Mammalian target of rapamycin complex 1 (mTORC1) is involved in anabolic metabolism in both osteoblasts and chondrocytes, but the role of mTORC1 in osteoclast biology in vivo remains to be elucidated. In this study, we showed that deletion of regulatory-associated protein of mTOR (Raptor) in osteoclasts led to an increase in bone mass with decreased bone resorption. Raptor-deficient bone marrow-derived macrophages exhibited lower mTORC1-S6K1 signaling and retarded osteoclast differentiation, as determined by the number of osteoclasts, tartrate-resistant acid phosphatase activity, and expression of osteoclast-specific genes. Enforced expression of constitutively active S6K1 rescued the impaired osteoclast differentiation in Raptor-deficient bone marrow-derived macrophages. Furthermore, pharmacological inhibition of mTORC1 signaling by rapamycin could also inhibit osteoclast differentiation and osteoclast-specific gene expression. Taken together, our findings demonstrate that mTORC1 plays a key role in the network of catabolic bone resorption in osteoclasts and may serve as a potential pharmacological target for the regulation of osteoclast activity in bone metabolic disorders.

Bone is a rigid yet metabolically active organ that is molded, shaped, and repaired continuously (1). After bone is formed, bone undergoes a process known as remodeling by which bone is turned over throughout life (2). Bone remodeling acts as the predominant metabolic regulator of both the physical structure and physiological function of bone. Remodeling is a complex process involving osteoclasts, which are responsible for removing old mineralized matrix, and osteoblasts, which synthesize and secrete new bone matrix (1, 3). An imbalance in bone remodeling can induce perturbation of bone structure and function and potentially result in disease (1). In adults, most bone diseases, such as osteoporosis, rheumatoid arthritis, and periodontal disease, are the result of bone loss secondary to excess osteoclast activity (4). Prevention and treatment of these pathological disorders highlight the study of the underlying mechanisms by which osteoclasts differentiate from their precursors.

Osteoclasts are tissue-specific giant multinucleated cells that differentiate from monocyte/macrophage precursor cells at or near the bone surface (1). It is known that the differentiation of osteoclasts is under the control of two important cytokines, receptor activator of nuclear factor κB ligand (RANKL) and M-CSF (3). RANKL and macrophage colony-stimulating factor (M-CSF) may activate a set of signaling pathways, including AKT and NF-κB, that promote the differentiation, multinucleation, activation, and survival of osteoclasts (5). However, the
precise mechanism regulating osteoclast differentiation is not fully understood.

Mammalian/mechanistic target of rapamycin (mTOR) is an evolutionarily conserved protein kinase (6, 7). mTOR functions in two structurally and functionally distinct multiprotein complexes, mTORC1 and mTORC2, that are distinct in their unique components and downstream targets (7). mTORC1 contains regulatory-associated protein of mTOR (Raptor) and is sensitive to rapamycin, whereas mTORC2 contains rapamycin-insensitive companion of mTOR (Rictor) and is resistant to rapamycin. mTORC1 constitutes a molecular node that regulates cell differentiation, growth, and survival though a series of downstream effectors, including S6 kinase (S6K1). The mTORC1/S6K1 axis transmits and integrates important signals that have been found to be critical for bone biology, including nutrients, growth factors, and energy metabolism.

Recently, mTORC1 has been found to play roles in bone biology and pathology (3, 8–10). Everolimus, a derivative of rapamycin, has been reported to have beneficial effects on bone when used as an anticancer ancillary in postmenopausal women with breast cancer (11, 12). Although the underlying mechanism of the protective effects of everolimus on bone is still unclear, osteoclasts may be its target, considering that blocking of mTORC1 in osteoblasts results in decreased bone mass in mice (4). It is reported that mTOR signaling is critical in osteoclast survival and bone resorption in vitro (13, 14). However, the role of mTORC1 in osteoclasts in vivo has not been completely elucidated. Here we report that osteoclast-specific deletion of Raptor (an indispensable component of mTORC1) results in increased bone mass with decreased bone resorption. We found that the Raptor/mTORC1-S6K1 axis played a determinant role in osteoclast differentiation and may be a potent drug target for regulation of osteoclasts.

Results

Raptor Deficiency in Osteoclasts Results in Increased Bone Mass with Impaired Osteoclast Differentiation—To determine the role of mTORC1 signaling in osteoclasts in vivo, we generated conditional Raptor knockout mice (RapBfl; Ctsk-cre, hereafter called RapCtsk) (Fig. 1A) by crossing RapBfl mice with Ctsk-cre mice, a transgenic line in which Cre expression is driven by the promoter of cathepsin K to achieve osteoclast-specific expression of Cre. To confirm the gene depletion of Raptor, we isolated BMMs from 4-week-old WT and RapCtsk mice and treated BMMs with 20 ng/ml M-CSF and 250 ng/ml RANKL. The cell lysates were collected and subjected to immunoblotting with Raptor antibody, which confirmed the loss of RANKL. The cell lysates were collected and subjected to immunoblotting with Raptor antibody, which confirmed the loss of RANKL.

We found that the Raptor/mTORC1-S6K1 axis played a determinant role in osteoclast differentiation and may be a potent drug target for regulation of osteoclasts.

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We next examined whether the reduced bone resorption in RapCtsk mice was a result of a change in bone formation. It is similar to the results of trabecular bone that the cortical bone of the femur from RapCtsk mice exhibited decreased osteoclast numbers (Fig. 3, A–D and G). Immunohistochemistry staining with an antibody specific to Ctsk demonstrated a robust Ctsk signal in osteoclasts of WT mice, whereas Ctsk staining was also reduced in RapCtsk mice (Fig. 3, E, F, and H). These results indicated reduced osteoclast activity and bone resorption in RapCtsk mice, which may be the main contributor to the increase in bone mass. We next analyzed the bone-forming activity by alizarin red and calcine double-labeling. As shown in Fig. 2, I–K, there was no significant difference in mineral apposition rate (MAR) and bone formation rate (BFR) between of the distal femur trabecular bone in WT and RapCtsk mice, indicating that the increased bone mass was not a result of a change in bone formation. This increase in bone mass in RapCtsk mice resulted from decreased bone resorption.

We next examined whether the reduced bone resorption in RapCtsk mice was a result of inadequate osteoclast differentiation. As shown in Fig. 4A, BMMs from WT mice fused to one another to form giant TRAP-positive multinucleated osteoclasts. In contrast, BMMs from RapCtsk mice showed impaired osteoclast differentiation and formation, as indicated by the decreased number of TRAP-positive osteoclasts and reduction in TRAP activity of the culture supernatant (Fig. 4, A and B). Because a specific set of genes is up-regulated during osteoclast differentiation (1), we used quantitative PCR (qPCR) to analyze and quantify the expression of the RANKL-induced osteoclast-specific genes Ctsk, Trap, Dc-stamp, Oscar, and Atp6v0d2. Consistent with the TRAP staining results, the expression of these osteoclast marker genes was suppressed in RapCtsk BMMs.
compared with WT cells (Fig. 4, C–J), confirming the inhibitory effects of Raptor deficiency on osteoclast differentiation.

Further, to determine whether Raptor deficiency affects osteoclast progenitor cell proliferation, the growth of BMMs from WT and RapCtsk mice was determined by crystal violet staining. As is showed in Fig. 4H, there was no significant difference in cell proliferation between WT and RapCtsk BMMs.

All of these results indicated that the increased bone mass in RapCtsk mice was due to reduced bone resorption secondary to impaired osteoclast differentiation.

S6K1 Is a Downstream Factor of Raptor/mTORC1 in Osteoclasts—S6K1 is the most important downstream regulator in mTORC1 signaling and plays crucial roles in development and

FIGURE 1. Deletion of Raptor in osteoclasts led to an increase of bone mass. A, illustration of Raptor deletion in Ctsk-expressing osteoclasts. B, Western blotting assay of Raptor of WT and RapCtsk BMMs cultured with osteoclast differentiation medium for 6 days. C, body weight of male WT and RapCtsk littermates measured at different age points. Data represent mean ± S.D. *, p < 0.05; n = 5. D, three-dimensional reconstruction of micro-CT images of trabecular bone close to the distal growth plate and cortical bone at the middle of femora from 4-week-old WT and RapCtsk littermates. E, representative view of micro-CT of femora from 8-week-old WT and RapCtsk mice. F–J, quantitative parameters of micro-CT. The trabecular bone close to the distal growth plate and cortical bone at the middle of the femur was analyzed. Data represent mean ± S.D. *, p < 0.05; n = 5. K–P, H&E staining of femora from 4-week-old male WT and RapCtsk littermates. M and N, high-power images of the secondary spongiosa marked in K and L, respectively. O and P, high-power images of the primary spongiosa marked in K and L, respectively. Ma, bone marrow.

FIGURE 2. Raptor deficiency in osteoclasts induced increased bone volume. A–D, H&E staining of femora from 4-week-old female WT and RapCtsk littermates. C and D, high-power images of the primary spongiosa marked in A and B, respectively. E–H, H&E staining of femora from 20-week-old male WT and RapCtsk littermates. G and H, high-power images of the primary spongiosa marked in E and F, respectively. Ma, bone marrow.
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The expression level of S6K1 in BMMs was comparable between WT and RapCtsk mice. Enforced expression of constitutively active S6K1 rescued osteoclast differentiation of RapCtsk-deficient BMMs and confirmed that Raptor/mTORC1-S6K1 signaling is essential for osteoclast differentiation.

Inhibition of mTORC1 by Rapamycin Suppresses Osteoclast Differentiation of BMMs—Our above data demonstrate that inactivation of mTORC1 signaling by Rapamycin treatment inhibited giant osteoclast formation, as the giant osteoclasts were barely detectable in the Rapamycin-treated mice, whereas giant osteoclasts were obvious in untreated mice (Fig. 6A). Notably, the decrease in phospho-S6K1 level was rapamycin dose-dependent and correlated with the decrease in the number of TRAP-positive osteoclasts and in TRAP activity in the rapamycin-treated groups. TRAP staining revealed that rapamycin treatment inhibited giant osteoclast formation, as the giant osteoclasts were barely detectable in the rapamycin-treated mice, whereas giant osteoclasts were obvious in untreated mice (Fig. 6A). Notably, the decrease in phospho-S6K1 level was rapamycin dose-dependent and correlated with the decrease in both the number of TRAP-positive osteoclasts and in TRAP activity (Fig. 6A and C). In addition, the expression level of Ctsk, Trap, Dc-stamp, Oscar, and Atp6v0d2 was significantly improved by CAS6K1 overexpression, as demonstrated by the increased number of TRAP-positive cells (Fig. 5C) together with elevated TRAP activity in the culture supernatant (Fig. 5D).

Consistent with this, the suppressed expression of osteoclast-specific genes Ctsk, Trap, Dc-stamp, Oscar, and Atp6v0d2 in RapCtsk-deficient BMMs was rescued by CAS6K1 overexpression, as shown in Fig. 5, E–I. The rescue of osteoclast differentiation of RapCtsk-deficient BMMs by CAS6K1 overexpression demonstrates that reduced S6K1 activity is responsible for the impaired osteoclast differentiation in RapCtsk-deficient BMMs and confirms that Raptor/mTORC1-S6K1 signaling is essential for osteoclast differentiation.

Discussion

This study provides several lines of evidence demonstrating that mTORC1 is required for osteoclast differentiation and bone metabolism. Conditional deletion of Raptor in osteoclasts led to increased bone mass accompanied by decreased osteoclast activity in mice. Osteoclast differentiation of RapCtsk-deficient BMMs was retarded with lower mTORC1-S6K1 signaling. Enforced expression of constitutively active S6K1 rescued the impaired osteoclast differentiation of RapCtsk-deficient BMMs. Further, pharmacological inhibition of mTORC1 signaling could also inhibit osteoclast differentiation.

The skeleton is a dynamic organ that undergoes continuous remodeling by osteoclasts (bone resorption) and osteoblasts (bone formation). The balance of bone resorption and bone formation enables bone to fulfill the physiological needs of the organism with the minimum of mass. To maintain bone homeostasis, the process of bone remodeling is regulated in an

FIGURE 3. Ablation of Raptor in osteoclasts resulted in decreased bone resorption. A–D, representative view of TRAP staining of distal femur trabecular bone of 4-week-old male WT and RapCtsk mice. E and F, immunohistochemical staining of Ctsk in distal femur trabecular bone of 4-week-old male WT and RapCtsk mice. Arrowheads indicate TRAP-positive osteoclasts. Maa, bone marrow. G and H, numbers of TRAP- and Ctsk-positive osteoclasts on the trabecular bone surface, measured as cells per millimeter of perimeter (μm/Pm). Data are mean ± S.D. *, p < 0.05; n = 3. I, alizarin red and calcein double-labeling of femora showed the bone turnover rate of distal femur trabecular bone in 8-week-old WT and RapCtsk mice. Arrowheads indicate Ctsk-positive osteoclasts. G and H, numbers of TRAP- and Ctsk-positive osteoclasts on the trabecular bone surface, measured as cells per millimeter of perimeter (μm/Pm). Data are mean ± S.D.; n = 3. J–O, quantitative parameters of MAR and BFR of trabecular bone in WT and RapCtsk mice. Data represent mean ± S.D. L–Q, quantitative parameters of MAR and BFR of trabecular bone in WT and RapCtsk mice. Data represent mean ± S.D.
orderly manner at both the systemic and local level, including nutrients, growth factors, and energy metabolism (4). On the other hand, an imbalance in bone remodeling can result in various metabolic diseases such as osteoporosis, rheumatoid arthritis, and periodontal disease, in which bone resorption disproportionately exceeds bone formation. Therefore, it is important for prevention and treatment of metabolic bone disease that the molecular mechanism that drives the differentiation of osteoclasts during both biological and pathological bone remodeling is better understood (5). In this study, we found that mTORC1 is a molecular node that is critical for osteoclast differentiation and bone metabolism.

mTORC1 signaling has been reported to regulate bone anabolism in both osteoblasts and chondrocytes (12, 15–18). However, evidence for the role of mTORC1 in osteoclast catabolism is limited. A recent study suggested that mTOR signaling in B cells could indirectly regulate osteoclast formation through regulation of β-catenin and RANKL/osteoprotegerin (OPG) (9) and another study showed everolimus can restrain the paracrine pro-osteoclast activity of breast cancer cells (19). mTOR signaling may also play an important role in osteoclast survival and differentiation of osteoclast progenitors (8, 20, 21). In this study, we found that mTORC1 regulates osteoclast differentiation and formation in a cell-autonomous manner with a conditional knockout mice model.

In the current mouse model, we found that blocking of mTORC1 in osteoclasts by deletion of Raptor led to increased bone mass, as determined by increases in BMD and BV/TV in the femur. Histological analysis showed that deletion of Raptor in osteoclasts did not influence osteoblast activity, as indicated by consistent MAR and BFR in Rap\textsuperscript{Ctsk} and WT mice. These results excluded the possibility that deletion of Raptor in osteoclasts promoted bone formation and increased bone mass indirectly through actions on osteoblasts, although mTORC1 may play an important but as yet undetermined role in osteoblasts (15, 16, 18). Further, reduced osteoclast activity, determined by decreased TRAP activity and Ctsk expression in Rap\textsuperscript{Ctsk} mice, was the chief cause of the increased bone mass. This is consistent with the observation that an mTOR inhibitor could rescue bone loss partly through suppression of bone resorption in ovariectomized rats (11) and protect bone health in postmenopausal women with breast cancer (12, 22). Meanwhile, Xian \textit{et al.} (23) reported that rapamycin decreased bone mass in WT mice, which may be caused by the fact that the effects of rapamycin on osteoblasts masked those on osteoclasts. Taken together, these data show that deletion of Raptor in osteoclasts impaired bone resorption but did not affect bone formation and, ultimately, led to increased bone mass in mice.

In our mouse model, we found that the Rap\textsuperscript{Ctsk} mice exhibited decreased body weight with increased bone density of distal femur. Raptor deletion in osteoclasts impaired bone resorption, which is important for bone modeling during bone development (3, 24), which may disturb the normal developmental process and, ultimately, induce decreased body weight. This phenomenon was found in other mouse models too (25, 26).

The differentiation and formation of osteoclasts from monocyte/macrophage precursor cells is regulated precisely to maintain the balance of bone remodeling (1–3). Our study found...
that primary Raptor-deficient BMMs displayed reduced osteoclast differentiation, as determined by decreased numbers of TRAP-positive osteoclasts, reduced TRAP activity, and lower expression of osteoclast-specific genes. This finding is supported by a previous in vitro study showing that mTORC1 plays a dominant role in osteoclast development as a nutrient sensor (21). There is limited information available concerning the molecular mechanisms by which mTORC1 controls osteoclast differentiation. It has been demonstrated that S6K1, a down-stream regulator of mTORC1, can positively regulate the differentiation of osteoblasts (15) and chondrocytes (17). In this study, we provide evidence that the mTORC1-S6K1 axis can promote osteoclast differentiation. S6K1 activity was reduced in Raptor-deficient BMMs, accompanied by impaired osteoclast differentiation in vitro. Moreover, impaired osteoclast differentiation in RapCtsk BMMs was rescued by enforced over-expression of CAS6K1, indicating that S6K1 acts as a major down-stream regulator of mTORC1 in osteoclasts.

mTORC1 signaling has been used as a target for drug treatment for a series of diseases, including cancer and aging (6). In this study, we found that rapamycin could inhibit osteoclast differentiation of primary BMMs in a dose-dependent manner, as determined by a decrease in the number of TRAP-positive osteoclasts, TRAP activity, and expression of osteoclast-specific genes. These findings suggest that mTORC1-S6K1 may be a drug target for the treatment of bone diseases related to osteoclasts. On the other hand, mTORC1 may play an important role in osteoblasts, although the results are still controversial. The mTORC1 inhibitor rapamycin can promote (27) or inhibit (28, 29) osteogenesis, which may be depended on cell type or cell differentiation stages. Further, blocking mTORC1 in preosteoblasts induced a decrease in bone mass in mice (15). Hence, further pharmacological studies about the effects of mTORC1 signaling on bone metabolism are needed in the future.

In summary, we reveal for the first time, to our knowledge, that specific inhibition of mTORC1 in osteoclasts results in increased bone mass in mice with decreased bone resorption. The Raptor/mTORC1-S6K1 axis constitutes a molecular node in the network of catabolic bone resorption of osteoclasts. These findings not only offer a mechanistic insight into the way in which mTORC1 drives osteoclast differentiation and bone resorption but also suggest an alternative pharmacological target for the regulation of osteoclasts in bone metabolic disorders.
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Experimental Procedures

**Mice—** Raptor^{fl/fl} mice bearing loxP sites flanking exon 6 of the Raptor gene (stock no. 013188) were purchased from The Jackson Laboratory. Raptor^{fl/fl} mice were crossed with Cathepsin K-Cre mice (Ctsk-cre; a gift from S. Kato, University of Tokyo, Tokyo, Japan) to generate Raptor^{fl/fl}; Ctsk-Cre (RapCtsk). All mice were bred and maintained under specific pathogen-free conditions in the institutional animal facility of the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. All experiments were performed with the protocol approved by the Animal Care and Use Committee of the Shanghai Institute of Biochemistry and Cell Biology.

**Micro-CT Analysis—** Femora from 4- and 8-week-old male mice were used for micro-CT analysis at a resolution of 10 μm (Skyscan μCT80, Bruker MicroCT, Kontich, Belgium). One hundred slices (total 1 mm) close to the distal growth plate of the femur were used to analyze trabecular microarchitectural parameters, including BMD, bone volume fraction (BV/TV), Tb.Th., and Tb.N. Fifty slices from the middle of the femur were used to analyze Ct.Th.

**Histological Analysis—** Femora from 4- and 200-week-old mice were fixed in 4% paraformaldehyde for 48 h, followed by decalcification in 10% EDTA for 4 weeks. Specimens were embedded in paraffin and cut into sections of 4-μm thickness. H&E staining and tartrate-resistant acid phosphatase (TRAP, Sigma) staining were performed according to methods described previously (31). Sections were dewaxed and rehydrated. A solution of 3% H₂O₂ was used to block the activity of endogenous peroxidase. Antigen retrieval was performed with protease K at 37 °C for 15 min. Antibodies against Ctsk (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) were added with protease K at 37 °C for 15 min. Corresponding biotinylated secondary antibodies were then added and incubated for 1 h at room temperature, followed by color development with an ABC kit (Vector Labs, Peterborough, UK).

To label the mineralization fronts, 8-week-old WT and RapCtsk mice received an intraperitoneal injection of 20 mg/kg Ctsk, 5'-GAAGCAG-3' and 5'-TCCAGGTTATGGGCAGAGATT-3'. The femora were embedded in paraffin and cut into sections of 4-μm thickness. H&E staining and tartrate-resistant acid phosphatase (TRAP, Sigma) staining were performed according to methods described previously (31).

**Immunohistochemical staining was performed following a protocol described previously (31).** Sections were dewaxed and rehydrated. A solution of 3% H₂O₂ was used to block the activity of endogenous peroxidase. Antigen retrieval was performed with protease K at 37 °C for 15 min. Antibodies against Ctsk (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) were added with protease K at 37 °C for 15 min. Corresponding biotinylated secondary antibodies were then added and incubated for 1 h at room temperature, followed by color development with an ABC kit (Vector Labs, Peterborough, UK).

**Cell Culture and Osteoclast Differentiation—** For osteoclast differentiation analysis, 4-week-old mice were used to obtain BMMs from whole bone marrow following a method described previously (26, 35). Briefly, bone marrow was washed out from the femora and tibiae and centrifuged at 500 g for 10 min. Cells were resuspended and cultured in α-MEM supplemented with 10% FBS, 1% penicillin/streptomycin in a 37 °C, 5% CO₂ incubator overnight. Then the non-adherent cells were seeded into α-MEM supplemented with 10 ng/ml M-CSF. After 48 h, the non-adherent cells were discarded and adherent cells were used as BMMs. For osteoclast differentiation, BMMs were reseeded into 24-well plate (for protein and RNA assay) and 96-well plate (for TRAP staining) at a density of 5 × 10⁴ cells/cm². The BMMs were cultured in osteoclast differentiation medium consisting of α-MEM supplemented with 20 ng/ml M-CSF and 250 ng/ml RANKL in the absence or presence of different concentrations of rapamycin. The culture medium was replaced every 2 days until formation of mature multinuclear osteoclasts was observed (5–7 days). Then the culture supernatant was harvested for examination of TRAP activity. Cells were washed twice with PBS, fixed with 4% paraformaldehyde for 5 min, and stained for TRAP using a diagnostic acid phosphatase kit (Sigma). TRAP-positive osteoclasts with more than three nuclei were counted in each well of a 96-well plate.

To determine the proliferation of BMMs, BMMs from WT and RapCtsk mice were seeded in 96-well plates (2.5 × 10⁴ cells/cm²) with 10 ng/ml M-CSF. Crystal violet staining was used to detect cell proliferation on days 0, 1, 3, and 7.

**Plasmid and Lentivirus—** cDNA of S6K1 was cloned into a phage-based plasmid. Constitutively active S6K1 was constructed following methods described previously by converting Thr³⁹⁰ to glutamic acid (36). The CAS6K1 vector and package vectors were transfected into HEK 293T/17 cells to generate a lentivirus expressing FLAG-CAS6K1 (Lenti-CAS6K1), and a lentivirus expressing green fluorescent protein (Lenti-GFP) was used as control. To force expression of CAS6K1, RapCtsk BMMs were infected with Lenti-CAS6K1 24 h after seeding and then cultured in osteoclast differentiation medium until formation of osteoclasts.

**TRAP Activity Quantification—** For the quantification of TRAP activity, cultured supernatant collected from the differentiated BMMs was incubated with a 0.33 m tartrate solution containing phosphatase substrate (Sigma) at 37 °C for 2 h before the reaction was terminated with 3 n NaOH. TRAP activity was measured by colorimetric analysis at a maximum wavelength of 405 nm.

**Western Blotting—** BMMs seeded into 24-well plates and cultured with osteoclast differentiation medium were used for Western blotting. Total proteins were extracted from cultured cells using 1× SDS lysis buffer (Takara Bio Inc., Shiga, Japan) containing protease inhibitor mixture. Lysates were centrifuged at 12,000 × g at 4 °C for 10 min, and the supernatants containing proteins were collected. Lysates containing 30 μg of protein were separated by 10% SDS-PAGE followed by Western blotting according to a standard protocol. The antibodies used were Raptor (Cell Signaling Technology, Danvers, MA, USA), P-S6K1 (Cell Signaling Technology, Santa Cruz Biotechnology), FLAG (Sigma), and β-actin (Santa Cruz Biotechnology).

**qPCR—** Total RNA was extracted from BMMs after osteoclastic differentiation using TRIzol following a standard protocol (Invitrogen). An aliquot of 500 ng of RNA was reverse-transcribed to cDNA using Takara PrimeScript reverse transcriptase (Takara Bio Inc., Shiga, Japan). qPCR was performed using a SYBR Green mixture (Takara) to detect the expression of osteoclast-specific genes. The primers used were displayed as follows: Ctsk, 5’-GAAGAAGACTCCA-GAAGCAG-3’ and 5’-GAGCAGTATGGGCGAGGATT-3’; Trap, 5’-GGGACATTGTGATCAGCAGATCAG-3’ and 5’-GGGACTTGTCGACAGACGGAT-3’; dendritic cell-specific transmembrane protein (Dc-stamp), 5’-GGGGACTTATGT-
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