INTRODUCTION

Multinucleated osteoclasts (OCs) can be differentiated from the macrophage lineage.\(^1,2\) OCs play important roles to regulate bone metabolism and homeostasis with OC-specific enzymes such as cathepsin K (CTSK) and tartrate-resistant acid phosphatase (TRAP).\(^2\) OCs can absorb the osteoid and dissolve the hydroxyapatite crystals, leading to diminished bone mineral density and bone growth.\(^2,3\) Several pathological conditions including postmenopausal osteoporosis are caused by reinforced OC activity.\(^4\) Therefore, modulation of OC differentiation and activity is crucial to maintain bone homeostasis.

Five or fewer carbons containing fatty acid are called short-chain fatty acids (SCFAs). The SCFAs are mainly derived from gut microbiota and regulate diverse pathophysiological responses in several different organs and tissues after being absorbed by the intestine.\(^5,6\) For example, previous reports demonstrated that several SCFAs regulate immune cell functions by regulating cytokine and chemokine production.\(^7\) SCFAs modulate...
neutrophil recruitment by regulating the expression of cytokine-induced neutrophil chemoattractant-2α and the adhesion molecule L-selectin. SCFAs also regulate cellular differentiation. Especially, butyric acid induces apoptosis to inhibit macrophage differentiation. Dendritic cells treated with butyric acid promote regulatory T-cell differentiation from naive T cells. Regarding the target receptors for the SCFAs, several G-protein coupled receptors (GPCRs) including Olfr78 have been reported to mediate biological responses caused by the SCFAs.

Branched-chain fatty acids (BCFAs) are made from essential amino acids including leucine by microbial fermentation, to form isobutyric acid with four carbons, or 2-methylbutyric acid and iso-valeric acid (IVA) with five carbons. The levels of the leucine metabolite IVA are increased by the consumption of high protein foods and certain pathological conditions including colon cancer. Previous reports showed that BCFAs affect energy metabolism and cause colon smooth muscle relaxation. Another report demonstrated that enterochromaffin cells are activated by a microbial metabolite, IVA, via Olfr558. However, much remains unknown regarding whether BCFAs regulate additional cellular activities and differentiation.

In this study, we examined whether IVA affects the activity of macrophages and their differentiation into OCs. The molecular mechanism involved in the modulation of OC differentiation by IVA was investigated as well. We also examined the in vivo effects of IVA on the regulation of osteoporosis, an OC-associated disorder.

2 | MATERIALS AND METHODS

2.1 | Generation of mouse bone marrow-derived macrophages (BMDMs)

The protocols for mouse experiments received the approval of the Institutional Review Committee for Animal Care and Use at Sungkyunkwan University (Suwon, Korea). Mouse BMDMs were generated as described before. Briefly, total bone marrow cells were obtained from the femur and tibia of 5-week-old C57BL/6 mice (Orient Bio). After culturing the bone marrow cells in α-MEM (Gibco) with 10% FBS (Access Biological) for 1 day, non-adherent cells were further cultured for 3 days in α-MEM with 10% FBS and 30 ng/mL of M-CSF (Peprotech).

2.2 | OC differentiation and TRAP staining

Isolated mouse BMDMs (1 × 10^4 cells/well) were cultured with 30 ng/mL of M-CSF and 100 ng/mL of RANKL (Peprotech) in 96-well plates for 3 days. Differentiated immature OCs were further differentiated into mature OCs by adding α-MEM with 10% FBS, 30 ng/mL of M-CSF and 100 ng/mL of RANKL for 2 days. Before TRAP staining, mature OCs were fixed with paraformaldehyde (4%) for 20 minutes, as described previously. Stained multinuclear cells (≥3 nuclei) were considered to be OCs and were counted.

2.3 | Cell viability assay

To determine cell viability, BMDMs were treated by IVA (Sigma-Aldrich), and culture media were applied to the LDH assay kit (Promega) at 490 nm.

2.4 | Chemotaxis assay

Chemotactic migration of BMDMs was conducted using multi-well chambers (Neuroprobe Inc) as reported previously. Briefly, 30 μL of chemotactants such as IVA and WKYMVm (Anycgen) were loaded on the lower well and then 25 μL of suspended BMDMs (1 × 10^6 cells/mL) in α-MEM were applied on the upper well, separated by a polycarbon filter. After 2 hours, cells that had migrated through the filter were stained with haematoxylin. Migrated cells in each well were counted under a microscope (Leica DM750).

2.5 | Western blot analysis

BMDMs were stimulated with 30 ng/mL of M-CSF and 100 ng/mL of RANKL in the absence or presence of 200 μM of IVA for the indicated lengths of time. Extracted proteins were separated by 10% SDS-PAGE and transferred to a 0.45 μm nitrocellulose membrane (Cytiva). After incubating the membranes with antibodies for target proteins, visualization with enhanced chemiluminescence followed. Antibodies used for Western blot analyses were obtained from Cell Signalling Technology or Santa Cruz Biotechnology.

2.6 | Bone resorption assay

The pit formation assay was performed on Corning 96-well plates (Corning). BMDMs (1 × 10^4 cells/well) were cultured with 30 ng/mL of M-CSF and 100 ng/mL of RANKL in 96-well plates for 7 days. The media containing M-CSF and RANKL was changed every 2 or 3 days after seeding the cells. After 7 days of induction, the media was removed and cells were incubated in 100 μL of 10% bleach solution for 5 minutes at room temperature. Wells were washed with dH2O 2 times and dried at room temperature for 4 hours. The pit clusters generated by OCs were photographed by a microscope.

2.7 | RNA isolation

Total RNA from mouse BMDMs and immature OCs were isolated using TRIzol reagent (Invitrogen). cDNAs were synthesized using the RT Premix Kit (iNTRON). Femurs and tibias of ovariectomy (OVX)
mouse models were rapidly frozen in liquid nitrogen and ground in a homogenizer to extract RNA using TRIzol reagent.

### 2.8 | Quantitative polymerase chain reaction (qPCR) analysis

qPCR analyses were conducted using the Rotor-gene Q (2plex on PC) instrument (QIAGEN) with SYBR Green qPCR Mix (Biofact). The primers used for qPCR analyses were presented in Table 1. Data were normalized against the expression of GAPDH as an internal control.

### 2.9 | OVX mouse model

OVX was performed with 8-week-old C57BL/6 female mice according to a previous report. Mice were anaesthetized by inhalation anaesthesia, and ovaries were bilaterally removed via a dorsal approach as described previously. To examine the effects of IVA on OVX mice, we added IVA to the drinking water (at a final concentration of 75 mM or 150 mM) from the day after surgery. The mice were sacrificed after 5 weeks, and the femurs were separated and used in subsequent experiments.

### 2.10 | Bone histology in OVX mice

All OVX model mice were sacrificed 5 weeks after surgery, and their bones were fixed in paraformaldehyde solution (4%) for 2 days. Next, the bones were decalcified in 10% EDTA solution for 3 weeks and embedded with paraffin. Bones were sectioned into 4 μm by a microtome. After staining the samples with haematoxylin and eosin (H&E) solution, morphological analysis such as bone density was conducted. TRAP staining was conducted to determine OC formation.

### 2.11 | Statistical analysis

GraphPad Prism software was used to evaluate results. Statistical analysis was performed using Student’s t-test or ANOVA (one-way or two-way). All results are expressed as the mean ± SEM. A P-value < .05 was considered statistically significant.

### 3 | RESULTS

#### 3.1 | IVA stimulates BMDM migration in a pertussis toxin (PTX)-sensitive manner

Various extracellular stimuli elicit the activation of crucial kinases including Akt and MAPKs during cellular activation. In this study, we investigated whether IVA stimulates BMDMs by measuring the phosphorylation of Akt and ERK. Stimulation of BMDMs with IVA caused apparent phosphorylation of Akt and ERK at 30 minutes (Figure 1A). These results suggested that IVA may activate the BMDMs. Chemotactic migration is one of the important functional activities of BMDM, which is needed for subsequent cellular responses. Next, we investigated the effects of IVA on macrophage chemotaxis at several different concentrations. Addition of IVA significantly elicited BMDM chemotactic migration, in an IVA concentration-dependent manner (Figure 1B), maximal migration-inducing activity was observed at 500 μM IVA, which is comparable

---

**TABLE 1** Primers used for qPCR analysis

| RNA   | Forward | Reverse  |
|-------|---------|----------|
| RANK  | 5′-AGAAGACGGTGCTGAGCT-3′ | 5′-TAGGAGCATGAACACGTGC-3′ |
| TRAF6 | 5′-GCCACGGCTGTGACATATG-3′ | 5′-TCGCCCACTGACTTCTACG-3′ |
| OSCAR | 5′-CTGCCGTTATAGGCGTCGCT-3′ | 5′-CCAGAGACCAAGCTCGTCG-3′ |
| TRAP  | 5′-CAGCCGGACGCTCAGGCTG-3′ | 5′-CCGAGCTTGACAGTGC-3′ |
| Blimp1| 5′-GGAGAAGAAACCCGCTG-3′ | 5′-ATTCTGGGACCTCAGGCTACG-3′ |
| c-fos | 5′-AGACGCGGAGAGTTGATAG-3′ | 5′-CGTCATAGAAGAGAAGAGGA-3′ |
| NFATc1| 5′-CAACGGGTCCTGCAAGGAT-3′ | 5′-GGAGGACCTGGAAGGGCT-3′ |
| Ctsk  | 5′-GGAGAGGACCCCTTGGCAG-3′ | 5′-ATCTGGCCGACTCAAGGC-3′ |
| MafB  | 5′-AGTGTGAGAGCCTCGTGG-3′ | 5′-CAGGAAAGACTCCAGGAGAG-3′ |
| IRF8  | 5′-AGACGAGTTGGACGCTG-3′ | 5′-TCCGGGACATTCGTTAAA-3′ |
| OC-STAMP | 5′-TGCGGCTCTCATGACTCTG-3′ | 5′-TCAAATGCGTTAGGAGGAT-3′ |
| DC-STAMP | 5′-GGGTCGTTGGTCCCGCTG-3′ | 5′-CGACTCTTGGTGTCCTCTG-3′ |
| Atp6v0d2| 5′-TTTGTGAGAGGGGACGGAG-3′ | 5′-CACCGGCTGTGACATG-3′ |
| PGC-1α | 5′-AGACACAGTGGTTACAGG-3′ | 5′-GCCGGCTCTCAGTAC-3′ |
| ALP   | 5′-TGAATGAGGCAGGCTATG-3′ | 5′-GTGACATCCACCTCAG-3′ |
| Osx   | 5′-AAAGAGAGGACCAAGAGGC-3′ | 5′-CAGGAAAGATGAGGGAGAG-3′ |
| Runx2 | 5′-CCCTGAACTCCTGACGTC-3′ | 5′-TTGAGGAGATGAGGGAGAT-3′ |
| GAPDH| 5′-CCACCCCTGGTGTGCTGTA-3′ | 5′-AAATGTGCTGGTGGATCT-3′ |
to that of a well-known macrophage chemoattractant, WKYMVm (Figure 1B). Previously, macrophage chemotactic migration was reported to be regulated by PTX-sensitive GPCRs. We also examined the possible role of PTX-sensitive GPCRs in IVA-induced BMDM chemotactic migration. Addition of PTX to BMDMs prior to the chemotaxis assay with IVA resulted in significant inhibition of BMDM chemotactic migration (Figure 1C). These results suggest that IVA stimulates BMDMs leading to chemotactic migration in a PTX-sensitive manner.

### 3.2 IVA inhibits RANKL-induced OC differentiation

Since IVA stimulates the activation of macrophages, which can differentiate into OCs, the effects of IVA on OC generation induced by RANKL was examined. Addition of M-CSF and RANKL to BMDMs generated multinucleated OCs, which can be observed by TRAP staining (Figure 2A). Under OC differentiation conditions, 200 μM IVA showed a strong inhibitory effect on osteoclastogenesis (Figure 2A). Multinucleated TRAP-positive OC numbers were significantly decreased upon IVA exposure (Figure 2B). LPS, a well-known inhibitor of osteoclastogenesis, also significantly blocked OC generation by inducing cell death, we conducted a cell viability analysis and found that IVA did not affect cell viability during OC generation at 100-500 μM (Figure 2E). Collectively, IVA strongly inhibits RANKL-induced OC differentiation without affecting cell viability. Mature OCs show bone resorbing activity. Since we found that IVA suppressed OC formation, we next tested the effects of IVA on OC function by using dentine slices. We found that OC generated by M-CSF + RANKL showed strong bone resorbing activity, while addition of IVA in the presence of M-CSF + RANKL blocked this activity (Figure 2F). Taking our results together, IVA appears to block OC differentiation, leading to inhibition of bone resorbing activity.

### 3.3 IVA inhibits OC-related gene expression

Our finding that IVA suppresses the differentiation of BMDMs into OCs led us to investigate if IVA affects the expression of OC-related genes. RANKL significantly augmented mRNA expression of OC-related genes including RANK, Blimp1, TRAF6, c-fos and NFATc1, a master transcriptional factor of the OCs (Figure 3A left). Addition of IVA to differentiation conditions significantly decreased the expression of OC-related genes (Figure 3A left). An additional set of OC-related genes including OSCAR, TRAP and Ctsk was also significantly suppressed by IVA (Figure 3A right). IVA strongly inhibits the protein expression of OC-related genes such as RANK, TRAF6, NFATc1 and c-fos (Figure 3B). IVA also downregulated the expression of fusogenic genes including OC-STAMP, DC-STAMP and Atp6v0d2 (Figure 3C). However, IVA did not affect the expression of negative regulator genes of osteoclastogenesis such as MafB and IRF8 (Figure 3D). Together,
these results indicate that IVA inhibits OC generation by suppressing the expression of not only OC-related genes but also fusogenic genes.

### 3.4 IVA-induced inhibitory effects on OC generation are partly mediated by PTX-sensitive signalling and AMPK activity

Since IVA has been reported to be recognized by Olfr558,20 and odorant receptors signal through the cAMP/protein kinase A (PKA) pathway,19 we examined whether IVA inhibits OC generation through PKA. Preincubation of BMDMs with a PKA inhibitor (H89) prior to IVA treatment did not block IVA-induced suppression of OC generation (Figure 4A). These results suggest that IVA inhibits OC generation in a cAMP/PKA-independent manner. Next, we checked whether PTX-sensitive signalling is involved in the IVA-induced inhibitory effects on OC generation, because IVA-induced BMDM chemotactic migration is blocked by PTX (Figure 1C). Preincubation of BMDMs with PTX prior to IVA stimulation partly blocked the inhibitory effects of IVA on OC generation, recovering multinucleated...
cell number (Figure 4B). Quantitative analysis showed that PTX significantly increased TRAP-positive multinuclear cell number compared to the vehicle control in IVA-treated groups (Figure 4C). The results suggest that IVA may inhibit OC generation in a PTX-sensitive manner.

Diverse metabolites absorbed in the gut are associated with host energy metabolism. AMPK plays an essential role in energy homeostasis. AMPK also has crucial roles in regulating osteoclastogenesis. Previous reports demonstrate that activated AMPK suppresses osteoclastogenesis. Here, we found that IVA elicits AMPK phosphorylation (Figure 4D). The role of AMPK in the IVA-elicited inhibitory effects on OC generation was also investigated using an AMPK inhibitor, compound C. Addition of compound C markedly reversed the inhibitory effects of IVA on OC generation (Figure 4E,F).

**FIGURE 3** IVA regulates the expression of OC-related genes induced by RANKL. A, C, D, Isolated BMDMs were treated with IVA (200 μM) in the presence of M-CSF (30 ng/mL) and RANKL (100 ng/mL) for 3 days. After RNA isolation, qPCR analyses were conducted using specific primers for OC-related genes (RANK, Blimp1, TRAF6, NFATc1, c-fos, OSCAR, TRAP, and CtsK) (A), fusogenic genes (OC-STAMP, DC-STAMP, and Atp6v0d2) (C), and negative regulator genes of osteoclastogenesis (MafB and IRF-8) (D) and GAPDH. (B) Western blot analysis was performed using antibodies against RANK, TRAF6, NFATc1, c-fos, and β-actin. Data are representative of three independent experiments (B). Data are presented as the mean ± SEM (n = 3 for A, C, D). *P < .05, **P < .01, ***P < .001. NS: not significant.

### 3.5 IVA attenuates OVX-induced osteoporosis

Since IVA inhibits OC generation induced by RANKL in vitro (Figure 2), the effects of IVA in an experimental osteoporosis model using OVX mice were examined. OVX surgery mimics postmenopausal osteoporosis showing body weight gain as well as bone density decrease and increased OC generation in bone tissue. We also found that OVX mice gained body weight compared to control mice. Addition of IVA at the final concentrations of 75 mM and 150 mM to drinking water significantly decreased the body weight gain in OVX mice (Figure 5A). At day 32, OVX mice gain about 25% body weight; however, sham mice gain about 10% body weight. OVX mice exposed to 75 mM and 150 mM IVA gain about 15% and 10.25% body weight, respectively (Figure 5B).
Five weeks after OVX surgery, femurs and tibias were isolated and the levels of OC-related genes were measured with qPCR analysis. Body weight increase in OVX mice is accompanied with decreased PGC-1α expression, which is reversed by IVA (Figure 5C). In addition, OVX surgery significantly augmented the expression of OC-related genes including NFATc1, Ctsk and TRAP, which was almost completely reversed by addition of IVA to the water drinking (Figure 5D). Fusogenic genes such as Atp6v0d2, DC-STAMP and OC-STAMP were also strongly increased by OVX surgery, and IVA exposure significantly decreased the levels of these genes (Figure 5D). However, the expression of MafB, a negative regulator of OC generation, was decreased by OVX and recovered by IVA exposure in OVX mice (Figure 5D). We also performed qPCR analysis of bone turnover markers such as ALP, Osx and Runx2. We found that addition of IVA in the OVX mice model up-regulates the expression of several bone formation-associated genes including Osx and Runx2 (Figure 5D). Histological analysis with H&E staining of the bone tissue of OVX mice showed that OVX surgery markedly increased the porous area, which was strongly decreased by IVA exposure (Figure 5E top). TRAP staining demonstrated that TRAP-positive OCs were increased upon OVX surgery; however, IVA exposure strongly decreased the numbers of TRAP-positive OCs in the bone tissue of OVX mice (Figure 5E bottom). The results suggest that IVA shows strong inhibitory effects against OVX-induced osteoporosis by suppressing osteoclastogenesis in vivo.

4 | DISCUSSION

Diverse metabolites of gut microbiota can regulate many pathophysiological responses. Among these gut microbiota-induced metabolites, SCFAs such as acetate, propionate and butyrate have been reported to regulate various biological functions including defence activity. A previous report demonstrated that three SCFAs, acetate, propionate and butyrate, improve systemic bone mass by attenuating OC formation in an experimental postmenopausal model as well as in a steady state. Here, we found that a BCFA, IVA, suppresses RANKL-induced OC generation without affecting cellular viability (Figure 2). The IVA-induced inhibitory effects on OC generation were also strongly supported by the suppressive activity of IVA on the expression of several OC-related genes (RANK, Blimp1, TRAF6, c-fos, NFATc1, OSCAR, TRAP and Ctsk) and fusogenic genes (OC-STAMP, DC-STAMP and Atp6v0d2) (Figure 3). Since the addition of IVA to BMDMs or pre-OCs resulted in suppression of RANKL-induced OC formation (Figure 2), and IVA stimulates BMDMs, causing crucial kinase activation (Akt and ERK) and chemotactic migration of the cells (Figure 1), IVA may regulate macrophage activity to suppress their differentiation into OCs.

Regarding the mechanism of action of IVA on macrophage activity and differentiation into OCs, we observed that PTX
Cho et al. significantly inhibits BMDM chemotaxis and OC formation induced by IVA (Figures 1C and 4B,C). The results suggest that IVA may act on PTX-sensitive G_{i}-protein-coupled receptors in BMDMs (Figure 6). In a separate experiment, we found that BMDMs express Olfr558 (data not shown), which has been reported to mediate IVA-induced cellular responses in enterochromaffin cells. 20 Although the functional role of Olfr558 in IVA-induced suppression of OC formation is yet to be clarified, IVA may act on G_{i}-coupled GPCRs in BMDMs to block osteoclastogenesis (Figure 6).

**FIGURE 5** IVA shows therapeutic effects against OVX-induced osteoporosis. A, B, Weight gain percentage was measured from the sham, OVX, OVX + IVA (75 mM), OVX + IVA (150 mM) groups for 32 days (A) and at the end of the experiment (B). C, D, Mice were sacrificed 5 weeks after OVX surgery. Femurs and tibias were harvested from each group [sham, OVX, OVX + IVA (150 mM)] to prepare RNA. qPCR analyses were performed using specific primers for PGC-1α (C), OC-related genes (TRAF6, NFATC1, and CtsK), fusogenic genes (OC-STAMP, DC-STAMP and Atp6v0d2), osteoclastogenesis negative regulator genes (MafB), bone turnover markers (ALP, Oxl and Runx2) and GAPDH (D). H&E and TRAP histological staining of mouse femurs of sham, OVX, and OVX + IVA mice 5 weeks after OVX surgery (E). Data are presented as the mean ± SEM (n = 4 for A-D). (A) Two-way ANOVA: *P < .05, **P < .01, ***P < .001 for sham compared with OVX model, #P < .05, ##P < .01 for OVX compared with OVX + IVA (75 mM), oP < .05, ooP < .01, oooP < .001 for OVX compared with OVX + IVA (150 mM). (B) One-way ANOVA: *P < .05, **P < .01, ***P < .001. (C, D) *P < .05, **P < .01, ***P < .001. (E top, E bottom) Data are representative of at least three independent experiments. Scale bar; 100 μm.
of action to suppress OC formation. Cellular signalling pathways involved in the IVA-induced suppression of OC formation were analysed. IVA-inhibited osteoclastogenesis was not affected by H89 (Figure 4A), ruling out a possible role of the cAMP/PKA pathway in the process. However, IVA markedly caused AMPK phosphorylation, and an AMPK inhibitor, compound C, significantly reversed the inhibitory effects of IVA on OC formation (Figure 4D-F), suggesting that the AMPK pathway is required for the inhibition of osteoclastogenesis by IVA (Figure 6). On the regulation of AMPK activity by GPCRs, many different GPCRs were previously reported to regulate AMPK activity. Several G_α-coupled receptors including the β-adrenoreceptor, several G_α-coupled receptors including the formyl peptide receptor and G_α_2-coupled receptors can induce AMPK activation. On the regulatory mechanism of AMPK by GPCR signalling, the G_α_β_γ adenylate cyclase/PKA/LKB1 cascade has been reported to cause phosphorylation of the Thr 172 residue of AMPK. The detailed molecular mechanisms involved in the regulation of IVA-induced AMPK activity is a topic for future investigation.

In this study, we found that IVA not only stimulates chemotactic migration of BMDMs but also suppresses OC formation (Figures 1 and 2). Regarding the functional relationship between the migration of OCs (or osteoclast precursors) and regulation of osteoclast differentiation, a previous report demonstrated that two chemokines, CCL19 and CCL21, stimulate osteoclast migration and bone resorption, mediating bone destruction. In this study, however, we found that IVA stimulates migration of osteoclast precursor cells, but suppresses osteoclast differentiation. At this point, it is not easy to connect the functional relationship of these two different findings. However, according to previous reports that demonstrate the functional relationship of the gut-bone axis, it might be possible that IVA produced by the gut as a commensal bacterial metabolite could regulate bone metabolism leading to inhibition of osteoclast differentiation. Further studies are necessary to clarify the relationship between macrophage migration and regulation of OC differentiation.

Previously, IVA was shown to be produced from a leucine-rich diet based on animal foods such as egg and cheese through fermentation by the human gut microbiome. Here, we observed that IVA suppresses in vitro osteoclastogenesis from BMDMs and elicits therapeutic effects against an in vivo osteoporosis model, OVX mice (Figures 2 and 5). Administration of IVA decreased the expression of OC-related genes and reduced OC generation in OVX mice, reducing bone porous area (Figure 5). In OVX mice, IVA administration also strongly increased expression of MafB, a negative regulator of osteoclastogenesis (Figure 5D). However, IVA did not affect MafB expression from BMDMs, the precursors of OCs (Figure 3D). Since MafB expression was analysed from the total bone of OVX mice, and total bone contains many different cell types including osteoblasts, and diverse leukocytes, as well as OCs, the contradictory effects of IVA on the expression of MafB in vitro and in vivo are likely caused by the complex cell types present in total bone as well as the complex regulation of MafB expression in vivo. In the OVX mice, IVA administration also induced PGC-1α expression (Figure 5C). Since a recent study showed that PGC-1α regulates osteoclastogenesis through metabolic regulation, it would be necessary to examine the effects of IVA on the regulation of metabolic activity in OC precursors. Our results suggest that IVA, a BCFA, can be used to control osteoporosis. In a previous paper, beta-hydroxy-beta-methylbutyrate, another leucine metabolite, was reported to be associated with bone growth in newborn pigs. Thus, we suggest that metabolites derived from leucine-rich diets may have potential beneficial effects against bone metabolic disorders including osteoporosis. In addition, isovaleric acidemia is an inborn error of leucine metabolism caused by a congenital deficiency of isovaleryl CoA dehydrogenase with elevated urinary IVA metabolites. Although the clinical syndrome of isovaleric acidemia is dominated by the neurologic findings, haematologic abnormalities are frequent. Also, the unpleasant odour of IVA in urine is typical. Our study may provide some molecular mechanisms to explain the clinical phenotype of isovaleric acidemia.

In conclusion, we found that IVA, a BCFA metabolite of a leucine-rich diet, can regulate macrophage activity and suppress osteoclastogenesis through G_α_2-coupled GPCR and AMPK-dependent, but
PKA-independent pathways. The therapeutic effects of IVA against an experimental osteoporosis O VX model suggest that IVA and a leucine-rich diet, which can be used to produce IVA, can be considered as a potentially new approach against OC-related bone diseases.

ACKNOWLEDGEMENTS
This study was supported by National Research Foundation of Korea (NRF) (NRF-2018R1A2B3003868, NRF-2017R1A5A1014560, NRF-2020M3A9D3038435).

CONFLICT OF INTEREST
The authors confirm that there are no conflicts of interest.

AUTHOR CONTRIBUTION
Kwang Min Cho: Conceptualization (supporting); Data curation (supporting); Formal analysis (supporting); Investigation (supporting); Funding acquisition (supporting); Project administration (supporting); Resources (supporting); Writing-review & editing (supporting). Ye Seon Kim: Data curation (supporting); Investigation (supporting). Mingyu Lee: Data curation (supporting); Formal analysis (supporting); Investigation (supporting). Ha Young Lee: Conceptualization (supporting); Data curation (supporting); Formal analysis (supporting); Writing-review & editing (supporting). Yoo-Sik Bae: Conceptualization (lead); Funding acquisition (lead); Project administration (lead); Resources (lead); Writing-review & editing (lead).

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

ORCID
Yoo-Sik Bae https://orcid.org/0000-0002-6198-4512

REFERENCES
1. Takahashi N, Ejiri S, Yanagisawa S, Ozawa H. Regulation of osteoclast polarization. Odontology. 2007;95:1-9.
2. Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature. 2003;423:337-342.
3. Kawana F, Sasaki T. Osteoclast differentiation and characteris- tic trabecular bone formation during growth plate destruction in osteoprotegerin-deficient mice. J Electron Microsc (Tokyo). 2003;52:515-525.
4. Zaidi M. Skeletal remodeling in health and disease. Nat Med. 2007;13:791-801.
5. Dallé B, Van Oudenhove L, Vervliet B, Urfer P. The role of short-chain fatty acids in microbiota–gut–brain communication. Nat Rev Gastroenterol Hepatol. 2019;16:461-478.
6. Zaiss MM, Jones RM, Schett G, Pacifici R. The gut-bone axis: How bacterial metabolites bridge the distance. J Clin Invest. 2019;129:3018-3028.
7. Corrêa-Oliveira R, Fachi JL, Vieira A, Sato FT, Vinolo MAR. Regulation of immune cell function by short-chain fatty acids. Clin Transl Immunol. 2016;5:73.
8. Vinolo MAR, Rodrigues HG, Hatanaka E, Hebeba CB, Farsky SHP, Curi R. Short-chain fatty acids stimulate the migration of neutrophils to inflammatory sites. Clin Sci. 2009;117:331-338.
9. Fu H, Shi YQ, Mo SJ. Effect of short-chain fatty acids on the proliferation and differentiation of the human colonic adenocarcinoma cell line Caco-2. Chin J Dig Dis. 2004;5:115-117.
10. Kim CH, Park J, Kim M. Gut microbiota-derived short-chain fatty acids, T cells, and inflammation. Immun Netw. 2014;14:277-288.
11. Bailón E, Cueto-Sola M, Utrilla P, et al. Butyrate in vitro immune-modulatory effects might be mediated through a proliferation-related induction of apoptosis. Immunobiology. 2010;215:863-873.
12. Gurav A, Sivaprakasam S, Bhutia YD, Boettger T, Singh N, Ganapathy V. Slt5a8, a Na+ coupled high-affinity transporter for short-chain fatty acids, is a conditional tumour suppressor in colon that protects against colitis and colon cancer under low-fibre dietary conditions. Biochem J. 2015;469:267-278.
13. Singh N, Gurav A, Sivaprakasam S, et al. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. Immunity. 2014;40:128-139.
14. Pluznick JL, Protzko RJ, Gevorgyan H, et al. Olfactfactory receptor responding to gut microbiotaderived signals plays a role in renin secretion and blood pressure regulation. Proc Natl Acad Sci U S A. 2013;110:4410-4415.
15. Lynch CJ, Adams SH. Branched-chain amino acids in metabolic signaling and insulin resistance. Nat Rev Endocrinol. 2014;10:723-736.
16. Weir TL, Manter DK, Shelifin AM, Barnett BA, Heuberger AL, Ryan EP. Stool microbiome and metabolome differences between colorectal cancer patients and healthy adults. PLoS One. 2013;8:e70803.
17. David LA, Maurice CF, Carmody RN, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014;505:559-566.
18. Heimann E, Nyman M, Pålbrink AK, Lindkvist-Pettersson K, Degerman E. Branched short-chain fatty acids modulate glucose and lipid metabolism in primary adipocytes. Adipocyte. 2016;5:359-368.
19. Blakeney BA, Crowe MS, Mahavadi S, Murthy KS, Grider JR. Branched short chain fatty acid isovaleric acid causes colonic smooth muscle relaxation via cAMP/PKA pathway. Dig Dis Sci. 2019;64:1171-1181.
20. Bellono NW, Bayrer JR, Leitch DB, et al. Enterochromaffin cells are gut chemosensors that couple to sensory neural pathways. Cell. 2017;170:185-198.
21. Lee Y, Kim HJ, Park CK, et al. MicroRNA-124 regulates osteoclast differentiation. Bone. 2013;56:383-389.
22. Park MY, Kim HS, Lee M, et al. FAM19A5, a brain-specific chemokine, inhibits RANKL-induced osteoclast formation through formyl peptide receptor 2. Sci Rep. 2017;7:15575.
23. Oh E, Lee HY, Kim HJ, et al. Serum amyloid A inhibits RANKL-induced osteoclast formation. Exp Mol Med. 2015;47:e194.
24. Bae YS, Park EY, Kim Y, et al. Identification of novel chemokine receptors signaling and insulin resistance. Immunity. 2015;469:267-278.
33. Väänänen HK, Zhao H, Mulari M, Halleen JM. The cell biology of osteoclast function. J Cell Sci. 2000;113:377-381.
34. Okamoto K, Takayanagi H. Osteoimmunology. Cold Spring Harb Perspect Med. 2019;9:a031245.
35. Kim K, Lee SH, Jung HK, Choi Y, Kim N. NFATc1 induces osteoclast fusion via up-regulation of Atpt6v0d2 and the Dendritic Cell-Specific Transmembrane Protein (DC-Stamp). Mol Endocrinol. 2008;22:176-185.
36. Miyamoto T. Regulators of osteoclast differentiation and cell-cell fusion. Keio J Med. 2011;60:101-105.
37. Zhao B, Takami M, Yamada A, et al. Interferon regulatory factor-8 regulates bone metabolism by suppressing osteoclastogenesis. Nat Med. 2009;15:1066-1071.
38. Kim K, Jung HK, Lee J, et al. MafB negatively regulates RANKL-mediated osteoclast differentiation. Blood. 2007;109:3253-3259.
39. Donohoe DR, Garge N, Zhang X, et al. The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. Cell Metab. 2011;13:517-526.
40. Wu L, Zhang L, Li B, et al. AMP-activated protein kinase (AMPK) regulates energy metabolism through modulating thermogenesis in adipose tissue. Front Physiol. 2018;9:122.
41. Hardie DG, Ross FA, Hawley SA. AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat Rev Mol Cell Biol. 2012;13:251-262.
42. Mai QQ, Zhang ZM, Xu S, et al. Metformin stimulates osteoprotgerin and reduces RANKL expression in osteoblasts and ovariectomized rats. J Cell Biochem. 2011;112:2902-2909.
43. Lee K, Chung YH, Ahn H, Kim H, Rho J, Jeong D. Selective regulation of MAPK signaling mediates RANKL-dependent osteoclast differentiation. Int J Biol Sci. 2016;12:235-245.
44. Kang N, Kim KW, Shin DM. Humanin suppresses receptor activator of nuclear factor-κB ligand-induced osteoclast differentiation via AMP-activated protein kinase activation. Korean J Physiol Pharmacol. 2019;23:411-417.
45. McCann RM, Colleary G, Geddis C, et al. Effect of osteoporosis on bone mineral density and fracture repair in a rat femoral fracture model. J Orthop Res. 2008;26:384-393.
46. Lucas S, Omata Y, Hofmann J, et al. Short-chain fatty acids regulate systemic bone mass and protect from pathologic bone loss. Nat Commun. 2018;9:55.
47. Alexeev EE, Lanis JM, Kao DJ, et al. Microbiota-derived indole metabolites promote human and murine intestinal homeostasis through regulation of interleukin-10 receptor. Am J Pathol. 2018;188:1183-1194.
48. Zelante T, Iannitti RG, Cunha C, et al. Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. Immunity. 2013;39:372-385.
49. Furusawa Y, Obata Y, Fukuda S, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature. 2013;504:446-450.
50. Hutchinson DS, Summers RJ, Bengtsson T. Regulation of AMP-activated protein kinase activity by G-protein coupled receptors: potential utility in treatment of diabetes and heart disease. Pharmacol Ther. 2008;119:291-310.
51. Gauthier MS, Miyoshi H, Souza SC, et al. AMP-activated protein kinase is activated as a consequence of lipolysis in the adipocyte: Potential mechanism and physiological relevance. J Biol Chem. 2008;283:16514-16524.
52. Thors B, Halldorsson H, Thorgerisson G. Thrombin and histamine stimulate endothelial nitric-oxide synthase phosphorylation at Ser1177 via an AMPK mediated pathway independent of PI3K-Akt. FEBS Lett. 2004;573:175-180.
53. Stahmann N, Woods A, Carling D, Heller R. Thrombin activates AMP-activated protein kinase in endothelial cells via a pathway involving Ca²⁺/calmodulin-dependent protein kinase j. Mol Cell Biol. 2006;26:5933-5945.
54. Lee J, Park C, Kim HJ, et al. Stimulation of osteoclast migration and bone resorption by c-c chemokine ligands 19 and 21. Exp Mol Med. 2017;49:e358.
55. Tyagi AM, Yu M, Darby TM, et al. The microbial metabolite butyrate stimulates bone formation via T regulatory cell-mediated regulated expression of WNT10B. Immunity. 2018;49:1116-1131.e7.
56. Baba M, Endoh M, Ma W, et al. Folliculin regulates osteoclastogenesis through metabolic regulation. J Bone Miner Res. 2018;33:1785-1798.
57. Blicharski T, Tomaszewksa E, Dobrowolski P, Hulas-Stasiak M, Muszyński S. A metabolite of leucine (β-hydroxy-β-methylbutyrate) given to sows during pregnancy alters bone development of their newborn offspring. PLoS One. 2017;12:e0179693.
58. Kelleher JF, Yudkoff M, Hutchinson R, August CS, Cohn RM. The Pancytopenia of isovaleric acidemia. Pediatrics. 1980;65:1023-1027.
59. Derksen M, Duran M, Jlst L, et al. Clinical variability of isovaleric acidemia in a genetically homogeneous population. J Inherit Metab Dis. 2012;35:1021-1029.

How to cite this article: Cho KM, Kim YS, Lee M, Lee HY, Bae Y-S. Isovaleric acid ameliorates ovariectomy-induced osteoporosis by inhibiting osteoclast differentiation. J Cell Mol Med. 2021;25:4287-4297. https://doi.org/10.1111/jcmm.16482