. RANKL activates canonical or non-canonical NF-κB signaling in pre-osteoclasts and osteoclasts. In the canonical pathway, inhibitor of κB (IκB) is phosphorylated and degraded by IκB kinase (IKK) complex. Proteasomal degradation of IκB then activates p50/p65 complex (6-8). In the non-canonical pathway, NF-κB-inducing kinase (NIK) and IKKα are phosphorylated and produce p52, processed from p100 by proteasome, and activate the p52/RelB complex (7, 9). NF-κB1/2 double knockout mice did not form osteoclast, resulting in an osteopetrosis phenotype (10). Inhibition of p65 nuclear translocation suppresses osteoclastogenesis *in vitro* (11). Bone marrow macrophages (BMMs) lacking RelB does not induce osteoclast formation upon RANKL stimulation (12). Furthermore, RANKL stimulation recruits the p50 and p65 subunits of NF-κB to the promoter of NFATc1, indicating that NF-κB is crucial for the early induction of NFATc1 (4).

Interferon regulatory factors (IRFs) are transcription factors, consisting of nine members (IRF1-9), generally contain a conserved N-terminal DNA-binding region and a C-terminal regulatory region (13). They play critical roles in immune cell development, differentiation, and responses to pathogens (13). IRF1 and IRF2 are identified in the late 1980s as transcription factors that regulate the interferon (IFN)-α/β gene (14). Although these two IRF proteins share a significant level of homology within the DNA binding domain, IRF2 is generally described as a transcription repressor because of its competition with the transcription activator IRF1 (15). However, IRF2 also acts as an activator for several genes such as the cell cycle-regulated histone H4, vascular adhesion molecular-1 (VCAM-1), and gp91phox (16-18). Among the IRF family, IRF1, IRF4, and IRF8 are reported

*Corresponding author. Tel: +82-61-379-2835; Fax: +82-61-373-6974; E-mail: nacksung@jnu.ac.kr

https://doi.org/10.5483/BMBRep.2021.54.9.070

Received 31 May 2021, Revised 22 June 2021, Accepted 19 August 2021

Keywords: IRF2, NFATc1, NF-κB, Osteoclast

ISSN: 1976-670X (electronic edition)

to be involved in bone metabolism. In IRF1 KO mice, osteoclast activity and bone resorption are enhanced (19). IRF8 blocks osteoclast differentiation by inhibiting transcriptional activity and expression of NFATc1, and IRF8 knockout mice exhibited severe osteoporosis due to increased osteoclast formation (20). In contrast, RANKL induces IRF4 expression in the nucleus, after which IRF4 accelerates the induction of NFATc1 by cooperating with NFATc2 and NF-κB within the promoter of NFATc1 (21). It has been reported that IRF2 recruits NF-κB subunit p65 into nucleus through physical interaction, and increases TNFα-dependent NF-κB transcription (22). These results postulated that IRF2 may modulate osteoclast differentiation through regulating NF-κB.

In this study, we explored the role of IRF2 in osteoclast differentiation induced by RANKL. Overexpression of IRF2 increased osteoclast differentiation, whereas downregulation of IRF2 using siRNA inhibited osteoclastogenesis. Our data revealed that IRF2 is associated with RANKL-induced osteoclastogenesis by mediating NF-κB-NFATc1 signaling pathway.

RESULTS

IRF2 overexpression enhances RANKL-mediated osteoclast differentiation

First, we examined the expression pattern of IRF2 during osteoclast differentiation. IRF2 was less expressed in the first day in BMMs, but gradually increased in the second and the third days during osteoclast differentiation (Fig. 1A). At this time, RANKL induced the expression of c-Fos, NFATc1, and TRAP during osteoclast differentiation (Fig. 1A).

Next, we overexpressed IRF2 using retroviral transduction to investigate the role of IRF2 in osteoclast differentiation. Interestingly, the number of TRAP-positive multinucleated osteoclasts was significantly increased upon IRF2 overexpression (Fig. 1B). We then examined whether IRF2 influences the expression of c-Fos, NFATc1, and TRAP, genes important for osteoclast formation. IRF2 overexpression strongly increased mRNA levels of NFATc1 and TRAP, but did not affect mRNA expression of c-Fos (Fig 1C). In addition, overexpression of IRF2 did not affect the protein level of c-Fos, while the amount of protein expression of NFATc1 was greatly increased (Fig. 1D). These data showed that IRF2 regulates RANKL-induced osteoclastogenesis by increasing NFATc1 expression, a master transcription factor of osteoclast differentiation.

IRF2 downregulation inhibits RANKL-mediated osteoclast differentiation

We confirmed the role of IRF2 in osteoclast differentiation by knockdown of IRF2 using siRNA. First, we examined whether IRF2-specific siRNA downregulates the expression of IRF2. The mRNA level of IRF2 was significantly decreased by transfection of IRF2 siRNA in BMMs compared to the control siRNA (Fig. 2A). Next, we examined the effect of IRF2 knockdown on RANKL-stimulated osteoclast formation. As expected, RANKL-induced osteoclast formation was significantly attenuated by IRF2 siRNA (Fig. 2B). Furthermore, IRF2 knockdown strongly reduced the mRNA levels of NFATc1 and TRAP, although there was no difference in c-Fos mRNA level compared to the control siRNA (Fig. 2C). In addition, when IRF2 expression was suppressed, the expression of c-Fos protein was comparable, but NFATc1 protein expression was greatly reduced (Fig. 2D). Collectively, these results indicated that IRF2 positively regulates osteoclast differentiation by regulating NFATc1 expression.

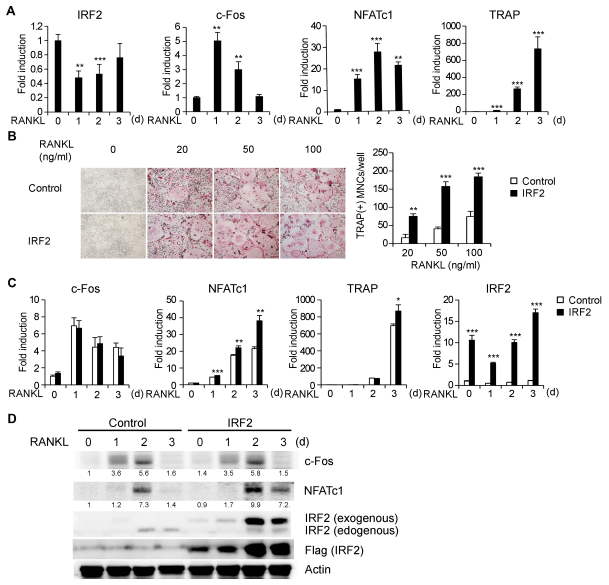

Fig. 1. Overexpression of IRF2 enhances RANKL-induced osteoclast differentiation. (A) BMMs were cultured in the presence of M-CSF and RANKL for the indicated days. Total RNA was collected and quantitative real-time PCR was performed to assess the expression of the indicated genes. (B-D) BMMs isolated from ICR were transduced with the control (pMX-FIG) or IRF2 (pMX-IRF2-FIG) and induced for osteoclast differentiation with M-CSF and RANKL at the indicated concentration. (B) TRAP stain of the control or IRF2 transduced osteoclasts (left panel). Quantification of TRAP-positive MNCs (right panel). (C) The mRNA expression levels of the indicated genes were determined by quantitative real-time PCR. (D) Whole cell lysates were harvested and immunoblotted against the indicated antibodies. Relative intensities of the bands on each gel measured by densitometry are shown below each lane. The data are presented as the mean means ± SDs. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus the control.

IRF2 modulates osteoclast differentiation through NF-κB and NFATc1 signaling

We investigated the mechanism by which IRF2 regulates NFATc1 expression during RANKL-mediated osteoclast differentiation. It has been reported that IRF2 recruits p65 into the nucleus and regulates NF-κB activity upon TNF-α stimulation (4). NF-κB controls the expression of NFATc1, one of the important signaling molecules activated by RANKL (4, 23). First,

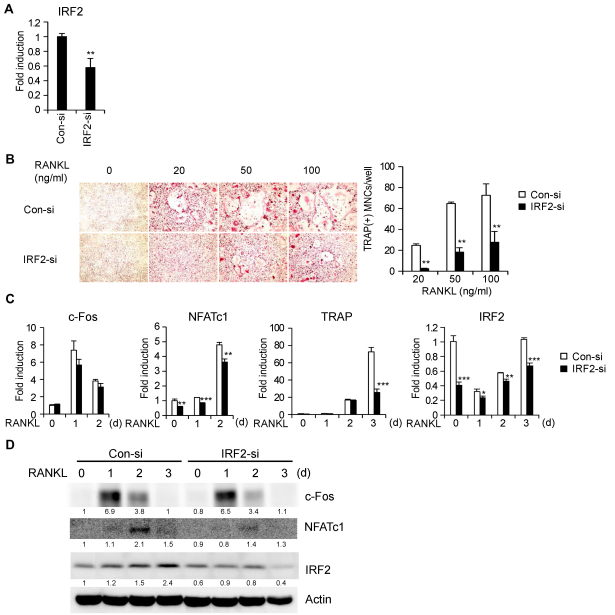

Fig. 2. Downregulation of IRF2 inhibits RANKL-induced osteoclast differentiation. (A) BMMs transfected with the control or IRF2 siRNA were cultured with M-CSF for 2 days. mRNA expression level of IRF2 was determined by quantitative real-time PCR. (B-D) BMMs transfected with the control or *Irf2* siRNA were differentiated to osteoclasts for the indicated days with various concentration of RANKL. (B) TRAP stain of the control or IRF2 siRNA transfected osteoclasts (left panel). Quantification of TRAP-positive MNCs (right panel). (C) The mRNA expression levels of the indicated genes were determined by quantitative real-time PCR. (D) Whole cell lysates were harvested and immunoblotted against the indicated antibodies. Relative intensities of the bands on each gel measured by densitometry are shown below each lane. The data are presented as the mean means ± SDs. **P < 0.01, and ***P < 0.001 versus the control.

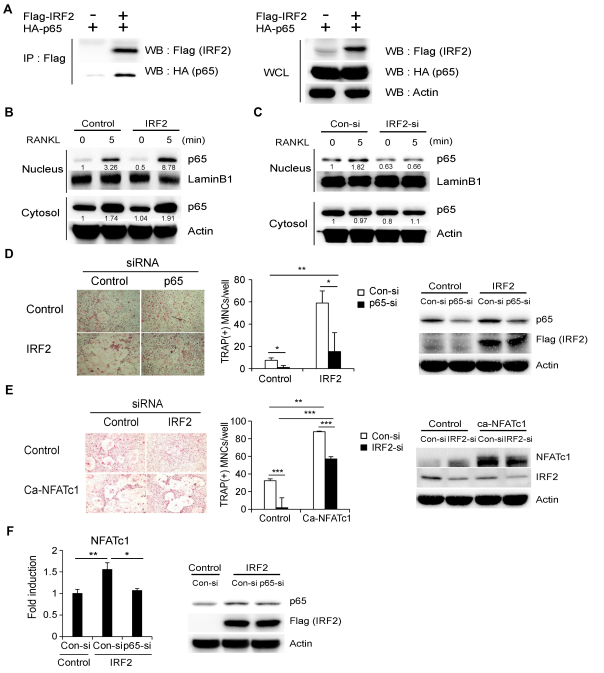

Fig. 3. IRF2 regulates osteoclast differentiation via increasing p65 nuclear translocation and NFATc1 expression. (A) 293T cells were transfected with HA-p65 with or without Flag-IRF2. Whole cell lysates were immunoprecipitated with a FLAG antibody and immunoblotted with the indicated antibodies. (B, C) Cytoplasmic fractions and nuclear fractions were harvested from cultured cells and immunoblotted with the indicated antibodies. Antibodies for actin and lamin B1 were used for the normalization of cytoplasmic and nuclear extracts, respectively. Relative intensities of the bands on each gel measured by densitometry are shown below each lane. (B) BMMs transduced with the control or IRF2 were serum starved for 6 hours followed by RANKL stimulation at the indicated times. (C) BMMs transfected with the control or Irf2 siRNA were serum starved for 6 hours followed by RANKL stimulation at the indicated times. (D) BMMs transduced with the control or IRF2 followed by transfection with the control or p65 siRNA were cultured with M-CSF and RANKL to differentiate into osteoclast. TRAP stain of the control or IRF2 followed by transfection with the control or p65 siRNA osteoclasts (left panel). Quantification of TRAP-positive MNCs (middle panel). Whole cell lysates were harvested and immunoblotted against the indicated antibodies (right panel). (E) BMMs transduced with the control or Ca-NFATc1 followed by transfection with the control or IRF2 siRNA were cultured with M-CSF and RANKL to differentiate into osteoclast. TRAP stain of BMMs transduced with the control or Ca-NFATc1 followed by transfection with the control or IRF2 siRNA osteoclasts (left panel). Quantification of TRAP-positive MNCs (middle panel). Whole cell lysates were harvested and immunoblotted against the indicated antibodies (right panel). (F) BMMs transduced with the control or IRF2 followed by transfection with control or p65 siRNA were cultured for 2 days with M-CSF and RANKL. The total RNA was isolated and the mRNA expression level of NFATc1 was determined by quantitative real-time PCR (left panel). Whole cell lysates were harvested and immunoblotted against the indicated antibodies (right panel). The data are presented as the mean means ± SDs. *P < 0.05, **P < 0.01, and ***P < 0.001 versus the control.

we examined whether IRF2 could directly interact with p65 by using an immunoprecipitation assay. 293T cells were transiently cotransfected with the HA-tagged p65 and Flag-tagged IRF2. As shown in Fig. 3A, IRF2 interacted with p65.

Next, we investigated whether the interaction between IRF2 and p65 could regulate the translocation of p65 to the nucleus by RANKL. Overexpression of IRF2 markedly increased RANKL-induced p65 nuclear translocation compared to the control (Fig. 3B). Conversely, knockdown of IRF2 by siRNA significantly inhibited translocation of p65 to the nucleus by RANKL (Fig. 3C). Moreover, increased osteoclast differentiation by overexpression of IRF2 was restored upon downregulation of p65 using siRNA (Fig. 3D). Therefore, we demonstrated that IRF2 acts as a positive regulator during osteoclastogenesis via increasing the nuclear translocation of p65.

Next, to determine whether IRF2 could regulate NFATc1 expression during osteoclast differentiation, we transfected BMMs with control or IRF2 siRNAs and then overexpressed a con-

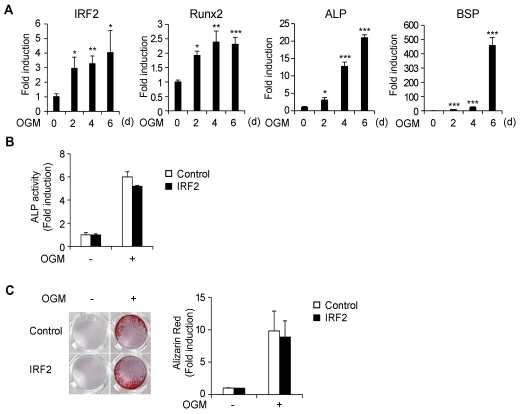

Fig. 4. IRF2 has no effect on osteoblast differentiation and function. (A) Primary calvarial osteoblast precursors were induced to osteoblast by culturing with OGM containing BMP2, ascorbic acid, and β-glycerophosphate for the indicated days. Total RNA was collected and quantitative real-time PCR was performed to assess the expression of the indicated genes. The data are presented as the mean means ± SDs. *P < 0.05, **P < 0.01, and ***P < 0.001 versus the control. (B, C) Primary calvarial osteoblast precursors transduced with control or IRF2 were induced to osteoblasts by culturing with OGM. (B) The cells cultured in OGM for 3 days were subjected to ALP assay and quantified by measuring the absorbance at 405 nm using a spectrophotometer. (C) The cells cultured in OGM for 6 days were stained for Alizarin red (left panel). Quantification of Alizarin red stain by measuring the absorbance at 562 nm using a spectrophotometer.

stitutively active form of NFATc1 (Ca-NFATc1). Knockdown of IRF2 in osteoclast precursors inhibited osteoclast differentiation and overexpression of Ca-NFATc1 restored this inhibitory effect (Fig. 3E). Furthermore, we found that upregulation of NFATc1 by IRF2 overexpression was restored by p65 knockdown (Fig. 3F). Taken together, these results suggest that IRF2 is a positive regulator of RANKL-induced osteoclast differentiation via modulation of NF-κB/NFATc1 signaling.

IRF2 does not affect the differentiation and function of osteoblasts

We next investigated the role of IRF2 in osteoblasts. To examine the expression pattern of IRF2 during osteoblast differentiation, primary calvarial osteoblast precursor cells were cultured in osteogenic medium (OGM). Expression of Runx2, ALP, and BSP as well as IRF2 was increased during osteoblast differentiation (Fig. 4A).

Next, we evaluated whether IRF2 affects osteoblastogenesis by overexpressing IRF2 using a retrovirus system. We assessed osteoblast differentiation and function through ALP assay and bone nodule formation, respectively. There was no difference in ALP activity and bone nodule formation by IRF2 overexpression (Fig. 4B, C). Thus, these results suggest that IRF2 is expressed in osteoblasts but does not affect osteoblastogenesis.

DISCUSSION

Osteoclasts and osteoblasts are the two main cells that maintain bone remodeling (24). Over-activation of osteoclasts is closely related to bone diseases such as osteoporosis, osteolysis, and rheumatoid arthritis (25).

IRF family members were originally identified as transcriptional regulators of type I interferons and regulate transcriptional activity, inflammatory response, and cellular responses involved in tumorigenesis (14, 26). In the IRFs, IRF1, IRF4, and IRF8 have been reported to be related to osteoclast differentiation, but the role of IRF2 in bone metabolism has not yet been elucidated. In this study, we revealed the role of IRF2 in RANKL-induced osteoclast differentiation. Overexpression of IRF2 enhanced RANKL-induced osteoclastogenesis via upregulation of NFATc1, whereas knockdown of IRF2 inhibited osteoclast differentiation through downregulation of NFATc1. Fusion analysis revealed that IRF2 overexpression/downregulation in committed preosteoclasts did not affect osteoclast maturation/fusion (data not shown). This finding suggests that IRF2 is involved in osteoclast commitment, rather than osteoclast maturation/fusion. In addition, IRF2 did not affect the differentiation and function of osteoblasts. Collectively, these results concluded that IRF2 is a positive regulator of RANKL-mediated osteoclast differentiation via upregulating NFATc1 expression similar to IRF4.

IRF2 mRNA expression was decreased after RANKL stimulation and subsequently increased (Fig. 1A). However, IRF2 protein expression was increased during osteoclast differentiation (Fig. 1D and 2D). This discrepancy might reflect the stability of IRF2 protein during osteoclast differentiation. IRF2 is known to be acetylated by p300/CBP-associated factor (PCAF), and protein acetylation by PCAF has been reported to increase the stability of acetylated proteins (16, 27). Therefore, IRF2 acetylation by PCAF may increase the protein stability of IRF2. Further, acetylated IRF2 interacts with other proteins that regulate gene transcription (15, 16). Therefore, although mRNA expression of IRF2 was decreased, increased IRF2 protein levels due to increased stability may contribute to the positive regulation of osteoclast differentiation.

IRF2 serves as the transcription repressor or the transcriptional activator. IRF2 acts as a transcriptional repressor, antagonizing the action of IRF1 within the same DNA binding specificity for IRF1 (13, 14, 26). In contrast, IRF2 acts as a transcriptional activator for interferon stimulated response element (IRSE)-like sequences such as VCAM-1, gp91phox, and Fas ligand (17, 18). In addition, IRF2 positively regulates the transcriptional activity of NF-κB by augmenting NF-κB activation by TNF-α (22). It has been reported that gp91phox deficiency strongly reduces the expression of NFATc1 induced by RANKL and causes impaired osteoclast differentiation, leading to osteopetrotic phenotype in gp91phox KO mice (28). Therefore, we investigated whether IRF2 enhances osteoclast differentiation by upregulating gp91phox. However, IRF2 did not significantly affect or rather decrease the gp91phox expression (Data not

shown). Next, since the putative ISRE-binding motifs exist within the NFATc1 promoter, we examined whether IRF2 directly induces NFATc1 expression by binding to the NFATc1 promoter region. As a result, IRF2 did not induce NFATc1 expression using NFATc1 reporter assay (data not shown). These results suggested that induction of NFATc1 expression mediated by IRF2 is not associated with induction of gp91hpox or direct transactivation of NFATc1.

NF-κB plays an important role in inducing NFATc1 expression during osteoclast differentiation (4, 29, 30). Deficiency of p50 and p52 subunits of NF-κB resulted in impaired osteoclastogenesis and consequently the osteopetrotic phenotypes (10, 29). The NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), inhibits osteoclast differentiation by reducing NFATc1 expression (31). Consistent with these results, the p50 and p65 subunits of NF-κB induce NFATc1 expression by binding to the NFATc1 promoter region in response to RANKL (2, 32). IRF2 directly interacts with p65, resulting in the enhanced translocation of NF-κB subunit p65 into the nucleus, and this migration increases NF-κB-dependent transcription by TNFα, whereas IRF2 mutant or IRF2 downregulation attenuates NF-κB activation by interfering with the nuclear translocation of p65 (22). Thus, IRF2 acts as a positive regulator of NF-κB activity through nuclear translocation of NF-κB subunit p65. Based on these reports, we explored whether IRF2 is involved in RANKL-induced osteoclast differentiation via NF-κB signaling. We observed that IRF2 increases the nuclear translocation of p65 by RANKL stimulation, and that IRF2 induces the expression of NFATc1 via activation of NF-κB subunit p65 during RANKL-induced osteoclastogenesis. Therefore, our results proposed that IRF2 regulates the expression of NFATc1 by increasing the accumulation of p65 in the nucleus.

In conclusion, we demonstrated that IRF2 enhances NFATc1 expression via p65 nuclear translocation and that this mechanism may contribute to the potential of IRF2 as a positive mediator of RANKL-induced osteoclastogenesis. Further, our findings suggest that IRF2 may be used as a suitable agent for the treatment of bone disease characterized by excessive osteoclast activity.

MATERIALS AND METHODS

Osteoclast and osteoblast differentiation

BMMs obtained from male 7-week-old ICR mice (Damul Science, Daejeon, Republic of Korea) were cultured with M-CSF (30 ng/ml) and RANKL (20-100 ng/ml) for 3 days. Cultured cells were fixed using 10% formalin and stained for TRAP. TRAP-positive cells with more than 3 nuclei were counted as osteoclasts. Primary calvairal osteoblast precursors prepared from neonatal ICR mice were induced to osteoblast differentiation by culturing in OGM, containing BMP2 (100 ng/ml) (Cowellmedi, Busan, Republic of Korea), ascorbic acid (50 ng/ml) (Junsei Chemical, Nihonbashi-honcho, Japan), and β-glycerophosphate (100 mM) (Sigma-Aldrich, St. Louis, MO, USA). ALP assay and Alizarin red stain were performed as previously described (33).

Retroviral gene transfection

Packaging cell line, Plat E, was transfected with retroviral vectors using FuGENE6 (Promega, Madison, WI, USA) according to the manufacturer's instruction. After 48 hours, the viral supernatant was collected and used for incubation of BMMs or osteoblasts for 6 hours in the presence of 10 μg/ml polybrene (Sigma-Aldrich).

Small interfering RNA (siRNA) transfection

BMMs were transfected with Control siRNA (Dharmacon, Lafayette, CO, USA) or IRF2 siRNA (siIRF2) (Dharmacon) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instruction.

Quantitative real-time PCR

The reverse transcribed cDNA was used for quantitative real-time PCR analyses using SYBR Green (Qiagen) and Rotor-Gene Q (Qiagen) in triplicates. The mRNA expression levels of the analyzed genes were normalized to the expression level of GAPDH. The primer sequences were as follows: c-Fos, 5′-ATGGGCTCTCCTGTCAACACA-3′ and 5′-TGGCAATCTCAGTCTGCAACGCAG-3′; NFATc1, 5′-CTCGAAAGACAGCACTGGAGCAT-3′ and 5′-CGGCTGCCTTCCGTCTCATAG-3′; TRAP, 5′-CTGGAGTGCACGATGCCAGCGACA-3′ and 5′-TCCGTGCTCGGCGATGGACCAGA-3′; IRF2, 5′- TCCAAGAAAGGAAAGAAACC-3′ and 5′- TCACTTCTACAACCTGGCAG-3′; GAPDH, 5′-TGACCACAGTCCATGCCATCACTG-3′ and 5′-CAGGAGACAACCTGGTCCTCAGTG-3′; Runx2, 5′-CCCAGCCACCTTTACCTACA-3′ and 5′-CAGCGTCAACACCATCATTC-3′; ALP, 5′-CAAGGATATCGACGTGATCATG-3′ and 5′-GTCAGTCAGGTTGTTCCGATTC-3′; BSP, 5′-GGAAGAGGAGACTTCAAACGAAG-3′ and 5′-CATCCACTTCTGCTTCTTCGTTC-3′.

Immunoprecipitation

The transfected 293T cells were lysed in extraction buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.01% protease inhibitor cocktail). The whole cell lysates were immunoprecipitated with the indicated antibodies.

Fractionation and western blot analysis

Cultured cells were fractionated using Nuclease and Cytoplasmic Extraction Reagents (Thermo Fisher Scientifics), according to the manufacturer's instruction. Fractionated cytoplasmic extracts and nuclear extracts were subjected to SDS-PAGE and transferred electrophoretically onto a polyvinylidene fluoride membrane (Millipore, MA, USA) followed by western blotting analysis and signals were detected by Azure c300 luminescent image analyzer (Azure Biosystems, Dublin, CA, USA).

Statistical analysis

Statistical analyses were performed using an unpaired Student's *t* test. All data are presented as the mean ± SD of three in-

dependent experiments. P values < 0.05 were considered statistically significant.

ACKNOWLEDGEMENTS

This work was supported by the National Research Foundation of Korea (NRF) grants funded by the Korea government (MIST) (NRF-2019R1A5A2027521 and 2020R1I1A1A01061781), and the Chonnam National University Hospital Biomedical Research Institute (BCRI20046).

CONFLICTS OF INTEREST

The authors have no conflicting interests.

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