

Tusc2/Fus1 regulates osteoclast differentiation through NF- κ B and NFATc1

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Tumor suppressor candidate 2 (Tusc2, also known as Fus1) regulates calcium signaling, and Ca²⁺-dependent nuclear factor of activated T-cells (NFAT) and nuclear factor kappa B (NF- κ B) pathways, which play roles in osteoclast differentiation. However, the role of Tusc2 in osteoclasts remains unknown. Here, we report that Tusc2 positively regulates the differentiation of osteoclasts. Overexpression of Tusc2 in osteoclast precursor cells enhanced receptor activator of nuclear factor κ B ligand (RANKL)-induced osteoclast differentiation. In contrast, small interfering RNA-mediated knockdown of *Tusc2* strongly inhibited osteoclast differentiation. In addition, *Tusc2* induced the activation of RANKL-mediated NF- κ B and calcium/calmodulin-dependent kinase IV (CaMKIV)/cAMP-response element (CRE)-binding protein CREB signaling cascades. Taken together, these results suggest that *Tusc2* acts as a positive regulator of RANKL-mediated osteoclast differentiation. [BMB Reports 2017; 50(9): 454-459]

INTRODUCTION

Bone is a dynamic organ which is continuously maintained by a balance between osteoclasts and osteoblasts. Osteoclasts are specialized cells derived from monocyte/macrophage hematopoietic progenitor cells (1, 2). Osteoclasts, which have bone-resorbing activity, are large multinucleated cells located on the trabecular and endosteal cortical bone surfaces (3). Meanwhile, osteoblasts, which synthesize and mineralize new bone, are derived from the mesenchymal lineage (4).

Receptor activator of nuclear factor κ B ligand (RANKL), a member of the tumor necrosis factor (TNF) superfamily, is an essential factor for osteoclast differentiation. Binding of RANKL to its receptor RANK activates nuclear factor kappa B (NF- κ B)

and mitogen-activated protein kinases (MAPKs), such as c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 MAPKs, which are involved in osteoclast differentiation (1, 2, 5). The RANKL-RANK interaction activates the nuclear factor of activated T-cells c1 (NFATc1), the master regulator of osteoclastogenesis (5, 6). Overexpression of NFATc1 induces osteoclast differentiation without RANKL stimulation (7-9). NFATc1 induces the expression of osteoclast-specific genes, including osteoclast-associated receptor (OSCAR), cathepsin K, and tartrate-resistant acid phosphatase (TRAP), which mediate the differentiation and functions of osteoclasts (5, 7, 8).

Calcium (Ca²⁺) serves as a ubiquitous second messenger and is crucial for bone homeostasis (10). Ca²⁺ signaling also regulates proliferation, differentiation, transcription, activation, and apoptosis in bone cells, including osteoclasts, osteoblasts, and osteocytes (11). RANKL induces Ca²⁺ signaling in osteoclasts via calmodulin (CaM), an intracellular Ca²⁺ receptor (12). On the binding of Ca²⁺, CaM activates calcineurin and a calcium/calmodulin-dependent kinase (CaMK), resulting in the activation and induction of NFATc1 (11, 13). Calcineurin is a Ca²⁺ and calmodulin-dependent serine/threonine protein phosphatase. Calcineurin dephosphorylates NFATc1, which allows nuclear translocation of NFATc1, thereby promoting osteoclastogenesis (11). CaMKs are important downstream mediators of the Ca²⁺ signaling pathway in osteoclast differentiation and bone resorption (14, 15). CaMKIV activates its downstream cAMP-response element (CRE)-binding protein (CREB), which induces the expression of NFATc1 and osteoclast-specific genes. Thus, Ca²⁺-related signal events are crucial for osteoclast differentiation and function.

Tumor suppressor candidate 2 (Tusc2), also known as Fus1, is a 110 amino acid mitochondrial protein. Tusc2 acts as a tumor suppressor and it is expressed in various tissues, such as the heart, kidney, liver, bone marrow, and lung. Tusc2 is frequently deleted in cancers, including lung cancer (16), mesotheliomas (17), and bone and soft tissue sarcomas (18). Uzhachenko *et al.* reported that Tusc2 is a novel regulator of mitochondrial Ca²⁺ handling and calcium-dependent mitochondrial and cellular functions (19). Tusc2 loss in activated CD4⁺ T cells decreased the activation of NFAT/NF- κ B-dependent pathways (19). Although Tusc2 regulates NFAT and NF- κ B signaling, which are important for osteoclast differentia-

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tion, the role of *Tusc2* in bone cells has not yet been described.

In this study, we investigated the role of *Tusc2* in osteoclasts. We observed that *Tusc2* could enhance RANKL-induced osteoclast differentiation via NF- κ B activation. Moreover, we showed that *Tusc2* promotes the activation of the CaMKIV-CREB signaling pathway. Taken together, our results suggested that *Tusc2* acts as a positive regulator of RANKL-induced osteoclast differentiation.

RESULTS

Overexpression of *Tusc2* enhances RANKL-induced osteoclast differentiation

First, we examined whether *Tusc2* is expressed in osteoclast lineage cells using real-time PCR analysis. BMMs were

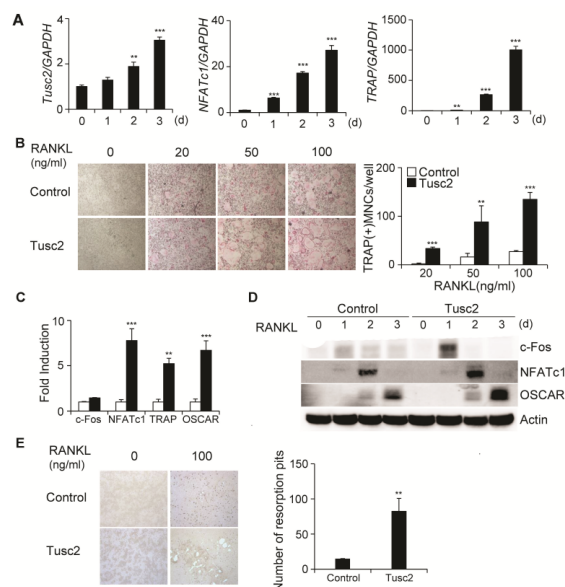


Fig. 1. Overexpression of *Tusc2* in bone marrow-derived macrophage-like cells (BMMs) enhances RANKL-induced osteoclast differentiation. (A) BMMs were cultured with M-CSF and RANKL for the indicated times. The mRNA levels of *Tusc2*, NFATc1, and TRAP were assessed by quantitative real-time PCR. ** $P < 0.01$, and *** $P < 0.001$ versus the control. (B) BMMs were transduced with pMX-IRES-EGFP (control) or *Tusc2* retrovirus, and cultured in the presence of M-CSF and various concentrations of RANKL for 3 days. Cultured cells were stained for TRAP (left panel). The TRAP-positive multinucleated cells (MNCs) per well were counted (right panel). ** $P < 0.01$, and *** $P < 0.001$ versus the control. (C, D) BMMs were transduced with pMX-IRES-EGFP (control) or *Tusc2* retrovirus and cultured in the presence of M-CSF and RANKL for the indicated times. (C) The mRNA levels of c-Fos, NFATc1, TRAP, and OSCAR were assessed by quantitative real-time PCR. Data represent the mean \pm standard deviation (SD) of triplicate samples. ** $P < 0.01$ and *** $P < 0.001$ versus the control. (D) Cell lysates were harvested from cultured cells and immunoblotted with the indicated antibodies. (E) Transfected BMMs were cultured with M-CSF alone or M-CSF and RANKL on inorganic crystalline calcium phosphate plates. Resorption lacunae were visualized by bright-field microscopy. ** $P < 0.01$ versus the control.

cultured with M-CSF and RANKL for 3 days to induce osteoclast differentiation. RANKL induced the expression of NFATc1, which is a key transcription factor in osteoclast differentiation. The induction of NFATc1 was followed by the expression of TRAP, which is an osteoclast marker gene (Fig. 1A). Expression of *Tusc2* increased gradually during RANKL-induced osteoclast differentiation (Fig. 1A).

To investigate the role of *Tusc2* in RANKL-mediated osteoclast differentiation, we retrovirally overexpressed *Tusc2* in BMMs. Transduced BMMs were cultured with various concentrations of RANKL in the presence of M-CSF for 3 days, and cultured samples were stained for TRAP (Fig. 1B). Overexpression of *Tusc2* in BMMs significantly enhanced RANKL-mediated osteoclast differentiation and strongly promoted the formation of TRAP-positive multinucleated cells (MNCs) compared with that in control cells (Fig. 1B). These results suggested that *Tusc2* expression in the osteoclast lineage contributed to osteoclast differentiation.

We next examined which osteoclast-specific genes are regulated by overexpression of *Tusc2* during osteoclast differentiation. Compared with the control, overexpression of *Tusc2* increased the expressions of c-Fos and NFATc1, and downstream target genes such as TRAP and OSCAR (Fig. 1C). We also confirmed the effect of *Tusc2* on the protein levels of these genes by western blot analysis. Overexpression of *Tusc2* increased the protein levels of c-Fos, NFATc1, TRAP, and OSCAR (Fig. 1D). These data suggested that *Tusc2* plays a role in RANKL-mediated osteoclast differentiation via regulating the expression of c-Fos and NFATc1.

Next, we determined whether overexpression of *Tusc2* affected bone resorption. Transduced BMMs were cultured on Osteo assay plates in the presence of M-CSF and RANKL for 5 days. The bone-resorbing ability of *Tusc2*-overexpressing osteoclasts was significantly increased compared with that in the control (Fig. 1E). Collectively, these results indicated that *Tusc2* regulates RANKL-mediated osteoclast differentiation.

Downregulation of *Tusc2* attenuates RANKL-induced osteoclast differentiation

We investigated the physiological role of *Tusc2* in osteoclast differentiation using small interfering RNA (siRNA)-mediated knockdown of *Tusc2*. Upon transfection with a *Tusc2*-specific siRNA, the expression of *Tusc2* was dramatically reduced in BMMs (Fig. 2A). Downregulation of *Tusc2* significantly reduced RANKL-induced osteoclast differentiation and attenuated the formation of TRAP-positive MNCs compared with that in the control cells (Fig. 2B). We then examined the expression levels of osteoclast marker genes such as c-Fos, NFATc1, TRAP, and OSCAR during osteoclast differentiation using real-time PCR and western blot analysis. As expected, downregulation of *Tusc2* suppressed the mRNA and protein levels of osteoclast marker genes (Fig. 2C, D). These results indicated that *Tusc2* plays a positive role in RANKL-mediated osteoclast differentiation.

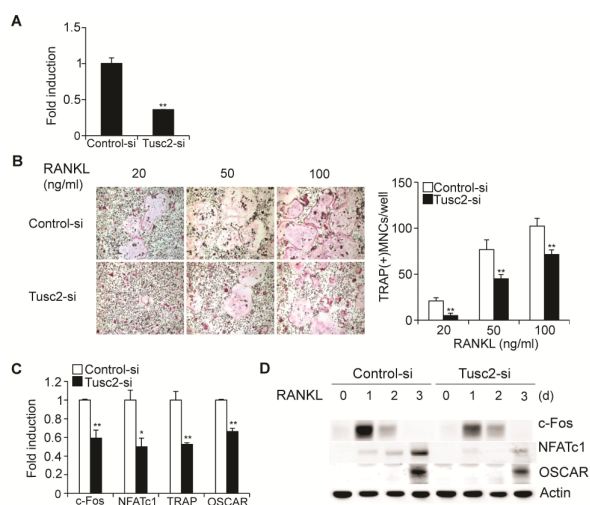


Fig. 2. Knockdown of Tusc2 in bone marrow-derived macrophage-like cells (BMMs) attenuates RANKL-induced osteoclast differentiation. (A) BMMs were transfected with control or Tusc2 siRNAs. The mRNA levels of Tusc2 were assessed by quantitative real-time PCR. Data represent the mean \pm SD of triplicate samples. ** $P < 0.01$ versus the control. (B) Transfected BMMs were cultured in the presence of M-CSF and various concentrations of RANKL for 3 days. Cultured cells were stained for TRAP (left panel). The number of TRAP-positive multinucleated cells (MNCs) per well were counted (right panel). ** $P < 0.01$ versus the control. (C, D) Transfected BMMs were cultured in the presence of M-CSF and RANKL for the indicated times. (C) The mRNA levels of c-fos, NFATc1, and TRAP were assessed by quantitative real-time PCR. Data represent the mean \pm standard deviation (SD) of triplicate samples. * $P < 0.05$ and ** $P < 0.01$ versus the control. (D) Cell lysates were harvested from cultured cells and immunoblotted with the indicated antibodies.

Overexpression of Tusc2 increases RANKL-induced NF- κ B and CaMKIV-CREB activation

RANKL activates multiple signaling pathways, including NF- κ B, p38, JNK, and ERK, which are essential for osteoclast differentiation. Tusc2 enhanced RANKL-induced osteoclast differentiation; therefore, we investigated the effect of Tusc2 on RANKL-induced early signaling pathways. BMMs were infected with control and Tusc2 retroviruses, respectively. Consistent with previous results (5, 20), RANKL induced the phosphorylation of I κ B, p38, JNK, and ERK (Fig. 3A). Overexpression of Tusc2 strongly enhanced RANKL-induced I κ B phosphorylation, whereas other signaling pathways, such as p38, JNK, and ERK, were not affected or even attenuated (Fig. 3A). These results indicated that Tusc2 plays a role in RANKL-induced NF- κ B activation.

Ca²⁺ serves as a second messenger and mediates its own signaling through Ca²⁺-binding proteins during osteoclast differentiation (21). The Ca²⁺/CaMKIV/CREB pathway is important for osteoclast differentiation and function (13). Tusc2 is a Ca²⁺-binding protein that has a Ca²⁺-binding domain (the EF-hand) (19); therefore, we investigated the role of Tusc2 in RANKL-induced Ca²⁺ signaling cascades. Overexpression of

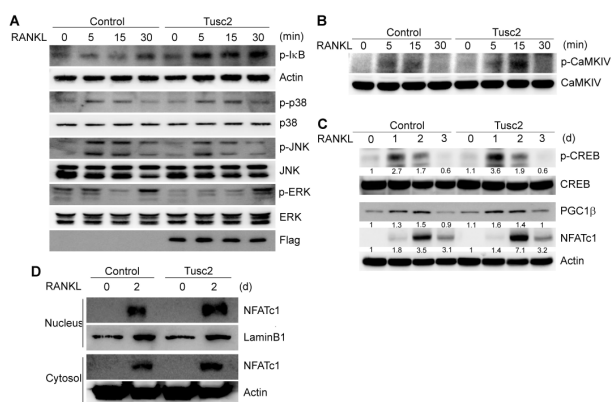


Fig. 3. Overexpression of Tusc2 in bone marrow-derived macrophage-like cells (BMMs) enhances RANKL-induced NF- κ B activation and CaMKIV/CREB signaling. (A) Bone marrow-derived macrophage-like cells (BMMs) were transduced with control or Tusc2 retrovirus. Transduced BMMs were serum-starved for 6 hours and stimulated with RANKL for the indicated times. Whole cell lysates were then harvested from the cultured cells and immunoblotted with the indicated antibodies. (B, C) BMMs were transduced with control or Tusc2 retrovirus. Transduced BMMs were serum-starved for 6 hours and stimulated with RANKL for the indicated durations. Whole cell lysates were then harvested from the cultured cells and immunoblotted with the indicated antibodies. (D) BMMs were transduced with pMX-IRES-EGFP (control) or Tusc2 retrovirus and cultured for 2 days with M-CSF and RANKL. Cytoplasmic fractions and nuclear fractions were harvested from cultured cells and immunoblotted with the indicated antibodies. Antibodies for actin and lamin B1 were used for the normalization of cytoplasmic and nuclear extracts, respectively.

Tusc2 increased the activation of CaMKIV after RANKL stimulation, as shown by its phosphorylation status (Fig. 3B). CaMKIV activates CREB through phosphorylation during osteoclast differentiation. Compared with the control, overexpression of Tusc2 enhanced the activation of CREB during RANKL-mediated osteoclast differentiation (Fig. 3C). In addition, activation of peroxisome proliferator-activated receptor- γ coactivator 1 β (PGC1 β), a target of CREB, was increased by Tusc2 overexpression. The expression of NFATc1 was increased when Tusc2 was overexpressed during osteoclast differentiation (Fig. 3C). Taken together, these results indicated that Tusc2 positively regulates the CaMKIV-CREB pathway during RANKL-mediated osteoclast differentiation.

RANKL-induced Ca²⁺ signaling regulates NFATc1 by activating the Ca²⁺/calmodulin-dependent phosphatase calcineurin (10). Dephosphorylation of NFATc1 by calcineurin leads to nuclear translocation of NFATc1, thereby promoting osteoclast differentiation (10). Next, we determined the localization of NFATc1 during osteoclast differentiation. Overexpression of Tusc2 induced enrichment of NFATc1 in the nuclear region of osteoclasts (Fig. 3D). These results indicated that Tusc2 activates the CaMKIV/CREB signaling pathway and enhances the nuclear localization of NFATc1 during RANKL-induced osteoclast differentiation.

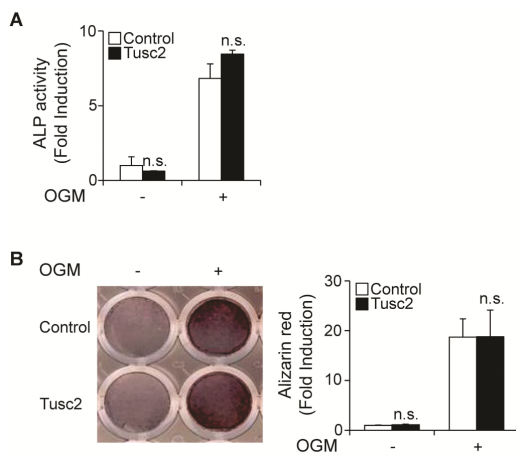


Fig. 4. Overexpression of *Tusc2* has no effect on osteoblast differentiation and function. (A, B) Osteoblasts were transduced with pMX-IRES-EGFP (control) or *Tusc2* retrovirus and cultured in an osteogenic medium (OGM) containing BMP2, ascorbic acid, and β -glycerophosphate. (A) Cells cultured for 3 days were subjected to an alkaline phosphatase (ALP) activity assay. ALP activity was measured as change in the absorbance at 405 nm. (B) Cells cultured for 9 days were fixed and stained for alizarin red (left panel), which was quantified by densitometry at 562 nm (right panel). n.s., not significant.

***Tusc2* overexpression does not affect osteoblast differentiation and function**

Next, we examined whether *Tusc2* has an effect on osteoblast differentiation. To test this assumption, we cultured primary osteoblast precursor cells infected with an empty retroviral vector or a vector containing *Tusc2* in an osteogenic medium (OGM) containing BMP2, ascorbic acid, and β -glycerophosphate. There was no difference in the alkaline phosphatase (ALP) activity between the control vector and *Tusc2*-infected osteoblasts (Fig. 4A). We then evaluated the effect of *Tusc2* on osteoblast function using mineralized nodule staining. Nodule formation in the *Tusc2*-infected osteoblasts was comparable to that in the control vector-infected cells (Fig. 4B). Therefore, these results indicated that *Tusc2* does not affect osteoblast differentiation and function.

DISCUSSION

In the present study, we report a novel role of *Tusc2* in RANKL-induced osteoclast differentiation. Overexpression of *Tusc2* in BMMs significantly enhanced RANKL-induced osteoclast differentiation. Ectopic expression of *Tusc2* in BMMs also increased the expression of osteoclast-related genes, including NFATc1, TRAP, and OSCAR. Conversely, knockdown of *Tusc2* using siRNA in BMMs inhibited osteoclast differentiation. Thus, we concluded that *Tusc2* is involved in RANKL-induced osteoclast differentiation.

Tusc2 is a Ca^{2+} /myristoyl switch protein that has a Ca^{2+} -binding domain and an N myristoylation domain (19).

Tusc2 acts by elevating Ca^{2+} , leading to Ca^{2+} accumulation in mitochondria, which is a major component of Ca^{2+} signals (22). Calcium serves as a signaling molecule that regulates osteoclast differentiation and function (23). These results suggested that *Tusc2* plays a role in osteoclast differentiation by regulating the calcium signaling pathway.

The activation of NF- κ B and NFAT pathways was reduced in *Fus1*-deficient T cells (19). NF- κ B is an important transcription factor that induces the expression of NFATc1 during osteoclast differentiation. Several papers have reported that NF- κ B is essential for RANKL-induced osteoclast differentiation. The disruption of the p50 and p52 subunits of NF- κ B resulted in impaired osteoclast differentiation, accompanied by an osteopetrotic phenotype (24). Takatsuna *et al.* reported that dehydroxymethylepoxyquinomicin (DHMEQ), an NF- κ B inhibitor, inhibited osteoclast differentiation by downregulating NFATc1 (25). Consistent with these results, NF- κ B components p50 and p65 are recruited to the NFATc1 promoter in response to RANKL (26). In addition, Ca^{2+} signaling activates the NF- κ B pathway in various cell lines. Ca^{2+} signals activate NF- κ B and NFAT, two transcription factors important for T cell activation (27). Ca^{2+} plays a crucial role in the activation of NF- κ B in cerebellar granule neurons and airway epithelial cells (28, 29). Increased cytosolic Ca^{2+} leads to phosphorylation of I κ B and activation of NF- κ B (30).

In this study, we demonstrated that *Tusc2* increased I κ B phosphorylation and NFATc1 induction in response to RANKL. Phosphorylation of I κ B leads to its degradation by the proteasome, which is important for p65 translocation into the nucleus and induction of NFATc1 expression (31). These results suggested that *Tusc2* might enhance NF- κ B activation through Ca^{2+} signaling, resulting in enhanced osteoclast differentiation.

Intracellular Ca^{2+} initiates NFATc1 activation and Ca^{2+} /CaM-mediated signaling events that regulate osteoclast differentiation and function (13). CaMK is a major downstream component of Ca^{2+} signaling and it is involved in osteoclast differentiation (26). Specific CaMK inhibitors, KN-62 and KN-63, disrupt osteoclast differentiation (14). CREB, a target of CaMKIV, interacts with NFATc1 (32). In addition, knockdown of CREB decreased NFATc1 expression (32). The CaMKIV/CREB pathway enhances induction of NFATc1 and NFATc1 downstream genes, and subsequently promotes osteoclastic bone resorption (13). We observed that *Tusc2* enhanced the activity of the CaMKIV-CREB signaling pathway. These data suggested that *Tusc2* activates a Ca^{2+} -mediated CaMKIV/CREB signaling cascade during osteoclast differentiation.

RANKL-induced Ca^{2+} signals have a crucial role in the activation of NFATc1 in BMMs. The calcium chelator, BAPTA-AM (a calcium-specific aminopolycarboxylic acid), suppresses RANKL-induced NFATc1 expression (9). Leflunomide blocks osteoclast differentiation through inhibition of expression of NFATc1 by inhibiting Ca^{2+} (14). Ca^{2+} -bound calmodulin activates calcineurin, which dephosphorylates NFATc1 and

translocates NFATc1 to the nucleus (10). We observed that Tusc2 activated the CaMKIV/CREB signaling pathway and induced nuclear localization of NFATc1 during RANKL-induced osteoclast differentiation. Thus, our data suggested that increased Ca^{2+} accumulation by Tusc2 affects the expression and localization of NFATc1 via activation of CaMKIV/CREB.

In conclusion, we identified Tusc2 as a positive regulator of RANKL-induced osteoclast differentiation. Tusc2 enhanced osteoclast differentiation via activation of NF- κ B and CaMKIV/CREB signaling cascades. Further studies examining the detailed mechanisms underlying Tusc2 regulation will provide a clearer understanding of the roles of Tusc2 and its potential as a therapeutic target for bone diseases such as osteoporosis.

MATERIALS AND METHODS

All materials and methods are shown in online supplementary data.

Osteoclast differentiation

Murine osteoclasts were prepared from bone marrow cells as described previously (33). Bone marrow cells were isolated from tibiae and femurs of 6-8 week old Institute of Cancer Research (ICR) mice by flushing the bone marrow with α -minimal essential medium (α -MEM). The cells were cultured in α -MEM containing 10% fetal bovine serum (FBS) with M-CSF (30 ng/ml) for 3 days. Floating cells were removed and adherent cells [bone marrow-derived macrophage-like cells (BMMs)] were used as osteoclast precursors. To generate osteoclasts, BMMs were cultured with M-CSF (30 ng/ml) and RANKL (20-100 ng/ml) for 3 days. Cultured cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP). TRAP-positive cells with more than three nuclei were counted as osteoclasts.

Osteoblast differentiation

Primary osteoblasts were isolated from neonatal mouse calvaria by successive enzymatic digestion with 0.1% collagenase (Thermo Fisher Scientific, MA, USA) and 0.2% dispase II (Roche Diagnostics GmbH, Mannheim, Germany). Osteoblasts were cultured in an osteogenic medium (OGM) containing BMP2 (100 ng/ml), ascorbic acid (50 ng/ml), and β -glycerophosphate (100 mM). To assess their differentiation, osteoblasts cultured for 3 days were stained for alkaline phosphatase (ALP). To assess ALP activity, cells were lysed in osteoblast lysis buffer [50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 150 mM NaCl, and 1 mM EDTA], and the lysates were incubated with p-nitrophenyl phosphate substrate (Sigma-Aldrich, MO, USA). ALP activity was then measured at an absorbance of 405 nm. To assess their function, osteoblasts cultured for 9 days were fixed with 70% ethanol and stained with 40 mM Alizarin red in 10% cetylpyridinium chloride (Sigma-Aldrich, MO, USA) (pH 4.2). Alizarin red staining was

measured at an absorbance of 562 nm.

Fractionation and Western blot analysis

Cultured cells were harvested after washing with ice-cold phosphate-buffered saline and then lysed in extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet p-40, 0.01% protease inhibitor mixture). Cells were fractionated using Nuclease and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, MA, USA), according to the manufacturer's protocol. Cell lysates, cytoplasmic extracts, and nuclease extracts were subjected to SDS-PAGE and transferred electrophoretically onto a polyvinylidene fluoride membrane (Millipore, MA, USA). The membranes were subjected to western blot analysis and signals were detected by a LAS3000 Luminescent image analyzer (GE Healthcare, NJ, USA) (34).

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CONFLICTS OF INTEREST

The authors have no conflicting financial interests.

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