Role of vasodilator-stimulated phosphoprotein in RANKL-differentiated murine macrophage RAW264.7 cells: Modulation of NF-κB, c-Fos and NFATc1 transcription factors

HAO HU[1,2], CHAO LI[1,2], HAITAO ZHANG[1,2], GANG WU[1,2] and YONG HUANG[1,2]

[1]Department of Traditional Chinese Traumatology, Hubei Provincial Hospital of Traditional Chinese Medicine, Wuhan, Hubei 430061; [2]Department of Traditional Chinese Traumatology, Hubei Province Academy of Traditional Chinese Medicine, Wuhan, Hubei 430070, P.R. China

Received May 24, 2020; Accepted September 22, 2020

DOI: 10.3892/etm.2021.9856

Abstract. Vasodilator-stimulated phosphoprotein (VASP) is essential for osteoclast differentiation, and reduced VASP expression results in depressed osteoclast differentiation. Previously, we demonstrated the importance of VASP and Ras-related C3 botulinum toxin substrate 1 interactions in osteosarcoma cell migration and metastasis using Mg-63 and Saos2 cells. However, the molecular details of the functional role of VASP in cell motility and migration remain to be elucidated. The present study demonstrated that VASP affects the expression of αV-integrin, tartrate-resistant acid phosphatase (TRAP) and lamellipodia protrusion in RAW 264.7 murine macrophage cells. The RAW 264.7 mouse monocyte macrophage cell line was used as an osteoclast precursor. RAW 264.7 cells were treated with 50 ng/ml of receptor activator of nuclear factor κ-B ligand (RANKL) in order to induce cell differentiation (osteoclastogenesis). Small interfering RNA (siRNA) was used to silence VASP, and RT-PCR and western blotting were used to determine the expression for genes and proteins, respectively. TRAP staining as a histochemical marker for osteoclast and fluorescent microscopy for lamellipodia protrusion was performed. RANKL treatment significantly increased the gene and protein expression of VASP, αV-integrin and TRAP in RAW 264.7 cells. Silencing of VASP significantly reduced the RANKL-induced expression of αV-integrin, TRAP and lamellipodia protrusion. In addition, knockdown of VASP attenuated RANKL-stimulated activation of NF-κB, c-Fos and nuclear factor of activated T cells cytoplasmic 1 transcription factors, and the phosphorylation of the p65 and IκBα. These results suggest the critical role of VASP in regulating osteoclast differentiation, which should be further explored in osteosarcoma research.

Correspondence to: Dr Gang Wu or Dr Yong Huang, Department of Traditional Chinese Traumatology, Hubei Provincial Hospital of Traditional Chinese Medicine, 4 Huayuanshan Road, Wuhan, Hubei 430061, P.R. China
E-mail: 24665088@qq.com
E-mail: 1187321355@qq.com

Key words: vasodilator-stimulated phosphoprotein, receptor activator of nuclear factor κ-B ligand, osteoclast, αV-integrin, tartrate-resistant acid phosphatase, lamellipodia, RAW264.7 cells, NF-κB, c-Fos, nuclear factor of activated T cells cytoplasmic 1

Introduction

Osteoclast function is important in clinical disorders of bone resorption, such as osteoarthritis, rheumatoid arthritis and osteoporosis. Vasodilator-stimulated phosphoprotein (VASP) has been demonstrated to be involved in several cell processes, including differentiation and mobility (1). VASP has been reported to serve an important role in cell migration and tumour metastasis (1). Previously, we demonstrated VASP protein regulates osteosarcoma cell migration and metastasis, potentially through interactions with Ras-related C3 botulinum toxin substrate 1 (Rac1) (2). VASP expression was significantly higher in patients with metastatic osteosarcoma compared with patients with non-metastatic osteosarcoma (2). VASP, a member of the Ena/VASP family, links the cytoskeletal system to signal transduction pathways and plays an essential role in cytoskeletal dynamics. VASP, therefore regulates cell morphology, adhesion and migration. In addition to its role in normal cell growth, embryonic development and homeostasis, VASP plays an essential role in numerous diseases, such as cancer metastasis, thrombosis, arteriosclerosis and nephritis (2-5). Notably, VASP has been shown to serve important roles in the stimulation and inhibition of cell migration (2,4). Osteoclasts are bone-resorbing cells that originate from hematopoietic precursor cells. Osteoclasts require colony stimulating factor and receptor activator of nuclear factor-κB ligand (RANKL) for their survival, proliferation, differentiation and activation. The binding of RANKL to its receptor RANK triggers osteoclast precursors to differentiate into osteoclasts (6,7). Thus, in the present study, the effects of VASP in osteoclast differentiation and mobility were investigated, providing a basis for exploring novel mechanisms of bone diseases. The molecular details of the functional role of VASP in cell motility and migration remain unknown. NF-κB

signalling mediates RANK ligand-induced osteoclastogenesis, and the inhibition of NF-κB has been proposed as an effective approach to inhibiting osteoclast formation and bone resorptive activity (8,9). The transcription factor NF-κB consists of several subunits capable of crossing the nuclear membrane, binding to specific promoters and regulating various signalling pathways, which are essential for normal cellular functions and development, including skeletal development (8,9). The present study investigated the effects of silencing VASP on the expression of αV-integrin and tartrate-resistant acid phosphatase (TRAP), and lamellipodia protrusion in murine macrophage RAW264.7 cells.

Materials and methods

Materials. Recombinant murine sRANKL (cat. no. 315-11C) was purchased from PeproTech, Inc. The antibodies used for fluorescent microscopy were as follows: Anti-VASP (cat. no. 13472-1-AP; 1:100; ProteinTech Group, Inc.), Phalloidin (cat. no. P5282; 1:100; Sigma-Aldrich; Merck KGaA), DAPI (1:1,000; cat. no. C1002; Beyotime Institute of Biotechnology). Antibodies used for western blotting experiments were anti-GAPDH (cat. no. 60004-1-Ig; 1:50,000; ProteinTech Group, Inc.), anti-VASP (cat. no. 13472-1-AP; 1:1,000; ProteinTech Group, Inc.), anti-TRAP (cat. no. sc-376875; 1:1,000; Santa Cruz Biotechnology, Inc.), anti-αV integrin (cat. no. 10569-1-AP; 1:1,000; ProteinTech Group, Inc.), anti-IκBα (cat. no. 10268-1-AP; 1:1,000; ProteinTech Group, Inc.), anti P-IκBα (cat. no. 13921-1; 1:1,000; Cayman Chemical Company), anti-NF-kB p65 (cat. no. ABP0167), anti-lamin A (cat. no. ABP0098), anti-nuclear factor of activated T cells cytoplasmic 1 (NFATc1) (cat. no. ABP53112) (all 1:1,000; Abbkine Scientific Co., Ltd.) and c-Fos (cat. no. AF6489; 1:1,000; Beyotime Institute of Biotechnology). Cy3-labeled goat anti-mouse was used as secondary antibody (cat. no. A0521; 1:500; Beyotime Institute of Biotechnology).

Cell culture and transfection. The RAW 264.7 mouse monocyte macrophage cell line was used as an osteoclast precursor. RAW 264.7 cells were obtained from the American Type Culture Collection and maintained in α-MEM (cat. no. PM150421; Procell Life Science & Technology Co., Ltd.) containing 10% FBS (cat. no. 10099-141; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (cat. no. ST488; Beyotime Institute of Biotechnology) in a humidified atmosphere (5% CO_2, 37˚C). Cells were checked for mycoplasma contamination on regular basis. After 3 days of cell growth, the RAW 264.7 cells were treated with 50 ng/ml sRANKL for 5 days in order to induce cell differentiation (i.e. osteoclastogenesis) (10,11). sRANKL was dissolved in PBS and PBS alone was used as the mock control.

For establishing stable clones of the VASP-siRNA, scrambled and empty vector, $5x10^3$ RAW 264.7 cells were seeded per well in 6-well plates (2). After the cells were grown to 70-80% confluence, transfection was performed using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.). After preparation of the Lipofectamine 2000/siRNA mix according to the manufacturer's protocol, 200 µl mix (5 µl Lipofectamine 2000 and 10 µl siRNA in MEM without FBS; total 200 µl) was added to each well and the cells were incubated at 37˚C in a 5% CO_2 incubator for 6 h. Stable clones were selected using blasticidin (cat. no. ST018; Beyotime Institute of Biotechnology) at 5 µg/ml for 3 weeks. VASP was silenced using specific siRNA (synthesized by Guangzhou RiboBio Co., Ltd.) which are as follows: forward, 5'-GGGCUACUG UGAUGCUUUATT-3' and reverse, 3'-TTCCCGAUGACA CUACGAAAU-5' and the scrambled sequence was used as a negative control (forward, 5'-UUCUCCGAACGUGUC ACGUTT-3' and reverse, 3'-TTAAGAGGCUUGCACAGU GCA-5'). Prior to subsequent experiments, cell were cultured in α-MEM containing 10% FBS for 2-6 h.

Isolation of RNA and RT-PCR. Total RNA was extracted from RAW 264.7 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. First-strand complementary DNA (cDNA) was synthesized from the total RNA in a final volume of 20 µl using Superscript II reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) containing 1 µl $Oligo(dT)_{12-18}$, 1 µl dNTP Mix, 4 µl 5X First-Strand Buffer and 1 µl reverse transcriptase. cDNA was amplified using gene specific primers via PCR. The PCR conditions were as follows: A holding stage of 94˚C for 3 min, followed by 40 cycles at 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec. Primers used are listed as follows: VASP forward, 5'-GGGCTACTGTGATGCTTTA-3' and reverse 5'-GAATGATGGCACAGTTGATA-3 (174 bp); αV integrin forward, 5'-TTCTCGGTGGTCCTGGTAG-3' and reverse, 5'-ACATCTGCGTAATCATCCCC-3' (370 bp); TRAP forward, 5'-CCCAGCCCTTACTACCGTTT-3' and reverse, 5'-TGCTTTTTGAGCCAGGACAG-3' (176 bp); and GAPDH forward, 5'-ATGGGTGTGAACCACGAGA-3' and reverse, 5'-CAGGGATGATGTTCTGGGCA-3' (229 bp). Relative gene expression was calculated using the standard $2^{-\Delta\Delta Cq}$ method (12). Data are presented as the fold-change in gene expression normalized to the level of GAPDH.

Microscopy. Cells were seeded onto glass coverslips in 24-well plates at concentration of $5x10^3$ cells/well in medium (α-MEM and 10% FBS) with 50 ng/ml sRANKL for 5 days with or without knockdown of VASP. Following incubation, coverslips were washed three times with PBS. Cells were fixed with 4% formaldehyde for 15 min and then washed with PBS thrice at room temperature. Before blocking with 5% BSA (cat. no. SW3015; Beijing Solarbio Science & Technology Co., Ltd.) for 30 min cells were immersed in 0.5% Triton X-100 for 20 min and washed thrice with PBS at room temperature. VASP antibody (1:100) was added and the cells were incubated overnight at 4˚C. The next day, the cells were incubated with goat anti-mouse polyclonal secondary antibody for 1 h at room temperature. After washing the cells with PBS, Phalloidin (5 µg/ml) was added for F-actin staining and the cells were incubated for 1 h at room temperature. Subsequently, cells were washed with PBS and DAPI (5 µg/ml) was added to stain the nuclei for 5 min. Representative images were captured using a C2+ confocal microscope system (Nikon Corporation) at x400 magnification.

Trypan blue exclusion assay (viability assay). RANKL-treated RAW264.7 cells were harvested and suspended with 0.4% trypan blue solution (cat. no. ST798; Beyotime Institute of

Biotechnology) for 3 min at room temperature. The cells were counted using a haemocytometer under an inverted microscope (CKX53; Olympus Corporation) and cells that were observed to exclude the dye were considered viable, as described previously (13).

TRAP staining. TRAP staining was performed to determine the pre-osteoclasts (RAW264.7 cells) and multinuclear mature osteoclasts using commercially available kit (Sigma-Aldrich; Merck KGaA, 387A). RAW 264.7 cells ($6x10^3$ cells/well) were plated into 24-well plates and allowed to attach overnight, and 50 ng/ml RANKL was added the next day. RAW 264.7 cells were allowed to differentiate for different numbers of days. After incubation, the RANKL-treated RAW 264.7 cells were fixed with fixative solution (25 ml citrate solution, 65 ml acetone and 8 ml of 37% formaldehyde) at room temperature for 30 sec and TRAP staining solution was added and incubated at 37˚C for 1 h in the dark. The morphology was observed using a Leica DMI6000 B microscope (Leica Microsystems GmbH) at x200 magnification. The number of TRAP-positive multinucleated cells was counted in 20 fields per treatment and the average number of TRAP-positive multinucleated cells in a triplicate experiment are shown in the results.

Western blotting. Cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer with a protease and phosphatase cocktail inhibitor (Thermo Fisher Scientific, Inc.). Cellular debris was cleared from lysates by centrifugation at 14,000 x g for 20 min at 4˚C, and the protein concentration was determined using a BCA protein assay (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of cell lysates containing 30 μg protein were separated on an 12% gel using SDS-PAGE, transferred to a PVDF membrane (Bio-Rad Laboratories, Inc.), blocked with 5% BSA (cat. no. SW3015; Beijing Solarbio Science & Technology Co., Ltd.) for 2 h at room temperature, and then visualized with enhanced chemiluminescence substrate (Pierce; Thermo Fisher Scientific, Inc.) as previously described (14,15). GAPDH was used as a reference protein and lamin was used as a reference for nuclear p65. Quantification of western blotting was performed using ImageJ software (National Institutes of Health).

Cell adhesion assays. RAW 264.7 cells were cultured on a 96-well plate at density of $5x10^4$ cells/well for 3 h. Unbound cells were removed by washing with PBS prior to the addition of 50 μl/well hexosaminidase substrate (3.75 mM 4-Nitrophenyl N-acetyl-β-D-glucosaminide, 0.25% Triton X-100, 0.05 M citrate buffer, pH 5.0). After 2 h, 75 μl/well development buffer (5 mM EDTA, 50 mM glycine, pH 10.4) was added and plates were read at 405 nm as previously described (2,16).

Statistical analysis. Statistical analysis was performed using a two-tailed unpaired Student's t-test or ANOVA with Tukey's post hoc test, and was based on a minimum of three replicates using Prism 5 for Mac statistical software (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

RANKL treatment increases osteoclast differentiation. RANKL treatment helps to induce cell differentiation (i.e. osteoclastogenesis) (10,17). Fig. 1 demonstrates that the RANKL treatment significantly increased the gene and protein expression of VASP, αV integrin and TRAP in RAW 264.7 cells over 7 days as determined by RT-PCR (Fig. 1A and B) and western blot analysis (Fig. 1B and C), respectively, when compared to basal levels (day 0). However, no significant differences in the expression of the three genes were observed during the 7 days in the negative control group (Fig. S1). Furthermore, the effect of RANKL treatment on osteoclast differentiation and maturation was explored using a TRAP assay. TRAP is an early enzyme marker of osteoclastogenesis, which is readily detectable in mononuclear pre-osteoclasts, through to mature osteoclasts (18). Fig. 1E and F shows the number of TRAP-positive multinucleated osteoclasts differentiated from RAW 264.7 cells in the presence of RANKL for a period of 7 days.

Knockdown of VASP downregulates the expression of osteoclast-specific genes. VASP has been reported to play an important role in cell behaviour, including in cell differentiation (1,2). To further elucidate the role of VASP in osteoclast differentiation, VASP expression was knocked down in RAW 264.7 cells using si-RNA post-RANKL treatment. Fig. 2 demonstrates a successful VASP-knockdown in the RAW cells as determined at the transcriptional (RT-PCR) and translation (western blotting) level when compared with the mock group.

Next, the effect of VASP-knockdown on the osteoclast-specific genes in differentiated RAW 264.7 cells was determined. A significant decrease in the gene (Fig. 3A-D) (compared with the mock group) and protein (compared with the siNC group) (Fig. 3E-F) expression of αV-integrin and TRAP was observed in the RANKL-treated VASP-knockdown cells. Furthermore, a significant decrease in multinucleated cell formation was observed in VASP-knockdown cells as determined by the TRAP staining ($P<0.05$; Fig. 4A and B). A Trypan blue exclusion assay (viability assay) was performed as a control before proceeding to the TARP staining to rule out the possibility of apoptosis or cell death. No significant differences in the viability of cells were observed following VASP-knockdown (Fig. S2). These results demonstrate the role of VASP in the osteoclast differentiation and its effect on osteoclast-specific genes. Future studies should explore the underlying mechanism behind the low level of cell differentiation following VASP silencing.

VASP-knockdown inhibits lamellipodia protrusion and cell adhesion of osteoclasts. VASP protein acts as an actin anti-capping protein and can therefore regulate the formation of membrane protrusions, such as lamellipodia and filopodia, which consequently affects cell motility and tumour metastasis (19,20). The effect of VASP-knockdown on the morphological changes of lamellipodia protrusion and actin fibbers was determined by microscopy in differentiated murine macrophage RAW 264.7 cells. A decrease in the length of F-actin fibers and lamellipodia protrusion in VASP-knockdown cells compared with their controls was

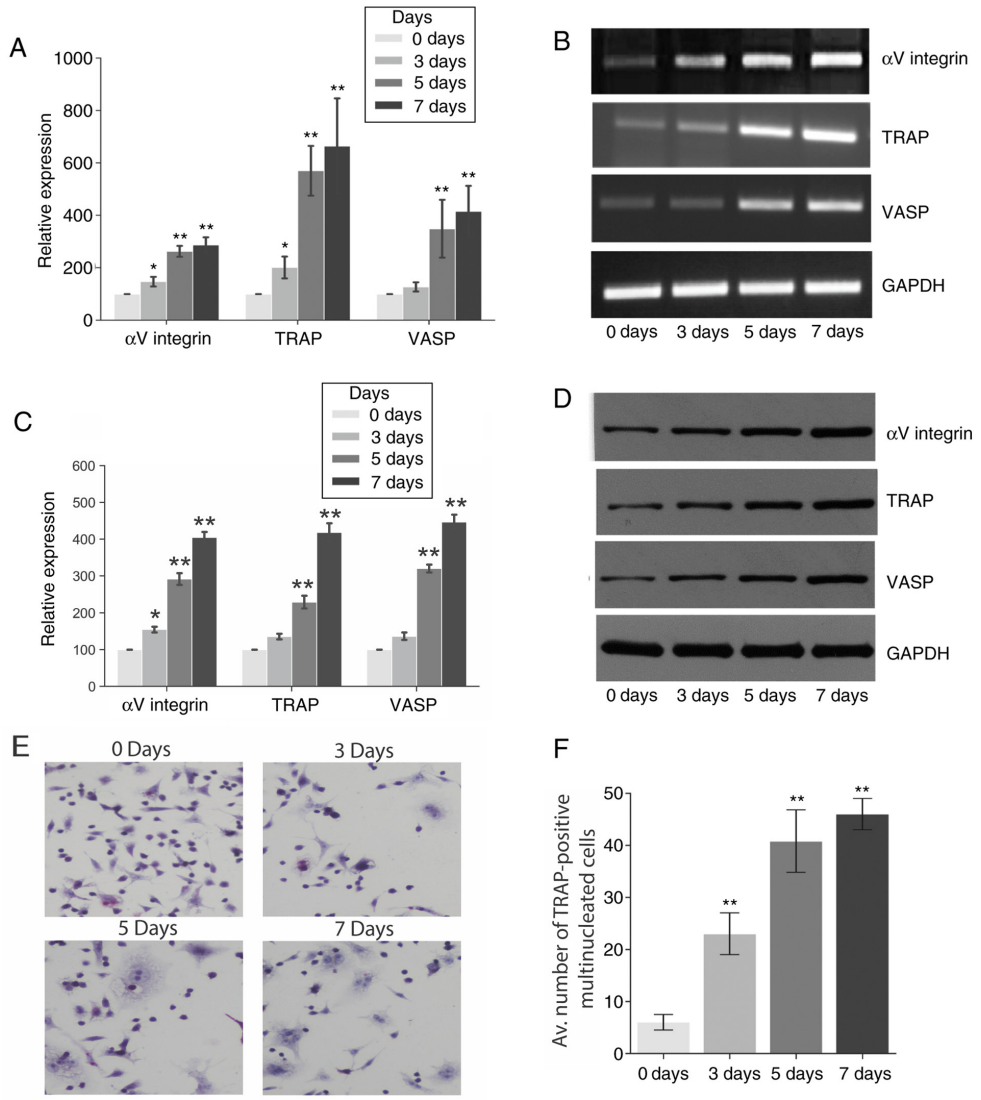

Figure 1. Differentiation of RAW 264.7 cells using RANKL showing a time-dependent increase in the expression of αV-integrin, TRAP and VASP post-RANKL treatment. (A) Quantification and (B) representative image of RT-PCR. (C) Quantification and (D) representative image of western blotting. (E) Representative images following TRAP staining showing the increase in the number of multinucleate cells (x200 magnification). (F) Quantification of the multinucleate cells as determined by TRAP staining. The number of TRAP-positive multinucleated cells was counted in 20 fields/treatment. Data are presented as the mean ± standard deviation (three repeats). *P<0.05 and **P<0.01 compared with day 0. VASP, vasodilator-stimulated phosphoprotein; RANKL, receptor activator of nuclear factor κ-B ligand; TRAP, tartrate-resistant acid phosphatase.

observed (Fig. 5). Additionally, VASP-knockdown significantly reduced the percentage of RAW 264.7 cell adhesion compared with the mock control group (Fig. 6).

VASP-knockdown reduces the activation of NF-κB signaling. The effects of VASP-knockdown on NF-κB signalling were determined. RANKL, a known activator of the NF-κB pathway triggers a rapid increase and mobilization of NF-κB components, and IκB kinase (IKK) complex formation (7). RANKL treatment increased the phosphorylation of IκBα in a time-dependent manner in RAW 264.7 cells (Fig. 7). Notably, the levels of RANKL-induced IκBα phosphorylation were low in the VASP-knockdown cells compared with the scrambled control cells. Additionally, RANKL-induced nuclear translocation of p65 was significantly reduced in the cells treated with si-VASP. In addition, the expression of NF-κB downstream signalling factors, including c-Fos and NFATc1, was also inhibited in VASP-siRNA cells compared with scrambled control cells. These results confirm the involvement of NF-κB signalling in the modulation of osteoclast differentiation by VASP.

Discussion

Osteoclasts play an essential role in bone homeostasis as characterized by their bone resorptive activity. RANKL, an indispensable cytokine for osteoclastogenesis, activates a series of intracellular signal pathways once it binds to its receptor, which in turn initiates monocyte/macrophage differentiation into multinucleated osteoclasts *in vitro* (7,21-23). The feasibility to generate osteoclast-type cells *in vitro* makes it easy for researchers to investigate the molecular mechanisms of this process (7,21-23). In the present study, RANKL-differentiated RAW 276.4 cells significantly increased the expression of VASP, TRAP and αV-integrin. These results are consistent with the previous findings, whereby TRAP mRNA expression

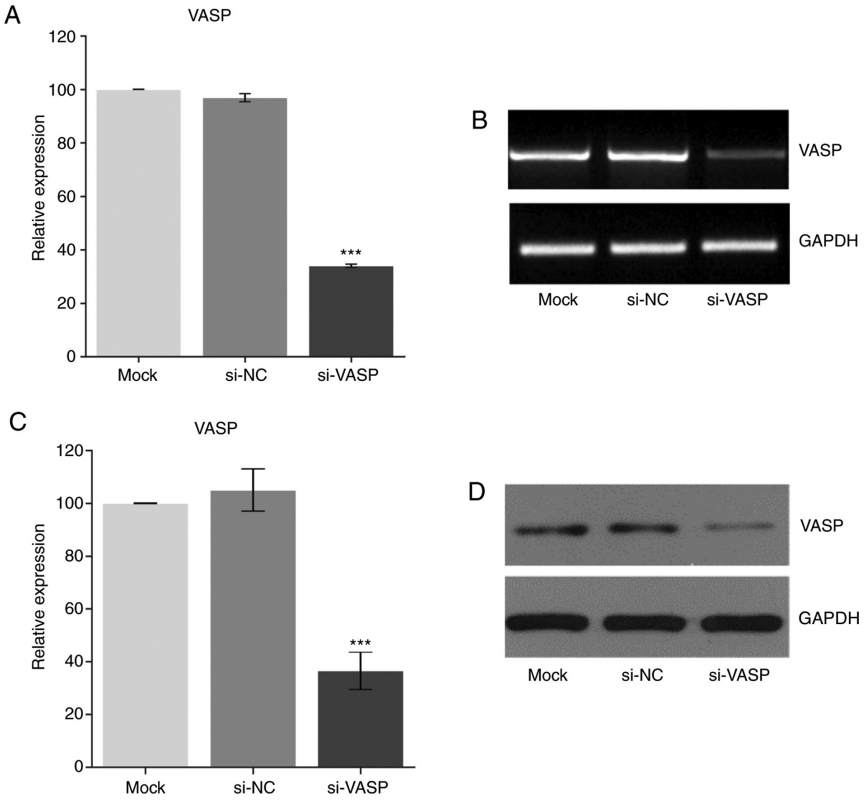

Figure 2. VASP expression after using si-RNA in RAW 264.7 cells. (A) Quantification and (B) representative image of RT-PCR. (C) Quantification and (D) representative image of western blotting. Data are presented as the mean ± standard deviation (three repeats). ***P<0.001 compared with mock. VASP, vasodilator-stimulated phosphoprotein; NC, negative control; si, small interfering.

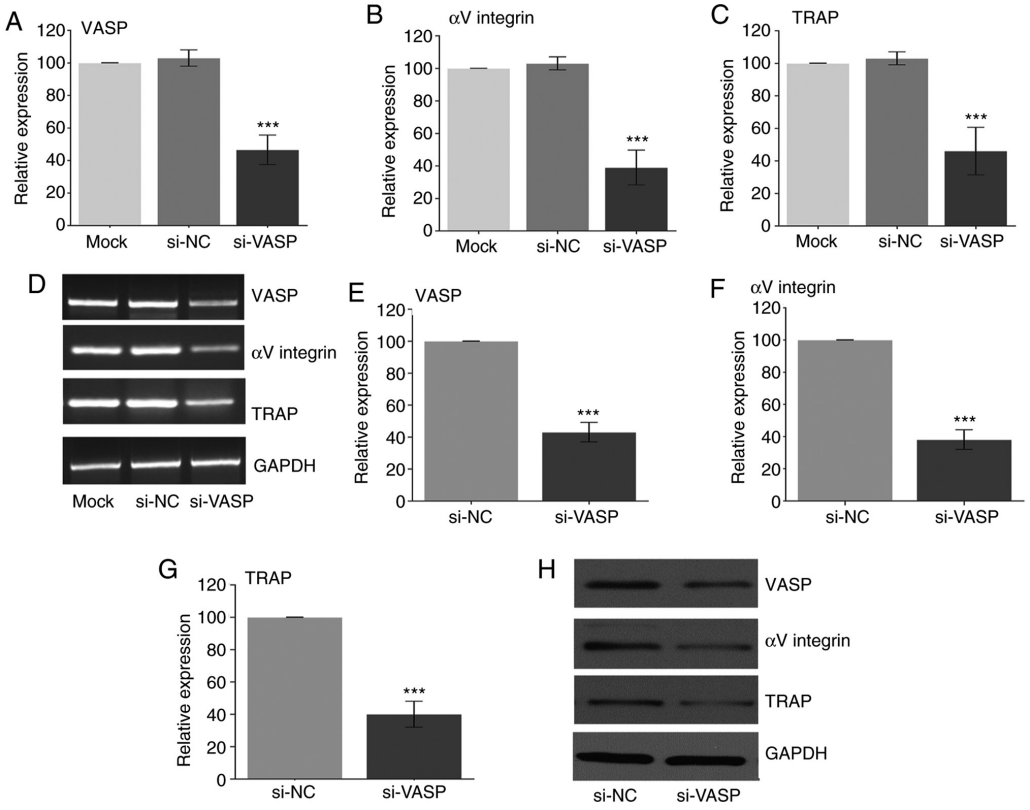

Figure 3. Effect of VASP silencing. Gene expression of (A) VASP, (B) αV-integrin and (C) TRAP after using siRNA as measured by RT-PCR. (D) Representative gel images of RT-PCR for VASP, αV-integrin, TRAP and GAPDH. Protein expression of (E) VASP, (F) αV-integrin and (G) TRAP after using si-RNA as measured by western blotting. (H) Representative western blotting for VASP, αV-integrin, TRAP and GAPDH. GAPDH was used as the loading control. Data are presented as the mean ± standard deviation (three repeats). ***P<0.001 compared with mock. VASP, vasodilator-stimulated phosphoprotein; TRAP, tartrate-resistant acid phosphatase; NC, negative control; si, small interfering.

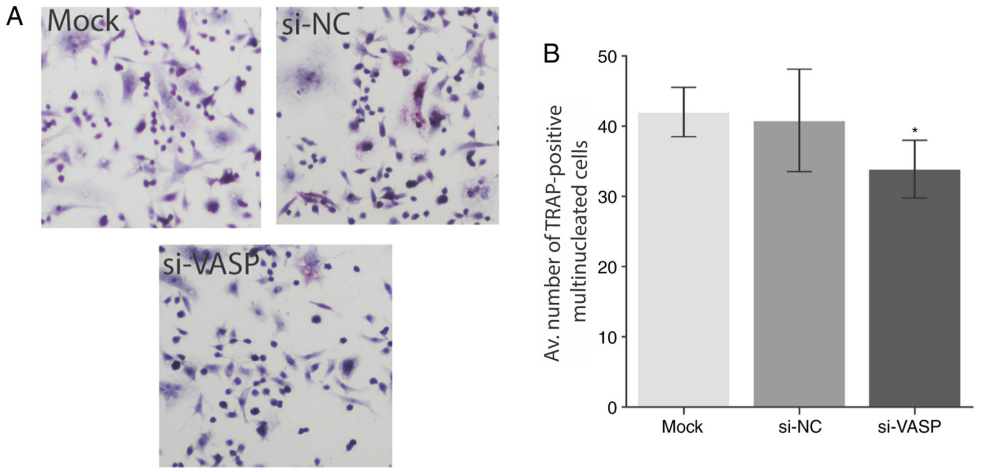

Figure 4. Effect of VASP-knockdown on TRAP staining in differentiated osteosarcoma cells. (A) Representative images following TRAP staining showing multinucleate cells (x200 magnification). (B) Quantification of the multinucleate cells as determined by TRAP staining. The number of TRAP-positive multinucleated cells was counted in 20 fields/treatment. Data are presented as the mean ± standard deviation (three repeats). *P<0.05 compared with mock. VASP, vasodilator-stimulated phosphoprotein; TRAP, tartrate-resistant acid phosphatase; NC, negative control; si, small interfering.

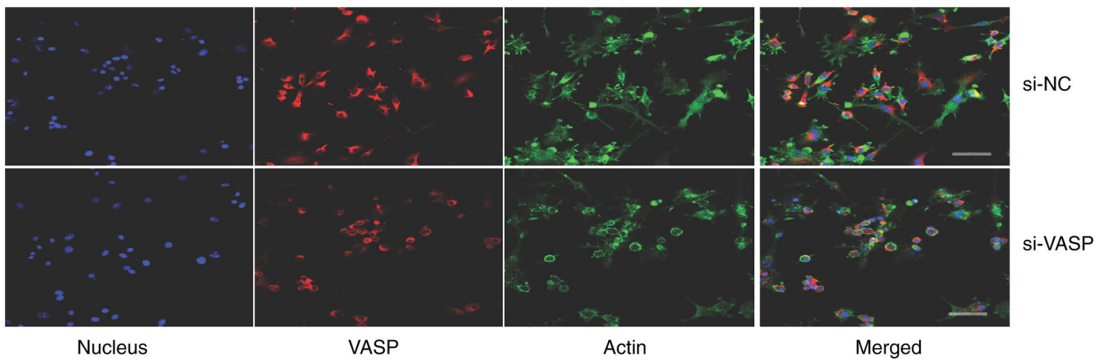

Figure 5. VASP-knockdown inhibits lamellipodia protrusion in receptor activator of nuclear factor κ-B ligand-differentiated RAW 264.7 cells osteoclasts. Fixed cells were stained using fluorescent antibodies as described in the methods section. The fluorescent staining was visualized and imaged using a confocal scanning microscope (x400 magnification; scale bar, 50 μm). VASP, vasodilator-stimulated phosphoprotein; NC, negative control; si, small interfering.

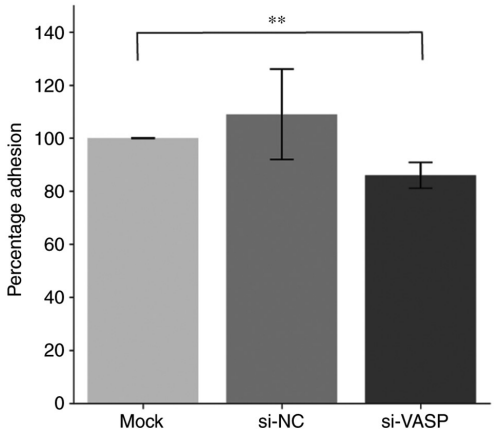

Figure 6. VASP-knockdown inhibits cell adhesion activity of receptor activator of nuclear factor κ-B ligand-differentiated RAW 264.7 cells. Data are presented as the mean ± standard deviation (three repeats). **P<0.01. VASP, vasodilator-stimulated phosphoprotein; NC, negative control; si, small interfering.

has been demonstrated to be upregulated by RANKL treatment in different experimental settings (24). Increased TRAP expression has been used as a marker of bone absorption of bone metastases in cancer (25).

VASP plays an important role in cellular activities that depend on cytoskeletal rearrangement. A positive correlation between VASP expression, and pathological stage and metastasis indicates its importance in various pathophysiology (2,5,7,26). Our previous work demonstrated a significant increase in VASP expression in samples from patients with metastatic osteosarcoma compared with patients with non-metastatic osteosarcoma (2). The data from the VASP-knockdown experiment in the present study demonstrated the importance of VASP in the regulation of osteoclast-specific genes in differentiated murine osteoclasts.

Osteoclasts are large, multinucleated cells that derive from the monocyte lineage, and express signalling molecules, such as integrins, paxillin, vinculin, talin, protein kinases and actin-associated molecules (27,28). Although mature osteoclasts express numerous integrins, it is considered that integrins of the β1, β2 and αv families are the major adhesion proteins (27,29). Integrins are highly induced in differentiating and mature osteoclasts, and bind to extracellular matrix components. VASP-knockdown significantly reduced the gene

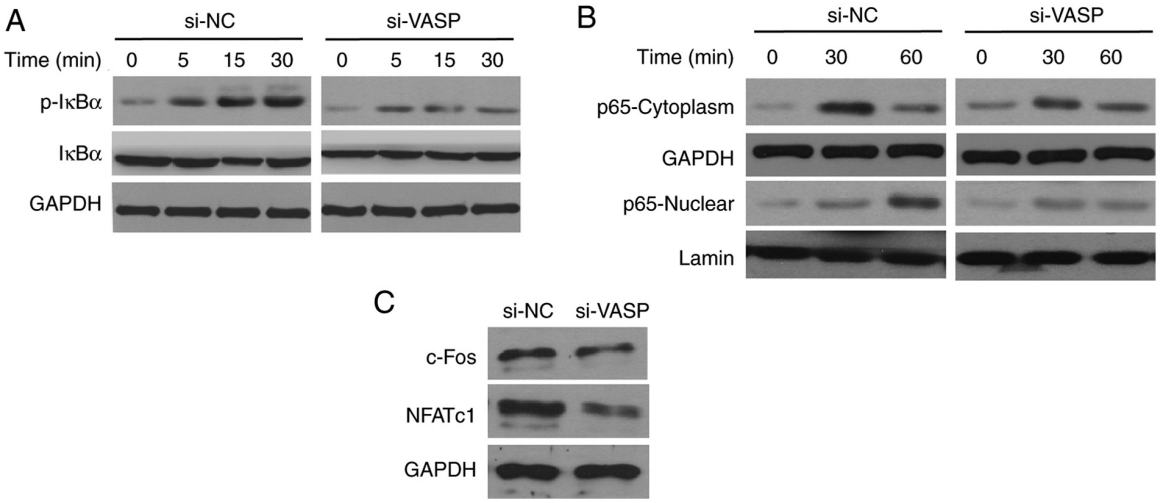

Figure 7. VASP-knockdown inhibits receptor activator of nuclear factor κ-B ligand-induced activation of NF-κB, cFos and NcFAT1 and phosphorylation of p65 and IκBα in 264.7 cells osteoclasts. Representative images of western blotting for (A) p-IκBα and IκBα, (B) cytoplasmic and nuclear p65, and (C) c-Fos and NFATc1. VASP, vasodilator-stimulated phosphoprotein; TRAP, tartrate-resistant acid phosphatase; NFATc1, nuclear factor of activated T cells cytoplasmic 1; NC, negative control; si, small interfering.

and protein expression of the αV-integrin in murine osteoclasts in the current study. These results further indicate the importance of VASP in osteosarcoma metastasis.

The importance of VASP proteins in facilitating actin-filament and membrane protrusions is well-established (19). However, the cell motility affected by VASP is not completely established and appears to be cell type-dependent. VASP-knockdown decreased filipodia formation and length of F-actin fibers when compared with the control in the current study. Day 5 was chosen to observe F-actin fibers and lamellipodia protrusion using a confocal microscope, because a significant increase in the expression of osteoclastogenesis genes at day 5 post RANKL treatment has been observed in the present study and other previous studies (11,30,31). These data further support VASP as a positive regulator of osteoclast cell migration. Overexpression of VASP in wild-type NIH 3T3 cells results in metaplasia to cancer cells (32,33). VASP-expressing Mg-63 osteosarcoma cells have high migration capacity compared with reduced VASP-expressing Saos-2 cells (2), which corroborates the results of the present study.

RANKL signalling is considered the main target of anti-resorptive agents that suppress osteoclast activation and bone loss. RANKL-induced osteoclast differentiation activates NF-κB and NFATc1. The activation of NF-κB and NFATc1 is mediated by adaptors, including TRAF6 and IKK, that leads to osteoclast differentiation (34). To understand the possible mechanisms by which VASP modulates osteoclast differentiation, the effect of VASP on the NF-κB activation was investigated in RAW 264.7 mouse monocyte macrophages. Post-translational modification of NF-κB subfamily proteins is critical in the modulation of NF-κB activation, especially, phosphorylation of p65 and IκB kinase to induce osteoclastogenesis (34,35). Osteoclastogenic genes, such as TRAP, are also activated via this pathway (36). Furthermore, the results of the present study demonstrated that the number of TRAP-positive osteoclasts decreased significantly with silencing of VASP. Additionally, RANKL treatment increased NF-κB expression and phosphorylation of the p65 and Iκk in RAW264.7 macrophages. Pre-treatment with si-VASP significantly inhibited the RANKL-induced expression of NF-κB complex subunits. Consequently, these results demonstrate the importance of NF-κB signaling in the modulation of osteoclast differentiation by VASP in RANKL-stimulated macrophages. In addition to the NF-κB signalling, we have previously demonstrated the role of the c-Fos/NFATc1 pathway in osteoclast development and arrest osteoclastogenesis (16).

In the present study, a decrease in the RANKL-induced c-Fos and NFATc1 expression was observed in VASP-knockdown cells. Taken together, the present findings demonstrate that silencing of VASP inhibits RANKL-induced osteoclast differentiation *in vitro*, and prevents activation of pivotal transcription factors, such as NF-κB, c-Fos and NFATc1. Caution should be extrapolated to interpret the results, as it is unclear whether these changes are directly caused by VASP by regulating the expression of effected proteins or indirectly as a product of upstream or downstream genes in the VASP signalling e.g. cyclic nucleotide-dependent kinases PKA and PKG (2,4,36) of VASP silencing. However, bearing in mind the diversity of the intracellular and intercellular signalling networks that are built around osteoclast maturation and differentiation, targeting VASP more specifically might be promising for the treatment or prevention of osteoclast-related diseases. Therefore, further investigations into the precise mechanisms by which VASP modulates the osteoclastogenesis are required.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Science Foundation of Hubei Provincial Hospital of Traditional Chinese Medicine (grant nos. 20190619 and 20191011) and the Hubei Provincial

Natural Science Foundation of China (grant nos. 2016CFB363 and 2016CFB187).

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HH performed the cell culture, the transfection and the TRAP-staining. CL and HZ performed the PCR and western blotting. YH conceived and designed the research study. GW performed the statistical analysis, and analysed and interpreted the data. HH and GW wrote the manuscripts and all authors read and approved it.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Rachubik P and Piwkowska A: The role of vasodilator-stimulated phosphoprotein in podocyte functioning. Cell Biol Int 43: 1092-1101, 2019.
2. Wu G, Wei L, Yu A, Zhang M, Qi B, Su K, Hu X and Wang J: Vasodilator-stimulated phosphoprotein regulates osteosarcoma cell migration. Oncol Rep 26: 1609-1615, 2011.
3. Li K, Ma YB, Tian YH, Xu XL, Gao Y, He YQ, Pan WT, Zhang JW, He CJ and Wei L: Silencing lncRNA SNHG6 suppresses proliferation and invasion of breast cancer cells through miR-26a/VASP axis. Pathol Res Pract 215: 152575, 2019.
4. Aszódi A, Pfeifer A, Ahmad M, Glauner M, Zhou XH, Ny L, Andersson KE, Kehrel B, Offermanns S and Fässler R: The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function. EMBO J 18: 37-48, 1999.
5. Bonello L, Camoin-Jau L, Armero S, Com O, Arques S, Burignat-Bonello C, Giacomoni MP, Bonello R, Collet F, Rossi P, *et al*: Tailored clopidogrel loading dose according to platelet reactivity monitoring to prevent acute and subacute stent thrombosis. Am J Cardiol 103: 5-10, 2009.
6. Park JH, Lee NK and Lee SY: Current understanding of RANK signaling in osteoclast differentiation and maturation. Mol Cells 40: 706-713, 2017.
7. van Dam PA, Verhoeven Y, Trinh XB, Wouters A, Lardon F, Prenen H, Smits E, Baldewijns M and Lammens M: RANK/RANKL signaling inhibition may improve the effectiveness of checkpoint blockade in cancer treatment. Crit Rev Oncol Hematol 133: 85-91, 2019.
8. Cheng AM, Rizzo-DeLeon N, Wilson CL, Lee WJ, Tateya S, Clowes AW, Schwartz MW and Kim F: Vasodilator-stimulated phosphoprotein protects against vascular inflammation and insulin resistance. Am J Physiol Endocrinol Metab 307: E571-E579, 2014.
9. Zeng XZ, He LG, Wang S, Wang K, Zhang YY, Tao L, Li XJ and Liu SW: Aconine inhibits RANKL-induced osteoclast differentiation in RAW264.7 cells by suppressing NF-κB and NFATc1 activation and DC-STAMP expression. Acta Pharmacol Sin 37: 255-263, 2016.
10. Brito C, Stavroullakis AT, Ferreira AC, Li K, Oliveira T, Nogueira-Filho G and Prakki A: Extract of acai-berry inhibits osteoclast differentiation and activity. Arch Oral Biol 68: 29-34, 2016.
11. Lee SH, Kim JK and Jang HD: Genistein inhibits osteoclastic differentiation of RAW 264.7 cells via regulation of ROS production and scavenging. Int J Mol Sci 15: 10605-10621, 2014.
12. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
13. Oliveira V, Mahajan N, Bates ML, Tripathi C, Kim KQ, Zaher HS, Maggi LB Jr and Tomasson MH: The snoRNA target of t(4;14) in multiple myeloma regulates ribosome biogenesis. FASEB Bioadv 1: 404-414, 2019.
14. Gang W, Tanjun W, Yong H, Jiajun Q, Yi Z and Hao H: Inhibition of miR-9 decreases osteosarcoma cell proliferation. Bosn J Basic Med Sci 20: 218-225, 2020.
15. Mahajan N, Hoover B, Rajendram M, Shi HY, Kawasaki K, Weibel DB and Zhang M: Maspin binds to cardiolipin in mitochondria and triggers apoptosis. FASEB J 33: 6354-6364, 2019.
16. Huang H, Chang EJ, Ryu J, Lee ZH, Lee Y and Kim HH: Induction of c-Fos and NFATc1 during RANKL-stimulated osteoclast differentiation is mediated by the p38 signaling pathway. Biochem Biophys Res Commun 351: 99-105, 2006.
17. Chen Y, Di Grappa MA, Molyneux SD, McKee TD, Waterhouse P, Penninger JM and Khokha R: RANKL blockade prevents and treats aggressive osteosarcomas. Sci Transl Med 7: 317ra197, 2015.
18. Zhao H, Gu J, Dai N, Gao Q, Wang D, Song R, Liu W, Yuan Y, Bian J, Liu X and Liu Z: Osteoprotegerin exposure at different stages of osteoclastogenesis differentially affects osteoclast formation and function. Cytotechnology 68: 1325-1335, 2016.
19. Applewhite DA, Barzik M, Kojima SI, Svitkina TM, Gertler FB and Borisy GG: Ena/VASP proteins have an anti-capping independent function in filopodia formation. Mol Biol Cell 18: 2579-2591, 2007.
20. Bear JE and Gertler FB: Ena/VASP: Towards resolving a pointed controversy at the barbed end. J Cell Sci 122: 1947-1953, 2009.
21. Yu J, Yun H, Shin B, Kim Y, Park ES, Choi S, Yu J, Amarasekara DS, Kim S, Inoue JI, *et al*: Interaction of tumor necrosis factor receptor-associated factor 6 (TRAF6) and Vav3 in the receptor activator of nuclear factor κB (RANK) signaling complex enhances osteoclastogenesis. J Biol Chem 291: 20643-20660, 2016.
22. Amarasekara DS, Yun H, Kim S, Lee N, Kim H and Rho J: Regulation of osteoclast differentiation by cytokine networks. Immune Netw 18: e8, 2018.
23. Feng X and Teitelbaum SL: Osteoclasts: New insights. Bone Res 1: 11-26, 2013.
24. Wittrant Y, Theoleyre S, Couillaud S, Dunstan C, Heymann D and Rédini F: Regulation of osteoclast protease expression by RANKL. Biochem Biophys Res Commun 310: 774-778, 2003.
25. Honig A, Rieger L, Kapp M, Krockenberger M, Eck M, Dietl J and Kämmerer U: Increased tartrate-resistant acid phosphatase (TRAP) expression in malignant breast, ovarian and melanoma tissue: An investigational study. BMC Cancer 6: 199, 2006.
26. Liu Z, Wang Y, Dou C, Xu M, Sun L, Wang L, Yao B, Li Q, Yang W, Tu K and Liu Q: Hypoxia-induced up-regulation of VASP promotes invasiveness and metastasis of hepatocellular carcinoma. Theranostics 8: 4649-4663, 2018.
27. Schmidt S, Nakchbandi I, Ruppert R, Kawelke N, Hess MW, Pfaller K, Jurdic P, Fässler R and Moser M: Kindlin-3-mediated signaling from multiple integrin classes is required for osteoclast-mediated bone resorption. J Cell Biol 192: 883-897, 2011.
28. Linder S and Kopp P: Podosomes at a glance. J Cell Sci 118: 2079-2082, 2005.
29. Helfrich MH, Nesbitt SA, Lakkakorpi PT, Barnes MJ, Bodary SC, Shankar G, Mason WT, Mendrick DL, Väänänen HK and Horton MA: Beta 1 integrins and osteoclast function: Involvement in collagen recognition and bone resorption. Bone 19: 317-328, 1996.
30. Feng MX, Hong JX, Wang Q, Fan YY, Yuan CT, Lei XH, Zhu M, Qin A, Chen HX and Hong D: Dihydroartemisinin prevents breast cancer-induced osteolysis via inhibiting both breast caner cells and osteoclasts. Sci Rep 6: 19074, 2016.
31. Chen Z, Andreev D, Oeser K, Krljanac B, Hueber A, Kleyer A, Voehringer D, Schett G and Bozec A: Th2 and eosinophil responses suppress inflammatory arthritis. Nat Commun 7: 11596, 2016.

32. Tian Y, Xu L, He Y, Xu X, Li K, Ma Y, Gao Y, Wei D and Wei L: Knockdown of RAC1 and VASP gene expression inhibits breast cancer cell migration. Oncol Lett 16: 2151-2160, 2018.
33. Liu K, Li L, Nisson PE, Gruber C, Jessee J and Cohen SN: Reversible tumorigenesis induced by deficiency of vasodilator-stimulated phosphoprotein. Mol Cell Biol 19: 3696-3703, 1999.
34. Abu-Amer Y: NF-κB signaling and bone resorption. Osteoporos Int 24: 2377-2386, 2013.
35. Mussbacher M, Salzmann M, Brostjan C, Hoesel B, Schoergenhofer C, Datler H, Hohensinner P, Basílio J, Petzelbauer P, Assinger A and Schmid JA: Cell type-specific roles of NF-κB linking inflammation and thrombosis. Front Immunol 10: 85, 2019.
36. Wada T, Nakashima T, Hiroshi N and Penninger JM: RANKL-RANK signaling in osteoclastogenesis and bone disease. Trends Mol Med 12: 17-25, 2006.