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RESEARCH ARTICLE

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Nobiletin, a NF-κB signaling antagonist, promotes BMP-induced bone formation

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Abstract

The NF-κB family of transcription factors plays an important role in skeletal development and bone homeostasis. In osteoblast cells, NF-κB signaling has been shown to suppress survival, proliferation, and differentiation. Furthermore, pharmacological suppression of NF-κB enhances osteoblast differentiation and bone formation. Thus, NF-κB antagonists are promising candidates as anabolic agents for enhancing bone mass. In this study, we describe the mechanism by which nobiletin, an inhibitor of NF-κB activity, regulates osteoblast differentiation and mineralization. We found that in MC3T3-E1 osteoblast cells, nobiletin inhibited a TNF-α responsive NF-κB luciferase reporter and also decreased the induction of classical NF-κB target genes by TNF-α. Consistent with this, nobiletin prevented TNF-α -mediated suppression of osteogenesis and potently enhanced the differentiation and mineralization of MC3T3-E1 cells. Likewise, in an in vivo BMP2-induced ectopic bone formation assay, nobiletin markedly enhanced ossicle bone volume. Western blotting and SMAD-responsive luciferase assays also demonstrated that NF-κB suppression of BMP signaling could be inhibited by nobiletin. Thus, our data suggest that mechanistically, nobiletin prevents the endogenous repression of BMP signaling by TNF-α, thereby enhancing osteoblast activity. In conclusion, nobiletin is a novel NF-κB antagonist that may be a useful anabolic agent for bone formation.

KEYWORDS

BMPs, bone formation, NF-κB signaling, Nobiletin, TNF-α

1 | INTRODUCTION

The inflammatory cytokines, tumor necrosis factor α (TNF-α), and interleukin-1 (IL-1) suppress osteoblast differentiation in multiple experimental models.[1] TNF-α and IL-1 activate the canonical NF-κB pathway, which in turn induces the phosphorylation of IκBs by the IκB kinase complex. Phosphorylated IκBs are ubiquitinated and degraded by the proteasome. Upon IκBs degradation, p50/p65 heterodimer enters the nucleus and regulates the

expression of genes that regulate immune and inflammatory responses.[2,3] Furthermore, TNF-α inhibits the expression of key transcriptional regulators of osteogenesis such as Runx2 and Osterix.[4] Several recent studies have described an inhibitory role for NF-κB on osteoblast differentiation. Expression of a dominant-negative mutant IKK enhanced osteogenesis in C2C12 cells.[5] In addition, osteoblast-specific expression of dominant-negative IKK increased trabecular bone mass.[5] We, as well as others, have previously demonstrated that NF-κB signaling suppresses BMP signaling and that p65, the main subunit of NF-κB, disrupts the association of R-Smad with Smad4.[6,7] Consistent with this, suppression of NF-κB blocks TNF-induced inhibition of Smad signaling and thereby enhances osteogenesis.[8] A selective inhibitor of the canonical NF-κB pathway (BAY11-70682) and peptides that interfere with Smad4-p65 interactions enhance BMP-2-induced ectopic bone formation.[6,7] Drugs inhibiting NF-κB activation are therefore considered promising candidates for augmenting bone formation.

Bone morphogenetic proteins (BMPs), members of the TGF-β superfamily, play an essential role in various biological processes and are critical for regulation commitment and differentiation of osteoblast precursors.[9] BMPs were initially discovered based on their ability to induce new bone formation in the skeletal muscle tissue of rodents. Amongst the approximately 20 kinds of BMPs, BMP-2, -4, and -7 are considered osteoinductive BMPs.[10] Indeed, BMP-2 and -7 have been approved for human bone regeneration by the Food and Drug Administration (FDA). BMP signal transduction is initiated by the binding of the BMP ligand to two types of BMP receptors (Type I and Type II receptor). The activated type I receptor phosphorylates the C-terminal of Smad1/5 (R-Smad) in the cytoplasm. The phosphorylated R-Smad is translocated into the nucleus together with Smad4 and regulates the expression of direct target genes such as Id-1 and Osterix.[11,12]

The small molecule nobiletin (NOB) is a polymethoxylated flavone extensively studied as a therapeutic agent in cancer and several other disease models, including inflammation, neurological and metabolic diseases. NOB improves motor deficits in Parkinson's disease models,[13] NOB can protect against metabolic syndrome[14] and can also attenuate cardiac dysfunction.[15] NOB has anti-inflammatory effects and downregulates interleukin-6 (IL-6) gene expression and TNF-α in mouse macrophages.[16,17] The effects of NOB on the skeletal system are not yet well understood. NOB has been shown to suppress LPS-induced osteoclast formation and bone resorption.[18] Interestingly, NOB also suppresses IL-1-induced osteoclast formation by inhibiting NF-kB transcription and NF-κB-dependent PGE_2 production in osteoblasts.[18] In in vitro experiments using MG-63 human osteosarcoma cells, NOB enhanced osteoblast differentiation by stimulating BMP-2 signaling.[19] However, the function of NOB on bone formation is still largely unknown. Here in this study, we examined the effect of NOB on NF-κB signaling, osteoblast differentiation, and bone formation induced by BMPs.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The mouse calvaria osteoblast cell line MC3T3-E1 cells from Riken BioResource Center (Tsukuba, Japan) were cultured. Cells were maintained in a 5% CO_2, 37°C with α-minimal essential medium (MEM-α) (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal bovine serum (Nichirei Biosciences Inc., Tokyo, Japan), 100 U/ml penicillin–streptomycin (Fujifilm Wako Pure Chemical Corporation).[20] Primary calvaria osteoblasts (POBs) were isolated from 3-day-old C57BL/6 mice. They were dissected and removed under general anesthesia using isoflurane, and soft tissue Calvariae were incubated at 37°C for 15 min in digestion medium (1 mg/ml Collagenase I [WAKO], Dispase 10 mg/ml [WAKO] αMEM [WAKO]). After digestion, the supernatant was discarded, and the process was repeated twice more for 15 and 45 min. Only cells released in the final digestion were collected. Cells were maintained in αMEM(WAKO) supplemented with 20% FBS (WAKO) and 1% penicillin–streptomycin (WAKO).[21] All animal protocols complied with Kyushu Dental University Guidelines' Animal Care and Use Committee (Approval number #19-011).

2.2 | Cell viability assay

The effects of NOB on the viability of cells were assessed by WST-8 assay with Cell Counting kit-8 assay kit (CCK-8, Dojindo, Kumamoto, Japan). MC3T3-E1 cells were cultured in 96-well plates at a density of 5.0×10^3 cells per well for 24 h. Then cells were pretreated with various concentrations (0, 10, 25 μM) of NOB for 3 days. After that, 10 μl CCK-8 was added to each well and incubated at 37°C for 30 min. The optical density was read at a wavelength of 450 nm with a microplate reader (BioRad).[22]

2.3 | Reagents for cell culture and osteoblast differentiation medium

MC3T3-E1 cells and POBs were treated with recombinant human (rh) BMP-4 (R&D Systems), rhTNF-α(R&D

Systems), and/or Nobiletin (NSC76751, Selleck. Co.jp, TX, USA). Induced osteoblast differentiation, cells were treated with αMEM (WAKO) supplemented with 10% FBS and 25 ng/ml rhBMP-4 as osteogenic medium.

2.4 | Alkaline phosphatase staining and activity

MC3T3-E1 and POBs were seeded in 6-well plates with density of 2×10^5 cells/well. When the density of cells was up to 80%, the medium was changed into the osteogenic medium. Alkaline phosphatase (ALP) staining was visualized by staining 70% ethanol-fixed cultures with 5-bromo-4-chloro-3-indolyl phosphate–Nitro Blue Tetrazolium (Sigma). Plates were imaged with a Keyence BZ-X800. ALP activity, after removing the culture medium, cell layers were washed with phosphate-buffered saline (PBS) and then treated with 0.1% Triton X-100 (WAKO) for 5 min. ALP activity in the cell lysate was assayed at 37°C in the buffer containing a substrate solution composed of 0.1 M diethanolamine, 1 mM $MgCl_2$, and 1 mg/ml p-nitrophenyl phosphate (WAKO). The reaction was stopped by adding 3 M NaOH. Absorbance was measured at 405 nm using an iMark Microplate Absorbance Reader (BioRad).[7]

2.5 | Mineral staining and quantification

MC3T3-E1 and POBs were seeded in 6-well plates at a density of 2×10^5 cells/well with osteogenic medium for 6 days. Calcein staining and quantification were performed.[23] After rinsing cells with PBS twice, cultures were incubated in 25 μg/ml calcein (Sigma) for 30 min. Calcein fluorescence was measured (excitation wavelength, 485 nm) using a fluorescence plate reader (TECAN Infinite 200 Pro).

2.6 | Western blot analyses

The cells were washed twice with ice-cold PBS and solubilized in lysis buffer supplemented with phosphatase (Roche) and protease inhibitor cocktails (Cell Signaling Technology). Cells were further lysed by sonication (TOMY Seiko), and lysates were clarified by centrifugation at 14000 RCF for 10 min at 4°C. Supernatants were collected, and protein concentrations were determined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). The supernatants were boiled in samples buffer containing 0.125 M Tris–HCl (pH 6.8), 40% glycerol, 4% sodium dodecyl sulfate (SDS), 0.2 M dithiothreitol, and 0.01% bromophenol blue, subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were probed with primary antibody followed by the appropriate horseradish peroxidase-conjugated secondary antibody. Using Immobilon Western Chemiluminescent HRP Substrate (Millipore), bands were detected by chemiluminescence and imaged using a LAS-4000 Imaging System (Fujifilm). The antibodies used were as follows: Rabbit anti-Phospho-NF-κB p65 (Cell Signaling; #3033), Rabbit anti-NF-κB p65 (Cell Signaling; #8242), Rabbit anti-IκBα (Cell Signaling; #4812), Rabbit anti-Phospho-Smad1/5 (Cell signaling; #9516), Rabbit anti-Smad1 (Cell signaling; #6944), Anti-GAPDH-HRP (MBL; #M171-7), Goat Anti-Rabbit HRP (Jackson ImmunoResearch; #111-035-144).

2.7 | RNA isolation and the quantitative real-time polymerase chain reaction

Total RNA was isolated from the cells using a FastGene™ RNA Basic Kit (Nippon Genetics, Tokyo, Japan) and reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). The SYBR green-based quantitative polymerase chain reaction was performed using PowerUp SYBR (ThermoFisher Scientific, Waltham, MA, USA) and the QuantStudio 3 Real-Time PCR System (ThermoFisher Scientific). Relative quantification was performed by the ΔCT method using *TBP* as the housekeeping gene for normalization. Primer sequences are shown in into Supporting Information.

2.8 | Luciferase assay

Luciferase reporter vectors and expression plasmids were transfected into MC3T3-E1 cells using Lipofectamine LTX (Life Technologies). Twenty-four hours after transfection, luciferase activity was measured with the Dual-Glo Luciferase Assay System (Promega). Firefly luciferase activity was normalized to renilla luciferase (pRL-TK). Transfected MC3T3-E1 cells were treated with Nobiletin (NOB) (10 μM) along with or without TNF-α (5 ng/ml) and BMP-4 (25 ng/ml). Cells were treated overnight before assay measurement.[11,21]

2.9 | Ectopic bone formation assay

BMP-2 (1 μg) (R&D Systems) and NOB (2.5 or 5 μg) were blotted onto a collagen sponge disk (6-mm diameter, 3-mm thickness) from commercially available

bovine collagen sheets (CollaCote, Zimmer dental) were soaked with BMP-2 along with or without Nobiletin and freeze-dried. All procedures were performed under sterile conditions. Eight-week-old male mice ($n = 10$) were anesthetized using Medetomidine (Domitol, Meiji Seika Pharma Co., Ltd., Tokyo, Japan), Midazolam (WAKO), and Butorphanol (Vetorphale, Meiji Seika Pharma Co., Ltd.), and implanted with pellets surgically into the fascia of back muscle. Before inserting pellets and 1 day after pellet implantation, mice were administered Meloxicam for analgesia.[7,24] All animal protocols complied with Kyushu Dental University Guidelines' Animal Care and Use Committee (Approval number #19-011).

2.10 | Simple X-ray, micro-CT analysis, histological analysis

Four weeks after surgery, the mice were euthanized, and the ectopic bones were harvested and processed for analysis. All the harvested samples were fixed in PBS-buffered glutaraldehyde (0.25%)-formalin (4%) fixative (pH 7.4) for 2 days at 4°C and washed with PBS for further studies. The longest diameters of the ectopic bones were measured (ImageJ). The ectopic bone's bone mineral density (BMD) was measured using dual-energy X-ray absorptiometry (DCS-600R; Aloka). In micro-CT (μCT) analysis, the bone mineral content (BMC) and BMD of the ectopic bone were measured. μCT also obtained three-dimensional reconstruction images of the ectopic bone (ScanXmate-E090; Comscan). For histology, decalcification with 20%EDTA until bones are soft and pliable after that embedded in paraffin wax. In each group, paraffin sections (4 μm) were stained with H&E. The sections were imaged with a Keyence BZ-X800.

2.11 | Statistical analyses

Comparisons were made using an unpaired analysis of variance (ANOVA) with the Tukey–Kramer post hoc or Wilcoxon signed-rank tests. Data are shown as mean, standard deviations.

3 | RESULTS

3.1 | Nobiletin (NOB) enhances BMP-Smad signaling by suppressing NF-κB signaling

To determine the effect of Nobiletin (NOB) on cell viability in MC3T3-E1 cells, cultures were treated with various concentrations of NOB, and the viability was assessed by a WST-8 assay. As shown in Figure 1A, NOB did not exhibit significant effects on viability or proliferation at the concentrations used (10, 25 μM) after 3 days of treatment (Figure 1A). 10 μM NOB was used for all further experiments.

We next examined the effect of NOB on NF-κB signaling in M3CT3-E1 cells. Western blotting showed that treatment of MC3T3-E1 cells with NOB suppressed p65 phosphorylation induced by TNF-α (Figure 1B). Furthermore, the reduction of IκBα by TNF-α could be reversed in NOB dose-dependent manner (Figure 1B). To determine whether the effects of NOB are reflected at the transcriptional level, we next examined the effect of NOB on TNF-α target gene expression. qPCR analysis revealed that NOB inhibited activation of *TNF-α* and *IL-1B* mRNA expression (Figure 1C). Consistent with these observations, NOB also decreased the activity of a TNF-responsive NF-κB luciferase reporter (Figure 1D).

TNF-α and NF-κB signaling has been shown to inhibit Bmp-Smad signaling. We hypothesized that inhibiting NF-κB by NOB will also prevent inhibition of Bmp-Smad signaling by TNF-α. Nobiletin canceled the suppressive effect of TNF-α on the induction of phosphorylated Smad 1/5 protein (Figure 1E) and *Id-1*, *Id-2* mRNA levels (Figure 1F) induced by BMP-4 treatment. Additionally, TNF-α suppression by NOB restored the activity of a BMP-responsive Id-1 luciferase reporter (Figure 1G). These data suggest that NOB inhibits NF-κB signaling in osteoblasts.

3.2 | Nobiletin improves BMP-induced osteoblastic differentiation in the presence of TNF-α

We next determined the effect of NOB on osteoblast differentiation induced by BMPs. MC3T3-E1 cells were induced to differentiate with BMP-4 and mRNA levels of osteoblast marker genes determined by qPCR. We found that NOB canceled the suppressive effect of TNF-α on differentiation (Figure 2A). NOB also strongly enhanced ALP staining (Figure 2B) and activity (Figure 2C) by canceling the negative effect of TNF-α in MC3T3-E1 cells. The effect of NOB on matrix mineralization was visualized and quantified by calcein staining. As shown in Figure 2D,E, NOB increased mineral accumulation in MC3T3-E1 cultures following BMP-4 treatment. These observations on the effects of NOB on osteogenesis were also confirmed using primary osteoblasts (POB). NOB also blocked the effects of TNF-α and increased osteoblast marker genes (Figure 2F) and ALP activity (Figure 2G,H) in POB cells. The effects of NOB on mineralization could also be observed in POB cultures, wherein NOB increased mineral accumulation in cultures treated with TNF-α (Figure 2I,J). Thus, NOB

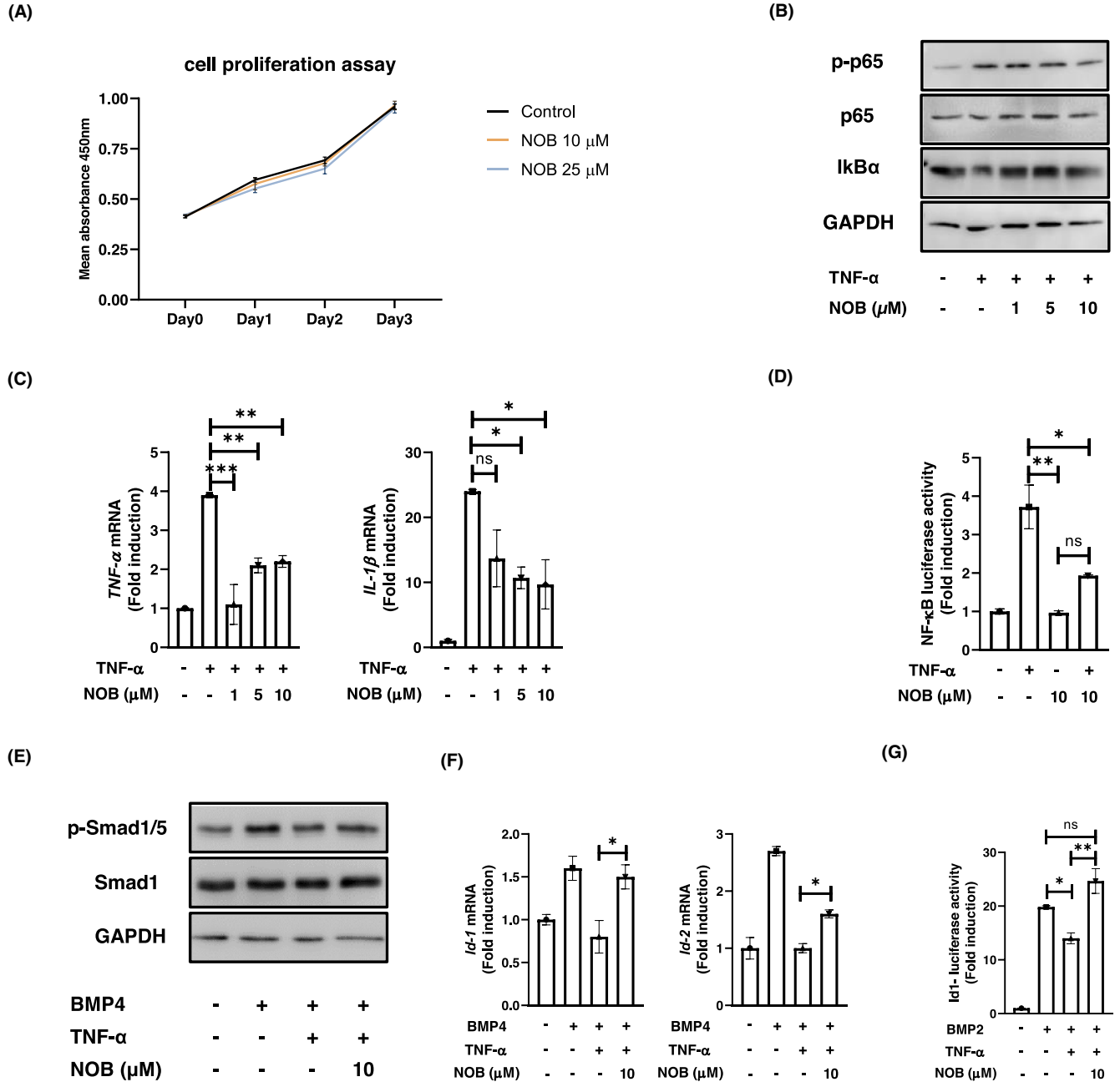

FIGURE 1 Nobiletin enhances BMP-Smad signaling by suppressing NF-κB signaling. MC3T3-E1 cells were treated with 0, 10, and 25 μM Nobiletin (NOB) for 3 days, during which cell proliferation was assessed by WST-8 assay (A). The cells were treated with various concentrations of NOB along with 5 ng/ml TNF-α. The protein levels of p65, phosphorylated p-p65, IκBα, and GAPDH were determined by western blot analysis. Images are representative of triple independent experiments (B). quantitative real-time PCR (qRT-PCR) shows NOB suppressed messenger RNA levels of *TNF-α* and *IL-1β* (C). MC3T3-E1 cells were transfected with both an NF-κB-luciferase reporter plasmid expressing a luciferase protein and a Renilla luciferase control reporter vector and then treated with TNF-α (5 ng/ml) and NOB 10 μM. The NF-κB luciferase activities were normalized using the Renilla luciferase activities, and the activity of non-treated cells was assigned as 1 (D). MC3T3-E1 cells were treated with Nobiletin (NOB) (10 μM), along with or without TNF-α (5 ng/ml) and BMP-4 (25 ng/ml) for 30 min. The protein levels of phosphorylated p-Smad1/5, Smad1, and GAPDH were determined by western blot analysis. Images are representative of triple independent experiments (E). Cells were treated with NOB, along with or without TNF-α and BMP-4, for 3 h, and total RNA was isolated. The messenger RNA level of *Id-1* and *Id-2* was determined by quantitative real-time PCR (qRT-PCR) (F). MC3T3-E1 cells were transfected with both an Id-1-luciferase reporter plasmid expressing a luciferase protein and a Renilla luciferase control reporter vector (G). Data are presented as means ± SD ($n \geq 3$). *$p < 0.05$; **$p < 0.005$; ***$p < 0.001$ (ANOVA with post hoc test).

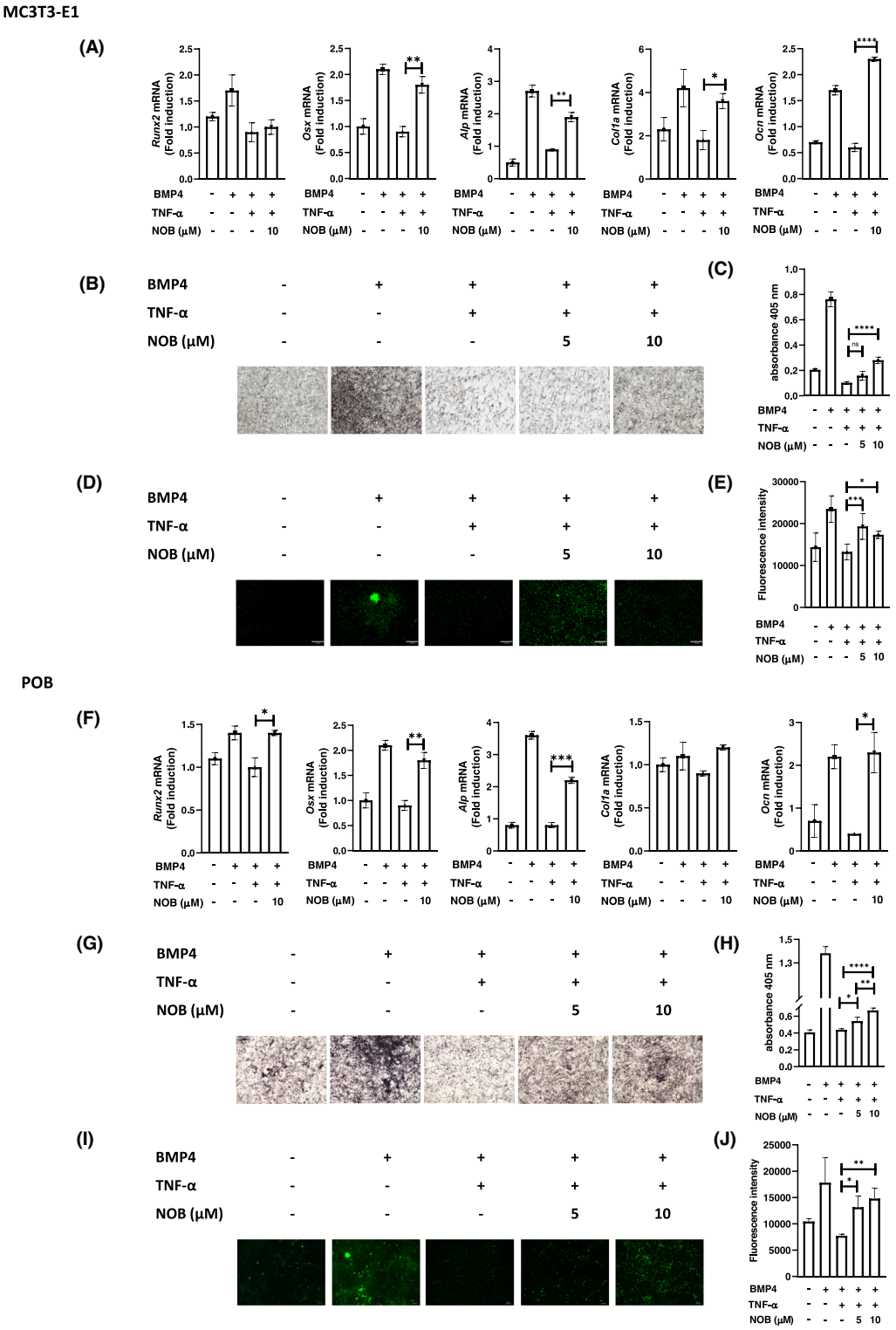

FIGURE 2 Nobiletin improves BMP-induced osteoblastic differentiation in the presence of TNF-α. MC3T3-E1 cells were treated with Nobiletin (NOB) (5 or 10 μM) along with or without TNF-α (5 ng/ml) and BMP-4 (25 ng/ml). Total RNA was isolated at 2 or 3 days, and the messenger RNA levels of osteoblast marker genes were by quantitative real-time PCR (qRT-PCR) (A). After 3 days, Alkaline phosphatase (ALP) activity was visualized by (B) staining and (C) quantified enzymatically. Mineral formed was (D) stained with calcein, and fluorescence intensity was quantified with a plate reader (E). Primally cultured murine calvarial osteoblast (POB)s were treated with NOB, with or without TNF-α and BMP-4. qRT-PCR analysis of osteoblast marker cells POB differentiated for 2 or 3 days (F), ALP staining (G), ALP activity (H), calcein staining (I), and calcein fluorescent intensity (J). Data are presented as means ± SD ($n \geq 3$). *$p < 0.05$; **$p < 0.005$; ***$p < 0.001$; ****$p < 0.0001$ (ANOVA with post hoc test).

improved BMP-induced osteoblastic differentiation in multiple bone cell systems in vitro.

3.3 | Nobiletin enhances BMP-induced bone formation

Finally, we examined the effect of NOB on BMP-induced ectopic bone formation in vivo. Collagen sponges adsorbed with BMP together with or without NOB were implanted into dorsal muscle pouches of 8-week-old mice. 4 weeks after implantation, the mineralized ossicles were dissected and examined. The combination of NOB with BMP-2 increased the diameter of ectopic bones (Figure 3A). Micro-computer tomography (μCT) analysis revealed that NOB increased the bone volume fraction (BV/TV%) and trabecular number (Tb. N) while reducing trabecular spaces (Tb. Sp) (Figure 3B). Histological analysis showed that implants with NOB had increased cortical bone width surrounding the ossicle (Figure 3C). These data indicate that NOB enhanced bone formation induced by BMP in vivo.

4 | DISCUSSION

NOB is a small molecule compound and polymethoxyflavone with multiple biological functions, including anti-inflammatory and NF-κB inhibitory properties. Several studies have evaluated the anabolic effects of NF-κB inhibition on osteogenesis. Here in this study, we examined the potential of NOB as an NF-κB antagonist and promoter of osteoblast differentiation. We showed that NOB can indeed suppress multiple functions of TNF-α and that NOB can abrogate the inhibitory effects of TNF-α on osteoblast differentiation and bone formation in vivo.

In the present in vitro experiments, we demonstrated that NOB enhanced BMP-induced osteoblast differentiation by suppressing NF-κB signaling induced by TNF-α (Figures 1 and 2). NOB has also been reported to stimulate osteoblast differentiation of MG63 human osteosarcoma cells.[19] This is in agreement with our observations in mouse POBs and MC3T3-E1 cells. We also observed that NOB could enhance in vivo BMP-2-induced bone formation even in the absence of supplemental TNF-α

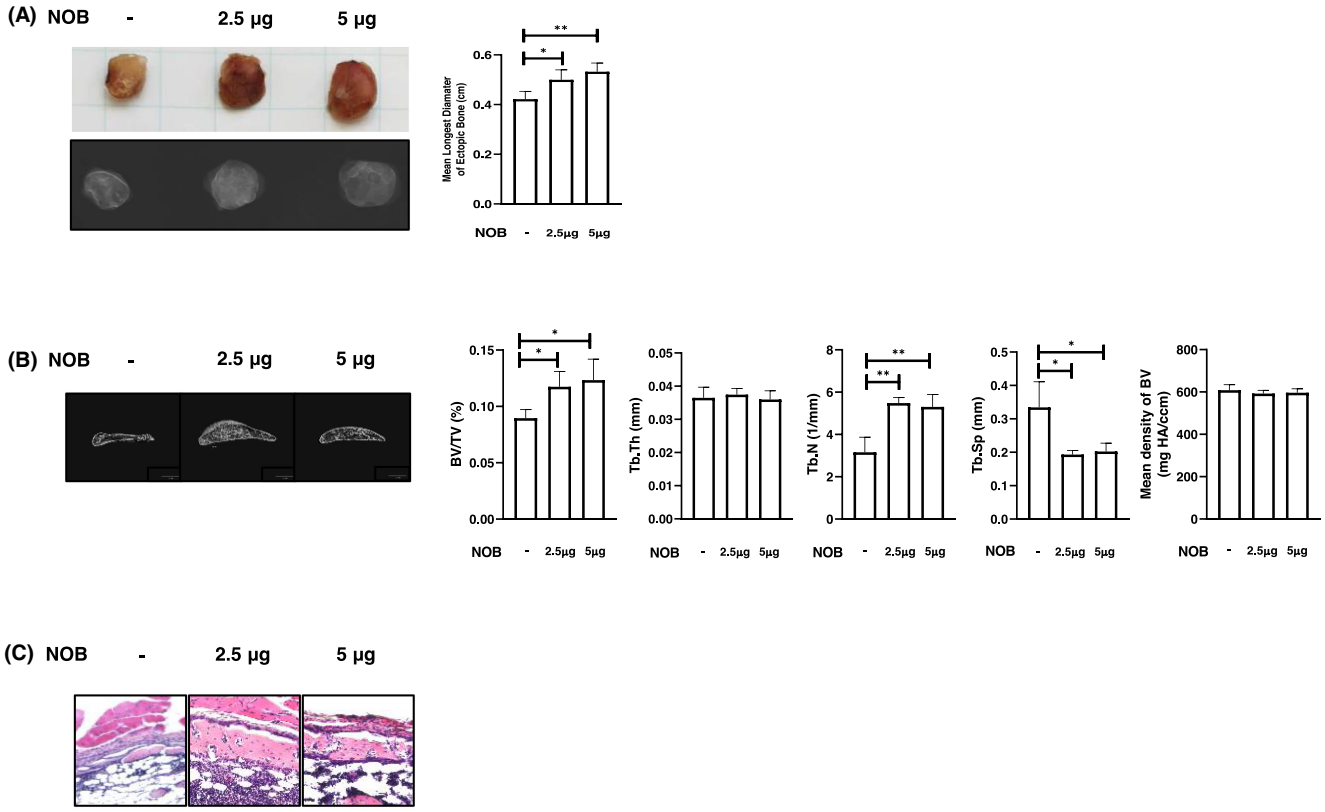

FIGURE 3 Nobiletin enhances bone formation. Ectopic bones induced by BMP-2 with various concentrations of Nobiletin (NOB) (0, 2.5, or 5 μg) were assessed by soft X-ray, and the longest diameters of them were measured (A). Micro-computer tomography (μCT) and μCT analysis generated the BV/TV (%), Tb.Th (mm), Tb.N (1/mm), Tb.Sp (mm), or Mean density of BV (mg HA/ccm) (B). Histology with HE staining analysis (C). Images are representative of triple-independent experiments: scale bar, 100 μm. Data are presented as means ± SD ($n \geq 5$). *$p < 0.05$; **$p < 0.005$ (ANOVA with post hoc test).

stimulation (Figure 3). Uncontrolled inflammation negatively affects bone regeneration.[25] In our animal model, local inflammation associated with pellet implantation surgery or recombinant protein might negatively affect the early stage of bone formation. Recent data that BMP-2 also induces inflammation have also been described.[26] One possible mechanism of inflammation induced by BMP-2 is the production of inflammatory cytokines such as interleukin (IL)-1α, IL-1β, and TNF-α.[27] We, therefore, speculate that NOB could enhance bone formation by reducing inflammation related to surgery and BMP-2. Osteoclasts also reside in BMP-2-induced ectopic bones where marrow cavities (Figure 3C) are present. NOB has also been reported to regulate bone resorption via suppression of osteoclastogenesis.[18,28–30] Therefore, it is possible that NOB not only promoted osteoblastogenesis but also suppressed osteoclastogenesis.

It has been reported that increased expression of TNF-α, IL-1, IL-6, and IL-7 has been found in several chronic inflammatory bone diseases,[31] including rheumatoid arthritis, osteoarthritis, osteoporosis, and periodontal disease.[32] These cytokines are produced by macrophages, lymphocytes, osteoblasts, and bone marrow stromal cells. We found that NOB suppressed inflammatory cytokine production in osteoblasts (Figure 1C and S1). Systemic administration of NOB suppresses systemic bone resorption and maintains bone mass in estrogen-deficient ovariectomized mice.[18,28,30] Future studies exploring NOB effects and mechanisms on bone formation and NF-κB signaling could be beneficial to revealing new therapeutic approaches for bone diseases.

AUTHOR CONTRIBUTIONS

Thira Rojasawasthien and Shoichiro Kokabu designed this experiment. Tomohiko Shirakawa and Shoichiro Kokabu performed the experiments. Thira Rojasawasthien, Michihiko Usui, William N. Addison, Takuma Matsubara, Tomohiko Shirakawa, Toshiyuki Tsujisawa, Keisuke Nakashima and Shoichiro Kokabu reviewed the intermediate draft. Thira Rojasawasthien and Shoichiro Kokabu performed the literature review, prepared the initial and final versions of the article, and submitted the document.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

DISCLOSURES

The authors declare that they have no conflict of interest with the contents of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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