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Vav1 inhibits RANKL-induced osteoclast differentiation and bone resorption

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Vav1 is a Rho/Rac guanine nucleotide exchange factor primarily expressed in hematopoietic cells. In this study, we investigated the potential role of Vav1 in osteoclast (OC) differentiation by comparing the ability of bone marrow mononuclear cells (BMMCs) obtained from Vav1-deficient (Vav1^{-/-}) and wild-type (WT) mice to differentiate into mature OCs upon stimulation with macrophage colony stimulating factor and receptor activator of nuclear kappa B ligand *in vitro*. Our results suggested that Vav1 deficiency promoted the differentiation of BMMCs into OCs, as indicated by the increased expression of tartrate-resistant acid phosphatase, cathepsin K, and calcitonin receptor. Therefore, Vav1 may play a negative role in OC differentiation. This hypothesis was supported by the observation of more OCs in the femurs of Vav1^{-/-} mice than in WT mice. Furthermore, the bone status of Vav1^{-/-} mice was analyzed *in situ* and the femurs of Vav1^{-/-} mice appeared abnormal, with poor bone density and fewer number of trabeculae. In addition, Vav1-deficient OCs showed stronger adhesion to vitronectin, an $\alpha_v\beta_3$ integrin ligand important in bone resorption. Thus, Vav1 may inhibit OC differentiation and protect against bone resorption. [BMB Reports 2019; 52(11): 659-664]

INTRODUCTION

Vav proteins are Dbl family guanine nucleotide exchange factors (GEFs) that activate Rho family GTPases by stimulating the release of GDP to allow GTP binding. There are three

members of the Vav protein family, Vav1, Vav2, and Vav3. Vav1 is expressed exclusively in hematopoietic cells (1), whereas Vav2 and Vav3 are expressed ubiquitously. Vav proteins are involved in essential cellular processes such as differentiation, proliferation, cytoskeletal organization, and vesicle trafficking. In addition, these proteins play an essential role in the coupling of β_2 integrin to Rho GTPases and regulate multiple integrin-induced events in leukocyte adhesion, migration, and phagocytosis (2, 3). Vav proteins are activated by growth factor receptors, chemokine receptors and integrins, and regulate multiple cellular processes such as osteoclast (OC) differentiation via the activation of Rho GTPases (4). Although Vav proteins share redundant functions, they also exhibit unique, isoform-specific roles. Vav1 regulates actin polymerization, cytoskeletal organization, adhesion, migration, and phagocytosis in hematopoietic cells. Vav1 is also required for optimal spreading and regulation by $\alpha_{IIb}\beta_3$ integrin (5), and upregulates $\alpha_4\beta_1$ -mediated T lymphocyte adhesion (6, 7) and β_1 integrin-mediated adhesion in dendritic cells (8).

Bone homeostasis is critical for the maintenance, repair and remodeling of bones, and depends on the balance between bone formation by osteoblasts and bone breakdown by OCs. OCs are a type of bone cell responsible for breaking down bone tissue via a process termed resorption, which occurs through physical association between OCs and the bone matrix (9, 10). OCs differentiate from the hematopoietic monocyte/macrophage lineage and are formed by the fusion of mononuclear OC precursor cells into multinucleate cells with abundant cytoplasmic azurophilic granules (11, 12). OCs can be produced from bone marrow mononuclear cells (BMMCs) *in vitro* by several cytokines, such as macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B (NF- κ B) ligand (RANKL) (13). OCs must tightly adhere to the bone surface to form a sealing zone which facilitates bone resorption (14). The binding of integrins to their corresponding ligands plays an important role in bone recognition by OCs. The transmembrane integrin $\alpha_v\beta_3$ is the most abundant integrin in OCs (15), and several studies have reported that $\alpha_v\beta_3$ integrin mediates the adhesion of OCs to bone surfaces.

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In this study, we explored the possible role of Vav1 in OC differentiation and bone resorption using Vav1-deficient (Vav1^{-/-}) mice. BMMCs differentiate into OC precursors in the presence of M-CSF *in vitro*, and subsequently develop into mature OCs in the presence of M-CSF and RANKL (16). Vav1^{-/-} mice were used to evaluate whether Vav1^{-/-} BMMCs differed from wild-type (WT) BMMCs in their ability to differentiate into OCs *in vitro* by measuring various OC-specific markers, such as tartrate-resistant acid phosphatase (TRAP), cathepsin K (CTSK), calcitonin receptor (CTR), and dendritic cell-specific transmembrane protein (DC-STAMP). We demonstrated that Vav1 suppressed OC differentiation, as indicated by the increased expression levels of OC markers in differentiating Vav1^{-/-} cells *in vitro*. Consistently, TRAP expression was more apparent in the femoral bones of Vav1^{-/-} mice than in those of WT mice. We observed abnormalities in the bone mineral density and number of trabeculae in the femurs of Vav1^{-/-} mice *in vivo*. This study reveals the important role of Vav1 in the prevention of bone resorption by suppressing OC differentiation.

RESULTS

Vav1 suppresses the differentiation of TRAP-positive multinucleated cells

We investigated the role of Vav1 in OC differentiation and bone absorption by comparing Vav1^{-/-} and WT mice. First,

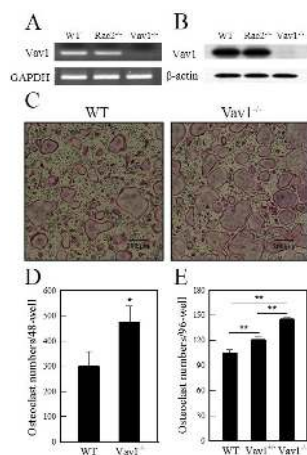


Fig. 1. TRAP-positive OC formation was increased in Vav1^{-/-} mice. (A) Total RNA was prepared from the OCs of WT and Vav1^{-/-} mice and the absence of Vav1 mRNA was verified by RT-PCR (n = 6). (B) Vav1 protein deficiency in the OCs was confirmed by western blotting using an anti-Vav1 polyclonal antibody (n = 3). RNA and protein samples from Rac2^{-/-} OCs were used as controls. (C, D) BMMCs were differentiated in the presence of 30 ng/ml M-CSF and 50 ng/ml RANKL, and TRAP-positive multinucleated OCs were counted (n = 10). (E) TRAP-positive OCs differentiated from littermate mice were counted (n = 3), *P < 0.05 and **P < 0.01.

we verified the disruption of Vav1 gene expression in the OCs of the Vav1^{-/-} mice used in this study (Fig. 1A, B). OC precursors were defined as the cells resulting from a 4-day culture with M-CSF, while mature OCs were defined as the cells obtained from an additional 4 days of culture with M-CSF and RANKL. TRAP activity is an important marker of mature and functional OCs. In the OC precursors, no noticeable differences were observed in the TRAP level between the Vav1^{-/-} and WT cells (data not shown). However, as the OC precursors differentiated into mature OCs the number of TRAP-positive multinucleated OCs was significantly higher in the Vav1^{-/-} cells than in the WT cells (Fig. 1C, D), while heterozygous Vav1^{+/-} mice produced intermediate levels of TRAP-positive OCs (Fig. 1E). This dose-dependent production of TRAP-positive OCs suggests that Vav1 suppressed OC differentiation. However, the suppressive role of Vav1 seems to be opposite to the effect of Vav3 on OC differentiation reported previously (17). The mechanisms underlying these opposing roles of Vav1 and Vav3 in OC differentiation are not yet understood.

Vav1 suppresses the expression of osteoclastogenic makers

We determined the mRNA levels of osteoclastogenic makers in WT and Vav1^{-/-} OCs. In addition to TRAP, CTSK, CTR, and DC-STAMP are essential for bone resorption (18, 19) and their expression levels increase during osteoclastogenesis. Quantitative RT-PCR analysis showed that the mRNA expression of these markers was significantly higher in Vav1^{-/-} OCs compared to WT OCs (Fig. 2A-D). Consistent with the increase in the number of TRAP-positive OCs, the enzymatic activity of TRAP was also higher in Vav1^{-/-} OCs than in WT OCs, confirming the negative effect of Vav1 on OC differentiation (Fig. 2E).

Vav1 suppresses α_vβ₃-mediated adhesion of OCs

The binding of integrins to their corresponding ligands plays an important role in the recognition of bone by OCs (3). Integrin α_vβ₃ is the most abundant integrin in OCs (14). Vav proteins are known to control integrin-mediated cell adhesion through "inside-out" signaling. However, the roles of Vav1 in

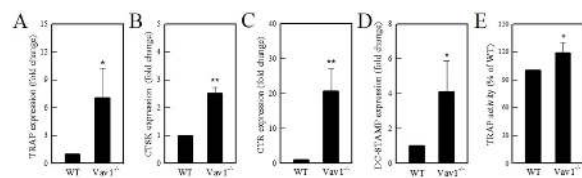


Fig. 2. OC-specific markers and TRAP activity were increased in Vav1^{-/-} mice. Expression level of mRNA of TRAP (A), CTSK (B), CTR (C), and DC-STAMP (D) in OCs was measured by qRT-PCR (n = 5). (E) TRAP activity in OCs was determined using p-nitrophenyl phosphate as the substrate (n = 3), *P < 0.05 and **P < 0.01.

integrin $\alpha_v\beta_3$ -mediated cell adhesion remain unclear. Vitronectin is a glycoprotein that binds to integrin $\alpha_v\beta_3$ as it contains an Arg-Gly-Asp sequence, a specific integrin $\alpha_v\beta_3$ binding site. Therefore, we examined whether Vav1 was involved in integrin $\alpha_v\beta_3$ -mediated adhesion by comparing the binding ability of Vav1^{-/-} and WT OC precursors on vitronectin-coated plates. Following the incubation of OC precursors on vitronectin-coated plates for 10 and 60 min, the number of adhered cells was counted and plotted as a histogram (Fig. 3). The Vav1^{-/-} OC precursors exhibited greater adherence to the vitronectin-coated plates than the WT cells. We also determined actin ring formation by Alexa-phalloidin staining, with Vav1^{-/-} OCs showing higher levels of actin ring formation than the WT cells (Supplementary Fig. 1). These results indicate that Vav1 suppresses the $\alpha_v\beta_3$ -mediated adhesion of OCs, presumably resulting in the attenuation of bone resorption activity.

Vav1 deficiency reduces bone volume fraction and number of trabeculae in femurs

As OCs are known to be involved in bone resorption, we compared the bone densities of Vav1^{-/-} and WT mice using μ CT. The femurs of Vav1^{-/-} mice appeared less dense than those of WT mice (Fig. 4A). To confirm this observation, the ratio of trabecular bone volume to total bone volume (BV/TV, %), trabecular thickness (Tb.Th, μ m), trabecular separation (Tb.Sp, μ m), and the number of trabeculae (Tb.N, mm⁻¹) were assessed. Trabeculae are composed of bone cells and collagenous tissue, and are an important skeletal element that supports the framework of organs and body. The BV/TV ratio is an important parameter for evaluating the microstructure of the trabecular bone (20). Vav1^{-/-} mice showed significantly lower BV/TV, Tb.Th, and Tb.N than WT mice (Fig. 4B-E), demonstrating that Vav1 prevents OC-mediated reductions in bone mass.

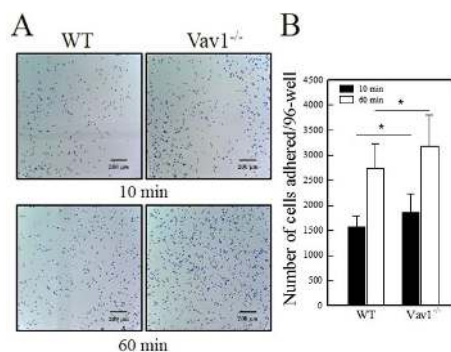


Fig. 3. Adhesion of OC precursors to vitronectin was increased in the absence of Vav1. The ability of OC precursors to adhere to vitronectin was determined in the presence of M-CSF and RANKL for 10 and 60 min (A), and the number of adhered cells was counted and plotted as a histogram (B) (n = 3), *P < 0.05.

Bones of Vav1^{-/-} mice contain more TRAP-positive cells than those of WT mice

As the results obtained with OCs *in vitro* suggested that Vav1 suppresses OC differentiation, we examined the effects of Vav1 on OC formation *in vivo*. We compared the number of TRAP-positive cells in the bone sections obtained from Vav1^{-/-} and WT mice. Consistent with the *in vitro* results, the femoral bone sections of Vav1^{-/-} mice had almost twice as many TRAP-positive cells than those of WT mice (Fig. 4F, G), confirming the suppression of OC differentiation by Vav1 *in vivo*.

DISCUSSION

Vav family GEFs are expressed as three isoforms, with Vav3 being the most abundant in OCs (17). Vav3 has been shown to stimulate OC differentiation and bone resorption by enhancing the affinity of integrin $\alpha_v\beta_3$ for vitronectin (17). Vav3-deficient OCs display defective resorption activity due to impaired signaling downstream of the M-CSF receptor and $\alpha_v\beta_3$ integrin. As a result, the bones of Vav3-deficient mice exhibit increased density.

In this study, we evaluated the role of Vav1 in OC differentiation. Surprisingly, Vav1 had the opposite effect of Vav3 by suppressing OC differentiation and bone resorption. To clarify the suppressive effect of Vav1 on OC differentiation, we determined the expression and activation levels of kinases known to be involved in OC differentiation, such as mitogen-activated protein kinases (MAPKs) and Akt (21-23). RANK signaling is initiated by cytoplasmic tumor necrosis factor receptor-associated factors that trigger the activation of signaling cascades downstream of these kinases (24, 25).

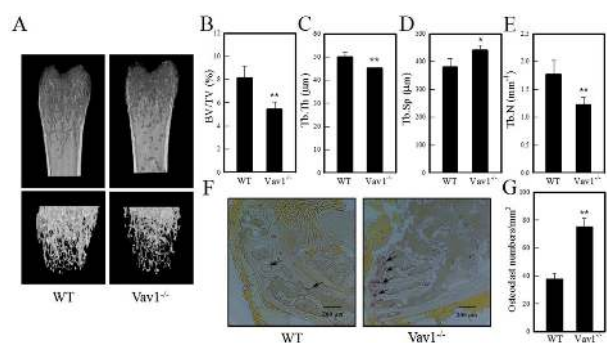


Fig. 4. Vav1 deletion leads to poor bone density *in vivo*. (A) Microcomputed tomography of the femurs of WT and Vav1^{-/-} mice (6-wk-old male mice, n = 4 per group). (B) Trabecular bone volumes of WT and Vav1^{-/-} mice were compared using quantitative μ CT. (C-E) Trabecular bone parameters of WT and Vav1^{-/-} mice were compared. (F) TRAP-positive cells in the femurs of 24-wk-old male mice were detected (n = 3 per group). (G) The number of TRAP-positive cells in selected femur fields was counted (n = 3), *P < 0.05 and **P < 0.01.

MAPKs play important roles in osteoclastogenesis and the expression of MAPKs has been shown to increase in response to RANKL in RAW 264.7 cells (21). Osteoclastogenesis is regulated by the balance between extracellular signaling-regulated kinase (ERK) and p38 MAPK signaling. ERK inhibitors enhance OC differentiation, whereas p38 MAPK inhibitors suppress OC differentiation (21). In this study, the activation of MAPKs by M-CSF and RANKL was inconsistent and the activation of MAPKs and Akt was unimpaired in Vav1^{-/-} OCs (data not shown). These results were consistent with our finding that MAPKs signaling was unaltered in the neutrophils of Vav1^{-/-} mice (data not shown).

The tentative targets for Vav1 are Rac1, Rac2, and RhoG (26), whereas Vav2 and Vav3 have a higher activity towards RhoA, RhoB, and RhoG (4, 27). RANKL selectively activates Rac1; hence, Rac1 may be a key regulator of OC differentiation via the activation of NF- κ B (28). Vav1 is known to preferentially activate Rac1 (26, 29); thus, we investigated Rac activation in Vav1^{-/-} OCs using an affinity precipitation assay for Rac-GTP (22). The levels of active Rac-GTP decreased with OC differentiation; however, no difference in Rac activation was observed between WT and Vav1^{-/-} OCs (data not shown). These results were inconsistent with previous studies, which showed that RANKL selectively activates Rac1 but not Rac2, and that Rac1 selectively regulates OC differentiation (28, 30). To determine the GEF role of Vav1 on hematopoietic-specific Rac2, we differentiated the BMMCs of Rac2^{-/-} mice (31) into OCs. The number of OCs in Rac2^{-/-} cells decreased to 40% of the number in WT cells, whilst, it increased in Vav1^{-/-} cells (data not shown). We expected that Vav1 and Rac2 would exert similar effects on OC differentiation; however, Vav1 suppressed OC differentiation whilst Rac2 was essential. This suggests that Vav1 is not an upstream regulator of Rac2-mediated OC differentiation.

Currently, there is no clear explanation for the opposing activities of Vav1 and Vav3 in OC differentiation and bone resorption. We speculate that Vav1 may co-exist with Vav3 and share common target molecules in OCs, and that Vav1 may inhibit or counterbalance Vav3-mediated OC differentiation by competitively inhibiting Vav3 binding to their common target molecules in response to "outside-in" signaling from extracellular signals. Vav1 deficiency may increase the effects of Vav3 on OC differentiation and bone resorption as observed in the femurs of Vav1^{-/-} mice. Rho-GEF kalirin has been reported to play a similar role to Vav1 in maintaining bone mass by controlling OC differentiation. Deletion of kalirin results in the decrease in the cortical bone mass of mice (32). Genomic studies have suggested that the function of Vav isoforms may have co-evolved with tyrosine kinase pathways to convert extracellular signals into biological responses in a competitive manner (33).

In summary, this study demonstrates that Vav1 suppresses OC differentiation, as shown by the increased potential of

Vav1^{-/-} BMMCs to differentiate into OCs than WT BMMCs. This was confirmed by the increased number of mature TRAP-positive OCs in the low density femoral bones of Vav1^{-/-} mice. Moreover, Vav1 inhibited the adhesion of OC precursors to vitronectin. Therefore, Vav1 may prevent bone resorption by inhibiting the interaction between OCs and bone tissues via integrins in order to maintain bone mass.

MATERIALS AND METHODS

Reagents

M-CSF and RANKL were purchased from PeproTech (London, UK). Phosphate-buffered saline (PBS), α -minimum essential medium (α -MEM), and fetal bovine serum (FBS) were obtained from HyClone (Logan, UT). PCR primers were procured from Bioneer (Daejeon, Korea). All other chemicals were purchased from Sigma (St. Louis, MO).

Mice

Vav1^{-/-} mice were generated previously (34) and the C57BL/6J mice used as the WT control were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed under pathogen-free conditions in the animal facility of Inha University. All procedures were conducted in accordance with the institutional guidelines approved by the Animal Care and Use Committee of Inha University (INHA 161214-465). To isolate bone marrow cells, mice were sacrificed by cervical dislocation and every effort was made to minimize their suffering.

Osteoclast differentiation from bone marrow cells

Bone marrow cells were prepared from 6 to 8-wk-old male mice and cultured overnight in α -MEM supplemented with 10% FBS and 10 ng/ml M-CSF. Non-adherent cells were separated and cultured continuously with 30 ng/ml M-CSF for more 3 days to induce differentiation into OC precursors. These cells were cultured for an additional 4 days in the presence of 30 ng/ml M-CSF and 50 ng/ml RANKL. The formation of multinucleated functional OCs was assessed by counting the number of TRAP-positive cells containing more than three nuclei.

TRAP staining

We stained TRAP-positive cells using a Leukocyte Acid Phosphatase Assay kit according to the manufacturer's instructions. Briefly, cells were washed with PBS and fixed using a fixative solution containing citrate solution, acetone, and 37% formaldehyde. Fixed cells were treated with TRAP staining solution for 60 min at 37°C and washed twice with water. After counterstaining with hematoxylin, the number of TRAP-positive cells containing three or more nuclei was counted.

Analysis of TRAP activity

TRAP enzyme activity was determined as described previously (35). Cells were incubated with 10 mM sodium tartrate and 5 mM p-nitrophenyl phosphate in 50 mM citrate buffer (pH 4.5) for 30 min at 37°C. The enzyme reaction was then terminated with 0.1 N NaOH. Absorbance was measured at 405 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA) equipped with SoftMax software.

RT-PCR and quantitative PCR

Total RNA was extracted from the OCs using TRI reagent (MRC, Cincinnati, OH) according to the manufacturer's instructions, and then reverse transcribed according to the protocol provided by Takara Bio (Japan). The PCR amplification of Vav1 mRNA was performed using the following primers; forward, 5'-GAC GAA GAT ATT TAC AGT GG-3' and reverse, 5'-GCT TAT CAT ACT CTG TCA TC-3'. Quantitative real time (qRT)-PCR was carried out on an Applied Biosystems StepOne unit (Foster City, CA) with SYBR Green PCR Master Mix (Toyobo, Japan) and the following primers (forward and reverse, respectively): TRAP, 5'-ACG GCT ACT TGC GGT TTC A-3' and 5'-TCC TTG GGA GGC TGG TCT T-3'; CTSK, 5'-GAA GAA GAC TCA CCA GAA GCA G-3' and 5'-TCC AGG TTA TGG GCA GAG ATT-3'; CTR, 5'-TGC TGG CTG AGT GCA GAA ACC-3' and 5'-GGC CTT CAC AGC CTT CAG GTA C-3'; DC-STAMP, 5'-TCCTCCATGAACAAACAGTTCCA A-3' and 5'-AGACGTGGTTTAGGAATGCAGCTC-3'; GAPDH, 5'-CCT TCC GTC CTA CCC C-3' and 5'-CCC AAG ATG CCC TTC ATG-3' (35). Amplification data were analyzed using the sequence detection software provided by Applied Biosystems.

Adhesion assay

OC precursors were seeded in 48-wells plates coated with 10 µg/ml vitronectin (R&D Systems, Minneapolis, MN) for 10 and 60 min at 37°C. Unattached cells were rinsed with PBS, stained with Diff-Quick solutions (Thermo, Rockford, IL), observed under the Axioskop2 Plus microscope (Carl Zeiss, Oberkochen, Germany), and then the number of adhered cells was counted.

Histology and microcomputed tomography (µCT) analysis

The femurs of 6-wk-old male mice were fixed with 10% formalin, decalcified with 10% EDTA, dehydrated, embedded in paraffin, and sliced into 5 µm sections. The sections were either stained for TRAP or hematoxylin and eosin. The trabecular microarchitecture of the distal femoral metaphysis was determined using SkyScan µCT (Bruker, Belgium) at 80 kV, 80 µA, and a 6.5-µm voxel size. Cortical bone parameters such as, BV/TV, Tb.Th, Tb.Sp, and Tb.N were analyzed by µCT at 3000 ms per projection (500 projections in total) (36).

Western blot analysis

Lysates from OCs were prepared as described previously (37) and electrophoresed on 10% SDS-PAGE. The resolved

proteins were transferred onto a PVDF membrane (Millipore, Bedford, MA) and probed with an antibody to Vav1 (C-14; Santa Cruz Biotech, Dallas, TX). Immunoblots were developed using ECL reagent (Thermo).

Statistical analyses

Two-tailed Student's *t*-test (paired) was performed using Microsoft Excel (Redmond, WA). Data are expressed as the mean ± standard deviation (SD) and *p* values of *P* < 0.05 were considered significant.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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