Molecules and Cells

PARK2 Induces Osteoclastogenesis through Activation of the NF-κB Pathway

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Osteoclast generation from monocyte/macrophage lineage precursor cells needs to be tightly regulated to maintain bone homeostasis and is frequently over-activated in inflammatory conditions. PARK2, a protein associated with Parkinson's disease, plays an important role in mitophagy via its ubiquitin ligase function. In this study, we investigated whether PARK2 is involved in osteoclastogenesis. PARK2 expression was found to be increased during the receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclast differentiation. PARK2 gene silencing with siRNA significantly reduced osteoclastogenesis induced by RANKL, LPS (lipopolysaccharide), TNFα (tumor necrosis factor α), and IL-1β (interleukin-1β). On the other hand, overexpression of PARK2 promoted osteoclastogenesis. This regulation of osteoclastogenesis by PARK2 was mediated by IKK (inhibitory κB kinase) and NF-κB activation while MAPK (mitogen-activated protein kinases) activation was not involved. Additionally, administration of PARK2 siRNA significantly reduced osteoclastogenesis and bone loss in an *in vivo* model of inflammatory bone erosion. Taken together, this study establishes a novel role for PARK2 as a positive regulator in osteoclast differentiation and inflammatory bone destruction.

Keywords: lipopolysaccharide-induced osteolysis, NF-κB signaling pathway, osteoclast, PARK2

INTRODUCTION

Throughout life, bone undergoes continuous remodeling, which is tightly regulated and maintained by a homeostatic balance between the activity of bone-resorbing osteoclasts and bone-forming osteoblasts. When these activities are out of balance, bone abnormalities or deformities occur (Boyle et al., 2003; Downey and Siegel, 2006). Numerous factors and mediators involved in the differentiation and function of osteoblasts and osteoclasts have been discovered over the last several decades. Among these factors, the receptor activator of nuclear factor-κB ligand (RANKL), a member of tumor necrosis factor (TNF) family, is the essential factor that drives generation of osteoclasts from monocyte-macrophage lineage progenitors (Yasuda et al., 1998). Inflammatory diseases such as rheumatoid arthritis, periodontitis, and inflammatory bowel disease are frequently associated with RANKL-induced osteoclastogenesis and subsequent bone resorption. Pro-inflammatory cytokines, such as interleukin (IL)-1β and TNFα, promote osteoclastogenesis either directly or indirectly through upregulation of RANKL expression in mesenchymal and immune cells (Amarasekara et al., 2018; Luo et al., 2018; Nakamura and Jimi, 2006). Pathogen-associated molecular patterns, such as lipopolysaccharide (LPS), also stimulate osteoclastogenesis in RANKL-primed committed cells, resulting in bone resorption (Zou and Bar-Shavit, 2002).

NF-κB transcription factor activation is a common response

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shared among cells receiving the IL-1β, TNFα, LPS, or RANKL signal. Although distinct pathways are engaged downstream of each receptor, NF-κB activation requires activation of the inhibitory κB (IκB) kinase (IKK) complex, which is composed of catalytic IKKα, IKKβ, and regulatory IKKγ/NEMO subunits. IKK-mediated phosphorylation of IκB results in K48 ubiquitination and subsequent proteasomal degradation of IκB, allowing for release and nuclear translocation of NF-κB. In RANKL-induced osteoclast differentiation, the tumor necrosis factor receptor-associated factor 6 (TRAF6), a RING type E3 ubiquitin ligase, is recruited to the cytoplasmic domain of RANK upon binding of RANKL. TRAF6 recruitment stimulates the mitogen-activated protein kinases (MAPK) and NF-κB signaling pathways (Chen and Chen, 2013; Kim and Kim, 2014; Park et al., 2017). In this NF-κB pathway, TRAF6 mediates non-degradative K63 ubiquitination of TAK1 complex, an upstream activator of IKK complex, and NEMO (Besse et al., 2007; Walsh et al., 2008). The lack of osteoclasts in nfkb1 and nfkb2 double knockout mice further demonstrates the critical role of NF-κB activity in osteoclastogenesis (Franzoso et al., 1997; Iotsova et al., 1997).

PARK2, also known as parkin or PRKN, has been shown to be strongly associated with Parkinson's disease (Kamienieva et al., 2021; Lubbe et al., 2021). PARK2 induces mitophagy through the ubiquitination of mitochondrial substrates, as it is a member of the RING/HECT type E3 ubiquitin ligase family (Harper et al., 2018; Quinn et al., 2020; Seirafi et al., 2015). Besides its role in mitophagy and Parkinson's disease, PARK2 has also been shown to regulate PI3K/AKT and Hippo/YAP pathways in cancers (Lin et al., 2015; Zhou et al., 2020). More recently, PARK2 was linked to the NF-κB signaling pathway. In mouse neuron cells, PARK2 activated NF-κB by promoting K63 ubiquitination of TRAF2 and NEMO (Henn et al., 2007). Another study found that PARK2 increased the linear ubiquitination of NEMO in response to stress stimuli, activating NF-κB (Muller-Rischart et al., 2013). Despite the implication of PARK2 in NF-κB signaling pathway and the importance of NF-κB in osteoclastogenesis, the role of PARK2 in osteoclast differentiation or function has not been investigated to date.

We hypothesized in this study that PARK2 may be involved in osteoclast formation and bone resorption. To explore this possibility, we exploited knockdown and overexpression of PARK2 in primary osteoclast culture. PARK2 gene silencing led to a marked reduction in osteoclast formation as a result of mitigated NF-κB activity. In comparison, overexpression of PARK2 had the opposite effect of PARK2 knockdown. We also demonstrated that inhibiting PARK2 *in vivo* could reduce bone destruction in LPS-treated mice. These findings suggest that PARK2 plays a critical role in the formation of RANKL-induced osteoclasts by modulating the NF-κB signaling pathway.

MATERIALS AND METHODS

Antibodies and reagents

All antibodies were purchased from Cell Signaling Technology (USA). PARK2 plasmid (#59416) was purchased from Addgene (USA). RNAiMAX was purchased from Invitrogen (USA). Recombinant human RANKL and macrophage colony-stimulating factor (M-CSF) were purchased from Pepro-Tech (USA).

Preparation of bone marrow macrophages and osteoclast differentiation

Tibia and femurs from 5-week-old male ICR mice were isolated and bone marrow cells were flushed with α-MEM (Welgene, Korea). After lysis of red blood cells, cells were cultured in α-MEM containing 10% fetal bovine serum (FBS) and M-CSF (30 ng/ml) for 24 h. Non-adherent cells were collected and cultured on Petri dishes with M-CSF for 3 days to obtain bone marrow macrophages (BMMs). To generate pre-fusion osteoclasts (pOCs) or mature osteoclasts (OCs), BMMs were cultured in α-MEM containing 10% FBS with RANKL (100 ng/ml) and M-CSF (30 ng/ml) for 2 or 4 days, respectively. After induction of osteoclast differentiation, cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100. Tartrate-resistant acid phosphatase (TRAP) staining was carried out using a leukocyte acid phosphatase kit (Sigma-Aldrich, USA) following the manufacturer's instructions. Cells were observed using a light microscope and TRAP-positive ($TRAP^+$) cells with more than three nuclei were considered as OCs.

Real-time polymerase chain reaction (PCR)

Using TRIzol reagent (Invitrogen), total RNA was isolated. Three micrograms of RNA were used to synthesize cDNA with Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Gene expression levels were analyzed with SYBR Green Master Mix reagents (Kapa Biosystems, USA) using an ABI 7500 instrument (Applied Biosystems, USA). The relative mRNA expression level was calculated using the Ct values. Primer sequences for real-time PCR were as follows: PRKN, 5'-GGTCGATTCTGACACCAGCA-3' (sense) and 5'-TGAACCGTCAGGTGATTCGG-3 (antisense)'; ACP5, 5'-CGACCATTGTTAGCCACATACG-3' (sense) and 5'-TCGTCCTGAAGATACTGCAGGTT-3' (antisense); MMP9, 5'-GACGGCACGCCTTGGTGTAG-3' (sense) and 5'-AGGAGCGGCCCTCAAAGATG-3' (antisense); OSCAR, 5'-AGGAGCGGCCCTCAAAGATG-3' (sense) and 5'-GGGTGACAAGGCCACTTT-3' (antisense); DC-STAMP, 5'-GGGTGCTGTTTGCCGCTG-3' (sense) and 5'-CGACTCCTTGGGTTCCTTGCT-3' (antisense); v-ATPase, 5'-GGGAGACCCTCTTCCCCACC-3' (sense) and 5'-CCACCGACAGCGTCAAACAAA-3' (antisense); OC-STAMP, 5'-ATGAGGACCATCAGGGCAGCCACG-3' (sense) and 5'-GGAGAAGCTGGGTCAGTAGTTCGT-3' (antisense); Hprt, 5'-CCTAAGATGAGCGCAAGTTGAA-3' (sense) and 5'-CCACAGGGACTAGAACACCTGCTAA-3' (antisense).

Gene knockdown

Both control and PARK2 small interfering RNA (siRNA) oligonucleotides were purchased from Invitrogen and Bioneer (Korea). pOCs were obtained by culturing BMMs with RANKL (100 ng/ml) and M-CSF (30 ng/ml) for 2 days. siRNA oligonucleotides (30 nM) were transfected into pOCs overnight using HiPerFect (Qiagen, Germany) following the manufacturer's instructions in the presence of M-CSF and RANKL.

Transfected pOCs were further cultured with M-CSF and RANKL until the indicated days. In some experiments, LPS, TNFα, or IL-1β was additionally included in this culture period.

Western blotting

Cells were lysed using RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP40, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, proteinase inhibitor cocktail, 0.5 mM PMSF, 1 mM NaF, and 1 mM Na_3VO_4) and centrifuged at 14,000 rpm for 20 min. After quantification, equal amounts of cell lysates were separated by 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham Pharmacia, Sweden). Using 5% fat-free milk/Tris-buffered saline, the membranes were blocked for 1 h and followed by incubation with a primary antibody and subsequently a secondary antibody conjugated to horseradish peroxidase. Primary antibodies against IKKα (2682), IKKβ (2684), ERK (9102), JNK (9252), p38 (9212), p65 (8242), IκB (9242), phospho-IKKα/β (2697), phospho-ERK (9101), phospho-JNK (9251), phospho-p38 (9211), phospho-p65 (3031), phospho-IκB (2859), and PARK2 (2132) at 1:1,000 dilution were used to detect their corresponding proteins. The anti-β-actin antibody was used at 1:10,000 dilution. Bound antibodies were detected using enhanced chemiluminescence reagents.

Bone resorption assay

BMMs seeded on dentin slices (Immunodiagnostic Systems, UK) were cultured in α-MEM with M-CSF (30 ng/ml) and RANKL (100 ng/ml) to form pOCs for 2 days. Cells were transfected with siRNA as described above and further cultured until day 12. After removing cells, the dentin slices were wiped with a cotton swab. Images of resorbed area and depth of the resorption pits were measured with a Zeiss LSM 800 laser-scanning microscope (Carl Zeiss, Germany).

Immunocytochemistry

To detect nuclear translocation of p65, BMMs on coverglasses were cultured with RANKL (100 ng/ml) and M-CSF (30 ng/ml) for 2 days and then transfected with siRNA. Transfected cells were serum-starved for 5 h and then stimulated with RANKL (500 ng/ml) for 30 min. Cells were fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton X-100. After permeabilization, cells were blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS), followed by incubation with p65 antibody (1:200) at 4°C overnight and then with a secondary antibody (1:200) for 1h. Coverglasses were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and observed under LSM700 (Carl Zeiss).

PARK2 overexpression

BMMs were seeded in 48 well plates and transfected with the PARK2 plasmid at a concentration of 200 ng DNA per well using Polyfect (Qiagen) according to the manufacturer's protocol.

Calvarial bone resorption experiment

All animal experiments were approved by the Institutional Animal Care and Use Committee at Seoul National Univer-

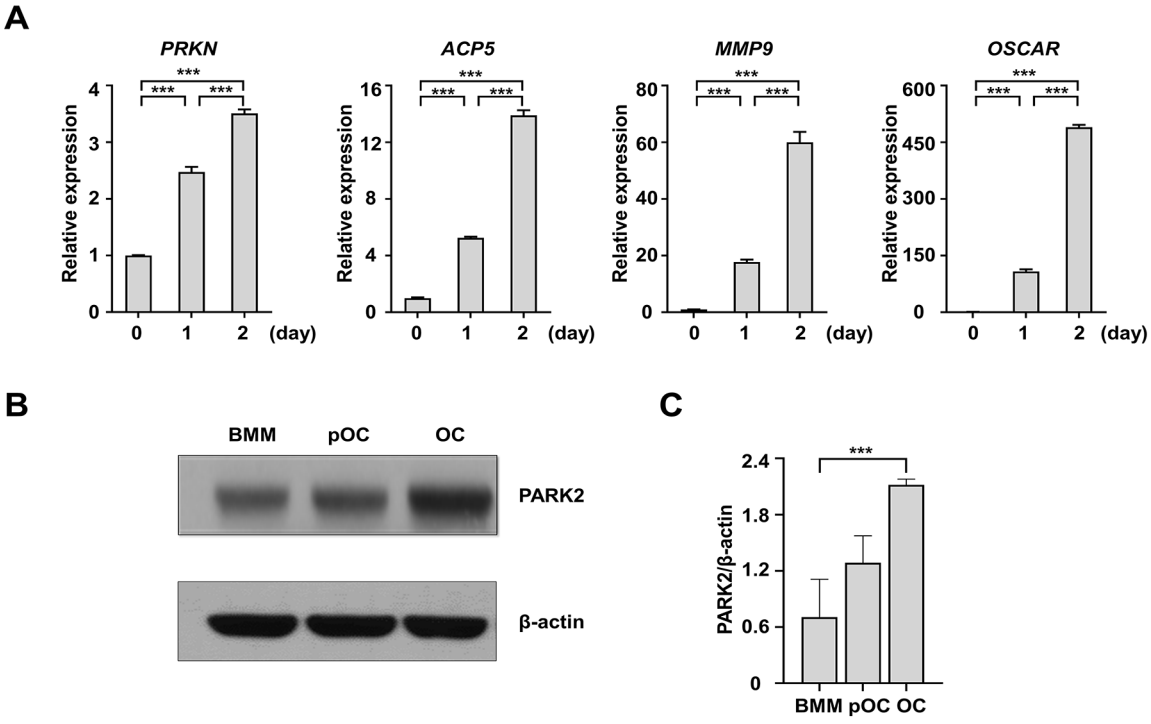

Fig. 1. PARK2 is up-regulated during osteoclastogenesis. (A) The mRNA levels of PARK2 and osteoclast marker genes were analyzed by real-time PCR in BMMs cultured with RANKL and M-CSF for the indicated days. ***$P < 0.001$ versus day 0. (B) PARK2 protein expression during osteoclast differentiation were analyzed by western blotting. (C) The relative level of PARK2 protein was determined by Image J software. ***$P < 0.001$ versus BMM. Data were presented as mean ± SD.

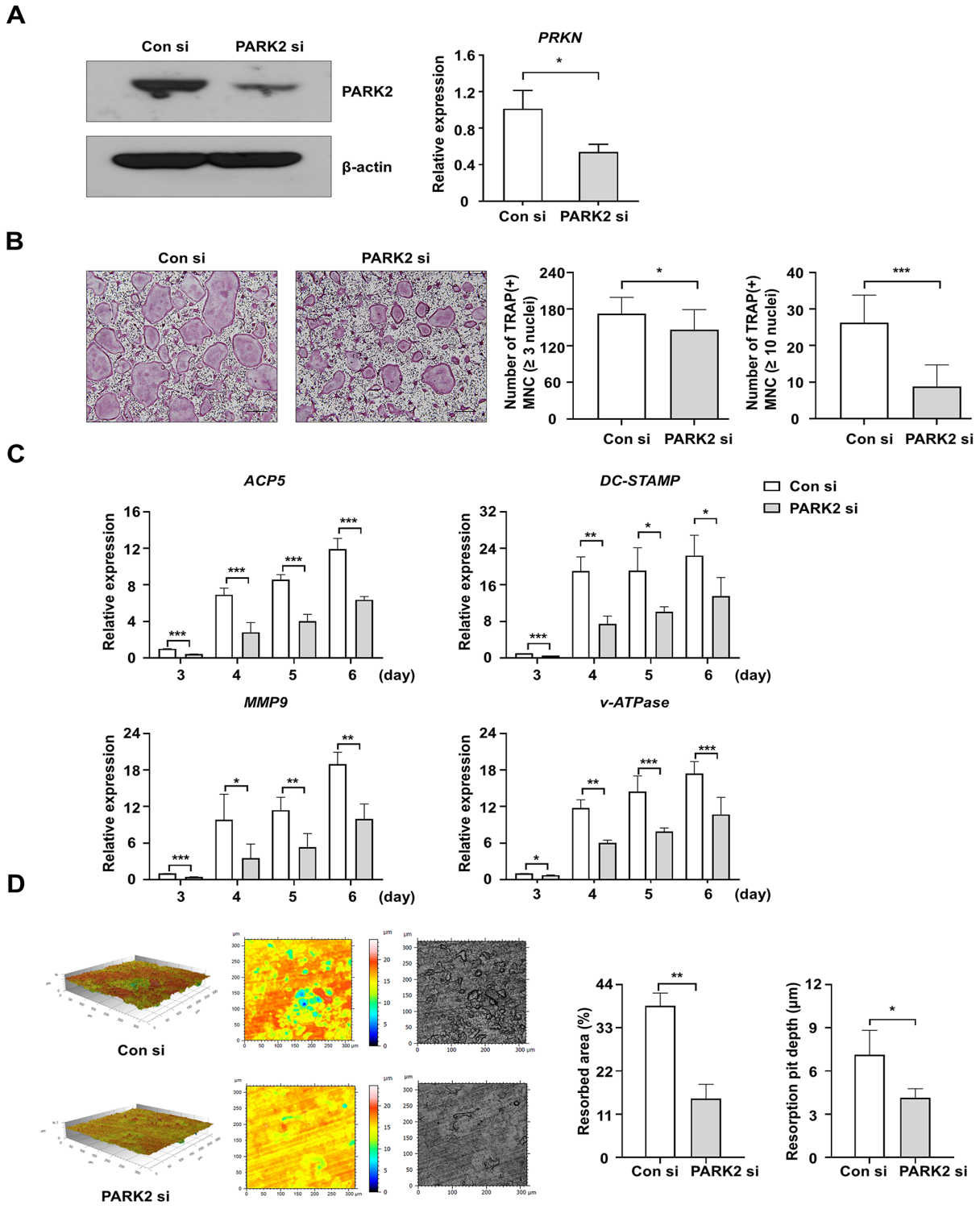

Fig. 2. PARK2 knockdown decreases osteoclast differentiation and function. (A) pOCs transfected with control (Con) or PARK2 siRNA were subjected to western blotting and real-time PCR analyses. $*P < 0.05$ versus the control siRNA group. (B) pOCs transfected with control or PARK2 siRNA were further cultured with RANKL and M-CSF for 3 days and then stained for TRAP. $TRAP^+$ multinucleated cells more than 3, 10 nuclei were counted. Scale bars = 200 μm. $*P < 0.05$, $***P < 0.001$. (C) pOCs transfected with control or PARK2 siRNA were cultured with RANKL and M-CSF until day 6. The mRNA expression levels of ACP5, DC-STMAP, MMP9 and v-ATPase were analyzed by real-time PCR. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Data were presented as mean ± SD. (D) pOCs transfected with control or PARK2 siRNA were cultured on dentine discs until day 12. The dark areas in gray images indicate the resorbed surface of dentin slices. The percentage of resorbed area and depth of resorption pits were presented as mean ± SD. $*P < 0.05$; $**P < 0.01$.

sity (SNU-211130-1) and performed under the approved guidelines. One day before LPS injection (2.5 mg/kg), complexes of control or PARK2 siRNA (20 μM; 30 μl) and Lipofectamine RNAiMAX (10 μl; Invitrogen) were injected to calvariae of 7-week-old ICR mice (n = 6). Subsequent siRNA injections were performed at 2-days intervals for 5 days after the LPS injection. After sacrifice, calvariae were collected for TRAP staining and micro-computed tomography (μCT) analyses using a high-resolution Skyscan 1172 (70 kV, 141 μA, 0.5 mm aluminum filter, 12.84 μm pixel size; SkyScan, Belgium).

Statistical analysis

Data are represented as the mean with SD. Analyses were performed using Student's *t*-test. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using using IBM SPSS Statistics (ver. 25.0; IBM, USA).

RESULTS

PARK2 is up-regulated during osteoclastogenesis

Based on the reported function of PARK2 in NF-κB signaling pathways, we hypothesized that PARK2 might act as a positive regulator of osteoclast differentiation and bone resorption. By using real-time PCR and western blotting, we first determined whether RANKL can regulate PARK2 expression levels during osteoclast differentiation from mouse BMMs. PARK2 mRNA expression was significantly increased in BMMs treated with RANKL for 1 or 2 days, along with ACP5, MMP9, and OSCAR, osteoclast marker genes (Fig. 1A). Additionally, PARK2 protein levels were significantly increased during osteoclastogenesis (Figs. 1B and 1C). These findings suggest that RANKL induces PARK2 expression during the early stages of osteoclast differentiation.

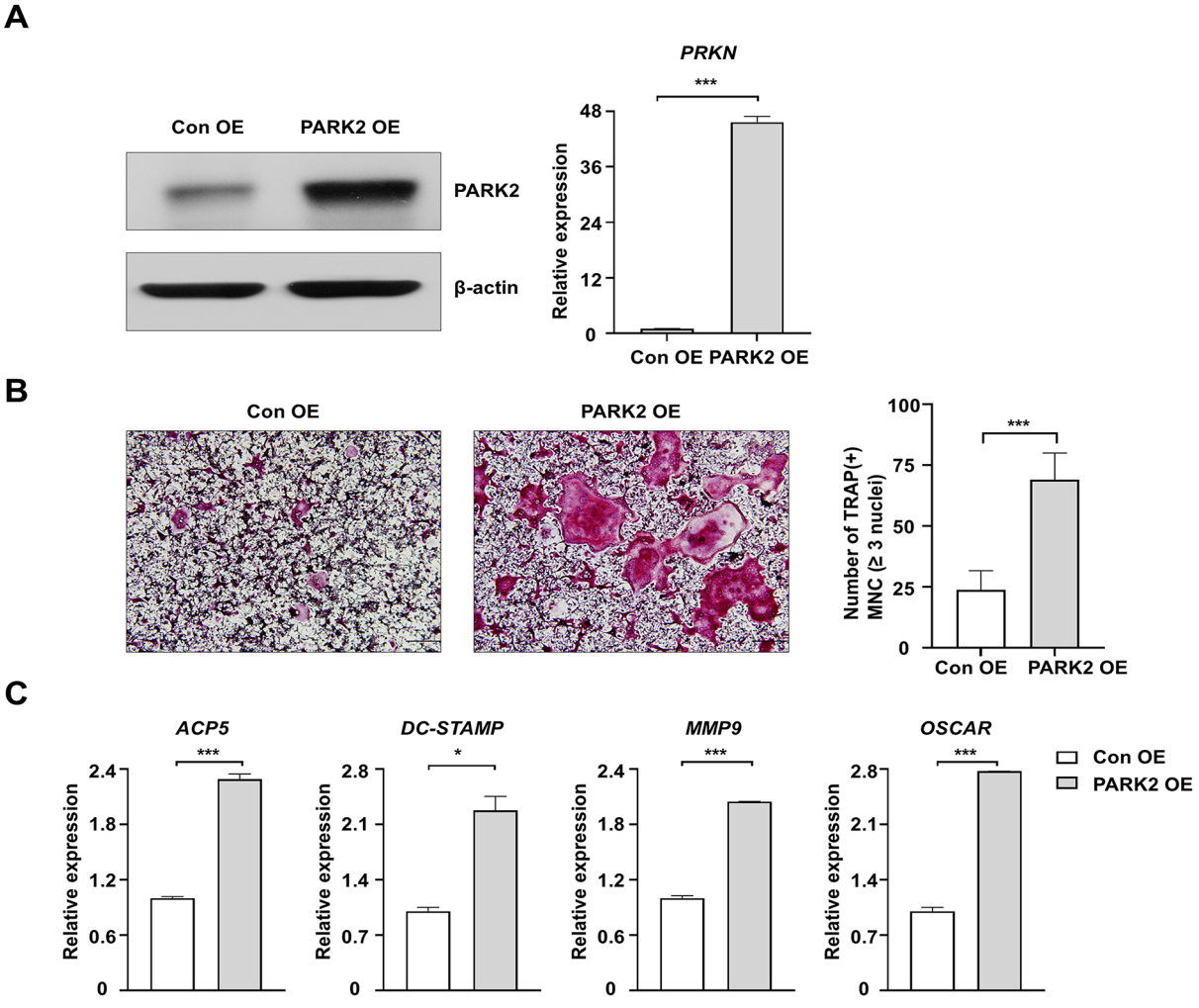

Fig. 3. PARK2 overexpression accelerates osteoclast differentiation. (A) BMMs transfected with control (Con) vector or PARK2 plasmid were subjected to western blotting (left panel) and real-time PCR (right panel) analyses. ***$P < 0.001$. (B) BMMs transfected with either control or PARK2 plasmid were cultured with RANKL and M-CSF for 5 days and stained for TRAP. Representative images (left panel) and quantification of $TRAP^+$ multinucleated cells are shown (right panel). Scale bars = 200 μm. ***$P < 0.001$. (C) mRNA levels of ACP5, DC-STAMP, MMP9 and OSCAR were measured by real-time PCR. *$P < 0.05$; ***$P < 0.001$.

Gene knockdown of PARK2 decreases osteoclast differentiation and activity

To investigate whether PARK2 functionally contributes to osteoclast differentiation, we knockdowned pOCs and further cultured the cells with RANKL and M-CSF until day 6. At both the transcript and protein levels, a significant decrease in PARK2 expression was observed (Fig. 2A). PARK2 siRNA-treated cells generated fewer multinucleated TRAP$^+$ cells than control knockdown cells (Fig. 2B). The effect of PARK2 knockdown was more pronounced in large TRAP$^+$ cells with more than 10 nuclei. In parallel with forming smaller osteoclasts, the up-regulation of late markers of osteoclast differentiation like DC-STAMP, MMP9, and v-ATPase by RANKL was also lower in PARK2 knockdown cells compared to the control knockdown cells (Fig. 2C). To determine the effect of PARK2 silencing on osteoclast function, pOCs were cultured on bovine bone slices with either PARK2 or control siRNAs and then cultured with RANKL and M-CSF until day 12. Both the resorption area and pit depth were reduced in the PARK2-deficient group (Fig. 2D). Cytoskeletal reorganization to generate the actin ring structure is a prerequisite for bone-resorbing function in OCs (Takahashi et al., 2007). When we assessed actin assembly in OCs by confocal microscopy, the deletion of PARK2 in pOCs exhibited a less clear actin ring formation with a reduced number of actin-ring positive OCs than the control knockdown cells (data not shown). Taken together, these findings indicate that PARK2 deficiency impairs the generation of functional osteoclasts.

Overexpression of PARK2 accelerates osteoclast differentiation

We next examined the effect of PARK2 overexpression on osteoclastogenesis. Both real-time PCR and western blotting analysis revealed that transfection of the PARK2 plasmid into BMMs increased the level of PARK2 expression (Fig. 3A). PARK2 transfected BMMs produced a greater number of TRAP$^+$ multinucleated osteoclasts after 5 days of osteoclastogenic culture (Fig. 3B). PARK2 overexpression consistently increased late-stage osteoclast differentiation markers such as ACP5, DC-STAMP, MMP9, and OSCAR (Fig. 3C). This result provides further evidence for the involvement of PARK2 in RANKL-induced osteoclast differentiation.

PARK2 activates osteoclastogenesis by enhancing NF-κB signaling

Given that ubiquitination is required for the regulation of NF-κB pathways and PARK2 functions as an E3 ubiquitin ligase, it is conceivable that PARK2 modulates NF-κB signaling via an ubiquitin-based mechanism. To investigate the possibility that PARK2 regulates NF-κB signaling during osteoclast differentiation, we examined RANKL-driven signaling pathways in either PARK2 or control knockdown pOCs. RANKL induced the phosphorylation of IKKα/β, IκB, and p65 NF-κB in control knockdown cells (Fig. 4A). PARK2 knockdown suppressed this RANKL-induced phosphorylation of NF-κB pathway components. In contrast, silencing PARK2 had no effect on the RANKL-induced phosphorylation of p38, JNK, or ERK (Fig. 4B). Notably, silencing PARK2 markedly reduced p65 nuclear translocation (Fig. 4C). To further validate the involvement PARK2 in NF-κB signaling, we next examined the effect of PARK2 overexpression on NF-κB in osteoclasts. PARK2 overexpression increased the level of phosphorylated p65, and Bay 11-7085, an NF-κB inhibitor, inhibited this response (Fig. 4D). Collectively, our results demonstrated that PARK2 promotes osteoclast differentiation via the NF-κB signaling pathway without interfering with MAPK signaling.

PARK2 silencing reduces osteoclastogenesis stimulated by inflammatory factors

Pro-inflammatory cytokines such as TNFα and IL-1β, as well as the bacterial pathogen LPS, induce inflammatory bone loss in osteoclasts by activating the NF-κB signaling cascade. Since our data showed that silencing PARK2 inhibits the phosphorylation of central NF-κB signaling molecules, we examined the role of PARK2 in inflammatory cytokine-induced osteoclastogenesis. PARK2 transcript levels increased in response to LPS, TNFα, and IL-1β, similar to the results obtained with RANKL treatment (Fig. 5A). Following that, we investigated PARK2's role in osteoclastogenesis under inflammatory conditions. pOCs were transfected with control or PARK2 siRNA and further cultured with LPS, TNFα, or IL-1β in the presence of RANKL and M-CSF. PARK2 deficiency abolished the stimulatory effect of LPS, TNFα, and IL-1β on osteoclastogenesis (Fig. 5B). Consistently, the elevation in mRNA levels of ACP5, DC-STAMP, OC-STAMP, and v-ATPase by LPS was attenuated by PARK2 siRNA (Fig. 5C). Given that PARK2 knockdown suppressed the generation of osteoclasts in response to LPS, we investigated whether PARK2 knockdown also inhibited LPS-induced NF-κB signaling. LPS increased the phosphorylation of IKKα/β, IκB, and p65 in control knockdown cells. PARK2 knockdown suppressed the phosphorylation of NF-κB signaling molecules induced by LPS (Fig. 5D). These data indicate that PARK2 controls the osteoclast formation in inflammatory conditions by regulating the NF-κB pathway.

PARK2 siRNA protects against LPS-induced inflammatory bone loss

To explore whether PARK2 plays a role in LPS-induced inflammatory bone loss *in vivo*, mice calvariae were injected with PARK2 or control siRNA complexes. At 5 days after LPS injection, TRAP staining and μCT analysis of whole calvariae were carried out. Real-time PCR analysis revealed a significant decrease in PARK2 expression in calvariae (Fig. 6A). LPS injection led to a prominent reduction in calvarial bone volume (BV/TV) in control mice, while the LPS-induced bone loss was significantly attenuated in mice injected with PARK2 siRNA (Fig. 6B). The percentage of area stained positive for TRAP was dramatically increased by LPS injection. This LPS-induced increase in TRAP$^+$ area was evidently ablated in the group received PARK2 siRNA (Fig. 6C). Taken together, these results demonstrate that local treatment of PARK2 siRNA has a potent protective effect against LPS-induced inflammatory osteolysis *in vivo*.

DISCUSSION

Some ubiquitin E3 ligases have been shown to regulate osteoclast function and differentiation. One example is the pro-

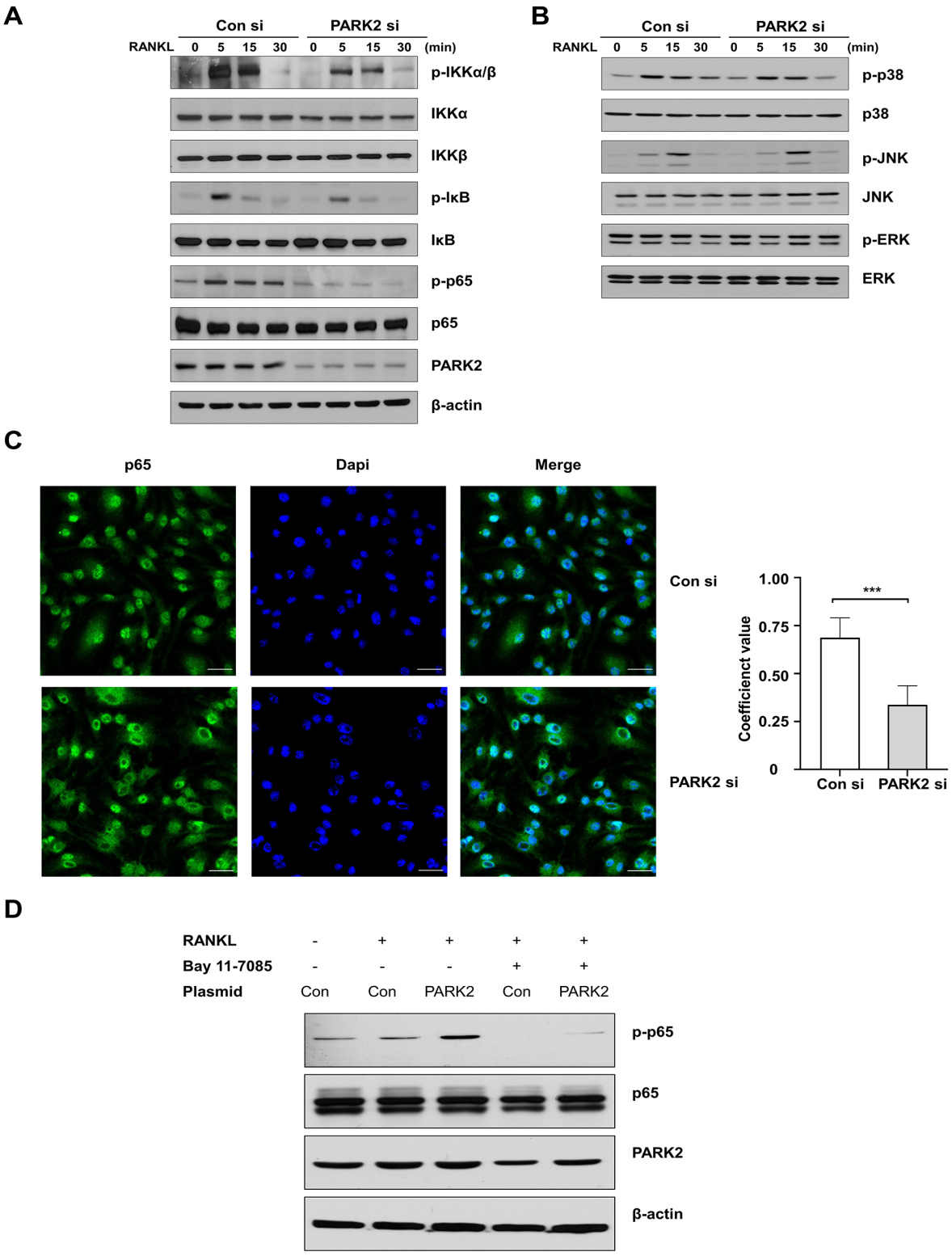

Fig. 4. PARK2 promotes osteoclast differentiation through NF-κB signaling. (A and B) pOCs transfected with control (Con) siRNA or PARK2 siRNA were starved with serum-free medium and then stimulated with RANKL (500 ng/ml) for the indicated time. Both phospho-forms and total forms of the NF-κB signaling components (A) and MAPKs (B) were determined by western blotting. (C) pOCs transfected with control siRNA or PARK2 siRNA were serum-starved and then stimulated with RANKL (500 ng/ml) for 15 min. Representative confocal images are presented. Scale bars = 20 μm. ***$P < 0.001$. (D) BMMs transfected with control or PARK2 plasmid were cultured with or without BAY 11-7085 (5 μM) for 2 days. Protein levels of phospho-forms and total forms of p65 were detected by western blotting.

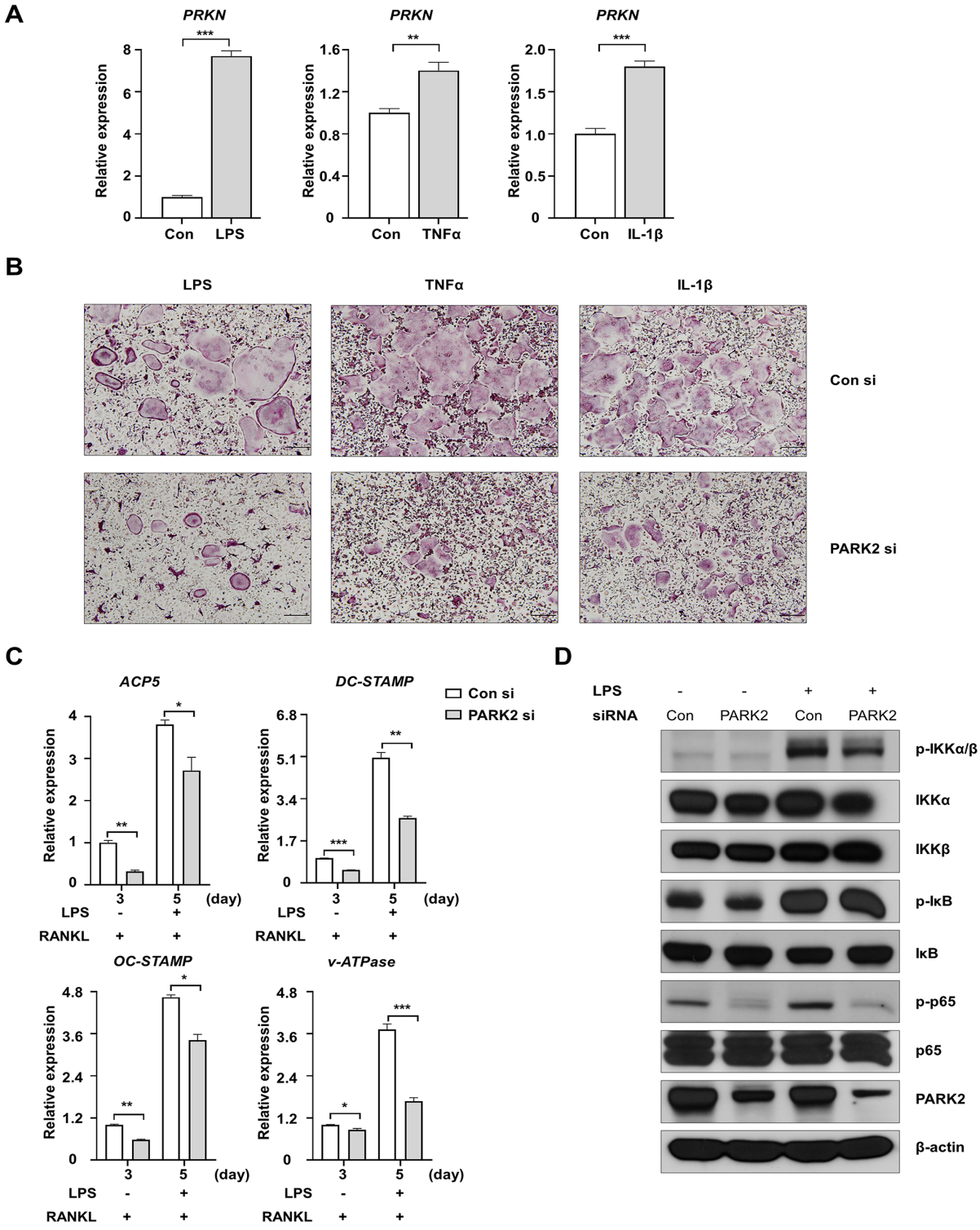

Fig. 5. PARK2 silencing reduces inflammatory factor-induced osteoclastogenesis. (A) BMMs were cultured in the presence of LPS (100 ng/ml), IL-1β (10 ng/ml), and TNFα (40 ng/ml) for 24 h and mRNA levels of PARK2 were determined by real-time PCR. $^{**}P < 0.01$; $^{***}P < 0.001$. Con, control. (B) pOCs transfected with control siRNA or PARK2 siRNA were cultured in the presence of LPS (100 ng/ml), IL-1β (10 ng/ml), or TNFα (40 ng/ml) with RANKL and M-CSF until day 4 and stained for TRAP. Scale bars = 200 μm. (C) mRNA levels of ACP5, DC-STAMP, OC-STAMP, and v-ATPase in PARK2 or control siRNA transfected pOC cultured with RANKL and LPS (100 ng/ml) were measured by real-time PCR. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (D) pOCs transfected with control siRNA or PARK2 siRNA were serum-starved and stimulated with LPS (200 ng/ml) for 15 min. Protein levels of the phospho-forms and total forms of IKK, IκB, and p65 were determined by western blotting.

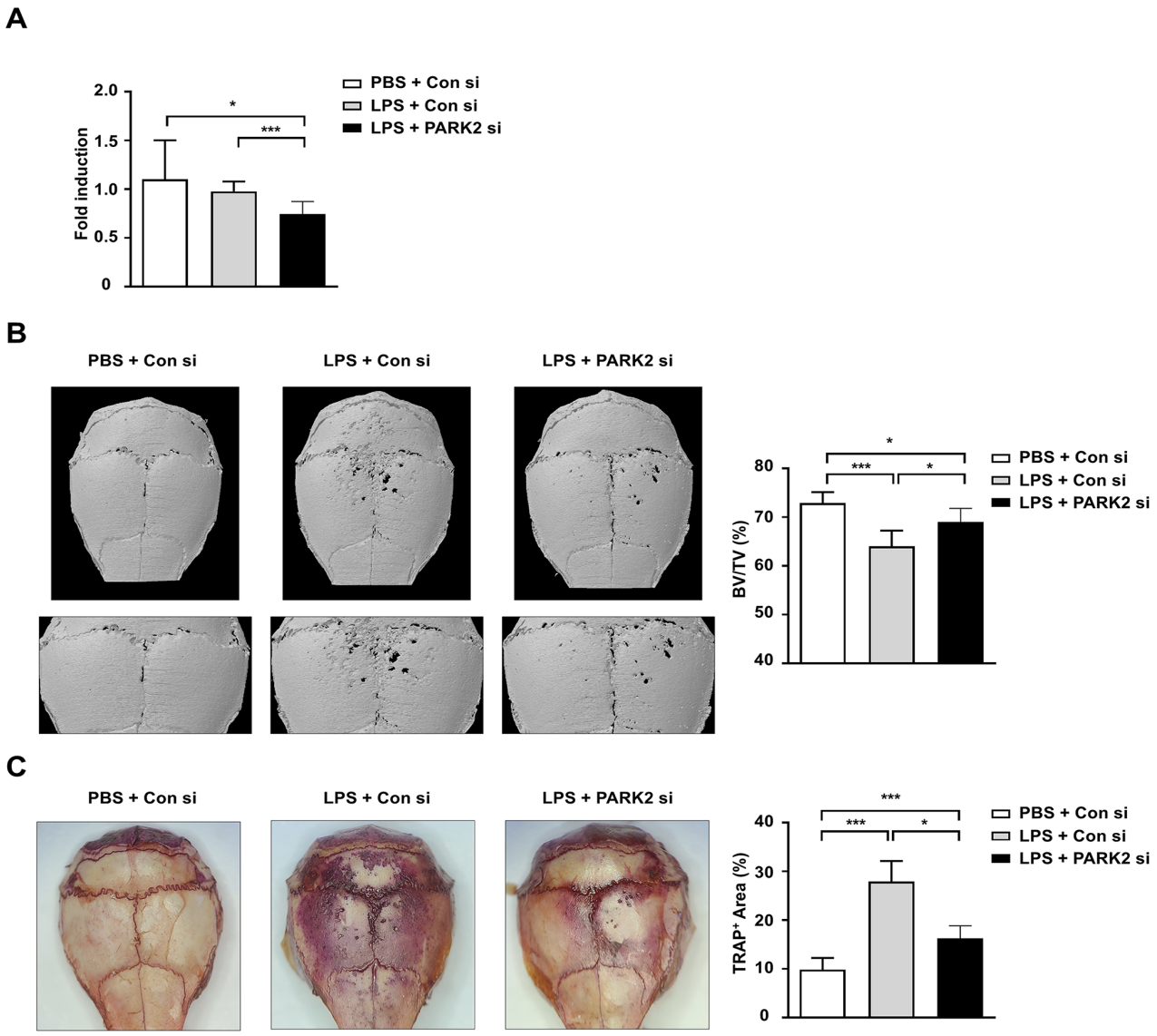

Fig. 6. PARK2 siRNA protected against LPS-induced bone loss. (A) LPS (2.5 mg/kg) or vehicle (PBS) was injected at the midline of calvariae. Either control (Con) or PARK2 siRNA was locally injected near the LPS-injected site. The knock-down efficiency was determined by real-time PCR. $*P < 0.05$; $***P < 0.001$. (B) Representative μCT images (left panel) and measurements of bone volume per tissue volume (BV/TV) (right panel) are shown. $*P < 0.05$; $***P < 0.001$. (C) Total calvariae were stained for TRAP activity (left panel). Quantitative values of $TRAP^+$ area (%) are shown at the right panel. $*P < 0.05$; $***P < 0.001$.

teins of Cbl family that act as signaling adaptors downstream of Src kinase and are involved in the resorption and migration of osteoclasts (Horne et al., 2005; Sanjay et al., 2001; Tanaka et al., 1996). Additionally, it was demonstrated that the ubiquitin E3 ligase TRAF6 plays a critical role in osteoclast differentiation via its K63-linked auto-ubiquitination (Lamothe et al., 2007). In the present study, we revealed that the E3 ligase PARK2 functions as a positive regulator in osteoclastogenesis. Inhibitory effects on osteoclast differentiation and bone resorption function were observed when PARK2 expression was decreased (Fig. 2). The activation of the NF-κB pathway induced by both RANKL and LPS was interfered with PARK2 knockdown (Figs. 4 and 5). Furthermore, *in vivo* injection of PARK2 siRNA significantly abated the bone loss induced by LPS in mice calvariae (Fig. 6). Based on our findings, we propose that PARK2 promotes osteoclastogenesis through the regulation of the NF-κB signaling pathway (Fig. 7).

In osteoclast differentiation, the RANKL-RANK engagement initiates multiple downstream signaling cascades, including the NF-κB, MAPK, and phosphatidylinositol pathways (Wada et al., 2006). Our study showed that PARK2 enhanced RANKL-induced NF-κB activation during osteoclast development. Similarly, Wang et al. (2018) reported that PARK2 stimulates NF-κB transcriptional activity in a retinal ganglion-like cell line in response to TNFα. However, it has not been investigated whether PARK2 exerts its effects on osteoclast

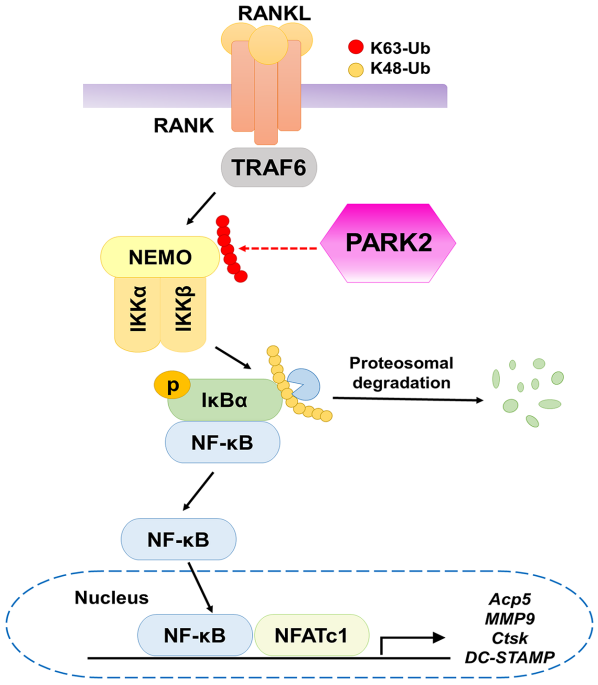

Fig. 7. Schematic drawing on the role of PARK2 in osteoclastogenesis. Upon activation of RANK by RANKL, TRAF6 is recruited and activated. Downstream of TRAF6, PARK2 may interact with NEMO and enhance its linear ubiquitination, resulting in the activation of the IKK complex. The activated IKK complex facilitates proteasomal degradation of IκB and triggers p65 translocation to the nucleus, leading to gene transcription of osteoclast marker genes like ACP5, MMP9, and Ctsk.

differentiation. The current study demonstrated that PARK2 silencing inhibited the phosphorylation of IKKα/β and IκBα (Fig. 4A), and decreased the nuclear translocation of p65 (Fig. 4C). Thus, PARK2 appears to control NF-κB-dependent osteoclast differentiation by modulating IKK activity. It is possible that this activation of IKK is mediated by NEMO ubiquitination, as has been demonstrated previously in other cell types (Henn et al., 2007; Muller-Rischart et al., 2013). However, since there are several proteins ubiquitinated by PARK2 (Henn et al., 2007; Laforge et al., 2016; Wang et al., 2018), additional studies on the direct molecular target of PARK2 are required to elucidate the molecular mechanisms for how PARK2 modulates NF-κB signaling pathways during osteoclastogenesis.

Osteoclasts share the same myeloid cell lineage with macrophages. Macrophages prior to polarization (M0) acquire distinct characteristics as a result of their microenvironment exposure—classically activated or inflammatory M1 macrophages versus alternatively activated or revolutionary M2 macrophages (Goodman et al., 2022; Martinez et al., 2008). It is unknown whether stimulating macrophage polarization would have a detrimental effect on osteoclastogenesis by driving precursor cells into committed M1 or M2 lineages, or whether M1 or M2 cells have a distinct capacity for osteoclastic differentiation (Li et al., 2019; Zhao et al., 2015). Regarding macrophage polarization, previous reports have shown that IL4-induced M2 polarization was enhanced in PARK2-deficient BMMs (Bhatia et al., 2019). In addition, Rictor expression was found to be increased in $CD11b^+$ monocytes and $F4/80^+$ macrophages isolated from the peritoneal cavity of PARK2 knockout mice (Bhatia et al., 2019). As a result, it is possible that PARK2's role in macrophage polarization is associated with osteoclastogenesis. LPS, a stimulator of M1 polarization (Zhu et al., 2015), has a dual role in osteoclast formation: it inhibits osteoclast differentiation in BMMs but promotes osteoclastogenesis in RANKL-primed pOCs (Liu et al., 2009). In the current study, we observed that silencing PARK2 in pOCs significantly inhibited both LPS-induced NF-κB activation (Fig. 5D) and osteoclast differentiation (Fig. 5B). Additionally, it was also observed that PARK2-knockdown reduced osteoclastogenesis in a calvarial LPS-injection model (Fig. 6). Thus, we believe that PARK2's control of osteoclast differentiation is primarily due to its regulation of the NF-κB signaling pathway, although we cannot rule out the possibility that PARK2's effect on macrophage polarization contributes to its role in osteoclastogenesis. In further studies, we intend to conduct additional research to determine whether PARK2 modulates macrophage polarization and function in inflammatory bone diseases such as periodontitis.

Mitophagy is a form of selective autophagy, which plays pivotal roles in modulating mitochondrial quality control by eliminating dysfunctional mitochondria (Ashrafi and Schwarz, 2013). When mitochondria are damaged, PINK1 is stabilized in the mitochondrial outer membrane (MOM), which promotes PARK2 retention on damaged mitochondria and induces its phosphorylation, activating E3 ligase activity of PARK2 (Jin and Youle, 2012). Upon activation, PARK2 ubiquitinates various proteins in MOM to recruit autophagy receptors, which binds to ubiquitin-tagged MOM proteins, and promotes mitochondrial clearance through autophagy (Gao et al., 2015; Geisler et al., 2010; Poole et al., 2010; Ziviani et al., 2010). However, the role of mitophagy in osteoclasts is not well defined. Therefore, the question of whether the observed PARK2-mediated regulation of osteoclast differentiation relies on the mitophagy dysfunction was intriguing. To address this question, we investigated whether PARK2 knockdown affects the co-localization of Tomm20 (mitochondria marker) with LC3B (autophagy marker) in pOCs. Between control and PARK2 knockdown cells, there was no difference in the co-localized regions of Tomm20 and LC3B (data not shown). Besides, contrary to previous reports that spermidine (mitophagy inducer) inhibits osteoclast differentiation (Madeo et al., 2018; Yamamoto et al., 2012), our study found that overexpression of PARK2, which is expected to stimulate mitophagy, promoted osteoclastogenesis in BMMs (Fig. 3). Therefore, we suppose that the role of PARK2-dependent mitophagy is only meager in osteoclast differentiation.

In conclusion, we established for the first time that PARK2 plays an important role in osteoclast differentiation by regulating the NF-κB signaling pathways. Additionally, we demonstrated that administration of PARK2 siRNA protected mice from bone loss caused by LPS. Agents that inhibit PARK2 expression or catalytic activity may have therapeutic potential

in bone diseases.

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AUTHOR CONTRIBUTIONS
S.J.H., M.K.K., S.J., and H.-H.K. perceived the experiments. S.J.H., S.J., J.S.J., and S.M. performed the experiments. S.J.H., S.J., J.S.J., and S.M. analyzed data. M.K.K. and J.O.K. provided expertise and feedback. S.J.H. and H.-H.K. wrote the manuscript.

CONFLICT OF INTEREST
The authors have no potential conflicts of interest to disclose.

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