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Metformin activates AMPK and mTOR to Inhibit RANKL-stimulated osteoclast formation

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Abstract. – OBJECTIVE: Metformin is a medication used to treat type 2 diabetes by inhibiting hepatic glucose production through adenosine monophosphate-activated protein kinase (AMPK) activation. Autophagy is closely related to the homeostasis and stress mechanisms of the body. In recent years, much research has arisen on therapeutic methods utilizing autophagy mechanisms to treat diagnoses such as metabolic diseases, tumors, and Alzheimer's disease. This study thus aimed to investigate the effects of metformin treatment on the differentiation of osteoclasts and changes in AMPK mechanisms, which play an important role in regulating energy homeostasis, and mTOR, a highly conserved kinase that regulates autophagy.

MATERIALS AND METHODS: Experimentation, including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, tartrate-resistant acid phosphate (TRAP) staining, pit formation assay, immunofluorescence, western blotting, and real-time polymerase chain reaction (PCR) was performed to investigate the effects of metformin on osteoclast differentiation. Additionally, to investigate its association with AMPK and pathways, the AMPK inhibitor compound C and mammalian targets of rapamycin (mTOR) activator leucine were used to examine the expression of osteoclast- or autophagy-related proteins, genes, and TRAP-positive cells.

RESULTS: Metformin showed no cytotoxic effects on mouse osteoblastic cell lines (MC3T3-E1) and murine macrophage cell lines (RAW264.7) but did inhibit osteoclast differentiation. Furthermore, metformin was found to inhibit osteoclast differentiation through AMPK activation and mTOR inhibition. In turn, AMPK inhibition using compound C promoted osteoclast differentiation, and mTOR activation using leucine inhibited autophagy and osteoclast differentiation.

CONCLUSIONS: Metformin activates the AMPK pathway while functioning as an activator of mTOR, thereby leading to the inhibition of autophagy and osteoclast differentiation.

Key Words:
Metformin, Autophagy, AMPK, mTOR, Osteoclast formation.

Introduction

Supported by skeletal muscles to protect important body organs, bones store calcium and other minerals[1] to regulate blood and tissue calcium levels, in turn allowing the control of numerous cellular functions such as skeletal and cardiac muscle contractions[2]. Bone remodeling is a normal physiological process that maintains the integrity of the skeleton by removing old bones, repairing them, and replacing them with new bones. In this process, bones are removed by osteoclasts and then replaced with new bones by osteoblasts. In total, 10% of the body's total bones are replaced and continuously renewed throughout the human lifespan[3,4]. An imbalance in osteoclast and osteoblast function and formation can result in bone diseases such as osteoporosis, osteopenia, osteomalacia, and Paget's disease. In particular, osteoporosis – a degenerative disease associated with aging – is caused by abnormal activation of osteoclasts, which is directly related to patient death. Several studies[5-7] have already been conducted on this topic, but more research is required to overcome this disease. When differentiating osteoclasts from bone marrow monocyte/macrophage lineage cells, a combination of receptor activator nuclear factor-κB (RANK) and nuclear factor-κB ligand (RANKL) is essential to

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stimulate nuclear factor-κB (NF-κB), which promotes osteoclast differentiation and resorption, as well as forms mature osteoclasts[8]. Osteoclast formation and osteolysis form an energy-consuming process supported by high metabolic activity[9]. Adenosine monophosphate-activated protein kinase (AMPK) plays a key role in regulating this energy homeostasis and is activated in response to environmental or nutritional stressors that deplete adenosine monophosphate (AMP) levels in cells[10]. AMPK is thus known to play an essential role in the high-energy environment of osteoclasts[11].

Metformin, a derivative of guanidine first extracted from plant isoamylenes in the 1920s, has been used for over 50 years in the treatment of hyperglycemia, type 2 diabetes, polycystic ovarian disease, diabetic nephropathy, and gestational diabetes[12,13]. Several studies[14,15] have revealed that the majority of metformin's effects depend on its ability to activate AMPK – a major cellular modulator of lipid and glucose metabolism. Metformin inhibits lipid synthesis and gluconeogenesis and promotes a glucose-lowering effect, as activated by AMPK and protein kinase A (PKA). The activation of AMPK suppresses PI3K, Akt, and mammalian targets of rapamycin (mTOR) complexes, which regulate T cell effector differentiation *in vitro* and *in vivo*[16]. Furthermore, AMPK directly modulates mTOR activity and phosphorylates Unc-51-like kinase 1 (ULK1) to regulate autophagic action by eliminating energy stress[17]. mTOR, a serine/treonine kinase, regulates cell growth and proliferation as a master regulator of cell metabolism[18].

Autophagy is a dynamic process in which unnecessary dysfunctional cargo proteins are transferred to lysosomes, where they are degraded to serve as an alternative energy source[19]. Autophagy is a self-digestion system that removes unnecessary substances and provides nutrients and energy for cell survival and maintenance10. There are many studies[21-24] on the important role of autophagy and autophagy-related proteins in osteoclast differentiation, bone resorption, and bone homeostasis in bone remodeling, as well as research that defends the association of their dysfunction with many bone-related disorders, including osteoporosis, osteopenia, and Paget's disease. Several researchers[25-27] have associated various signaling pathways with the regulation of autophagy, and a few of these pathways converge on targets of rapamycin (TOR), a highly conserved kinase important to autophagy. When mTOR is activated by nutrients and growth factors in cells, autophagy is suppressed by the phosphorylation of ULK1, autophagy-related protein (ATG), and activating molecule in beclin1-regulated autophagy (AMBRA1), all proteins associated with autophagy initiation and nucleation[18]. A recent study[28] reported that the autophagic process plays a rather important role in osteoclast formation and bone resorption *in vitro* and *in vivo*, as ATGs and microtubule-associated proteins 1A/1B light chain 3 (LC3) – essential autophagy-related proteins – enable osteoclasts' secretory functions. Therefore, the inhibition of autophagy in osteoclasts has been recognized as an important component of osteoporosis[29-31].

Metformin has preventative effects in osteoclast differentiation and bone resorption *in vivo* and *in vitro*[32-34]. However, the effects of metformin treatment on the relationship between AMPK and autophagy in osteoclast differentiation are still poorly understood. In this study, we investigate the role of metformin in osteoclast differentiation and elucidate its effects on mTOR and AMPK pathways.

Materials and Methods

Cell Culture and Osteoclast Differentiation

MC3T3-E1 (mouse osteoblastic cell line) cells were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were routinely cultured at 37°C in a humidified atmosphere of 5% CO_2 and maintained in an alpha modification of Dulbecco's Modified Eagle's medium (α-MEM without ascorbic acid; GIBCO, Grand Island, NY, USA) with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin. RAW264.7 cells (murine macrophage cell line) were purchased from the Korean Cell Line Bank (Seoul, South Korea). These cells were also cultured at 37°C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle's medium (DMEM; GIBCO, Grand Island, NY, USA) with 10% FBS (GIBCO, Grand Island, NY, USA) without antibiotics. To induce osteoclast differentiation, RANKL (10 ng/ml; R&D systems, Minneapolis, MN, USA) was added to the culture medium of the RAW264.7 cells and cultured in an incubator at 37°C with 5% CO_2 for up to 5 days. After 5 days, multinucleated osteoclasts were observed.

MTT Assay

The MC3T3-E1 and RAW264.7 cells' viability was determined via MTT (Sigma Aldrich, Louis, MO, USA) assay. The MC3T3-E1 cells were

seeded in a 96-well plate (1×10^4 cells/well) and incubated for 24 h to be attached and stabilized. In contrast, the RAW264.7 cells (2×10^4 cells/well) were cultured for 2 h because they were sufficiently stabilized within that time. Then, both sets of cells were treated with metformin (0-300 μM; Sigma Aldrich, Louis, MO, USA) for 48 h. After removing the supernatant, 100 ml (0.5 mg/ml) MTT solution was added to each well and incubated at 37°C for 4 h. The insoluble purple formazan product produced in the cells was lysed by adding Dimethyl sulfoxide (DMSO) (200 μl/well; Sigma Aldrich, Louis, MO, USA) and constantly shaken for 10 min. After the supernatant was transferred to a new 96-well plate, the plate was read with a microplate spectrophotometer at a 570-nm wavelength using the SpectraMax iD3 micro reader (BioTek, Winousk, VT, USA). The results were presented in the form of the percentage of change in activity compared to untreated controls.

TRAP-Positive Cell Staining

RAW264.7 cells were seeded for 2 h and stabilized in a 24-well plate (2×10^5 cells/well). Thereafter, the cells were treated with 0-300 μM metformin in the presence of 10 ng/ml RANKL and incubated for 5 days. The medium was replaced each day. After osteoclast differentiation, the cells were fixed in 4% paraformaldehyde (PFA; Sigma Aldrich, Louis, MO, USA) for 10 min at room temperature and then stained for tartrate-resistant acid phosphate (TRAP) using an acid phosphatase leukocyte kit (Sigma Aldrich, Louis, MO, USA) according to the manufacturer's instructions. Multinucleated TRAP-positive cells with three or more nuclei were considered osteoclasts under microscopic examination using an inverted microscope (Olympus CX41, Japan).

Pit Formation Assay

To measure the osteoclasts' resorption capacity, we performed a pit formation assay. RAW264.7 cells were seeded for 2 h and stabilized in a 24-well plate coated with calcium phosphate (2×10^5 cells/well; Osteo Assay Plate Corning, NY, USA). Thereafter, the cells were treated with 0-300 μM metformin in the presence of 10 ng/ml RANKL and incubated for 5 days. The medium was replaced each day. The cells were lysed with 10% NaOCl, and the Osteo Assay Plate were washed with distilled water. The resorbed areas on the plates were observed with an inverted microscope (Olympus CX41, Japan), and each plate's pit area was measured using Image J software.

Immunofluorescent Staining

RAW264.7 cells (2×10^5 cells) were seeded for 2 h and stabilized on a Lab-TekTM II Chamber Slide (Nunc; Thermo Fisher Scientific, Rochester, NY, USA). The cells were then treated with 200 μM metformin in the presence of 10 ng/ml RANKL and incubated for 5 days. The medium was replaced each day. The cells were fixed in 4% paraformaldehyde (Sigma Aldrich, Louis, MO, USA) and maintained in 0.1% Triton X-100 for 10 min at room temperature. The cells were then incubated with 1% Bovine serum albumin (BSA) in Phosphate-buffered saline (PBS) for 1 h. After washing the sample with PBS three times, it was treated with primary antibodies NFATc1, TRAF6, and mTOR (1:200 dilution; Abcam, USA) and incubated overnight at 4°C. After once more washing with PBS three times, a secondary antibody (Alex Fluor 488 anti-mouse, Invitrogen, Eugene, OR, USA) was diluted at a ratio of 1:200 for 10 min at room temperature. The sample was stained using rhodamine phalloidin (Invitrogen, Eugene, OR, USA) for 30 min. Fluorescent images were observed and analyzed using a Zeiss LSM 750 laser scanning confocal microscope (Göttingen, Germany).

Western Blot Assay

For western blotting, RAW264.7 cells were prepared as samples under various conditions and treated with metformin and RANKL. The cells were lysed in RIPA Lysis Buffer (Sigma Aldrich, Louis, MO, USA) containing a protease inhibitor cocktail buffer (Sigma Aldrich, Louis, MO, USA), incubated at 4°C for 1 h, and centrifuged at 13,200 rpm for 30 min at 4°C. Protein concentrations of the cell lysates were determined via Bradford protein assay (Bio-Rad, Richmond, CA, USA), and 10 μg proteins were resolved using 10% SDS/PAGE gel before being transferred into polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies (1:1000 dilution; NFATc1, TRAF6, mTOR, p-mTOR MMP9, cathepsin K, AMPK, p-AMPK, ULK, p-ULK, Beclin-1, ATG5, p62 LC3B, and β-actin; purchased from Abcam and Cell Signaling Technology, USA). Immunostaining with secondary antibodies took place using the Chemiluminescent HRP Substrate Kit (Millipore, Billerica, MA, USA) and Super-SignalWest Femto substrate (Pierce, Rockford, IL, USA). Protein expression was detected using ImageQuant LAS 500 chemiluminescence (GE Healthcare, Chicago, IL, USA).

Real-Time PCR

RAW264.7 cells were seeded in six-well plates at a density of 2×10^5 cells per well. After seeding, the cells were treated with 1.25 μM compound C (Com C), 10 μM leucine, and 200 μM metformin either alone or in combination in a medium containing 10 ng/ml RANKL. The cells were then incubated for 5 days. Total RNA was prepared using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). RNA was also extracted from each sample using the RNeasy Mini Kit and quantified using a SpectraMax iD3 micro reader (BioTek, Winousk, VT, USA). The RNA concentration was set to 100 nM for each sample. The RNA from each sample was synthesized into cDNA using a RevertAid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Pittsburgh, PA, USA) according to the manufacturer's protocol and as previously described[35]. Real-time quantitative PCR was performed using Maxima™ SYBR Green/ROX qPCR Master Mix (Fermentas); the primers (sequenced by Macrogen Institute, Seoul, Korea), which were diluted with cDNA, are listed in Table I. The reactions were run on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems 7500 System, Foster City, CA, USA) using Sequence Detection System software version 2.0.1. Relative mRNA levels were normalized using GAPDH as a housekeeping gene.

Statistical Analysis

Statistical analysis was performed using Prism 5 (GraphPad Software Inc., La Jolla, CA, USA) with one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. All experimental data were independently repeated three times. Statistical significance was set at $p < 0.05$.

Results

Metformin Is Not Cytotoxic, but Attenuates RANKL-Induced Osteoclast Differentiation

We first measured cytotoxicity *via* MTT assay to identify the appropriate concentration of metformin. Then, the MC3T3-E1 cells (osteoblast-like cells) and RAW264.7 (macrophages) were treated with metformin at concentrations of 0-300 μM and observed for 48 h for changes in cell viability. Metformin had no effect on RAW264.7 cell viability and triggered weakly increasing cell proliferation at 200 μM in the MC3T3-E1 cells (Figure 1A-B). Next, we performed TRAP staining[36] and pit formation assay to identify how metformin at the same concentrations (0-300 μM) affects osteoclast formation. RANKL (10 ng/mL) was applied to RAW264.7 cells to induce osteoclast differentiation. Metformin significantly inhibited the formation of osteoclasts (TRAP-positive cells) in the 100-μM treatment group (18.7 cells), and almost no osteoclast formation occurred in the 200- (12.1 cells) and 300-μM (5.3 cells) treatment groups. In contrast, the number of osteoclasts formed in the control group (RANKL-only) was 53.6 cells (Figure 1C and 1E).

Table I. Primer Sequences.

Target gene		Primer sequence (5′ to 3′)
ACP5	Forward	CTACCTGTGTGGACATGACCA
	Reverse	GCACATAGCCCACAC CGTTC
Cathepsin K	Forward	TACCCATATGTGGGCCAGGA
	Reverse	TTCAGGGCTTTCTCGTTCCC
Calcitonin receptor	Forward	TAGTTAGTGCTCCTCGGGCT
	Reverse	AGTACTCTCCTCGCCTTCGT
TRAF6	Forward	ATATGACAGCCACCTCCCCT
	Reverse	GGCAAGCAGTTCTGGTTTGG
c-Fos	Forward	TACTACCATTCCCCAGCCGA
	Reverse	GCTGTCACCGTGGGGATAAA
NFATc1	Forward	CCAGCTTTCCAGTCCCTTCC
	Reverse	AGGTGACACTAGGGGACACA
OSCAR	Forward	CGTGCTGACTTCACACCAAC
	Reverse	GGTCACGTTGATCCCAGGAG
MMP9	Forward	AAAACCTCCAACCTCACGGA
	Reverse	CACAGCGTGGTGTTCGAATG
ATP6V0d2	Forward	CTGGTTCGAGGATGCAAAGC
	Reverse	TCCAAGGTCTCACACTGCAC
GAPDH	Forward	CCGCATCTTCTTGTGCAGT
	Reverse	TCAATGAAGGGGTCGTTGAT

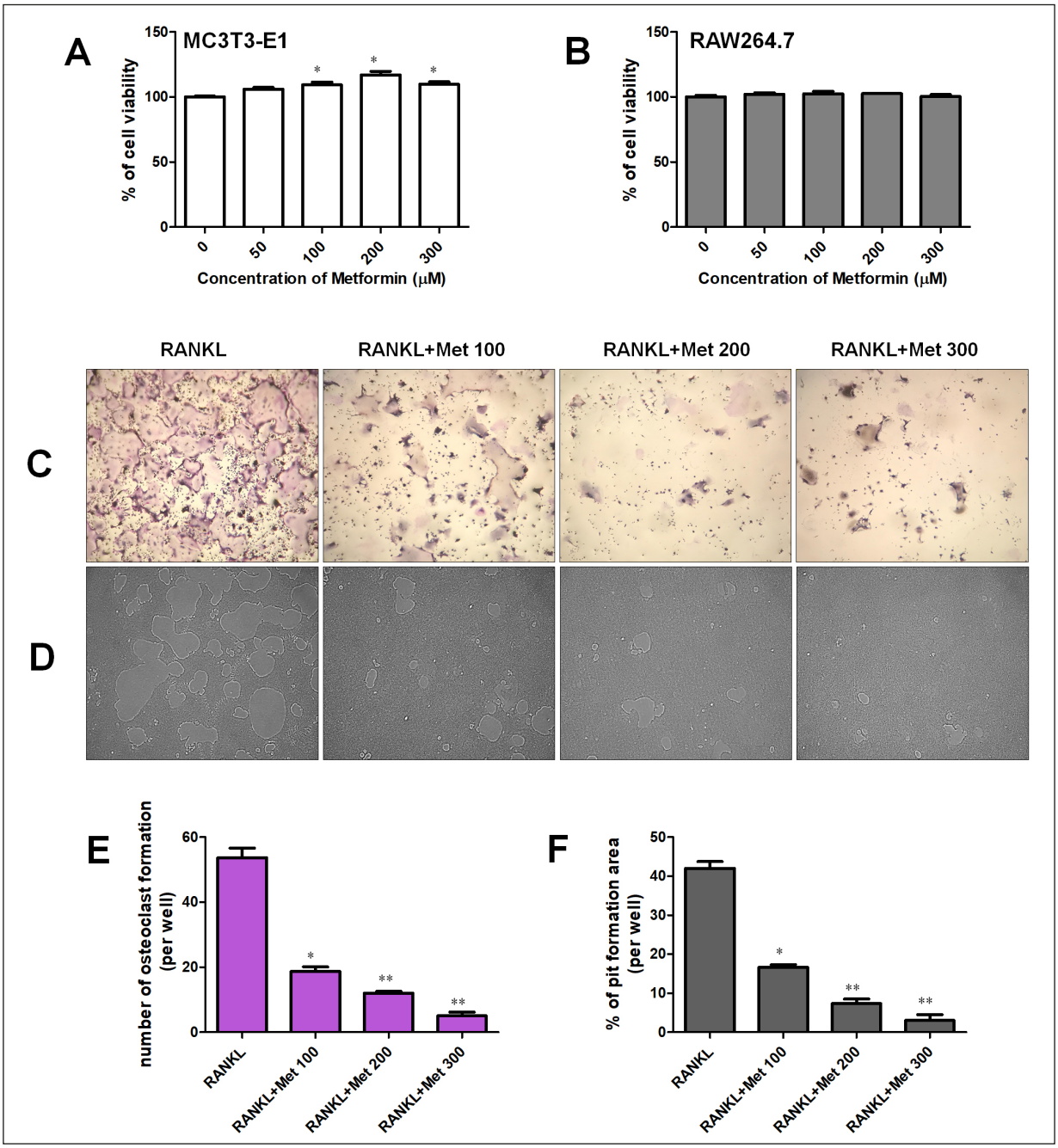

Figure 1. Metformin showed low cytotoxicity but attenuating RANKL-induced osteoclast differentiation at the same concentrations in RAW264.7 cells. **A-B**, Metformin (0-300 μM) was used on MC3T3E1 and RAW264.7 cells for 48 h, and cytotoxicity was measured *via* MTT assay. Each experiment was performed at least three times, and data were calculated as a percentage of control (nontreated: 0 μM; *$p < 0.05$). RAW264.7 cells then received a single treatment of RANKL or a combination treatment of RANKL and metformin for 5 days. **C**, TRAP staining of RANKL-induced osteoclast with metformin treatment (0-300 μM). **D**, Resorption pit formation of RANKL-induced osteoclast with metformin treatment (0-300 μM) observed under light microscopy. Images were captured at 40x. **E**, Ratio of osteoclast formation in metformin-treated cells. **F**, Ratio of the resorption pit in metformin-treated cells. Each experiment was performed at least three times, and data were calculated as a percentage of control (RANKL-only treatment; *$p < 0.05$, **$p < 0.01$).

Bone resorption of osteoclasts occurs when cells enter the differentiation process and activity occurs[37]. We thus investigated how metformin affects the functional outcome of osteoclast formation on bone resorption. While resorption pits were observed upon osteoclast differentiation

[RANKL-only treated group (control): 42.2% pit area], the addition of metformin significantly reduced the area of pit formation by up to 3% (metformin 300-μM treatment group; Figure 1D and 1F). These results indicate that metformin can significantly inhibit osteoclast differentiation through RANKL, even though there is no cytotoxicity.

Metformin Inhibits NFATc1 and TRAF6 Expression, including Osteoclast-Specific Genes

RANKL-induced macrophage stimulation, in turn, induces the accumulation of tumor necrosis factor (TNF) receptor-related factor (TRAF), which leads to the nuclear factor of activated T cells (NFATc1); these factors are ultimately considered markers for mature osteoclast generation[38]. Based on this knowledge, we set the metformin concentration at 200 μM and proceeded with the next experiment. RAW264.7 cells were treated with 200 μM metformin and RANKL, and osteoclasts matured for 3 days. Then, the expression of NFATc1 and TRAF6 was confirmed *via* western blotting and confocal microscopy. The protein expressions of NFATc1 and TRAF6 were significantly decreased in the metformin-treated group compared to the RANKL-treated positive control group (Figure 2A-B), as defended by immunofluorescence after confocal microscopy (Figure 2C-D). These results suggest that metformin inhibits the expression of sub-factor NFATc1 by interfering with the accumulation of TRAF6 and makes osteoclast maturation difficult.

How metformin affects osteoclast-specific genes Metalloproteinase-9 (MMP9), cathepsin K (CTSK), ACP5, calcitonin receptor (Calr), and osteoclast associated receptor (OSCAR) were also evaluated using western blotting and qPCR. As shown in Figure 2A-B, the RANKL-only treatment group upregulated the protein expression of *MMP9* and *CTSK*, but metformin significantly inhibited this upregulation. Similarly, Figure 2E features the confirmation of *MMP9* and *CTSK* gene expression *via* qPCR, with metformin significantly suppressing their upregulation compared to the RANKL-only treatment group. Additionally, metformin was shown to inhibit the gene expression of *ACP5, Calr, CTSK,* and *OSCAR*. These data suggest that metformin inhibits hydroxyapatite reuptake by attenuating messenger RNA (mRNA) expression in osteoclast-specific genes.

Metformin Activates AMPK and mTOR in RANKL-Stimulated RAW264.7 Cells

The most extensively studied of metformin's mechanism is its activation of the signaling kinase AMPK[39]. To investigate the effects of metformin treatment on the protein changes of AMPK, mTOR, and ULK1 during osteoclast differentiation, we treated RAW264.7 cells with RANKL and metformin and incubated them for 0, 10, 30, and 60 min. As a control group, we used cells treated with RANKL only. In the group treated with both RANKL and metformin, the phosphorylation of AMPK increased rapidly over time, thereby leading to its activation in comparison to the control group (Figure 3).

In addition, mTORC1 plays a critical role in regulating cell growth and metabolism and is normally kept in an active state during regular metabolic processes. However, when mTORC1 is inactivate, autophagy becomes fully active[40]. Research has established that AMPK promotes autophagy by phosphorylating ULK1, while mTORC1 inhibits autophagy by phosphorylating ULK1 under nutrient-rich conditions[26]. Accordingly, we observed that the phosphorylation of AMPK, mTOR, and ULK1 [p-AMPK (Thr172), p-mTOR (Ser2481), and p-ULK (Ser555)] was significantly increased in the group treated with RANKL and metformin, while the group treated with RANKL alone showed decreased mTOR phosphorylation and almost no change in the phosphorylation of AMPK and ULK1 compared to the metformin-treated group (Figure 3).

AMPK Activation via Metformin Suppresses Osteoclast Differentiation

Next, we treated RAW264.7 cells with an AMPK inhibitor, Com C, and investigated how the activation of AMPK *via* metformin was affected. Furthermore, we examined the impact of differentiation factors of osteoclasts. After treating the RAW264.7 cells with RANKL, we allowed them to culture for 3 days with Com C (RANKL + Com C; Com C, 1.25 μM), metformin (RANKL + Met; metformin, 200 μM), or a combination of Com C and metformin (RANKL + Com C + Met; Com C, 1.25 μM; metformin, 200 μM) to induce osteoclast differentiation. Thereafter, we examined the expression of osteoclast-related proteins and genes using western blotting and qPCR. Com C increased the protein expression of TRAF6, NFATc1, and ATP6v0d2, as well as the gene expression of osteoblast differentiation-related genes such as *ACP5, calcitonin*

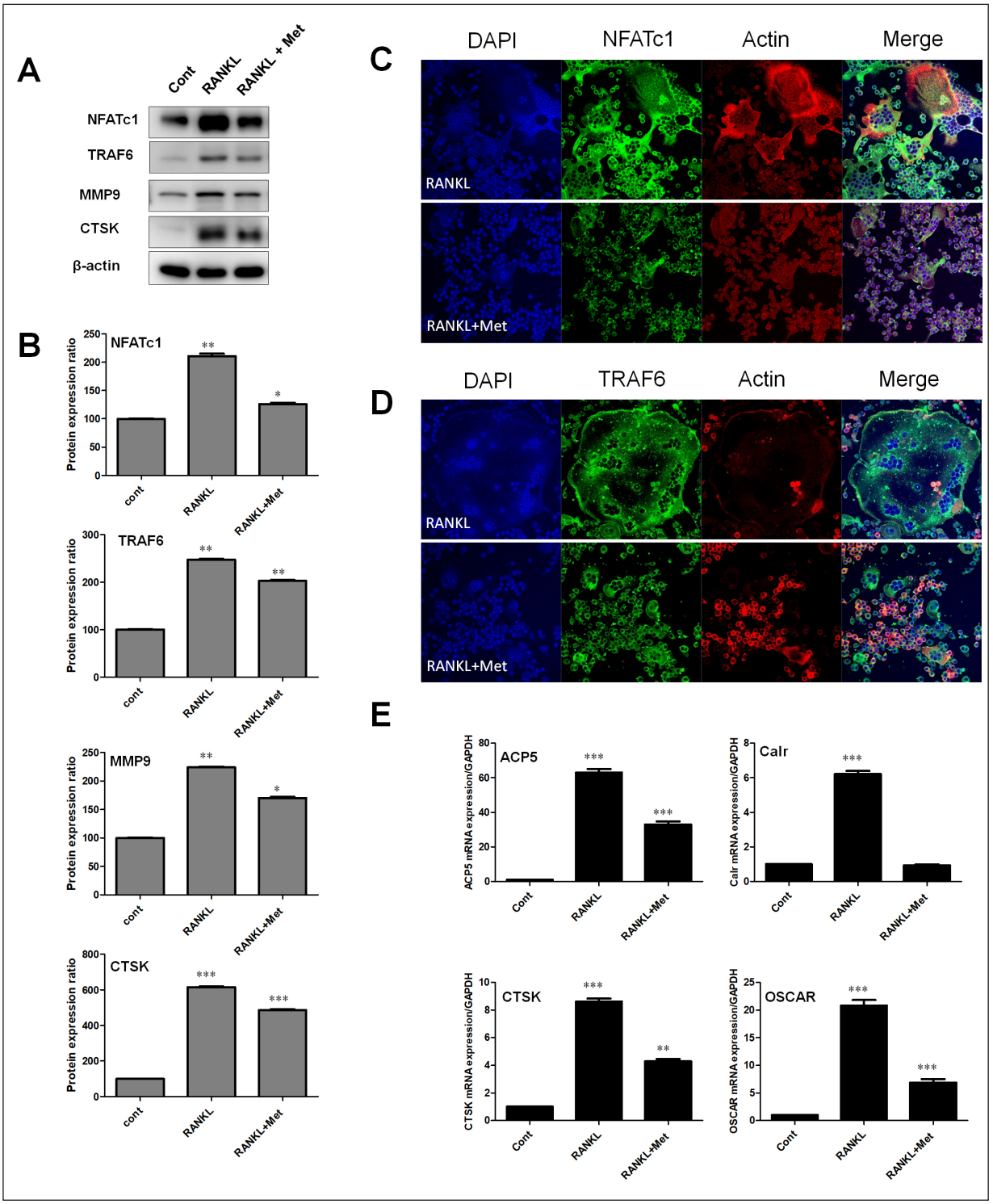

Figure 2. Metformin reduced NFATc1 and TRAF6 protein expression. RAW264.7 cells were treated with 200 μM metformin for 5 days. **A**, Metformin treatment led to decreased protein expression of NFATc1, TRAF6 MMP9, and CTSK. **B**, Protein expression density after each experiment was performed at least three times, with data calculated as a percentage of control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **C-D**, Immunofluorescence images obtained *via* confocal microscopy, with NFATc1 and TRAF6 expression decreasing in the metformin treatment group. Images were captured at 40x. **E**, Graph of metformin-inhibited osteoclast transcription genes, such as *ACP5, Calr, CTSK,* and *OSCAR*, per mRNA expression rates. Each experiment was performed at least three times, and data were calculated as a fold of control (**$p < 0.01$, ***$p < 0.001$).

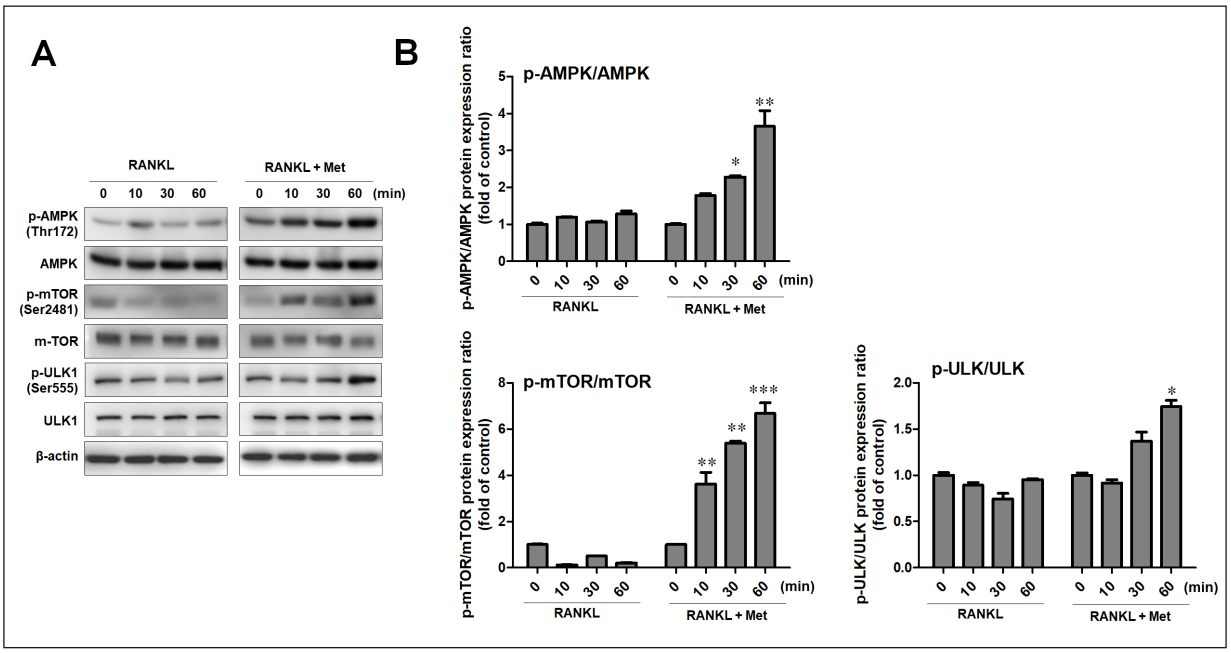

Figure 3. Metformin increased the phosphorylation of AMPK, ULK1, and mTOR in RANKL-treated RAW264.7 cells. The cells were treated with either RANKL only or a combination of RANKL and metformin for 0-60 min. **A**, The expressions of p-AMPK (Thr172), AMPK, p-mTOR (Ser2481), mTOR, p-ULK1 (Ser555), and ULK1 analyzed *via* western blotting. **B**, p-AMPK/AMPK, p-mTOR/mTOR, and p-ULK1/ULK1 ratios calculated and analyzed using Image J software. Each experiment was performed at least three times, with data calculated as a percentage of control (RANKL only; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

receptor, CTSK, TRAF6, cFos, NFATc1, OSCAR, MMP9, and *ATP6v0d2*. In contrast, metformin significantly decreased the expression of these proteins and genes. This indicates that AMPK inhibition promotes osteoclast differentiation, while its activation *via* metformin suppresses osteoclast differentiation (Figure 4).

Metformin Inhibits Autophagy through mTOR Activation, Leading to Suppressed Osteoclast Differentiation

We examined the protein expression of autophagy-related factors under the above-mentioned conditions to determine the effects of AMPK inhibition *via* Com C and AMPK activation *via* metformin on autophagy. RANKL treatment alone and with Com C (RANKL + Com C) weakly increased the expression of beclin1, ATG5, and p62 and resulted in complete conversion of LC3I to LC3II, thereby indicating that AMPK inhibition activates autophagy and promotes osteoclast differentiation. In contrast, metformin suppressed the expression of beclin1, ATG5, and p62 and inhibited the conversion of LC3I to LC3II, suggesting that AMPK activation *via* metformin suppresses autophagy and ultimately leads to the inhibition of osteoclast differentiation (Figure 5).

The autophagy pathway is further inhibited by the mTOR protein. RAW264.7 cells were induced to differentiate into osteoclasts *via* RANKL and treated with 0-500 μM metformin. We confirmed that phosphorylated mTOR protein expression significantly increased in the groups treated with metformin at 200 μM or higher (Figure 6A). The increase in mTOR protein expression after metformin treatment was also confirmed *via* immunohistochemistry staining (Figure 6B). Based on these results, we discovered that mTOR activation *via* metformin inhibits the differentiation of osteoclasts by suppressing autophagy.

From here, we were curious about what would happen to osteoclast differentiation if we used an mTOR activator instead of metformin. To investigate this, we applied leucine (Leu), a well-known mTOR activator, to RAW264.7 cells and examined its effects. We treated RAW264.7 cells with either leucine (RANKL + Leu, 10 μM), metformin (RANKL + Met, 200 μM), or a combination of leucine and metformin (RANKL + Leu + Met; Leu, 10 μM; Met, 200 μM); TRAP staining was performed after 5 days. All groups were co-treated with RANKL (10 ng/ml), with the RANKL-only treated group used as a control. In all groups treated with leucine, metformin, or

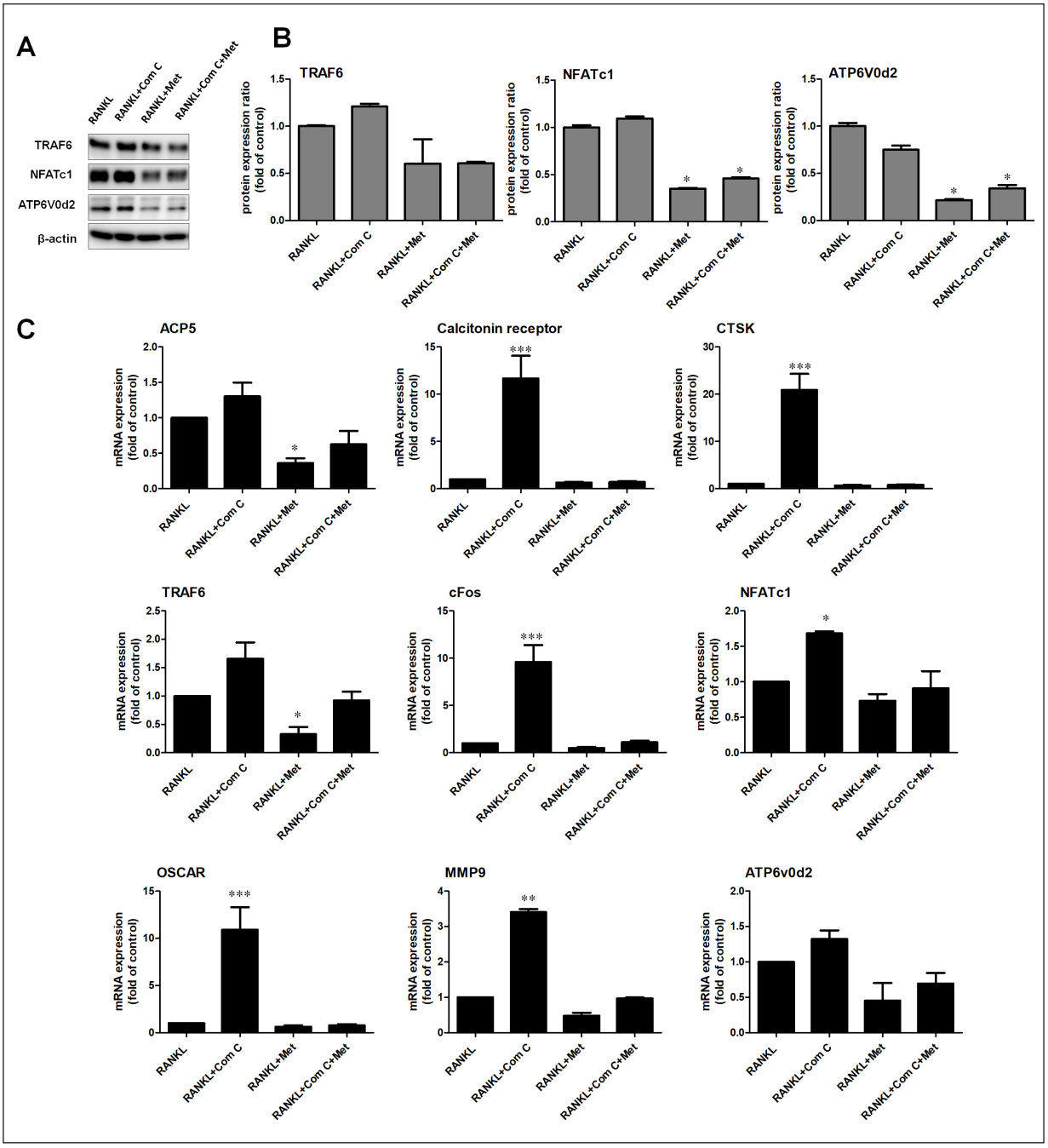

Figure 4. Metformin's activation of AMPK suppressed osteoclast differentiation-related factors. RAW264.7 cells were classified into four groups: the RANKL-treated control group, Com C group, metformin (Met) group, and combination Com C and Met treatment group. The cells were treated with each reagent and cultured for 5 days. **A**, The protein expressions as analyzed using western blotting. **B**, Graph of protein density. **C**, mRNA expression of osteoclast differentiation-related factors measured *via* qPCR. Each experiment was performed at least three times, and data were calculated as a fold of control (RANKL only; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

a combination thereof, we observed a significant reduction in the number of TRAP-positive osteoclasts (Figure 6C). The protein expression of osteoclast differentiation and autophagy-related factors was also examined using western blotting under the same conditions. In all groups treated with Leu, Met, or a combination of them, we noted a significant decrease in the expression of the differentiation factors TRAP6, NFATc1, and ATP6v0d2, as well as autophagy-related factors

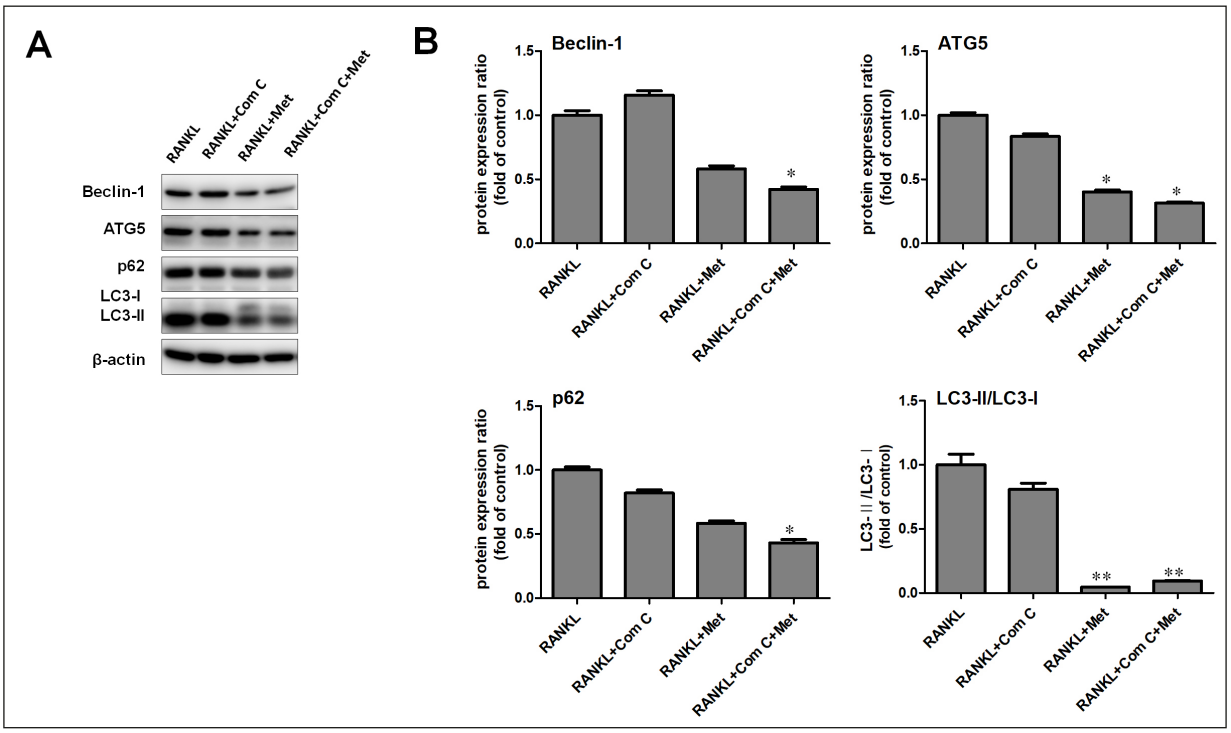

Figure 5. Activation of AMPK by metformin led to inhibited autophagy factors. RAW264.7 cells were classified into four groups: the RANKL-treated control group, Com C group, Met group, and combination Com C and Met treatment group. The cells were treated with each reagent and then cultured for 5 days. **A**, Protein expressions analyzed using western blotting. **B**, Graph illustrating protein density. Each experiment was performed at least three times, and data were calculated as a fold of control (RANKL only; $*p < 0.05$, $**p < 0.01$).

Beclin-1, ATG5, and P62 *via* western blotting. We barely observed the conversion from LC3I to LC3II (Figures 6D-E). Based on these results, we confirmed that the activation of AMPK and mTOR *via* metformin inhibits osteoclast differentiation through the suppression of autophagy.

Discussion

Many kinds of stimuli – including hormones, cytokines, and mechanical stimuli – affect bone turnover, which in turn affects the amount and quality of tissue produced[41]. In particular, in patients with type 2 diabetes who are affected by insulin secretion, bone turnover is low, which increases the prevalence of osteopenia and osteoporosis, thereby leading to an increased risk of bone fracture[42-44]. Given these concerns, it is essential to have more effective treatments and prophylaxis to control bone metabolism in diabetic osteoporosis patients. Bone metabolism itself is largely dynamically balanced by osteoclasts and osteoblasts. Osteolysis occurs when excessive osteoclasts trigger bone resorption and weaken bone formation[45]. The ideal bone disease treatment improves bone quality by promoting osteoblast differentiation, thereby inhibiting osteoclast formation and destruction[46].

Metformin is one of the most effective therapeutic reagents for treating type 2 diabetes[47]. Recently, studies[33,48,49] on metformin have been extended to various disease models, such as Alzheimer's disease, cancer prevention, and bone healing. In particular, Ribeiro Serrão et al[50] found that metformin did not restore the damaging effects of diabetes on peri-implant bone repair in a diabetes-induced rat model, but increased the expression of OPG and decreased the ratio of RANKL/OPG in the peri-implant medulla bone.

Due to their protease release activity, osteoclasts produce an acidic microenvironment in which bone demineralization and substrate degradation occur[51]. TRAP is a histochemical marker of osteoclasts expressed by the presence of osteoclasts, macrophages, dendritic cells, and numerous other cell types. TRAP plays a crucial role in numerous biological processes, including skeletal development, collagen synthesis and degradation, bone mineralization, and macrophages' production of

cytokines[52]. In particular, lysosomes containing TRAP and CTSK are abundantly present in the ruffled border region of osteoclasts, and they enable the resorption of the bone matrix[53].

In this study, metformin significantly reduced the number and shape of TRAP-positive osteoclasts in the early stages of osteoclast differentiation and weakened the resorption of calcium phosphate. A key cytokine for osteoclastogenesis, receptor activator of NF-κB ligand (RANKL) – which binds to its receptor, RANK – is essential for bone marrow progenitor cells or macrophages to differentiate into mature osteoclasts54. These essential interactions lead to the activation of TRAF6, mitogen-activated protein kinase (MAPK), and NF-κB, which induce the self-amplification of NFATc1; then, NFATc1 translocates to the nucleus and promotes the expression of osteoclast-labeled genes TRAP, MMP9, *CTSK*, Calcr, and OSCAR[55]. MMP9 degrades the extracellular matrix (ECM) in various organs. Several studies[56] have reported that MMP9, as activated

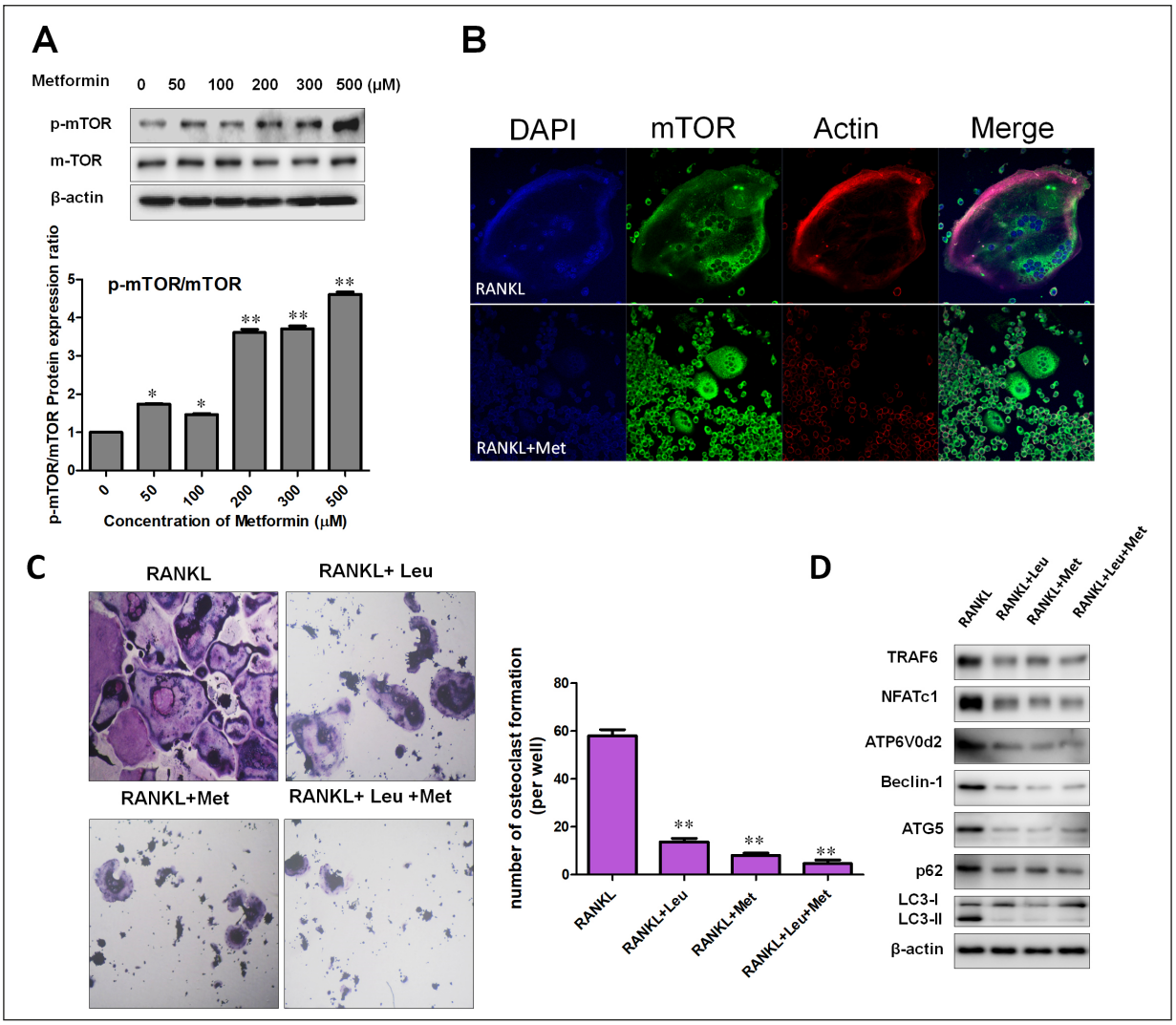

Figure 6. Metformin activated mTOR, inhibited autophagy, and suppressed osteoclast differentiation. **A**, Cells treated with RANKL, exposed to varying concentrations of metformin (0-500 μM), and cultured for 5 days, then analyzed for protein expression *via* western blotting. **B**, Cells treated with RANKL and metformin, followed by a 5-day culture and immunofluorescence imaging using confocal microscopy. mTOR expression was found to be increased in the metformin treatment group. **C**, RAW264.7 cells treated with RANKL and categorized into four groups: RANKL only, Leucine (Leu), Met, and Leu + Met groups. The cells were then cultured for 5 days, followed by TRAP staining and graphing of TRAP-positive cells. Images were captured at 40x. **D-E**, Protein expression levels of osteoclast differentiation-related factors and autophagy-related factors analyzed using western blotting, followed by quantification and graphing of the results. Each experiment was performed at least three times, and data were calculated as a fold of control (RANKL only; $*p < 0.05$, $**p < 0.01$).

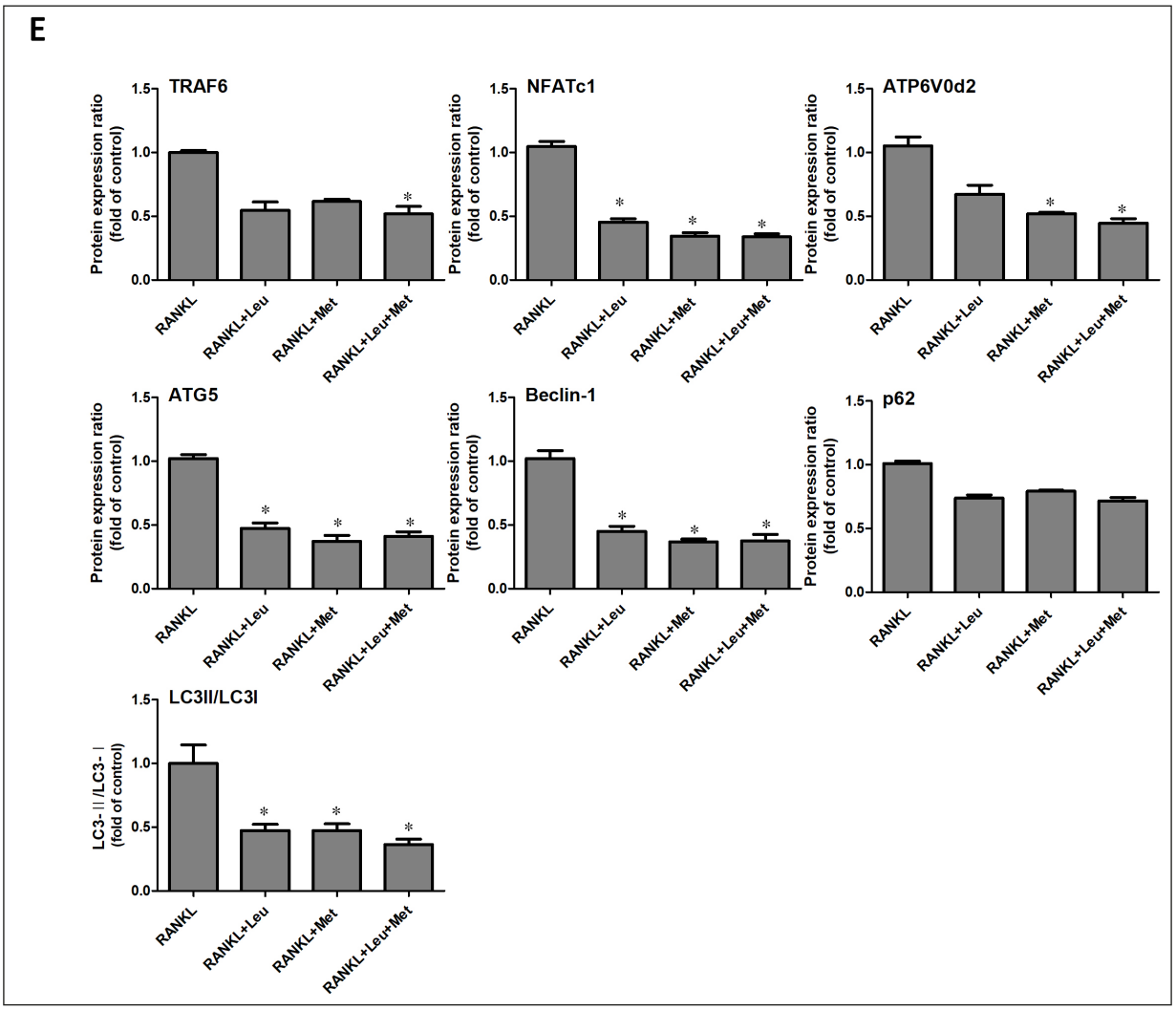

Figure 6. ***(continued)*** Metformin activated mTOR, inhibited autophagy, and suppressed osteoclast differentiation. **A**, Cells treated with RANKL, exposed to varying concentrations of metformin (0-500 μM), and cultured for 5 days, then analyzed for protein expression *via* western blotting. **B**, Cells treated with RANKL and metformin, followed by a 5-day culture and immunofluorescence imaging using confocal microscopy. mTOR expression was found to be increased in the metformin treatment group. **C**, RAW264.7 cells treated with RANKL and categorized into four groups: RANKL only, Leucine (Leu), Met, and Leu + Met groups. The cells were then cultured for 5 days, followed by TRAP staining and graphing of TRAP-positive cells. Images were captured at 40x. **D-E**, Protein expression levels of osteoclast differentiation-related factors and autophagy-related factors analyzed using western blotting, followed by quantification and graphing of the results. Each experiment was performed at least three times, and data were calculated as a fold of control (RANKL only; $*p < 0.05$, $**p < 0.01$).

by osteoclasts, promotes the recruitment and migration of pre-osteoclastic cells, and increased MMP9 protein expression accelerates osteoclastogenesis and bone resorption. Since OSCAR activates NFATc1 and functions as a costimulatory receptor for osteoclast differentiation, stimulation via RANKL in the process of osteoclast differentiation upregulates NFATc1 expression to induce OSCAR expression; when osteoclasts enter the mature stage, the induced OSCAR then causes a positive feedback loop that activates NFATc1[57]. Similar to TRAP expression and multinucleation during osteoclast differentiation, CTSK receptors are specific to osteoclasts, making them a useful marker of osteoclast identification and enumeration[58]. Overall, our results revealed that metformin could affect osteoclast differentiation and osteoclast bone resorption by repressing osteoclast marker genes such as *TRAP, MMP9, CTSK, Calcr,* and *OSCAR*.

AMPK belongs to the metabolite-sensing protein kinase family, which has the ability to regulate

cell differentiation and has a beneficial effect on vascular function[59]. However, the role of AMPK in osteoclast differentiation and bone resorption is controversial. Some studies[60,61] have suggested that AMPK plays an important role in osteoclast differentiation, while others have reported that RANKL expression significantly increases when AMPK is inhibited, in addition to AMPK activation negatively inhibiting RANKL-induced osteoclast formation[11]. Metformin is a known AMPK agonist[61]. Recently, researchers[62] showed that metformin enhances the osteogenic differentiation of stem cells in human exfoliated deciduous teeth *via* the AMPK pathway.

AMPK and mTOR are important proteins that play complementary roles in metabolic regulation. AMPK is activated during energy-depleted conditions to regulate metabolism, while mTOR responds to nutritional and hormonal signals to promote protein synthesis. Studies[63,64] have suggested that the activation of AMPK is closely related to the activation of mTOR as well. Another study[65] revealed that metformin regulates differentiation and adipogenesis in bone tissue through the AMPK and mTOR pathways. Moreover, metformin treatment was shown to activate AMPK and suppress mTOR in bone cells, thereby promoting differentiation and inhibiting adipogenesis. However, findings on the correlation between the activation of AMPK and mTOR during osteoclast differentiation are limited, and there is a lack of related studies on this topic.

Further, therapeutic metformin/AMPK activation has been reported to stimulate angiogenic phenotypes in an ERα-negative MDA-MB-435 breast cancer model[66], as well as to inhibit lymphoma cell growth through inhibition of the mTOR pathway and induction of autophagy[67]. AMPK and mTOR pathways are indeed interrelated, and generally, the activation of the AMPK pathway is associated with the inhibition of the mTOR pathway. However, the AMPK and mTOR pathways can also function independently of each other. One study[68] indicated that mTORC1 induces phosphorylation of AMPK alpha1 at Ser487, which promotes the activation of AMPK alpha1 in contrast to the inhibition of the AMPK pathway. Still, this phenomenon has not been extensively studied under various conditions, thus requiring more research. In this study, we confirmed that metformin inhibits osteoclast differentiation and decreases autophagy not only through AMPK activation but also mTOR activation.

Autophagy is a highly organized intracellular degradation process that is activated by a variety of environmental stressors[69]. The main mechanism of autophagy is to suppress mTOR, a highly conserved serine/threonine kinase that functions as a sensor for energy status[70]. The activation of two other protein kinases, AMPK and ULK1, inhibits mTOR, thereby stimulating autophagy[71, 72]. In a recent study[73], a question was raised of whether the antiproliferative effects of the AMPK agonists AICAR and metformin in brain tumor cells are independent of AMPK. Both AMPK agonists inhibited cell proliferation, but through unique AMPK-independent mechanisms, they also reduced tumor growth *in vivo* independently of AMPK. In particular, metformin directly inhibited mTOR by enhancing the association of PRAS40 with RAPTOR. These results revealed that AICAR and metformin are potent AMPK-independent antiproliferative agents.

Recently, several studies[28,70,74] have demonstrated the role of autophagy in bone cells and showed that essential autophagy proteins – such as ATG5, SQSTM1/p62, and LC3 – are important for osteoclast ruffled border generation, osteoclast secretion, and bone resorption *in vivo* and *in vitro*. In this study, we observed that metformin not only activated AMPK and ULK but also increased mTOR expression in RANKL-stimulated RAW264.7 macrophages. Therefore, to investigate whether metformin directly affects osteoclast differentiation and autophagic activity as an AMPK activator, we used the AMPK inhibitor Com C. As depicted in Figures 4 and 5, AMPK inhibition promoted the expression of osteoclast differentiation markers, and metformin had a competitive advantage over Com C in preventing the inhibitory effect of AMPK inhibition. In addition, metformin also induced the inhibition of autophagic activity factors.

Autophagy is essential during osteoclast differentiation. Some other studies[68,69] have shown that AMPK negatively regulates RANKL, though Com C, an AMPK inhibitor, has been found to activate osteoclast differentiation[75] and enhance autophagic activity[76]. In the present study, we found that AMPK inhibition *via* Com C activated autophagy, while treatment with metformin, an AMPK activator, suppressed autophagy and inhibited osteoclast differentiation. Along with the results depicted in Figure 3, we thus confirm that metformin treatment induces phosphorylation and AMPK, mTOR, and ULK activation.

Therefore, we speculate that metformin acts independently of the mTOR inhibition mechanism,

which is known to be associated with AMPK activation. As the cells were exposed to metformin for less than 60 min, as depicted in Figure 3, we were curious about changes in mTOR over the long term. We observed a concentration-dependent increase in mTOR expression when treated with RANKL and metformin. Moreover, in osteoclasts induced by RANKL, we found that metformin treatment led to a greater accumulation of mTOR in the cytoplasm (Figure 6A-B). Further, we confirmed that the activation of mTOR *via* metformin inhibits osteoclast differentiation and suppresses autophagic factors. To further investigate the effect of mTOR agonists, not merely metformin, on osteoclast differentiation and autophagy, we conducted experiments using leucine, a well-known mTOR agonist. Both metformin and leucine suppressed RANKL-induced osteoclastogenesis, as well as autophagic factors such as Beclin 1, ATG5, and p62 and the conversion from LC3I to LC3II. Furthermore, the expressions of TRAF6, NFATc1, and ATP6v0d2 – all related to osteoclast differentiation – were also suppressed. These results suggest that the activation of mTOR during the process of RANKL-induced osteoclastogenesis inhibits autophagy and ultimately leads to the suppression of osteoclast differentiation.

Conclusions

We conclude that metformin independently activates mTOR while activating AMPK, thereby inhibiting autophagy and subsequently suppressing the differentiation and maturation of osteoclasts. However, the relationship between metformin, an anti-diabetic drug, and mTOR gene expression remains unclear. Thus, additional studies are necessary to evaluate the autophagy suppression caused by metformin as a drug with infinite potential, particularly in the treatment of various diseases beyond diabetic osteoporosis.

Ethics Approval
Not applicable.

Informed Consent
Not applicable.

Availability of Data and Materials
The experimental data used to support the findings of this study are available from the corresponding author upon request.

Conflict of Interest
The authors declare that they have no conflict of interest.

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Authors' Contributions
I-R.K. designed this study. Y-S.K and H-M.K. performed the relevant analyses. Y-S.K., H-S.B, and B-S.P. wrote the manuscript. J-M.O. edited the English in this manuscript. All authors read and approved the final version of the manuscript.

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