Lack of NOD2 attenuates ovariectomy-induced bone loss via inhibition of osteoclasts

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Abstract

Nucleotide-binding oligomerization domain-2 (NOD2) is a pattern recognition receptor of the innate immune system. It interacts with serine–threonine kinases to induce activation of nuclear factor κ B (NF- κ B), which is important for receptor activator of nuclear factor kappa-B ligand (RANKL) signaling. We tested the idea that NOD2 modulates bone metabolism via an action on osteoclasts (OCs). The absence of NOD2 reduced ovariectomy-induced bone loss in mice, and lowered the area and the activity of OCs, by impairing RANKL signaling. It also reduced the level of reactive oxygen species (ROS), as well as of NF- κ B-DNA binding upon RANKL exposure. NOD2 was found to physically interact with nicotinamide adenine dinucleotide phosphate oxidase 1, and this led to increased production of ROS in OCs. Our data suggest that NOD2 contributes to bone loss in estrogen deficiency by elevating ROS levels in OCs.

Key Words

- ▶ nucleotide-binding oligomerization domain 2
- ▶ osteoclast
- ▶ receptor activator of nuclear factor- κ B ligand signaling
- ▶ reactive oxygen species
- ▶ nicotinamide adenine dinucleotide phosphate oxidase 1

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Introduction

Bone is a dynamic tissue, aging bone being constantly replaced by new tissue. Bone remodeling occurs via the balanced action of osteoblasts and OCs. Loss of ovarian function increases the rate of bone remodeling and results in a loss of bone mass due to excessive bone resorption (Weitzmann & Pacifici 2006). The enhanced bone remodeling enlarges the remodeling space and increases cortical porosity and the resorption area on trabecular surfaces (Eriksen *et al.* 1999). This is primarily due to elevated osteoclastogenesis involving neighboring cells (Weitzmann & Pacifici 2006), as well as increased resorption due to longer OC survival (Hughes *et al.* 1996). Since recent findings suggest a close interrelationship

between bone and immune system, postmenopausal osteoporosis could be an example reflecting mutual influence of immune system, bone and endocrine system (Ginaldi & De Martinis 2016). Ovariectomy (OVX) in mice is a commonly accepted model reflecting the human menopause. Loss of ovarian function has been reported to be associated with metabolic pathologies mainly due to chronic inflammation (Rogers *et al.* 2009, Choi *et al.* 2013, 2015, Kim *et al.* 2013), suggesting that chronic inflammation contributes to the metabolic complications such as bone loss observed upon OVX.

OCs, multinucleated giant cells responsible for bone resorption, are formed from hematopoietic cells

of the monocyte/macrophage lineage and share some of their morphological and functional properties. They are responsible not only for physiological bone remodeling, but also for the bone destruction associated with chronic inflammatory disease (Crotti *et al.* 2015, Ginaldi & De Martinis 2016). However, OCs are affected by other OCs as well as by cross-talk with neighboring cells. To promote osteoclastogenesis, two essential molecules are generated by bone marrow mesenchymal cells: macrophage-colony stimulating factor (M-CSF) and RANKL, a member of the tumor necrosis factor (TNF) family (Kong *et al.* 1999, Suda *et al.* 1999). Binding of RANKL to its receptor, RANK, on OC precursor cells induces and activates transcription factors including NF- κ B (Cappellen *et al.* 2002) needed to initiate signals for OC differentiation, and this results in the expression of OC-specific genes such as tartrate-resistant acid phosphatase (TRAP) and calcitonin receptor.

Different pathogen-associated molecular patterns (PAMPs) such as flagellin, which are important microbial components, unique nucleic acid structures and bacterial cell wall constituents, are recognized by the corresponding specific receptors. Toll-like receptors (TLR) sense PAMPs on the cell surface and within endosomes, whereas a cytosolic pattern recognition receptor (PRR), nucleotide-binding and oligomerization domain 2 (NOD2) recognizes muramyl dipeptides (MDPs), breakdown products of bacterial cell walls in the cytosol (Girardin *et al.* 2003), suggesting another way that invading microorganisms can be recognized. Activation of NOD2 is triggered by direct or indirect recognition of MDPs through its C-terminal leucine-rich repeat region (LRR) in the cytosol, and is followed by NOD-mediated oligomerization and direct interaction with receptor-interacting protein 2, leading to the formation of a NOD2 'signalosome complex' and further activation of NF- κ B (Rosenstiel *et al.* 2008). Mutation of *NOD2*, which is involved in susceptibility to Crohn's disease, a chronic inflammatory intestinal disorder, results in impaired MDP recognition and attenuated NF- κ B activation (Hugot *et al.* 2001), indicating that NOD2 is involved in many aspects of chronic inflammation. In the present study, we investigated the hypothesis that NOD2 promotes OVX-induced bone loss by amplifying RANKL-induced signaling in OCs.

Materials and methods

Animals and study design

Nod2^{-/-} (*Nod2*-knockout (KO)) mice were purchased from the Jackson Laboratory. The mice were bred with C57BL/6J

mice and maintained by crossing in the animal facility of the University of Ulsan. Six-week-old *Nod2*^{+/+} (WT) and *Nod2*^{-/-} (*Nod2*-KO) mice on a background of C57BL/6J mice were subjected to OVX (*n*=6) or sham operation (*n*=6) under anesthesia using a mixture of Zoletil and Rompun. Eight weeks after surgery, bone density was analyzed, and genomic DNA was extracted from the tails of the mice for genotyping by PCR. All mice were housed in a specific pathogen-free animal facility, and animal care and all procedures were conducted according to protocols and guidelines approved by the University of Ulsan Animal Care and Use Committee (UOUACUC). The standards employed were approved by the latter Committee (UOU2011-009). For visualizing the architecture of the long bone, femurs were analyzed by scanning with a high-resolution micro-computed tomography (μ CT) imaging system in a SkyScan 1072 System (SkyScan, Kontich, Belgium) set to 6.9 μ m effective detector pixel size and a threshold of 77–255 mg/cc. Trabecular bone was assessed in a 1.5 mm region 0.2 mm below the distal growth plate of the femur. Three-dimensional analyses were performed with CT volume software (ver. 1.11; SkyScan) using a total of 250–300 tomographic slices. To examine *in vivo* TRAP-positive OCs, mouse femora were excised, cleaned with a soft tissue and decalcified in EDTA. Representative histological sections of the distal femoral metaphyses of WT and *Nod2*-KO mice were stained for TRAP to identify OCs (original magnification, $\times$ 200). As *in vivo* markers of bone resorption, serum C-telopeptide fragments of collagen type 1 (CTX-1) were determined with a RatLaps enzyme immunoassay (EIA) according to the manufacturer's instructions (Immunodiagnostic Systems Inc., Woburn, MA, USA). Serum osteocalcin was measured with an osteocalcin EIA kit (Biomedical Technologies Inc., Stoughton, MA, USA) and alkaline phosphatase (ALP) by a colorimetric kinetic assay (BioAssay Systems, Hayward, CA, USA). Serum H₂O₂ was evaluated with an OxiSelect hydrogen peroxide assay kit (Cell Biolabs, Inc., San Diego, CA, USA).

OC formation

Bone marrow cells were isolated from mice as described previously (Van Phan *et al.* 2013). Cells from sham and OVX mice were obtained for *ex vivo* OC formation 7–8 weeks after surgery. Femora and tibiae were removed aseptically and dissected free of adherent soft tissue. The marrow cavity was washed with α -MEM from one end of the bone using a sterile 21-gauge needle after dissecting the bone ends, and a single-cell suspension was prepared with

a Pasteur pipette. The resulting bone marrow suspension was washed twice and incubated on plates with M-CSF (20 ng/mL) for 16 h. Floating cells were harvested and loaded on a Ficoll-Hypaque gradient and the cells at the interface were harvested. Two more days of incubation resulted in large populations of monocyte/macrophage-like cells adhering to the culture plates. Floating cells were discarded by washing the dishes with phosphate-buffered saline (PBS), and the adherent cells (bone marrow-derived macrophages (BMMs)) were collected and seeded on plates. These cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and found to be negative for CD3 and CD45R and positive for CD11b (Ke *et al.* 2015). The lack of growth without M-CSF confirmed the absence of contaminating stromal cells. M-CSF and RANKL (40 ng/mL) were added to the cells, and the incubation medium was replaced on day 3. When MDP (10 µg/mL, Sigma Chemical) was treated, it was added to the cells 10 min before RANKL treatment. After incubation for the indicated times, the cells were fixed in 10% formalin for 10 min and stained for TRAP as described (Van Phan *et al.* 2013). OC numbers were evaluated blind by counting TRAP-positive multinucleated cells (MNCs) (three or more nuclei) per well using an eye piece graticule at a magnification of ×100. Cultures in the presence of M-CSF with and without RANKL were used as positive and negative control, respectively. Whole bone marrow cells from sham and OVX mice were stimulated with 1,25(OH)₂D₃ (10 nM) and PGE₂ (1 µM) for *ex vivo* OC formation for 7–8 days, and the incubation medium was replaced every 3 days. For E₂ treatment *in vitro*, BMMs were cultured in α-MEM without phenol red (Life Technologies) containing 10% charcoal-treated FBS (de Faria *et al.* 2016). The resorptive activity of OCs was determined with a lacunar resorption assay on dentine disks placed in wells (Jimi *et al.* 1999). Mature OC cells were generated by incubation with M-CSF and RANKL in the presence or absence of MDP on dentine slices for 6–7 days. Mature OCs with and without RANKL in the presence of M-CSF were used as positive and negative controls, respectively. The slices were cleaned by ultrasonication in 1 M NH₄OH to remove adherent cells and stained with Mayer's hematoxylin (Sigma) to visualize resorption pits. Resorption pit areas were measured with ImageJ 1.37v.

Transfection of BMMs with siRNA

The BMMs were transfected with small interfering RNAs (siRNA) against *Nod2* (sc-30199; Santa Cruz

Biotechnology), *Nox1* (sc-25545) or scrambled siRNA (scRNA) (Santa Cruz) using Lipofectamine RNAiMAX (Invitrogen). Lipofectamine diluted in α-MEM was mixed with an equal volume of α-MEM containing the siRNA. After 20 min of incubation, 100 µL of RNAiMAX/siRNA were added to the cells, to a final volume of 700 µL. After 8-h incubation, the cells were replated in serum-containing medium, cultured for another 2 days, and mRNA levels were analyzed by quantitative polymerase chain reaction (qPCR).

RNA isolation and qPCR

Total RNA was reverse-transcribed with random primers and M-MLV reverse transcriptase (Promega). qPCR used SYBR Green 1 Taq polymerase (Qiagen) and appropriate primers on a StepOnePlus Real-Time System (Applied Biosystems). Primer specificity was confirmed by melting curve analysis and agarose-gel electrophoresis. The housekeeping gene for GAPDH was amplified in parallel with genes of interest. Relative copy numbers compared to GAPDH were calculated using $2^{-\Delta\Delta Ct}$. Primer sequences were 5'-ggcaccctgaagtgacatttgc-3' and 5'-acatctcccacagagtgtaatcc-3' (NOD2); 5'-tccatttcctctctggagtgcat-3' and 5'-ggcattggtagtgctgttgc-3' (NOX1); 5'-ctccaacaagtgcttgga-3' and 5'-gaagcagtagatagtcgca-3' (calcitonin receptor); 5'-gaccaccttgcaatgtctctg-3' and 5'-tggctgaggaagtcattgagtg-3' (TRAP); 5'-gtgggtgtcaagtttctgc-3' and 5'-ggtagtcttctccatagc-3' (cathepsin K); 5'-aataacatgcgagccatcac-3' and 5'-tcaccctgtgttctctc-3' (nuclear factor of activated T cells, cytoplasmic 1 (NFAT2)); 5'-agacgtggttaggaatgcagctc-3' and 5'-tcctccatgaacaacagttcca-3' (DC-STAMP); 5'-ttcagtgctatccaggactcgga-3' and 5'-gcatgtcatgtagtgagaaatgtgctca-3' (ATP6v0d2) and 5'-accagaagactgtggatgg-3' and 5'-cacattggggtaggaacac-3' (GAPDH). Primer sequences for RT-PCR were 5'-atgggaactggctggttaaccac-3' and 5'-ggcattggtagtgctgttgc-3' (NOX1); 5'-accacagtcctgcatcac-3' and 5'-tccaccacctgttgctga-3' (GAPDH), respectively.

Electrophoretic mobility shift assays (EMSA)

Biotinylated double-stranded oligonucleotides were synthesized by Bioneer Co. (Korea) as follows: NF-κB, 5'-agttgaggggacttcccaggc-3'; NF-Y, 5'-agaccgtacgtgattggttaattctt-3'. Nuclear extracts were prepared from BMM cells stimulated with RANKL

(40 ng/mL) using NE-PER nuclear and cytoplasmic extraction reagents (Pierce). Binding reactions were carried out for 20 min at room temperature in the presence of 50 ng/mL poly(dI-dC), 0.05% Nonidet P-40, 5 mM MgCl₂, 10 mM EDTA and 2.5% glycerol in 1× binding buffer using 20 fM of biotin-end-labeled target DNA and 3 µg of nuclear (LightShift Chemiluminescent EMSA kit; Pierce). Samples were loaded onto native 6% polyacrylamide gels pre-electrophoresed for 60 min in 0.5× Tris borate/EDTA and electrophoresed at 100 V before being transferred onto positively charged nylon membranes (Hybond-N+) in 0.5× Tris borate/EDTA at 100 V for 30 min. Transferred DNAs were cross-linked to the membrane at 10 mJ/cm² and detected using horse radish peroxidase (HRP)-conjugated streptavidin.

Plasmid construction

The *NOX1* gene was V5-tagged by subcloning into the pcDNA6-V5-His-A vector (Invitrogen) containing a C-terminal V5 tag between NheI and NotI. The FLAG-tagged *Nod2* gene was subcloned into pcDNA3.1 vector (Addgene, MA, USA) between BamHI and EcoRI. All constructs were sequenced to verify 100% agreement with the original sequence.

Co-immunoprecipitation and Western blots

HEK293T cells (8×10⁶) grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum were transfected with 5 µg samples of the expression vectors using Lipofectamine 3000 (Invitrogen). After 48 h, cells were harvested and lysed by incubating with RIPA buffer (50 mM Tris-HCl pH 7.5, 1% Triton-X 100, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1% protease inhibitor mixture) for 20 min. Cell pellets were removed by centrifugation at 16,500g for 10 min. The lysates were incubated with Anti-V5-tag mAb-Magnetic beads (MBL, Nagoya, Japan) O/N with rotation at 4°C. Immunoprecipitates were then washed three times with RIPA buffer and eluted with SDS sample buffer by incubation at 100°C for 2 min. Proteins were separated by SDS-PAGE, and western blotting was performed using monoclonal HRP-conjugated anti-FLAG M2 (Sigma).

Measurement of intracellular reactive oxygen species

Intracellular ROS were detected using the fluorescent probe 2',7'-dichlorofluorescein diacetate (H₂DCFDA)

(Molecular Probes). BMMs were cultured under various experimental conditions for 48 h, harvested, suspended in PBS, loaded with H₂DCFDA and incubated at 37°C for 30 min. Intracellular ROS were measured by flow cytometry with a FACSCalibur.

Statistical analysis

Values are expressed as means±standard error of mean (S.E.M.). Student's *t*-test was used to evaluate the differences between samples and corresponding controls. Differences between groups were assessed by one-way ANOVA, followed by Bonferroni posttests. A *P* value of less than 0.05 was considered statistically significant.

Results

NOD2 deficiency increases bone density upon OVX-induced bone loss

We hypothesized that NOD2 reduces bone mass by acting on OCs, since it interacts with serine-threonine kinases to induce the activation of NF-κB (Ogura *et al.* 2001), which is important for osteoclastogenesis (Iotsova *et al.* 1997). To test this hypothesis, we set up a bone loss model involving OVX in mice and compared the femurs of *Nod2*-KO mice and their littermates by µCT. The absence of NOD2 attenuated the bone loss induced by OVX and had no significant effect on sham-operated mice (Fig. 1A). It also increased bone mineral density (BMD), bone volume (BV/TV), trabecular number (Tb. N.) and trabecular thickness (Tb. Th.) and reduced the enlargement of trabecular space (Tb. Sp.) (Fig. 1B). As shown in Fig. 1C, OC.N/BS (OC number over total bone surface) in *in vivo* TRAP staining was also reduced in the absence of NOD2 upon OVX (Fig. 1C). Consistent with this, OC formation increased significantly in *ex vivo* cultures of BMM-enriched populations derived from WT OVX mice, while there were fewer OC in parallel cultures from *Nod2*-KO OVX mice (Fig. 1D). NOD2 deficiency also significantly reduced the large OCs that were evaluated by the area of OCs compared to sham-operated WT. *Ex vivo* cultures of whole bone marrow cells showed a similar trend to that of enriched BMMs in the absence of NOD2 (Fig. 1E). Consistent with this, serum CTX-1, an *in vivo* bone resorption marker, was also reduced in the absence of NOD2 upon OVX (Table 1). OVX also increased levels of the *in vivo* bone formation markers serum ALP and osteocalcin in WT mice, but lack of NOD2

Figure 1

Absence of NOD2 protects against bone loss induced by OVX in mice. Representative μCT images of distal mouse femora of WT mice 1.0 mm from the growth plate (OVX, $n=6$; sham, $n=6$) and *Nod2*-KO mice (OVX, $n=6$; sham, $n=6$) eight weeks after OVX and sham surgery (A). Trabecular BMD, trabecular BV/TV, Tb. N., Tb. Th. and Tb. Sp. were measured by μCT (B). To examine *in vivo* TRAP-positive OCs, mouse femora were excised, cleaned with a soft tissue and decalcified in EDTA. Representative histological sections of the distal femoral metaphysis of WT and *Nod2*-KO mice were stained for TRAP to identify OCs (original magnification $\times 200$) with OC.N/BS (OC number over total bone surface) (C). Enriched BMMs were stimulated with RANKL (40 ng/mL)/M-CSF (20 ng/mL) for 3 days, and TRAP-positive MNCs per well were counted after fixation. Thereafter, more than 70 TRAP-positive MNCs in each culture were randomly selected, and the area of the formed OCs were measured (D). Whole bone marrow cells were stimulated with $1,25(\text{OH})_2\text{D}_3$ (10 nM) and PGE_2 (1 μM) for 7 days (E). Data are expressed as means $\pm$ s.e.m. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with corresponding SHAM. Differences between groups were analyzed by two-way ANOVA, followed by Bonferroni posttests to compare the effect of genotype (Tb. Sp.; $P < 0.05$, BV/TV; $P < 0.01$, Tb. Th. and OC. N/BS; $P < 0.001$), the effect of surgery (Tb. Sp., Tb. Th.; $P < 0.01$, BMD, BV/TV, and Tb. N.; $P < 0.001$) and interaction (BMD and OC.N/BS; $P < 0.01$, Tb. N.; $P < 0.001$). Similar results were obtained in three independent experiments.

Table 1 Biochemical markers of OVX and SHAM mice in WT and *Nod2*-KO mice at 8 weeks after surgery.

	WT		<i>Nod2</i> -KO	
	SHAM	OVX	SHAM	OVX
CTX (ng/mL)	17.72 ± 1.188	30.73 ± 1.853***	14.89 ± 1.124	24.16 ± 1.050***
ALP (U/L)	35.29 ± 1.606	53.24 ± 4.223**	40.66 ± 1.931	42.81 ± 4.049
OCN (ng/mL)	21.76 ± 2.450	42.01 ± 1.450***	22.65 ± 1.682	36.14 ± 1.723***

Data are represented as mean ± S.E.M. Differences between groups were analyzed by two-way ANOVA, followed by Bonferroni posttests to compare the effect of genotype (CTX, $P < 0.01$) and the effect of surgery (ALP, $P < 0.01$; CTX and OCN, $P < 0.001$), and interaction between genotype and surgery (ALP, $P < 0.05$). Similar results were obtained in three independent experiments. ** $P < 0.01$; *** $P < 0.001$ compared with SHAM.

did not reduce these levels. NOD2 deficiency also did not significantly affect serum ALP and osteocalcin after sham surgery (Table 1).

NOD2 deficiency decreases osteoclastogenesis *in vitro*

To further examine the role of NOD2 in OCs, *Nod2* expression was evaluated upon RANKL stimulation. Stimulation of BMMs with RANKL to induce their differentiation into OCs appeared to slightly increase transcripts of *Nod2*, but the effect was not significant (Fig. 2A). In addition, *Nod2* mRNA levels in OC were not significantly changed upon OVX (Fig. 2A). To see whether NOD2 affected osteoclastogenesis *in vitro*, we

examined OC formation in cultures of BMMs from WT and *Nod2*-KO mice, the BMMs were free of stromal cells and lymphocytes. The two factors necessary for OC differentiation, M-CSF and RANKL, induced maximal OC formation after 3-days exposure, and OC formation in the *Nod2*-KO BMMs was 28% lower than that in the WT BMMs, as measured by counting TRAP-positive MNCs (Fig. 2B and C). NOD2 deficiency reduced the area of OCs with a greater extent (45% reduction) than the number of OCs (Fig. 2C). Since the area of OCs was more prominent than the number of OCs in the absence of NOD2, we used the area of OCs to evaluate the effect of NOD2 in further studies. To confirm the effects of NOD2, we used MDP to stimulate endogenous NOD2 in WT cells. MDP,

Figure 2

NOD2 deficiency decreases the formation of OCs. RNA from BMMs stimulated with RANKL (40 ng/mL) and M-CSF (20 ng/mL) at indicated time points was measured by qPCR for *Nod2*. Expression before RANKL treatment was set at 1 (A). BMMs from WT and *Nod2*-KO mice were incubated in the presence of M-CSF and stimulated with RANKL plus or minus MDP (10 $\mu\text{g/mL}$, Sigma Chemical) (B, C). Scale bar; 100 μm in representative photos of OCs (B). Cells were fixed after 3 days, more than 70 TRAP-positive MNCs in each culture were randomly selected, and the area of the formed OCs was measured. RNA from BMMs stimulated with RANKL and M-CSF at 48 h was measured by qPCR for OC-specific genes (D) * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with WT cells. Similar results were obtained in three independent experiments.

a ligand of NOD2, did not increase the number of OC, but the area of OCs was increased. No further changes with MDP were found in the absence of NOD2 (Fig. 2C). As shown in Fig. 2D, transcript levels of TRAP, cathepsin K, calcitonin receptor, NFAT2, ATP6v0d2 and DC-STAMP were significantly reduced in response to RANKL in the OCs from the *Nod2*-KO mice.

NOD2 deficiency decreases OC function *in vitro*

To examine whether NOD2 affects OC activity, we examined bone resorption *in vitro* using dentine slices. Mature OCs generated from the cells transfected with siNOD2 gave rise to a significantly lower total pit area/number of OC compared to those from the cells transfected with scRNA (Fig. 3A). Stimulation of endogenous NOD2 by MDP in mature OCs increased total pit area/number of

OC significantly (Fig. 3B), suggesting that stimulation of NOD2 augments the resorptive capacity of mature OCs.

NOD2 affects RANKL signaling by activating NF- κ B during osteoclastogenesis

To gain insight into how NOD2 elevates the number and activity of OCs, we investigated the effect of NOD2 deficiency on RANKL-induced signaling. Cross-linking between RANK and RANKL activates the key transcription factor, NF- κ B (Iotsova *et al.* 1997), resulting in the expression of OC-specific genes. We asked whether NOD2 affected the activation of NF- κ B. As shown in Fig. 4A, stimulation of WT BMMs with RANKL for 1 h induced NF- κ B DNA-binding activity and stronger NF- κ B DNA binding was observed after treatment with MDP. Similar pattern was observed with 2-h stimulation. NF- κ B DNA-binding activity was lower in the absence of NOD2, and there was no further change in response to MDP. The specificity of the binding activity was confirmed by competition assays using excess unlabeled probe. To clarify the mode of action of NOD2 in NF- κ B activation, we compared the effects of a pharmacological inhibitor of the nuclear translocation of p65 with those of an inhibitor of I κ B α phosphorylation on OC formation in response to NOD2 activation by MDP. Both inhibitors decreased the area of OC significantly and abolished the increase induced by MDP (Fig. 4B). A similar pattern was observed with the expression of OC-specific genes including TRAP, calcitonin receptor and ATP6v0d2, supporting the morphological evaluation of OCs. Next, knockdown of *Nod2* by siNOD2 reduced OC-specific genes as well as the area of OCs. Those reductions were attenuated by blockade of NF- κ B activation (Fig. 4C), confirming that NOD2 plays a role via NF- κ B activation.

Figure 3 NOD2 deficiency decreases OC activity. BMMs were transfected with siNOD2 or scRNA for 8 h, and then cultured with M-CSF (20 ng/mL) and RANKL (40 ng/mL) on whole dentine slices to generate mature OCs for 7 days (A). Mature OCs were generated on whole dentine slices with M-CSF and RANKL with or without MDP (10 μ g/mL) for 6 days (B). Cells were fixed with formalin and stained for TRAP. Then, cells were removed and the slices were stained with Mayer's hematoxylin. Representative photos of TRAP-positive OCs and resorption pits formed are shown. Scale bar: 50 μ m. Total pit area/number of TRAP-positive OC was measured. * P <0.05; *** P <0.001 compared with vehicle-treated or scRNA-transfected cells. Similar results were obtained in three independent experiments.

NOD2 deficiency decreases RANKL-induced ROS

Since our previous finding showed OVX-induced oxidative stress (Van Phan *et al.* 2013), we tested whether NOD2 was implicated in the induction of oxidative stress. The absence of NOD2 decreased the elevation of serum ROS in response to OVX (Fig. 5A). Then, we examined whether NOD2 affected the sustained level of ROS in OCs stimulated by RANKL, since ROS plays an important role in RANKL signaling during OC formation (Lee *et al.* 2005). Treatment with RANKL induced a sustained level of ROS over 48 h of exposure, but NOD2 deficiency reduced the RANKL-induced ROS level significantly

Figure 4

Attenuated RANKL signaling in OCs in the absence of NOD2. BMMs from WT and *Nod2*-KO mice were stimulated with vehicle (lane 1, 4), RANKL (100 ng/mL) (lane 2, 5) or RANKL along with MDP (10 µg/mL) (lane 3, 6) for 1 h. A 100-fold excess of unlabeled probe (lane 7) was added as a negative control (Left panel). BMMs from WT mice were stimulated with vehicle (lane 1'), RANKL (100 ng/mL) (lane 2', 4') or RANKL along with MDP (10 µg/mL) (lane 3', 5') for 1 h (1', 2', 3') and 2 h (4', 5') (Right panel). NF-γ DNA binding activity served as an internal control (A). BMMs were treated with BAY 11-7082 (5 µM) or JSH-23 (10 µM), and stimulated with RANKL or RANKL+MDP (10 µg/mL) (B) for 48 h and 72 h to measure the expression of TRAP, calcitonin receptor, and ATP6v0d2 and the area of OCs, respectively. BMMs were transfected with siNOD2 or siRNA for 8 h before receiving above treatment (C). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with RANKL-treated cells receiving vehicle (V) or siRNA-transfected RANKL-treated cells. Similar results were obtained in three independent experiments.

(Fig. 5B). The sustained level of ROS is the net outcome of production and removal of ROS; to evaluate whether NOD2 affected the production or the removal of ROS, we determined RANKL-induced ROS levels in the presence of N-acetylcysteine (NAC), a general antioxidant, and diphenyliodonium (DPI), a selective inhibitor of NOX, in the absence of NOD2. A further decrease of ROS level in the absence of NOD2 was seen in the presence of NAC, but not of DPI (Fig. 5C), suggesting that the NOD2-induced increase in ROS was due to increased production of ROS rather than reduced removal of ROS. To confirm that NOD2 was associated with RANKL-induced ROS production, we knocked down *Nox1* by transfection with siNOX1. As shown in Fig. 5D, downregulation of the RANKL-specific NADPH oxidase by siNOX1 decreased ROS, but the extent of the reduction was not affected by the absence of NOD2. A similar pattern was observed when the area of OCs was measured (Fig. 5E).

Since oxidative stress induced by OVX was attenuated in the absence of NOD2, we wondered whether NOD2 was implicated in the effects of estrogen (E_2) on ROS levels. As shown in Fig. 5F, E_2 reduced RANKL-induced ROS levels in WT cells, but not in *Nod2*-KO cells, suggesting that NOD2 is associated with the E_2 effect in modulating ROS levels in OCs. A similar pattern was found for decreasing the area of OCs in response to E_2 (Fig. 5G).

Since there are reports of a direct interaction between TLR components and NOX family members (Lipinski 2009, Park 2004), we examined whether NOD2 physically interacted with NOX1 in OCs. Co-expression of FLAG-tagged NOD2 with V5-tagged NOX1 in HEK293 cells was analyzed by co-immunoprecipitation. As shown in Fig. 6, precipitation of overexpressed V5-tagged NOX1 resulted in co-precipitation of FLAG-tagged NOD2, supporting a direct physical interaction between NOX1 and NOD2.

Discussion

We have shown that absence of NOD2 reduces the bone loss induced by OVX in mice. OVX has been shown to induce a modest amount of bone loss when 5- to 6-week-old mice (Lee *et al.* 2006a), 8-week-old mice (Lee *et al.* 2006b) and 16-week-old mice (Gao *et al.* 2007) were subjected to OVX and analyzed 4 weeks later. OVX induced an immediate decrease of bone formation after 2 weeks (Li *et al.* 2011), but thereafter, there was a sustained increase (Cenci *et al.* 2000). In the present work, we carried out OVX on 6-week-old *Nod2*-KO mice and analyzed its effect 8 weeks later. The OVX induced significant bone loss in WT mice, but not in *Nod2*-KO mice. As all the mice used were not raised under germ-free condition, but under

Figure 5

NOD2 deficiency decreases RANKL-induced ROS. Serum ROS was determined from OVX and SHAM mice in WT and *Nod2*-KO mice at 8 weeks after surgery (A). Differences between groups were analyzed by two-way ANOVA, followed by Bonferroni posttests to compare the effect of genotype ($P < 0.05$) and the effect of surgery ($P < 0.01$), and interaction between genotype and surgery ($P < 0.05$). BMMs from WT and *Nod2*-KO mice were stimulated with RANKL along with M-CSF (B) and treated with NAC (3 mM), DPI (5 nM) (C), or E₂ (10 nM) (F, G). For E₂ treatment, BMMs were cultured in α-MEM without phenol red containing 10% charcoal-treated FBS. Cultures were incubated for 48 h to determine intracellular levels of ROS by flow cytometry using H₂DCF-DA (B, C, D, F) and for 72 h to determine the area of OCs (E, G). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with WT cells. + $P < 0.05$ compared with *Nod2*-KO cells. # $P < 0.05$; ### $P < 0.001$ compared with the corresponding WT cells. BMMs were transfected with siNOX1 or scRNA. Downregulation of *Nox1* by siRNA was confirmed by RT-PCR and qPCR. After 8 h, cells were stimulated with RANKL to determine intracellular ROS using H₂DCF-DA for 48 h (D) and to determine the area of OCs for 72 h (E), respectively. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with RANKL-treated scRNA-transfected cells (V). Similar results were obtained in three independent experiments.

specific pathogen-free condition, microbiota could help MDP to activate NOD2 in WT mice. We focused on the trabecular microarchitecture of the ovariectomized mice, since postmenopausal women have been reported to undergo trabecular bone loss induced by elevated bone

remodeling with excess bone resorption exceeding bone formation (Riggs & Melton 1992, Recker *et al.* 2004). *Nod2*-KO OVX mice exhibited a significant increase in trabecular BMD, BV/TV, Tb. N. and Tb. Th. and a decrease of Tb. Sp. compared with their littermates. The absence of NOD2 reduced the number of OCs derived from BMMs in *ex vivo* cultures upon OVX as well as histological sections of femur from OVX mice, suggesting that the effect of NOD2 was through OCs. In accordance with this view, lack of NOD2 decreased the level of an *in vivo* marker of bone resorption, serum CTX-1, which is elevated upon OVX. However, no significant change in bone mass was observed in the absence of NOD2 after sham surgery. This discrepancy can be explained by stimulus-induced signal augmentation that are found in CD44 (Li *et al.* 2015) and leukotriene B4 receptor (Hikiji *et al.* 2009). It is likely that the protective effect of NOD2 deficiency upon OVX is due to preventing the increased activity of NOD2 in OCs that occurs in WT mice, hence implying that NOD2 is involved in the decrease of bone density upon OVX. At the same time, NOD2 deficiency did not increase bone formation, as indicated by serum ALP and osteocalcin levels, which implies that the observed bone phenotype of *Nod2*-KO mice is mainly due to decreased bone resorption rather

Figure 6

NOD2 interacts physically with NOX1. NOD2-FLAG and NOX1-V5 constructs were overexpressed in HEK293 cells. Immunoprecipitates and cell lysates were assayed for co-precipitated proteins as indicated. Similar results were obtained from three independent experiments.

than increased bone formation. Although lack of NOD2 did not alter bone formation *in vivo*, we cannot exclude a contribution of stromal cells/osteoblasts in the increased OC formation. In fact, increased osteoclastogenesis has been reported in response to MDP activation in osteoblasts (Yang *et al.* 2005).

The contribution of OCs to the attenuated bone loss in the absence of NOD2 is supported by our *in vitro* observation that, in the absence of NOD2, the number of OCs decreased as the result of decreased commitment to the OC lineage. Interestingly, the decrease of OC formation in the absence of NOD2 was more prominent in the area of OCs, but a decrease of ATP6v0d2 and DC-STAMP transcripts was similar to that of other OC-specific genes in the absence of NOD2, suggesting that NOD2 may not specifically participate in fusion. In addition, the activity of OCs was reduced in the absence of NOD2. These results suggested that NOD2 augments the resorptive capacity of OCs as well as enhances large OCs. Since our *in vitro* studies (except for Fig. 2A) were performed with cells from male mice to avoid periodic E₂ effects, we examined the expression of RANKL-induced OC-specific genes in cells from female mice. The absence of NOD2 reduced the expression of these genes but to a lesser extent than that in the cells from male mice (Supplementary Fig. 1, see section on supplementary data given at the end of this article). This agrees with the finding that NOD2 deficiency had less effect on *ex vivo* OC formation in cells from female mice, as well as the finding that NOD2 deficiency abolished the effect of E₂ on OC formation in E₂-free conditions. Taken together, these results suggest that NOD2 is responsible, at least in part, for the increased OC formation upon OVX.

We found that *Nod2* mRNA levels did not change upon OVX and that they increased only modestly, if at all, during OC formation, whereas others (Prates *et al.* 2014) have reported that the levels increased significantly, and that bacterial stimulation also elevated them (Liu *et al.* 2014). This discrepancy suggests *Nod2* expression in OCs does not change very much. It is well known that NOD2 levels are regulated by posttranslational modification (Tigno-Aranjuez & Abbott 2012), not by transcript levels. While we were investigating the effect of NOD2 in our OVX-induced bone loss model, Prates and coworkers (Prates *et al.* 2014) reported that lack of NOD2 led to lower bone resorption in an experimental model of periodontitis 4 weeks after *Porphyromonas gingivalis* (*P. gingivalis*) infection. On the other hand, absence of NOD2 along with ApoE deficiency led to alveolar bone loss as well as atherosclerosis and an increase of inflammatory cytokines

after 15 weeks of *Porphyromonas gingivalis* infections (Yuan *et al.* 2013). Dual deficiency of NOD2 and ApoE appears to exacerbate impairing lipoprotein profiles, exhibiting chronic inflammation. The discrepancy between two findings could be due to the differences of environment and/or duration of stress. It is likely that NOD2 may behave oppositely under different microenvironments upon same stress, although it is not clear yet to define the role of NOD2 in inflammatory bone loss.

We showed that NOD2 deficiency antagonized RANKL signaling by decreasing the area of OCs. In the absence of NOD2, NF-κB DNA binding activity was attenuated (Fig. 4A). The specific inhibitors of NF-κB, JSH-23 (inhibitor of p65 nuclear translocation) and BAY 11-7082 (IκBα phosphorylation inhibitor) abolished the effects of NOD2 on the area of OC during NOD2 activation and NOD2 deficiency (Fig. 4B and C), suggesting that NOD2 plays a critical role upstream of cytoplasmic phosphorylation in the NF-κB signaling pathway.

We also showed that NOD2 deficiency reduced OVX-induced oxidative stress. Our *in vitro* data demonstrated that NOD2 acted to increase the sustained ROS level induced by RANKL but not the level induced by M-CSF (Fig. 5B) and that inhibition of NOX or knockdown of *Nox1* abolished the decrease in ROS seen in the absence of NOD2 (Fig. 5D and E). It is well known that elevation of ROS upon RANKL stimulation contributes to OC formation (Lee *et al.* 2005). Our previous studies also showed that ROS participates in enhancing the number and activity of OCs by oxidation of c-Src and SHP-1 (Ke *et al.* 2014). The finding that NOD2 increased the differentiation and function of OCs (Figs 2 and 3), at least part, by increasing ROS is in agreement with findings that implicate other NLR family members in intracellular redox control. Thus, the assembly and activation of the NALP3 inflammasome is reported to be stimulated by ROS (Dostert *et al.* 2008), and NOD2-dependent ROS formation via dual oxidase 2 (DUOX2) has been shown to protect against bacterial infections (Lipinski *et al.* 2009). Binding of TLR4 to LPS stimulates NF-κB activation and ROS generation via NOX4 (Park *et al.* 2004). In addition, our data clearly show that NOD2 physically interacts with NOX1, thus paralleling the complex formation between NOX4 and TLR4 (Park *et al.* 2004) and between NOD2 and DUOX2 (Lipinski *et al.* 2009). However, it is possible that NOD2 interacts with NOX4, which regulates bone mass by generating hydrogen peroxide in OCs (Goettsch *et al.* 2013).

Taken together, we demonstrate that NOD2 deficiency attenuates the bone loss caused by OVX in mice. Our data also point to previously unrecognized effects of

NOD2-dependent ROS formation on osteoclastogenesis. They indicate that NOD2 acts as a molecular switch that modulates differentiation and activity of OCs by increasing ROS generation in OCs. These results suggest NOD2 as a potential therapeutic target to reduce bone loss and oxidative stress due to loss of ovarian function.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-16-0591>.

Declaration of interest

The authors declare that they have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

The study was designed by K K, O J S and H S C and was performed by K K, O J S and S W C. The manuscript was written by K K, O J S and H S C and revised by K K, O J S and H S C.

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