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NDRG2 Expression Decreases Tumor-Induced Osteoclast Differentiation by Down-regulating ICAM1 in Breast Cancer Cells

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Abstract

Bone matrix is properly maintained by osteoclasts and osteoblasts. In the tumor microenvironment, osteoclasts are increasingly differentiated by the various ligands and cytokines secreted from the metastasized cancer cells at the bone metastasis niche. The activated osteoclasts generate osteolytic lesions. For this reason, studies focusing on the differentiation of osteoclasts are important to reduce bone destruction by tumor metastasis. The N-myc downstream-regulated gene 2 (NDRG2) has been known to contribute to the suppression of tumor growth and metastasis, but the precise role of NDRG2 in osteoclast differentiation induced by cancer cells has not been elucidated. In this study, we demonstrate that NDRG2 expression in breast cancer cells has an inhibitory effect on osteoclast differentiation. RAW 264.7 cells, which are monocytic preosteoclast cells, treated with the conditioned media (CM) of murine breast cancer cells (4T1) expressing NDRG2 are less differentiated into the multinucleated osteoclast-like cells than those treated with the CM of 4T1-WT or 4T1-mock cells. Interestingly, 4T1 cells stably expressing NDRG2 showed a decreased mRNA and protein level of intercellular adhesion molecule 1 (ICAM1), which is known to enhance osteoclast maturation. Osteoclast differentiation was also reduced by ICAM1 knockdown in 4T1 cells. In addition, blocking the interaction between soluble ICAM1 and ICAM1 receptors significantly decreased osteoclastogenesis of RAW 264.7 cells in the tumor environment. Collectively, these results suggest that the reduction of ICAM1 expression by NDRG2 in breast cancer cells decreases osteoclast differentiation, and demonstrate that excessive bone resorption could be inhibited via ICAM1 down-regulation by NDRG2 expression.

Key Words: NDRG2, ICAM1, Osteoclast, Osteolytic metastasis, Tumor environment

INTRODUCTION

Bone remodeling is usually regulated by the resorption of osteoclasts and the synthesis of osteoblasts interacting with each other. The osteoclast is a tissue-specific multinucleated cell created by the fusion of myeloid hematopoietic precursors at or near the bone surface (Boyle *et al*., 2003; Boyce, 2013). Osteoclast precursors in their normal state are attracted from the bone marrow to the bloodstream by a variety of chemokines and circulate until they are resorbed into the bone by various factors (Boyle *et al*., 2003; Weilbaecher *et al*., 2011; Boyce, 2013). However, in the tumor environment, the disseminated tumor cells are attracted to the bone matrix by the released factors, such as chemokine (C-C motif) ligand 2 (CCL2) and vascular endothelial growth factor (VEGF), from the bone stromal cells and osteoblasts. The tumor cells form a bone metastatic niche, which recruits and interacts with osteoclast precursor cells. They release proinflammatory cytokines and soluble factors including RANKL, tumor necrosis factor (TNF), matrix metalloproteinase (MMP) and interleukin-6 (IL-6). Additionally, they promote the differentiation and activation of osteoclasts. The bone matrix degraded by the activated osteoclasts secretes several factors including transforming growth factor β (TGF-β) and insulin-like growth factor (IGF) that enhance tumor cell proliferation and survival. These processes induce a "vicious cycle" and increase bone resorption at the tumor-bone interface (Boyle *et al*., 2003; Weilbaecher *et al*., 2011; Ell and Kang, 2012).

It has been reported that breast cancer cells also inhibit osteoblast differentiation and activity (Mercer *et al*., 2004; Gregory *et al*., 2013). Therefore, the imbalance between bone formation and bone resorption in the tumor environment is

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increasingly aggravated. The imbalance in bone remodeling causes skeletal diseases and osteolytic bone metastases. Approximately 80% of breast cancer patients have bone metastasis causing pain, bone fraction, hypercalcemia and nerve compression (Coleman, 2001; Weilbaecher *et al*., 2011). The inhibition of osteoclasts or the restoration of osteoblasts has been regarded as notable therapeutic targets. Among the many therapies for osteolytic bone metastasis in breast cancer, molecules expressed at the surface of the osteoclast have been investigated as novel therapeutic targets (Clezardin, 2009; Desgrosellier and Cheresh, 2010).

Previous research established that intercellular adhesion molecule 1 (ICAM1) is implicated in osteoclast development (Harada *et al*., 1998). The interaction between ICAM1 and its receptors induces a high-affinity adhesion between cells and an increase in soluble factors necessary for osteoclast differentiation (Harada *et al*., 1998; Tani-Ishii *et al*., 2002). Soluble ICAM1 (sICAM1) released from breast cancer cells is involved in osteoclast differentiation and bone metastasis of cancer cells (Ell *et al*., 2013).

ICAM1 is a highly glycosylated immunoglobulin super-family molecule expressed in a wide variety of cell types. It consists of the five Ig-like domains on the extracellular surface, a hydrophobic transmembrane region and a short cytoplasmic tail of 28 amino acids. ICAM1 has binding sites for the integrin LFA-1 ($\alpha_L\beta_2$) in domain 1 and Mac-1 ($\alpha_M\beta_2$) in domain 3 (Jun *et al*., 2001; Tsakadze *et al*., 2004). ICAM1 undergoes proteolytic cleavage by specific protease and is shed in the soluble form of ectodomain from cell surface. Other studies have shown that sICAM1 is created from specific mRNA transcripts. The expression level of sICAM1 is regulated by several cytokines and other various factors. The increase in sICAM1 expression is induced by TNF-α and interferon-γ (INF-γ) secreted from tumor cells as well as oxygen radicals and hypoxia (Whiteman *et al*., 2003; Witkowska and Borawska, 2004). In addition, ICAM1 is elevated in a variety of inflammatory diseases, such as atherosclerosis, ischemia and asthma (Vainer and Nielsen, 2000; Tang and Fiscus, 2001; Lu *et al*., 2002). However, studies about the role of ICAM1 in the interplay between the metastatic breast cancer cells and osteoclasts are still lacking.

N-myc downstream-regulated gene 2 (NDRG2) is a member of the NDRG family, which consists of 4 members (NDRG1-4) that show high level of homology, and is involved in cell proliferation, differentiation, development and stress responses. NDRG2 is highly expressed in the heart, brain, kidney, skeletal muscle, cartilage, and epidermis, but it is weakly expressed or undetectable in the several human cancer cell lines and primary tumors (Hu *et al*., 2006; Melotte *et al*., 2010). NDRG2 has been shown to have a suppressive effect on many types of cancer cells, such as breast cancer (Oh *et al*., 2012), pancreatic cancer (Yamamura *et al*., 2013), liver cancer (Hu *et al*., 2004) and colon cancer (Kim *et al*., 2009). Recently, it has been reported that NDRG2 inhibits the epithelial-mesenchymal transition (EMT) through the down-regulation of signal transducer and activator of transcription 3 (STAT3)/Snail signaling (Kim *et al*., 2014b) and attenuates metastasis and invasion via the suppression of cyclooxygenase-2 (COX-2) expression in malignant human breast cancer cells (Kim *et al*., 2014a). NDRG2 also suppresses metastasis in mouse breast cancer cells by decreasing active TGF-β (Oh *et al*., 2012). In particular, it was shown that NDRG2 expression is implicated in the regulation of the activated leukocyte cell adhesion molecule (ALCAM) in the differentiation of monocytic cells to dendritic cells (Choi *et al*., 2008). However, the exact role of NDRG2 expressed in breast cancer in osteoclast differentiation has not yet been elucidated.

In this study, we examined whether the expression of NDRG2 in breast cancer cells affects ICAM1 expression in tumor cells, and we identified the NDRG2 effect on the osteoclast differentiation in the tumor environment via the regulation of ICAM1 expression.

MATERIALS AND METHODS

Cell culture and reagents

Mouse metastatic breast cancer cells (4T1-WT, mock and NDRG2) were obtained from Prof. KD Kim at Gyeongsang National University in Jinju, Korea. 4T1 and mouse leukemic monocyte/macrophage (RAW 264.7) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with a 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco/Invitrogen), 100 U/ml of penicillin and 100 μg/ml of streptomycin (Gibco/Invitrogen) at 37°C in a humidified 5% CO_2 incubator. Recombinant murine RANKL was purchased from PeproTech, Inc (Rocky Hill, NJ, USA). Mouse anti-ICAM1 antibody was obtained from Abcam (Cambridge, UK) and mouse anti-β2 antibody was purchased from BD Biosciences (San Jose, CA, USA).

Knockdown of ICAM1 by small interfering RNA (siRNA)

Murine control-siRNA and ICAM1-siRNA were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). The control- and ICAM1-siRNAs were transfected into 4T1 cells using Lipofectamin™ RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Preparation of the tumor conditioned media (CM)

4T1-WT, 4T1-mock and 4T1-NDRG2 cells were cultured for 48 h; then, the culture media were harvested to use for the osteoclast differentiation. The harvested culture media were passed through a 0.2 μm syringe filter (Sartorius, Goettingen, Germany), and then, concentrated using an Amicon® Ultra-15 centrifugal filter 3K devices (Merck Millipore, Billerica, MA, USA). The concentrates of the cultured media were diluted with minimum essential media (α-MEM; Gibco/Invitrogen) supplemented with 5% FBS (Gibco/Invitrogen) to produce 30% concentrated media (CM; conditioned media).

Osteoclast differentiation

RAW 264.7 cells were seeded in a 96-well plate at a density of 4,000 cells/well and in a 24-well plate at a density of 2×10^4 cells/well. After one day, the media were changed in α-MEM supplemented with 5% FBS (control) and 4T1-CM with 100 ng/ml RANKL. The cells were differentiated into osteoclasts for 4 days, and the media and recombinant protein were replenished daily.

Tartrate-resistant acid phosphatase (TRAP) staining

TRAP staining was performed using an acid phosphatase leukocyte kit (Sigma-Aldrich, St. Louis, MO, USA). The cells were washed using Dulbecco's phosphate-buffered saline (DPBS; WELGENE, Daegu, Korea) and fixed in 4% paraformaldehyde (Sigma-Aldrich) for 10 min at room temperature.

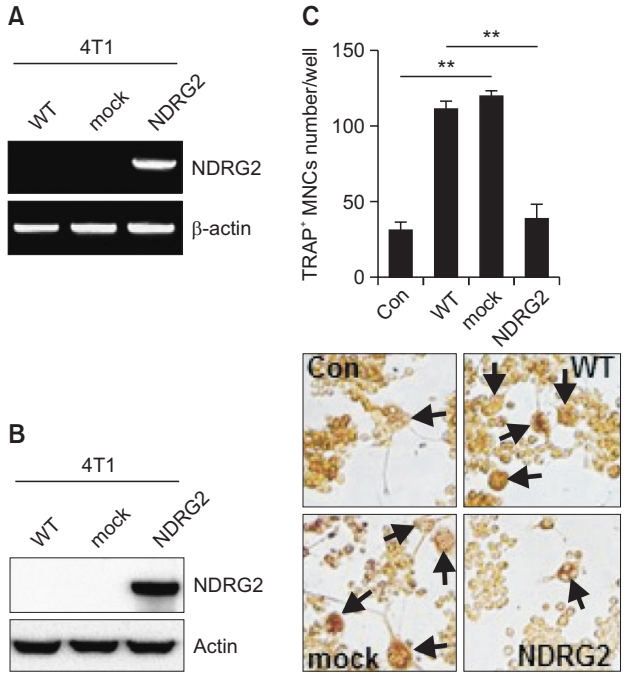

Fig. 1. Conditioned media of 4T1 cells overexpressing NDRG2 demonstrate a reduced activity for osteoclast differentiation. (A, B) 4T1 cells were stably transfected with the empty vector (mock) and NDRG2 cDNA. The NDRG2 mRNA level was measured by RT-PCR (A). The expression level of NDRG2 protein was confirmed by Western blot analysis (B). (C) RAW 264.7 cells were seeded in a 96-well plate (4×10^3). After one day, the media were changed to α-MEM (Control) and the CM of 4T1-WT, 4T1-mock and 4T1-NDRG2 cells in the presence of RANKL (100 ng/ml). RAW 264.7 cells were differentiated into osteoclasts for 4 days, and then the cells were stained using the TRAP assay kit. The media and RANKL (100 ng/ml) were changed daily. The graphs indicate the average number of TRAP-positive multinucleated cells showing three or more nuclei per well. The images were captured in representative staining under a microscope (16×) (**p<0.01).

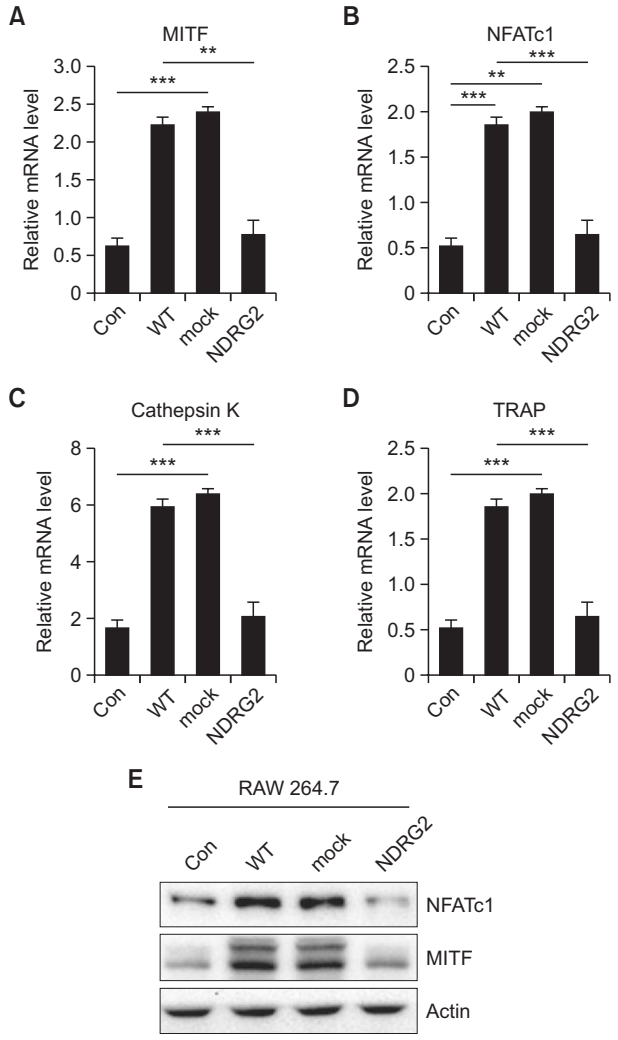

Fig. 2. Expression of osteoclast differentiation markers in RAW 264.7 cells decreases after culture with the conditioned media of 4T1 cells overexpressing NDRG2. (A-D) RAW 264.7 cells were seeded into 6-well plate (2×10^5) and differentiated in α-MEM (Control) and the CM of 4T1, 4T1-mock and 4T1-NDRG2 cells in the presence of RANKL (100 ng/ml) for 4 days. The media and RANKL were changed daily. The mRNA expression levels of Cathepsin K, MITF, NFATc1 and TRAP in RAW 264.7 cells were confirmed by quantitative real time PCR. The protein expression of NFATc1 and MITF was measured by Western blot analysis (**p<0.01, ***p<0.001).

After washing, the cells were stained with a TRAP solution for 30 min at 37°C. The images were captured by Olympus fluorescence microscope (Model IX71 with fluorescence system, Olympus Corp., Tokyo, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time PCR

Total RNA from harvested cells was isolated using the TRI reagent (Molecular Research Center Inc, Cincinnati, OH, USA). To synthesize cDNA, 5 μg of total RNA was first incubated with an oligo-dT primer at 70°C to denature the secondary structure of the RNA and then incubated at room temperature for 10 min to allow the annealing of the primer. Then, dNTPs (Bioneer, Daejeon, Korea), M-MLV reverse transcriptase (Promega, Madison, WI, USA) and RT buffer (Promega) were added to the reaction. The mixture was incubated for 3 h at 37°C and then boiled at 100°C for 3 min. β-actin sequences were amplified for 22 cycles to use as loading control. Other sequences were amplified by PCR for 35-40 cycles in a 20 μl reaction mixture containing cDNA, 10 pmol of each primer, 10 mM dNTP and 0.5 U of Top DNA polymerase. The PCR primers and reagents were purchased from Bioneer. The PCR products were electrophoresed on 1% agarose gels containing ethidium bromide. Quantitative real time PCR was performed using an ABI StepOnePlus™ real time PCR thermal cycler with Power SYBR Green PCR Master Mix according to the manufacturer's protocol (Life Technology, Carlsbad, CA, USA). The target mRNA levels were normalized to cyclophilin. The experiments were performed in triplicate.

Western blot analysis

The cells were washed using DPBS, and the total protein was isolated by a protein extraction solution (iNtRON Biotechnology, Seongnam, Korea). The supernatant fractions were obtained by centrifugation at 12,000 rpm for 15 min at 4°C.

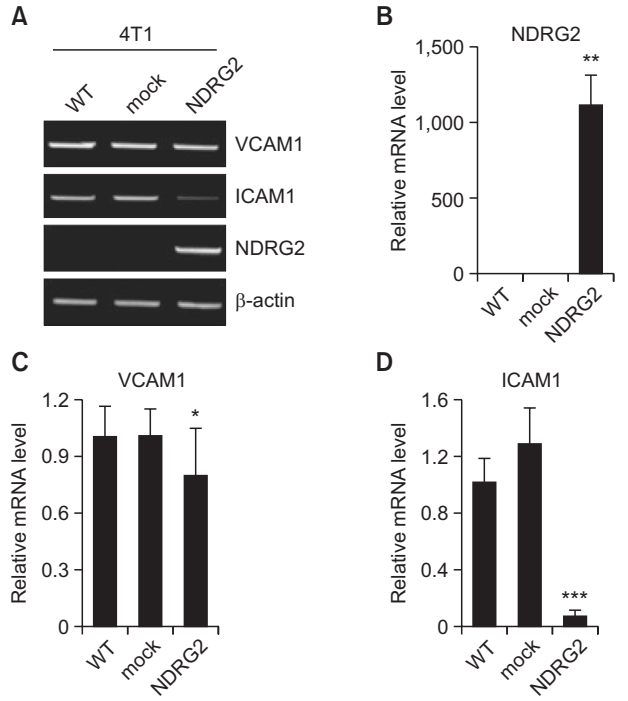

Fig. 3. NDRG2 overexpression modulates the mRNA level of cell adhesion molecules in 4T1 cells. (A-D) 4T1 cells were stably transfected with the empty vector (mock) and NDRG2 cDNA. The mRNA expression level of NDRG2, VCAM1 and ICAM1 in the 4T1-WT, 4T1-mock and 4T1-NDRG2 cells was determined by RT-PCR (A) and quantitative real time PCR (B-D) (**p*<0.05, ***p*<0.01, ****p*<0.001).

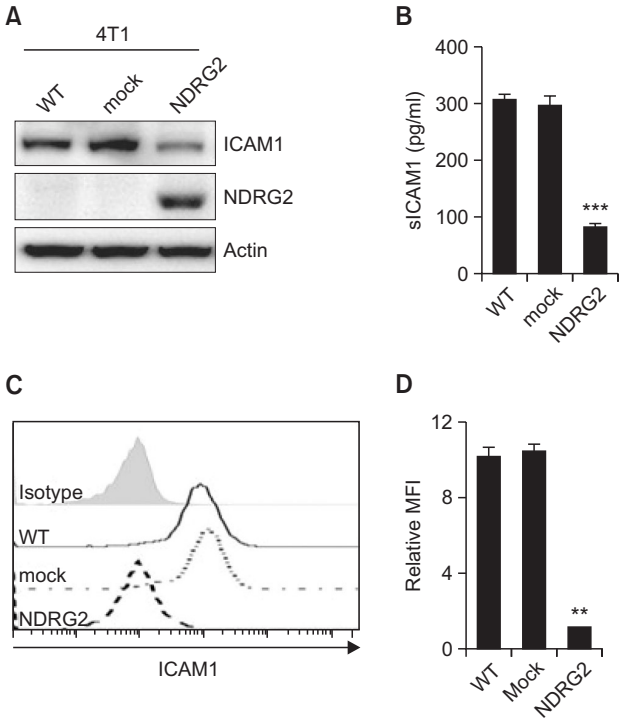

Fig. 4. ICAM1 protein level is reduced by NDRG2 overexpression in 4T1 cells. (A) Total ICAM1 protein levels in 4T1-WT, 4T1-mock and 4T1-NDRG2 were measured by Western blot analysis. (B) The 4T1-WT, 4T1-mock, 4T1-NDRG2 cells were cultured for 48 h, and then soluble ICAM1 (sICAM1) levels in the supernatants were determined by ELISA. (C, D) The 4T1-WT, 4T1-mock and 4T1-NDRG2 cells were stained with specific antibodies against ICAM1 for measuring the membrane-bound form (mICAM1), and then the expression level was detected by flow cytometry. The histograms represent the expression of mICAM1 in 4T1 cells. The graphs represent an average of the relative mean fluorescence intensity (MFI) (***p*<0.01, ****p*<0.001).

The protein was mixed with a 5X sample buffer and separated on 9% or 12% SDS-polyacrylamide gel. Then, gel was transferred to a PVDF blotting membrane (GE healthcare Life Science, Wauwatosa, WI, USA), and the membrane was blocked with Tris-buffered saline plus 0.1% Tween-20 (TBS-T) containing 5% skim milk (BD Biosciences) or BSA (Gibco). The membrane was incubated with specific antibodies overnight at 4°C and washed by TBS-T. The antibodies against actin, α-actinin, NDRG2 and ICAM1 were purchased from Santa Cruz Biotechnology and the antibodies against NFATc1, MITF, STAT3, p-STAT3, JNK, p-JNK, ERK, p-ERK, p38, p-p38, AKT and p-AKT were obtained from Cell Signaling (Danvers, MA, USA). After attaching the secondary antibodies coupled to horseradish peroxidase, the blots of the membrane were visualized with an EZ-Western Lumi Plus solution (ATTO Corporation, Tokyo, Japan), pico EPD solution (ELPIS-Biotech., Inc, Daejeon, Korea) and Ez-Capture MG (ATTO Corporation).

Enzyme-linked immunosorbent assay (ELISA)

After the 4T1-WT, 4T1-mock and 4T1-NDRG2 cells were cultured for 48 h, the level of soluble ICAM1 (sICAM1) in the supernatant was measured by the mouse sICAM1/CD54 immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The absorbance at 450 nm was quantified with a VICTOR^3 1420 multi-label counter (PerkinElmer, Waltham, MA, USA).

Flow cytometry

The cells were stained with a PE-conjugated mouse anti-ICAM1 antibody (eBioscience, San Diego, CA, USA) or a control IgG_1-PE (eBioscience) at 4°C in DPBS for 30 min to observe the surface antigen expression. After washing the non-binding antibodies twice using DPBS, the cells were detected by a FACSCanto™II flow cytometry (BD Biosciences). The imaging graphs and the mean fluorescence intensity (MFI) were analyzed by the FlowJo program (Ashland, OR, USA).

Statistical analysis

Student's *t*-test and one-way ANOVA followed by Bonferroni test were used for statistical analysis. Values are represented as the mean ± SD. A value of $p<0.05$ was considered significant.

RESULTS

Osteoclast differentiation is reduced in the conditioned media of 4T1 cells overexpressing NDRG2

To examine effects of NDRG2 on the osteoclast differentia-

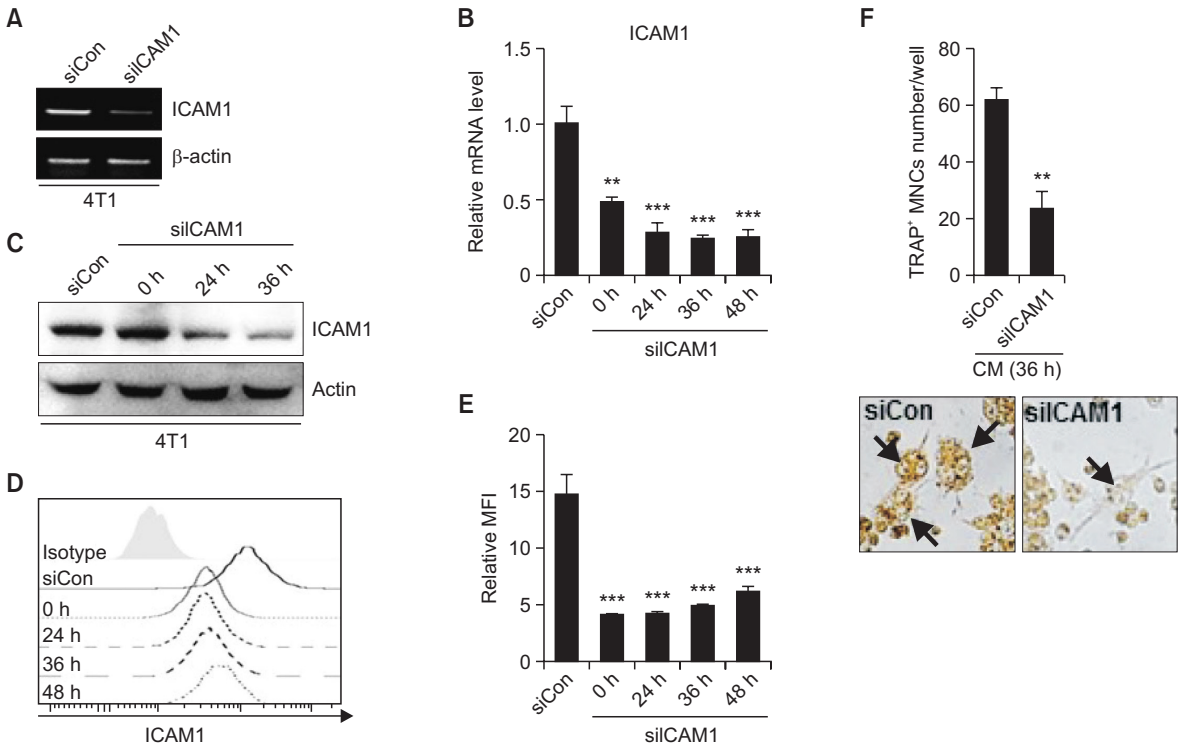

Fig. 5. ICAM1 knockdown in 4T1 cells decreases osteoclast differentiation. (A-E) 4T1 cells were transfected with control- and ICAM1-siRNA for 48 h. The cells were washed out and cultured for 48 h in fresh media. After transfection, ICAM1 mRNA levels were confirmed by RT-PCR (A). The ICAM expression at the indicated time after changing the media was quantified by quantitative real time PCR (B), Western blot analysis (C) and flow cytometry (D, E). The histograms represent the expression of mICAM1 in 4T1 cells (D). The graphs indicate an average of the relative mean fluorescence intensity (MFI) (E). (F) RAW 264.7 cells were seeded in a 96-well plate (4×10^3) and differentiated into osteoclasts in the CM of the control-siRNA and ICAM1-siRNA transfected 4T1 cells. After 4 days, the cells were stained using the TRAP assay kit. The graphs represent the average number of TRAP-positive multinucleated cells showing three or more nuclei per well. The images were captured in representative staining under a microscope (16×) (**p<0.01, ***p<0.001).

tion by metastatic breast cancer cells, 4T1 cells were transfected with an empty vector or NDRG2 cDNA. Expression levels of the NDRG2 mRNA and protein were confirmed by RT-PCR and Western blot analysis, respectively (Fig. 1A, B). The culture media of 4T1-WT, 4T1-mock and 4T1-NDRG2 cells were harvested and used to make tumor conditioned media (CM). RAW 264.7 cells, a preosteoclast cell line, were differentiated into osteoclasts in the CM to identify how NDRG2 affects osteoclast maturation. TRAP-positive multinucleated cells containing three or more nuclei were measured using the TRAP staining. As shown in Fig. 1C, RAW 264.7 cells were differentiated into osteoclasts to a greater extent in the CM of the 4T1-WT and 4T1-mock than in the control without the tumor culture medium. Osteoclast differentiation was reduced in the CM of 4T1-NDRG2 cells compared with that in the CM of 4T1-WT and 4T1-mock cells (Fig. 1C). These results suggest that the CM of the 4T1-NDRG2 cells can reduce osteoclast differentiation, likely via modulation of the soluble factor expression or secretion.

Expression level of genes related with osteoclast differentiation of RAW 264.7 cells is reduced in the conditioned media of 4T1 cells overexpressing NDRG2

We raised the question of whether the CM of the 4T1-NDRG2 cells can regulate the expression of genes related with osteoclastogenesis, because osteoclast maturation was decreased in the CM of 4T1-NDRG2 in previous results. As shown in Fig. 2A, B, E, the mRNA and protein expression of microphthalmia-associated transcription factor (MITF) and nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), which are known as essential transcription factors for the osteoclast development, was significantly increased in RAW 264.7 cells after the treatment with the CM of 4T1-WT and 4T1-mock cells. Interestingly, we confirmed that MITF and NFATc1 expression was not increased after culture with the CM of 4T1-NDRG2 cells. mRNA levels of cathepsin K and TRAP as mature osteoclast markers were also enhanced in RAW 264.7 cells cultured with the CM of 4T1-WT and 4T1-mock cells, whereas they were little affected after the culture with the CM of the 4T1-NDRG2 cells (Fig. 2C, D). These findings suggest that NDRG2-overexpressing 4T1 cells lose the ability to induce the osteoclast maturation likely by modulating the expression of some factors related with differentiation.

Expression of cell adhesion molecules in 4T1 cells is regulated by NDRG2

Because ICAM1 and VCAM1, among various cell adhesion molecules, are known to be involved in osteoclast differentiation (Fernandes *et al*., 2008; Lu *et al*., 2011), we tried to discover whether the mRNA expression of ICAM1 and VCAM1 in 4T1 cells can be regulated by NDRG2. ICAM1 and VCAM1 mRNA levels decreased in 4T1-NDRG2 cells (Fig. 3A-D). The

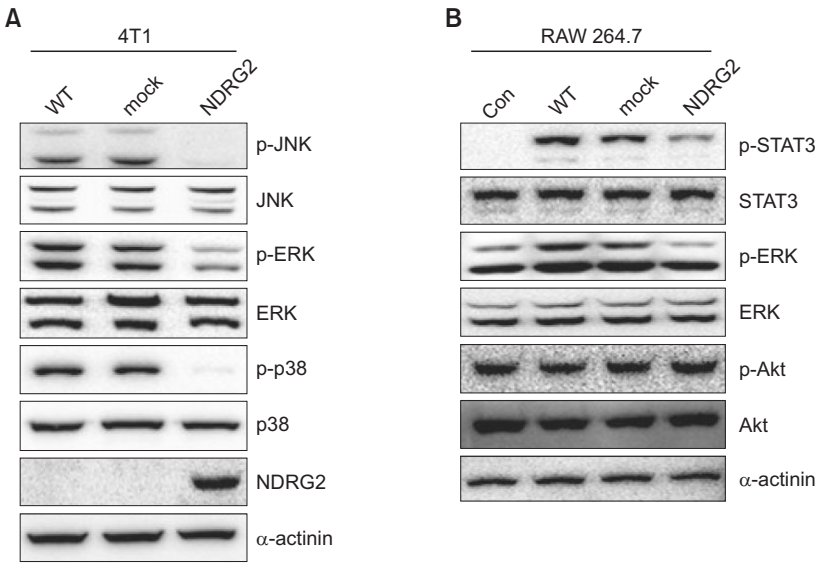

Fig. 6. MAPK signaling is inhibited in 4T1-NDRG2 cells and β2 integrin downstream signaling is decreased in RAW 264.7 cells treated with CM of 4T1-NDRG2 cells. (A) 4T1 cells were stably transfected with the empty vector (mock) and NDRG2 cDNA. Expression of p-JNK, JNK, p-ERK, ERK, p-p38, p38 and NDRG2 proteins was confirmed by Western blot analysis. (B) RAW 264.7 cells were treated with the CM of 4T1-WT, 4T1-mock and 4T1-NDRG2 cells for indicated time periods. The expression of p-STAT3, STAT3, p-ERK, ERK, p-AKT and AKT protein was detected by Western blot analysis.

decrease in the ICAM1 mRNA was more significant than that in the VCAM1 mRNA, suggesting that the ICAM1 level was more strongly affected by the NDRG2 expression than VCAM1, and the decreased ICAM1 level was probably correlated with the low level of osteoclastogenesis in RAW 264.7 cells.

ICAM1 expression is lower in the breast cancer cells overexpressing NDRG2

It has been well known that ICAM1 is present in both the membrane-bound form (mICAM1) of cells and soluble form. To investigate whether NDRG2 expression affects the level of ICAM1 protein, mICAM1 and sICAM1 were analyzed. As shown in Fig. 4A, ICAM1 expression in the total protein was inhibited in the 4T1-NDRG2 cells. sICAM1 (Fig. 4B) and mICAM1 expression (Fig. 4C, D) was significantly decreased in the 4T1-NDRG2 cells. These results support the notion that ICAM1 expression is negatively regulated by NDRG2, and osteoclast differentiation can be decreased by the down-regulation of ICAM1 level in the 4T1 cells.

siRNA-mediated knockdown of ICAM1 in 4T1 cells inhibits osteoclast differentiation

To examine whether the ICAM1 affects osteoclastogenesis, we also investigated the change in osteoclast differentiation by ICAM1 down-regulation. ICAM1 expression was knocked down using siRNA (Fig. 5A), and then, the media were exchanged for fresh media. As a result, ICAM1 mRNA expression decreased, and the inhibition was maintained for 36 h (Fig. 5B). Similarly, it was shown that total ICAM1 and mICAM1 were inhibited for 36 h (Fig. 5C-E). To identify whether osteoclast development could be regulated in the CM containing a low level of ICAM1, similar to the CM of the 4T1-NDRG2 cells, RAW 264.7 cells were cultured in the CM of 4T1 cells transfected with siRNA. As a result, RAW 264.7 cells were significantly less differentiated into osteoclasts in the CM from the ICAM1-silenced 4T1 cells in comparison with the CM from the control-siRNA transfected 4T1 cells (Fig. 5F). These results demonstrate that ICAM1 in 4T1 cells is a crucial stimulating factor for osteoclastogenesis.

MAPK signaling and β2 integrin downstream signaling are decreased in the NDRG2-overexpressing 4T1 cells and RAW 264.7 cells treated with CM from the same cells, respectively

Previous reports have shown that the protein kinase C (PKC), mitogen-activated protein kinases (MAPK) and NF-κB signaling primarily regulate ICAM1 expression (Roebuck and Finnegan, 1999) and NDRG2 modulates MAPK signaling (Liu *et al*., 2010). To identify how ICAM1 is regulated by NDRG2 expression in 4T1 cells, the MAPK (JNK, ERK, p38) expression was investigated using Western blot analysis. As shown in Fig. 6A, the phosphorylation of JNK, ERK and p38 was significantly suppressed by NDRG2 expression in 4T1 cells. These results demonstrated that the ICAM1 level could be inhibited by NDRG2 via the down-regulation of MAPK protein expression. In addition, we investigated whether the downstream signals of the β2 integrin, the ICAM1 receptor subunit, are regulated in RAW 264.7 cells treated with the CM of 4T1. Furthermore, β2 integrin engagement induces the phosphorylation of ERK in leukocytes and triggers STAT3 activation (Dib, 2000; Verma and Kelleher, 2014). As expected, phosphorylation of STAT3 and ERK increased after treatment with the CM of the 4T1-WT and 4T1-mock cells relative to that in the absence of CM (Fig. 6B). However, activation of STAT3 and ERK was markedly decreased in the CM of 4T1-NDRG2 cells compared with those in the CM of 4T1-WT and 4T1-mock cells. AKT activation was not significantly altered in the cells in the presence or absence of CM. These results suggest that ICAM1 down-regulated by NDRG2 less activated β2 integrin signaling in RAW 264.7 cells and osteoclast differentiation

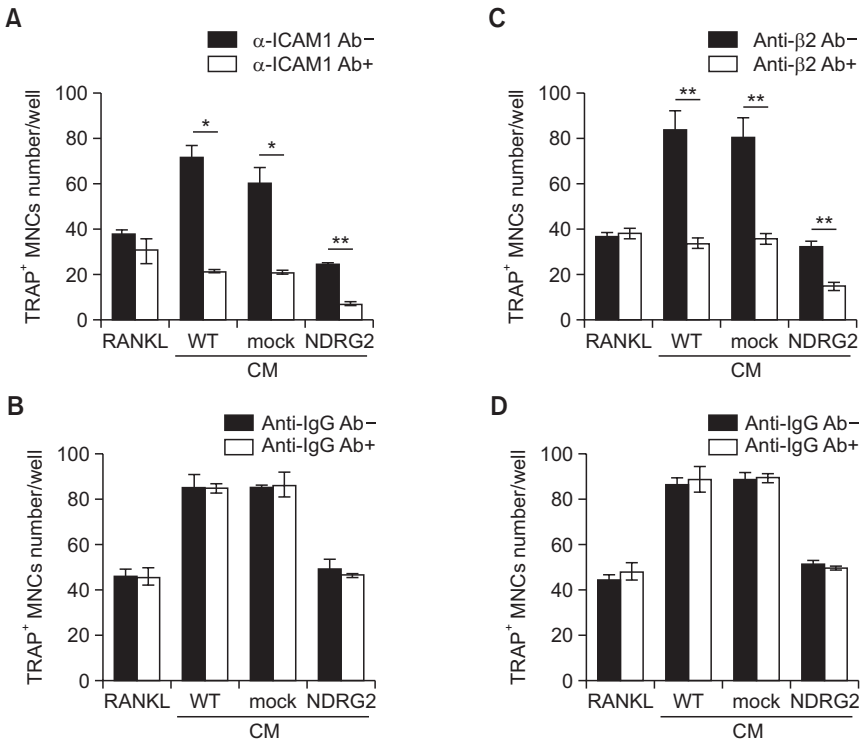

Fig. 7. Blocking of ICAM1 derived from the tumor-conditioned media reduces osteoclast differentiation. (A, B) The concentrated culture media from 4T1-WT, 4T1-mock and 4T1-NDRG2 cells were pre-treated with anti-ICAM1 antibody (10 μg/ml) for 1 h at room temperature (A) or pre-treated with anti-IgG antibody (10 μg/ml) for 1 h at room temperature (B). The concentrated media were diluted to 30% with α-MEM. RAW 264.7 cells were seeded in a 96-well plate (4×10^3). After one day, the media were changed to α-MEM with RANKL (100 ng/ml) or pre-treated CM with or without the antibody. (C, D) RAW 264.7 cells were seeded in a 96-well plate (4×10^3). After one day, the media were changed to α-MEM with RANKL (100 ng/ml) or CM of 4T1-WT, 4T1-mock and 4T1-NDRG2 cells with or without the anti-β2 antibody (10 μg/ml) (C). RAW 264.7 cells were seeded in a 96-well plate (4×10^3). After one day, the media were changed to α-MEM with RANKL (100 ng/ml) or the CM of 4T1-WT, 4T1-mock and 4T1-NDRG2 cells with or without the anti-IgG antibody (10 μg/ml) (D). The culture media were added or changed daily. RAW 264.7 cells were differentiated into osteoclasts for 4 days, and the cells were stained using the TRAP assay kit. The graphs represent the average number of TRAP-positive multinucleated cells counted showing three or more nuclei per well (*p<0.05, **p<0.01).

was, therefore, reduced due to the decreased signaling of the β2 downstream pathway.

Inhibition of binding between ICAM1 and receptors suppresses osteoclastogenesis in the tumor environment

Previous research has demonstrated that osteoclast-like cell formation is modulated by the LFA-1/ICAM1 interaction (Kurachi *et al*., 1993; Harada *et al*., 1998). In addition, osteoclast differentiation and activity are suppressed by the blocking of the ICAM1 interaction (Fernandes *et al*., 2008; Suzuki *et al*., 2011; Ell *et al*., 2013). To identify whether the interaction between ICAM1 and its receptors directly regulates osteoclast differentiation in the tumor environment, osteoclastogenesis was investigated using blocking antibodies. As shown in Fig. 7A, C, osteoclast differentiation in the presence of RANKL was not inhibited by both the anti-ICAM1 antibody and the anti-β2 antibody, showing the ICAM-1-independent osteoclast differentiation by RANKL. To investigate the effect of the ICAM1 blocking on the osteoclastogenesis in the tumor environment, RAW 264.7 cells were cultured in the presence of the neutralizing antibody without RANKL. Osteoclast development was stimulated in the CM of 4T1-WT and 4T1-mock in the absence of additional RANKL. This result suggests that the CM of the 4T1 cells may contain various factors that enhance osteoclast maturation including sICAM1. Osteoclast maturation was almost completely inhibited by the neutralization of ICAM1 in the CM of 4T1-WT and 4T1-mock cells compared to that in the presence of the anti-IgG control antibody and it was similar to the osteoclast development in the absence of CM (Fig. 7A, B). Moreover, osteoclastogenesis inhibition by the anti-ICAM1 antibody was also noticeable in the CM of the 4T1-NDRG2 cells (Fig. 7A), suggesting that sICAM still remained in the CM of 4T1-NDRG2 cells. Additionally, sICAM1 interacts with LFA-1 (αLβ2) and Mac-1 (αMβ2) (Jun *et al*., 2001). LFA-1 and Mac-1 are expressed on preosteoclast cells, suggesting a crucial role in osteoclastogenesis (Smith, 2008). Thus, the anti-β2 antibody was used to block the interaction between ICAM1 and its receptors. As shown in Fig. 7C, D, osteoclast differentiation was decreased by the anti-β2 antibody in the CM of 4T1-WT and 4T1-mock, whereas the control antibody did not affect it. Osteoclast development was again significantly inhibited by blocking β2 receptors in the CM of the 4T1-NDRG2 cells compared to that in a normal maturation state, indicating a potent inhibition of ICAM-mediated β2 receptor stimulation by anti-β2 antibody. Therefore, these results suggest that the neutralization of ICAM1 or the blocking of ICAM1 receptors in tumor

environment can inhibit osteoclast differentiation.

DISCUSSION

Cell adhesion molecules (CAM) including the epithelial cell adhesion molecule (EpCAM), VCAM1, ALCAM and ICAM1 have been implicated with clinical significance and prognostic factors in patients with several cancers, such as breast, ovarian and lung cancer (Karabulut *et al*., 2014; Kotteas *et al*., 2014; Lee, 2014). In particular, the sICAM1 level in serum of the lung and breast cancer patients is correlated to the cancer stage and its aggressiveness degree (Schroder *et al*., 2011; Thielemann *et al*., 2014). It was reported that ICAM1 is highly expressed in triple negative breast cancers (TNBC) cells compared to the non-TNBC and normal cells (Guo *et al*., 2014). In addition, ICAM1 plays an inducer role in tumor cell metastases and invasion in a variety of cancers. For example, the down-regulation of ICAM1 in human metastatic breast cancer cells (MDA-MB-435) strongly suppresses invasion through the Matrigel matrix (Rosette *et al*., 2005). Treatment of anti-ICAM1 antibody significantly reduces the invasion and migration of human TNBC cells (Rosette *et al*., 2005; Guo *et al*., 2014). The increase of ICAM1 expression by TGF-α promotes cell migration in osteosarcoma cells (Hou et al., 2014). Highly metastatic murine breast cancer cells (4T1E/M3) strongly attach to the plastic plate and bone marrow-derived endothelial cells. The 4T1E/M3 cells show remarkable bone marrow metastases in vivo and express a high level of ICAM1. The migration activity of the cells is inhibited by the anti-ICAM antibody (Takahashi *et al*., 2008). In fact, the current experiment and our previous work demonstrate that the metastatic 4T1 cells (Fig. 3, 4) and malignant human breast cancer cells (MDA-MB-231) strongly express ICAM1 (Kim *et al*., 2014b). It is conceivable that ICAM1 will provide useful information and become a critical marker for patients, and it can be used for clinical application in patients with metastatic and malignant cancer. The inhibition of ICAM1 expression provides the possibility of attenuating the metastasis, aggressiveness and malignancy of cancer cells. Therefore, it is certainly important to understand how ICAM1 expression could be inhibited by specific factors and how the down-regulation of ICAM1 affects the process of metastasis.

It has been suggested that ICAM1 could be related to the bone metastasis of breast cancer because ICAM1 signal stimulates osteoclast differentiation more efficiently (Ell *et al*., 2013). In fact, the CM of 4T1 cells that express high level of sICAM1 more efficiently induced TRAP-positive multinucleated osteoclast-like cells than ordinary maturation environment without CM (Fig. 1C). However, treatment of the CM derived from the 4T1-NDRG2 cells that exhibit low levels of sICAM1 did not induce the enhancement of osteoclast differentiation. These results support the notion that NDRG2 might have an inhibitory effect on osteoclast differentiation through the down-regulation of sICAM1 expression. In fact, the level of decreased osteoclastogenesis in the CM of the 4T1-NDRG2 cells was similar to the level of differentiation in the CM of 4T1-WT and 4T1-mock in the presence of blocking antibodies (Fig. 6). It is assumed that the sICAM1 stimulation inhibited by the anti-ICAM1 antibody, anti-β2 antibody or NDRG2 expression suppresses the downstream pathway of the β2 integrin in RAW 264.7 cells. It has been demonstrated that signaling through the stimulation of β2 integrin activates the NF-κB pathway in neutrophils (Kettritz *et al*., 2004; Kim *et al*., 2004). Therefore, the gene expression necessary for a mature osteoclast might be inhibited by blocking the signal through β2 integrin, such as NF-κB signaling, and ultimately, osteoclast development would decline in RAW 264.7 cells.

Previous research has indicated that the β2 subunit is composed of four types of integrins (αLβ2, αMβ2, αXβ2 and αDβ2). It was reported that an analysis of RAW 264.7 cells shows strong expression of the αM and β2 subunits but a lower expression of the αL subunit (Ell *et al*., 2013). Because RAW 264.7 cells often express Mac-1 only, the anti-β2 antibody used in this study is sufficient in blocking ICAM1 receptors. For this reason, it is assumed that the blocking of ICAM1 signaling using the anti-β2 and anti-ICAM1 antibodies during osteoclastogenesis shows similar results. The anti-β2 antibody might be inappropriate to target osteoclasts specifically in clinical trials or in vivo experiments because different types of cells containing other subunits may exist in the body. Taken together, it is evident that ICAM1 plays an important role during osteoclast maturation. Nevertheless, many experiments are required to know whether ICAM1 is able to exclusively induce osteoclast differentiation and whether osteoclast differentiation is dependent on sICAM1 concentration in the CM. Furthermore, the mechanism by which ICAM1 is regulated by NDRG2 expression in 4T1 cells should be investigated. Previous studies have demonstrated that nuclear transcription factors for ICAM1 expression are AP-1, NF-κB, C/EBP, Ets, STAT and Sp1 (Roebuck and Finnegan, 1999). In particular, it has been reported that the release of sICAM1 is regulated by the MAPK pathway, NF-κB activation and MMP-9 in osteoblast-like cells (Tsai et al., 2014). Therefore, NDRG2 may decrease related signaling and some factors including NF-κB, STAT and AP-1 that induce ICAM1 expression in 4T1 cells. In fact, it was confirmed in this study that MAPK protein expression is suppressed by NDRG2. Thus, sICAM1 inhibited by NDRG2 expression in cancer cells would lead to the reduction in the osteoclast differentiation. These findings suggest that NDRG2 indirectly inhibits osteoclastogenesis through the down-regulation of sICAM1 secretion from cancer cells.

In summary, we identified that NDRG2 overexpression in breast cancer cells affects the expression and secretion of ICAM1 occurring in the tumor microenvironment. Consequentially, osteoclast differentiation is reduced by the down-regulation of ICAM1 in metastatic breast cancer cells overexpressing NDRG2. This study is the first attempt to elucidate how NDRG2 expression in metastatic breast cancer affects osteoclast differentiation. These findings strongly suggest that NDRG2 could have inhibitory effects on bone metastasis in breast cancer and could be a novel therapeutic target of excessive osteoclast-mediated bone resorption.

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