Increased S1P expression in osteoclasts enhances bone formation in an animal model of Paget’s disease

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Abstract
Paget’s disease (PD) is characterized by increased numbers of abnormal osteoclasts (OCLs) that drive exuberant bone formation, but the mechanisms responsible for the increased bone formation remain unclear. We previously reported that OCLs from 70% of PD patients express measles virus nucleocapsid protein (MVNP), and that transgenic mice with targeted expression of MVNP in OCLs (MVNP mice) develop bone lesions and abnormal OCLs characteristic of PD. In this report, we examined if OCL-derived sphingosine-1-phosphate (S1P) contributed to the abnormal bone formation in PD, since OCL-derived S1P can act as a coupling factor to increase normal bone formation via binding S1P-receptor-3 (S1PR3) on osteoblasts (OBs). We report that OCLs from MVNP mice and PD patients expressed high levels of sphingosine kinase-1 (SphK-1) compared with wild-type (WT) mouse and normal donor OCLs. SphK-1 production by MVNP-OCLs was interleukin-6 (IL-6)-dependent since OCLs from MVNP/IL-6−/− mice expressed lower levels of SphK-1. Immunohistochemistry of bone biopsies from a normal donor, a PD patient, WT and MVNP mice confirmed increased expression levels of SphK-1 in OCLs and S1PR3 in OBs of the PD patient and MVNP mice compared with normal donor and WT mice. Further, MVNP-OCLs cocultured with OBs from MVNP or WT mice increased OB-S1PR3 expression and enhanced expression of OB differentiation markers in MVNP-OCLs precursors compared with WT-OBs, which was mediated by IL-6 and insulin-like growth factor 1 secreted by MVNP-OCLs. Finally, the addition of an S1PR3 antagonist (VPC23019) to WT or MVNP-OBs treated with WT and MVNP-OCL-conditioned media (CM) blocked enhanced OB differentiation of MVNP-OBs treated with MVNP-OCL-CM. In contrast, the addition of the S1PR3 agonist, VPC24191, to the cultures enhanced osterix and Col-1A expression in MVNP-OBs treated with MVNP-OCL-CM compared with WT-OBs treated with WT-OCL-CM. These results suggest that IL-6...
produced by PD-OCLs increases S1P in OCLs and S1PR3 on OBs, to increase bone formation in PD.

**KEYWORDS**

bone formation, osteoclasts, Paget’s bone disease, S1P, S1PR3, SphK-1

1 | INTRODUCTION

Paget’s disease (PD) is characterized by increased numbers of abnormal osteoclasts (OCLs) that drive exuberant bone formation, but the mechanisms responsible for the increased bone formation remain unclear.1–3 Both environmental and genetic factors have been implicated in the etiology of PD.4 We previously reported that OCLs from the majority of PD patients we studied express measles virus nucleocapsid protein (MVNP), and that transgenic mice with targeted expression of MVNP in OCLs (MVNP mice) develop bone lesions and abnormal OCLs characteristic of PD. MVNP expression in OCLs from PD patients or MVNP mice induced high levels of interleukin-6 (IL-6) in OCLs,5,6 which was required for MVNP mice to develop pagetic-like bone lesions that demonstrated highly localized increased bone resorption and formation.7,8 Although these results suggest that the high IL-6 levels in OCLs expressing MVNP increased local bone formation, it was unclear if the effects of IL-6 on bone formation in PD were direct or mediated indirectly via other factors. More recently, we found that the high levels of IL-6 in OCLs from PD patients and MVNP mice induced increased levels of insulin-like growth factor 1 (IGF-1) and ephrinB2 in OCLs and increased EphB4 expression on osteoblasts (OBs).9 IGF-1 enhanced OB differentiation in MVNP mice.9 However, it is unknown if additional OCL-derived factors also play a role in the exuberant bone formation characteristic of PD.

Sphingosine-1-phosphate (S1P) has fundamental functions in development, immunity, and as recently recognized, bone metabolism.10–17 Extracellular S1P signals through G protein-coupled receptors, S1PR1–S1PR5.15 S1P is synthesized by ceramide deacylation followed by phosphorylation by sphingosine kinase 1 (SphK-1) and SphK-2. Its concentrations in vivo are tightly regulated by dephosphorylation by two S1P-specific phosphatases and three lipid phosphate phosphatases. S1P also undergoes irreversible degradation to phosphoethanolamine and 2-hexadecanal by a single enzyme, S1P lyase.12 S1P’s effects on bone homeostasis have been reported to be mainly through its effects on bone remodeling by regulating the migration of OCL progenitors in the circulation to the bone.10,11,16 Ishii et al.18,19 showed that an S1P gradient between bone and plasma regulates OCL precursor migration. S1P in the plasma attracts OCL progenitors away from the bone by binding S1PR1 on OCL precursors, while S1P binding to S1PR2 on OCL precursors inhibits OCL precursor migration from the bone.

Numerous studies reported that S1P could affect OB behavior. OCL-secreted S1P stimulates receptor activator of NF-κB-ligand (RANKL) production in OBs and promotes OB differentiation.20 Further, cathepsin K deletion in OCLs stimulates SphK-1 activity that increases S1P expression and bone formation,21 via S1P binding to S1PR3 on OBs.10 These results suggest that S1P plays an important role in regulating OCL and OB activity. However, the role of S1P in PD is unclear. Therefore, in this report, we investigated the role and regulation of S1P and S1PRs in OCL and OB differentiation in PD, using OCLs and OBs from MVNP mice, and bone samples from a PD patient and a normal donor.

2 | MATERIALS AND METHODS

2.1 | Antibodies and reagents

Runx2 (sc-10758), NFATc-1 (sc-7294, clone 7A6), EDG-3 target for S1PR3 (sc-30024, clone [H-70]), and α-tubulin (sc-58666, clone 4G1) antibodies were from Santa Cruz. Anti–osteosterix antibody (ab22552) was purchased from Abcam Inc. Antibodies recognizing phospho-Akt (Ser473) (#9271), Akt (#9272), phospho-Erk1/2 (Thr202/Tyr204) (#9101), Erk1/2 (#9102), and glyceraldehyde 3-phosphate dehydrogenase (#2118) on Western blots were purchased from Cell Signaling Technology. Anti-phospho-SphK1 (Ser225) antibody (LS-C26925) was purchased from Life Span Bioscience. Anti–SphK-1 antibody (#ABN435) and anti-collagen type1A (Col-1A) (#AB765P) were obtained from Millipore. VPC 23019 (Cat# 4195) was purchased from TOCRIS Bioscience and VPC24191 (857365 P) was purchased from Sigma-Aldrich.

2.2 | Generation of MVNP, MVNP/IL-6−/−, and IL-6−/− mice

Animal studies were approved by the Institutional Animal Care and Use Committees at Virginia Commonwealth
University and Indiana University School of Medicine. Transgenic mice expressing MVNP under the control of the mouse TRAP promoter were generated as previously described. Global IL-6−/− mice were generated by Kopf et al. and obtained from the Jackson Laboratory (stock number 002650). Mice were interbred with MVNP to generated MVNP/IL-6−/− mice. All data are from mice generated from this MVNP/IL-6−/− colony (not from the parental colonies).

2.3 Bone sections from a PD patient and a normal donor

Resin-embedded bone sections from a PD patient and a healthy donor were generously provided by Dr. David D. Dempster (Department of Clinical Pathology and Cell Biology, Columbia University, New York, NY, USA). A transiliac crest bone biopsy was taken from a 58-year-old female patient with PD and a 30-year-old female healthy control who were treated with calcine and tetracycline before sampling. The biopsy sample was embedded without decalcification in methyl methacrylate. The bone sections were then processed as previously described by Gomes et al. SphK-1 was detected with an anti-SphK-1 antibody (Life Span Bioscience) and S1PR3 was detected with an anti-S1PR3 antibody (Santa Cruz). Briefly, bone sections were deacylated in a 1:1 mixture of xylene and chloroform, rehydrated in graded alcohol solutions, and then decalcified with 1% acetic acid. After rinsing with distilled water, and treatment with 0.1% Tween 20 in phosphate-buffered saline, endogenous peroxidase activity was inhibited by a mixture of 3% hydrogen peroxide in methanol for 30 min, followed by two water washes. The samples were incubated with avidin-biotin solutions and with 5% normal serum from the same species of secondary antibody with 1% bovine serum albumin to block nonspecific bindings. Histologic evaluation was performed under bright-field microscopy. The detailed histological examinations of bone sections from this PD patient and normal donor were previously reported. Human studies were approved by the Indiana University Institutional Review Board.

2.4 Immunohistochemistry analyses

Femurs from male and female WT or MVNP mice (12 months of age) were fixed in 10% buffered formalin and completely decalcified in 10% ethylenediaminetetraacetic acid (EDTA) at 4°C for 20 days and embedded in paraffin. Five-micrometer longitudinal sections were cut and mounted on glass slides. Deparaffinized sections were treated with 1% horse serum for one hour, then primary antibodies for SphK-1 and S1P3 or control immunoglobulin G (IgG) (Cell Signaling) were added and the slides incubated overnight. The sections were then stained with anti-rabbit IgG conjugated to peroxidase (Vector Laboratories). The representative image was semiquantitated using ImageJ. (https://imagej.net/Colour_Deconvolution). Preliminary experiments showed that SphK-1 expression by OCLs or S1PR3 expression by OBs in bone sections from male versus female WT mice or male versus female MVNP mice did not differ significantly (Table S1). Therefore, marrow cells or bones from WT male and female or male and female MVNP mice were combined in the experiments below to minimize the number of mice used.

2.5 Preparation of bone lysates for protein analysis

Tibias and femurs were dissected from 12-month-old male and female WT and MVNP mice, frozen immediately in liquid nitrogen, and reduced to powder using a small mortar and pestle. Total proteins were extracted from WT or MVNP-derived bones in lysis buffer overnight at 4°C, the male or female WT or MVNP lysates combined and 25 μg of protein/lane subjected to Western blot analysis using appropriate antibodies.

2.6 Formation of mature OCLs in mouse bone marrow cultures

Bone marrow cells flushed from long bones of WT, MVNP, MVNP/IL-6−/−, or IL-6−/− mice were cultured with 10% fetal calf serum (FCS) in α-minimum essential medium (αMEM) in 10-cm dishes (2.5 × 10⁷ cells/dish) for overnight, then nonadherent marrow cells from these mice were harvested and enriched for CD11b+ mononuclear cells using the Miltenyi Biotec MACS (Magnetic Cell Sorting) system. These cells were incubated with hM-CSF (10 ng/ml) (R&D System) for 3 days (CFU-GM), then the cells treated with hRANKL (50 ng/ml) for 4 days as previously described. At the end of cultures, trypsin-EDTA (Corning) was added and cultures incubated for 3 min to remove nonosteoclastic cells and thereby enrich the concentration of OCLs in the cultures. The cultures were stained for TRACP, and TRACP-positive multinucleated cells (≥3 nuclei/cell) were scored as OCLs.

2.7 Isolation and culture of primary OB-precursors

After flushing the bone marrow from tibias of WT or MVNP mice, tibias were cultured in αMEM with 10% FCS for 7–10 days. The bones were then placed in 60-mm dishes, and the cultures continued in αMEM containing 10% FCS.
until cells growing out of the bones formed a confluent monolayer. The original bones were removed and the outgrowth cells from the bones were treated with 0.25% trypsin and 0.05% EDTA for 10 min at 37°C. These cells were used as primary OBs without further passage. The primary OBs (2 × 10^5 cells/well in six-well plate) were cultured in αMEM containing 10% FCS for 7–14 days.25 The cell lysates were collected and analyzed for protein expression.

### 2.8 Coculture of OCLs and OBs from WT and MVNP mice

OCLs (2.0 × 10^4/well) derived from marrow cultures of WT or MVNP-mice (as described in Section 2.6) were scraped with a rubber policeman, replated, and cultured with 50 ng/ml RANKL overnight. OBs (1.0 × 10^5/well) were plated on top of the OCLs the next day, and the cells cocultured for 72 h.

### 2.9 Treatment of primary OB precursors with OCL-CM in the presence or absence of a S1PR3 antagonist or agonist

Primary OB precursors (1 × 10^5/well) derived from WT- or MVNP-mice bones as described above were cultured in αMEM with 10% FCS containing 30% (v/v) of OCL-CM (conditioned for 48 h by OCLs from WT or MVNP mice) or with vehicle (αMEM) for 48 h. Then, an S1PR3 antagonist (10 µM) or agonist (10 µM) was added and the OB precursors cultured for 72 h.

### 2.10 Immunoblotting of OCL lysates from WT, MVNP, MVNP/IL-6^−/−, and IL-6^−/− mice

Total proteins were extracted from OCLs formed from OCL precursors derived from these genotypes, and the cell lysates (25 µg/lane) loaded on sodium dodecyl sulfate (SDS) gels using the Bio-Rad Mini-gel System (Bio-Rad Laboratories). The resolved proteins were transferred onto nitrocellulose membranes (TGX-Membrane; Bio-Rad) using Trans-Blot Turbo System Bio-Rad) and incubated in blocking solution (5% nonfat dry milk in TBST) for 1 h. Membranes were then exposed to primary antibodies overnight at 4°C and incubated with IgG horseradish peroxidase-conjugated antibody for 1 h. The blots were washed and visualized by an Immobilon Western Chemiluminescent detection system (Thermo Fisher Scientific). Protein expression levels were quantitated by densitometry using ImageJ software (NIH).

### 2.11 The measurement of phosphorylation of SphK-1 in OCLs

Preformed OCLs from WT or MVNP mice were cultured in αMEM + 10% FCS for 3 days. Cells were then starved by culturing in αMEM + 2% FCS for 24 h. Cells were then exposed to RANKL (50 ng/ml) for the indicated times and then lysed, fractionated by SDS-polyacrylamide gel electrophoresis, and analyzed by immunoblot using antibodies recognizing phosphorylated Sphk-1 and total SphK-1.

### 2.12 S1P enzyme-linked immunosorbent assay (ELISA) assays

OCLs (2.0 × 10^4/ml) from WT and MVNP mice were isolated as described above and cultured with RANKL for 4 days. Conditioned media (CM) from these cultures were harvested at the end of the culture period, and the concentration of S1P was determined using an ELISA kit for mouse/human S1P (Echelon Biosciences, Inc.) according to the manufacturer’s instructions.26 The assay was calibrated based on a standard curve using the S1P protein that was included in the ELISA kit. The results were determined by subtracting the S1P level of the culture media containing serum without cultured cells.

### 2.13 Statistical analysis

Significance was evaluated using a two-tailed Welch’s t test. When more than two treatment groups were compared, a one-way analysis of variance with Tukey’s test for repeated measures was used. Differences with p < .05 were considered significant.

### 3 RESULTS

#### 3.1 SphK-1 expression in OCLs from MVNP mice and a patient with PD disease

Twelve months old mice were used for these studies because OCLs from MVNP mice at that age and older consistently express a Pagetic phenotype in vitro.5–9 Therefore, we determined SphK-1 expression levels in bone extracts (tibia and femur) from 12 months old WT and MVNP mice. SphK-1 was detected in both WT and MVNP mice, and was increased 2.6-fold in MVNP mice compared with WT (Figure 1A). We next determined SphK-1 expression in distal femur sections from 12 months old WT and MVNP mice by immunohistochemistry. We found that SphK-1 expression was increased in OCLs from
FIGURE 1  SphK-1 expression is increased in bone lysates and OCLs from MVNP mice and a patient with Paget’s disease. (A) SphK-1 expression in bones from 12-month-old of WT or MVNP mice. The bone extracts from the femur and tibia of one female and one male WT or MVNP mouse were combined and loaded on SDS gels, and SphK-1 expression analyzed by Western blot analysis using an anti-SphK-1 antibody. α-Tubulin was used as the loading control. The relative expression levels of SphK-1 expressed compared with α-tubulin with the basal ratio of SphK-1/α-tubulin for WT mice set at 1.0. Results are the mean ± SEM from three biological replicates. (B) SphK-1 expression in OCLs in the distal femur from 12 months old WT and MVNP mice. Decalcified sections from femurs were stained with anti-SphK-1 antibody. The upper panels show low power views and the lower panels show high power views. The arrow indicates the OCLs. Immunostaining for anti-SphK-1 is brown. All scale bars represent 10 µm. (C) SphK-1 expression in OCLs of transiliac crest bone sections from a normal donor and a patient with Paget’s disease. The upper panels show low power views and the lower panels show high power views. The arrow indicates the OCLs. Immunostaining for anti-SphK-1 is brown. All scale bars represent 10 µm. MVNP, measles virus nucleocapsid protein; OCLs, osteoclasts; PD, Paget’s disease; SphK-1, sphingosine kinase-1; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; WT, wild type
MVNP mice compared with WT mice (Figure 1B). We semiquantified the Shp-K-1 staining in randomly selected 20 OCLs in three sections from the individual WT and MVNP mice. The OCLs from MVNP mice displayed a 2.4-fold higher staining level than WT (p < .01 two-tailed Welch’s t test). We next examined Shp-K-1 expression in transiliac crest bone sections from a normal donor and a PD patient by immunohistochemistry. We found that Shp-K-1 expression was increased in OCLs from the PD patient compared with the normal donor (Figure 1C).

### 3.2 SphK-1 expression is increased in OCLs from MVNP mice

We then determined SphK-1 expression levels in purified OCLs derived from MVNP or WT mouse bone marrow cultures. SphK-1 expression was increased 1.5-fold in OCLs from MVNP mice compared with WT mice (Figure 2A). Further, S1P levels in media conditioned by purified OCLs for 3 days showed that OCLs from MVNP mice released 1.5-fold more S1P compared with WT OCLs (Figure 2B).

### 3.3 IL-6 increases SphK-1 expression in OCLs from MVNP mice

OCLs from patients with PD express high levels of IL-6, and MVNP expression in OCLs induces high IL-6 expression levels that are essential for the formation of pagetic-like OCLs and bone lesions in MVNP-expressing mice. However, it is unknown if IL-6 increases SphK-1/S1P expression, which would in turn increase the S1P levels in MVNP mouse. We found that IL-6 treatment (10 ng/ml) of OCLs derived from CD11b+ OCL precursors from WT and MVNP mice increased SphK-1 expression in WT OCLs and in MVNP-expressing OCLs by 1.5-fold compared with vehicle-treated cultures (Figure 3A). Since the loss of IL-6 prevents the development of pagetic OCLs and pagetic bone lesions in MVNP mice, we tested if IL-6 regulates SphK-1 expression in OCLs by measuring SphK-1 expression in OCLs from

![Figure 2](link-to-figure-image)

**Figure 2** Expression levels of SphK-1 and S1P in OCLs derived from WT- or MVNP-mice bone marrow cultures. (A) SphK-1 expression in lysates of OCLs derived from cocultures of equal numbers of male and female marrow cells from one male and one female WT or one male or female MVNP mouse was determined by Western blot analysis as described in Materials and Methods. The basal ratios of SphK-1/GAPDH levels for OCLs from WT mice were set at 1.0. Results are the mean ± SEM from three biological replicates. The data were analyzed using a two-tailed Welch’s t test. (B) S1P levels in media conditioned for 3 days by purified OCLs (2.0 × 10⁴/ml) from cultures of marrow cells from 12 months old mice as described above. S1P levels were determined as described in Materials and Methods. The results are shown as the mean ± SEM for four biological replicates. The data were analyzed using a two-tailed Welch’s t test. ELISA, enzyme-linked immunoabsorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MVNP, measles virus nucleocapsid protein; OCLs, osteoclasts; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; S1P, sphingosine-1-phosphate; SphK-1, sphingosine kinase-1; WT, wild type
WT, MVNP, MVNP/IL-6−/−, and IL-6−/− mice. SphK-1 expression in lysates of OCLs from MVNP/IL-6−/− and IL-6−/− mice was significantly decreased compared with WT and MVNP-mouse lysates (Figure 3B). Loss of IL-6 also decreased the expression of phospho-SphK1 in RANKL-treated OCLs derived from WT and MVNP mice (Figure 3C).

### 3.4 | S1PR3 expression in OBs from WT and MVNP mice

We then examined S1PR3 expression on OBs from WT and MVNP mice. We found that S1PR3 was increased in OBs from a PD patient compared with a normal donor (Figure 4A). Further, when we examined S1PR3 expression in OBs from WT and MVNP mouse, the OBs in 12 months old MVNP mice were more strongly stained (Figure 4B). We semiquantified the S1PR3 staining in 30 randomly selected OBs from each section from three WT and MVNP mice. The OBs from MVNP mice had 2.5-fold higher stain than WT (p < .01 two-tailed Welch’s t test). We then determined S1PR3 expression levels in bone extracts (tibia and femur) from 12 months old WT and MVNP mice. S1PR3 expression was increased by twofold in bone extracts of MVNP-mice compared with WT (Figure 4C). To determine if these results reflect S1PR3 expression on OBs in the bone, we then examined the expression levels of S1PR3 in purified OBs and OCLs from WT and MVNP mice. OBs from WT and MVNP mice expressed similar levels of S1PR3, but S1PR3 expression in OCLs of WT and MVNP mice was very low compared with OBs (Figure 4D). Although S1PR3 in OBs was detectable in both WT and MVNP mice, only very low levels of S1PR1 and S1PR2 were found in OBs from both genotypes (data not shown). Because S1PR3 was increased in OBs in bone sections from a PD patient and MVNP mice, we then examined S1PR3 expression levels in cocultures of WT-OCLs/WT-OBs, WT-OCLs/MVNP-OBs, MVNP-OCLs/WT-OBs, and MVNP-OCLs/MVNP-OBs. As expected, cocultures of OCLs/OBs from MVNP mice induced the highest S1PR3 levels on OBs compared with other coculture combinations (Figure 4E).

We next determined if IL-6 or IGF-1 induced S1PR3 expression in the OCLs/OBs cocultures since IL-6 and IGF-1 are produced by MVNP-OCLs. Western blot analysis of OBs from bone explants of WT and MVNP mice showed that S1PR3 expression was higher in OBs from MVNP mice and was further increased in MVNP-OBs by either IL-6 (10 ng/ml) or IGF-1 (10 ng/ml). In contrast, IGF-1 had no effect on S1PR3 expression by WT-OBs (Figure 4F), consistent with the minimal increase in S1PR3 expression in cocultures of MVNP-OCL with WT-OBs.

### 3.5 | S1P induced the expression of differentiation markers in OBs from WT and MVNP mice

We then determined if S1P induces OB differentiation in WT and MVNP OBs by treating OB precursors from WT and MVNP mice with S1P for 3 days. S1P (10 μM) increased Runx2 expression in OBs from MVNP mice approximately twofold compared with controls without S1P treatment (Figure 5A). Since S1P enhanced Runx2 expression in OBs derived from WT and MVNP mice, we assessed if S1P activates MAPK and PI3K to induce OB differentiation and bone formation. As shown in

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**FIGURE 3** IL-6 increases SphK-1 expression in OCLs of WT and MVNP mice. (A) Effects of IL-6 treatment on SphK-1 expression in mature OCLs. Preformed OCLs derived from cocultures of CD11b (+) bone marrow cells from one female and male WT or MVNP mouse were cultured with/without 10 ng/ml of IL-6 for 48 h. The cell lysates were then loaded onto SDS gels, and SphK-1 expression analyzed as described in Materials and Methods. Results are the mean ± SEM from three biological replicates. The data were analyzed using ANOVA with Tukey’s test. (B) SphK-1 expression in bone extracts from 12 months old WT, MVNP, MVNP/IL-6−/−, and IL-6−/− mice. Bone extracts derived from the femurs and tibia of one female and male mouse of each genotype were loaded onto SDS gels, and SphK-1 expression analyzed as described in Materials and Methods. Results shown are representative of those from three biological replicates. The ratio of SphK1/α-tubulin was evaluated by ImageJ. The basal ratio of SphK-1/α-tubulin in WT mice was set at 1.0. The data were analyzed using one-way ANOVA with Tukey’s test. (C) Effects of RANKL on SphK-1 phosphorylation in OCLs from 12 months old WT, MVNP, MVNP/IL-6−/−, and IL-6−/− mice. OCLs precursors from these mice were incubated with RANKL (50 ng/ml) for 5 or 30 min. and Phospho-SphK-1 was measured in cell lysates as described in Materials and Methods. These experiments were performed by combining lysates derived from one female and male mouse of each of the four genotypes. The results of the experiment shown were similar in three biological replicates. *p < .01 compared with 0 min treatment of OCLs precursors from each genotype. The data were analyzed using ANOVA with Tukey’s test. ANOVA, analysis of variance; CD, cluster of differentiation; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-6, interleukin-6; MVNP, measles virus nucleocapsid protein; OCLs, osteoclasts; SEM, standard error of the mean; SphK-1, sphingosine kinase-1; WT, wild type
In contrast, VPC24191 increased osterix levels in MVNP-OB treated with WT-OCL-CM and MVNP-OCL-CM (p < .01) (Figure 6A). As expected, VPC24191 enhanced Col-1A expression to a greater extent in MVNP-OBs treated with MVNP-OCL-CM compare with vehicle treatment (p < .01) (Figure 6B).

### 4 | DISCUSSION

We previously showed that MVNP expression in OCLs from MVNP mice and PD patients increases IL-6 production, which upregulates expression of ephrinB2 and IGF-1 in OCLs and EphB4 in OBs. These results suggest that enhanced coupling factor expression and increased IGF-1 production by pagetic OCLs may contribute to the rapid bone formation that occurs in PD. To determine if additional OCL-derived factors contributed to the increased bone formation in PD, we tested if S1P was also involved because OCL-derived S1P was recently reported to increase bone formation by binding the S1PR3 on OBs.

We found that SphK-1 and S1P production was increased in MVNP-expressing OCLs (Figures 1 and 2). Further, S1P production by MVNP-OCLs was IL-6-dependent since SphK-1 expression was markedly decreased in OCLs from MVNP/IL-6−/− mice (Figure 3). Thus, S1P production was regulated by the autocrine/paracrine
effects of OCL-IL-6, and decreasing OCL number and activity in PD should reduce S1P secretion in vivo. These results further support our previous findings that increased IL-6 in PD-OCLs plays a critical role in the enhanced OCL and OB activities in PD since IL-6 produced by MVNP-OCLs increased S1P production as well as expression of ephrinB2 and IGF-1 production by OCL and EphB4 by OB.

Additionally, immunohistochemical analysis of bones from a normal donor, a PD patient, WT and MVNP mice showed that OBs from the PD patient and MVNP mice expressed high levels of S1PR3 in vivo (Figure 4A,B), and that bone lysates from MVNP mice had increased expression of S1PR3 (Figure 4C). Our coculture studies of OCLs and OBs supported these in vivo findings and showed that OCLs from MVNP mice enhanced S1PR3
expression levels in OBs from MVNP but not WT mice (Figure 4E). As shown Figure 4D, S1PR3 levels in WT and MVNP mice OBs were similar, but S1PR3 expression was increased by treatment of MVNP but not WT OBs with IL-6 or IGF-1 (Figure 4F). These results suggest that OCL-derived IGF-1 induced by IL-6 also increased S1PR3 in addition to their potential effects on OB differentiation via Erk and Akt pathways (Figure 5B). The inability of IL-6 or IGF-1 to increase S1PR3 in WT OBs suggests that additional factor(s) in vivo may prime OBs in MVNP mice to respond to IL-6 and/or IGF-1 to increase S1PR3 expression in PD-OBs. The identity of this factor(s) is currently unknown.

Since increased S1PR3 mediated signaling via increased S1P and S1PR3 may contribute to the enhanced OB differentiation in MVNP mice, we determined if treating OBs from WT and MVNP mice with WT or MVNP-OCL-CM, which contained S1P (as shown in Figure 2B) in the presence of S1PR3 antagonist (VPC23019) or agonist (VPC24191) could block or enhance the effect of OCL-CM on the expression of osterix and Col1A, factors required for bone formation. The S1PR3 agonist-stimulated expression of these markers of OB differentiation in MVNP-OB to a greater extent than WT-OB treated with OCL-CM from MVNP mice. In contrast, the S1PR3 antagonist inhibited the expression of these OB differentiation markers in WT-OB to a greater extent than in MVNP-OB (Figure 6). These results are consistent with our hypothesis that S1P increases new bone formation in PD patients expressing MVNP in OCLs, and that enhanced OB differentiation is due in part to increased levels of S1PR3 on OB precursors. A recent study by Keller et al. supports our results that S1P from OCLs can regulate bone formation. They showed that calcitonin negatively regulates bone formation by inhibiting the release of the anabolic bone factor, S1P, from OCLs.

Our current model for the potential action of S1P in PD is depicted in Figure 7. IL-6 produced by MVNP-expressing OCLs increases S1P production by OCLs as well as in combination with IGF-1 promotes S1PR3 expression on OBs to increase bone formation. In this model, IL-6 increases ephrinB2, IGF-1, and SphK-1/S1P in MVNP mice. These results suggest that OCL-IL-6...
contributes to bone formation in PD by inducing OCL-IGF1, which enhances bone formation via the upregulation of ephrinB2/EphB4 in OCLs and OBs, respectively.\textsuperscript{9,27} and increases Shpk1/S1P/S1PR3 in OCLs and OBs in MVNP mice. These results further suggest that antagonists of S1PR3 may be useful to control the increased bone formation in patients with PD, who are unable to receive bisphosphonates. Our results also support recent reports by Weske et al.\textsuperscript{28} who suggested that S1P-based drugs could be a promising anabolic treatment for bone loss.

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**CONFLICT OF INTERESTS**

The authors declare that there are no conflict of interests.

**AUTHOR CONTRIBUTIONS**

*Designed the study, interpreted the data, and wrote the manuscript:* G. David Roodman and Noriyoshi Kurihara.  
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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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