Mutation in Osteoactivin Promotes Receptor Activator of NFκB Ligand (RANKL)-mediated Osteoclast Differentiation and Survival but Inhibits Osteoclast Function*

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Background: The importance of osteoactivin has emerged from its role in osteogenesis.

Results: Loss-of-function mutation of osteoactivin stimulates osteoclast differentiation and survival, with a defect in bone resorption.

Conclusion: Osteoactivin acts as a negative regulator of osteoclastogenesis and a positive regulator of osteoclast function.

Significance: This study helps to identify the possible mechanisms and targets of osteoactivin during osteoclast differentiation with impact on bone remodeling.

We previously reported on the importance of osteoactivin (OA/Gpnmb) in osteogenesis. In this study, we examined the role of OA in osteoclastogenesis, using mice with a nonsense mutation in the Gpnmb gene (D2J) and wild-type controls (D2J/Gpnmb⁺). In these D2J mice, micro-computed tomography and histomorphometric analyses revealed increased cortical thickness, whereas total porosity and eroded surface were significantly reduced in D2J mice compared with wild-type controls, and these results were corroborated by lower serum levels of CTX-1. Contrary to these observations and counterintuitively, temporal gene expression analyses supported up-regulated osteoclastogenesis in D2J mice and increased osteoclast differentiation rates ex vivo, marked by increased number and size. The finding that MAPK was activated in early differentiating and mature D2J osteoclasts and that survival of D2J osteoclasts was enhanced and mediated by activation of the AKT-GSK3β pathway supports this observation. Furthermore, this was abrogated by the addition of recombinant OA to cultures, which restored osteoclastogenesis to wild-type levels. Moreover, mix and match co-cultures demonstrated an induction of osteoclastogenesis in D2J osteoblasts co-cultured with osteoclasts of D2J or wild-type. Last, in functional osteo-assays, we show that bone resorption activity of D2J osteoclasts is dramatically reduced, and these osteoclasts present an abnormal ruffled border over the bone surface. Collectively, these data support a model whereby OA/Gpnmb acts as a negative regulator of osteoclast differentiation and survival but not function by inhibiting the ERK/AKT signaling pathways.

Osteoclasts are large multinucleated cells, derived from hematopoietic/monocytic lineage and are specialized in bone resorption (1–3). Osteoclasts are involved in bone remodeling, which is a physiological process regulated by the tight coupling between bone-resorbing osteoclasts and the primary osteogenic cells, osteoblasts (4, 5). Macrophage colony-stimulating factor (MCSF)3 and receptor activator of NFκB ligand (RANKL) are among the major cytokines released by osteoblasts that initiate osteoclast differentiation and stimulate their bone resorptive function (3, 6–8). The use of genetically engineered mouse strains has elucidated the importance of several transcription factors in the regulation of osteoclastogenesis; null mutations of PU.1, c-FOS, and NFκB p50/p52 have all resulted in an increased skeletal mass due to failure of bone resorption (9–12). Osteopetrosis is a congenital skeletal disorder in which excessive bone accumulates and ablates the formation of bone marrow space (13). This condition is generally the result of impaired osteoclast differentiation and/or function and occurs in many species, including humans (13). Several different osteopetrotic mutants have been described. For example, the toothless (t/l/tl) rat possesses a genetic mutation, characterized by failure of tooth eruption and moderate skeletal sclerosis (14). Another model of osteopetrosis is the incisor absent (ia/ia) rat, characterized by increased bone mass with fair reduction of bone marrow cavities (15). The osteopetrotic

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3 The abbreviations used are: MCSF, macrophage colony-stimulating factor; BM, bone marrow; OCP, osteoclast precursors; Oc, osteoclasts; RANK, receptor activator of NFκB; RANKL, RANK ligand; μCT, micro-computed tomography; OA, osteoactivin; rOA, recombinant OA; MMA, methylmethacrylate resin; PGE2, prostaglandin E2; TRAP, tartrate resistant acid phosphatase; OPG, osteoprotegerin.
(op/op) rat model represents the most severe osteopetrotic phenotype due to the absence of marrow cavities in long bones (16). All types of osteopetrosis resulted from heterogeneous mutations assigned to a single locus on the proximal end of rat chromosome 10 with homology to the proximal end of chromosome 16 in the human genome (17). Such mutations negatively regulate osteoclast and macrophage maturation, morphology, and their function, resulting in osteopetrosis with few osteoclasts in vivo and failure of osteoclast precursors (OCP) to differentiate into osteoclasts ex vivo (17). It was also reported that bone resorption can be restored in ia/ia and op/op rats following bone marrow transplantation in which the donor cell population contains normal osteoclast progenitors (15, 18).

Our group previously identified osteoactivin (OA/Gpnmb) as a novel osteogenesis gene in (op/op) rat long bones and calvaria (19, 20) and also in (tltl) and (ia/ia).4 The Gpnmb gene has high homology to Pmel-17 and qnr-71 genes that are important in melanocyte differentiation and melanosome formation (21, 22). The OA/Gpnmb protein is a type I trans-membrane glycoprotein of 562 amino acids that has been assigned several names in other species, such as DC-HIL (dendritic cell heparan sulfate proteoglycan integrin-dependent ligand) in mouse dendritic cells (23), GPNMB (glycoprotein nm) in human melanoma cell lines and melanocytes (24), and HGFIN (hematopoietic growth factor-inducible neurokinin) in human tumor cells (25). The importance of OA/Gpnmb in osteogenesis emerged from several of our previous reports in which we tested the effects of OA/Gpnmb, using several approaches, on mesenchymal stem cells and osteoblast differentiation and function (26–28). For example, exogenous OA/Gpnmb markedly enhanced osteoblast differentiation and matrix mineralization (29, 30). Furthermore, we reported on the increased expression of OA/Gpnmb not only into osteoblasts but also into osteoclasts during fracture healing (31). Other reports demonstrated the temporal expression of OA/Gpnmb during osteoclastogenesis (32). In this report, we examine the role of OA/Gpnmb in osteoclastogenesis using the previously described DBA/2J (D2J) mouse, a strain characterized by a nonsense mutation of the Gpnmb gene in all cell types, including osteoblasts and osteoclasts (33). Mesenchymal stem cells and osteoclasts isolated from D2J mice failed to differentiate and mineralize matrix ex vivo due to autonomous defect in cell proliferation (29). Moreover, bone mass in D2J mice was significantly reduced and remained relatively constant with age, which may indicate a decrease in bone remodeling capability (34). In this study, the mutant D2J and the wild-type D2J/Gpnmb+ mice were used to gain a thorough insight into the role of OA/Gpnmb in osteoclastogenesis and bone remodeling. Here, we report that loss of function mutation of Gpnmb gene promoted osteoclast differentiation and survival by enhancing RANKL-mediated MAPK-AKT activation. On the other hand, mutation of the Gpnmb gene suppressed osteoclast activity in bone resorption. Thus, our data suggest that OA/Gpnmb functions as a negative regulator of osteoclastogenesis but not function.

**Experimental Procedures**

*Mice—*Mutant DBA/2J (D2J) mice were obtained from Jackson Laboratory, as well as control DBA/2J with wild-type Gpnmb alleles (DBA/2J-Gpnmb+/SJl), referred to as D2J/Gpnmb+, as described previously (34). Because the Gpnmb+ is the ancestral D2J allele, the homozygous Gpnmb nonsense mutation is the only known genetic difference between the D2J and D2J/Gpnmb+ strains (35). All mice colonies were housed and maintained at Northeast Ohio Medical University according to the guidelines of the Institutional Animal Care and Use Committee.

*Biochemical Analysis—*Sera were prepared from 4- and 8-week-old D2J/Gpnmb+ and D2J (n = 4) male mice were scanned using the SkyScan 1172 micro-CT system (Micro Photonics Inc.), following the protocol described previously (34). Briefly, cortical measurements of femoral diaphyses were taken 2000 μm proximal to the distal growth plate in 200 consecutive slices of 5 μm resolution over a distance of 1000 μm, and volumetric regions were rendered as three-dimensional arrays, using SkyScan NRecon software. Guidelines for cortical analysis were followed as described by Bouxsein et al. (36). Image processing was performed by filtration using a Gaussian filter and automated algorithms on a slice-by-slice basis with shrink wrap feature applied to the region of interest. Image segmentation was performed using a lower gray threshold set on 72 and upper gray threshold set on 255. Parameters, including total cross-sectional area (mm2), cortical bone area (mm2), cortical area fraction, cortical bone thickness (mm), medullary marrow area (mm2), percentage of cortical porosity (%), cortical pore number (mm−1), total pore volume (mm3), and average pore volume (mm3), were accurately determined by using the shrink wrap option in the SkyScan CT analyzer. Three-dimensional reconstructed images of the sagittal and axial planes of the femoral metaphysis were generated using SkyScan CTvox software.

*Micro-computed Tomography (μCT)—*Femurs from 4-, 8-, and 16-week-old D2J/Gpnmb+ and D2J (n = 4) male mice were embedded undecalcified in plastic methylmethacrylate resin (MMA) as described previously (34). Five-micrometer sagittal sections were stained with TRAP and counterstained with toluidine blue. Quantitative histomorphometry of osteoclasts and resorption pits was performed at femoral metaphysis between 100 and 600 μm proximal to the growth plate and also at femoral diaphysis (subperosteal).
between 600 and 1000 μm, using OsteoMeasure software (Osteometrics). Two-dimensional parameters, including total osteoclast number per bone perimeter (mm⁻¹), number of multinucleated osteoclasts (mm⁻²), average size of multinucleated osteoclasts (× 10³ mm²), percentage of eroded surface over bone surface (%), number of resorption pits per bone perimeter (mm⁻¹), and average pit depth (μm), were calculated in three sections at different levels per animal.

**Generation of Murine Osteoclasts from Bone Marrow Cells**—Mouse bone marrow-derived cells (BM cells) were isolated from 8-week-old D2J/Gpnmb⁻ and D2J male mice as described (37). To generate osteoclasts, BM cells were plated at 5.6 × 10⁵ cells/cm² in a 96-well plate and primed with M-CSF (20 ng/ml; R&D Systems) for 72 h. OCP were then treated with RANKL (20 ng/ml) and M-CSF (20 ng/ml) (R&D Systems). Cells were fed every other day with a fresh medium containing RANKL and M-CSF. In parallel cultures, OCP of D2J mice were treated with recombinant OA protein (100 ng/ml; R&D Systems) and/or RANKL. At day 4, cells were fixed, and TRAP activity, TRAP positive (TRAP⁺) osteoclast count, and size were calculated, using a live image Nikon Ti Eclipse inverted microscope and automatic object capture of NIS-Elements software (Nikon).

**Flow Cytometry**—Freshly isolated bone marrow-derived cells from D2J/Gpnmb⁻ and D2J mice were extracted from the mouse hind limb. Red blood cells were lysed using ammonium chloride, and the cells were resuspended in washing buffer containing 1% FBS, 0.1% NaN₃ in PBS at a concentration of 1.0 × 10⁶ cells/ml. The bone marrow suspension was incubated with CD3 FITC, CD45R FITC, and CD11b phycoerythrine-conjugated antibodies for 30 min in the dark. The cells were washed twice and then analyzed using the BD Accuri C6 flow cytometer (BD Biosciences).

**Osteoclast Formation Using Co-culture Assay**—To generate osteoclasts in co-culture with osteoblasts, primary calvarial osteoblasts were plated at 1.8 × 10⁴ cells/cm² in a 48-well plate with vitamin D₃ (10⁻⁸ M) and prostaglandin E₂ (PGE₂) (10⁻⁶ M) (Sigma). The next day, BM cells were plated at 2.8 × 10⁵ cells/cm² on top of osteoblasts. Cells were fed every other day with a fresh medium containing vitamin D₃ and PGE₂. Large osteoclasts were evident by day 7, then cells were fixed for measurement of TRAP activity, cell count, and size of TRAP⁺ osteoclasts.

**Quantitative TRAP Activity Assay and TRAP Staining**—Osteoclasts plated in 96- and 48-well plate were fixed, permeabilized, and then air-dried. For TRAP activity assays, formalin-fixed osteoclasts were incubated with a TRAP buffer (52 mM of sodium tartrate in 0.1 M sodium acetate buffer, pH 5.2) containing 0.1 mg/ml p-nitrophenyl phosphate (Sigma). The reaction was stopped by adding 1 M NaOH to the reaction mixture and read for optical density at 405 nm using a BioTek Synergy H4 microplate reader (BioTek). For TRAP staining, osteoclasts were incubated with TRAP buffer, containing 1.5 mM naphthol AX-MX phosphate and 0.5 mM Fast Red Violet LB salt (Sigma). TRAP⁺ cells with more than three nuclei were counted as osteoclasts. TRAP activity and staining data were averages of six replicates per condition per experiment.

**Survival and Apoptosis Assays**—For the osteoclast survival assay, OCP from D2J/Gpnmb⁻ and D2J were differentiated with RANKL into osteoclast on 6-well collagen-precoated plates for 4 days. Mature osteoclasts were collected using 2.5 mg/ml collagenase in dissociation buffer (Life Technologies, Inc.) and seeded on a 96-well plate. Mature osteoclasts were then treated with RANKL only in the presence or absence of AKT inhibitor, LY294002 (1–5 μM) (Cell Signaling), for 48 h. Osteoclasts were fixed for TRAP activity and staining. For the cell apoptosis assay, OCP were differentiated into osteoclasts for 4 days. Media was then replaced with RANKL only (20 ng/ml) for an additional 16 h. Caspase-3/7 activity was measured using caspase-Glo 3/7 (Promega), as described previously (34).

**Osteoclast Activity Assay**—OCP derived from D2J/Gpnmb⁻ and D2J were differentiated into osteoclasts for 4 days on a Corning Osteo Assay surface (Corning, Inc.) with RANKL (20 or 50 ng/ml) and M-CSF (20 ng/ml). Osteoclasts were removed by bleach, and bone resorption was analyzed by quantification of the total pit area as a percentage threshold using a live image Nikon Ti Eclipse microscope and automatic object capture using NIS-Elements software. In a parallel experiment, OCP were cultured on an OsteoAssay human bone plate (Lonz) coated with a thin layer of adherent human bone particles. Bone resorption was assessed by measuring CTX-1 in conditioned medium harvested from 2- and 4-day differentiated osteoclasts. In parallel experiments, OCP collected from D2J/Gpnmb⁻ and D2J mice were differentiated with RANKL on 6-well collagen-precoated plates for 4 days. Mature osteoclasts were collected as described previously and seeded on bovine cortical slices in a 96-well plate with RANKL for 72 h. CTX-1 was measured in conditioned media as described above.

**Scanning Electron Microscopy**—For scanning electron microscopy, osteoclasts were fixed in a solution containing 1.25% glutaraldehyde, 1% paraformaldehyde, 0.1 M sucrose, 2 mM MgCl₂, 2 mM CaCl₂ in HEPES buffer for 3 h at 4 °C. Cells were rinsed and fixed with 2% osmium tetroxide and then dehydrated in ethanol and allowed to dry. Samples were viewed using a Hitachi S-2600N scanning electron microscope. In order to visualize actin ring formation, parallel bone slices were used, and osteoclasts were cultured as described above. Cells were fixed with methanol and incubated with rhodamine phalloidin for 1 h to visualize actin ring formation using an Olympus Fluoview FV1000 confocal microscope. Resorption pits were stained using a 1% toluidine blue solution. The resorption pit area was captured using Nikon Ti Eclipse microscope and automatic object capture as described above.

**Transmission Electron Microscopy**—Transmission electron microscopy was performed as described previously (34). Briefly, femurs from 8-week-old D2J/Gpnmb⁻ and D2J were fixed and embedded in Spurr resin. Two-micrometer bone sections of femurs were stained with uranyl acetate and lead citrate prior to examination with a Philips CM10 electron microscope (FEI).

**Quantitative Real-time RT-PCR**—Total RNA was extracted directly from total bone marrow, BM cells, OCP, 2- and 4-day differentiated osteoclasts, 8-week-old tibia, calvarial osteoblasts of D2J/Gpnmb⁺, and D2J (n ≥ 3), using an RNA extraction kit (Qiagen). RNA quantities were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Cloned cDNA was prepared using a high capacity cDNA reverse tran-
scription kit (Life Technologies). Quantitative RT-PCR was performed with the Step-one quantitative PCR system in duplicate with 2× SYBR® Green PCR Master Mix (Life Technologies), as described previously (34). Using GAPDH as the internal control, relative gene expression among samples was determined using the ΔΔC_{T} method. All quantitative PCR mouse primers used in this study, including Gpnmb, PU.1, MITF, TRAP, DC-STAMP, cFMS receptor, RANK receptor, c-FOS, IKKß, NFκB, RelA/p65, NFATc1, RANKL, and GAPDH, were purchased from Qiagen.

Osteogenesis Array—Total RNA was isolated from 8- and 16-week-old D2J/Gpmb−/− and D2J mice using TRI Reagent® (Sigma), as described previously (34). Gene expression profiling using the Osteogenesis RT® Profiler PCR Array was conducted. Relative gene expression was determined using the ΔΔC_{T} method.

Protein Isolation and SDS-PAGE—Total proteins were isolated from differentiated osteoclasts treated with RANKL for 0, 5, 15, 30, 60, and 120 min. Total proteins were also isolated from primary osteoblasts of D2J/Gpmb−/− and D2J mice. Samples were subjected to SDS-PAGE electrophoresis as described previously (29, 34, 38). The blot was incubated in blocking buffer using the SNAP i.d. system (EMD Millipore) and then incubated with polyclonal anti-OA/Gpmb (Bioss), anti-phospho-ERK1/2 (p44/p42 MAPK), anti-phospho-P38, anti-phospho-SAPK/JNK (Thr-183/Tyr-185), anti-phospho-AKT (Thr-308), anti-Bim (Cell Signaling), and anti-RANKL (Abcam). The blot was incubated with HRP-conjugated secondary antibody (R&D Systems). The signal was detected using SYNGENE PXi System (SYNGENE). The blot was developed with chemiluminescent substrate (Pierce) and anti-RANKL (Abcam). The blot was incubated with HRP-conjugated secondary antibody (R&D Systems). The signal was detected using SYNGENE PXi System (SYNGENE). The blot was stripped and reprobed for ERK1/2, P38, SAPK/JNK, AKT, glycogen synthase kinase (GSK)-3β (Cell Signaling), and actin (Bioss). Densitometric analysis was performed by quantitative measurement of the optical density of phosphorylated protein relative to the density of corresponding total protein, using Image Studio Lite software (LI-COR).

Statistical Analysis—For all quantitative generated data, differences between individual groups were analyzed for statistical significance using Prism 5 software version 5.04 (GraphPad). In most cases, when the data follow a normal distribution, one-factor or two-factor analysis of variance was employed, followed by a Bonferroni post hoc test. For comparisons between two group means, an unpaired Student’s t test was performed. Any differences with a probability value less than 0.05 were considered statistically significant. Group means or means ± S.E. were plotted in graphs. All in vitro experiments were repeated (n ≥ 3) with six replicates per experiment.

Results

Mutation in OA/Gpmb Inhibits Bone Remodeling in Vivo—Given that OA/Gpmb is expressed during osteoclast differentiation, here we further examined the role of OA in osteoclastogenesis and bone remodeling in vivo. Using a D2J mouse model with a natural nonsense mutation of the Gpmb gene, we have previously identified the positive role of Gpmb in osteoclastogenesis and bone formation in vivo and ex vivo (29, 34). In this study, a μCT two-dimensional sagittal plane of the femoral diaphysis in 16-week-old D2J mice showed a significant increase of the cortical thickness compared with the D2J/Gpmb−/− wild type (Fig. 1A). The μCT-reconstructed three-dimensional axial plane of the femoral diaphysis showed the decrease in cortical pores on the cross-section (Fig. 1B) and on the endosteal surface (Fig. 1C). μCT findings showed that total cross sectional area was not significant (Fig. 1D), whereas cortical bone area was increased (p < 0.001) in D2J compared with D2J/Gpmb−/− (Fig. 1E). Moreover, the cortical area fraction was increased at 4 and 8 weeks (Table 1) and also at 16 weeks (p < 0.0001) of D2J age (Fig. 1F). Afterward, cortical thickness was not different at 4 weeks, whereas it was increased (p < 0.001) at 8 weeks (Table 1) and 16 weeks of age in D2J compared with D2J/Gpmb−/− (Fig. 1G). Additionally, the marrow medullary area of D2J femur was reduced at 16 weeks of age (p < 0.01) compared with D2J/Gpmb−/− (Fig. 1H). Because it is well documented that cortical porosity is the result of osteoclast activity-induced bone erosions (39, 40), we sought to measure the cortical porosity in D2J mice. The percentage of cortical porosity was lower (p < 0.0001) in D2J at 16 weeks of age (Fig. 1I). Furthermore, cortical pore number, total pore volume, and average pore size were decreased (p < 0.001) at 16 weeks in D2J compared with D2J/Gpmb−/− (Fig. 1, J–L). Similar μCT findings of cortical analyses were shown in D2J mice at 4 and 8 weeks old compared with D2J/Gpmb−/− (Table 1). Evaluation of cortical thickness over 16 weeks of age showed a significant increase in D2J mice (Fig. 1M). In contrast, cortical porosity was markedly decreased over 16 weeks in in D2J compared with wild-type mice (Fig. 1N). Taken together, these data suggest that mutation in Gpmb in D2J mice inhibits bone remodeling.

To investigate the calcitropic role of OA/Gpmb on cytokines that stimulate bone resorption, we measured serum ELISA of RANKL in D2J mice, which was not higher compared with D2J/Gpmb−/− at 4 and 8 weeks of age (Fig. 2A). Next, we examined the serum levels of OPG, a cytokine that antagonizes RANKL and inhibits bone resorption (41). As found with RANKL, there were no significant differences in the levels of OPG in the serum of D2J and D2J/Gpmb−/− mice (Fig. 2B). The ratio of RANKL/OPG was slightly higher (p < 0.072) in D2J compared with D2J/Gpmb−/− at 8 weeks of age (Fig. 2C). Moreover, serum ELISA of the osteoclastogenesis marker, TRAP5b, reflective of an increased OC number in vivo, was higher (p < 0.01) in D2J compared with D2J/Gpmb−/− at 4 and 8 weeks of age (Fig. 2D), whereas the levels of the bone resorption marker, carboxyl-terminal collagen cross-links (CTX-1), were lower (p < 0.05) in D2J mice (Fig. 2E). The average osteoclast resorption function, marked by the ratio CTX-1/TRAP5b, was significantly lower (p < 0.0001) in D2J compared with D2J/Gpmb−/− mice (Fig. 2F). Taken together, these data suggest that the Gpmb mutation results in suppressed bone resorption with an apparent increase in osteoclast numbers.

To further confirm our μCT and ELISA analyses, histological analyses of the femurs was carried out and demonstrated an increased cortical width and decreased marrow medullary area of femoral diaphysis in D2J compared with D2J/Gpmb−/− at 8 weeks of age (Fig. 3A). Additionally, numerous larger TRAP-positive osteoclasts were found on the bone surface at femoral metaphysis of D2J compared with D2J/Gpmb−/− (Fig. 3B).
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![Image of D2J/Gpnmb+ and D2J mice](image)

**FIGURE 1. Cortical bone mass is increased in D2J mice.** Shown is μCT analysis of femoral diaphyses of 16-week-old OA/Gpnmb mutant (D2J) and wild-type (D2J/Gpnmb+™) mice. A, representative two-dimensional μCT sagittal section of femoral diaphysis showing decreased width of marrow medullary cavity. Dotted line, 1000 μm of the diaphysis subjected to μCT analysis. B, representative μCT images of cross-sections of femoral diaphysis from 16-week D2J and D2J/Gpnmb+™ showing decreased width of medullary cavity (double-headed arrows), increased cortical width (opposite arrowheads), and decreased cortical porosity (small arrows) in D2J compared with D2J/Gpnmb+™ mice. C, representative three-dimensional μCT reconstructed images of the axial plane of femoral diaphysis at two different angles showing decreased resorption pits and tracks on the endosteal surface in D2J mice (black arrows). D–N, μCT parameters (femur total cross sectional area (Tt.Ar), cortical bone area (Ct.Ar); cortical area fraction (Ct.Ar/Ct.Ar), cortical bone thickness (Ct.Th), marrow area (Ma.Ar), cortical porosity (Ct.Po), pore number (Po.N), total pore volume (Po.V), and average pore volume (avg. Po.V) were calculated in D2J and D2J/Gpnmb+™ (n = 4). M and N, μCT parameters in 4-, 8-, and 16-week D2J and D2J/Gpnmb+™. D2J samples overall show an increased trend over time in cortical thickness (M) and decreased cortical porosity (N) compared with D2J/Gpnmb+™. The mean value is represented in all graphs. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. Error bars, S.E.

**TABLE 1**

μCT analysis of femoral diaphyses in 4- and 8-week-old OA/Gpnmb mutant (D2J) and wild-type (D2J/Gpnmb+™) mice

| μCT parameters | Genotype | 4 weeks | 8 weeks |
|----------------|----------|---------|---------|
| Tt. Ar (mm²)   | D2J/Gpnmb+™ | 0.52 ± 0.02a | 0.65 ± 0.06 |
| Ct. Ar (mm²)   | D2J/Gpnmb+™ | 0.47 ± 0.01a | 0.49 ± 0.03b |
| % Ct. Ar / Tt. Ar | D2J/Gpnmb+™ | 44.65 ± 4.5 | 35.01 ± 7.8 |
| Ct. Th (mm)    | D2J/Gpnmb+™ | 0.153 ± 0.51 | 0.224 ± 0.57 |
| Ma. Ar (mm²)   | D2J/Gpnmb+™ | 0.979 ± 0.04a | 1.117 ± 0.02 |
| % Ct. Po       | D2J/Gpnmb+™ | 1.681 ± 0.25 | 1.995 ± 0.11 |
| Po. N (1/mm)   | D2J/Gpnmb+™ | 8.726 ± 0.20 | 12.550 ± 0.18 |
| Po. V (mm³)    | D2J/Gpnmb+™ | 2.544 ± 0.07 | 3.293 ± 0.07 |
| Avg. Po. V (mm³) | D2J/Gpnmb+™ | 0.039 ± 0.01 | 0.104 ± 0.01 |

* p < 0.05.
** p < 0.01.
*** p < 0.001.
**** p < 0.0001.

Interestingly, subperiosteal TRAP-positive large osteoclasts were more evident on the cortical bone surface with reduced eroded surfaces in D2J mice compared with normal size osteoclasts with prominent resorption pits in wild-type D2J/Gpnmb+™ mice (Fig. 3C). Osteoclast morphometric analyses showed that total osteoclast numbers on the trabecular and cortical bone surfaces were not statistically different at 4 weeks (Table 2) and 8 weeks between D2J and wild type (Fig. 3, D and E). However, the size of multinucleated osteoclasts on trabecular and cortical surfaces was larger (p < 0.05) in D2J of 4-week-old (Table 2) and 8-week-old mice (Fig. 3F). Because it has been well reported that bone erosions result from osteoclast activity (42–44), we sought to measure eroded surface and resorption pits. Further analysis of metrics indicative of osteoclast function supported the decreased bone resorption as marked by eroded surface, resorption pit number, and average pit depth in D2J compared with D2J/Gpnmb+™ at 8 weeks of age (Fig. 3, G–I), respectively. Collectively, these data suggest that the mutation in Gpnmb results in increased size and reduced resorptive activity of osteoclasts in vivo.

**D2J Osteoclast Precursors Are Hyperresponsive to RANKL ex Vivo**—To further understand the importance of OA/Gpnmb in osteoclastogenesis, we differentiated OCP derived from D2J

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and D2J/Gpnmb + of 8-week-old mice, with MCSF and RANKL, into osteoclasts ex vivo. Interestingly, OCP from D2J showed enhanced differentiation in response to RANKL (1–50 ng/ml) with more and larger TRAP-positive osteoclasts compared with the wild-type counterparts (Fig. 4A). The TRAP activity, osteoclast number, and size of D2J osteoclasts were increased and showed a hyperresponse to RANKL (5–50 ng/ml) (p < 0.01) compared with D2J/Gpnmb + (Fig. 4, B–D).

Due to the small osteoclast size at 5 ng/ml RANKL, data were omitted from the graph in Fig. 4D. In addition, a differential count of osteoclasts in D2J showed a 3-fold increase (p < 0.05) of osteoclast number containing more than 50 nuclei compared with D2J/Gpnmb + (Fig. 4E). These data suggest a hyperresponsiveness of D2J osteoclasts to RANKL treatment.

To test the osteoclast hyperresponsiveness over time, D2J and D2J/Gpnmb + OCP were treated with MCSF and RANKL
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TABLE 2

Histomorphometric analyses of the trabecular and cortical osteoclasts in 4-week-old D2J and wild-type mice

The total number of TRAP-positive osteoclasts (N.Oc)/bone perimeter (B.Pm); number of multinucleated osteoclasts (N.m.Oc)/bone perimeter (B.Pm), size of mOc × 10³ (mm²); percentage of eroded surface (ES)/bone surface (BS); number of resorption pits (N.Pit)/B.Pm; and average depth of resorption pit (µm), were calculated in D2J and D2J/Gpnmb⁺ (n ≥ 4). Values are mean ± S.E.

| Histomorphometric parameters | Genotype     | Trabecular | Cortical |
|------------------------------|--------------|------------|----------|
| N.Oc/B.Pm (1/mm)             | D2J/Gpnmb⁺   | 3.20 ± 0.32| 1.32 ± 0.42|
| N.m.Oc/B.Pm (1/mm)           | D2J          | 3.98 ± 0.37| 1.88 ± 0.24|
| m.OCSize × 10³ (mm²)         | D2J/Gpnmb⁺   | 0.92 ± 0.1  | 0.42 ± 0.15|
| % ES/ BS                     | D2J/Gpnmb⁺   | 0.82 ± 0.07| 0.56 ± 0.37|
| N.Pit/B.Pm (1/mm)            | D2J/Gpnmb⁺   | 0.21 ± 0.01| 0.11 ± 0.04|
| Pit depth (µm)               | D2J          | 0.24 ± 0.01| 0.18 ± 0.05|

* p < 0.05.

for either 2, 3, or 4 days. Interestingly, D2J osteoclasts showed a temporal increase in number and size over 4 days (Fig. 4F). Differentiation parameters of D2J osteoclasts, including TRAP activity, showed a significant increase (p < 0.01) at days 2, 3, and 4 compared with wild type (Fig. 4G). Although the differential count of osteoclasts (<20 nuclei) was similar at day 2 and 4, D2J osteoclast number was higher at day 3 (Fig. 4H). Moreover, the osteoclast count (>20 nuclei) was higher (p < 0.01) at days 2, 3, and 4 (Fig. 4I). In addition, D2J osteoclasts showed a temporal increase (p < 0.05) in size, peaking at day 4 compared with wild type (Fig. 4J). Increased osteoclast numbers of D2J in vitro could be due to changes in the proportion of osteoclast progenitor/hematopoietic stem cells in the bone marrow. It has been previously identified that an OCP cell population lies within the CD45⁻CD3⁻CD11b⁺/- bone marrow population (45). Flow cytometry was used to assess differences in the population of hematopoietic stem cells and showed that triple negative and CD45⁻CD3⁻CD11b⁻/low fractions in D2J bone marrow were similar to wild type (Fig. 4, K and L). This suggests that the increased osteoclastogenesis was not caused by differences in the osteoclast progenitor population within the D2J bone marrow.

Next, we examined whether adding recombinant OA (rOA) would inhibit osteoclast formation. OCP from wild type and D2J were treated with rOA with/without RANKL after priming with MCSF. Interestingly, rOA treatment decreased the number and size of TRAP-positive osteoclasts (Fig. 4M) as well as the TRAP activity of wild-type and D2J osteoclasts compared with untreated osteoclasts (p < 0.01) (Fig. 4N). Osteoclast number and size were also decreased (p < 0.01) when treated with rOA compared with untreated osteoclasts (Fig. 4, O and P). Collectively, these data demonstrate that Gpnmb mutation induces osteoclast hyperresponsiveness to RANKL and temporal increase of D2J osteoclasts. Moreover, this response was ameliorated by exogenous rOA treatment.

D2J Osteoblasts Stimulate Osteoclastogenesis ex Vivo—To test the contribution of D2J osteoblasts in osteoclastogenesis, we examined RANKL expression by osteogenesis array in D2J and D2J/Gpnmb⁺ calvaria. Surprisingly, RANKL showed a 2-fold increase in D2J compared with wild-type calvaria in 8- and 16-week-old mice (Fig. 5A). Quantitative RT-PCR analysis also showed a ~1.5-fold increase of RANKL expression in D2J tibia compared with wild type (Fig. 5B). Next, we investigated the contribution of osteoblasts in D2J osteoclastogenesis using a mix and match osteoblast-BM cell co-culture system, stimulated with vitamin D3 and PGE2 for 7 days. Interestingly, D2J osteoblasts markedly stimulated differentiation of OCP of either D2J or D2J/Gpnmb⁺ into large TRAP-positive osteoclasts, compared with D2J/Gpnmb⁺ osteoblasts (Fig. 5C). TRAP activity of mature osteoclasts differentiated in co-culture with D2J osteoblasts was significantly increased (p < 0.0001), compared with co-culture with D2J/Gpnmb⁺ osteoblasts (Fig. 5D). Osteoclast cell count and size were also increased (p < 0.001) when BM cells from both genotypes were co-cultured with D2J compared with wild-type osteoblasts (Fig. 5, E and F). These data suggest that the increased number of D2J osteoclasts is mediated at least in part by D2J osteoblasts. Next, we examined expressions of MCSF and RANKL mRNA in D2J and D2J/Gpnmb⁺ osteoblasts. Quantitative RT-PCR analysis showed a significant increase of MCSF and RANKL expressions in D2J osteoblasts compared with wild type (Fig. 5, G and H). These data were supported by ELISA of RANKL and OPG in neonatal calvarial and adult 8-week-old long bone osteoblasts isolated from D2J mice. Our data showed that ELISA ratios of RANKL/OPG were higher (p < 0.001) in D2J osteoblasts compared with wild type (Fig. 5I). Western blot analysis confirmed ELISA data and showed higher levels of RANKL proteins in D2J calvarial osteoblasts (p < 0.01) compared with wild type (Fig. 5, J and K). Collectively, these data showed clear evidence of increased levels of RANKL in D2J osteoblasts that is responsible, at least in part, for increased osteoclastogenesis.

RANK-RANKL Signaling Is Up-regulated in D2J Osteoclasts—Next, we explored the downstream signaling that mediates enhanced osteoclast differentiation in D2J mice. Quantitative RT-PCR analyses showed an abrogation of OA/Gpnmb expression in D2J/BM cells, OCP, and differentiated osteoclasts, compared with OA/Gpnmb in wild type (Fig. 6A). The transcription factors PU.1 and MITF, responsible for hematopoietic stem cell commitment into osteoclast progenitors (46), showed an up-regulation in both genotypes but were higher in D2J compared with wild type (Fig. 6, B and C). TRAP, an osteoclast differentiation marker, and DC-STAMP, an osteoclast fusion marker, showed similar patterns of up-regulation in 2–4-day osteoclasts and were higher in D2J (Fig. 6, D and E). Interestingly, the expression of cFOS (AP-1 heterodimeric complex), which is downstream of MAPK signaling, also showed higher expression in D2J osteoclasts over the 4-day differentiation (Fig. 6H). By examining the NFκB signaling pathway, our findings showed a latent increased expression of IKKβ, NFκB (P50/P65), and RelA (P65) in D2J compared with D2J/Gpnmb⁺ (Fig. 6, I–K), respectively. Finally, NFATc1, a transcription factor that mediates osteoclast differentiation, showed a significantly increased expression in D2J osteoclasts from priming to full maturity compared with D2J/Gpnmb⁺ (Fig. 6L). These data supported the enhanced osteoclast differentiation in D2J.
Next, we examined OA production during osteoclast differentiation. ELISA of OA in the cell lysates and conditioned medium showed a temporal and significant increase of OA in the wild type compared with D2J osteoclasts (Fig. 7A). Afterward, we examined the levels of RANK receptors, NFκB, and NFATc1 during osteoclastogenesis. Western blot analyses showed a significant increase of RANK (Fig. 7, B and C) and NFATc1 (Fig. 7, F and G) levels in D2J osteoclasts at days 2 and 4 compared with wild type. In contrast, phosphorylation of NFκB was not different between D2J and wild-type osteoclasts (Fig. 7, D and E). Next, we examined the activation of the MAPK pathway, downstream of RANK receptors in D2J and D2J/
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FIGURE 5. D2J osteoblasts stimulate osteoclastogenesis in a mix and match experiment. A, osteogenesis array of tibia showing increased RANKL expression in the calvaria of D2J compared with D2J/Gpnmb-/- in 8- and 16-week-old mice. B, quantitative PCR analysis showing increased RANKL in D2J tibia of 8-week-old mice. C–F, co-culture of D2J and D2J/Gpnmb-/- osteoblasts and BM cells, in mix and match, was treated with vitamin D3 (10^{-8} M) and PGE2 (10^{-6} M) for 7 days to stimulate osteoclast differentiation. C, microscopic pictures show increased TRAP-positive osteoclasts (arrows) and their size (double-headed arrows) in D2J osteoblast co-cultured with D2J or wild-type BM. Osteoclast differentiation parameters that include TRAP activity (D), count of TRAP-positive osteoclasts (E), and osteoclast size (F) were exponentially increased in D2J osteoblast co-cultures compared with D2J/Gpnmb-/-. G and H, comparative quantitative PCR analysis of calvarial osteoblasts showing increased MCSF (G) and RANKL (H) in D2J osteoblasts compared with D2J/Gpnmb-/-. J, ELISA showing increased RANKL/OPG ratios in D2J osteoblasts of neonatal calvaria and adult long bones compared with wild type. J, Western blot and densitometry (K) of calvarial osteoblasts showing increased production of RANKL in D2J compared with D2J/Gpnmb-/-. Experiments were repeated at least three times and showed similar results. The mean value is represented in all graphs ± S.E. (error bars). **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. Scale bars, 200 μm.

FIGURE 4. Enhanced differentiation of D2J osteoclasts ex vivo. A–E, OCP from D2J and D2J/Gpnmb-/- were differentiated into osteoclasts with RANKL (1, 5, 10, 20, and 50 ng/ml) and TRAP-stained. A, microscopic images show hyperresponsiveness to RANKL of TRAP-positive osteoclasts (arrows) and their size (double-headed arrows) in D2J compared with D2J/Gpnmb-/-. Parameters of osteoclast differentiation that include TRAP activity (B), count of TRAP-positive osteoclasts (≥3 nuclei) (C), size of TRAP-positive osteoclasts (D), and differential count of osteoclasts at 20 ng/ml RANKL (E) are significantly increased in D2J compared with wild type. Parameters of osteoclast differentiation that include TRAP activity (G), count of TRAP-positive osteoclasts (3–19 nuclei) (H), osteoclast count (≥20 nuclei) (I), and osteoclast size (J) are significantly increased in D2J compared with D2J/Gpnmb-/-. K and L, hematopoietic stem cells from D2J and D2J/Gpnmb-/- mice were immunostained for CD11b, CD3, and CD45R and subjected to flow cytometry. The data are presented in pseudocolor density plots, whereas the green box represents CD11b+/CD3-CD45R- cells and the red box represents CD11b+/CD3-CD45R+ cells (K). Data were quantified (TN, triple negative) (L). M–P, OCP from D2J and D2J/Gpnmb-/- were differentiated into osteoclasts with RANKL and MCSF in the presence or absence of rOA protein (10 and 100 ng/ml). M, microscopic images show decreased number and size of D2J osteoclasts in RANKL and rOA compared with D2J with no rOA treatment. TRAP activity (N), count of TRAP-positive osteoclasts (O), and size of osteoclasts (P) were reduced in D2J osteoclasts differentiated with RANKL and rOA compared with D2J without rOA. Data presented in all graphs represent mean ± S.E. (error bars) in six replicates per condition. The experiment was repeated six times and showed similar patterns. The mean value is represented in all graphs ± S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. Scale bars, 200 μm.
Interestingly, phosphorylation of ERK, P38, and JNK was significantly higher in D2J control osteoclasts and over 2 h of RANKL stimulation at day 2 (Fig. 7, H, J, and L), and day 4 (Fig. 7, N, P, and R). Moreover, a day 2 phosphorylation of ERK, P38, and JNK in D2J osteoclasts reached a maximum after 5 min, compared with D2J/Gpnmb+ (Fig. 7, I, K, and M), whereas at day 4, activation of ERK, P38, and JNK in D2J osteoclasts reached the highest at 15 min compared with D2J/Gpnmb+, following RANKL treatment (Fig. 7, O, Q, and S). Taken together, these data suggest an enhanced differentiation of D2J osteoclasts in response to RANKL that is mediated, in part, by RANK receptors, leading to activation of the downstream MAPK signaling pathway and resulting in stimulation of NFATc1.

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Gpnmb Mutation Activated AKT Pathway and Enhanced Survival of D2J Osteoclasts—Because we showed above that mutation of OA/Gpnmb+ promoted D2J osteoclast differentiation in response to RANKL, we investigated other signaling pathways downstream of RANKL that might be involved in osteoclast cell survival. D2J and D2J/Gpnmb+ OCP were differentiated with MCSF and RANKL for 4 days. Interestingly, D2J osteoclasts showed increased expression of BCL2, a pro-survival transcription factor, as early as the priming stage compared with wild type (Fig. 8A). On the other hand, BAX, a pro-
apoptotic regulating transcription factor, was down-regulated in D2J osteoclasts compared with the wild type (Fig. 8B).

Because activation of AKT signaling pathway is critical for osteoclast survival (47), we examined AKT phosphorylation in mature osteoclasts, treated with RANKL for up to 2 h. Interestingly, our data showed that AKT phosphorylation is significantly increased endogenously in D2J osteoclast control (no RANKL) and in response to RANKL treatment, reaching the highest level after 5 min when compared with wild-type controls (Fig. 8, C and D). Of several AKT substrates, phosphorylation of GSK-3β was also increased in D2J osteoclasts, reaching the highest level after 5 min of stimulation with RANKL. GSK-3β is a well characterized substrate of AKT and exists in an unphosphorylated form in resting cells. Similar to the enhanced activation of AKT, phosphorylation of GSK-3β was augmented in RANKL-treated D2J osteoclasts compared with the wild type (Fig. 8, E and F). Taken together, these data indicate that mutation of Gpnmb promoted AKT activation and the downstream GSK-3β in mature osteoclasts, in response to RANKL treatment. Next, we examined the significance of AKT activation in the survival of D2J osteoclasts. D2J and D2J/Gpnmb OCP were differentiated into osteoclasts for 4 days and then treated with AKT inhibitor (LY2940) with/without RANKL for 48 h. Interestingly, D2J osteoclasts treated with
FIGURE 8. **Enhanced survival of D2J osteoclasts ex vivo.** A and B, comparative quantitative RT-PCR analyses of RNA collected from BM cells, OCP, and 2- and 4-day RANKL-differentiated osteoclasts, showing decreased BCL2 expression (survival) (A) and increased BAX pro-apoptotic (B) transcription factors in D2J compared with D2J/Gpnmb+.

C–F, mature osteoclasts from D2J and D2J/Gpnmb+ were serum-starved for 1 h prior to treatment with RANKL (20 ng/ml) at the indicated time points. Blots were probed with anti-phospho-AKT (p-AKT) and anti-phospho-GSK-3β (p-GSK-3β) in the top panel. The blots were stripped and reprobed with the respective total AKT and GSK-3β proteins to determine loading (bottom panels). The ratio of phosphorylated proteins to total proteins was measured to correct for experimental variability: phospho-AKT/AKT and phospho-GSK-3β/GSK-3β.

G–I, mature osteoclasts from D2J and D2J/Gpnmb+ were treated with AKT inhibitor LY2940 (1–5 μM) with or without RANKL (20 ng/ml) for 48 h. G, microscopic image shows the surviving osteoclasts. Parameters of osteoclast differentiation that include TRAP activity of surviving osteoclasts (H) and count of TRAP-positive surviving osteoclasts (I) were increased in D2J compared with D2J/Gpnmb+.

J–M, mature osteoclasts are serum-starved for 1 h before treatment with LY2940 and/or RANKL (20 ng/ml) for 16 h. Cell lysates were collected for Western blot analysis. Blots were probed with anti-phospho-AKT and anti-Bim apoptotic marker. Experimental variability was corrected by quantifying total proteins and measuring the ratio of phospho-AKT/AKT and Bim/actin.

N and O, mature osteoclasts from D2J and D2J/Gpnmb+ were treated with RANKL in the presence/absence of rOA. Proteins from cell lysates were subjected to SDS-PAGE and probed for phospho- and total AKT and quantified by densitometry.

P, Caspase-Glo 3/7 activity of mature osteoclasts without MCSF for 16 h is decreased in D2J compared with D2J/Gpnmb+. Experiments were repeated at least three times and showed similar results. Data presented in all graphs represent mean ± S.E. (error bars) in six replicates per condition. The experiment was repeated three times and showed similar results. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.
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RANKL showed enhanced cell survival (Fig. 8G) marked by TRAP activity and count of surviving osteoclasts after 48 h when compared with the wild type (Fig. 8, H and I). Moreover, the AKT inhibitor LY2940 decreased cell survival of D2J cells and wild-type osteoclasts; however, D2J osteoclasts showed improved cell survival ($p < 0.01$) when compared with the wild-
type D2J/Gpnmb+ osteoclasts (Fig. 8, H and I). Additionally, we tested AKT activation in mature osteoclasts treated with AKT inhibitor with/without RANKL treatment. Our data showed increased AKT phosphorylation in D2J osteoclasts treated with RANKL alone. Moreover, LY2940 treatment suppressed AKT phosphorylation in D2J/Gpnmb+ osteoclasts compared with AKT partial suppression in D2J osteoclasts (Fig. 8, J and K). These data suggest that AKT activation is cell-autonomous and is involved in survival of D2J osteoclasts and thus might contribute to increased number and size of osteoclasts ex vivo. To confirm our hypothesis that D2J osteoclasts have enhanced cell survival, we measured Bim expression in osteoclasts treated with LY2940 with/without RANKL. Bim is a member of the BCL2 family of transcription factors that promotes cell apoptosis (48). Bim expression showed a 3-fold increase, as expected in wild-type osteoclasts treated with LY2940, compared with RANKL-treated or untreated control (Fig. 8, L and M). On the other hand, Bim expression was not altered in D2J osteoclasts treated with LY2940, compared with RANKL alone (Fig. 8, L and M). Afterward, we investigated the role of rOA in AKT activation. Interestingly, rOA marked inhibitory AKT phosphorylation not only in wild type but also in D2J osteoclasts (Fig. 8, N and O). Next, we tested osteoclast apoptosis of D2J and their wild-type counterparts. Seven-day differentiated osteoclasts of D2J and D2J/Gpnmb+ were either left untreated or treated with RANKL alone, and cell apoptosis was determined by caspase-Glo 3/7 activity. Our findings demonstrate that D2J osteoclasts are less apoptotic (p < 0.001) when compared with D2J/Gpnmb+ at 0 and 16 h following RANKL treatment (Fig. 8P). These results indicate that osteoclast apoptosis is suppressed in D2J, which probably contributes to prolonged osteoclast survival.

**Gpnmb Mutation Attenuates D2J Osteoclast Function ex Vivo**—Given that mutation of Gpnmb promoted osteoclast differentiation and survival of D2J OCP, we explored the osteolytic activity of those osteoclasts. OCP of D2J and D2J/Gpnmb+ were differentiated into osteoclasts over osteo-assay discs to test their function. Photomicrographs of TRAP-positive cells showed larger D2J osteoclasts with decreased resorption areas compared with wild type (Fig. 9A). Photomicrographs of osteo-assay discs, after removal of osteoclasts, confirmed the reduced matrix resorption by D2J osteoclasts (Fig. 9B). Moreover, the resorbed surface by D2J osteoclasts was smaller in size with less complexity of resorption tracks compared with wild type (Fig. 9B). Measurement of the resorption area showed a significant decrease (p < 0.001) in osteolysis of D2J osteoclasts up to 50% when compared with D2J/Gpnmb+ in contrast, adding rOA enhanced the resorptive activity of wild-type and D2J osteoclasts (Fig. 9C). These data were supported by testing the osteolytic activity of D2J osteoclasts on a human bone osteoassay. Measurements of CTX-1 in the conditioned medium of D2J osteoclasts were significantly lower compared with wild-type controls (Fig. 9D). To further determine the role of OA in osteoclast function, the activity of differentiated osteoclasts was tested on cortical bone slices. We found that mutation of Gpnmb in D2J mice resulted in impaired osteoclastic bone resorption (Fig. 9, E and H). Despite the larger size of TRAP-positive D2J osteoclasts (Fig. 9E), they were ineffective in bone resorption, as reflected by resorptive track formation (Fig. 9F).

The average bone resorption pit area was lower (p < 0.01) in D2J as compared with wild-type osteoclasts (Fig. 9G). Moreover, levels of CTX-1 were measured in medium from osteoclasts cultured on bone slices and revealed a decrease (p < 0.05) in D2J osteoclasts (Fig. 9H). To gain further insight into the underlying cause of defective bone resorption by D2J osteoclasts, we examined the osteoclast cytoskeleton. D2J/Gpnmb+ osteoclasts exhibited characteristic cytoskeleton organization, as evidenced by the presence of a tight ring of actin at the site of bone attachment called the “F-actin ring” or “sealing zone.” In contrast, D2J osteoclasts showed an abnormal cytoskeleton, with F-actin diffusely distributed through the cytoplasm (Fig. 9I). Next, we further examined the osteoclastic cell morphology over bone slices. Wild-type osteoclasts showed normal cell polarization over the bone surface. Next to the wild-type osteoclast is a resorption pit with exposed collagen fibers. In contrast, D2J osteoclasts were larger in size with a flattened surface and abnormal spreading of the ruffled border (Fig. 9J). Next, we examined in vivo osteoclast cell morphology and ultrastructure. Analysis showed that D2J osteoclasts were larger in size and less polarized over the bone surface (Fig. 9K). In addition, D2J osteoclasts were more elongated over the bone surface with a less developed ruffled border compared with D2J/Gpnmb+ osteoclasts (Fig. 9K, insets). Collectively, these results showed that osteoclasts with Gpnmb mutation are less effective in bone resorption, and this is partially due to aberrant cell morphology and cytoskeleton reorganization.

**FIGURE 9. Decreased resorptive activity of D2J osteoclasts.** A–C, OCP were differentiated into osteoclasts over osteo-assay discs with RANKL for 4 days. A, microscopic images show larger rounded D2J osteoclasts, surrounded by a minimal clear zone, compared with wild type. B, microscopic images show less resorbed tracks (representative analyses are shown within the blue dotted circle) in D2J compared with D2J/Gpnmb+. Scale bars, 200 μm. C, percentage area fraction of the clear resorbed area over non-resorbed area in D2J and D2J/Gpnmb+ osteoclasts in the presence or absence of rOA (100 ng/ml). Note the decrease in resorption in D2J compared with wild type. D, CTX-1 values in the conditioned medium of D2J and wild-type osteoclasts, differentiated over a human osteo-assay for 2 and 4 days. E–I, OCP were differentiated on collagen-precocated 6-well plates. Mature osteoclasts were seeded with RANKL on cortical bone slices for 72 h. E, microscopic images show TRAP-stained large D2J osteoclasts compared with D2J/Gpnmb+ (yellow arrows). F, microscopic images of resorption pits after removal of osteoclasts and staining with toluidine blue. Note the smaller resorption pit in D2J (yellow arrows). G, average resorption area per osteoclast with a significant reduction in D2J compared with D2J/Gpnmb+ osteoclasts (yellow arrows). H, CTX-1 values in the conditioned media of D2J and D2J/Gpnmb+ osteoclasts. I, microscopic images of the F-actin ring in osteoclasts (yellow arrows). Note the abnormal localization of F-actin in D2J compared with wild type. J, scanning electron micrographs of D2J and D2J/Gpnmb+ osteoclasts on bone slices. Note the significant resorption pit (yellow arrow) made by wild-type osteoclast compared with D2J. K, transmission electron microscopy of D2J osteoclast in vivo showing the larger osteoclast setting on the bone surface (no lacuna) with large intracellular vesicles (V), absence of secretory domain (SD), and few short ruffled borders (inset) compared with the D2J/Gpnmb+ osteoclast with the normal appearance of a ruffled border (RB) and the presence of a secretory domain. Experiments were repeated three times and showed similar results. Data presented represent mean ± S.E. (error bars) in triplicate per strain. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
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Discussion

It is well established that OA/Gpnmb is a positive regulator of osteoblast differentiation and is a potent stimulator of bone formation in physiological and pathological conditions (19, 29–31, 34, 38). We recently reported on the positive effects of OA/Gpnmb in osteogenesis, using D2J mice, and showed that mutation of Gpnmb resulted in substantial loss of trabecular bone mass (34). Moreover, Gpnmb mutation inhibited the differentiation of D2J mesenchymal stem cells and osteoblast progenitors, resulting in decreased matrix mineralization ex vivo (29, 34). Previous studies reported on the importance of OA/Gpnmb in osteoclastogenesis and bone remodeling (32). This led us to further investigate the role of OA/Gpnmb in osteoclast differentiation and bone resorption, using D2J mice as a model of loss of function. These mice are a unique animal model in which to examine skeletal phenotype due to a loss of function resulting from the natural nonsense homozygous mutation of the Gpnmb alleles, leading to a remarkable decrease of bone remodeling (based on μCT findings). Initial characterization of osteoclastogenesis in D2J mice showed hyperresponsiveness to RANKL, which might be explained by the high expression of RANK receptor mRNA in D2J osteoclast precursors. Previous reports have shown that overexpression of RANK receptor hyperstimulated osteoclast differentiation in response to RANKL (49), whereas RANK knock-out mice were characterized by profound osteopetrosis resulting from an apparent block in osteoclast differentiation (50). Our data suggest that RANKL-induced signaling in D2J osteoclasts is enhanced. Conversely, a separate report, in which the OA protein was blocked with neutralizing antibody, inhibiting osteoclast differentiation and function in C57/Bl6 mice, these data suggested that OA/Gpnmb may act a positive regulator of osteoclastogenesis (32). However, in our report, we observed a marked increase in osteoclast number and size in D2J mice (Gpnmb mutant), and this was abrogated by treatment with rOA. This discrepancy in data might be explained by the fact that short term treatment with an anti-OA blocking antibody in the aforementioned study inhibited not only the OA protein but also inhibited the β3 integrin-mediated osteoclast attachment and not differentiation, as previously reported (32). However, in our study, longer treatment of Gpnmb mutant osteoclast precursors with rOA rescued their differentiation to the wild-type level. Another possibility is that the OA antibody and rOA protein may have atypical impacts on osteoclastogenesis in different genetic background, such as C57/Bl6 compared with D2J/Gpnmb+ mice. In osteoclasts, expression of RANK receptors is regulated by the transcription factors PU.1 and MITF (51). Interestingly, our quantitative PCR analysis showed an increased expression of PU.1 in D2J osteoclasts, which is correlated with increased RANK expression. Expression analysis of overall MITF mRNA was relatively high in D2J osteoclasts. Interestingly, MITF was also reported to significantly enhance OA/Gpnmb transcriptional regulation in melanocytes and macrophages (52, 53). Because we propose that OA/Gpnmb is a suppressor of osteoclastogenesis, it would be expected that mutation/deletion of the OA/Gpnmb gene will relieve suppression and augment other positive effectors down-stream of MITF on osteoclastogenesis. It would be very interesting to study the impact of MITF activation on OA/Gpnmb during osteoclastogenesis in D2J and D2J/Gpnmb+ mice. Next, our unpublished data5 showed that proliferation of BM cells of D2J mice was significantly higher compared with the wild-type counterparts. Additionally, a previous study (54) reported on the subtle changes in myeloid differentiation in bone marrow and spleen, resulting in alteration in the proportion of CD11b-positive cells between D2J and their wild-type controls. CD11b is an adhesion molecule and an early marker of mononuclear cells (55). These changes in myeloid cell proliferation and differentiation in D2J mice may contribute to their osteoclast number. From our data, we showed that there was no difference in the populations of OCP between D2J and WT, suggesting that increased osteoclastogenesis in the D2J mice is not due to an inherited defect in mutant osteoclast precursors and could be due to an autonomous effect later in differentiation.

Next, our findings showed that the serum TRAP5b biomarker was significantly increased in D2J mice at 4 and 8 weeks of age. In fact, the total number of osteoclasts did not differ between D2J mice and their wild-type controls; however, the size of multinucleated osteoclasts was significantly increased in D2J mice in vivo. TRAP is highly expressed by osteoclasts and, to a lesser extent, by osteoblasts, osteocytes, activated macrophages, monocytes, and lymphocytes (56–59). These findings were supported by ex vivo experiments that showed a larger size of D2J osteoclasts when differentiated in co-culture with D2J osteoblasts or with RANKL independently. These data suggest that increased osteoclastogenesis in D2J mice is due, at least in part, to RANKL overexpression by D2J osteoblasts. These data can also be explained by the increased cell fusion of OCP in the D2J lineage (in the absence of Gpnmb). Interestingly, our results showed a marked increase in DC-STAMP mRNA expression during osteoclast differentiation in D2J mice compared with wild-type controls. These data were also supported by the decreased size of wild-type and D2J osteoclasts when treated with rOA protein. Collectively, we conclude that OA/Gpnmb reduced osteoclast differentiation, at least in part, by inhibition of cell fusion. A previous study (60) reported that DC-STAMP-deficient mice have an osteopetrotic phenotype due to the absence of multinucleated osteoclasts. The total number of nuclei in cultures of osteoclasts was comparable between DC-STAMP-deficient and wild-type mice, indicating that the inhibition of multinucleation was not caused by reduced cell density of OCP in DC-STAMP-deficient mice (60, 61). DC-STAMP gene expression is regulated by activation of RANK signaling and the downstream NFATc1 (61, 62). In our study, we showed that NFATc1 is highly up-regulated in early and mature D2J osteoclasts, corresponding with DC-STAMP mRNA expression. It would be interesting to study the mechanism of Gpnmb in relation to the RANK/NFATc1/DC-STAMP axis in fusion of osteoclast precursors. Next, we also showed an increased RANKL signaling of D2J osteoclasts resulting from activation of MAP kinase pathways through ERK, P38, and JNK phosphory-

5 S. M. Abdelmagid, G. R. Sondag, F. M. Moussa, J. Y. Belcher, B. Yu, H. Stinnett, K. Novak, T. Mbimba, M. Khol, K. D. Hankenson, C. Malcuit, and F. F. Safadi, unpublished data.
It has been reported that RANKL signaling activates MAP kinase, which in turn activates AP-1 transcription factors (members of the Fra, FOS, and Jun family) that activate downstream MITF and, thus, regulate osteoclastogenesis (63). It was also shown that ERK1 positively regulates osteoclast development and bone resorptive activity, because genetic disruption of ERK1 reduced osteoclast progenitor numbers and compromised pit formation (64). Moreover, it was found that P38 MAPK activation is required for inducing osteoclast differentiation but not for osteoclast function (65). A previous report (66) has demonstrated that deletion of the c-FOS gene resulted in osteopetrosis by halting osteoclast differentiation at the macrophage stage, and this defect was completely rescued by expressing FOS protein. It would be interesting to examine the role of OA in activation of AP-1 transcription factor complex that regulates osteoclast differentiation.

Because enhanced osteoclast survival could potentially contribute to the increased number and size of D2J osteoclasts, we examined osteoclast survival programs in these Gpnmb mutant cells. Our data showed that survival of D2J osteoclasts is mediated by AKT-GSK3β phosphorylation. ERK1 and AKT activation is reported to be involved in osteoclast survival downstream of PI3K phosphorylation (67). Although controversial (68), phosphorylation of AKT (Thr-308) is probably a principal mediator of RANKL-stimulated osteoclast survival (69). On the other hand, AKT also promotes osteoclast differentiation, perhaps by activating the NFκB signaling pathway (68). GSK-3β is a well characterized substrate of AKT that exists in a persistently active condition (unphosphorylated) in resting cells, and inactivation of GSK-3β is required for osteoclast differentiation (70). Moreover, inactivation of GSK-3β is reported to attenuate cell apoptosis (71) by promoting pro-survival transcription factors, such as CREB and heat shock factor-1 (72), and inhibiting pro-apoptotic transcription factors, such as P53 (73). In our study, similar to the enhanced AKT activation by phosphorylation in D2J osteoclasts, inactivation of GSK-3β by phosphorylation was also augmented in RANKL-treated D2J osteoclasts. Taken together, we conclude that the enhanced survival of D2J osteoclasts is mediated by the AKT-GSK-3β pathway. Moreover, our results show up-regulation of the anti-apoptotic BCL-2 and down-regulation of the pro-apoptotic BAX transcription factors in D2J osteoclast precursors. These data are supported by the lower levels of the pro-apoptotic marker Bim in mature D2J osteoclasts, which was not increased after treatment with AKT inhibitor (LY2940) compared with wild type.

Previous studies have demonstrated that ubiquitination-dependent regulation of Bim levels are critical for controlling apoptosis and activation of osteoclasts (74). Another study demonstrated that Ser-87 of Bim is an important regulatory site that is targeted by AKT to attenuate the pro-apoptotic function of Bim, thereby promoting cell survival (75). Indeed, AKT inhibitor (LY2940) decreased AKT phosphorylation and increased Bim expression in wild-type osteoclasts but not in D2J osteoclasts. Collectively, survival of D2J osteoclasts is enhanced, perhaps due to controlled regulation of both the AKT-GSK-3β and AKT-Bim pathways.

Next, we tested the function of D2J osteoclasts in osteolysis by osteo-assay and demonstrated a reduction in their resorptive activity, as evidenced by the decreased CTX-1 and reduced number and size of resorptive pits and their inability to form complex resorption tracks. However, these D2J osteoclasts did display an increase in overall size compared with wild-type osteoclasts. These findings were supported by the in vivo evidence of increased cortical thickness, decreased pore number and volume, decreased eroded surface, and decreased serum CTX-1 in D2J mice. Taken together, we conclude that although D2J osteoclasts are larger than those of the wild type, they fail to appropriately stimulate bone resorption. In a similar study multinucleated osteoclasts that are defective in bone resorption was reported in ClC-7 chloride channel knock-out osteoclasts, leading to osteopetrotic bone phenotype (76). Other interesting studies showed that targeted deletion of Cathepsin-K or carbonic anhydrase II in osteoclasts increased their number but with a functional deficit (77, 78). In our study, the in vivo and ex vivo functional defect could be explained by the abnormal morphology of D2J osteoclasts with an underdeveloped ruffled border and large vesicles trapped within the osteoclasts. The reduced size of resorptive lacunae might be explained by inadequate formation of the sealing zone constructed by α, β integrin-binding to bone matrix proteins like osteopontin (79). Recent evidence has shown that OA binds to β3 integrins in differentiated osteoclasts (32); thus, the absence of OA might diminish sealing zone formation and osteoclast function. Moreover, impaired polarization of D2J osteoclasts might contribute to the insufficient sealing zone. In a previous study, we reported on OA expression in osteoclasts during active bone remodeling of the hard callus at 14 and 21 days postfracture (31). Moreover, targeted over-expression of Gpnmb under control of the TRAP promoter in an osteoclastic lineage promoted their bone resorptive activity (80). Collectively, we conclude that OA/Gpnmb positively regulates osteoclast function. Recent studies (81, 82) demonstrated that down-regulation of small GTPase RAB7 decreased osteoclast polarization and ruffled border formation. It has been hypothesized that RAB7 is required for the transfer of vesicles over microtubules to actin cytoskeleton for subsequent fusion to the plasma membrane of the ruffled border (82). Given the abnormal actin ring formation in D2J osteoclasts and its abnormal spreading over the bone matrix, this might contribute to the defective D2J osteoclast function. Additionally, we noticed that rOA stimulated SRC phosphorylation in osteoblasts. Previous studies also reported on the osteopetrotic phenotype of SRC-deficient mice that is due to osteoclasts with fewer podosomes, indicating the critical role of SRC kinase activity in actin ring formation, assembly, and stability of podosomes that is crucial for bone resorption (83, 84). Future studies will focus on studying the contribution of RAB7 and SRC activity in D2J osteoclasts and also investigating the possible role of OA in vesicle trafficking and cytoskeletal organization in osteoclasts. In this study, we were able to identify the role(s) of OA/Gpnmb in osteoclast differentiation and survival and their function in bone resorption (Fig 10). Generation of other genetically

G. Sondag, F. Moussa, T. Mbimba, and F. Safadi, unpublished observations.
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![Diagram of osteoactivin effects](image)

FIGURE 10. Schematic diagram of osteoactivin effects on osteoclast differentiation, survival, and function. Mutation of Gpnmb (XGpnmbX) promotes osteoclast differentiation mediated by MAPK phosphorylation and up-regulation of osteoclastogenic factors: PU.1, RANK receptors, DC-STAMP, and NFATc1. Mutation of Gpnmb also promotes osteoclastogenesis by stimulating RANKL production of osteoblasts. Moreover, mutation of Gpnmb enhances osteoclast survival, perhaps by phosphorylation of the AKT-GSK3β pathway and regulated expression of BCL2 and Bim. Finally, mutation of Gpnmb inhibits osteoclast activity in bone resorption by altering the cytoskeletal organization through an unknown mechanism yet to be determined.

engineered mouse models, such as an osteoclast-specific knockout of Gpnmb, will help us to understand and identify the precise mechanism(s) of action of OA in osteoclast differentiation and function in bone remodeling.

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