Early estrogen-induced gene 1 facilitates osteoclast formation through the inhibition of interferon regulatory factor 8 expression

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Abstract
Osteoclast-mediated inflammatory bone resorption is a major cause of many inflammatory bone disorders, including rheumatoid arthritis and periodontitis. However, the mechanisms regulating osteoclast differentiation in inflammatory settings are not well understood. We demonstrate here that early estrogen-induced gene 1 (EEIG1)-deficient mice are protected from inflammatory bone loss as determined with the use of models of lipopolysaccharide (LPS)-induced bone destruction. EEIG1-deficient macrophages markedly decreased RANKL- and TNFα-mediated osteoclastogenesis due to the downregulation of the nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), which is an essential transcription factor for osteoclast formation. In contrast, expression of interferon regulatory factor 8 (IRF8), a transcriptional repressor that blocks osteoclast differentiation, is elevated in EEIG1-deficient macrophages relative to wild-type cells. We found that reduced expression of B lymphocyte-induced maturation protein-1 (Blimp1) by siRNA downregulated RANKL-induced EEIG1 levels, whereas overexpression of Blimp1 potentiated EEIG1 levels. Mechanistic studies revealed that EEIG1 forms a complex with Blimp1 to negatively regulate the expression of the anti-osteoclastogenic gene, Irf8. We elucidated a novel mechanism by which EEIG1 restricts IRF8 expression and function, thereby enhancing the osteoclast formation by contributing to Blimp1-mediated IRF8 regulation. Together, these findings identify EEIG1 as a key regulator of osteoclastogenesis and a possible therapeutic target for pathological bone destruction.

Keywords
bone, bone loss, osteoclast formation, osteoclastogenesis, osteoclasts

Abbreviations: Atp6v0d2, v-type proton ATPase subunit d2; Blimp1, B lymphocyte-induced maturation protein-1; BMM, bone marrow-derived macrophages; ChIP, chromatin immunoprecipitation; EEIG1, early estrogen-induced gene 1; IRF8, interferon regulatory factor 8; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; MNC, multi-nucleated cells; NFATc1, nuclear factor of activated T cell; PBS, phosphate-buffered saline; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RANKL, receptor activator of nuclear factor-κB; siRNA, short interfering RNA; TNFα, tumor necrosis factor alpha; TRAP, tartrate-resistant acid phosphatase.

Eutteum Jeong and Jihee Kim contributed equally to this work.
1  |  INTRODUCTION

The balance between bone-forming osteoblasts and bone-resorbing osteoclasts maintains bone homeostasis, and disturbance to this balance can lead to various bone diseases, such as rheumatoid arthritis and osteoporosis.\(^1\) The differentiation of osteoclasts, which are multinucleated myeloid lineage cells capable of bone resorption, is triggered by the essential cytokines, macrophage colony-stimulating factor (M-CSF), and receptor activator of nuclear factor-κB ligand (RANKL).\(^3,4\) Binding of RANKL to its receptor, RANK, initiates the recruitment of tumor necrosis factor (TNF) receptor-associated factor 6, and mitogen-activated protein kinase (MAPK) pathways. During osteoclastogenesis, RANKL activates various transcription factors, including microphthalmia transcription factor, c-Fos, and nuclear factor of activated T cells (NFATc1), which is known as a master regulator of osteoclast differentiation.\(^5-7\) RANKL also acts together with co-stimulatory immunoreceptor tyrosine-based activation motif-containing adaptors, which in turn activate the spleen tyrosine kinase with Btk/Tec kinases, leading to the activation of phospholipase C gamma 2 (PLCγ2) and subsequent calcium signaling.\(^8,10\)

TNFα, which has been implicated in the pathogenesis of inflammatory diseases plays an important role in immunity,\(^11,12\) promotes inflammatory bone resorption, and acts directly on osteoclast precursors to promote osteoclastogenesis in synergy with RANKL, thereby accompanying inflammatory arthritis and periodontitis.\(^13-15\) Although the activating signaling pathways are similar to RANKL, TNFα does not effectively induce osteoclastogenesis without RANKL stimulation. However, underlying mechanisms have not been fully explored.

Recent studies have demonstrated that transcriptional repressors expressed by osteoclast precursors negatively regulate osteoclastogenesis. Interferon regulatory factor 8 (IRF8), v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (MafB), and B cell lymphoma 6 (Bcl6) all suppress osteoclast differentiation by inhibiting the expression and function of NFATc1.\(^16-19\) B lymphocyte-induced maturation protein-1 (Blimp1) is a transcriptional repressor crucial for the repression of anti-osteoclastogenic genes that encode IRF8, MafB, and Bcl6, thereby playing important roles in the differentiation and function of osteoclasts.\(^15,18,20\) However, the roles of these transcription repressors in regulating the mechanisms underlying RANKL- and TNFα-mediated osteoclast differentiation and inflammatory bone resorption remain mostly unknown.

Originally, early estrogen-induced gene 1 (EEIG1) was identified as an early estrogen-inducible gene in breast cancer cell lines.\(^21\) In previous work, we identified EEIG1 as a novel RANK signaling component controlling RANK-mediated osteoclast formation. Upon RANKL signaling, EEIG1 interacts with the RANK signaling complex and facilitates RANKL-stimulated PLCγ2 phosphorylation and NFATc1 induction.\(^22\) We suggested that targeting EEIG1 may be a new therapeutic strategy for treating bone disease, but the specific mechanism by which EEIG1 contributes to inflammatory osteoclastogenesis and pathological bone resorption has yet to be elucidated.

In the current study, we investigate the role of EEIG1 in osteoclastogenesis and suggest a new model by which EEIG1 may contribute to RANKL- and TNFα-induced osteoclastogenesis by downregulating inhibitory factors, such as IRF8. We also demonstrate how the loss of EEIG1 results in markedly reduced RANKL- and TNFα-induced osteoclastogenesis and show that the lack of EEIG1 can reduce excessive bone resorption in inflammatory settings and that EEIG1 cooperates with Blimp1 to restrict IRF8 expression and function, thereby enhancing osteoclastogenesis. Overall, our results indicate that the Blimp1-EEIG1-IRF8 axis is crucial to inflammatory bone resorption and bone homeostasis.

2  |  MATERIALS AND METHODS

2.1  |  Mice

The EEIG1 gene of embryonic stem (ES) cells was targeted by homologous recombination. A replacement targeting scheme was designed to delete the first exon of the EEIG1 gene, including the transcription start site, which was replaced with an MC1-Neo Cassette. Targeted ES cell clones were confirmed by Southern blot analysis. The resulting chimeras were then mated to C57BL/6 females to obtain germ-line transmission. Wild-type (WT) and knockout genomic DNA was digested with the BglII restriction enzyme. Genomic DNA isolated from the tail was analyzed by polymerase chain reaction (PCR) using the specific forward primer, 5’-TTGAGACACCTCTGCGATTGT-3’ and reverse primers specific for EEIG1 (ER: 5’-CGGATGGCTTCTGCCTCGGTTAG-3′) and the Neo cassette (NR: 5’-CGARRGCRGRRG RGCCCCAAGRCAT-3′). The EEIG1 mutation was crossed onto a C57BL/6 background for more than six generations and was confirmed by western blotting and real-time quantitative PCR (RT-PCR). The protocols of all animal and primary cell experiments were approved by the Institutional Animal Care and Use Committee of Ewha Laboratory Animal Genomics Center and were conducted in accordance with the approved guidelines.

2.2  |  Lipopolysaccharide (LPS)-induced bone destruction

LPS (Sigma-Aldrich Corporation, St. Louis, MO, USA; 02:B6, L8274, 12.5 mg/kg body weight) or phosphate-buffered
saline (PBA; sham group) was injected into the calvaria of EEIG1+/+ and EEIG1−/− male mice (6 weeks old, n = 10 for each of four groups). The injection site was at a point on the midline of the skull located between the ears and eyes. All mice were euthanized and serum was collected at 5 days after the initial LPS injection. Tartrate-resistant acid phosphatase (TRAP) levels were determined using a Mouse TRAP Assay kit (Immunodiagnostics Systems, Tyne and Wear, UK) in accordance with the manufacturer’s protocol. For histological analysis, the calvaria were fixed in formaldehyde (Sigma-Aldrich Corporation) for 24 hours and then decalcified in 0.5 M ethylenediaminetetraacetic acid (pH 7.4) (EDTA; LPS solution) at 4°C for 7 days. After decalcification, the calvaria were embedded in paraffin and cut into 5 μm-thick sections, which were then stained for TRAP. Data were obtained by an OsteoMeasureXP bone histomorphometry analysis system (Osteometrics, Inc, Atlanta, GA, USA) and an Olympus DP72 charge-coupled device camera (Olympus Corporation, Tokyo, Japan).

2.3 | Reagents and plasmids

Recombinant human M-CSF and recombinant mouse IL-1β and TNFα were purchased from R&D Systems (Minneapolis MN, USA), and RANKL was purchased from PeproTech EC (London, UK). Antibodies used in the study include the following: anti-IRF8, anti-Blimp1, anti-ERK1/2, anti-phospho-ERK1/2, anti-SAPK/JNK, anti-phospho-SAPK/JNK, anti-p38, anti-phospho-p38, anti-IκBα, anti-phospho-IκBα, anti-p65, anti-phospho-p65, anti-phospho-IKKαβ, anti-EEIG1 (Cell Signaling Technology Inc Danvers, MA, USA), monoclonal anti-FLAG, rabbit polyclonal antibodies to Atp6v0d2, Atp6v0d1, c-Fms, Mafb, Actin, c-Myb, AKT, β-actin, NFATc1, IKKαβ, p65, phospho-p65, phospho-IκBα, phospho-IRF8, phospho-Blimp1 (Santa Cruz Biotechnology, Inc Dallas, TX, USA), and RANKL. Mouse EEIG1 was generated by PCR and cloned into the plasmids pFLAG-CMV2 and pcDNA3.1-HA. The pMX-puro and pMX-puro-Flag-Blimp1 plasmids are described elsewhere.22,23 The retroviral vector containing a constitutively active form of NFATc1 (NFATc1-CA) has been previously described.24

2.4 | Bone marrow macrophages’ (BMMs) cultures and osteoclast generation

BMMs were prepared from bone marrow cells using a standard method.25 Briefly, primary BMMs were isolated from the femurs and tibias of 4 to 6-weeks-old C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME, USA) by flushing with medium using a sterile 21-gauge syringe, and then cultured in α-minimum essential medium (α-MEM; HyClone Laboratories, Inc, South Logan UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Inc) and 1% penicillin/streptomycin. After 24 hours, unattached non-adherent cells were harvested and cultured with 30 ng/mL of M-CSF. After 3 days, adherent BMMs were harvested to obtain osteoclast precursor cells of the monocyte/macrophage lineage. For osteoclast generation, BMMs were cultured in the presence of M-CSF (30 ng/mL) and RANKL (50 ng/mL) or TNFα (30 ng/mL) for 3-5 days.

2.5 | Reverse transcription and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cultured BMMs with RNAiso Plus (Takara Bio, Inc, Kusatsu, Shiga Prefecture, Japan) in accordance with the manufacturer’s instructions. Reverse transcription of purified RNA was performed using the DiaStar Reverse Transcription kit (SolGent Co., Ltd., Seoul, Korea). All qRT-PCR reactions were performed in triplicate using an ABI PRISM 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the SYBR Green Master kit (Kapa Biosystems, Wilmington, MA, USA) with the following primers: EEIG1, 5′TTTTGTCAGTTGTGTCGCAACA-3′ and 5′-CGCACAGACACTGGAAGAT-3′; Blimp1, 5′-TTCTTTGTTGTTATTTG-3′ and 5′-TTGAGGACACTCTTGGTAC-3′; Irf8, 5′-GTCACAGATCGACACA3′ and 5′-CTGGGGCTTTGTTCAGA-3′; Mafb, 5′-AGTGTTGGAGGAGCCCTGTT-3′ and 5′-CAGAAAAGAGACTCAGGAG-3′; Nfatc1, 5′-CCA GAAAATAACATGCA3′ and 5′-GGTGGATGTGAACCTCTG-3′; Atp6v0d2, 5′-CAGAGATGGAAGCTGT-3′ and 5′-TGCCCAAATGAGTTCAGA-3′; RANK, 5′-TCTGGCGTTTACTACAGGA3′ and 5′-CATTGGACCAATATGCCAAA3′; c-Fms, 5′-TCCACCCGGAGCTGAAGCA-3′ and 5′-CCAGTCCACCCATCT-3′; and Actin, 5′-GCTTCTTCTTTGCACTTCTT-3′ and 5′-ATCGTCATCCATGGCGA-3′.

2.6 | Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed with an EZ-Chip kit (EMD Millipore, Bedford, MA, USA) in accordance with the manufacturer’s instructions. In brief, BMMs were cultured in the presence of M-CSF (30 ng/mL) with RANKL (50 ng/mL) or TNFα (30 ng/mL) for 2 days, and then fixed with formaldehyde and lysed with SDS lysis buffer containing protease inhibitors for 10 minutes on ice. Afterward, the lysates were sonicated to reduce the DNA length to 200-1000 base pairs and centrifuged. Supernatant fractions were diluted in the ChIP
dilution buffer. The chromatin solution was precleared with salmon sperm DNA/protein agarose slurry for 1 hour at 4°C with rotation. After preclearing, the supernatant was used for ChIP analysis with anti-Blimp1 (Cell Signaling Technology Inc) or the control rabbit IgG (Santa Cruz Biotechnology, Inc) overnight at 4°C with rotation. The final immunoprecipitated DNA was analyzed by qRT-PCR. An Irf8 promoter primer pair (5′-ATCAAAACTCTGTGAGGAACA-3′ and 5′-AGCTCTTCCCTGACCCCCTGT-3′) was generated to detect DNA segments located near the Blimp1 binding sites at nucleotides −5920 (AGAGA) and −5953 (GAAA).

2.7 | Retroviral infection

PLAT-E retrovirus packaging cells were transfected with the pMX-puro empty, pMX-puro-Flag-Blimp1, or pMX-puro-Flag-EEIG1 retroviral vector using polyethylenimine reagent (Sigma-Aldrich Corporation). At 36-48 hours after transfection, and supernatant was collected. The BMMs were infected with the retroviral supernatant in the presence of M-CSF (30 ng/mL) and polybrene (10 μg/mL) for 5 hours. After a 24 hours infection period, medium containing M-CSF (30 ng/mL) and puromycin (2 μg/mL) was refreshed over a period of 2 days to select for the infected cells. Puromycin-resistant BMMs were used for osteoclast differentiation in the presence of M-CSF (30 ng/mL) with RANKL (50 ng/mL) or TNFα (30 ng/mL) for additional days.

2.8 | Transfection with short interfering RNA (siRNA)

Control, Blimp1, and IRF8 siRNAs were purchased from Genolution, Inc (Seoul, Korea). BMMs were plated 1 day before siRNA transfection. The BMMs were transfected with siRNAs using Lipofectamine RNAiMAX reagent (Invitrogen Corporation, Carlsbad, CA, USA) in accordance with the manufacturer’s instructions. After 1 day, siRNA-transfected BMMs were stimulated with RANKL- or TNFα for the indicated time periods.

2.9 | GST pulldown assay

293T cells were cultured in DMEM (HyClone Laboratories, Inc) containing 10% FBS and antibiotics and re-plated 24 hours prior to transfection with the indicated combinations of expression vectors using polyethylenimine. After 48 hours, cell extracts were obtained using 1% NP-40 lysis buffer (20 mM HEPES pH 7.0, 10 mM EDTA, 150 mM NaCl, 150 mM KCl, 10% glycerol, 1% Nonidet P-40) with protease inhibitors. The whole-cell lysates were pulled down with the use of Glutathione Sepharose 4B (GE Healthcare Life Science, Chicago, IL, USA) for 3 hours at 4°C and then subjected to western blot analysis.

2.10 | Western blot analysis and immunoprecipitation

BMMs were incubated with M-CSF (30 ng/mL) in the presence or absence of RANKL (50 ng/mL) and TNFα (30 ng/mL) for the indicated periods, and then lysed in cell extraction buffer (20 mM HEPES pH 7.9, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 10% glycerol) with protease inhibitors. After harvesting, the cell lysates were subjected to western blot analysis with the use of appropriate antibodies. Whole-cell extracts were incubated with specific antibodies overnight, followed by incubation with Protein G-Sepharose beads (GE Healthcare Life Science) for 2 hours at 4°C, and then immunoprecipitated. Afterward, the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and western blot analysis. 293T cells were harvested at 48 hours after transfection and then lysed in 1% NP-40 lysis buffer. The whole-cell lysates were subjected to western blot analysis or immunoprecipitation.

2.11 | Micro-computed tomography (micro-CT) analyses

To analyze bone parameters, samples were isolated from 10 to 12-weeks-old male WT and EEIG1−/− mice, fixed in 10% formaldehyde, and scanned using a SkyScan 1176 in vivo micro-CT scanner (Bruker micro-CT, Kontich, Belgium) at 75 kV and 333 μA. Total of 360° views were acquired at 0.7° increments, each for an exposure time of 260 ms, to obtain a resolution of 18 μm. The raw data from the tibias acquired by micro-CT were translated into two-dimensional cross-sectional gray-scale image slices using the NRecon reconstruction software (Bruker micro-CT). From the acquired two-dimensional images, structural parameters of the tibial trabecular bone were evaluated with the use of a CT Analyzer (CT-AN; Bruker micro-CT).

2.12 | Quantification and statistical analysis

Statistical analysis was performed using GraphPad Prism software version 8 (GraphPad Software, Inc, San Diego, CA, USA). All data were assembled from at least three independent experiments. The Student’s two-tailed t-test was used to analyze differences between two samples. Analysis of more than two samples was performed using one-way or two-way analysis of variance (ANOVA), followed by pairwise
multiple comparisons if significant. As appropriate, post hoc analysis was performed using Fisher’s protected least-squares difference test. The error bars represent the standard deviation (SD) of parametric data. Also, 95% confidence intervals were calculated for non-parametric data.

3 | RESULTS

3.1 | Generation of EEIG1 knockout mice

To determine the functional role of EEIG1 in vivo, EEIG1 knockout mice (EEIG1−/−) mice were generated. The EEIG1 gene of ES cells was targeted by homologous recombination. A replacement targeting scheme was designed to delete the first exon of the EEIG1 gene, including the transcription start site, which was replaced with a neomycin gene cassette to inactivate the EEIG1 gene (Figure 1A). The EEIG1 mutation was crossed onto a C57BL/6 background for more than six generations, as confirmed by Southern blot analysis (Figure 1B) and PCR genotyping (Figure 1C). EEIG1 deficiency was confirmed at the RNA and protein levels in BMMs (Figure 1D,E). EEIG1−/− mice are viable, fertile, and normal in size with a normal lifespan, and do not display any gross morphological or histological abnormalities (data not shown).

3.2 | EEIG1 deficiency protects bone loss against LPS-induced bone destruction

The relationship between EEIG1 expression and bone phenotype was examined in EEIG1−/− mice. Since EEIG1 deficiency had no effect on bone mass under homeostatic conditions (Figure S1A,B), the effects of EEIG1 deficiency on pathological bone loss were tested with the use of inflammatory bone destruction models by LPS injection into the calvaria of EEIG1+/+ and EEIG1−/− mice to induce bone destruction. TRAP staining revealed that the amount of osteoclasts in EEIG1−/− mice under this pathological condition was significantly reduced as compared to the control mice (Figure 2A,B). Histological analysis showed that bone erosion was markedly reduced in EEIG1−/− mice. Quantitative analysis revealed a greater than the twofold decrease of the osteolytic cavities in EEIG1−/− mice compared with the control WT mice (Figure 2C). Consistently, administration

**FIGURE 1** Genetic ablation of EEIG1. A, Schematic map of the EEIG1 targeting construct of knockout mice. B, Southern blot of BglII digested tail DNA of EEIG1 F1 heterozygous (+/−) and WT (+/+ ) mice. Southern blot analysis revealed the expected 8.0-kb band (KO) in addition to the WT 4.4-kb band. C, Genotyping of the EEIG1 locus was performed by PCR with a high-fidelity polymerase using a combination of primers targeting Exon 1; F + ER [576 bp] or Neo cassette; F + NR [1017 bp]. The WT allele produced a 576-bp band, whereas the KO allele produced a 1017-bp band. D, Protein expression of EEIG1 by BMMs isolated from 4-weeks-old EEIG1+/+ and EEIG1−/− mice. BMMs derived from EEIG1+/+ and EEIG1−/− mice were cultured with M-CSF and RANKL for 3 days. Western blot analysis showed that EEIG1 was undetectable in the EEIG1−/− BMMs. E, mRNA expression levels of EEIG1 relative to β-actin were analyzed by qRT-PCR. ***P < .005. Values are expressed as the mean ± SD (two-way ANOVA)
of LPS to the calvarial periosteum resulted in high levels of serum TRAP in \textit{EEIG1}^{+/+} mice as compared to \textit{EEIG1}^{−/−} mice (Figure 2D). These results show that EEIG1 mediates inflammation-induced osteolysis in vivo.

### 3.3 EEIG1 mediates RANKL- and TNFα-induced osteoclast formation

Because inflammatory cytokines, such as TNFα and IL-1β, play important roles in pathological bone loss under inflammatory conditions,\textsuperscript{26-28} we explored whether these cytokines regulate EEIG1 expression in osteoclast precursors, BMMs and found that the EEIG1 mRNA expression was clearly induced by both RANKL and TNFα, but not IL-1β (Figure S2A). We further confirmed that EEIG1 protein expression was markedly upregulated during RANKL- and TNFα-induced osteoclast differentiation (Figure S2B,C), suggesting a potential role of EEIG1 in RANKL- and TNFα-mediated osteoclast formation.

To clarify the mechanism of increased bone loss by EEIG1 deletion, in vitro experiments were conducted using BMMs isolated from \textit{EEIG1}^{+/+} and \textit{EEIG1}^{−/−} mice. We first evaluated whether EEIG1 deficiency affects RANKL- and TNFα-mediated osteoclast formation and found that BMMs derived from \textit{EEIG1}^{−/−} mice exhibited a significant decrease in RANKL-induced osteoclast differentiation (Figure 3A). In parallel with the decrease in TRAP-positive multinucleated cells (MNCs), mRNA expression levels of osteoclast marker genes, such as NFATc1 and Atp6v0d2, were also decreased in \textit{EEIG1}^{−/−} cells relative to control cells during RANKL-induced osteoclast differentiation (Figure 3B). To further confirm that EEIG1 is essential for osteoclastogenesis, \textit{EEIG1}^{−/−} BMMs were infected with empty or EEIG1-expressing retroviruses, which resulted in the restoration of TRAP-positive MNC formation and increased expression levels of NFATc1 (Figure 3C,D), indicating that EEIG1 acts as a positive regulator of RANKL-induced osteoclast formation.

Next, we examined whether EEIG1 affects TNFα-mediated osteoclast formation. Interestingly, we found that the absence of EEIG1 markedly decreased the formation of TRAP-positive osteoclasts in response to TNFα (Figure S3A). Likewise, the mRNA expression levels of osteoclast marker genes were decreased in \textit{EEIG1}^{−/−} cells relative to control

**FIGURE 2** EEIG1 deficiency reduces LPS-induced bone destruction in vivo. A-D, The calvaria of 4-weeks-old \textit{EEIG1}^{+/+} and \textit{EEIG1}^{−/−} male mice were injected with PBS (sham group) or LPS (12.5 mg/kg body weight) twice at 48 hours intervals. A, TRAP staining of mouse whole calvaria. Scale bar, 0.5 cm. n = 5 per group. B, TRAP staining was performed on histological sections of the calvarial bone. Scale bar, 200 μm. n = 10 per group. C, Histomorphometric analysis of calvaria from \textit{EEIG1}^{+/+} and \textit{EEIG1}^{−/−} mice. Quantification of the percentage of the eroded surface over the total bone surface (left), osteoclast surface per bone surface (middle), osteoclast number per bone perimeter (right). D, Serum concentrations of TRAP of 4-weeks-old \textit{EEIG1}^{+/+} and \textit{EEIG1}^{−/−} male mice injected with PBS or LPS (12.5 mg/kg body weight). n = 10 per group. ES/BS, eroded surface per bone surface; Oc.S/BS, osteoclast surface per bone surface; N.Oc/B.Pm, number of osteoclasts per bone perimeter. *P < .05, **P < .01, ***P < .005, ****P < .0005, ####P < .0001. Values are expressed as the mean ± SD (two-way ANOVA)
**FIGURE 3** EEIG1 deficiency decreases RANKL-induced osteoclast formation. A, BMMs derived from EEIG1+/+ and EEIG1−/− littermates were stimulated with RANKL in the presence of M-CSF. After 4 days, osteoclasts were fixed and stained for TRAP. Then, the number of TRAP-positive MNCs (>5 nuclei per cell) per well was counted. Scale bar, 200 μm. B, qRT-PCR of the mRNA expression levels of EEIG1, NFATc1, and Atp6v0d2 relative to β-actin in RANKL-stimulated BMMs for the indicated times. C, D, BMMs derived from EEIG1−/− were transduced with retrovirus expressing an empty vector (EV) or Flag-tagged EEIG1 (EEIG1). Cells were cultured for 4 (C) or 2 (D) days with M-CSF and RANKL. C, Osteoclasts were fixed and stained for TRAP. Then, the number of TRAP-positive MNCs (>5 nuclei per cell) per well was counted. Scale bar, 200 μm. D, Cells were lysed and immunoblotted with NFATc1, EEIG1, and GAPDH antibodies. Data are representative of at least three (A, C, D) or at least five (B) independent experiments. Data are presented as the mean ± SD Student’s two-tailed t-test (A, C). Values are expressed as the mean ± SD two-way ANOVA (B). EV, empty vector. *P < .05, **P < .01, ***P < .005, ¶P < .001, ¶¶P < .0005.

**FIGURE 4** EEIG1 inhibits the expression of IRF8 by RANKL during osteoclastogenesis. A, C, BMMs derived from EEIG1+/+ and EEIG1−/− were cultured with M-CSF and RANKL or TNFα for the indicated times. Whole-cell lysates were obtained and analyzed by western blotting with the indicated antibodies. B, D, mRNA expression levels of NFATc1, Blimp1, and IRF8 relative to β-actin were analyzed by qRT-PCR. Data are the representative of at least five independent experiments (A-D). ns, not significant, *P < .05, **P < .01, ***P < .005, ¶P < .001, ¶¶P < .0005. Statistical differences were assessed by two-way ANOVA.
cells (Figure S3B). Under inflammatory conditions, TNFα acts on osteoclast precursors in synergy with RANKL to promote osteoclastogenesis.29,30 To further explore the role of EEIG1 during osteoclast formation under inflammatory conditions, we examined osteoclastogenesis under RANKL priming conditions and found that EEIG1 deletion dramatically decreased TNFα-induced osteoclast formation in the presence of RANKL (Figure S3C), indicating that EEIG1 may play a synergistic role during osteoclast formation induced by RANKL and TNFα.

3.4 EEIG1 downregulates the osteoclastogenic repressor IRF8

To understand how EEIG1 regulates both RANKL- and TNFα-induced osteoclastogenesis, we tested whether EEIG1 affects signaling cascades, including MAPKs and NF-κB, upon stimulation of RANKL or TNFα, and found that EEIG1 had no significant effect on these signaling cascades (Figure S4A,B). We further demonstrated that mRNA expression levels of c-fms and Rank were comparable in EEIG1+/+ and EEIG1−/− osteoclast precursors, indicating that attenuated osteoclast differentiation in EEIG1−/− osteoclast precursors was not due to changes in receptor levels (data not shown).

Next, we investigated the expression of various osteoclast markers during RANKL-induced osteoclast differentiation. Intriguingly, we found that mRNA and protein expression levels of IRF8, an important regulatory transcription factor for osteoclastogenesis, were higher in EEIG1−/− cells than WT cells during RANKL-induced osteoclast differentiation (Figure 4A,B). However, the induction of Blimp1, which is known as a transcription repressor of anti-osteoclastogenic genes, such as Irf8 and Bcl6,20,31 was not changed. Consistent with these results, mRNA and protein expression levels of IRF8 were increased in EEIG1−/− cells, while the expression of Blimp1 was unchanged during TNFα-induced osteoclastogenesis (Figure 4C,D). We then attempted to examine the relationship between EEIG1 and IRF8 during osteoclastogenesis. To investigate whether IRF8-induced suppression of osteoclastogenesis is regulated by EEIG1, siRNA-mediated knockdown of IRF8 in RANKL-stimulated EEIG1-deficient cells was performed, which showed that the knockdown of IRF8 expression significantly increased NFATc1 expression and

**FIGURE 5** EEIG1 inhibits IRF8 expression and function. A, EEIG1+/− BMMs were transfected with Irf8-specific siRNA (IRF8) or non-targeting control siRNA (Con) and cultured with M-CSF and RANKL for 24 hours. The protein levels of NFATc1 and IRF8 determined by western blot analysis. B, EEIG1+/+ and EEIG1−/− BMMs were transfected with siIRF8 (IRF8) or control siRNAs (Control) and cultured with M-CSF and RANKL. After 4 days, osteoclasts were fixed and stained for TRAP. Then, the number of TRAP-positive MNCs (≥5 nuclei per cell) per well was counted. Scale bar, 200 μm. Data are the representative of at least four (A) or five (B) independent experiments. *p < .05, **p < .005, ***p < .001, ##p < .0005. Data are presented as the mean ± SD (two-way ANOVA).
osteoclast differentiation by RANKL in EEIG1-deficient cells (Figure 5A,B). Taken together, these results show that EEIG1, which might be upstream of IRF8, enhances osteoclast formation by downregulating IRF8 expression.

3.5 | Blimp1 upregulates EEIG1 expression

Recently, Blimp1 has been implicated as an upstream transcriptional repressor of several anti-osteoclastogenic factors during osteoclast differentiation.18,20,23 Since EEIG1 negatively regulates IRF8 expression, we investigated whether EEIG1 is involved in Blimp1-mediated IRF8 repression during osteoclastogenesis. Because our previously mentioned results showed that EEIG1 deletion had no effect on Blimp1 expression (Figure 4), EEIG1 was overexpressed with the use of a retrovirus to confirm that EEIG1 has no effect on Blimp1 expression. Stimulation of transduced BMMs with or without RANKL for 24 hours showed no change in Blimp1 protein expression by EEIG1 overexpression, whereas IRF8 expression had slightly decreased (Figure S5).

We then examined whether Blimp1 regulates the expression of EEIG1 using Blimp1-specific siRNA. Interestingly, knockdown of Blimp1 expression significantly reduced RANKL-induced EEIG1 expression. Consistent with the levels of Blimp1 and EEIG1, RANKL-induced NFATc1 expression was decreased in cells transfected with Blimp1 siRNA relative to control cells (Figure 6A,B). Moreover, retroviral overexpression of Blimp1 accelerated the induction of EEIG1 after RANKL stimulation (Figure 6C,D). These results indicate that Blimp1 positively regulates EEIG1 expression during RANKL-induced osteoclast differentiation.

3.6 | EEIG1 contributes to Blimp1-mediated IRF8 inhibition

To investigate the molecular mechanism by which Blimp1 and EEIG1 suppress IRF8 expression, we tested the interaction between EEIG1 and Blimp1 and found that EEIG1 interacts with Blimp1 after co-transfection in 293T cells (Figure 7A). To further examine the interaction between Blimp1 and EEIG1, BMMs were incubated with M-CSF,
with or without RANKL, for 3 days. Afterward, endogenous Blimp1 was immunoprecipitated with anti-Blimp1 antibody. As demonstrated by the immunoprecipitation results presented in Figure 7B, endogenous EEIG1 interacted with Blimp1 following RANKL stimulation, suggesting that EEIG1 may be involved in Blimp1-mediated IRF8 regulation by interacting with Blimp1.

Consistent with the nuclear expression of Blimp1, we observed that EEIG1 was expressed in both the nuclear and cytosolic fractions (Figure 8A). Based on these results, we then investigated whether EEIG1 affects Blimp-mediated repression of IRF8 transcription. Indeed, the results of the ChIP assay confirmed that RANKL-induced recruitment of Blimp1 to its target Irf8 promoter was markedly decreased in EEIG1−/− cells (Figure 8B,C), suggesting that EEIG1 interacts with Blimp1 and plays an important role in Blimp1-mediated regulation of IRF8.

4 | DISCUSSION

Osteoclast-mediated inflammatory bone resorption is a major cause of skeletal diseases, such as rheumatoid arthritis and periodontitis. However, the mechanisms regulating osteoclastogenesis and bone resorption under inflammatory conditions are complex and have not been well elucidated. In this study, EEIG1 was identified as a positive regulator of osteoclast formation by counteracting IRF8 under inflammatory conditions. Our results indicate that EEIG1 promotes inflammation-induced osteolysis and mediates TNFα-induced osteoclast formation. The role of EEIG1 in osteoclastogenesis and EEIG1 upregulation by TNFα suggests that EEIG1 presents an attractive therapeutic target to prevent bone destruction in inflammatory diseases associated with enhanced osteoclast formation and function.

The balance between positive and negative regulation of osteoclastogenesis is important for bone homeostasis and the prevention of excessive bone resorption in inflammatory and other diseases. Positive signaling pathways and transcription factors that promote osteoclastogenesis have been extensively studied and are well characterized. A typical example is NFATc1, whose activity and expression are maintained at an extremely high level by RANKL stimulation, thereby promoting osteoclastogenesis. The negative regulation of osteoclast formation is also important for the control of healthy skeletal remodeling and preventing the development of bone-related diseases. Initiation and termination of the signaling pathways underlying the formation of mature osteoclasts from mononuclear precursors is a critical checkpoint that is part of this control. The complicated network of transcriptional repressors, including IRF8, Bcl6, and MafB, and extracellular secreted molecules, such as osteoprotegerin and interferon-β, are tightly regulated to control osteoclast formation. Recent studies have revealed...
that RANKL downregulates gene expression levels of Id and Lhx2, which also act as osteoclast differentiation repres-
sors. Nevertheless, in many pathological situations, es-
pecially under inflammatory conditions, there are cases that
counteract this negative regulation, but little is known about
the mechanisms regulating osteoclast formation. In this re-
gard, our results suggest that EEIG1 targets IRF8 to reverse
the inhibition of osteoclast formation.

How is the balance between positive- and negative-reg-
ulation released within RANK signaling and the tran-
scriptional network? To this end, a signaling molecule or
pathway usually may stimulate the negative feedback reg-
ulatory pathways to keep in check any excesses during cell
differentiation. In this context, we propose two roles of
EEIG1, either in the cytosol or nucleus, during osteoclast
formation. We previously showed that EEIG1 is induced
by RANKL stimulation via NFATc1 and positively regu-
lates osteoclast formation. In this case, the interaction
between EEIG1 in the cytosol and RANK signaling com-
ponents, such as Gab2, PLCγ2, and Btk/Tec, is necessary
for PLCγ2 phosphorylation required for the induction of
NFATc1. In this study, in vivo deletion of EEIG1 further
demonstrated that the loss of EEIG1 in the nucleus causes
NFATc1 downregulation, which is due to decreased bind-
ing of Blimp1 to the IRF8 promoter. Thus, EEIG1 func-
tions not only as a component of RANK receptor signaling
component in the cytosol, but also partly as a transcrip-
tional regulator that cooperates with Blimp1 to restrain
IRF8. However, it remains to be determined how EEIG1
shuttles between the cytosol and nucleus, and which sig-
nals trigger or regulate trafficking.

Given the role of EEIG1 as a RANK signaling compo-
nent in the cytosol, EEIG1 may regulate RANKL-induced
Blimp1 expression. However, there was no difference in
Blimp1 expression under the condition of EEIG1 deficiency
or overexpression. This discrepancy between the expected
and observed results suggests that there may be additional
regulatory mechanisms or signals involved in the temporal
and spatial control of osteoclast formation. An in-depth un-
derstanding of the gene regulatory programs between EEIG1
and Blimp1 will require further investigations.

In summary, the results of the present study revealed an
additional layer of controlling negative regulation during
osteoclastogenesis. Furthermore, we found that EEIG1 is
involved in both RANKL- and TNFα-induced osteoclast
differentiation, suggesting that EEIG1 could be an attractive

**FIGURE 8** EEIG1 contributes to Blimp1-mediated IRF8 regulation. A, EEIG1+/+ and EEIG1−/− BMMs were cultured with RANKL for
the indicated time periods and the nuclear and cytoplasmic fractions were harvested. Cell lysates were subjected to western blot analysis with the
indicated antibodies. B, Schematic diagram of the murine Irf8 promoter sequences. Locations of the putative Blimp1 binding sites at nucleotides
−5920 and −5953 relative to the transcription sites are indicated. C, ChIP analysis of Blimp1 occupancy at the Irf8 promoter in EEIG1+/+ and
EEIG1−/− treated with RANKL for 2 days in the presence of M-CSF. Input corresponded to PCR containing 2% of the total amount of chromatin
used in the immunoprecipitation reactions. Data are the representative of at least three (A) or five (B, C) independent experiments. *P < .05,
**P < .01, ***P < .005, **P < .001, ***P < .0005. Data are presented as the mean ± SD (two-way ANOVA)
target for inflammatory bone resorption. Therefore, further elucidation and multiple approaches targeting EEIG1 would have therapeutic implications for suppressing osteoclast differentiation and related pathological bone disorders.

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interests.

AUTHOR CONTRIBUTIONS
Study design: E. Jeong, J. Kim, and S.Y. Lee; Study conduct: E. Jeong, J. Kim, and M. Go; Data analysis and interpretation: E. Jeong, J. Kim, and S.Y. Lee; Drafting of the manuscript: E. Jeong, J. Kim, and S.Y. Lee; All authors approved the final version of this manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available within the article and the accompanying Supplementary Information.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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