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14. ABSTRACT

Paget's disease (PD) of bone occurs in 3-4% of population over the age of 50. We have identified expression of measles virus nucleocapsid transcripts in osteoclast (OCL) precursors and that MVNP expression induces pagetic phenotype in osteoclasts with increased bone resorption activity as seen in patients with Paget's disease. We previously cloned and identified osteoclast inhibitory peptide-1 (OIP-1/hSca) which inhibits osteoclast formation and bone resorption. We hypothesize that MVNP expression in osteoclast precursors modulates RANK receptor signaling leading to Pagetic OCL development. OIP-1 blocks these signaling events and inhibits MVNP induced osteoclastogenesis and elevated bone resorption activity. We demonstrated that MVNP increases TNF-alpha induced OCL differentiation and activation by increasing NF-kB signaling through increased expression of p62, and IKK-gama and increased MAPK signaling. Our results also suggest that MVNP's effects on TNF-alpha signaling contribute to the increased OCL formation in PD. Furthermore, expression of MVNP gene in OCL in vivo induces a pagetic-like phenotype. RANKL stimulation of OIP-1 mice derived bone marrow cells resulted in significantly decreased osteoclast formation. Furthermore, OIP-1 transgenic mouse bones demonstrated an osteopetrotic phenotype. These data suggest that OIP-1 is an important physiologic regulator of osteoclast development and bone resorption in vivo and may have therapeutic utility to control excess bone turnover in patients with Paget's disease.

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INTRODUCTION:

Paget's disease affects approximately 2-3 million people in the United States and is the second most common bone disease after osteoporosis. We shown that bone marrow cells from patients with Paget's disease express measles virus nucleocapsid protein (MVNP) transcripts and further demonstrated that expression of the Edmonston MVNP gene in normal osteoclast (OCL) precursors results in formation of OCL that share many of the characteristics of OCL from Paget's patients. The MVNP gene contained several sense mutations, which constituted 1% of the nucleotide sequence. The pathologic significance of MVNP and associated mutations to induce abnormal OCL formation and activity in Paget's disease, is unknown (1). RANKL is a member of Tumor necrosis factor (TNF) family member that is expressed on stromal/osteoblast cells and RANK receptor is expressed on committed osteoclast precursor cells. RANKL/RANK signaling is critical for osteoclast differentiation and bone resorption activity in vitro and in vivo (2,3). We have recently cloned and identified the Ly-6 family member, osteoclast inhibitory peptide-1 (OIP-1/hSca) which inhibits osteoclast formation and bone resorption activity. We have further demonstrated that OIP-1 significantly inhibits TNF receptor associated factor-2 (TRAF-2) and c-Jun kinase activity in osteoclast precursor cells (4). Our hypothesis is that MVNP expression in osteoclast precursors modulates the status of RANK receptor signaling molecules leading to Pagetic OCL development in Paget's disease. OIP-1 blocks RANK receptor signaling events and inhibits MVNP induced osteoclastogenesis and elevated bone resorption activity in Paget's patients.

BODY:

The final progress report of work is as follow:

Task 1. Determine the sensitivity of MVNP transduced osteoclast precursors to RANK Ligand (RANKL) and TNF-alpha stimulation to form pagetic osteoclasts (Months 1-24): We identifid that RANKL enhanced MVNP stimulation of osteoclastogenesis in a dose-dependent manner. MVNP transduced osteoclast precursors resulted in formation of pagetic osteoclasts. The nuclear number in the MVNP transduced osteoclasts were increased significantly. Similarly, MVNP transduced normal human CFU-GM showed increased responsivity to TNF-alpha approximately two-fold. Human bone marrow derived osteoclast precursor cells (CFU-GM) cells were cultured with and without MVNP transduction. TNF-alpha stimulation further enhanced by a two-fold increase in osteoclast precursor growth in MVNP transduced cells. We did not detect a significant effect of OPG on MVNP or TNF stimulated osteoclast precursor growth or osteoclast development in MVNP enhanced osteoclast formation. We observed a significant increase in the levels of IL-6 in the conditioned media obtained from MVNP transduced cultures in the presence of RANKL. We did not see significant effect of TNF-alpha to enhance IL-6 levels in MVNP transduced osteoclast precursor cells in the presence/absence of RANKL. These results suggest that MVNP transduction will increase IL-6 production significantly and that IL-6 may play an important role in MVNP stimulated osteoclast formation.

Task 2. Determine the RANK receptor signaling in MVNP transduced osteoclast precursors (Months 24-36). Our results revealed that MVNP transduction into human bone marrow derived osteoclast precursors significantly increased the levels of c-Fos and NFATc1 transcription factor expression. In contrast, there was no significant change in the levels of TRAF2 adaptor protein expression levels in RANKL or TNF-alpha stimulated osteoclast precursor cells. Human bone marrow derived non-adherent mononuclear cells transduced MVNP identified significant increase (2.5 fold) in the levels phospho-c-jun levels in response to RANKL stimulation. TRAP-MVNP transgenic mouse bone marrow stimulated with RANKL resulted in significantly increased in number and size of osteoclasts compared to wild-type mice. Furthermore, MVNP derived from paget's patient also demonstrated similar results. These data suggest that MVNP induce pagetic phenotype in osteoclasts. We have also identified high levels expression of kininogen (KNG) in serum samples from patients with paget's disease. KNG had no significant effect on osteoclast stimulation and did not enhance RANKL expression in marrow stromal/preosteoblast cells. However, it does enhance survival of these cells. Transfection of CFU-GM from wild-type (WT) and TRAP-$p62^{P392L}$ mice with the MVNP gene further increased levels of NF-κB in TRAP-$p62^{P392L}$ osteoclast precursors compared to WT precursors. Expression of MVNP in osteoclast precursors from WT or TRAP-$p62^{P392L}$ mice did not increase expression of c-fos. These data suggested that p62 contributes to significantly increased NF-kB activation during osteoclast differentiation in patients with paget's disease.

Task 3. Determine the effects of OIP-1 on MVNP altered RANK receptor signaling in osteoclast precursor cells (Months 29-48). We have previously cloned and characterized the osteoclast inhibitory peptide-1 (OIP-1) (Ref.4). We identified that OIP-1 inhibited MVNP stimulated osteoclast differentiation in human bone marrow cultures in a dose-dependent manner. Similarly, OIP-1 significantly inhibited bone resorption capacity of MVNP stimulated osteoclasts cultured on dentine slices. We further identified that both wild-type *p62* and mutant *p62P392L* transduced OCL precursors formed significantly larger osteoclasts compared to empty vector (EV) transduced cells. However, our results concluded that mutation of the *p62* gene increases osteoclastogenesis but do not induce Paget disease. We have characterized transgenic mice targeted with OIP-1 expression to the cells of osteoclast linage using the mouse tartrate resistant acid phosphatase (TRAP) gene promoter. The OIP-1 mice demonstrated osteopetrotic bone phenotype. OIP-1 transgenic mouse lines #5 and #13 derived cells showed a significant decrease in the number of CFU-GM colonies by 35% and 41% respectively, compared to control mice (Fig.1). The number of OCLs formed in OIP-1 #5 and OIP-1 #13 mouse bone marrow cultures was significantly decreased by 39% and 42% respectively, compared to control mice in response to RANKL (100 ng/ml) treatment (Fig.2). Western blot analysis of total cel lysates obtained from osteoclast progenitor cells revealed that OIP-1 did not affect the levels of RANK receptor expression in these cells. However, there is a significant increase (2.5 fold) in the levels of RANK adaptor protein TRAF2 expression in MVNP transduced wild-type mouse preosteoclast cells, but not TRAF 6. OIP-1 mice derived preosteoclast cells demonstrated no significant increase in the levels of TRAF2 in response to MVNP expression. In addition, transcription factors such as c-Fos, NFATc1 critical for OCL

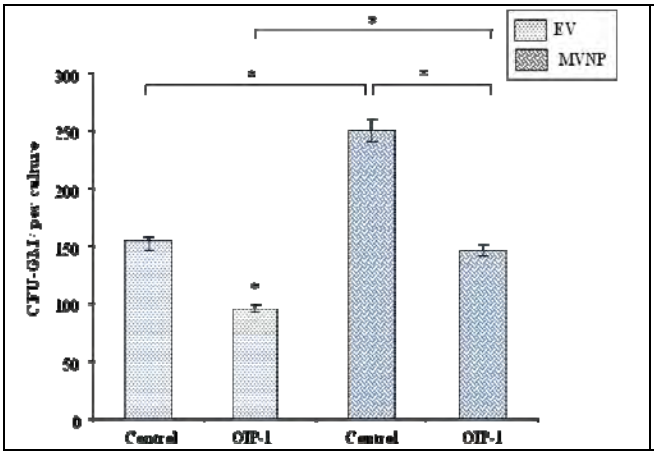

Fig.1. OIP-1 inhibits MVNP stimulated CFU-GM formation in human bone marrow cultures. Non-adherent human (n=6) bone marrow cells (4×105/ml) transdued with MVNP or empty vector (EV) were cultured with GM-CSF (10 ng/ml) in the presence or absence of OIP-1 (100 ng/ml) in methyl cellulose to form CFU-GM colonies. At the end of a 7 day culture period, CFU-GM colonies formed in these cultures were scored using a light microscope. The results represent quadruplicate cultures of five independent experiments and data shown as mean ± SD, ($p < 0.05$).

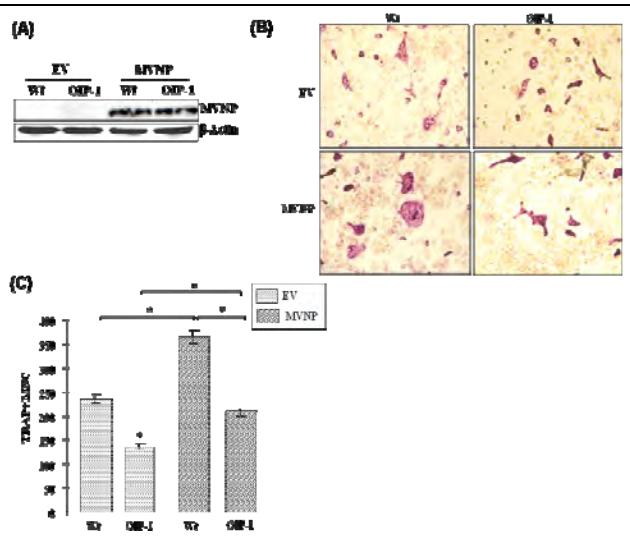

Fig.2. Osteoclast formation in wild-type (Wt) and OIP-1 transgenic mouse bone marrow cultures transduced with MVNP or empty vector (EV). (A) The non- adherent bone marrow cells from Wt and OIP-1 mice were transduced with EV or MVNP retroviral vectors as described in the methods and cultured with 10 ng/ml mM-CSF, 100 ng/ml mRANKL for 2 days. Total cell lysates were prepared from these preosteoclast cells and MVNP expression was confirmed by Western blot analysis using rabbit anti-MVNP antibody. (B) Bone marrow cells from Wt and OIP-1 transgenic mice were transduced with MVNP or EV and cultured for OCL formation in the presence of 10 ng/ml mM-CSF and mRANKL (100 ng/ml) for 4 days. At the end of the culture period the cells were fixed and stained for TRAP activity. (C) The TRAP (+) multinucleated cells (MNC) formed were scored under a light microscope. The results represent quadruplicate cultures of three independent experiments and data shown as mean ± SD, ($p < 0.05$).

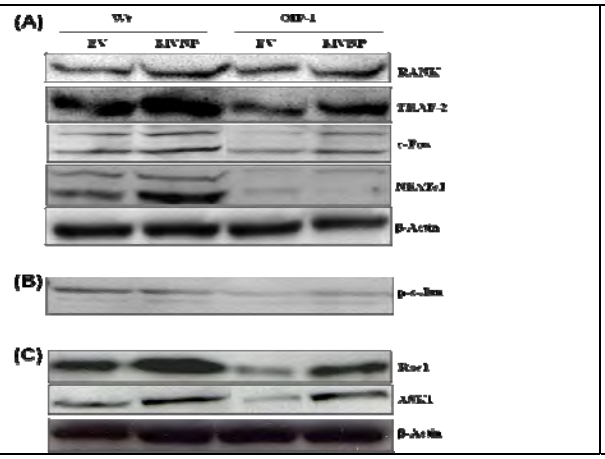

Fig.3. Western blot analysis of RANK receptor signaling molecules in MVNP transduced OIP-1 mice derived preosteoclast cells. Bone marrow cells from Wt and OIP-1 mouse were transduced with MVNP or EV and cultured in the presence of 10 ng/ml mMCSF and 100 ng/ml mRANKL for 2 days and the total cell lysates prepared were subjected to Western blot analysis for (A) RANK receptor signaling molecules. (B) phosphor-c-Jun levels (C) Rac1 and ASK1 expression.

differentiation were significantly decreased in OIP-1 transgenic mice derived preosteoclast cells compared to wild-type mice (Fig.3A). OIP-1 mouse derived preosteoclast cells transduced with MVNP showed a significant inhibition of JNK phosphorylation in response to RANKL stimulation compared to wild-type mice (Fig.3B). Furthermore, the JNK activators such as Rac1 and ASK1 expression was also inhibited in the OIP-1 derived preosteoclast cells stimulated with MVNP (Fig.3C). These results suggest that OIP-1 inhibits MVNP stimulated RANK-RANKL signaling during osteoclast differentiation in vivo.

KEY RESEARCH ACCOMPLISHMENTS:

- We have identified MVNP transduction will enhance RANK signaling molecules such as c-Fos and NFATc1 during osteoclast differentiation.
- We have shown that P62 enhance NF-kB activation during osteoclast differentiation.
- P62 contributed to enhanced NF-kB activation in pagetic osteoclasts.
- We have developed MVNP retroviruses expression constructs for transduction into osteoclast precursor cells.
- We have identified MVNP transduction will enhance IL-6 production by the osteoclast precursor cells.
- We have shown anti-IL-6 significantly decrease MVNP stimulated osteoclastogenesis.
- We identified that OIP-1 expression significantly decreased MVNP enhanced levels of TRAF2, c-Fos, p-c-Jun and NFATc1. However no change in the levels of RANK and TRAF6 expression in RANKL stimulated mouse bone marrow cultures.
- Our data suggest that OIP-1 may have therapeutic utility against excess bone resorption activity in Paget's disease.

REPORTABLE OUTCOMES:

Published articles relevant to the proposal:

1. **Reddy SV**. Etiologic factors in Paget's disease of bone. Cellular Molec. Life Sci. 63:391-398, 2006.
2. Tsuruga E, Rao DS, Baatz JE and **Reddy SV**. Elevated serum Kininogen in patients with Paget's Disease of Bone: A role in marrow stromal/preosteoblast cell proliferation. J Cell. Biochem. 98:1681-88, 2006.
3. Kurihara N, Zhou H, **Reddy SV**, Garcia Palacios V, Subler MA, Dempster DW, Windle JJ, Roodman GD. Expression of measles virus nucleocapsid protein in osteoclasts induces Paget's disease-like bone lesions in mice. J Bone Miner. Res. 21:446-55, 2006.
4. **Reddy SV**. Etiology of Paget's disease and osteoclast abnormalities. J. Cellular Biochem., 93:688-696, 2004.

5. Kurihara N, Hiruma Y, Zhou H, Subler MA, Dempster DW, Singer, FR, **Reddy SV**, Gruber HE, Windle JJ and Roodman GD. Mutation of the sequestosome-1 gene (p62) increases osteoclastogenesis but does not induce paget disease. J Clin Invest. 117: 133–142, 2007.
6. Srinivasan S, Irie K, Musselwhite C, Key Jr, LL, Ries WL and **Reddy SV**. Transgenic mice with OIP-1/hSca over-expression targeted to the osteoclast lineage develop an osteopetrosis bone phenotype. J Pathol. 213:420-428, 2007.
7. Srinivasan S, Youssef RF, Pati P, Ries WL, Rao DS and **Reddy SV**. Osteoclat inhibitory peptide-1 (OIP-1) inhibits measles virus nucleocapsid protein stimulated osteoclast formation/activity. J Cell. Biochem. 104:1500–1508, 2008.

CONCLUSIONS:

In conclusion, our results demonstrate that MVNP significantly enhanced RANK receptor signaling molecules such as c-Fos and NFATc1. Thus, OIP-1 inhibits MVNP stimulated Pagetic osteoclast formation/activity through suppression of RANK signaling. OIP-1 may have therapeutic utility against excess bone turnover associated with Paget's disease.

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APPENDICES:
Reprints of all articles published during total grant period are enclosed as listed under reportable outcomes.

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Cellular and Molecular Life Sciences

Visions & Reflections

Etiologic factors in Paget's disease of bone

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Abstract. Paget's disease of bone is a chronic focal skeletal disorder characterized by increased bone resorption by the osteoclasts. Paramyxoviral gene products have been detected in pagetic osteoclasts. Paget's disease is an autosomal dominant trait with genetic heterogeneity. Several mutations in the ubiquitin-associated (UBA) domain of sequestosome 1 (SQSTM1/p62) have been identified in patients with Paget's disease. Similarly, mutations in the valosin-containing protein (VCP) gene have been shown to cause inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia. In addition, gene polymorphisms and enhanced levels of cytokine/growth factors associated with Paget's disease have been identified. However, the etiologic factors in Paget's disease remain elusive. A cause and effect relationship for the paramyxoviral infection and SQSTM1/p62 gene mutations responsible for pagetic osteoclast development and disease severity are unclear. This article will highlight the etiologic factors involved in the pathogenesis of Paget's disease.

Key words. Paget's disease; osteoclast; measles virus; sequestosome (p62); RANK ligand (RANKL).

Paget's disease of bone is a chronic focal skeletal disease that affects 2–3% of the population over the age of 60, with an increased incidence in Caucasians. The disease is associated with deformity and enlargement of single or multiple bones, among which the skull, clavicles, long bones and vertebral bodies are the most frequently involved [1]. Patients with Paget's disease are frequently asymptomatic, but approximately 10–15% have severe symptoms including bone pain, fractures, neurological complications due to spinal cord compression or nerve entrapment syndromes, deafness, and dental abnormalities. Paget's disease is a highly localized disease, and new lesions rarely develop during the course of the disease. It can be monostotic or polyostotic and the bone lesions continue to progress in size if untreated. Studies have also indicated that patients with Paget's disease have an increased incidence of osteosarcoma, approximately 1% of them developing osteosarcoma in an affected bone. Paget's disease has a very unusual geographic distribution, with an increased incidence in Caucasians of European descent, but it also occurs in African Americans. It is rare in those of Asian descent. Studies have also suggested high prevalence rates of radiographic Paget's disease in Britain, Australia, North America and western Europe. The incidence of Paget's disease appears to have been decreasing over the last several decades [2, 3], but the basis for this decrease is unknown.

Familial expansile osteolysis (FEO) is a rare disease related to Paget's disease, but occurs in patients at a much younger age and is a much more severe disease linked to activating mutations in the gene encoding the receptor activator of nuclear factor κB (RANK) on chromosome 18q [4].

Juvenile Paget's disease is characterized by widespread involvement of the skeleton, distinguishing it from Paget's disease of adults. It is caused by a homozygous deletion of the gene on chromosome 8q24.2 that encodes osteoprotegerin (OPG), a member of the tumor necrosis factor receptor family [5].

The primary pathologic abnormality in patients with Paget's disease is increased bone resorption, followed by abundant new bone formation that is disorganized and of

poor quality. Paget's disease has been described as a slow paramyxoviral infection process, suggesting a viral etiology for the disease. Familial incidence is common in Paget's disease and 40% of patients with the disease have an affected first-degree relative. Familial Paget's disease has an equal incidence in males and females. It is also evident that genetic factors play an important role in the familial and sporadic forms of Paget's disease of bone. Genetic linkage analysis has further indicated that Paget's disease is an autosomal dominant trait with genetic heterogeneity and incomplete penetrance. In this review, viral, genetic, and other etiologic factors that play an important role in the pathogenesis of Paget's disease of bone will be discussed.

Paramyxoviral etiology

A viral etiology has been proposed for Paget's disease due to an initial description of nucleocapsid-like structures in the nuclei and cytoplasm of pagetic osteoclasts by electron microscopy [6]. Immunocytochemical studies further confirmed that these nuclear inclusions cross-reacted with antibodies that recognized measles virus (MV) or respiratory syncytial virus (RSV) nucleocapsid antigens [7]. In situ hybridization techniques also identified the presence of MV messenger RNA sequences in up to 90% of osteoclasts and other mononuclear cells in pagetic bone specimens. Similarly, canine distemper virus (CDV) nucleocapsid antigens were also detected in osteoclasts from patients with Paget's disease [8]. These paramyxoviral-like nuclear inclusions are not unique to Paget's disease and were reported in patients with FEO and rarely in patients with osteopetrosis, pycnodysostosis, otosclerosis, and oxalosis [9]. This has raised the possibility that the virus may be a non-etiologic agent in a cell altered by a genetic defect. Alternatively, there may be sequence homologies between viral and cellular proteins. Since paramyxoviruses are RNA viruses, it is most unlikely that part of the viral genome is integrated into the genome of the affected population.

We have previously identified the expression of MV nucleocapsid (MVNP) transcripts in freshly isolated bone marrow cells from patients with Paget's disease. These MVNP transcripts contain mutations which resulted in amino acid substitutions clustered at the C-terminal end [10]. The mutations occured at a 1% rate in the total MVNP gene isolated from a patient with Paget's disease. We further demonstrated that osteoclast precursors, the granulocyte macrophage colony-forming unit (CFU-GM), as well as mature osteoclasts from patients with Paget's disease, expressed MVNP transcripts. We also detected expression of MVNP transcripts in peripheral blood-derived monocytes from these patients, indicating that MV infection occurs in early osteoclast lineage cells [11]. MV infection has a similar incidence worldwide and occurs in very young patients, whereas Paget's disease is a disease of the elderly. These observations suggest that if paramyxoviruses have an etiologic role in Paget's disease, these viral infections must persist for long periods of time. Pluripotent hematopoietic stem cells, which can persist for long periods of time in a quiescent phase, may be the initial target for the paramyxoviral infection in patients with Paget's disease. We found that other hematopoietic lineages from patients with Paget's disease in addition to the osteoclast lineage, including the erythroid and the erythroid precursors, burst-forming unit-erythroid (BFU-E), and multipotent myeloid precursors (CFUGEMM) also express MVNP transcripts [11]. Thus, if the initial site of infection occurs in a small number of primitive pluripotent hematopoietic stem cells that predominantly remain in Go, this might explain the chronicity of the infection. Also, there may be a genetic predisposition for chronic paramyxoviral infections of hematopoietic precursors in patients with Paget's disease. However, a cause and effect relationship of paramyxoviruses in Paget's disease remains to be proven, as no infectious virus has been isolated from pagetic cells. Also, it is not clear how the focal lesions are initiated in Paget's disease. In contrast to these results, other workers have been unable to detect paramyxoviral nucleocapsid transcripts in samples obtained from patients with Paget's disease [12, 13].

The presence of paramyxoviral transcripts in osteoclasts and osteoclast precursors from patients with Paget's disease suggests a pathophysiologic role for the viral genes in the development of the pagetic lesions. In studies using normal osteoclast precursors (CFU-GM) transduced with retroviral vectors expressing the MVNP gene, the cells formed pagetic-like osteoclasts more rapidly, with an increased number of nuclei, hypersensitivity to 1,25-dihydroxyvitamin D3 (1,25-$[OH]_2D_3$), and an increased bone-resorbing capacity compared to normal osteoclasts. In contrast, normal osteoclast precursors transduced with the MV matrix gene did not express an abnormal phenotype [14]. Furthermore, infecting canine bone marrow cells with CDV results in the development of multinucleated cells that share some of the phenotypic characteristics of pagetic osteoclasts [15]. More recently, CDV was shown to be infectious to human osteoclast precursors and to enhance osteoclast differentiation and function. Previously, we have targeted CD46, the human MV receptor, to cells of the osteoclast lineage in transgenic mice and demonstrated that MV infection of osteoclast precursors from CD46 transgenic mice form osteoclasts, which express a pagetic phenotype *in vitro* [16]. However, TRAP-CD46 mice do not develop sustained MV infection, most likely reflecting the need for blocking interferon production for development of persistent MV infection in these mice. Transgenic mice targeted with

MVNP expression to cells of the osteoclast lineage in vivo results in a bone phenotype that is characteristic of Paget's disease and supports a pathophysiologic role for MVNP in Paget's disease. However, these studies do not exclude genetic factor(s) that may play an important role in disease severity and pathogenesis.

Taken together, these data suggest a potential pathophysiologic role for the paramyxoviral nucleocapsid gene that is expressed in patients with Paget's disease. Mouse models of MV infection were also developed in which CD46 is introduced into transgenic mice and bred to another transgenic mouse lacking the alpha-beta interferon receptor. Upon exposure to MV, these mice developed immune suppression similar to patients with acute MV infection. The mice lacking the alpha-beta interferon receptor demonstrated persistence of MV infection for at least 12 days [17]. Although several lines of evidence support a viral etiology for Paget's disease, it is still unclear how this is related to the late onset and focal nature of Paget's disease.

Genetic linkage of sequestosome 1/p62 and molecular signaling

A genome-wide search in familial Paget's disease of bone indicated genetic heterogeneity of the disease, with candidate loci on chromosomes 2q, 5q, 6p, 10p, and 18q [18, 19, 20]. Linkage studies, coupled with mutation screening, have excluded involvement of RANK and also osteoprotegerin in the majority of patients with Paget's disease of bone [21]. Genetic studies have demonstrated linkage in 7 of 7 patients with osteosarcoma to loss of heterozygosity in a region of 18q that is adjacent to or within a locus for Paget's disease on 18q [22].

Recently, the sequestosome 1 gene encoding the protein p62 (SQSTM1/p62) mapped within the critical region on chromosome 5q35-qter identified a proline-leucine amino acid change at codon 392 (P392L) in French-Canadian patients with Paget's disease of bone [23]. The frequency of the mutation was 16% and 46% for sporadic and familial cases tested, respectively. Further studies also identified different mutations affecting the highly conserved ubiquitin-associated (UBA) domain of the SQSTM1/p62 protein in patients with familial and sporadic Paget's disease [24–26]. In addition to the P392L mutation, two novel mutations (M404V and G425R) were also identified in exon 8 of the SQSTM1 gene in Italian sporadic patients; however, no significant differences in the clinical history was observed in these patients [27]. Studies with patients with familial disease in The Netherlands further identified three new mutations, S399P, M404T, and G425R, which correlated with serum alkaline phosphatase activity similar to patients with P392L mutations [28]. Insertion mutations introducing a stop codon or abolishing the splice donor site at the start of the intron 7 region of SQSTM1 were also identified in UK-derived familial and sporadic Paget's disease cases [29].

Structural analysis studies have classified p62 mutations that retain or abolish the ability of the isolated UBA domain to bind to K48-linked polyubiquitin [30]. Cavey et al. [31] have recently studied the effects of various p62 mutants associated with Paget's disease on the *in vitro* ubiquitin-binding properties of p62 protein. These studies indicated that several SQSTM1 mutations associated with Paget's disease impair p62 binding to ubiquitinylated targets at physiological temperature, suggesting that p62 mutations predispose to Paget's disease through a common mechanism that depends on loss of ubiquitin binding by p62. Using an *in vitro* expression cloning approach, 11 proteins that interact with the p62 UBA domain that are associated with neurodegenerative disorders have been identified [32]. These studies have shown that the heat shock protein-70 (HSP70) interacts with the p62 UBA domain. HSPs are well known to play a role in protein binding, assembly, intracellular transport and degradation within cells. Development of methods such as the yeast two-hybrid screening of an osteoclast cDNA library using the p62 UBA domain as bait should identify further genes that play an important role in pagetic osteoclast development.

The atypical protein kinase C (aPKC) interaction with SQSTM1/p62 has been implicated in signaling cascades that control NF-κB activation (fig. 1). It is evident that p62 provides a scaffold linking the aPKCs to the tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1) receptor signaling complexes through its interaction with RIP and TRAF-6, respectively [33]. Thus, SQSTM1/p62 mediates IL-1 and TNF-α cytokine signaling to activate NF-κB. TRAF-6, plays an essential role in receptor activator of NF-κB ligand (RANKL) signaling during osteoclastogenesis. Recently, RANKL stimulation has been shown to result in upregulation of p62 expression in osteoclast precursor cells, and the genetic inactivation of p62 in mice impaired PTHrP-induced osteoclastogenesis *in vivo*. However, p62 null mice have a grossly normal skeletal phenotype and no alterations were found in the trabecular size and number of osteoclasts compared to wild type mice. In vitro studies demonstrated that p62 deficiency leads to inhibition of IKK activation and NF-κB nuclear translocation during osteoclastogenesis [34]. These studies also demonstrated that RANKL stimulation induces formation of a ternary complex involving TRAF-6, p62 and aPKC during osteoclastogenesis. Recent evidence indicates that TNF-α stimulation of osteoclast precursors in the presence of cofactors such as transforming growth factor-beta (TGF-β) results in osteoclastogenesis independent of the RANKL-RANK-TRAF-6 axis [35]. Also, TRAF-2 has been shown to be essential for TNF-α signaling to induce osteoclastogenesis [36]. It is unclear if p62

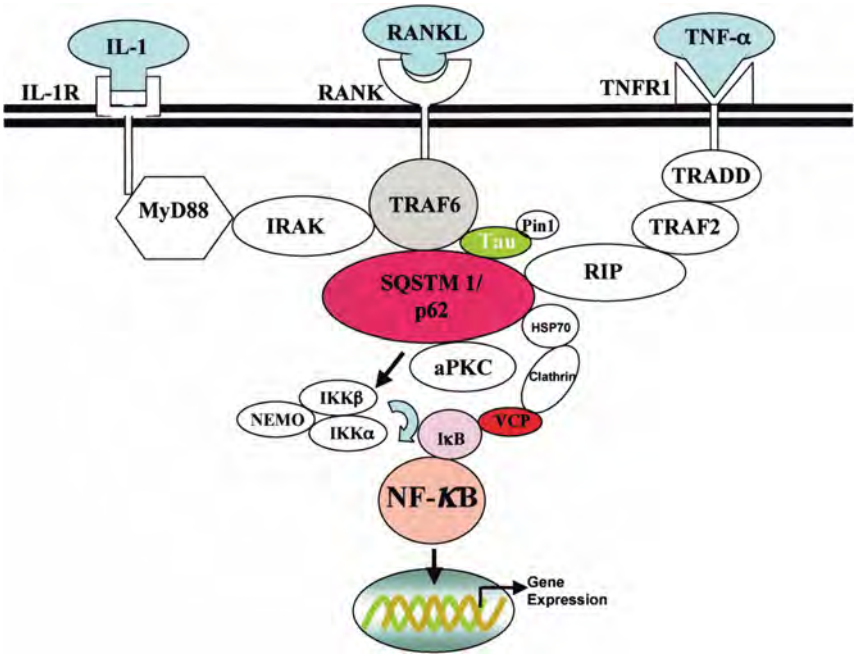

Figure 1. Sequestosome 1 (SQSTM1/p62) protein-protein interactions and associated cellular signaling cascades. RANKL-RANK signaling induces p62 to form a ternary complex with TRAF-6 and aPKCs during osteoclast differentiation. Several mutations in the UBA domain of SQSTM1/p62 have been identified in patients with Paget's disease. Similarly, mutations in VCP, a multiubiquitin chain-targeting factor required in ubiquitin-proteasome degradation have been shown to cause inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia.

UBA mutants affect the status of NF-κB activation and result in a pagetic phenotype of the osteoclast. However, it is reasonable to assess ligand specificity and alternative signaling mechanisms or alternative protein-protein interactions in pagetic-like osteoclast development. For example, it has been shown that inositol 5′ phosphatase-deficient mice are severely osteoporotic with an increased number of osteoclast precursors and hyperactive osteoclasts. In addition, serum levels of IL-6 are markedly increased in these mice as in Paget's disease [37].

p62 was shown to bind ubiquitin non-covalently and sequester into cytoplasmic aggregates in some systems. p62 has been proposed to function as a polyubiquitin shuttling factor for proteasomal degradation through its interaction with the proteasome. Therefore, it is reasonable to speculate that the occurrence of mutations in the UBA domain of p62 in patients with Paget's disease results in cellular accumulation of insoluble polyubiquinated protein aggregates due to failure of proteasomal degradation. For example, accumulation of hyperphosphorylated Tau, the microtubule-associated protein in the brain of patients with Alzheimer's disease, contributes to neurodegeneration. Recently Tau has been identified as a K63-polyubiquitinated substrate of TRAF6 that interacts with the UBA domain of p62 and is targeted for proteasomal degradation [38]. Furthermore, recent evidence also suggests that ubiquitin-proteasome regulatory mechanisms play an important role in osteoblast differentiation [39]. Smad ubiquitin regulatory factor-1 (Smurf1) ubiquitin ligase deficiency results in an age-dependent increase in bone mass due to accumulation of phosphorylated MEKK2 and activation of the JNK signaling cascade in osteoblast cells [40]. Although patients with Paget's disease demonstrate high levels of alkaline phosphatase activity, the molecular defect or alteration in osteoblast cells and the role that the p62 UBA mutant may play in osteoblast activity in these patients is not clear. Osteoblasts are also increased in lesions in patients with Paget's disease, and they appear to be morphologically normal.

Normal human osteoclast precursors transduced with a P392L mutant p62 retroviral expression vector displayed enhanced sensitivity to RANKL and increased osteoclast formation. However, the osteclast precursors demonstrated no pagetic characteristics such as hypersensitivity to 1,25-$(OH)_2D_3$ and increased number of nuclei in the osteoclasts formed *in vivo*. Furthermore, transgenic mice with the P392L mutant p62 gene targeted to cells in the osteoclast lineage using the tartrate-resistant acid phosphatase (TRAP) promoter demonstrated increased osteoclast numbers and were osteopenic but did not develop the increased osteoblast activity that is characteristic of pagetic lesions. These studies suggested that the P392L mutation in p62 enhances osteoclast formation, possibly through increased RANK signaling. Therefore, the precise role that SQSTM1/p62 and signaling mechanisms

may play in pagetic-like osteoclast development and pathogenesis of disease remain to be elucidated.

Inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia (IBMPFD) were recently reported to be caused by mutant valosin-containing protein (VCP) that maps to chromosome 9p21.1-p12 [41]. VCP, a member of the AAA-ATPase superfamily, is a multiubiquitin chain targeting factor for proteasome degradation. VCP is known to function in cell cycle control, membrane fusion, and the ubiquitin-proteasome degradation pathway. It has also been shown that VCP may provide a physical and functional link between IKB-α and the 26S proteasome and play an important role in the proteasome degradation of IKB-α [42, 43]. Formation of protein aggregates and or accumulation of cellular signaling molecules may occur upon failure of proteasomal degradation due to mutations in the N-terminal ubiquitin-binding domain of VCP or UBA domain of p62. This ubiquitous mechanism does not explain specific molecules associated with the pathogenesis of Paget's disease or dominant negative effects on gene expression which regulate osteoclastogenesis and bone resorption activity. However, identification of molecules which interact with p62/VCP provide further insights into ligand specificity for altered signaling cascades responsible for pagetic-like osteoclast development. Recent evidence suggests that a fraction of IKB-α physically associates with nuclear corepressors and histone acetylases. It has further been shown that recruitment of IKKs to the nucleus in response to TNF-α may induce chromatin-associated IKB-α release and gene activation [44]. However, genetic linkage analysis indicated that mutations in the p62 gene may not completely account for the pathogenesis of Paget's disease. The severity of disease in family members carrying the same mutation can vary widely, and up to 20% of individuals who harbor p62 mutations and are older than 55 years do not have PD. Therefore, it is reasonable to hypothesize that other gene loci may be involved in the genetic predisposition and osteoclast abnormalities associated with Paget's disease of bone.

Gene polymorphisms and mRNA expression in Paget's disease

Several studies have examined the linkage of HLA begenes cause of their highly polymorphic nature, and significant associations were observed between class II antigens and Paget's disease [45]. Similarly, studies also indicated that the TNFRSF11B gene encoding OPG with lysine at the codon 3 position predisposes to the development of sporadic and familial forms of Paget's disease that is not caused by SQSTM1 mutations [46]. Recent studies also identified significant variations in genotype frequency of polymorphisms in estrogen receptor-alpha and calcium-sensing receptor genes in patients with Paget's disease compared to normal subjects, which may contribute genetic susceptibility to Paget's disease [47]. Allelic association determines the risk of disease severity and susceptibility but does not explain the focal nature of the disease. Inhibition of apoptosis has been hypothesized to lead to an increased osteoclast lifespan resulting in an increase in the size and number of osteoclasts responsible for enhanced bone resorption activity in patients with Paget's disease. In support of this, in situ hybridization studies have identified increased levels of Bcl2 mRNA expression in pagetic osteoclasts. Further studies indicated that the polymorphic mutations present in the Bcl2 gene promoter region are responsible for elevated Bcl2 expresssion in patients with paget's disease [48]. In situ hybridization studies have also identified increased levels of IL-6 and c-fos proto-oncogene mRNA expression in pagetic osteoclasts. IL-6 receptor and NF-IL-6 mRNA levels were also increased in osteoclasts from bone samples from patients with Paget's disease compared to those with osteoarthritis [49]. It is essential to delineate whether enhanced gene expression or half-life of mRNA and recruitment of coactivators for gene transcription play a pivotal role in pagetic osteoclast development. This is evident from the studies which indicated that pagetic osteoclast precursors are hypersensitive to 1,25-$(OH)_2D_3$ compared to normals. The increased sensitivity of osteoclast precursors from Paget's patients to 1,25-$(OH)_2D_3$ is mediated through the vitamin D3 receptor (VDR); however, this is not due to increased numbers of VDR in pagetic osteoclast precursors compared to normals, but appears to be due to enhanced affinity of the VDR in pagetic cells for its ligand compared to normals [50]. In support of a viral etiology, MVNP gene expression in osteoclast precursors was recently demonstrated to result in increased levels of TAF_{II}-17 transcription factor gene expression. The high levels of TAF_{II}-17 permit formation of a VDR transcription complex at low levels of receptor occupancy by 1,25-$(OH)_2D_3$ [51]. These results support the hypothesis that part of the pathophysiology underlying the increased osteoclast activity in Paget's disease is due to increased levels of VDR coactivators that enhance VDR-mediated gene transcription at low levels of 1,25-$(OH)_2D_3$. Enhanced levels of general transcription factors such as TAF_{II}-17 may not explain the cellular specificity and chronic pathogenesis. However, this does not exclude the possibility of their involvement in pagetic osteoclast development at the involved sites.

Systemic factors

Bones not clinically involved with Paget's disease appear to show increased bone remodeling. This increased bone remodeling in unaffected bones has been ascribed to sec-

ondary hyperparathyroidism rather than to subclinical involvement of the bones with Paget's disease. However, less than 20% of patients with Paget's disease have elevated parathyroid hormone (PTH) levels [2]. Enhanced levels of IL-6, RANKL, M-CSF and endothelin-1 have been associated with Paget's disease. These systemic factors are implicated in the pathogenesis and as an indicator of disease activity [52, 53]. We have recently detected elevated levels of high molecular-weight serum kininogen in patients with Paget's disease [unpublished data]. Because Paget's lesions are focal, pagetic cells may be more sensitive to the elevated systemic factors. The increased levels of IL-6 in the peripheral blood of patients with Paget's disease may in part explain the increased bone remodeling seen in bones not clinically involved with Paget's disease. We need, therefore, to define a pathologic role of systemic factors that are upregulated in patients with Paget's disease. Identification of such factors will also provide further insights into the localized nature and progression of pagetic lesions and disease activity. Osteoclasts from patients with Paget's disease also appear to produce increased levels of IL-6 and express higher levels of IL-6 receptors than normal osteoclasts. IL-6, which is a stimulator of human osteoclast formation, may act as an autocrine/paracrine factor to enhance osteoclast formation in patients with Paget's disease and increase the osteoclast precursor pool. The number of early osteoclast precursors, CFU-GM, was increased significantly in marrow aspirates from patients with Paget's disease compared to normals [54]. The osteoclast precursors from patients with Paget's disease also appear to be hyperresponsive to RANKL and marrow stromal cells from pagetic lesions have increased RANKL expression [55, 56]. RANKL is a critical osteoclast differentiation factor that is expressed on marrow stromal and osteoblast cells in response to several osteotropic factors. The increased sensitivity of osteoclast precursors from Paget's patients to RANKL appears to be due to interactions of these precursors with IL-6. Therefore, it has been hypothesized that Pagetic osteoclasts expressing the MVNP gene produce high levels of cytokines that increase the osteoclast precursor pool. Chronic exposure to cytokines produced by the pagetic osteoclasts results in constitutive overexpression of RANKL in stromal/osteoblast cells further enhancing the abnormal osteoclast development and localized nature in pagetic bone lesions in patients with Paget's disease [57]. Although osteoclasts are thought to be the primary cells affected in Paget's disease, osteogenic cells may be either indirectly or directly affected by the elevated systemic factors or intrinsic genetic defect. Immature osteoblasts are the major responders to RANKL-inducing cytokines and studies have also suggested that expression of RANKL decreases with osteoblast maturation [58]. Therefore, the increased numbers of highly active osteoblasts rapidly form large amounts of woven bone in patients with Paget's disease.

Conclusions and perspectives

In recent years significant progress has been made with respect to etiologic factors associated with Paget's disease of bone, an autosomal dominate trait with genetic heterogeneity. Although recurrent mutations in the UBA domain of sequestosome 1 (SQSTM1/p62) in patients with Paget's disease have been identified and implicated as a common cause of familial and sporadic Paget's disease, it is still unclear if mutant p62 is sufficient to cause Paget's disease, and what its precise role is in osteoclast abnormalities. Future perspectives are to identify novel cellular protein interactions with the ubiquitin-binding domain of VCP or the UBA domain of p62 and to develop animal models to further delineate the role of SQSTM1/p62-associated signaling cascades in pagetic osteoclast development. Lack of skeletal abnormalities in p62-deficient mice further suggests a potential role for genes present in other candidate loci that have been linked with Paget's disease. Alternatively, a genetic defect may favor environmental factors such that MV infection plays a potential role in the pathogenesis of the disease. However, the molecular basis for the abnormalities associated with osteoclasts, the role of paramyxoviral infection, and the persistence of the virus in patients with Paget's disease are unclear. It will be important to determine a cause and effect relationship for the persistence of paramyxoviral infection and genetic predisposition in patients with Paget's disease. Essential also, will be to define the role of elevated systemic factors and underlying molecular mechanisms in the initiation and progression of focal lesions in patients with Paget's disease.

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Elevated Serum Kininogen in Patients with Paget's Disease of Bone: A Role in Marrow Stromal/ Preosteoblast Cell Proliferation

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Abstract Paget's disease (PD) of bone is a chronic focal skeletal disorder characterized by excessive bone resorption followed by abundant new bone formation. Enhanced levels of IL-6, RANKL, M-CSF, and endothelin-1 have been associated with PD. In the present study, we identified increased serum levels (2 to 5-fold) of inflammatory cytokine, kininogen (KNG) in patients with PD compared to normal subjects. Treatment of pagetic bone marrow derived stromal/ preosteoblast cells with recombinant KNG (25 ng/ml) for 24 h period resulted in a 5-fold increase in the levels of phospho-HSP27 and a 3-fold increase in ERK1/2 phosphorylation in these cells. However, pagetic stromal cells stimulated with KNG in the presence of ERK activation inhibitor peptide did not significantly affect the levels of phospho-HSP27. KNG increased normal and pagetic marrow stromal cell proliferation at 1.4-fold and 2.5-fold, respectively. KNG in the presence of an ERK inhibitor peptide did not stimulate pagetic marrow stromal cell proliferation. Furthermore, siRNA suppression of HSP27 expression significantly decreased KNG inhibition of etoposide-induced caspase-3 activation and apoptosis in these cells. In summary, KNG modulate bone marrow derived stromal/preosteoblast cell proliferation and suppress etoposide-induced apoptosis through ERK and HSP27 activation, respectively. These results implicate a pathophysiologic role for KNG in patients with PD. J. Cell. Biochem. 98: 1681–1688, 2006.

Key words: kininogen (KNG); Paget's disease; stromal/preosteoblast cells; extracellular signal-regulated kinase (ERK); heat-shock protein

Paget's disease (PD) of bone is a chronic focal skeletal disorder that affects up to 2%–3% of the population over the age of 60 years. The pathologic abnormality in patients with PD involves increased bone resorption by the osteoclasts, followed by abundant new bone formation that is of poor quality [Roodman and Windle, 2005]. Genetic linkage analysis indicated that 40% of patients with PD have an affected first degree relative and 1% of patients develop osteosarcoma [Hansen et al., 1999]. PD is an autosomal dominant trait with genetic heterogeneity. Recurrent mutations in the ubiquitin-associated domain of Sequestosome 1 (SQSTM1/p62) are identified in patients with PD [Laurin et al., 2002; Johnson-Pais et al., 2003; Hocking et al., 2004]. Osteoclasts and osteoclast precursors from patients with PD contain paramyxoviral transcripts and appear hyperresponsive to 1,25-$(OH)_2D_3$ and RANK ligand (RANKL) [Neale et al., 2000; Roodman and Windle, 2005]. However, a cause and effect relationship for the paramyxoviral infection and SQSTM1/p62 gene mutations associated with this disease and osteoclast abnormalities are unclear.

The biochemical markers provide an integrated assessment of the cellular events occurring throughout the skeleton of patients with PD. Interleukin-6 (IL-6) levels were shown to increase in bone marrow plasma and peripheral blood of patients with PD. These studies further indicated that IL-6 is an autocrine/paracrine factor, which stimulates human osteoclast formation and increase the osteoclast precursor

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pool in patients with PD. In addition, RANKL, a critical osteoclastogenic factor that is expressed on marrow stromal/osteoblast cells is also upregulated in areas involved with PD. The increased sensitivity of osteoclast precursors from Paget's patients to RANKL appears to be due to interactions of these precursors with IL-6. Addition of neutralizing antibodies to IL-6 decreased the RANKL sensitivity of osteoclast precursors to normal levels. Similarly, addition of IL-6 to cultures of normal osteoclast precursors enhanced the responsivity of these precursors to RANKL to the levels seen with pagetic osteoclast precursors. The enhanced expression of RANKL and IL-6 in pagetic lesions could contribute to the abnormal osteoclast development and highly localized nature of PD [Roodman and Windle, 2005]. In situ hybridization studies have further identified increased levels of IL-6, c-fos proto-oncogene, Bcl-2 anti-apoptotic gene mRNA expression in pagetic osteoclasts [Hoyland et al., 1994; Brandwood et al., 2003].

Urinary *N*-telopeptide, pyridinoline, and deoxypyridinoline have all been reported to be more specific indices of skeletal matrix resorption and are not influenced by dietary gelatin. Furthermore, serum calcium levels are typically normal in PD and also serum osteocalcin levels appear to be a poor index of the progression of the disease. The increased bone remodeling in unaffected bones has been ascribed to secondary hyperparathyroidism rather than to subclinical involvement of the bones with PD. However, less than 20% of patients with PD have elevated parathyroid hormone (PTH) levels [Siris, 1998]. Serum tartrate resistant acid phosphatase (TRAP), presumably released by osteoclasts, appears to be an index of bone resorption in PD but is not routinely used. The most useful markers for the increased osteoblast activity in PD are the total alkaline phosphatase and bone-specific alkaline phosphatase activity levels in serum [Reddy, 2004]. It has been reported that serum M-CSF levels are significantly elevated in patients with PD, however not significantly different in patients under treatment compared to normal subjects [Neale et al., 2002]. Patients also showed significantly higher endothelin-1 circulating levels than controls with a positive correlation with serum alkaline phosphatase, but not with urinary hydroxyproline [Tarquini et al., 1998]. In the present study, we identified high-level expression of an inflammatory cytokine, kininogen (KNG) in patient's sera compared to normal subjects, and further demonstrated potential role that KNG may play in marrow stromal cell proliferation. Our results further implicate a pathophysiologic role for KNG in PD.

MATERIALS AND METHODS

Materials

Recombinant human KNG was purchased from R&D systems, Inc. (Minneapolis, MN). Extracellular signal-regulated kinase (ERK) activation inhibitor peptide I was obtained from Calbiochem (Darmstadt, Germany).

Western Blot Analysis

Normal and pagetic bone marrow derived stromal/preosteoblast cells were isolated as described previously [Roccisana et al., 2004]. The cells were seeded in six-well plates at a density of 10^6 cells in 10 ml of α-minimal essential medium (MEM) containing 10% fetal calf serum (FCS) and cultured for 24 h in the presence of human recombinant KNG. The cells were lysed in a buffer containing 20 mM Tris, pH 7.4, NaCl 150 mM, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 200 μM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and aprotinin (1 mg/ml). The protein content of the samples was measured using the BCA method as per the manufacturer's protocol (Pierce, Rockford, IL). Serum (4 μg total protein) or cell lysates (15 μg protein) samples were then subjected to SDS–polyacrylamide gel electrophoresis (PAGE), using 12% Tris-HCl gels. The proteins were transferred from SDS gels onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA) for immunoblot analysis. Blocking was performed with 5% non-fat dry milk in 150 mM NaCl, 50 mM Tris, pH 7.2, 0.05% Tween-20 (TBST) buffer. The membrane was then incubated for 1 h with anti-KNG antibody (The Binding Site, Birmingham, UK), and anti-ERK1/2, phospho-ERK1/2, HSF-2 (heat-shock factor-2), RANKL mouse monoclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-HSP27 (heat-shock protein-27), phospho-HSP27 (P-HSP27) antibodies (Cell signaling technology, Inc., Beverly, MA) diluted 1:500 in 5% non-fat dry milk-TBST. The blots then were incubated for 1 h with horseradish

peroxidase-conjugated goat anti-mouse IgG, diluted 1:2,500 in 5% non-fat dry milk-TBST, and developed using an ECL system (Amersham Biosciences, Little Chalfont, UK). NIH image program (National Institutes of Health, Bethesda, MD) was used for quantification analysis after digital scanning of the exposed X-ray films.

Inhibition of ERK Activation

Inhibition of ERK activation was performed as described previously [Kelemen et al., 2002]. Briefly, serum-starved pagetic bone marrow stromal/preosteoblast cells were treated with the ERK activation inhibitor peptide (25 μM) for 4 h at 37°C and then stimulated with KNG (25 ng/ml) for an additional 24 h in α-MEM containing 10% FCS. Cells were washed with ice-cold phosphate buffered saline, suspended in 0.5 ml of icecold lysis buffer, and scraped from flasks. Cell lysate was sonicated, centrifuged to remove remaining insoluble material, and measured protein concentration. The protein concentration in the cell lysate was determined by BCA protein assay system (Pierce Chemical Co.).

Cell Proliferation Assay

Normal and pagetic human bone marrow stromal/preosteoblast cell proliferation was determined using a CellTiter 96 non-radioactive cell proliferation assay kit (Promega, Madison, WI). The cells were seeded at 5×10^3 cells/well on 96-well plates, and incubated in the presence or absence of KNG (0–50 ng/ml) for 48 h at 37°C in humidified, 5% CO_2 atmosphere. After 48 h period, cell proliferation rate was assayed following the manufacturer's protocol.

To determine the role of HSP27 in cell proliferation, we have used siRNA to suppress HSP27 expression in pagetic stromal cells. The cells were seeded at 5×10^3 cells/well in a 96-well plate and transiently transfected with HSP27 siRNA or control siRNA (10 nM) (Santa Cruz Biotechnology, Inc.) by Lipofectamine method. Briefly, 1 μl of 10 μM siRNA, 100 μl of α-MEM media, and 6 μl of Lipofect AMINE Plus reagent (Invitrogen, Grand Island, NY) were premixed for 15 min at room temperature. During this time, 5 μl of Lipofect AMINE transfection reagent was mixed with 100 μl of α-MEM media. The two mixtures were then combined and incubated for 15 min at room temperature to form a complex. The reaction mixture was diluted with 800 μl of α-MEM medium and 100 μl aliquot of the entire mixture was added to each well. After 12 h, the cells were treated with α-MEM containing 10% FCS with KNG (0.5, 25 ng/ml) for additional 48 h, and subjected to the proliferation assay.

Inhibition of ERK phosphorylation was performed by seeding the pagetic stromal cells at 5×10^3 cells/well in 96-well plates, and treated with the ERK activation inhibitor peptide (25 μM) for 4 h. The cells were then stimulated with KNG (0–25 ng/ml) for an additional 48 h in α-MEM containing 10% FCS, and subjected to the proliferation assay. Each treatment was analyzed in triplicate and the results represent mean values of three independent experiments ($P < 0.05$).

Apoptosis Assay

To determine the effect of KNG and role of HSP27 in stromal/preosteoblast cell apoptosis, Paget's bone marrow stromal cells were transiently transfected with HSP27 siRNA or control siRNA as described above. The cells were stimulated with KNG (25 μM) for 24 h and cultured in the presence of 25 μM etoposide (Sigma-Aldrich, St. Louis, MO) at 37°C for additional 24 h period. Total cell lysates were prepared with a lysis buffer containing 25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin. The supernatant of the cell lysate (100 μg of protein) was then assayed for caspase-3 activation to detect cell apoptosis using the CaspACE assay system (Promega) following the manufacturer's protocol.

Etoposide-induced apoptosis in pagetic stromal/preosteoblast cells was confirmed by immuno-fluorescence method. Briefly, paget's bone marrow stromal cells were cultured on glass coverslips in the presence of KNG (25 ng/ml) or etoposide (25 μM) alone, and in combination for 4 h. The cells were fixed in 4% paraformaldehydephosphate buffered saline buffer and permeabilized with 0.1% Triton X-100 in PBS. The cells were incubated overnight with a mouse anti-cytochrome c antibody (PharMingen, San Diego, CA) diluted 1:1,000 in PBS containing 1% bovine serum albumin. We used an Alexa ($\lambda_{ex} = 488$ nm and $\lambda_{em} = 519$ nm)-coupled anti-mouse IgG (1:600 dilution) to detect cytochrome c release using a confocal microscope.

RESULTS

Identification of KNG Overexpression in the Serum from Patients with PD

Enhanced levels of KNG (63 kDa) are associated with inflammatory conditions [Colman and Schmaier, 1997; Carretero, 2005]. In the present study, we examined KNG expression in serum samples obtained from normal subjects and patients with PD by Western blot analysis. As shown in Figure 1, Western blot analysis of serum samples (4 μg total protein) of five representative individuals from a total of nine PD patients and normals analyzed further indicated 2 to 5-fold increases in levels of KNG (63 kDa) in patients with PD compared to normal subjects. These data are consistent with a potential pathologic role for KNG in PD.

KNG Enhances ERK and HSP27 Phosphorylation in Pagetic Marrow Stromal/Preosteoblast Cells

RANKL, a critical osteoclastogenic factor that is expressed on marrow stromal/osteoblast cells is upregulated in PD. We have recently demonstrated that HSF-2 is a downstream target of b-FGF-induced RANKL expression in SAKA-T normal human bone marrow derived stromal cells [Roccisana et al., 2004]. We therefore, further examined the effects of KNG on HSP27 and ERK phosphorylation in pagetic marrow stromal cells. As shown in Figure 2, Western blot analysis of total cell lysates obtained from the pagetic stromal cells treated with KNG (25 ng/ml) for a period of 24 h demonstrated a significant increase (5-fold) in the levels of HSP27 phosphorylation compared to untreated cells. However, there was no significant change in the levels of HSF-2 expression in these cells. In addition, KNG enhanced a 3-fold increase in ERK1/2 phosphorylation compared with untreated cells (Fig. 3A). To further delineate if ERK signaling is involved in HSP27 phosphorylation, we used ERK1/2 activation inhibitor. Pagetic stromal cells were stimulated with

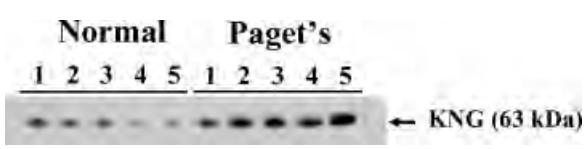

Fig. 1. Western blot analysis for KNG expression in normal and Paget's patient's serum. Serum samples (4 μg) from normal and Paget's patients shown are representative of total nine subjects of each analyzed. Sample loading was normalized for protein concentration.

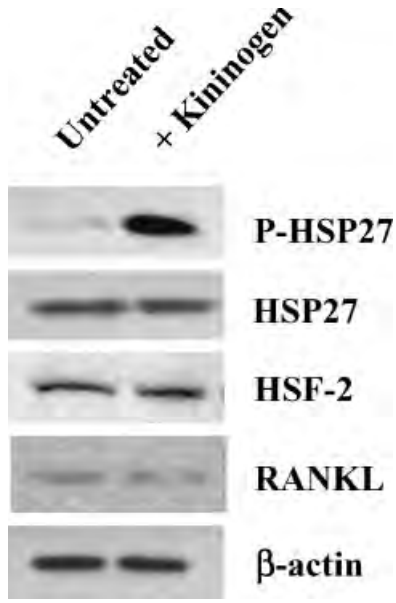

Fig. 2. KNG induces HSP27 phosphorylation. Pagetic marrow stromal/preosteoblast cells were treated with KNG (25 ng/ml) for 24 h, and total cell lysates (15 μg) were subjected to Western blot analysis using anti-human HSP27, P-HSP27, HSF-2, and RANKL antibodies as described in methods.

KNG in the presence of ERK activation inhibitor peptide-1 (25 μM), which binds to ERK2 and prevents interaction with MEK (mitogen-activated protein kinase kinase). Western blot analysis of total cell lysates obtained from

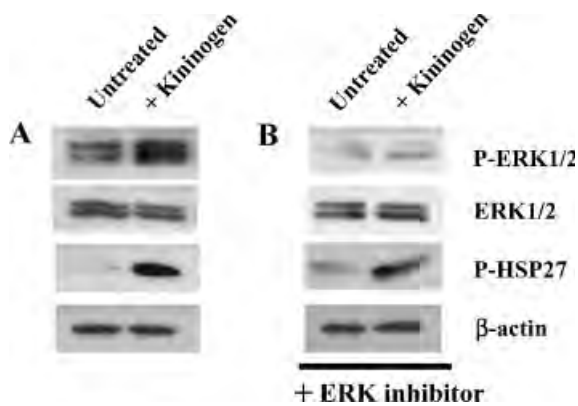

Fig. 3. KNG promotes ERK phosphorylation and ERK activation inhibitor did not affect HSP27 phosphorylation. **A**: KNG enhanced ERK and HSP27 phosphorylation. Pagetic stromal cells were stimulated with KNG (25 ng/ml) for 24 h, and total cell lysates (15 μg) prepared were subjected to Western blot analysis using anti-human ERK1/2, phospho-ERK1/2, and phospho-HSP27 as described. **B**: HSP27 phosphorylation is independent of ERK1/2 signaling pathway. Serum-starved pagetic stromal cells were treated with the ERK activation inhibitor peptide (25 μM) for 4 h at 37°C and then stimulated with KNG (25 ng/ml) for an additional 24 h. Total cell lysates (15 μg) obtained were subjected to Western blot analysis.

KNG stimulated cells did not demonstrate a significant change in the levels of phospho-HSP27 (Fig. 3B), suggesting that HSP27 phosphorylation is independent of ERK signaling pathway. We have observed similar effect of KNG using normal human bone marrow derived stromal cells (data not shown).

KNG Enhances Proliferation of Paget's Bone Marrow Stromal Cells Through ERK Activation

Since ERK signaling is associated with cellular proliferation, we have examined the potential of KNG to stimulate normal and pagetic bone marrow stromal/preosteoblastic cell growth. As shown in Figure 4, KNG treatment significantly increased normal and pagetic bone marrow stromal cells proliferation in a dose-dependent manner. KNG (25 ng/ml) increased normal and pagetic marrow derived stromal cell proliferation at 1.4-fold and 2.5-fold compared to untreated control cells, respectively. Since KNG significantly enhanced HSP27 and ERK phosphorylation in pagetic bone marrow derived stromal cells, we further examined if HSP27 and ERK signaling is involved in KNG stimulation of cell proliferation. We used siRNA and ERK inhibitor peptide

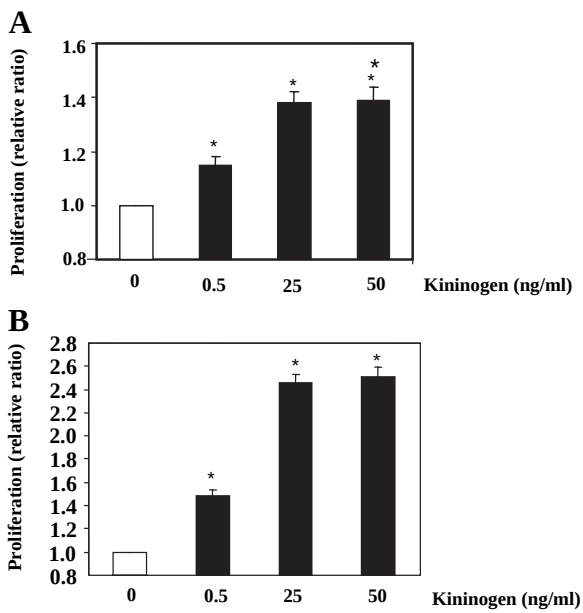

Fig. 4. Effect of KNG on proliferation of bone marrow stromal/preosteoblast cells. **A**: Normal human bone marrow derived stromal cells and (**B**) Pagetic marrow derived stromal/preosteoblastic cells were seeded at 5×10^3 cells/well in 96-well plates, and incubated in the presence or absence of KNG (0.5–50 ng/ml) for 48 h, and proliferation assay was performed as described in methods.

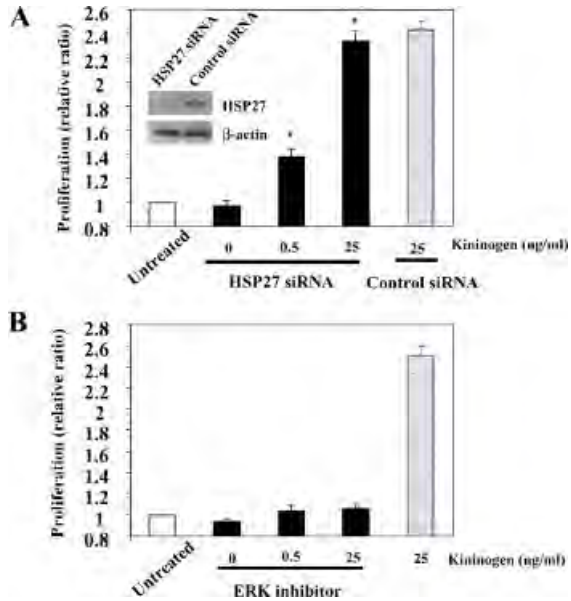

Fig. 5. KNG stimulate pagetic stromal cell proliferation through ERK signaling pathway. **A**: siRNA suppression of HSP27 expression did not affect KNG stimulated proliferation of pagetic marrow stromal cells. HSP27 or control siRNA (10 nM) was transiently transfected into pagetic stromal cells, and examined cell proliferation. Western blot analysis confirms the suppression of HSP27 expression (Inset). **B**: ERK activation inhibitor peptide suppressed the KNG stimulated proliferation of pagetic stromal cells. Pagetic marrow stromal cells were treated with ERK activation inhibitor peptide (25 μM) for 4 h at 37°C, and then stimulated with KNG (0.5, 25 ng/ml) for an additional 48 h and assayed for cell proliferation as described ($P < 0.05$).

to block the expression of HSP27 and ERK activation in these cells, respectively. As shown in Figure 5A, siRNA suppression of HSP27 expression did not affect the KNG stimulated proliferation of these cells. However, inhibition of ERK phosphorylation completely abolished the KNG stimulated cell proliferation (Fig. 5B). These results suggest that KNG enhances proliferation of Paget's bone marrow stromal/preosteoblast cells through ERK signaling pathway.

KNG Inhibits Etoposide-Induced Apoptosis in Paget's Bone Marrow Stromal Cells

It has been reported that HSP27 inhibits cellular apoptosis by preventing cytochrome c-triggered caspase-3 activation [Garrido et al., 1999]. Therefore, we further examined the effect of KNG on etoposisde-induced apoptosis and the role of HSP27 in Paget's bone marrow derived stromal/preosteoblast cells. Paget's stromal cells were transiently transfected with HSP27 siRNA and treated with KNG (25 ng/ml)

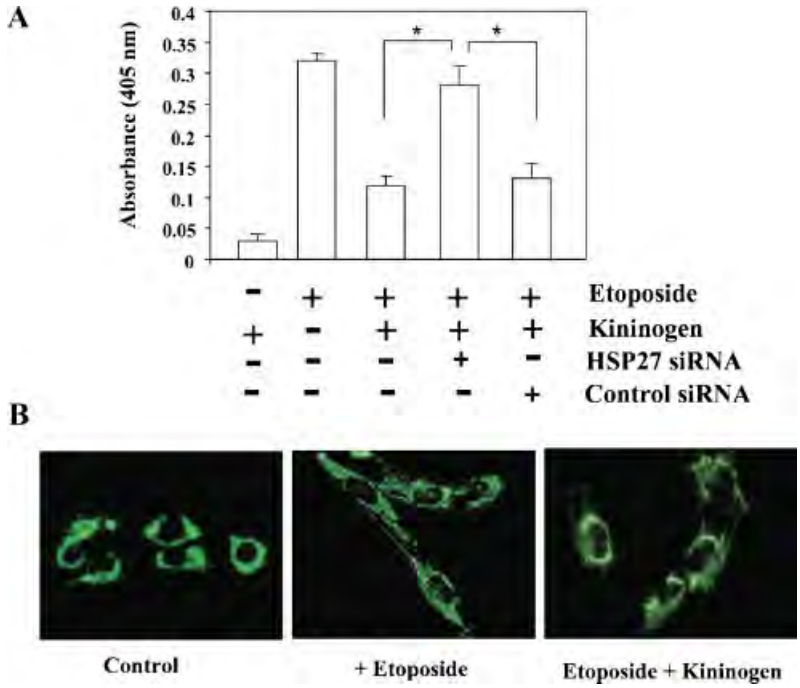

Fig. 6. Effect of KNG on etoposide-induced apoptosis in pagetic bone marrow stromal cells. **A**: Paget's bone marrow stromal cells were transiently transfected with HSP27 siRNA or control siRNA, and stimulated with or without KNG in the presence or absence of etoposide (25 μM) for 24 h. Total cell lysates obtained were assayed for caspase-3 activity was measured as described in methods. **B**: Immunofluorescence staining of cytochrome c distribution in KNG treated control cells, etoposide alone and KNG + etoposide combination treatment to pagetic marrow stromal cells.

in the presence or absence of etoposide (25 μM) and assayed for caspase-3 activation. As shown in Figure 6A, KNG treatment significantly inhibits etoposide-induced caspase-3 activity in pagetic stromal cells. Furthermore, siRNA suppression of HSP-27 expression significantly decreased KNG inhibition of etoposide-induced caspase-3 activity in these cells. Evident from the immunoflurenscence staining, etoposide treatment to pagetic marrow stromal cells demonstrated a diffused pattern of cytochrome c staining further confirms apoptosis in these cells. However, etoposide treatment in the presence of KNG (25 μM) demonstrates low levels of cytochrome c diffusion compared to etoposide alone treated cells (Fig. 6B). These results indicate functional role for HSP27 in KNG inhibition of apoptosis in pagetic marrow stromal/preosteoblastic cells.

DISCUSSION

Enhanced levels of IL-6, M-CSF, and endothelin-1 have been associated with PD, implicated in its pathogenesis and indicator of disease activity. Although PD is localized, bones not clinically involved with PD appear to show increased bone remodeling. For example, the increased levels of IL-6 in the peripheral blood of patients with PD may in part explain the increased bone remodeling seen in bones not clinically involved with PD. Therefore, it is necessary to define a pathologic role of systemic factors that are upregulated in patients with PD. In the present study, we have identified elevated levels of KNG expression in serum of patients with PD. Since KNG appears to be the major glycosylated peptide that is detected at the level of total protein analysis of patient's serum, we further determined KNG influence on pagetic bone marrow derived stromal/pre-osteoblast cells and associated signaling mechanism. The KNG is a multifunctional inflammatory cytokine which is composed of a 362-amino acid heavy chain, the 9-residue bradykinin sequence, and one 255-amino acid light chain [Takagaki et al., 1985]. KNG being a glycosylated secretory molecule, posttranslational regulatory mechanisms may be responsible for enhanced levels of serum KNG in patients with PD. The KNG localizes on the surface of endothelial cells, platelets, and

neutrophils. It has also been demonstrated that KNG purified from bovine milk stimulates proliferation of osteoblastic cells; however, the molecular signaling mechanism is unclear [Yamamura et al., 2000]. Although our results indicate KNG treatment results in high levels of HSP27 phosphorylation in pagetic stromal cells, we observe no significant change in RANKL expression in these cells. We have recently demonstrated that HSF-2 is a downstream target of fibroblast growth factor-2 (FGF-2) to induce RANKL expression in stromal/preosteoblast cells [Roccisana et al., 2004]. HSP are molecular chaperones activated upon cellular stress/stimuli [Snoeckx et al., 2001]. HSPs have been shown to prevent inflammatory damage through production of antiinflammatory cytokines [van Eden et al., 2005]. Several members of the *HSP* gene family have been reported to exhibit differential expression during stromal/preosteoblast differentiation. The differences in HSP expression are consistent with involvement in mediating a series of regulatory events functionally related to the physiologic control of cell growth and differentiation [Shakoori et al., 1992]. Our results using siRNA suppression of HSP27 did not significantly affect KNG stimulation of pagetic marrow stromal cell proliferation. However, inhibition of ERK phosphorylation completely abolished the KNG stimulated cell proliferation suggesting that KNG enhances proliferation of pagetic stromal cells through ERK signaling pathway. Therefore, KNG may play an important role in modulating marrow stromal cell proliferation/differentiation. Although KNG exerts similar effect on normal and pagetic marrow derived stromal cells with respect to HSP27 and ERK activation, our results indicate that KNG stimulates pagetic marrow derived stromal cell proliferation efficiently compared to normal bone marrow derived cells. It is possible that the pagetic marrow stromal cells are either indirectly or directly affected by the elevated systemic factors and chronic exposure to cytokines produced in the focal lesions. Alternatively, pagetic cells may be more sensitive to KNG stimulation due to an intrinsic genetic defect in patients with PD.

HSPs have been implicated with anti-apoptotic role in mammalian cells. Recent evidence further indicates that rapid phosphorylataion of HSP27 is required for cell adhesion and suppression of apoptosis in renal epithelial cells [de Graauw et al., 2005]. In the present study, KNG-induced HSP27 activation and suppression of etoposide-induced caspase-3 activity suggest antiapoptotic role for KNG in pagetic marrow stromal/preosteoblastic cells. In support of our results, recently Kaschina et al. (2004), demonstrated that KNG deficiency results in enhanced caspase-3 mediated cellular apoptosis. In summary, KNG modulate pagetic bone marrow stromal/preosteoblast cell proliferation through ERK signaling pathway and suppress etoposide-induced apoptosis through enhanced HSP27 phosphorylation. Therefore, enhanced levels of KNG in patients with PD further implicate a pathophysiologic role for KNG in PD.

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Expression of Measles Virus Nucleocapsid Protein in Osteoclasts Induces Paget's Disease-Like Bone Lesions in Mice

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ABSTRACT: We targeted the *MVNP* gene to the OCL lineage in transgenic mice. These mice developed abnormal OCLs and bone lesions similar to those found in Paget's patients. These results show that persistent expression of MVNP in OCLs can induce pagetic-like bone lesions in vivo.

Introduction: Paget's disease (PD) of bone is the second most common bone disease. Both genetic and viral factors have been implicated in its pathogenesis, but their exact roles in vivo are unclear. We previously reported that transfection of normal human osteoclast (OCL) precursors with the measles virus nucleocapsid (MVNP) or measles virus (MV) infection of bone marrow cells from transgenic mice expressing a MV receptor results in formation of pagetic-like OCLs.
Materials and Methods: Based on these in vitro studies, we determined if the *MVNP* gene from either an Edmonston-related strain of MV or a *MVNP* gene sequence derived from a patient with PD (P-MVNP), when targeted to cells in the OCL lineage of transgenic mice with the TRACP promoter (TRACP/MVNP mice), induced changes in bone similar to those found in PD.
Results: Bone marrow culture studies and histomorphometric analysis of bones from these mice showed that their OCLs displayed many of the features of pagetic OCLs and that they developed bone lesions that were similar to those in patients with PD. Furthermore, IL-6 seemed to be required for the development of the pagetic phenotype in OCLs from TRACP/MVNP mice.
Conclusions: These results show that persistent expression of the *MVNP* gene in cells of the OCL lineage can induce pagetic-like bone lesions in vivo.
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Key words: Paget's disease, osteoclast, TAF_{II}-17, IL-6, measles virus nucleocapsid

INTRODUCTION

Paget's disease (PD) of bone is the second most common bone disease, affecting 1–2 million patients in the United States. Although the etiology of PD is unknown, both genetic and nongenetic factors have been implicated. Studies of large families with PD have shown an autosomal dominant mode of inheritance, and recently, several loci have been linked to PD.[1–5] Mutations in the *sequestosome-1* (*SQSTM1*) gene occur in ~30% of patients with familial or 10% of patients with sporadic PD,[6] although the penetrance of PD in families with these mutations is variable. In addition, previous studies have suggested a viral etiology for PD. Electron microscopic studies first showed nuclear inclusions in pagetic osteoclasts (OCLs), which were similar to paramyxoviral nucleocapsids.[7] Immunohistochemical studies subsequently identified both respiratory syncytial virus and measles virus nucleocapsid proteins (MVNPs) in pagetic OCLs.[8] In situ hybridization studies also showed MVNP transcripts in cells from bone biopsy specimens from patients with PD,[9] and RT-PCR studies identified MVNP or canine distemper virus nucleocapsid transcripts in OCLs from patients with PD.[10,11] However, others have been unable to detect viral transcripts in pagetic OCLs.[12,13] Thus, the role of paramyxoviruses in the pathogenesis of PD is unclear.

We previously reported that transfection of normal human OCL precursors with the *MVNP* gene results in formation of OCLs that have many of the abnormal features of pagetic OCLs.[14] Both pagetic and MVNP-transfected normal OCL precursors form markedly increased numbers of OCLs in vitro, which contain many more nuclei per OCL and have an increased resorption capacity compared with normal OCLs. Furthermore, both pagetic and MVNP-transfected normal OCL precursors display marked hyperresponsivity to $1,25(OH)_2D_3$, forming OCLs at concentrations that are one to two logs lower than required for

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normal OCL formation.[15] In addition, both pagetic and MVNP-transfected OCL precursors express high levels of TAF_{II}-17, a member of the TF-IID transcription complex, which acts as a coactivator of vitamin D receptor–mediated gene transcription.[16] OCL from PD patients and OCL precursors transduced with the *MVNP* gene also secrete large amounts of IL-6.[14,17] Finally, when bone marrow cells from transgenic mice in which the CD46 MV receptor[18] is targeted to cells in the OCL lineage are infected in vitro with MV, they form OCLs that have the abnormal characteristics of pagetic OCLs.[19] However, it is unknown if MV can induce pagetic-like bone lesions in vivo that are similar to the abnormal bone present in PD.

In this study, we determined if persistent expression of the nucleocapsid gene from either an Edmonston variant of MV (E-MVNP) or the nucleocapsid sequence derived from a patient with PD (P-MVNP)[20] could induce changes in bone similar to those found in PD. The *E-MVNP* or *P-MVNP* gene was targeted to cells in the OCL lineage in transgenic mice using the TRACP promoter. These mice were analyzed at 4–16 months of age to determine if they developed bone abnormalities similar to those seen in PD.

MATERIALS AND METHODS

Development of TRACP/E-MVNP and TRACP/P-MVNP transgenic mice

These studies were approved by the IACUCs at both the University of Pittsburgh School of Medicine and Virginia Commonwealth University. The E-MVNP cDNA, originally derived from a measles patient, was generously provided by Dr Chris Richardson of the University of Toronto. Sequence analysis of this cDNA showed that it was from a virus belonging to the Edmonston group of MV strains, which is the most widespread group of MV strains and the origin of the majority of MV vaccines.[21] This cDNA encodes a protein that differs from the Edmonston strain wildtype MVNP (GI: 1041617)[22] at five amino acid residues: 26 (G to E), 453 (E to G), 467 (L to P), 473 (L to P), and 525 (D to G). Sequence analysis of the *P-MVNP* gene showed that it also encodes a closely related Edmonston strain MVNP that differs from the wildtype Edmonston MVNP at seven residues: 26 (G to E), 435 (K to R), 453 (E to G), 467 (L to P), 473 (L to P), 494 (A to T), and 525 (D to E). The majority of the amino acid differences between either the E-MVNP or P-MVNP and the wildtype MVNP fall within the hypervariable carboxy terminus of this protein. When we initially reported the detection of MVNP transcripts from the bone marrow of several Paget's patients,[20] the sequence from patient 1 (P1) diverged from the consensus MVNP sequence beginning at amino acid 497. It was subsequently determined that this apparent divergence was caused by a DNA sequencing error that produced a frameshift in P1 relative to the consensus MVNP sequence and that the P1 sequence in fact matches wildtype MVNP with the exceptions noted above. To generate the *TRACP/E-MVNP* and *TRACP/P-MVNP* transgenes, the E-MVNP and P-MVNP cDNA was inserted into the unique *Eco*RI site of the pBSmTRACP5′ plasmid.[23,24] This resulted in the addition of a 25 amino acid C-terminal tag to the P-MVNP but not the E-MVNP construct. The transgenes were excised with *Xho*I, and transgenic mice were generated by standard methods[25] in a CB6F1 (C57Bl/6 × Balb/c) genetic background. Transgenic founders were identified by Southern blot analysis of tail DNA, and transgenic mice of subsequent generations were identified by PCR analysis. Two TRACP/E-MVNP and four TRACP/P-MVNP founder mice were generated and bred to establish multiple independent lines of mice. OCL formation assays were performed on marrow cultures from all transgenic lines, and the TRACP/E-MVNP and TRACP/P-MVNP line that had the highest level of MVNP expression and OCL formation were selected for further characterization and longitudinal studies. This was necessitated by the large numbers of mice that had to be maintained and the extensive histomorphometric analysis that was required. All data presented here were derived from one line for each transgene that expressed the highest levels of the transgene, although increased levels of OCL formation and hypersensitivity to $1,25(OH)_2D_3$ were found in marrow cultures from the additional transgenic lines.

Immunohistochemical detection and Western blot analysis of MVNP expression in marrow cells from TRACP/MVNP and WT mice and PD patients

OCLs from nonadherent mouse bone marrow cells (2×10^5 cells/well) from TRACP/MVNP or WT mice cultured for 7 days with 10^{-8} M $1,25(OH)_2D_3$ were tested for cross-reactivity with a monoclonal antibody against the MVNP protein (Gene Tex, San Antonio, TX, USA) or mouse IgG (60 ng/ml) using a Vectastatin-ABC-AP kit (Vector Laboratories, Burlingame, CA, USA) as previously described.[19] For Western blot analysis, nonadherent mouse bone marrow cells (1.2×10^6 cells) from TRACP/MVNP or WT mice were cultured with macrophage-colony stimulating factor (M-CSF; 10 ng/ml)/RANKL (25 ng/ml) in α-MEM–10% FBS for 48 h. Cell lysates were prepared and processed for Western blot analysis as previously described.[14] The MVNP monoclonal antibody or β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at 1:1000 dilution in Tris-buffered saline containing 1% BSA. Nonadherent marrow mononuclear cells (5×10^5 cells) from patients with PD or normals were cultured for 3 weeks with 10^{-8} M $1,25(OH)_2D_3$, to induce OCL formation, and the cells were processed for Western blot analysis as described above.

PCR analysis for TAF_{II}-17 expression

MVNP and WT mice OCL precursors were cultured for 2 days with $1,25(OH)_2D_3$ (10^{-8} M) and subjected to RT-PCR for TAF_{II}-17 mRNA as previously described.[16] The mouse TAF_{II}-17 sense primer was 5′-CAGATTATGAACCAGTTTGGCCCTTCA-3′ and was derived from the *TAF_{II}-17* gene sequence (GenBank accession no. AL590442). The TAF_{II}-17 antisense primer was 5′-CCTGTGTTATTTCTTGGTTGTTTTCCG-3′. The actin sense and antisense primers were 5′-GGCCGTACCACTGGCATCGTGATG-3′ and 5′-CLTGGCCGTCAGGCAGCTCGTAGC-3′ and were derived from the actin gene sequence (GenBank accession no. NM000492).

In vitro analysis of OCL formation by bone marrow cells from TRACP/MVNP or WT mice

Nonadherent marrow cells from long bones of TRACP/E-MVNP, TRACP/P-MVNP, or WT mice at 4–16 months of age were cultured for OCL formation in the presence of varying concentrations of $1,25(OH)_2D_3$ (Roche, Indianapolis, IN, USA) or with 10 ng/ml of murine M-CSF and 25 ng/ml RANKL (R&D Systems, Minneapolis, MN, USA) for 7 days as previously described.[26] The cells were stained for TRACP activity using a commercial kit (Sigma, St Louis, MO, USA), and the number of $TRACP^+$ multinucleated cells that contained at least three nuclei, as well as the number of nuclei per multinucleated cell, were scored microscopically. IL-6 levels in conditioned media from these marrow cultures were determined using a commercial ELISA kit (R&D Systems). In selected experiments, marrow cultures were treated with vehicle or $1,25(OH)_2D_3$ in the presence of a neutralizing antibody to murine IL-6 (50 ng/ml) or isotype specific mouse IgG (R&D Systems) to assess the effects of IL-6 on OCL formation.

Histologic analysis of TRACP/E-MVNP and TRACP/P-MVNP vertebral bones

The first to fourth lumbar vertebrae from TRACP/E-MVNP, TRACP/P-MVNP, and WT mice were fixed in 10% buffered formalin and completely decalcified in 10% EDTA at 4°C and embedded in paraffin. Five-micrometer longitudinal sections were cut and mounted on glass slides. Deparaffinized sections were stained for TRACP as described by Liu et al.[27] OCLs containing active TRACP were stained red. Another set of sections was stained with 0.1% toluidine blue.

Histomorphometry was performed on the region of cancellous bone between the cranial and caudal growth plates of the third lumbar vertebral body under bright field and polarized light at a magnification of ×200, using the OsteoMeasure 4.00C morphometric program (OsteoMeasure; OsteoMetrics, Atlanta, GA, USA). Osteoclast perimeter (Oc.Pm) was defined as the length of bone surface covered with $TRACP^+$ mono- and multinuclear cells. Osteoblast perimeter (Ob.Pm), cancellous bone volume (BV/TV), trabecular width (Tb.Wi), trabecular number (Tb.N), and trabecular separation (Tb.Sp) were also quantified and calculated.

To examine bone formation parameters, animals were given calcein (10 mg) subcutaneously on days 7 and 1 before death. Bones from these animals were embedded, undecalcified, in methyl methacrylate and sections were examined under fluorescent light for quantification of mineralized perimeter, mineral apposition rate, and bone formation rate. All variables were expressed and calculated according to the recommendations of the ASBMR Nomenclature Committee.[28,29]

Statistical analysis

In vitro culture results were analyzed by a two-way ANOVA. Histomorphometric variables were analyzed by two-factor ANOVA using NCSS 2004 software (NCSS Statistical Software, Kaysville, UT, USA). Genotype and age were assigned as factors and the responses of the measured variables were tested. A p value of <0.05 was considered statistically significant.

RESULTS

Analysis of OCLs from TRACP/E-MVNP and TRACP/P-MVNP mice for expression of MVNP

Immunohistochemical analysis showed that OCL formed in marrow cultures from TRACP/E-MVNP and TRACP/P-MVNP mice expressed MVNP (Fig. 1A). Similar levels of staining were detected in both the TRACP/E-MVNP and TRACP/P-MVNP OCLs, and no staining was seen in OCLs from nontransgenic control (WT) or normal human marrow cultures. These results were confirmed by Western blot analysis of MVNP expression, which showed expression of MVNP in TRACP/MVNP and PD patient samples but not in WT mice or normal marrow (Figs. 1B and 1C). Importantly, the levels of MVNP expression in both lines of transgenic mice was roughly comparable with that seen in OCL formed from marrow cultures of patients with PD (Fig. 1C).

Characterization of OCLs formed in marrow cultures of TRACP/E-MVNP and TRACP/P-MVNP mice

Significantly more OCLs were formed in marrow cultures from TRACP/E-MVNP and TRACP/P-MVNP mice than from WT mice in response to $1,25(OH)_2D_3$ (Fig. 2A). Furthermore, both TRACP/E-MVNP and TRACP/P-MVNP marrow cultures formed OCLs at concentrations of $1,25(OH)_2D_3$ that were significantly lower than those required for WT marrow cultures, with OCL formation occurring in MVNP cultures at 10^{-11} to 10^{-12} M $1,25(OH)_2D_3$. In addition, the OCL precursors from TRACP/P-MVNP and E-MVNP, but not WT mice, expressed high levels of TAF_{II}-17 mRNA (Fig. 1D). The number of nuclei per OCL was also significantly increased in marrow cultures from TRACP/E-MVNP and TRACP/P-MVNP mice compared with WT mice (Fig. 2B), and the OCLs that formed were larger than those formed in WT marrow cultures (Fig. 2C). In contrast, there was no significant difference in the sensitivity of OCL precursors from WT, TRACP/E-MVNP, and TRACP/P-MVNP mice to RANKL or expression of RANK mRNA in OCL precursors from these mice (data not shown).

IL-6 production and effects of anti-IL-6 in bone marrow cultures from TRACP/P-MVNP or WT mice

Low levels of IL-6 were detected in bone marrow–conditioned media from marrow cultures of TRACP/P-MVNP and WT mice treated with vehicle. In contrast, high levels of IL-6 were present in conditioned media of cultures of TRACP/P-MVNP, but not WT mice, treated with $1,25(OH)_2D_3$ to induce OCL formation (Fig. 3A). In contrast, IL-11 levels were similar in conditioned media from WT and TRACP/P-MVNP or TRACP/E-MVNP marrow cultures (data not shown).

We determined if IL-6 also played a role in the formation of pagetic-like OCLs in TRACP/P-MVNP marrow cultures. Addition of an anti-IL-6 antibody (50 ng/ml) to marrow cultures of TRACP/P-MVNP mice treated with $1,25(OH)_2D_3$ significantly decreased OCL numbers and the number of nuclei per OCL (Fig. 3B; Table 1). In contrast,

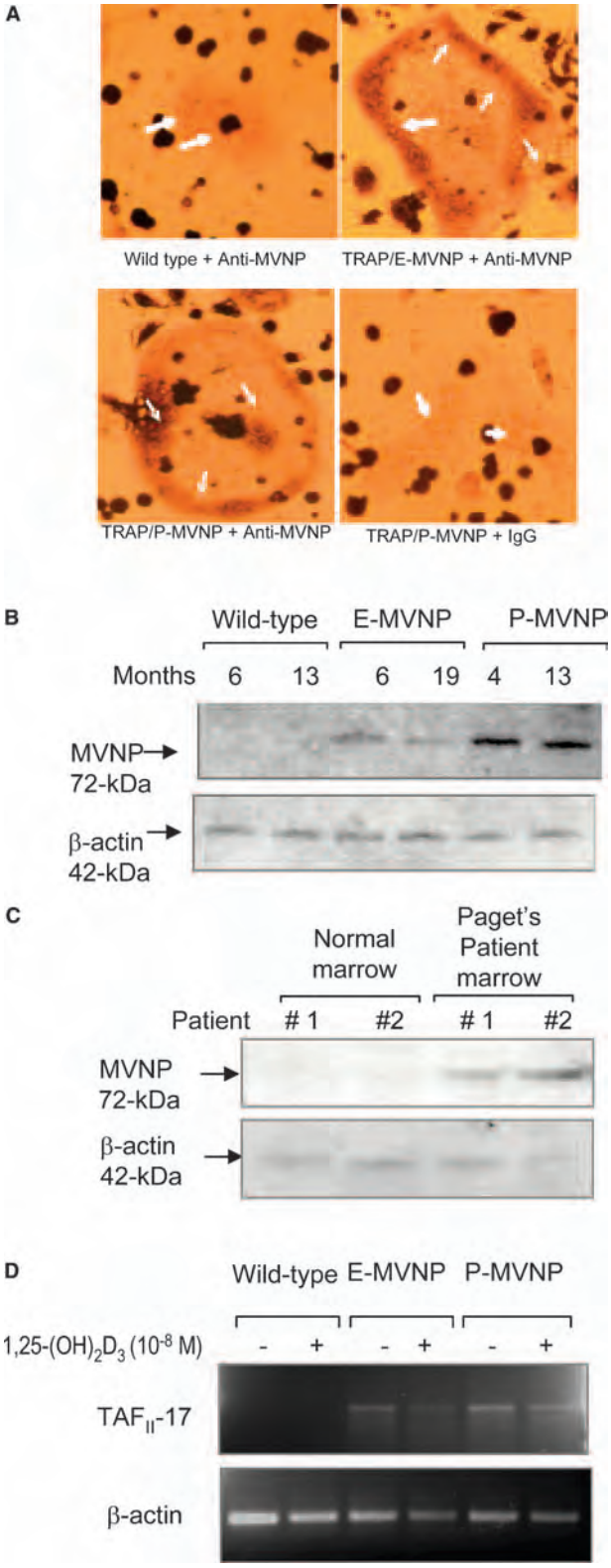

FIG. 1. (A) Expression of MVNP in OCLs formed from TRACP/MVNP mice. Nonadherent mouse bone marrow cells from WT, TRACP/E-MVNP, or TRACP/P-MVNP mice were cultured for 7 days with 10^{-8} M $1,25(OH)_2D_3$ and tested for cross-reactivity with a monoclonal antibody against the MVNP protein or control IgG using a Vectastatin-ABC-AP kit. Mouse IgG (60ng/ml) was used as a negative control. Arrows denote nuclei. Cross-reactivity with the anti-MVNP results in nuclei that are stained brown. The dark particles throughout are precipitated alkaline phosphatase stain. Magnification, ×100. (B) Western blot analysis of MVNP expression by nonadherent mouse bone marrow cells from TRACP/MVNP and WT mice and (C) MVNP in OCLs from marrow cultures from normal individuals and patients with PD. Lysates were prepared and analyzed. The gels were reprobed for β-actin expression to control for loading. (D) TAF_{II}-17 mRNA expression by MVNP and WT mice OCL precursors. MVNP and WT mice OCL precursors were cultured for 2 days with $1,25(OH)_2D_3$ (10^{-8} M) and subjected to RT-PCR analysis using the primers listed for TAF_{II}-17 or actin mRNA. The conditions for amplification were as follow: 94°C for 5 minutes, 35 (TAF_{II}-17) or 28 cycles (β actin) at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, followed by extension at 72°C for 7 minutes. PCR products were separated by 2% agarose gel electrophoresis.

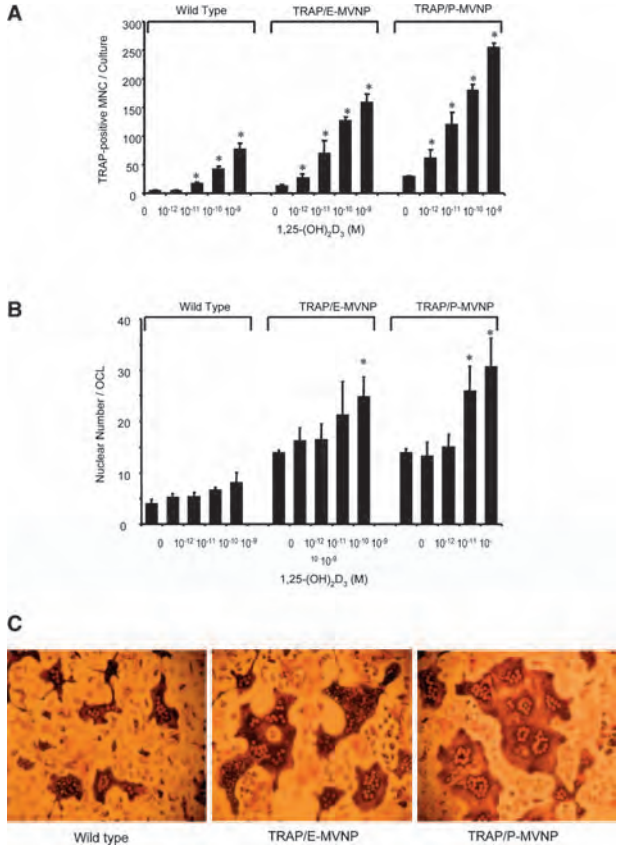

FIG. 2. OCLs formed in marrow cultures from TRACP/MVNP mice. (A) OCL formation in TRACP/MVNP mice. OCL precursors (10^5 cells/well) from WT, TRACP/E-MVNP, or TRACP/P-MVNP mice were cultured in the presence of varying concentrations of $1,25(OH)_2D_3$. After 7 days of culture, the cells were fixed with 1% formaldehyde and stained for TRACP using a leukocyte acid phosphatase kit (Sigma). The $TRACP^+$ multinucleated cells were scored using an inverted microscope. The results are expressed as mean ± SE for quadruplicate cultures from a typical experiment. *Significantly different from marrow cultures treated with vehicle alone ($p < 0.01$). Similar results were obtained in five independent experiments. (B) Nuclear number per $TRACP^+$ OCLs. The number of nuclei per OCL was determined in 20 random $TRACP^+$ OCLs for each treatment group, and the results are expressed as mean ± SE. *Significantly different from cultures treated with vehicle alone ($p < 0.01$). (C) Morphology of multinucleated cells formed from TRACP-MVNP mice by OCL precursors treated with 10^{-8} M $1,25(OH)_2D_3$ for 9 days and stained for TRACP.

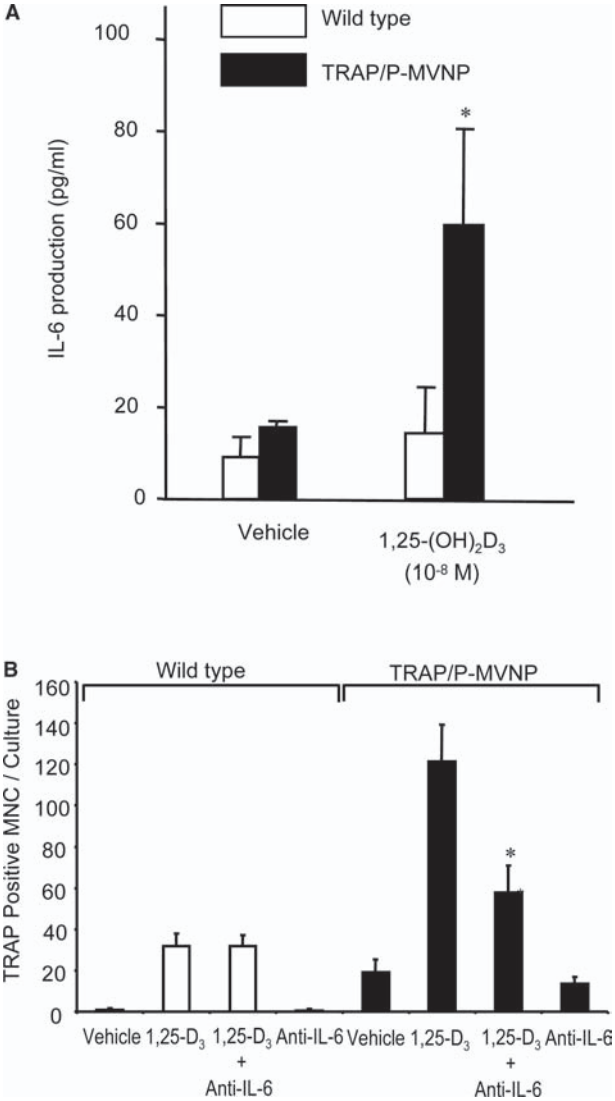

FIG. 3. (A) IL-6 production and (B) effects of anti-IL-6 on bone marrow cultures from TRACP/MVNP or WT mice. (A) Mouse bone marrow cells were treated with vehicle (media alone) or 10^{-8} M $1,25(OH)_2D_3$ for 7 days. Conditioned media were harvested at 7 days, and the concentration of IL-6 was determined using ELISA kits for mouse IL-6 (R&D Systems). Results are reported as IL-6 concentration (pg/ml) and are the mean ± SD of triplicate samples. A similar pattern of results was seen in two independent experiments. *Significantly different from WT marrow treated with the same concentration of $1,25(OH)_2D_3$ ($p < 0.001$). (B) Bone marrow cells (10^5 cells/well) from TRACP/P-MVNP or WT mice were cultured with 10^{-8} M $1,25(OH)_2D_3$ in the presence or absence of 50 ng/ml anti-mouse IL-6 or isotype specific mouse IgG (R&D Systems). This concentration of anti-IL-6 can neutralize 5 ng/ml of IL-6. After 7 days of culture, the cells were fixed with 1% formaldehyde and stained for TRACP using a leukocyte acid phosphatase kit (Sigma). The $TRACP^+$ multinucleated cells were scored using an inverted microscope. The results are expressed as mean ± SE for quadruplicate cultures from a typical experiment. *Significantly different from TRACP/P-MVNP cultures treated with $1,25(OH)_2D_3$ alone ($p < 0.001$). Similar results were obtained in three independent experiments.

anti-IL-6 had no effect on OCL formation or nuclear number per OCL in WT bone marrow cultures (Fig. 3B; Table 1).

TABLE 1. EFFECTS OF ANTI-IL-6 ON NUCLEAR NUMBER/OCLS IN TRACP/MVNP MICE

Cell type	*Vehicle*	*Anti-IL-6 (50 ng/ml)*	*$1,25(OH)_2D_3$ (10^{-8} M)*	*$1,25(OH)_2D_3$ (10^{-8} M) + anti-IL-6 (50 ng/ml)*
WT mice	3 ± 1	4 ± 1	4 ± 1	4 ± 1
TRACP/P-MVNP	6 ± 1	5 ± 1	16 ± 5	6 ± 1*

Bone marrow cells from TRACP/P-MVNP or WT mice were treated with or without $1,25(OH)_2D_3$ and anti-IL-6. At the end of the culture period, the cells were stained for TRACP activity, and the number of nuclei per OCL was assessed in 15 random $TRACP^+$ OCLs for each treatment group, in quadruplicate cultures, from two independent experiments. The results are expressed as mean ± SD.

* Significantly different from cultures treated with $1,25(OH)_2D_3$ ($p < 0.01$).

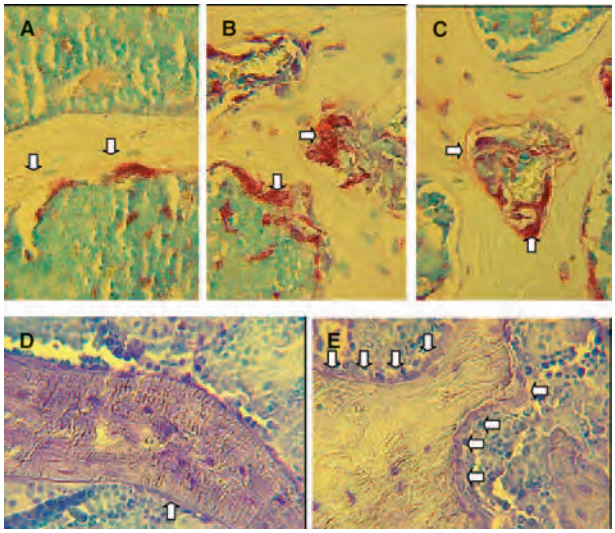

FIG. 4. OCL morphology in TRACP/MVNP and WT controls. OCL morphology in (A) WT controls and (B and C) TRACP/MVNP. Note larger OCLs in deeper resorption cavities and tunneling resorption in (B and C) MVNP (arrows) compared with fewer, smaller OCL in shallow resorption cavities in (A) WT controls (arrows). Original magnification, ×400. The number of mice analyzed is shown in Table 2. Osteoblast morphology in TRACP/MVNP and WT controls. Osteoblast morphology in (D) WT controls and (E) TRACP/MVNP. Note increased numbers of plump, cuboidal osteoblasts (arrows) in (D) TRACP/MVNP bones compared with fewer, flattened osteoblasts (arrow) in (E) WT bone. Original magnification, ×400.

Histology and histomorphometry of bones from TRACP/E-MVNP and TRACP/P-MVNP mice

There were no significant differences in the measured histomorphometric variables between bones from TRACP/E-MVNP and P-MVNP mice, and their histological features were qualitatively very similar. The data from these two groups were therefore pooled as one MVNP group and compared with WT animals.

MVNP OCLs were larger in size and had more nuclei per cell and the resorption cavities were deeper in MVNP bone

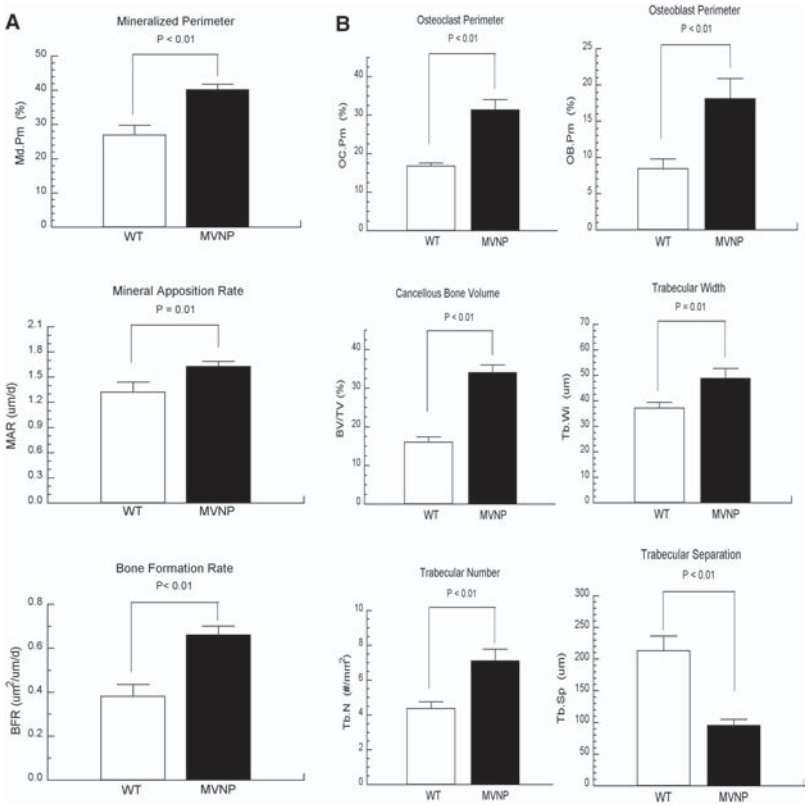

FIG. 5. (A) Mineralized perimeter, mineral apposition rate, and bone formation in 12 MVNP (age, 4–20 months; mean age, 13 months) and 10 WT mice (age, 4–19 months; mean age, 9 months). Data are expressed as mean ± SE. Note increased mineralized perimeter, mineral apposition rate, and bone formation rate in the MVNP mice. (B) OCL perimeter, osteoblast perimeter, cancellous bone volume, trabecular width, trabecular number, and trabecular separation in 4 12-month-old MVNP mice compared with 12 age-matched WT mice. Data are expressed as mean ± SE. Note increased OCL perimeter, osteoblast perimeter, cancellous bone volume, trabecular width, and trabecular number, but decreased trabecular separation in the MVNP mice. Data from all 14 12-month-old MVNP mice, including the 4 shown here, are shown in Table 3.

than in WT bone. Furthermore, tunneling resorption was present in MVNP bone but was rarely seen in WT bone (Figs. 4A–4C). Plump, cuboidal osteoblasts were more common in the MVNP than in the WT bone (Figs. 4D and 4E). Dynamic histomorphometric variables from nonlesioned bone in the calcein-labeled animals are shown in (Fig. 5A). Mineralized perimeter, mineral apposition rate, and bone formation rate were all significantly higher in the MVNP mice than in WT controls.

Markedly abnormal bone structure was seen in at least two of the four vertebrae examined in a subset of 4 of the 14 MVNP mice (29%) at 12 months of age. Two of these animals were TRACP/E-MVNP mice and two were TRACP/P-MVNP mice. These lesions were histologically similar to those seen in PD and were characterized by focally thickened and irregular trabeculae composed mainly of woven bone (Fig. 6). Cancellous bone volume, trabecular number, trabecular width, OCL perimeter, and osteoblast perimeter were all significantly increased in these four animals compared with age-matched WT controls, whereas trabecular separation was significantly reduced (Fig. 5B). None of these histological features was seen in WT controls.

To determine whether the dramatic changes seen in the vertebrae from these four animals were localized to individual vertebrae, we measured the histomorphometric variables in adjacent vertebrae that, qualitatively, did not appear to be as severely affected. These data are shown in Table 2. Whereas bone microarchitecture and turnover variables were not as abnormal in the adjacent vertebrae, they were still significantly different from those in WT animals. Data from the animals studied at 4, 8, and 12 months of age, including the lesioned bone from the four 12-month-old mice in Fig. 6, are given in Table 3. OCL perimeter was increased by 20–58% in MVNP mice compared with those from WT, and osteoblast perimeter was increased by 26–61%. The magnitude of the differences between MVNP and WT in OCL and osteoblast perimeters increased with age.

DISCUSSION

In this study, we determined the capacity of two different *MVNP* genes, one from an Edmonston group of MV originally isolated from a measles patient and one derived from a patient with PD, to induce a Paget's-like phenotype in transgenic mice. Expression of the two nucleocapsid genes was directed to cells in the OCL lineage using the TRACP promoter, which is highly expressed in OCLs and OCL precursors and has been used previously to target expression of multiple genes to cells in the OCL lineage.[30] TRACP is also expressed in chondrocytes and occasional osteoblasts in bone[31] but at very low levels compared with OCLs.

OCL precursors from TRACP/E-MVNP and TRACP/P-MVNP mice were found to be similar to each other and express almost all the features of pagetic OCL precursors. These include increased levels of OCL formation and a marked hyper-responsivity to $1,25(OH)_2D_3$. In addition, the OCLs that form are larger and contain many more

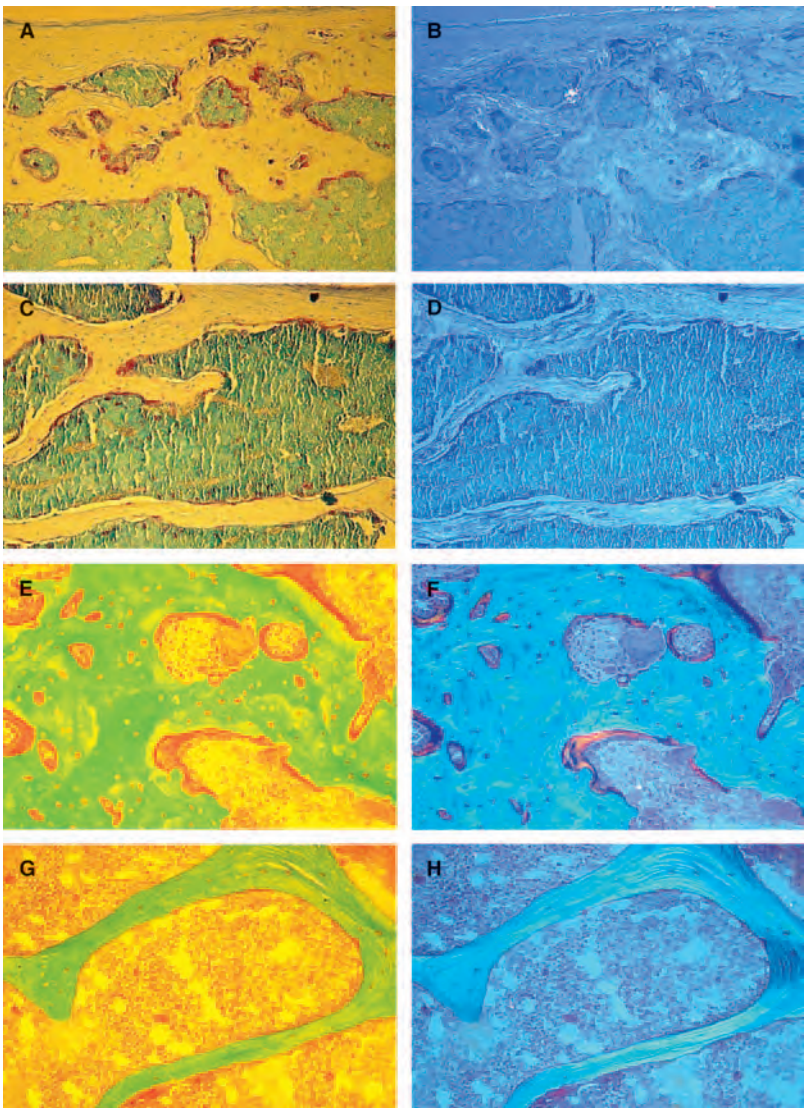

FIG. 6. Histological features of a 12-month-old TRACP/E-MVNP mouse compared with WT control. Histological features in (A and B) a TRACP/E-MVNP mouse compared with (C and D) WT controls at 12 months of age. Note thickened, irregular trabeculae, increased OCL number, (A) tunneling resorption, and (B) increased amounts of woven bone in the TRACP/MVNP mouse compared with the (C and D) WT control. Provided for comparison are sections from (E and F) a 58-year-old woman with PD and (G and H) a 58-year-old normal subject. (A and C) TRACP stain, counterstained with methyl green-thionin. (C and D) Same sections viewed under polarized light to reveal woven bone. (E and G) Goldner's trichrome stain. (G and H) Same sections viewed under polarized light. Original magnification, ×100.

nuclei per OCL. The only phenotypic difference that distinguished OCL precursors from TRACP/E-MVNP or TRACP/P-MVNP mice from OCL precursors from PD patients is that TRACP/MVNP OCL precursors are not hyper-responsive to RANKL.[32,33] These data suggest that additional factors, possibly genetic factors linked to PD, may be responsible for the hyper-responsivity of pagetic OCLs to RANKL.

Importantly, bones from the TRACP/E-MVNP or TRACP/P-MVNP mice displayed many of the histologic and histomorphometric features of bone lesions from patients with PD. These include an increase in mineralized perimeter, mineral apposition rate, bone formation rate, an increase in OCL and osteoblast perimeters, increases in the number and size of OCLs with more nuclei/OCL, deeper resorption cavities and tunneling resorption, and abundant large cuboidal osteoblasts. Furthermore, the bone that was formed was abnormal and was woven in character, similar to that seen in pagetic lesions. These changes were particu-

TABLE 2. CANCELLOUS BONE IN 12-MONTH MVNP AND WT MICE

Variables	*WT (n = 12)*	*MVNP lesion (n = 4)*	*MVNP adjacent (n = 4)*
BV/TV (%)	16.0 ± 1.4	34.1 ± 1.9*	24.9 ± 1.1*
Tb.Wi (μm)	37.1 ± 2.2	48.7 ± 4.0*	45.8 ± 2.7
Tb.N (#/mm^2)	4.4 ± 0.4	7.1 ± 0.7*	5.5 ± 0.2
Tb.Sp (μm)	213.2 ± 22.8	95.4 ± 9.3*	137.8 ± 5.9
Oc.Pm (%)	16.8 ± 0.8	31.4 ± 2.7*	28.3 ± 1.9*
Ob.Pm (%)	8.5 ± 1.4	19.9 ± 1.8*	17.8 ± 2.6*

Data are expressed as mean ± SE. The data were analyzed using ANOVA with Duncan's multiple-comparison.

* $p < 0.01$ vs. WT. MVNP data were pooled from two TRACP/E-MVNP and two TRACP/P-MVNP mice.

MVNP lesion, data from vertebrae with Paget's-like lesion; MVNP adjacent, data from vertebrae adjacent to those with Paget's-like lesion; BV/TV, cancellous bone volume; Tb.W, trabecular width; Tb.N, trabecular number; Tb.Sp, trabecular separation; Oc.Pm, osteoclast perimeter; Ob, osteoblast perimeter.

TABLE 3. CANCELLOUS BONE STRUCTURE IN MVNP AND WT MICE

Variables	*WT at 4 months (n = 18)*	*MVNP at 4 months (n = 17)*	*WT at 8 months (n = 12)*	*MVNP at 8 months (n = 10)*	*WT at 12 months (n = 12)*	*MVNP at 12 months (n = 14)*
BV/TV (%)	18.3 ± 0.8	16.9 ± 1.2	17.1 ± 1.9	17.8 ± 0.8	16.0 ± 1.4	20.4 ± 2.6
Tb.Wi (μm)	34.0 ± 1.0	31.9 ± 1.1	35.0 ± 2.28	36.7 ± 1.1	37.1 ± 2.2	39.9 ± 2.3
Tb.N (#/mm^2)	5.4 ± 0.2	5.3 ± 0.3	4.8 ± 0.4	4.9 ± 0.2	4.4 ± 0.4	5.0 ± 0.5
Tb.Sp (μm)	155.7 ± 6.7	163.9 ± 9.7	184.7 ± 24.1	171.8 ± 7.0	213.2 ± 22.8	182.8 ± 21.9
Oc.Pm (%)	17.5 ± 1.1	21.0 ± 0.8*	17.0 ± 1.3	21.3 ± 1.3*	16.8 ± 0.8	26.5 ± 1.9*
Ob.Pm (%)	11.6 ± 1.1	14.6 ± 1.3*	6.9 ± 1.0	10.1 ± 1.2*	8.5 ± 1.4	13.6 ± 1.5*

Data are expressed as mean ± SE.
See Table 2 for abbreviations.
* Significant differences between MVNP and MVNP ($p < 0.01$, by two-factor ANOVA).

larly evident in 30% of the animals at 12 months of age. In addition to the marked increase in turnover, the lesions in these animals displayed a dramatic increase in bone volume and trabecular thickness. The abnormally thickened and coarse trabeculae were remarkably similar to those seen in PD, and the fact that these dramatic lesions were only observed in the oldest animals is also consistent with the slow development of pagetic lesions. Furthermore, not all of the bones in these animals were as severely affected, consistent with a variable rate of expression of the phenotype in different bones, although adjacent bones showed increased OCL and osteoblast activity (Table 2). These data suggest that the rate of development of the pagetic-like lesions differed in the individual vertebral from these mice. In contrast to these findings, high turnover states are generally associated with reduced, rather than increased, bone volume in both humans and experimental animals. For example, transgenic mouse models of both primary and secondary hyperparathyroidism display increased resorption and formation, but bone volume is reduced.[34,35] Also, unlike the histological changes accompanying chronic PTH excess,[36] the MVNP mice did not show any peritrabecular or marrow fibrosis. Increased bone volume does accompany high bone turnover states, but only when the stimulus is anabolic (e.g., intermittent, exogenous administration of PTH or PGE_2).[37,38] Woven bone formation is also seen under such circumstances but requires very high doses.[37,38] Furthermore, such anabolic effects occur throughout the skeleton, rather than being restricted to individual bones, as seen in the TRACP/MVNP mice. Furthermore, other transgenic mouse models in which the TRACP promoter has been used to target genes to the OCL lineage do not develop pagetic-like bone lesions similar to those found in TRACP/MVNP mice.[30] When taken together, these observations strongly suggest that the bone lesions observed in the MVNP mice are the result of osteoclastic expression of the nucleocapsid genes rather than being caused by a generalized high turnover state.

We previously hypothesized that the sequence variants of the MVNP transcripts isolated from Paget's patients might contribute to the unique pathogenic role of MVNP in PD.[20] However, in this study, we introduced two MVNP variants into transgenic mice, one originally derived from a MV patient and one derived from a Paget's patient, and the resulting phenotype is indistinguishable. The majority of the amino acid substitutions between either E-MVNP or P-MVNP and the Edmonston WT MVNP, as well as the differences between E-MVNP and P-MVNP, fall within the C-terminal region of the protein, which is known to be hypervariable.[21] Thus, it now seems unlikely that the unique amino acid substitutions seen in P-MVNP specifically contribute to its pathogenic role in PD.

The *MVNP* gene can have effects on other human cells in addition to OCLs. During initial infection by MV, transient profound immune suppression occurs, followed by development of long-term immunity to MV.[39] The mechanism for this immunosuppression involves binding of the nucleocapsid protein to the Fc-γ receptors on dendritic cells, resulting in suppression of IL-12 expression and increased IL-6 expression.[40] These data suggest that MVNP can have profound effects on cellular function in cells of the monocyte macrophage lineage, and it is the same precursor cell in this lineage that gives rise to both OCL and dendritic cells. Thus, it is reasonable that the OCL precursors in the monocyte macrophage lineage could be affected by MV.

The mechanism(s) underlying the capacity of the *MVNP* gene to induce pagetic-like OCLs in vitro and in vivo are still being defined. Previous reports have shown that chronic infection of human glial cells with MV markedly increases IL-6 production with little or no increase in IL-1β or TNF-α expression.[41] Induction of IL-6 expression in OCL seems to play a role in the abnormal OCLs formed in TRACP/MVNP mice. lL-6 levels were increased in conditioned media from marrow cultures of TRACP/P-MVNP mice induced to form OCLs, and an anti-IL-6 antibody decreased both OCL formation and nuclei/OCL in TRACP/P-MVNP marrow cultures. In contrast, IL-6 levels were not increased in WT marrow cultures treated with $1,25(OH)_2D_3$, and anti-IL-6 had no effect on OCL formation in WT cultures. We previously showed that OCLs formed in cultures of marrow from PD patients also produce high levels of IL-6.[17] Taken together, these data support an important role for IL-6 in the abnormal OCL formation in PD and suggest that MVNP may be responsible for the increased IL-6 expression in pagetic OCLs. These results further suggest that other factors yet to be defined are involved in the abnormal OCL formation in TRACP/MVNP mice because anti-IL-6 did not reduce OCL formation to control levels.

However, this factor does not seem to be IL-11, because IL-11 levels were not increased by MVNP expression in OCL precursors.

Although the experiments reported here do not prove that MV can cause PD, they clearly show that expression of MVNP in cells of the OCL lineage can result in bone lesions and abnormalities in OCL precursors that are very similar to those found in patients with PD. In addition, just as in patients with a genetic predisposition to PD (e.g., an inherited *SQSTM1* mutation), the development of PD is variable, not all of the mice in our cohorts developed PD-like lesions. Furthermore, like patients with PD, the lesions that did arise were focal despite the forced expression of MVNP in the majority of OCLs in the transgenic mice.

Thus, persistent expression of MVNP in cells of the OCL lineage can induce pagetic-like lesions in vivo. These results suggest that persistent expression of MVNP in OCLs is an important component in the complex etiology of PD.

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Etiology of Paget's Disease and Osteoclast Abnormalities

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Abstract Paget's disease of bone is a chronic focal skeletal disorder that affects up to 2–3% of the population over the age of 60 years. Paget's disease is primarily a disease of the osteoclast. The pathologic abnormality in patients with Paget's disease involves increased bone resorption by the osteoclasts, followed by abundant new bone formation that is of poor quality. Genetic linkage analysis indicated that 40% of patients with Paget's disease have an affected first degree relative and 1% of patients develop osteosarcoma. Paget's disease is an autosomal dominant trait with genetic heterogeneity. Recurrent mutations in the ubiquitin-associated (UBA) domain of sequestosome 1 (SQSTM1/p62) are identified in patients with Paget's disease. Osteoclasts and osteoclast precursors from patients with Paget's disease contain paramyxoviral transcripts and appear hyperresponsive to 1,25-$(OH)_2D_3$ and RANK ligand (RANKL). It has been suggested that the enhanced sensitivity of osteoclast precursors for 1,25-$(OH)_2D_3$ in Paget's disease results from increased expression of coactivators of vitamin D receptor (VDR). However, a cause and effect relationship for the paramyxoviral infection and SQSTM1/p62 gene mutations associated with this disease and osteoclast abnormalities are unclear. Therefore, the etiology of Paget's disease remains uncertain. J. Cell. Biochem. 93: 688–696, 2004.

Key words: Paget's disease; osteoclast; measles virus (MV); sequestosome (p62); tartrate resistant acid phosphatase (TRAP); RANK ligand (RANKL)

Sir James Paget first described Paget's disease of bone in 1877 as Osteitis Deformans, a chronic focal skeletal disease that can be monostotic or polyostotic. Paget's disease is the second most common metabolic bone disease and affects between 2% and 3% of the population over the age of 60. The disease been associated with deformity and enlargement of single or multiple bones among which, the skull, clavicles, long bones, and vertebral bodies are the most frequently involved [Paget, 1877]. Patients with Paget's disease are frequently asymptomatic, but approximately 10–15% of the patients have severe symptoms including bone pain, fractures, neurological complications due to spinal cord compression, deafness, and dental abnormalities. Paget's disease is a highly localized disease, and new lesions rarely develop during the course of the disease. Rather, lesions continue to progress in size unless treated. The primary pathologic abnormality in patients with Paget's disease is increased bone resorption, followed by abundant new bone formation. The bone that is formed is disorganized and of poor quality, resulting in bowing of the bone, stress fractures, and arthritis in joints contiguous to the involved bones. In addition, patients with Paget's disease can develop hypercalciuria and hypercalcemia, due to accelerated bone resorption induced by immobilization. Another interesting feature of Paget's disease is that bones not clinically involved with Paget's disease appear to show increased bone remodeling. This increased bone remodeling in unaffected bones has been ascribed to secondary hyperparathyroidism rather than to subclinical involvement of the bones with Paget's disease. However, less than 20% of patients with Paget's disease have elevated parathyroid hormone (PTH) levels [Siris, 1999]. A juvenile form of Paget's disease also called hyperostosis corticalis deformans juvenilis or hereditary hyperphosphatasia is very different than the adult form of

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the disease. It is characterized by widespread involvement of the skeleton and has histological and radiological features that distinguish it from Paget's disease of the adults, including the absence of viral-like nuclear inclusions in osteoclasts present in the bone microenvironment [Whyte et al., 2002].

Bone scans are the most sensitive method of detecting pagetic lesions and can be used to follow the activity of the disease in these patients. Initial lesions appear osteolytic, followed by a chaotic sclerotic appearance, and finally become osteosclerotic. Considerable thickening of the sclerotic bone results in bone deformity. Serum tartrate resistant acid phosphatase (TRAP), presumably released by osteoclasts, appears to be an index of bone resorption in Paget's disease but is not routinely used. The most useful markers for the increased osteoblast activity in Paget's disease are the total alkaline phosphatase and bone-specific alkaline phosphatase activity levels in serum. Patients also showed significantly higher endothelin-1 circulating levels than controls with a positive correlation with serum alkaline phosphatase, but not with urinary hydroxyproline [Tarquini et al., 1998]. Furthermore, serum calcium levels are typically normal in Paget's disease and also serum osteocalcin levels appear to be a poor index of the progression of the disease. Bisphosphonates are the most common treatment for patients with Paget's disease. These inorganic phosphate compounds inhibit osteoclast-mediated bone resorption and induce osteoclast apoptosis [Siris, 1999]. It has been reported that serum M-CSF levels are significantly elevated in patients with Paget's disease, however not significantly different in patients under treatment compared to normal subjects [Neale et al., 2002].

Paget's disease has a very unusual geographic distribution, with an increased incidence in Caucasians of European descent, but it also occurs in African Americans. It is rare in those of Asian descent. Studies also suggested high prevalence rates of radiographic Paget's disease in Britain, Australia, North America, and Western Europe. Interestingly, even within Britain there is a marked geographic variation in the incidence of Paget's disease, with an increased incidence in the Western portion of England and a much lower incidence in the Southern portion of England (8% vs. 4%) [Cooper et al., 1999]. The prevalence of the disease is 2.5% among men and 1.6 % among women aged 55 years and above in British towns. The level of prevalence in Spain is estimated to be at 1.5%. A radiographic survey of 24 patients with Paget's disease in Ireland further revealed monostotic disease in 8 and polyostotic disease in 16. There have been reports that one to three million patients over the age of 55 are affected with Paget's disease in the United States. This is in contrast with the extreme rarity of the disease in Scandinavia, Ireland, and Southern Europe. This unusual geographic distribution for the incidence of Paget's disease is not attributable to geographic, environmental, or industrial exposures in these areas and currently cannot be explained. Furthermore, the incidence of Paget's disease appears to be decreasing over the last several decades [Siris, 1999; Doyle et al., 2002], but the basis for this decrease in the incidence of Paget's disease is unknown. However, it is evident that genetic factors play important role in the familial and sporadic forms of Paget's disease. Furthermore, Paget's disease has been described as a slow paramyxoviral infection process, suggesting a viral etiology for the disease. Therefore, this review will focus on the etiology of Paget's disease with an emphasis on the role that genetic and paramyxoviral infection may play in abnormal osteoclast development responsible for excess bone resorption in patients with Paget's disease.

GENETICS OF PAGET'S DISEASE

Familial incidence is common in Paget's disease and 40% of patients with the disease have an affected first-degree relative. Therefore, genetic factors play an important role in the pathogenesis of Paget's disease of bone. The disease often is inherited in an autosomal dominant manner manifesting genetic heterogeneity and incomplete penetrance. Familial Paget's disease has an equal incidence in males and females. A genetic locus for Paget's disease has been identified on chromosome 18q [Leach et al., 2001; Good et al., 2002] in several large families with Paget's disease in a region near the familial expansile osteolysis (FEO) locus. FEO is a disease related to Paget's disease but occurring in patients at a much younger age and being a much more severe disease. FEO is an extremely rare disease, affecting only a very limited number of kindreds in the world, also mapped to chromosome 18q and is linked to activating mutations in the TNFRSF11A gene

which encodes receptor activator of nuclear factor κB (RANK) [Hughes et al., 2000]. Recently, in patients with Juvenile Paget's disease, a homozygous deletion of the gene on chromosome 8q24.2 that encodes osteoprotegerin, member of the superfamily of tumor necrosis factor receptors, has been reported [Whyte et al., 2002]. However, linkage studies, coupled with mutation screening have excluded involvement of RANK and also osteoprotegerin in the majority of patients with Paget's disease of bone [Sparks et al., 2001]. Studies also indicated that patients with Paget's disease have an increased incidence of osteosarcoma, with approximately 1% of patients with Paget's disease developing osteosarcoma in an affected bone. This incidence of osteosarcoma is 1,000 times higher than that in the general population for this age group. Recent genetic studies have demonstrated linkage in seven of seven patients with osteosarcoma to loss of heterozygosity in a region of 18q that is adjacent to or within a locus for Paget's disease on 18q [Hansen et al., 1999].

A genome wide search in familial Paget's disease of bone further indicated genetic heterogeneity of the disease with candidate loci on chromosomes 2q, 10q, and 5q [Hocking et al., 2001]. More recently, the gene encoding sequestosome 1 (SQSTM1/p62) mapped within the critical region on chromosome 5q35-qter identified a proline-leucine amino acid change at codon 392 (P392L) in French-Canadian patients with Paget's disease of bone [Laurin et al., 2002]. The frequency of mutation was 16% and 46% for sporadic and familial cases tested, respectively. Further studies also identified different mutations affecting the highly conserved ubiquitin-binding domain of SQSTM1/p62 protein in patients with familial and sporadic Paget's disease [Johnson-Pais et al., 2003; Good et al., 2004; Hocking et al., 2004].

ROLE OF SQSTM1/P62 IN OSTEOCLASTOGENESIS

The atypical PKC (aPKC) interaction with SQSTM1/p62 has been implicated in signaling cascades that control NF-κB activation. It is evident that p62 provides a scaffold linking the aPKCs to the tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1) receptor signaling complexes through its interaction with RIP and TRAF-6, respectively [Moscat and Diaz-Meco, 2000]. Thus, SQSTM1/p62 mediate IL-1 and TNF-α cytokine signaling to activate NF-κB (Fig. 1). TRAF-6 plays an essential role in RANK ligand (RANKL) signaling during osteoclastogenesis. More recently, it has been shown that RANKL stimulation results in upregulation of p62 expression in osteoclast precursor cells and that the genetic inactivation of p62 in mice impaired PTHrP induced osteoclastogenesis in vivo. However, p62 null mice have grossly normal skeletal phenotype and that no alterations were found in the trabecular size and number of osteoclasts compared to wild-type mice. In vitro studies further demonstrated that p62 deficiency leads to inhibition of IKK activation and NF-κB nuclear translocation during osteoclastogenesis [Duran et al., 2004]. These studies also demonstrated that RANKL stimulation induces formation of a ternary complex involving TRAF-6, p62, and aPKC during osteoclastogenesis. Recent studies also identified novel mutations in ubiquitin-associated (UBA) domain of SQSTM1/p62, however, genotype-phenotype analysis indicated that there is no correlation with respect to different mutations in UBA and disease occurrence [Hocking et al., 2004]. Therefore, the precise role that SQSTM1/p62 may play in the pathogenesis of Paget's disease of bone remains to be elucidated.

VIRAL ETIOLOGY

Since the early 1970s, a variety of studies have implicated paramyxoviruses in Paget's disease. The viral etiology has been proposed for Paget's disease with an initial description of nucleocapsid-like structures in the nuclei and cytoplasm of pagetic osteoclasts by electron microscopy [Mills and Singer, 1976]. Immunocytochemical studies further confirmed that these nuclear inclusions cross-reacted with antibodies that recognized measles virus (MV) or respiratory syncytial virus (RSV) nucleocapsid antigens. In situ hybridization techniques also identified the presence of MV messenger RNA (mRNA) sequences in up to 90% of osteoclasts and other mononuclear cells in pagetic bone specimens. Similarly, canine distemper virus (CDV) nucleocapsid antigens were also detected in osteoclasts from patients with Paget's disease. These paramyxoviral-like nuclear inclusions are not unique to Paget's disease and were reported in patients with FEO and rarely in patients with osteopetrosis, pycnodysostosis, and otosclerosis, oxalosis [Singer, 1999]. This has raised the

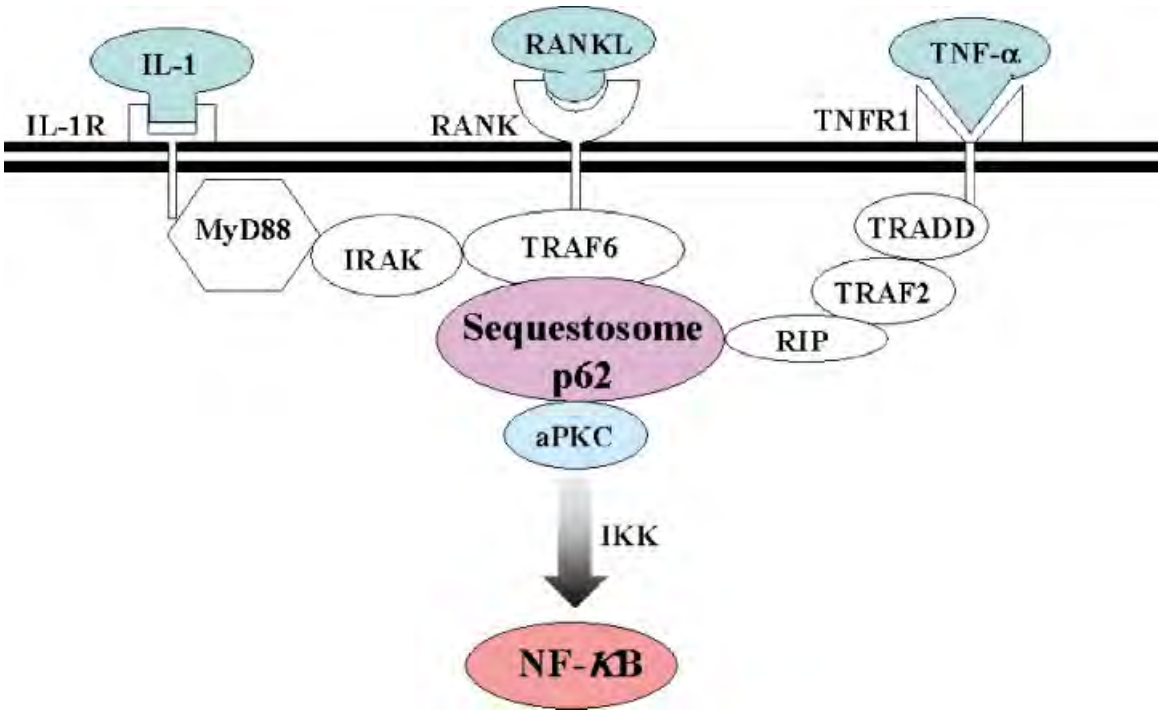

Fig. 1. Signaling cascades associated with sequestosome/p62. Sequestosome provides a scaffold linking the aPKCs to the TNF-α and IL-1 receptor signaling complexes through its interactions with RIP and TRAF-6, respectively resulting in phosphorylation of IKK and activation of NF-κB. RANKL-RANK signaling induce p62 to form a ternary complex with TRAF-6 and aPKCs during osteoclast differentiation [Duran et al., 2004]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

possibility that that the virus may be a non-etiologic agent in a cell altered by a genetic defect.

To further explore the viral etiology, using reverse transcription-polymerase chain reaction (RT-PCR) analysis, we have amplified the MV nucleocapsid (MVNP) transcripts from freshly isolated bone marrow cells from patients with Paget's disease. These MVNP transcripts contain mutations clustered at the c-terminal end of the mRNA [Reddy et al., 1995]. All these mutations were sense mutations and resulted in amino acid substitutions in the nucleocapsid gene product. The mutations occurred at 1% rate in the total MVNP gene isolated from a patient with Paget's disease. We further demonstrated that osteoclast precursors, the granulocyte macrophage colony-forming unit (CFU-GM), as well as mature osteoclasts from patients with Paget's disease, expressed MVNP transcripts. Since CFU-GM circulate and also give rise to monocytes and granulocytes in the peripheral blood, we then examined peripheral blood mononuclear cells from patients with Paget's disease and normals for expression of MVNP transcripts. We found by RT-PCR analysis that peripheral blood samples from 9 of 10 patients with Paget's disease contain MVNP transcripts, while none of the 10 normals tested expressed MVNP transcripts [Reddy et al., 2001a]. We were unable to find CDV or RSV nucleocapsid transcripts in patients we have studied. In contrast, CDV nucleocapsid transcripts were detected in affected bones from 100% of patients tested using in situ RT-PCR techniques. Furthermore, it has also been demonstrated that infecting canine bone marrow cells with CDV results in development of multinucleated cells that share some of the phenotypic characteristics of pagetic osteoclasts [Gordon et al., 1992]. However, other workers have been unable to detect paramyxoviral nucleocapsid transcripts in samples obtained from patients with Paget's disease [Helfrich et al., 2000; Ooi et al., 2000].

The presence of MV or CDV transcripts in osteoclasts and osteoclast precursors from patients with Paget's disease does not infer a

pathophysiologic role for these genes in the development of the pagetic lesions. It is possible that these paramyxoviral transcripts and paramyxoviral-like inclusions are simply markers for the disease and have no pathophysiologic significance. In studies, using normal osteoclast precursors (CFU-GM) transduced with retroviral vectors expressing the MVNP gene formed large osteoclasts more rapidly with an increased numbers of nuclei, hypersensitive to 1,25-dihydroxyvitamin D3 (1,25-$[OH]_2D_3$) and had increased bone resorbing capacity compared to normal osteoclasts. In contrast, normal osteoclast precursors transduced with the MV matrix gene did not express an abnormal phenotype [Kurihara et al., 2000]. In further studies, we have targeted CD46, human MV receptor to cells of the osteoclast lineage in transgenic mice and demonstrated that MV infection of osteoclast precursors from CD46 transgenic mice form osteoclasts, which express a pagetic phenotype in vitro [Reddy et al., 2001b]. Taken together, these data suggest a potential pathophysiologic role for the paramyxoviral nucleocapsid gene that is expressed in patients with Paget's disease. Mouse models of MV infection were also developed in which CD46 is introduced into transgenic mice and has been bred to another transgenic mouse lacking the alpha-beta-interferon receptor. Upon exposure to MV, these mice developed immune-suppression similar to patients with acute MV infection. The mice lack the alpha-beta interferon receptor demonstrated persistence of MV infection for at least 12 days [Peng et al., 2003]. However, TRAP-CD46 mice do not develop sustained MV infection, most likely reflecting the need for blocking interferon production for development of persistent MV infection in these mice.

MV infection has a similar incidence worldwide and occurs in very young patients, whereas Paget's disease is a disease of the elderly. These observations suggest that if paramyxoviruses have an etiologic role in Paget's disease, these viral infections must persist for long periods of time. To further investigate a potential site for the initial infection of osteoclast precursors with Paget's disease, we tested the hypothesis that very early pluripotent hematopoietic stem cells, which can persist for long periods of time in a quiescent phase, may be the initial target for the paramyxoviral infection in patients with Paget's disease. We found that other hematopoietic lineages from patients with Paget's disease in addition to the osteoclast lineage, including the erythroid and the erythroid precursors, burst-forming unit-erythroid (BFU-E), and multipotent myeloid precursors (CFU-GEMM), which give rise to megakaryocytes, monocytes, erythroid cells, and granulocytes, also contain paramyxoviral nucleocapsid transcripts [Reddy et al., 2001a]. Thus, if the initial site of infection occurs in a small number of primitive pluripotent hematopoietic stem cells that predominantly remain in Go, this might explain the chronicity of the infection. Furthermore, there may be a genetic predisposition for chronic paramyxoviral infections of hematopoietic precursors in patients with Paget's disease. However, a cause and effect relationship of paramyxoviruses in Paget's disease remains proven as yet no infectious virus been isolated from pagetic cells and also, it is not clear how the initial lesion occurs in Paget's disease.

PAGETIC OSTEOCLASTS

Histologic examination of pagetic bone biopsy revealed abundant structurally abnormal osteoclasts. Osteoclasts are increased in number and size, and contain as many as 100 nuclei per multinucleated cell compared to three to five nuclei for a normal osteoclast. These osteoclasts have characteristic ultrastructural abnormalities including microfilaments, paracrystalline arrays located in the nucleus and sometimes in the cytoplasm that are absent in non-pagetic bone or bone marrow cells. These inclusions closely resemble nucleocapsids of viruses of the paramyxoviridae family [Mills and Singer, 1976]. Osteoblasts are also increased in lesions in patients with Paget's disease, and they appear to be morphologically normal. Osteoblasts contain abundant rough endoplasmic reticulum and mitochondria in a well-developed Golgi zone, consistent with the increased bone formation activity that occurs in the active lesions. In advanced lesions in patients with Paget's disease, the marrow is also abnormal. The bone matrix in Paget's disease is highly abnormal in structure due to disordered bone remodeling. The bone matrix consists of erratic patterns of "cement lines" and demonstrates a "mosaic" pattern. The matrix is interspersed with numerous foci of woven bone, reflecting the increased rates of bone deposition that is of poor quality.

The bone marrow culture techniques identified several abnormalities in osteoclast formation and osteoclast precursors from patients with Paget's disease. Osteoclast-like multinucleated cells formed more rapidly with increased numbers (10–100-fold) and nuclei per osteoclast, expressed high levels of TRAP in marrow cultures from patients with Paget's disease compared to normals. In addition, osteoclast formation in pagetic bone marrow cultures was induced at concentrations of 1,25-$(OH)_2D_3$ that were 10–100 times lower than those required in normal marrow cultures. Structural examination of the osteoclast-like cells formed in bone marrow cultures also showed that they had many of the features of pagetic osteoclasts but lacked the characteristic nuclear and cytoplasmic inclusions. Immunocytochemical studies confirmed that MV and RSV nucleocapsid antigens were expressed in osteoclasts formed in vitro in these cultures. Osteoclasts from patients with Paget's disease also appear to produce increased levels of IL-6 and express high levels of IL-6 receptors compared to normal osteoclasts. In situ hybridization studies have further identified increased levels of IL-6, c-fos proto-oncogene, Bcl 2 anti-apoptotic gene mRNA expression in pagetic osteoclasts. IL-6 receptor and NF-IL-6 mRNA levels were also increased in osteoclasts from bone samples from patients with Paget's disease compared to those with osteoarthritis [Hoyland et al., 1994]. These data suggest that IL-6, which is a stimulator of human osteoclast formation, may act as an autocrine/paracrine factor to enhance osteoclast formation in patients with Paget's disease and increase the osteoclast precursor pool. IL-6 levels were also shown to increase in bone marrow plasma and peripheral blood of patients with Paget's disease [Roodman et al., 1992]. In addition, the increased levels of IL-6 in the peripheral blood of patients with Paget's disease may in part explain the increased bone remodeling seen in bones not clinically involved with Paget's disease.

To further investigate the potential abnormalities in osteoclast precursors in patients with Paget's disease, the number of osteoclast precursors in marrow aspirates from involved bones from patients with Paget's disease were assessed. It has been found that the number of early osteoclast precursors, CFU-GM, was increased significantly in marrow aspirates from patients with Paget's disease compared to normals. Interestingly, when the osteoclast precursors were separated from the marrow microenvironmental elements present in the marrow aspirates, similar numbers of osteoclast precursors were detected in these aspirates. These data suggested that the marrow microenvironment enhanced osteoclast precursor growth compared to the normal marrow microenvironment.

To determine the potential role of the marrow microenvironment and the enhanced osteoclast formation in patients with Paget's disease, reconstitution experiments were conducted using highly purified populations of osteoclast precursors from patients with Paget's disease or normals and marrow stromal cells from patients with Paget's disease and normals. Coculture of normal osteoclast precursors with marrow stromal cells from patients with Paget's disease resulted in enhanced growth of the osteoclast precursors from normals. Interestingly, when osteoclast precursors from patients with Paget's disease were cocultured with marrow stromal cells from normals, they also showed increased growth. These data suggest that both the marrow microenvironment, as well as the osteoclast precursors, are abnormal in patients with Paget's disease.

These studies also confirmed that the osteoclast precursors were hypersensitive to 1,25-$(OH)_2D_3$ compared to normals. The increased sensitivity of osteoclast precursors from Paget's patients to 1,25-$(OH)_2D_3$ is mediated through the vitamin D3 receptor (VDR). This was confirmed by upregulation of 24-hydroxylase mRNA expression in pagetic osteoclast precursors at concentrations of 1,25-$(OH)_2D_3$ that are one log less than that required for normal osteoclast precursors. The increased sensitivity to 1,25-$(OH)_2D_3$ was not due to increased numbers of vitamin D receptors in pagetic osteoclast precursors compared to normals, but appeared to be due to enhanced affinity of the VDR in pagetic cells for its ligand compared to normals [Menaa et al., 2000a]. Recently, it has been demonstrated that MVNP gene expression in osteoclast precursors results in increased levels of TAF_{II}-17 transcription factor gene expression. The high levels of TAF_{II}-17 permit formation of VDR transcription complex at low levels of receptor occupancy by 1,25-$(OH)_2D_3$ [Kurihara et al., 2004]. These results support the hypothesis that part of the pathophysiology underlying the increased osteoclast activity in Paget's

disease is due to increased levels of VDR coactivators that enhance VDR-mediated gene transcription at low levels of 1,25-$(OH)_2D_3$. These studies suggested that Paget's disease may be a VDR coactivator disease.

The osteoclast precursors from patients with Paget's disease also appear to be hyperresponsive to receptor activator of NF-κB ligand (RANKL) and that marrow stromal cells from pagetic lesions have increased RANKL expression [Neale et al., 2000; Menaa et al., 2000b]. RANKL is a critical osteoclast differentiation factor that is expressed on marrow stromal and osteoblast cells in response to several osteotropic factors. The increased sensitivity of osteoclast precursors from Paget's patients to RANKL appears to be due to interactions of these precursors with interleukin-6 (IL-6). Addition of neutralizing antibodies to IL-6 decreased the sensitivity of the osteoclast precursors from patients with Paget's disease to RANKL to normal levels. Similarly, addition of IL-6 to cultures of normal osteoclast precursors enhanced the responsivity of these precursors to RANKL to the levels seen with pagetic osteoclast precursors. Pagetic osteoclasts expressing MVNP gene produce high levels of cytokines that increase osteoclast precursor pool as well as osteoblast precursor proliferation and constitutive expression of RANKL, which contribute to the abnormal osteoclast development and highly localized nature of Paget's disease (Fig. 2). Immature osteoblasts are the major responders to RANKL inducing cytokines and studies also suggested that expression of RANKL decreases with osteoblast maturation [Gori et al., 2000]. Therefore, the increased numbers of highly active osteoblasts rapidly form large amounts of woven bone in patients with Paget's disease. Furthermore, the prodigious amounts of cytokine production by the pagetic osteoclasts result in continued stimulation of osteoblast precursors growth making the local microenvironment in the pagetic lesion progressively more osteoclastogenic resulting in elevated levels of bone resorption in these patients. Consistent with this hypothesis are findings of high levels of cytokines such as IL-6 being produced by pagetic osteoclasts and the increased RANKL protein levels in marrow adherent cells from pagetic lesions compared to normal marrow and uninvolved bones from the same patient [Menaa et al., 2000b]. In addition, the clinical observation that inhibiting osteoclast formation with bisphosphonates results in a dramatic fall in alkaline phosphatase in

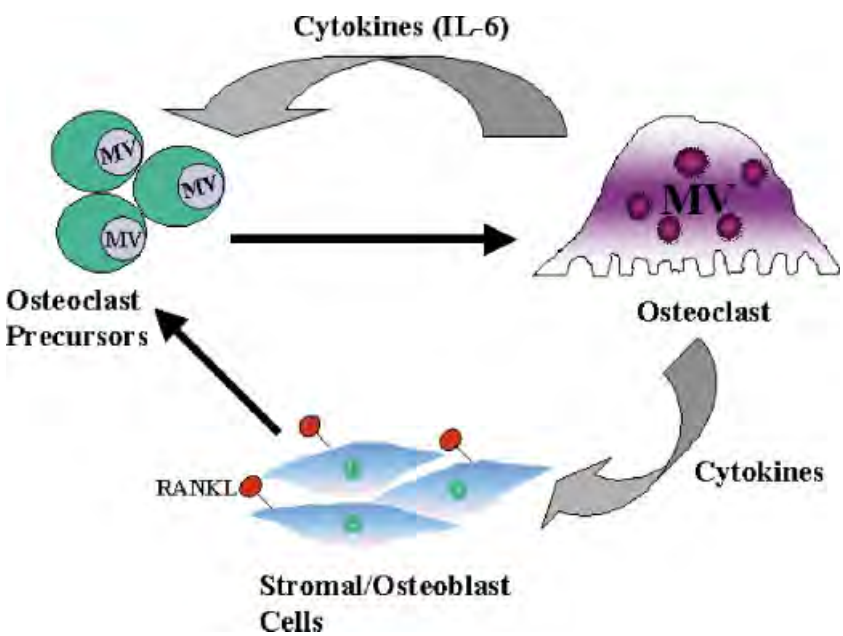

Fig. 2. Osteoclastogenesis in pagetic bone microenvironment. The osteoclast precursors contain measles virus (MV) transcripts and are hyperresponsive to RANK ligand (RANKL). The pagetic osteoclasts produce increased levels of cytokines such as IL-6, which enhance osteoclast formation. Chronic exposure to cytokines produced by the pagetic osteoclasts results in constitutive overexpression of RANKL in stromal/osteoblast cells further enhancing the abnormal osteoclast development in pagetic bone lesions. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

patients with Paget's disease, demonstrates that osteoclasts are driving the osteoblastic response in Paget's disease. Alternatively, pagetic osteoclasts may live for very long periods of time compared to normal osteoclasts and persist in the lesion. It has been reported that the anti-apoptotic gene Bcl-2 is overexpressed in pagetic osteoclasts, suggesting that the osteoclasts lifespan may be prolonged in pagetic lesions. More recently, it has also been reported that mutations in the Bcl-2 gene promoter are responsible for upregulation of Bcl-2 expression leading to enhanced osteoclastogenesis in patients with Paget's disease [Brandwood et al., 2003]. Recently, it has been shown that SHIP, inositol 5′ phosphatase deficient mice are severely osteoporotic with an increased numbers of osteoclast precursors and hyperactive osteoclasts. In addition, serum levels of IL-6 are markedly increased in these mice as in Paget's disease [Takeshita et al., 2002]. However, the basis for these abnormalities in both osteoclasts and osteoclast precursors from patients with Paget's disease is still unknown.

SUMMARY AND FUTURE DIRECTIONS

Paget's disease of bone is the second most disorder of bone after osteoporosis. The disease is an autosomal dominant trait with genetic heterogeneity. The recent discovery of recurrent mutations occurring in the UBA domain of SQSTM1/p62 in patients with Paget's disease suggests that genetic factors may play an important role. Although SQSTM1/p62 mutations are implicated as a common cause of familial and sporadic Paget's disease, there is no correlation among different mutant forms of p62 and disease severity. Lack of skeletal abnormalities in p62 null mice further suggests a potential role for genes present in other candidate loci that were linked with Paget's disease. Alternatively, a genetic defect may favor the environmental factors such as MV infection to have potential role in pathogenesis of the disease. However, the molecular basis for the abnormalities associated with osteoclasts, the role of paramyxoviral infection and persistence of the virus in patients with Paget's disease is unclear. Targeting the expression of candidate genes to the cells of osteoclast lineage in transgenic mouse that are permissive to MV infection may allow better understanding of the pathobiology of Paget's disease. It is important to determine a cause and effect relationship for persistence of paramyxoviral infection and genetic predisposition in these patients. Therefore, the etiology of Paget's disease remains uncertain.

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Mutation of the sequestosome 1 (*p62*) gene increases osteoclastogenesis but does not induce Paget disease

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Paget disease is the most exaggerated example of abnormal bone remodeling, with the primary cellular abnormality in the osteoclast. Mutations in the *p62* (sequestosome 1) gene occur in one-third of patients with familial Paget disease and in a minority of patients with sporadic Paget disease, with the P392L amino acid substitution being the most commonly observed mutation. However, it is unknown how $p62^{P392L}$ mutation contributes to the development of this disease. To determine the effects of $p62^{P392L}$ expression on osteoclasts in vitro and in vivo, we introduced either the $p62^{P392L}$ or WT *p62* gene into normal osteoclast precursors and targeted $p62^{P392L}$ expression to the osteoclast lineage in transgenic mice. $p62^{P392L}$-transduced osteoclast precursors were hyperresponsive to receptor activator of NF-κB ligand (RANKL) and TNF-α and showed increased NF-κB signaling but did not demonstrate increased 1,25-$(OH)_2D_3$ responsivity, TAF_{II}-17 expression, or nuclear number per osteoclast. Mice expressing $p62^{P392L}$ developed increased osteoclast numbers and progressive bone loss, but osteoblast numbers were not coordinately increased, as is seen in Paget disease. These results indicate that $p62^{P392L}$ expression on osteoclasts is not sufficient to induce the full pagetic phenotype but suggest that *p62* mutations cause a predisposition to the development of Paget disease by increasing the sensitivity of osteoclast precursors to osteoclastogenic cytokines.

Introduction

Paget disease (PD) is the second most common bone disease in persons of Anglo-Saxon descent over the age of 55 (1). It is the most exaggerated example of disordered bone remodeling, with abnormalities in all phases of the bone remodeling process (2). The primary cellular abnormality resides in the osteoclast (OCL). OCLs in PD are increased in number and size and have increased numbers of nuclei (3). In addition, they are hyperresponsive to 1,25-$(OH)_2D_3$ and receptor activator of NF-κB ligand (RANKL) (4, 5) and show increased expression of TAF_{II}-*17*, a member of the $TF_{II}D$ transcription complex, which acts as a coactivator of vitamin D receptor–mediated transcription and is upregulated in pagetic osteoclasts (6).

Both genetic and environmental factors have been proposed as contributing to the etiology of PD, and multiple families with an autosomal dominant mode of inheritance have been described (7, 8). Recently, mutations in the *p62* (sequestosome 1) gene have been linked to approximately 30% of patients with familial PD and to a minority of patients with sporadic PD (8). All of the mutations identified to date lie within or near the ubiquitin-binding domain in the carboxyterminal region of the protein, with the P392L amino acid substitution representing the most frequently observed mutation (8). *p62* plays a critical role in NF-κB activation induced by TNF-α, CD40, and IL-1 through its interactions with the atypical protein kinases ζPKC and λPKC (9, 10). However, the role of *p62* mutations in PD is unclear since not all individuals carrying a *p62* mutation have PD (11–13).

It is our hypothesis that both genetic and nongenetic factors are required for the development of PD and that the genetic factors, such as *p62* mutations, function to increase OCL formation but are not sufficient to induce the abnormal OCLs or pagetic bone lesions characteristic of PD. To test this hypothesis, we characterized OCL precursors from Paget patients carrying the $p62^{P392L}$ mutation as well as control human OCL precursors transduced with either *p62* or $p62^{P392L}$ expression vectors. We also determined whether the $p62^{P392L}$ gene can induce pagetic-like OCLs or bone lesions in vivo when expressed in OCL precursors of transgenic mice.

Results

OCL precursors from PD patients carrying the $p62^{P392L}$ gene are hyperresponsive to RANKL, TNF-α, and 1,25-$(OH)_2D_3$ and express increased levels of TAF_{II}-17. OCL precursors from PD patients carrying the $p62^{P392L}$ mutation or from controls were compared for their capacity to form OCLs over a range of concentrations of RANKL, TNF-α, and 1,25-$(OH)_2D_3$. OCL precursors from patients carry-

Nonstandard abbreviations used: ERK1/2, extracellular signal–regulated kinase 1/2; EV, empty vector; IκB, inhibitor of NF-κB; MVNP, measles virus nucleocapsid protein; OCL, osteoclast; PD, Paget disease; RANKL, receptor activator of NF-κB ligand; TRAP, tartrate-resistant acid phosphatase.

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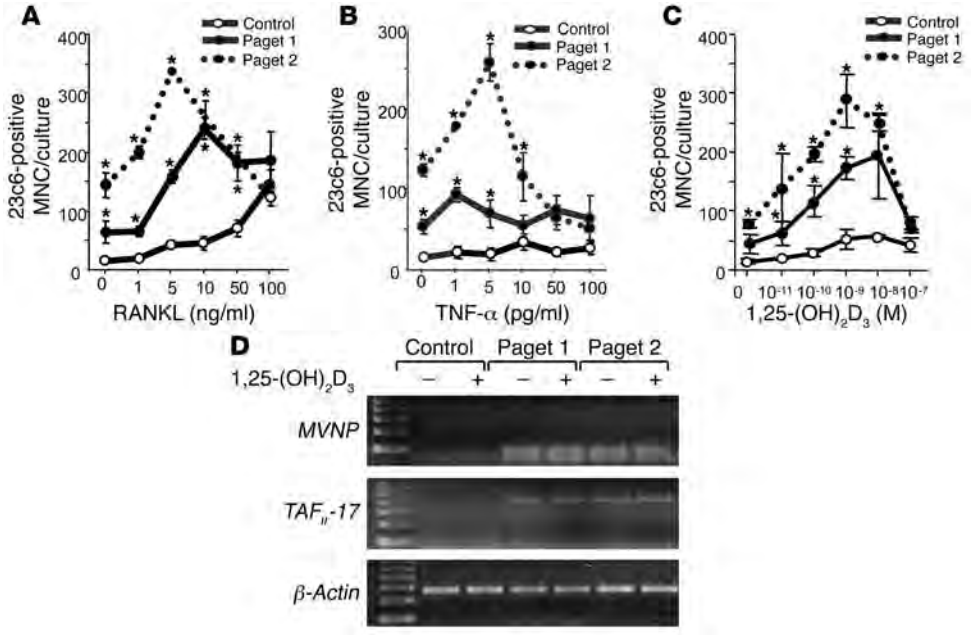

Figure 1
OCL formation and expression of *MVNP* and *TAF_{II}-17* in GM-CFU from *$p62^{P392L}$*-positive PD patients and controls. (**A**–**C**) GM-CFU (10^5 cells/well) from Paget patients known to harbor the *$p62^{P392L}$* mutation and from normal individuals were cultured for OCL formation in the presence of RANKL (**A**), TNF-α (**B**), or 1,25-$(OH)_2D_3$ (**C**). After 3 weeks of culture, cells were fixed and stained with the 23c6 monoclonal antibody, which identifies OCLs. Results are expressed as the mean ± SEM for quadruplicate determinations. *Significant differences ($P < 0.001$) compared with results from cultures of normal GM-CFU treated with the same concentration of each factor. A similar pattern of results was seen in 2 independent experiments. Paget 1 and Paget 2 refer to PD patient sample groups 1 and 2. MNC, multinuclear cell. (**D**) RT-PCR analysis of *MVNP* and *TAF_{II}-17* mRNA expression in GM-CFU from *$p62^{P392L}$*-positive PD patients and controls cultured for 2 days with 1,25-$(OH)_2D_3$. RT-PCR analysis of β-*actin* expression was included as a control for mRNA quality and amplification.

ing the *$p62^{P392L}$* mutation were hyperresponsive to RANKL and TNF-α as well as 1,25-$(OH)_2D_3$ (Figure 1, A–C); this is similar to previous findings from patients with sporadic PD (3–5). In cultures of OCL precursors from PD patients with *$p62^{P392L}$* mutations, maximum OCL formation was obtained at RANKL and TNF-α concentrations of 1–10 ng/ml and 1–10 pg/ml, respectively, compared with 100 ng/ml and 50 pg/ml for normal OCL precursors. Similar results were obtained with 3 individual PD patients. Further, the OCLs that formed contained increased numbers of nuclei per OCL (Table 1) and elevated expression of *TAF_{II}-17* (Figure 1D). However, *measles virus nucleocapsid protein* (*MVNP*) transcripts were also present in OCLs from these patients (Figure 1D), raising the possibility that a subset of the phenotypic characteristics of these OCLs might be caused by the presence of *MVNP* rather than *$p62^{P392L}$*.

OCL formation by p62- and $p62^{P392L}$-transduced normal OCL precursors. To further dissect the role of *$p62^{P392L}$* in PD, we transduced normal human OCL precursors with the WT *p62* or *$p62^{P392L}$* gene or *empty vector* (*EV*). The total p62 expression levels were determined by immunoblotting extracts from transduced GM-CFU–derived cells with an antibody that recognizes both WT and mutant *p62*. The *p62* protein was detected in both *p62*- and *$p62^{P392L}$*-transduced GM-CFU without treatment with RANKL. In contrast, *p62* protein was only detected in *EV*-transduced GM-CFU after 1 day of treatment with RANKL (data not shown).

$p62^{P392L}$-transduced OCL precursors treated with varying concentrations of RANKL or TNF-α were found to be hyperresponsive to both cytokines and formed increased numbers of OCLs (Figure 2, A and B). While overexpression of WT *p62* resulted in a degree of hyperresponsivity to RANKL similar to that of *$p62^{P392L}$*, the *$p62^{P392L}$* cells were much more hyperresponsive to TNF-α than WT *p62* cells. Both *p62*- and *$p62^{P392L}$*-transduced OCL precursors formed significantly larger OCLs than EV cells (Figure 2D), although nuclear number per OCL was not increased in *p62*- or *$p62^{P392L}$*-derived OCLs regardless of treatment (Table 1). Also, a 7-fold increase in bone resorption was observed when OCLs formed by *$p62^{P392L}$*-transduced human GM-CFU were treated with RANKL (50 ng/ml) and cultured on dentin, as compared with RANKL-treated *EV*-transduced OCLs (Figure 3, A and B).

In contrast with the results with RANKL and TNF-α, neither *p62*- nor *$p62^{P392L}$*-transduced OCL precursors were hyperresponsive to 1,25-$(OH)_2D_3$ (Figure 2C) or formed increased numbers of OCLs compared with EV-transduced cells. In addition, neither *p62*- nor *$p62^{P392L}$*-transduced GM-CFU–derived cells showed elevated expression of *TAF_{II}-17* in the presence or absence of 10^{-10} M 1,25-$(OH)_2D_3$ (Figure 4).

Effects of $p62^{P392L}$ expression on OCLs in vivo. Since OCLs harbor the primary cellular abnormality in PD, we used the tartrate-resistant acid phosphatase (TRAP) promoter to target expression of the human *$p62^{P392L}$* gene to the OCL lineage in transgenic mice (TRAP-*$p62^{P392L}$* mice) to determine the effects of *$p62^{P392L}$* expression in OCLs in vivo. Eight lines of TRAP-*$p62^{P392L}$* transgenic mice were generated from independent founder mice, and each of these was characterized with regard to transgene expression level, TNF-α responsivity, and histology. The results described below were obtained from a single line (Tp62m2), although similar results were observed in multiple other lines as well. The expression levels of total *p62* protein in mice of the Tp62m2 line, as determined by immunoblotting of extracts from bone marrow cells with an antibody that detects both murine and human *p62*, were found to be approximately 2.5-fold higher than *p62* levels in WT mice (data not shown).

Histomorphometric evaluation of vertebral bones from TRAP-*$p62^{P392L}$* mice at 4, 8, 12, and 16 months of age revealed an increase in OCL perimeter (the amount of bone surface covered with TRAP-positive, mono-, and multinuclear cells) and a progressive reduction in cancellous bone volume when compared with that of age-matched WT controls (Table 2 and Supplemental Figure 1; supplemental material available online with this article;

Table 1
Nuclear number per OCL

Cell type	Vehicle	1,25-$(OH)_2D_3$ (10^{-8} M)	RANKL (50 ng/ml)	TNF-α (50 pg/ml)
Control human	10 ± 3	13 ± 2	12 ± 2	13 ± 2
Familial PD	24 ± 3	52 ± 13[A]	32 ± 5[A]	25 ± 4[A]
Human GM-CFU–*EV*	6 ± 3	7 ± 2	12 ± 5	7 ± 2
Human GM-CFU–*p62*^{P392L}	5 ± 3	7 ± 2	10 ± 2	8 ± 2
WT mice	5 ± 3	6 ± 2	9 ± 2	5 ± 2
TRAP-*p62*^{P392L} mice	5 ± 3	6 ± 2	9 ± 2	5 ± 2

The number of nuclei per OCL was determined in 20 random 23c6-positive or TRAP-positive OCLs for each treatment group. Results are expressed as mean ± S.D. [A]Significantly different from the same treatment as the normal donor, $P < 0.01$.

doi:10.1172/JCI28267DS1). The decrease in cancellous bone volume was associated with decreases in both trabecular width and number. Although OCL perimeter was elevated, there was no coupled increase in osteoblast perimeter, as is seen in PD lesions.

Electron microscopic examination of OCLs from TRAP-*p62*^{P392L} mice demonstrated that the cells did not contain the nuclear inclusions characteristic of pagetic OCLs. OCLs were similar to those in WT controls in terms of nuclear and cytoplasmic ultrastructure as well in the morphology of the ruffled border (data not shown).

OCL precursors from TRAP-p62^{P392L} *mice are hyperresponsive to RANKL and TNF-α but not 1,25-$(OH)_2D_3$.* When marrow cells from TRAP-*p62*^{P392L} mice were cultured with RANKL and TNF-α to induce OCL formation, they were found to be hyperresponsive to both cytokines, and they formed increased numbers of OCLs as compared with nontransgenic littermates (Figure 5, A and B). Similarly, treatment of TRAP-*p62*^{P392L} mice with TNF-α (0–1.5 μg/d) significantly increased OCL formation compared with that of WT mice at all concentrations tested (Figure 5, E and G). Further, the dose-response curves for NF-κB reporter gene activity in TRAP-*p62*^{P392L} OCL precursors for both RANKL and TNF-α were shifted to the left compared with cells from WT mice (Figure 5, D and E). However, OCL precursors from TRAP-*p62*^{P392L} mice were not hyperresponsive to 1,25-$(OH)_2D_3$ (Figure 5C) and did not express detectable *TAF_{II}-17* (data not shown). They also did not have increased nuclear number per OCL (Table 1). These results are consistent with our results from *p62*^{P392L}-transduced human OCL precursors and indicate that expression of *p62*^{P392L} induces a subset of pagetic characteristics, including hyperresponsivity to the osteoclastogenic cytokines RANKL and TNF-α, but does not result in development of OCLs that express the complete pagetic phenotype or development of pagetic-like lesions.

To further examine the mechanisms responsible for the increased levels of OCL formation in marrow cultures from TRAP-*p62*^{P392L} mice, we determined the time course for OCL formation, the rates of proliferation of OCL precursors and of OCL apoptosis, and the expression levels of OCL differentiation markers. As shown in Figure 6A, OCL formation in marrow cultures from TRAP-*p62*^{P392L}

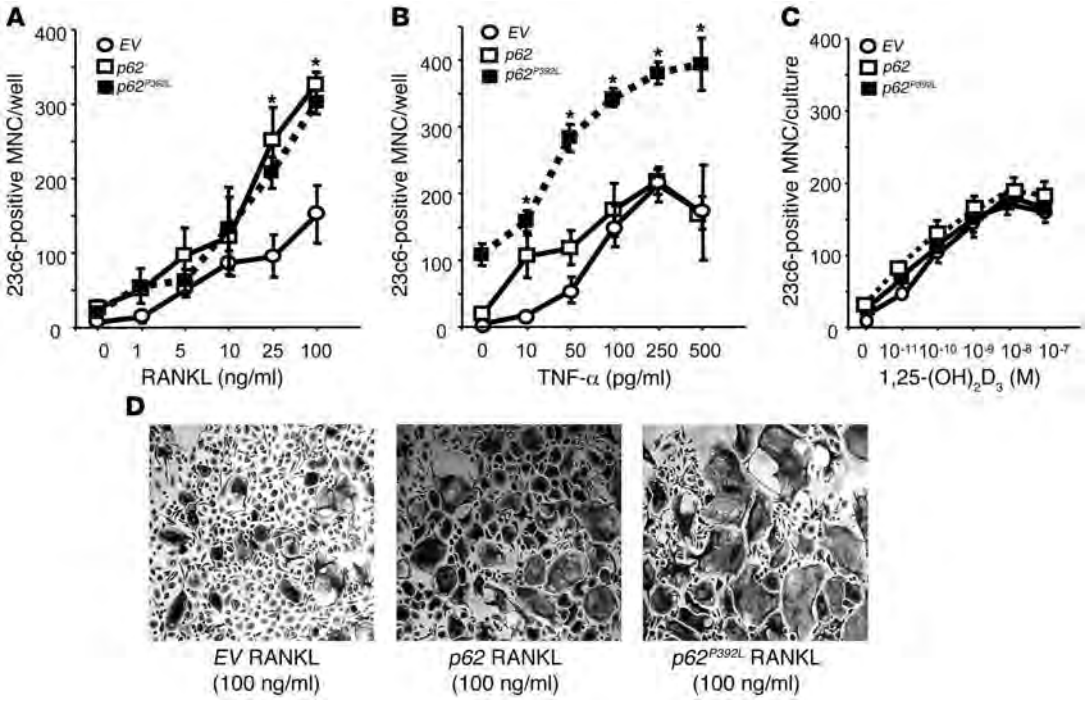

Figure 2
OCL formation by *p62*- and *p62*^{P392L}-transduced human OCL precursors. GM-CFU–derived cells (10^5 cells/well) transduced with *p62*, *p62*^{P392L}, or *EV* were cultured with RANKL (**A**), TNF-α (**B**), or 1,25-$(OH)_2D_3$ (**C**). After 3 weeks of culture, cells were fixed and stained with the 23c6 monoclonal antibody, which identifies OCLs. Results are expressed as the mean ± SEM for quadruplicate cultures from a typical experiment. *Significant differences ($P < 0.001$) compared with results of *EV*-transduced cell cultures treated with the same concentration of individual factors. A similar pattern of results was seen in 3 independent experiments. (**D**) Morphology of OCLs formed by *p62*^{P392L}- and *EV*-transduced GM-CFU–derived cells. Original magnification, ×100.

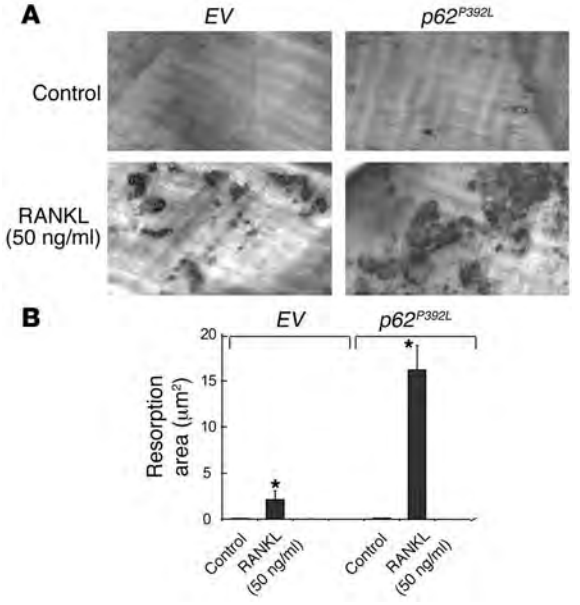

Figure 3
Resorption lacunae formed by OCLs from *p62^{P392L}*- and *EV*-transduced human GM-CFU. (**A**) Resorption lacunae formed on dentin by OCL. Original magnification, ×100. (**B**) Resorption areas per dentin slice for each treatment group. Results represent mean ± SEM for quadruplicate determinations for a typical experiment. Similar results were seen in 3 independent experiments. *Significant differences ($P < 0.05$).

mice was maximum after 6 days of culture while OCL formation in WT cultures was maximum at 9 days of culture. Further, OCL precursor proliferation was significantly increased in marrow cultures from *p62^{P392L}* mice compared with WT cultures but followed a similar time course (Figure 6B). In contrast, although the number of apoptotic OCLs was increased in TRAP-*p62^{P392L}* marrow cultures compared with WT marrow cultures, the percentages of apoptotic OCLs were similar (18% versus 10%; $P > 0.05$) in TRAP-*p62^{P392L}* and WT cultures after 9 days (Figure 6C). Apoptotic OCLs were not detected in cultures of mice from either genotype at day 3 or 6 of culture. Marrow cultures from TRAP-*p62^{P392L}* mice expressed relatively higher levels of TRAP, cathepsin K, and calcitonin receptor mRNA compared with WT cultures (Figure 7).

Since OCL precursors from Paget patients carrying the P392L mutation were hyperresponsive to 1,25-$(OH)_2D_3$ and also expressed *MVNP*, we transfected OCL precursors from TRAP-*p62^{P392L}* mice with *MVNP* and determined their responsivity to 1,25-$(OH)_2D_3$. As shown in Figure 8, OCL precursors from TRAP-*p62^{P392L}* mice transfected with *MVNP* were hyperresponsive to 1,25-$(OH)_2D_3$ and formed OCL at concentrations that were 1 to 2 logs lower than those of *EV*-transfected cells.

To further delineate the mechanisms responsible for the enhanced OCL formation in TRAP-*p62^{P392L}* mice, we examined inhibitor of NF-κB (IκB), p38 MAPK, and extracellular signal–regulated kinase 1/2 (ERK1/2) signaling in nonadherent marrow cells from WT and TRAP-*p62^{P392L}* mice. As shown in Supplemental Figure 2A, p-ERK1/2 was increased in marrow cells treated with RANKL or TNF-α while only modest changes were seen in p38 MAPK and p-IκB activity. Transfection of GM-CFU from WT and TRAP-*p62^{P392L}* mice with the MVNP gene further increased levels of NF-κB in TRAP-*p62^{P392L}* OCL precursors compared with those of WT precursors (Supplemental Figure 2B). Expression of MVNP in OCL precursors from WT or TRAP-*p62^{P392L}* mice did not increase expression of c-Fos.

Discussion

Mutations in the *p62* gene have been linked to PD in approximately one-third of patients with familial PD and a minority of patients with sporadic PD. However, it is unlikely that these mutations are sufficient to induce the OCL abnormalities and bone lesions that are characteristic of PD, since pagetic lesions are focal even in patients carrying germline *p62* mutations and some individuals harboring *p62* mutations fail to develop PD (11–13). To determine the role of *p62* mutation in PD, we characterized OCL precursors from familial PD patients carrying the most common PD-associated *p62* mutation (P392L) and compared them with normal OCL precursors. OCL precursors from *p62^{P392L}*-positive PD patients formed OCLs at lower concentrations of RANKL, TNF-α, and 1,25-$(OH)_2D_3$ than controls (Figure 1, A–C) and had increased TAF_{II}-17 expression (Figure 1D), all characteristic of PD; this was similar to our previous findings in OCL precursors from patients with sporadic PD (3–6). However, OCL precursors from these patients also expressed MVNP, which we have previously reported results in increased OCL formation in response to RANKL and 1,25-$(OH)_2D_3$ and to increased expression of *TAF_{II}-17* both in vitro and in vivo (6, 14).

To further characterize the contributions of *p62^{P392L}* to PD in the absence of *MVNP*, we transduced normal human OCL precursors with vectors encoding WT *p62* or *p62^{P392L}* or with *EV*. As with OCL precursors from PD patients, *p62^{P392L}*-transduced OCL precursors showed increased TNF-α and RANKL sensitivity (Figure 2, A and B) but, in contrast, did not demonstrate increased 1,25-$(OH)_2D_3$ responsivity (Figure 2C) or increased expression of *TAF_{II}-17* (Figure 4). In addition, nuclear number per OCL was not increased in *p62^{P392L}*-transduced OCLs (Table 1). Thus, these OCLs express only a subset of the characteristics of pagetic OCLs (15). Similar results were obtained when expression of the *p62^{P392L}* gene was targeted to cells in the OCL lineage in vivo. OCL precursors from TRAP-*p62^{P392L}* mice were hyperresponsive to RANKL and TNF-α but not to 1,25-$(OH)_2D_3$ (Figure 5, A–C) and did not express increased levels of *TAF_{II}-17*. Further, OCL formation in TRAP-*p62^{P392L}* mice was significantly increased in vivo by treatment with TNF-α (Figure 5, F and G).

Both RANKL and TNF-α activate signaling pathways involving p62 that ultimately lead to the activation of NF-κB, p38 MAPK, and ERK1/2, which are important for OCL formation and function.

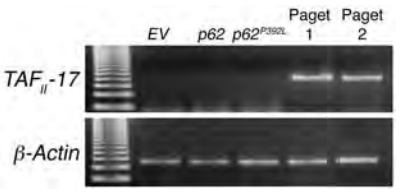

Figure 4
TAF_{II}-17 expression in *p62*-, *p62^{P392L}*-, and *EV*-transduced human OCL precursors.GM-CFU–derived cells (10^6 cells) transduced with *p62*, *p62^{P392L}*, and *EV* were cultured for 2 days with 10^{-8} M 1,25-$(OH)_2D_3$. RNA was then prepared and subjected to RT-PCR analysis of *TAF_{II}-17* or β-*actin* expression. The 2 PD patient samples shown in Figure 1 were used as positive controls.

Table 2
Cancellous bone structure and bone turnover in TRAP-*p62^{P392L}* mice

Variable	WT 4 mo	p62 4 mo	WT 8 mo	p62 8 mo	WT 12 mo	p62 12 mo	WT 16 mo	p62 16 mo
	n = 16	*n* = 9	*n* = 20	*n* = 11	*n* = 15	*n* = 3	*n* = 5	*n* = 11
F:M	14:2	7:2	15:5	6:5	13:2	3:0	3:2	1:10
BV/TV (%)	19.1 ± 0.8	15.4 ± 1.3[B]	15.3 ± 1.0	13.6 ± 0.7[B]	16.2 ± 1.1	11.5 ± 1.7[B]	17.6 ± 2.3	12.7 ± 1.0[B]
Tb.Wi (μ*m*)	36.6 ± 1.0	33.6 ± 0.9[B]	35.5 ± 1.3	32.9 ± 0.7[B]	39.0 ± 1.7	35.3 ± 3.7[B]	39.9 ± 3.3	32.2 ± 1.0[B]
Tb.N (per *mm*^{2})[C]	5.2 ± 0.2	4.5 ± 0.2[A]	4.3 ± 0.2	4.1 ± 0.2[A]	4.2 ± 0.3	3.2 ± 0.2[A]	4.4 ± 0.4	3.9 ± 0.3[A]
Tb.Sp (μ*m*)	158.2 ± 6.6	189.9 ± 10.1[A]	212.0 ± 14.0	216.0 ± 12.9[A]	214.8 ± 17.5	277.0 ± 22.6[A]	198.6 ± 29.1	235.6 ± 20.3[A]
Oc.Pm (%)	19.4 ± 0.8	22.7 ± 1.7[B]	18.0 ± 0.9	20.8 ± 2.0[B]	17.6 ± 0.7	27.4 ± 3.9[B]	16.8 ± 1.7	20.6 ± 2.3[B]
Ob.Pm (%)	12.3 ± 1.3	10.8 ± 1.6	8.4 ± 0.9[D]	7.4 ± 0.8[D]	8.8 ± 1.0[D]	4.5 ± 1.9[D]	8.9 ± 1.7[D]	13.0 ± 0.9[D]

Data are expressed as mean ± SEM. P62, TRAP-*p62^{P392L}* mice; BV/TV, cancellous bone volume; Tb.Wi, trabecular width; Tb.N, trabecular number; Tb.Sp, trabecular separation; Oc.Pm, OCL perimeter; and Ob.Pm, osteoblast perimeter. Data were analyzed using 2-way ANOVA. Significant differences are indicated as follows: [A]$P < 0.05$ and [B]$P < 0.01$ versus WT; [C]$P < 0.05$ and [D]$P < 0.01$ versus 4-month group. A significant interaction ($P < 0.05$) between the factors of treatment (*p62* or WT) and age (4, 8, 12, and 16 months) is noted in the variable of osteoblast perimeter.

Our results demonstrate elevated NF-κB activation as well as p38 MAPK and ERK1/2 signaling in OCLs expressing *p62^{P392L}*, strongly suggesting that Paget-associated mutations in p62 lead to increased osteoclastogenesis by stimulating signaling pathways that activate NF-κB (Supplemental Figure 2A). However, the detailed mechanisms by which these p62 mutations activate signaling remain to be determined. All of the PD-associated *p62* mutations reside in or near the ubiquitin-binding domain and result in loss of the ubiquitin-binding capacity of *p62* (16). Thus, it is possible that the ubiquitin-binding domain of *p62* normally mediates a protein/protein interaction that dampens NF-κB signaling in OCLs in response to inflammatory cytokines, such that loss of this interaction leads to increased activation of these pathways.

It is interesting to note that in both transduced human OCL precursors and in transgenic mouse OCL precursors, expression of *p62^{P392L}* had a much more dramatic effect on responsivity to TNF-α than to RANKL. Consistent with our results, Duran et al. have reported that the P392L mutation in *p62* increased NF-κB reporter activity (17). These results suggest that TNF-α, in addition to RANKL and 1,25-$(OH)_2D_3$, may be involved in the increased osteoclastogenesis in PD and should be studied further.

Histomorphometric analysis of vertebral cancellous bone in the TRAP-*p62^{P392L}* transgenic mice revealed a phenotype that was characterized by low bone volume with reduced trabecular number and width. OCL perimeter was increased at all ages examined, but there was no coupled increase in osteoblast perimeter. This marked imbalance between OCL formation and new bone formation is analogous to imbalance in inflammatory diseases of bone, such as rheumatoid arthritis and lytic bone metastases, in which bone formation is suppressed, rather than PD, in which OCL activity is closely coupled to new bone formation. This phenotype is very different from that observed in TRAP-*MVNP* transgenic mice, which persistently express the gene encoding the MVNP (14). In contrast with TRAP-*p62^{P392L}* mice, TRAP-*MVNP* mice displayed a bone phenotype that closely resembled PD in humans. This included a coupled increase in both bone resorption and new bone formation and enlarged OCLs with increased nuclear number (14). Moreover, a subset (30%) of 12-month-old TRAP-*MVNP* mice displayed pagetic-like lesions, with increased bone volume and dramatically thickened and disorganized trabeculae composed primarily of woven bone. No such lesions were observed in the TRAP-*p62^{P392L}* mice, which were examined up until 18 months of age. In addition, OCL precursors from the TRAP-*MVNP* mice showed increased sensitivity to 1,25-$(OH)_2D_3$ and expressed increased levels of *TAF$_{II}$-17*, findings that were not observed in the TRAP-*p62^{P392L}* mice unless the cells were transfected with *MVNP* (Figure 8). Finally, transfection of OCL precursors from TRAP-*p62^{P392L}* mice with *MVNP* further increased levels of NF-κB, suggesting that *MVNP* can increase the enhanced OCL formation induced by the *p62^{P392L}* mutation (Supplemental Figure 2B).

One possible explanation for the failure of the TRAP-*p62^{P392L}* mice to display increased osteoblast activity is that expression of *p62^{P392L}* is restricted to cells of the OCL lineage in this model while familial PD patients carrying a *p62* mutation express the mutant protein in all cell types. It remains to be determined whether the *p62* mutation plays a direct role in other cell types besides OCL in PD. It will therefore be of interest to evaluate the bone phenotype in knockin mice carrying the analogous *p62* mutation in the germline, which are currently being developed.

Taken together, these results demonstrate that the expression of *p62^{P392L}* in OCLs increases OCL formation but is not sufficient to induce PD. Thus, *p62* mutations may predispose to the development of PD by increasing basal OCL activity, but 1 or more additional factors are required for development of the full PD phenotype.

Methods

Chemicals. FBS was purchased from Invitrogen. All other chemicals and media were purchased from Sigma-Aldrich, unless otherwise noted. RANKL, TNF-α, IL-3, IL-6, and GM-CSF were purchased from R&D Systems. The 1,25-$(OH)_2D_3$ was generously provided by Teijing Corp. Polyclonal anti-IκBα, anti–p-IκBα, anti-p38, anti–p-p38, anti-ERK, and anti–p-ERK antibodies were from Cell Signaling Technology. Protease inhibitor mixtures and SB203580 were from Calbiochem.

Isolation of OCL precursors from PD patients carrying the p62^{P392L} mutation and from normal individuals. After obtaining informed consent, we obtained heparinized peripheral blood from 3 patients with PD and 3 age-matched controls. These studies were approved by the Institutional Review Boards at the University of Pittsburgh and the John Wayne Cancer Institute. Nonadherent peripheral blood mononuclear cells were isolated as previously described (18). The cells were cultured in methylcellulose in the presence of 100 pg/ml of recombinant human GM-CSF to form GM-CFU colonies. Individual colonies were pooled after 7 days of culture. Aliquots from the

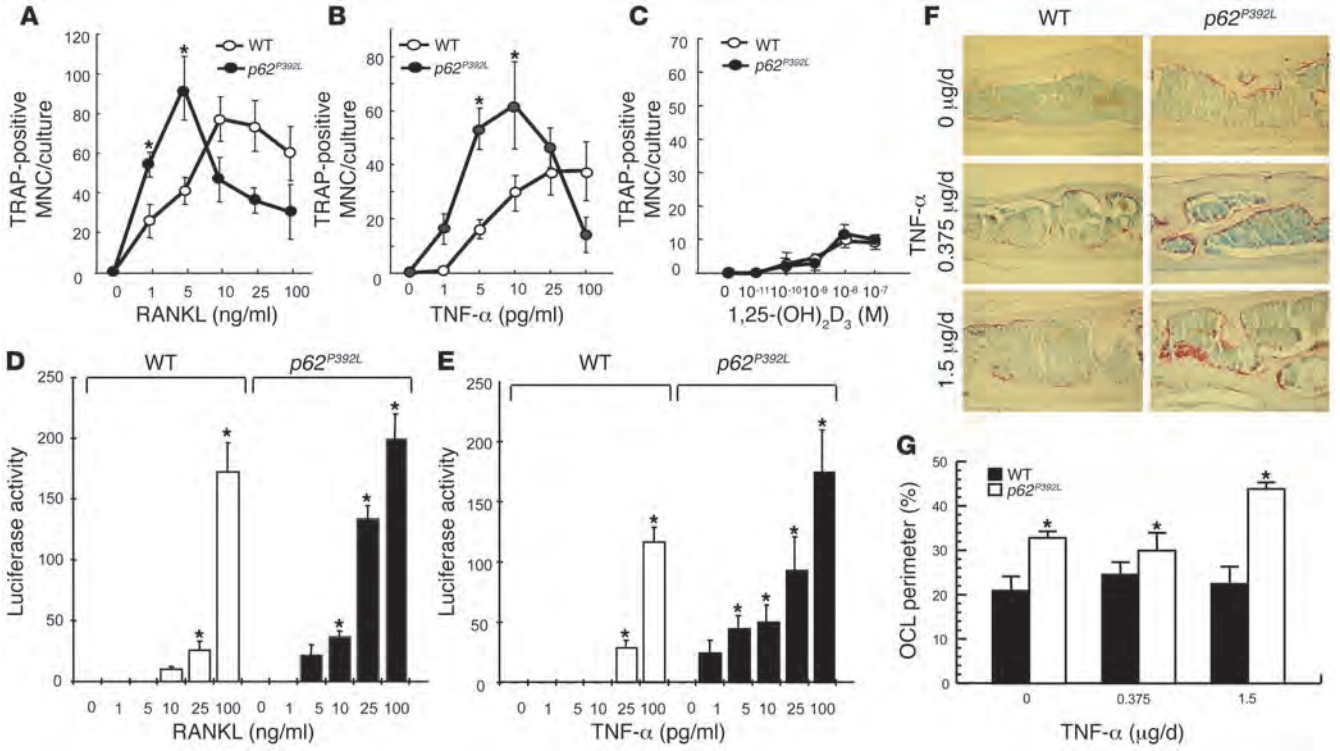

Figure 5

OCL formation and NF-κB gene reporter activity in OCL precursors from TRAP-*p62*^{P392L} and WT mice. (**A**–**C**) OCL precursors (10^5 cells/well) from TRAP-*p62*^{P392L} and WT mice were cultured in the presence of RANKL (**A**), TNF-α (**B**), or 1,25-$(OH)_2D_3$ (**C**). After 6 days of culture, cells were fixed and stained for TRAP activity. Results are expressed as mean ± SEM for quadruplicate cultures from a typical experiment. *$P < 0.001$ compared with results in WT cell cultures. Similar results were obtained in 5 independent experiments. Activation of NF-κB was measured by cotransfection of an NF-κB reporter plasmid and a β-gal expression vector into TRAP-*p62*^{P392L} or WT OCL precursors following 24 hours of treatment with RANKL (**D**) or TNF-α (**E**). The results are expressed as the mean ± SEM for the ratio of NF-κB reporter activity to β-gal activity for quadruplicate determinations. *Significant differences ($P < 0.001$) compared with results with WT cultures. A similar pattern of results was seen in 2 independent experiments. (**F**) Six-month-old TRAP-*p62*^{P392L} and WT mice were injected over the calvaria for 5 days with TNF-α (0, 0.375 μg, and 1.5 μg/day). Calvaria were harvested and tissue sections stained for TRAP activity (red color). Original magnification, ×100. (**G**) TRAP-positive OCL perimeter on the endosteal surface of calvaria in WT and TRAP-*p62*^{P392L} mice. *Significant differences ($P < 0.01$) between TRAP-*p62*^{P392L} and WT mice were found by 2-way ANOVA.

pooled colonies were cultured in the presence of RANKL, TNF-α, or 1,25-$(OH)_2D_3$ to induce OCL formation as described below.

Long-term cultures for OCL formation. For experiments employing highly purified OCL precursors, GM-CFU–derived cells, prepared as described above, were cultured in 96-well plates in α-MEM containing 20% horse serum and varying concentrations of RANKL, TNF-α, or 1,25-$(OH)_2D_3$. Every 3 days, half the medium was replaced, and after 21 days of culture, the cells were fixed with 1% formaldehyde and tested using a VECTASTAIN ABC-AP kit (Vector Laboratories) for cross-reactivity with the monoclonal antibody 23c6 (CD51), which recognizes the OCL vitronectin receptor. The 23c6-positive multinucleated cells were scored using an inverted microscope by an observer without knowledge of the treatment group.

Polymerase chain reaction amplification of RT-PCR. GM-CFU–derived cells were cultured for 2 days and subjected to RT-PCR analysis for expression of *MVNP* and *TAF*$_{II}$*17*. The gene-specific primers for human *MVNP* were 5′-CAGATTATGAACCAGTTTGGCCCTTCA-3′ (sense) and 5′-CCTGTGTTATTTCTTGGTTGTTTTCCC-3′ (antisense). The gene-specific primers for human *TAF*$_{II}$*17* were 5′-CATGCCATGGCTATGAACCAGTTTGGCCCCTCA-3′ (sense) and 5′-ATACTGCAGTTATTTCTTGGTTGTTTTCCG-3′ (antisense). The gene-specific primers for β-*actin* were 5′-GGCCGTACCACTGGCATCGTGATG-3′ (sense) and 5′-CTTGGCCGTCAGGCAGCTCGTAGC-3′ (antisense). Conditions for amplification were as follows: 94°C for 5 minutes, 35 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, followed by extension at 72°C for 7 minutes. PCR products were separated by 2% agarose gel electrophoresis and were visualized by ethidium bromide staining with ultraviolet light illumination (6).

Production of WT p62 and p62^{P392L} *vectors*. A plasmid containing the full-length human *p62* cDNA was kindly provided by J. Moscat (University of Cincinnati, Cincinnati, Ohio, USA), and the P392L mutation (a C-to-T transition) was introduced by PCR-based site-directed mutagenesis. The mutagenized cDNA was fully sequenced to verify correct introduction of the P392L mutation. Retroviral constructs containing the *p62* or *p62*^{P392L} cDNAs under the control of the CMV promoter were prepared and transfected into normal human OCL precursors as previously described (15). In brief, the *p62* or *p62*^{P392L} cDNAs were inserted into the *Xho*I site of the *p-LXSN* retroviral vector, and the recombinant plasmid constructs were transfected into the PT67 amphotropic packaging cell line using calcium phosphate. Stable cloned cell lines producing recombinant retrovirus at 10^6 virus particles/ml were established by selecting for resistance to neomycin (600 μg/ml). Similarly, a control retrovirus producer cell line was also established by transfecting the cells with the *p-LXSN EV*. Producer cell lines were maintained in DMEM containing 10% FBS, 100 U/ml streptomycin/penicillin, 4 mM L-glutamine,

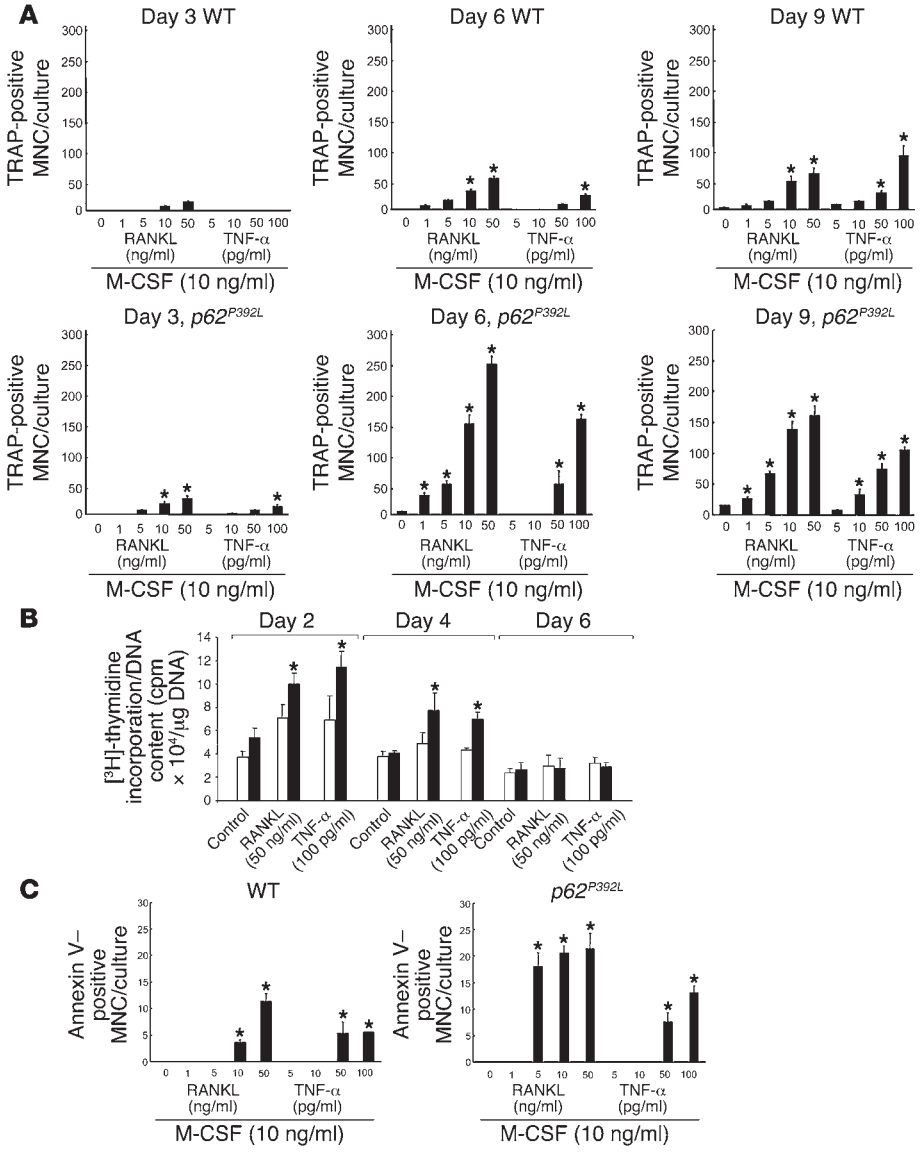

Figure 6
Time course for OCL formation, OCL precursor proliferation, and OCL apoptosis by marrow cells from TRAP-$p62^{P392L}$ or WT mice. (**A**) OCL precursors (10^5 cells/well) from TRAP-$p62^{P392L}$ and WT mice were cultured in the presence of RANKL or TNF-α. After 3, 6, or 9 days of culture, cells were fixed and number of TRAP-positive OCLs counted. (**B**) OCL precursors (1×10^6 per culture) from WT (white bars) and TRAP-$p62^{P392L}$ (black bars) littermate mice were cultured for 2, 4, or 6 days and were pulsed at the end of the culture period for 1 hour with 1 mCi [^{3}H]-thymidine. Radioactivity was counted by liquid scintillation spectrometry. Results are expressed as the mean ± SD for quadruplicate cultures. *$P < 0.001$ compared with results of WT cultures treated with the same concentration of RANKL or TNF-α. (**C**) OCL precursors (10^5 cells/well) from TRAP-$p62^{P392L}$ and WT mice were cultured in the presence of RANKL or TNF-α. After 9 days of culture, cells were fixed and number of apoptotic OCLs were counted using a commercial Annexin V kit (Promega). All results are expressed as the mean ± SD for quadruplicate cultures.

and high glucose (4.5 g/l). Retroviral supernatants from the producer cell cultures were collected and filtered (0.45 μm) for immediate use. The retrovirus stocks were demonstrated to be helper free by a marker assay. Viral titers present in the culture supernatants were determined by testing for multiplicity of infection with serial dilutions of the supernatants on NIH3T3 cells and scoring the number of G418-resistant CFUs formed (following exposure to 250 μg/ml G418) as described (15).

Overexpression of p62 and $p62^{P392L}$ in normal human OCL precursors. After obtaining informed consent, we obtained bone marrow aspirates from normal volunteers as previously described (15). These studies were approved by the Institutional Review Board at the University of Pittsburgh. Human bone marrow mononuclear cells were cultured for 2 days in α-MEM containing 10% FBS and 10 ng/ml each of IL-3, IL-6, and SCF. The bone marrow cells were cultured for an additional 48 hours with varying amounts of viral supernatant (1–10% v/v) containing the *p62 or $p62^{P392L}$* vectors or *EV*. Cultures were supplemented with 4 μg/ml of polybrene, 20 ng/ml of IL-3, 50 ng/ml of IL-6, and 100 ng/ml of SCF. We previously determined that this was the optimum cytokine combination that supported the highest transduction efficiency. After 24 hours, cells were centrifuged, spent supernatant was removed, and freshly prepared viral supernatant supplemented with 4 μg/ml of polybrene and growth factors was added; then the cultures were continued for an additional 24 hours. After 48 hours, cells were harvested for short-term GM-CFU assays in methylcellulose as previously described (15, 19), and an aliquot of the cells was evaluated for p62 expression by immunostaining with an anti-p62 monoclonal antibody (BD Biosciences).

Bone resorption assays. GM-CFU–derived cells transduced with *$p62^{P392L}$* or *EV* (10^5 cells/well) were cultured with RANKL (50 ng/ml) or TNF-α (100 pg/ml) on mammoth dentin slices (Wako). After 3 weeks of culture, cells were removed, dentin slices were stained with acid hematoxylin, and areas of dentin resorption were determined using image analysis techniques, as previously described (20).

Construction of the TRAP-$p62^{P392L}$ hybrid transgene. We have previously described construction of the p-BSmTRAP5′ plasmid, which contains 1294 bp of the 5′ flanking sequence as well as the entire 5′ untranslated region of the murine *TRAP* gene (21). The full-length human *$p62^{P392L}$* cDNA described above was inserted into the unique *EcoR*I site of *p-BSpKCR3* (21), which contains part of the second exon, the second intron, and the third exon, including the polyadenylation site of the rabbit β-globin gene. There are

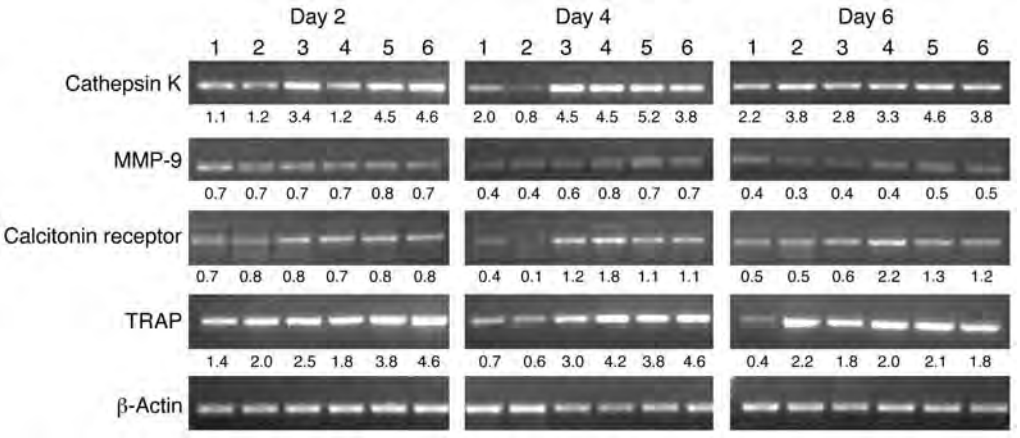

Figure 7
Expression of markers of OCL differentiation by TRAP-*p62*^{P392L} and WT OCL precursors. OCL precursors (10^6 cells) from WT and TRAP-*p62*^{P392L} littermates were cultured for 2, 4, or 6 days with 50 ng/ml of RANKL or 100 pg/ml TNF-α. RNA was prepared and subjected to RT-PCR analysis for cathepsin K, MMP-9, calcitonin receptor, and TRAP as described in Methods. Ratio of marker mRNA expression to β-*actin* is shown below each lane. Lane 1, WT M-CSF (10 ng/ml); lane 2, WT M-CSF (10 ng/ml) plus RANKL (50 ng/ml); lane 3, WT M-CSF (10 ng/ml) plus TNF-α (100 pg/ml); lane 4, *p62*^{P392L} M-CSF (10 ng/ml); lane 5, *p62*^{P392L} M-CSF (10 ng/ml) plus RANKL (50 ng/ml); lane 6, *p62*^{P392L} M-CSF (10 ng/ml) plus TNF-α (100 pg/ml).

no AUG initiation codons within the β-globin sequences upstream of the cDNA insertion site, so translation of the *p62*^{P392L} protein starts at the normal *p62* initiation codon. The murine TRAP promoter was then inserted into the multiple cloning site immediately upstream of the rabbit β-globin sequences. The *TRAP-p62*^{P392L} transgene was excised by XhoI digestion from the resulting plasmid, p–KCR3-TRAP-*p62*^{P392L}, and was agarose gel purified before microinjection.

Production and identification of TRAP-p62^{P392L} *transgenic mice*. These studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. The *TRAP-p62*^{P392L} transgene was microinjected at a concentration of 3 μg/ml into the male pronucleus of fertilized 1-cell mouse embryos by standard methods (22). The embryos were obtained from mating $CB6F_1$ (C57BL/6 × BALB/c) males and females (Harlan). The injected embryos were then reimplanted into the oviducts of pseudopregnant CD-1 female mice. The presence of the transgene was identified in resulting offspring by Southern blot analysis of genomic DNA prepared from a small tail-tip biopsy taken at the time of weaning. Probes for Southern blot analysis were generated by random oligonucleotide labeling (Amersham Biosciences) using [α-^{32}P] dCTP (DuPont/NEN). Transgenic mice of subsequent generations were identified by PCR analysis using transgene-specific primers. The upstream primer was derived from the murine TRAP5′ untranslated region: 5′-GTCCTCACCAGAGACTCTGAACTC-3′ (sense); and the downstream primer was derived from the human p62 cDNA: 5′-TGAGCGACGCCATAGCGAGCGG-3′ (antisense). The conditions for amplification were as follows: 94°C for 2 minutes, 35 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 2 minutes, followed by extension at 72°C for 7 minutes. PCR products were separated by 1.25% agarose gel electrophoresis and were visualized by ethidium bromide staining with ultraviolet light illumination.

Histologic analysis of TRAP-p62^{P392L} *vertebral bones*. The first through fourth lumbar vertebrae from 4-, 8-, 12-, and 16-month-old *TRAP-p62*^{P392L} and WT littermates were fixed in 10% buffered formalin for 24–48 hours, then completely decalcified in 10% EDTA at 4°C, processed through graded alcohols, and embedded in paraffin. Longitudinal sections of 5 μm were cut and mounted on glass slides. Deparaffinized sections were stained for TRAP as described by Liu et al. (23). OCLs containing active TRAP were stained red. Another set of sections was stained with 0.1% toluidine blue. Histomorphometry was performed on the region of cancellous bone between the cranial and caudal growth plates of the third lumbar vertebral body under bright field and polarized light at a magnification of ×200, using the OsteoMeasure 4.00C morphometric program (OsteoMeasure; OsteoMetrics). OCL perimeter was defined as the length of bone surface covered with TRAP-positive and mono- and multinuclear cells. Osteoblast perimeter, cancellous bone volume, trabecular width, trabecular number, and trabecular separation were also quantified and calculated. All variables were expressed and calculated according to the recommendations of the American Society for Bone and Mineral Research Nomenclature Committee (24, 25).

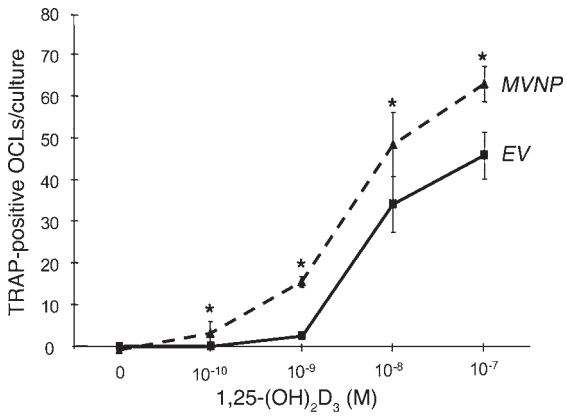

Figure 8
MVNP-transduced TRAP-*p62*^{P392L} mouse GM-CFU cells are hyperresponsive to 1,25-$(OH)_2D_3$. OCL precursors (5×10^5 cells/well) from *MVNP*- or *EV*-transduced TRAP-*p62*^{P392L} mouse GM-CFU were cultured in the presence of 1,25-$(OH)_2D_3$. After 9 days of culture, cells were fixed and stained for TRAP activity. Results are expressed as the mean ± SEM for quadruplicate cultures. *$P < 0.001$ compared with results of cultures of EV-transduced GM-CFU treated with the same concentration of 1,25-$(OH)_2D_3$.

NF-κB gene reporter activity in p62^{P392L} and WT marrow cells. For reporter gene assays, GM-CFU–derived cells from TRAP-*p62^{P392L}* mice or WT littermate controls were cotransfected with a luciferase reporter plasmid containing an NF-κB–responsive promoter (Clontech; Cambrex) and a β-gal expression plasmid using the FuGENE 6 Reagent (Roche Diagnostics). Sixteen hours after transfection, varying concentrations of RANKL or TNF-α were added. Twenty-four hours later, cells were harvested and lysed in the cell lysate solution provided with the luciferase assay kit (Promega). The luciferase activities of the cell lysates were measured with the luciferase assay kit according to the manufacturer's instructions and were normalized to β-gal activities of the same cell lysates using a β-gal assay kit (Promega).

Measurement of OCL differentiation markers in TRAP-p62^{P392L} and WT marrow cells by polymerase chain reaction amplification of RT-PCR. Marrow cells from TRAP-*p62^{P392L}* or WT mice were cultured for 2, 4, or 6 days with M-CSF (10 ng/ml) alone, RANKL (50 ng/ml) and M-CSF, or TNF-α (100 pg/ml) and M-CSF. Total RNA was extracted using RNAzol B solution (Tel-Test Inc.) and reverse transcribed as follows: 5% of the first-strand cDNA pool was subjected to PCR amplification using gene-specific PCR primers following standard PCR protocols. The gene-specific primers for mouse *cathepsin K* were 5′-GCAGAACGGAGGCATTGAC-3′ (sense) and 5′-TGGCTGGAATCACATCTTGG-3′ (antisense). The gene-specific primers for mouse *MMP-9* were 5′-TTGGTTTCTGCCCTAGTTAG-3′ (sense) and 5′-TGCCCAGGAAGACGAAGG-3′ (antisense). The gene-specific primers for mouse *calcitonin receptor* were 5′-CCCAGACATCCAGCAAGAG′ (sense) and 5′-CAGCACATCCAGCCATCC-3′ (antisense). The gene-specific primers for mouse *TRAP* were 5′-GAACTTCCCCAGCCCTTA-3′ (sense) and 5′-CCCACTCAGCACATAGCC-3′ (antisense). The gene-specific primers for mouse β-*actin* were 5′-GGCCGTACCACTGGCATCGTGATG-3′ (sense) and 5′-CCTGGCCGTCAGGCAGCTCGTAGC-3′ (antisense). The conditions for amplification were as follow: 94°C for 5 minutes, 35 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, followed by extension at 72°C for 7 minutes. PCR products were separated by 2% agarose gel electrophoresis and were visualized by ethidium bromide staining with ultraviolet light illumination.

Transduction of OCL precursors from TRAP-p62^{P392L} or WT mice with MVNP. Bone marrow cells were obtained by flushing the femurs of TRAP-*p62^{P392L}* or WT mice with α-MEM, and cells were collected by centrifugation at 300 *g* for 10 minutes. The cells were resuspended at 2.5×10^6 cells/ml and cultured in α-MEM containing 10 ng/ml each of IL-3, IL-6, and SCF for 2 days to induce proliferation of hematopoietic precursors. The marrow cells were then transduced with retroviral vectors that contained a neomycin resistance gene and the *MVNP* gene or *EV* (15). The transduced cells were cultured in methylcellulose with mouse GM-CSF (200 pg/ml) in the presence of 250 μg/ml G418 to select for GM-CFU colonies that expressed *MVNP* or *EV*. GM-CFU colonies were scored after 7 days of culture, using an inverted microscope. Colonies were individually picked, using finely drawn pipettes. GM-CFU–derived cells used for OCL formation assays are described below. We have previously demonstrated that these cells are highly purified early OCL precursors (15). GM-CFU–derived cells (10^6 cells /ml) obtained as described above (15) were plated in 96-well plates in α-MEM containing 10% FBS and cultured in the presence of 1,25-$(OH)_2D_3$ to induce OCL formation. The cultures were fed every 3 days by replacing half the medium, and after 6 days of culture, the cells were fixed with 1% formaldehyde and stained for TRAP using a leukocyte acid phosphatase kit (Sigma-Aldrich). The TRAP-positive multinucleated cells were scored using an inverted microscope.

Immunoblotting of OCL precursors from TRAP-p62^{P392L} or WT mice. Cytokine-treated or control OCL precursors from TRAP-*p62^{P392L}* or WT mice were washed twice with ice-cold PBS. Cells were lysed in the buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, and ×1 protease inhibitor mixture. Fifty micrograms of cell lysates were boiled in the presence of SDS sample buffer (0.5 M Tris-HCl, pH 6.8, 10% [w/v] SDS, 10% glycerol, 0.05% [w/v] bromphenol blue) for 5 minutes and subjected to electrophoresis on 7.5% SDS-PAGE. Proteins were transferred to nitrocellulose membranes using a semi-dry blotter (Bio-Rad) and incubated in blocking solution (5% nonfat dry milk in TBS containing 0.1% Tween-20) for 1 hour to reduce nonspecific binding. Membranes were then exposed to primary antibodies overnight at 4°C, washed 3 times, and incubated with secondary goat anti-mouse or rabbit IgG HRP–conjugated antibody for 1 hour. Membranes were washed extensively, and enhanced chemiluminescence detection assay was performed following the manufacturer's directions (Bio-Rad). All blots were densitometrically quantitated and the results expressed relative to control and normalized to β-*actin*.

Statistics. Significance was evaluated using a 2-tailed, unpaired Student's *t* test, with $P < 0.05$ considered to be significant.

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Original Paper

Transgenic mice with OIP-1/hSca overexpression targeted to the osteoclast lineage develop an osteopetrosis bone phenotype

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Abstract

Regulatory mechanisms operative in bone-resorbing osteoclasts are complex. We previously defined the *Ly-6* gene family member OIP-1/hSca as an inhibitor of osteoclastogenesis *in vitro*; however, a role in skeletal development is unknown. In this study, we developed transgenic mice with OIP-1/hSca expression targeted to the osteoclast lineage that develop an osteopetrotic bone phenotype. Humeri from OIP-1 mice showed a significant increase in bone mineral density and bone mineral content. μCT analysis showed increased trabecular thickness and bone volume. OIP-1 mice have dense sclerotic cortical bone with absence of spongiosa and inadequate formation of marrow spaces compared to wild-type mice. Moreover, complete inhibition of osteoclasts and marrow cavities in calvaria suggests defective bone resorption in these mice. OIP-1 mouse bone marrow cultures demonstrated a significant decrease (41%) in osteoclast progenitors and inhibition (39%) of osteoclast differentiation/bone resorption. Western blot analysis further demonstrated suppression of TRAF-2, c-Fos, p-c-Jun, and NFATc1 levels in RANKL-stimulated osteoclast precursors derived from OIP-1 mice. Therefore, OIP-1 is an important physiological inhibitor of osteoclastogenesis and may have therapeutic value against bone loss *in vivo*.

Keywords: osteoclast inhibitory peptide-1/human Sca; osteoclast; osteopetrosis; bone resorption; osteoblast

Introduction

The osteoclast (OCL) is the bone resorbing cell derived from the monocyte/macrophage lineage. OCL formation and bone resorption are regulated by local factors present in the bone marrow micro-environment. Tumour necrosis factor (TNF) gene family member, receptor activator of nuclear factor κB (RANK), expressed on OCL precursors, and RANK ligand (RANKL), expressed on osteoblast/stromal cells, interaction is critical for OCL differentiation and bone resorption [1]. RANK–RANKL signalling promotes the binding of TNF receptor associated factor (TRAF) family proteins such as TRAF-6 to RANK, which results in the activation of Jun N-terminal kinase (JNK) pathways [2]. We have previously identified and characterized a novel autocrine/paracrine inhibitor of OCL formation termed osteoclast inhibitory peptide-1 (OIP-1/hSca) [3]. OIP-1/hSca is a *Ly-6* gene family member expressed on immature thymocytes and thymic epithelial cells [4]. OIP-1/hSca is a glycophosphatidylinositol (GPI)-linked membrane protein (16 kD) containing a 79-amino acid extracellular peptide and a 32-amino acid carboxy-terminal GPI-linked peptide (c-peptide). OIP-1/hSca is a human homologue of mouse *Sca-2* with 65% identity at the nucleotide level, with a conserved pattern of cysteine residues in protein structure [5]. Previously, we have demonstrated that the OIP-1 c-peptide region is critical for OCL inhibitory activity and that a neutralizing antibody against the c-peptide completely blocks OIP-1 activity to inhibit OCL formation *in vitro*; however, a role in skeletal development is unknown [6]. We have also shown that OIP-1/hSca mRNA is highly expressed in osteoblastic cells, OCLs, and bone marrow cells, and that the hSca protein is cleavable from the OCL surface [3]. It has been demonstrated that Sca-2 functions as a modulator of the T-cell receptor (TCR) signalling pathway [7]. In addition, it has been shown to be physically and functionally associated with CD3 ζ chains of the TCR complex [8]. Furthermore, *Ly*-6A knock-out mice demonstrated a significant decrease in bone mineral density and bone mineral content compared with wild-type mice [9].

These studies implicate an essential role for the *Ly-6* gene family in normal bone remodelling. In this study, we show that transgenic mice with OIP-1/hSca over-expression targeted to the osteoclast lineage develop an osteopetrotic bone phenotype through inhibition of OCL formation/activity *in vivo*.

Materials and methods

Development of OIP-1 transgenic mice

Human OIP-1 cDNA was excised from the CDS 5-3 plasmid by BamH1 and PmeI digestion. The resulting DNA fragment (430 bp) encoding the complete coding sequence of OIP-1 was sub-cloned into the pKCR3ΔR1-mTRAP gene promoter containing plasmid (Figure 1A). The transgene fragment from the plasmid construct (pKCR3ΔR1-mTRAP-OIP-1#4) was micro-injected into the male pronucleus of fertilized one-cell mouse embryos at 3 μg/ml concentration and re-implanted into the oviducts of pseudo-pregnant female mice as previously described [10]. The presence of the transgene was identified in resulting offspring by polymerase chain reaction (PCR) screening with OIP-1 gene-specific primers [6] using template genomic DNA purified from a small piece of tail taken from each animal at the time they were

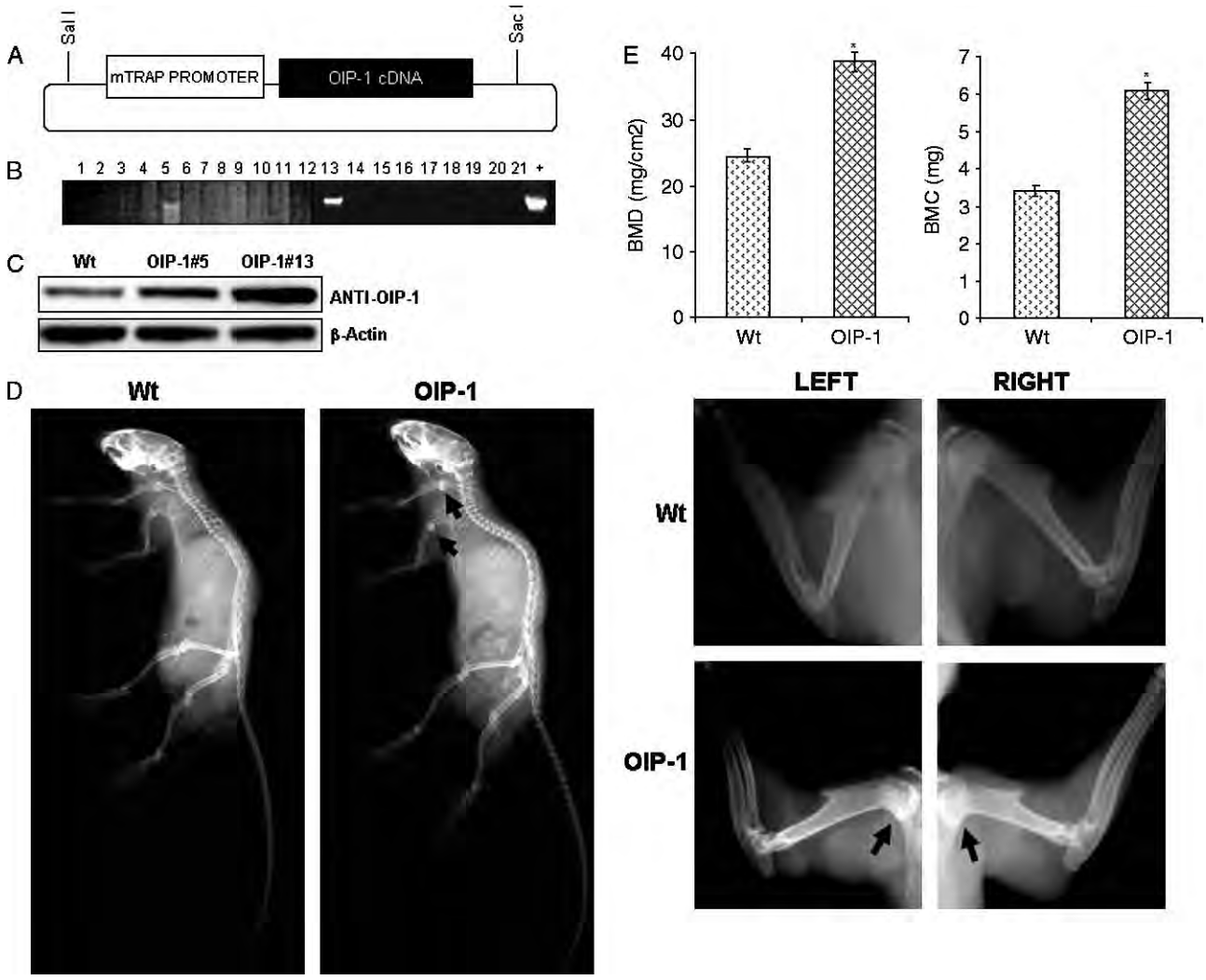

Figure 1. OIP-1 transgenic mice exhibit focal osteopetrotic bone phenotype. (*A*). mTRAP-OIP-1 transgene plasmid map. The mTRAP-OIP-1 transgene fragment (4 Kb) was excised by Sal-I-Sac 1 digestion from the plasmid PKCR3ΔR1 mTRAP-OIP#4 for micro-injection into the mouse embryos to develop OIP-1 transgenic mice. (*B*). PCR screening of potential founder mice with OIP-1 gene specific primers as described in methods. PCR product amplified using the transgene plasmid served as a positive control (+). We thus identified two potential founder mouse lines #5 and#13. (C) Western blot analysis of OIP-1 expression in transgenic mouse derived preosteoclast cells. Non-adherent bone marrow cells obtained from wild-type (Wt) and OIP-1 transgenic mice were cultured at a density of 1×10^6 cells for 4 days in the presence of 10 ng/ml M-CSF and 100 ng/ml RANKL to obtain preosteoclast cells. Total cell lysates obtained were subjected to Western blot analysis using rabbit anti-OIP-1 antibody. (D) Radiological analysis of the wild-type (Wt) and OIP-1 transgenic mice were performed using a Faxitron MX20 equipped with a FPX-2 Imaging system. Magnified radiographs of left and right humerus of Wt and OIP-1 mice (4 week old) showed increased radiodensity in the proximal humeral region as pointed by arrows. (E) Bone mineral density (BMD) and Bone mineral content (BMC) of Wt and OIP-1 mice humerus bones at 4 weeks of age as measured by dual energy X-ray absorptiometry (DEXA) and values represent mean ± SD ($n = 5$; $p < 0.05$)

weaned. We thus identified two potential founder lines (Nos 5 and 13) of OIP-1 transgenic mice (Figure 1B). Western blot analysis further confirmed high levels of OIP-1 expression in pre-osteoclast cells derived from these founder lines compared with wild-type mice (Figure 1C). All experiments were performed using OIP-1 mice, line 13 (3 to 4 weeks old), unless otherwise specified, with appropriate littermate controls, following the Institutional Animal Care and Use Committee (IACUC) procedure approved by the Institutional Review Board.

Micro-computed tomography (μCT) analysis

The bones collected from 4-week-old mice were fixed in 70% ethanol and the distal metaphyses were scanned with a Skyscan 1072 μCT instrument and analysed by CT-Analyzer software (SkyScan). Two-dimensional images were used to generate three-dimensional reconstructions and to calculate morphometric parameters.

Histology and histomorphometric analysis

The wild-type and OIP-1 mouse bone specimens were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), decalcified in 0.5 M EDTA (pH 7.4) for a 1 to 3-week period, and processed for paraffin embedding. Serial 5-μm sections were cut on a modified Leica RM 2155 rotary microtome (Leica Microsystems, Ontario, Canada) and stained with haematoxylin and eosin [11].

In order to perform histochemical staining, bone specimens were fixed overnight in 70% ethanol and embedded in methyl methacrylate (MMA). Serial 4 to 6-μm sections of MMA embedded calvaria were sectioned from anterior to posterior through frontal and parietal bone tissues and humerus sections were stained for tartrate resistant acid phosphatase (TRAP) activity using a histochemical kit (Sigma). Alkaline phosphatase activity and Goldner trichrome staining were performed as previously described [12,13]. Histomorphometric analysis was performed with OsteoMeasure version 2.2 software.

Osteoclast culture and bone resorption activity assays

Wild-type and OIP-1 transgenic mouse bone marrow cells were cultured to form OCLs as previously described [6]. Bone marrow-derived non-adherent cells (1.3×10^6 per ml) were cultured in 24-well plates in the presence of mRANKL (5–100 ng/ml) and M-CSF (10 ng/ml) for 5 days. At the end of the culture period, the cells were fixed with 2% glutaraldehyde in PBS for 20 min and stained for TRAP activity. TRAP-positive multinucleated OCLs containing three or more nuclei were scored microscopically.

To determine the bone resorption activity, wild-type and OIP-1 transgenic mouse bone marrow cells treated with 10 ng/ml mM-CSF for 12 h, and non-adherent bone marrow mononuclear cells (1×10^6 cells per well) were cultured to form OCLs on sterile dentine slices for 10 days as previously described [6]. The cells were removed using 1 M NaOH and stained with 0.1% toluidine blue. The areas of resorption lacunae on the digital images were quantified. The percentage of resorbed area was calculated relative to the total dentine disc area.

Western blot analysis for RANK receptor signalling molecules in OIP-1 transgenic mouse-derived pre-osteoclasts and immunocytochemical staining of OIP-1 expression in osteoclast cells were performed using rat anti-mouse TSA-1/Sca MAb (PharMingen, CA, USA) [14].

Osteoblast differentiation

Wild-type and OIP-1 transgenic mouse bone marrow-derived stromal/pre-osteoblast cells were cultured with medium containing 10 mM glycerophosphate and 50 μg/ml ascorbic acid for an indicated period (0–12 days). The total RNA (2 μg) isolated was reverse-transcribed using random hexamers and Moloney murine leukaemia virus reverse transcriptase (Applied Biosystems, CA, USA). The resulting cDNAs were then amplified by PCR using osteoblast marker gene-specific primers for osteocalcin, alkaline phosphatase, collagen type I, and β-actin control primers as previously described [15].

Statistical analysis

Data are presented as mean and the statistical analysis between the wild-type and OIP-1 transgenic mice for a given parameter was established by Student's *t*-test, with $p < 0.05$ considered statistically significant. Statistical analysis of skeletal parameters was also applied by one-way ANOVA.

Results

OIP-1 mice show osteopetrotic bone phenotype

We have previously identified and characterized the osteoclast inhibitory peptide-1 (OIP-1/hSca), which inhibits osteoclast formation and bone resorption activity *in vitro*; however, a role for OIP-1 in skeletal development is unknown [6]. To define a functional role for OIP-1 in bone remodelling, we developed transgenic mice in which OIP-1 expression is targeted to the osteoclast lineage using the mouse TRAP gene promoter as described in the Materials and methods section. OIP-1 transgenic mice that are heterozygous were born normal and fertile; however, there was no significant difference in overall body size compared with non-transgenic littermates. OIP-1 mice showed normal tooth eruption and no visible deformity, or in the growth of the skeleton, compared with wild-type mice. Interestingly, the radiographic analysis of OIP-1 transgenic mice showed an increased

radiodensity of the proximal humerus when compared with wild-type mice of 4-week-old littermates, indicating an osteopetrotic bone phenotype in OIP-1 mice (Figure 1D). To confirm the increased radio-opacity in the humerus of OIP-1 mice, bone mineral density (BMD) and bone mineral content (BMC) were measured by DEXA. The humeral region of 4-week-old OIP-1 mice showed a significant increase in BMD (58.36%) and in BMC (79.41%) (Figure 1E). μCT analysis also demonstrated a significant increase in trabecular thickness, bone volume, and bone surface in the humeral region of OIP-1 mice (Figures 2A–2D) compared with wild-type littermates. Furthermore, μCT analysis of the vertebral region of OIP-1 mice showed an increased trabecular thickness, trabecular bone volume, and bone surface compared with wild-type mice (Figures 2E–2H).

To analyse the osteopetrotic bone phenotype further, we examined the humeri from the OIP-1 mice histologically. The altered growth zone at the proximal end in the humerus of 4-week-old OIP-1 transgenic mice is shown in Figure 3A. OIP-1 mice exhibited absence of primary and secondary spongiosa and inadequate formation of marrow spaces. Much of the epiphysis consisted of dense sclerotic cortical bone, suggesting defective bone resorption in OIP-1 mice. The solid block of bone below the growth plate obliterating the marrow cavity varied in severity in the majority (>85%) of OIP-1 mice at 3 to 4 weeks old. The growth plate region appeared distorted due to the decreased marrow space rather than a significant change in the height or premature closure. To clarify further that the osteopetrotic bone phenotype in OIP-1 mice was due to OIP-1 inhibition of OCL development, we evaluated the OCL numbers in histological specimens by staining for TRAP activity (Figure 3B). Histomorphometric analysis identified a significant decrease (46.16%) in the number of TRAP-positive OCLs in the humeri from OIP-1 mice compared with wild-type littermates (Figure 3C). However, the number of osteoblast cells per bone perimeter area was not affected in humeri from OIP-1 mice compared with wild-type mice (Figure 3D). In addition, calvaria from the OIP-1 mice demonstrated inhibition of TRAP-positive OCLs at the endosteal bone surfaces and underdeveloped bone marrow spaces compared with wild-type littermates (Figure 4A). Furthermore, as evident from the alkaline phosphatase activity staining, there was no significant change in osteoblastic activity on periosteal and endosteal bone surfaces (Figure 4B). Also, calvarial sections showed normal osteoid seams

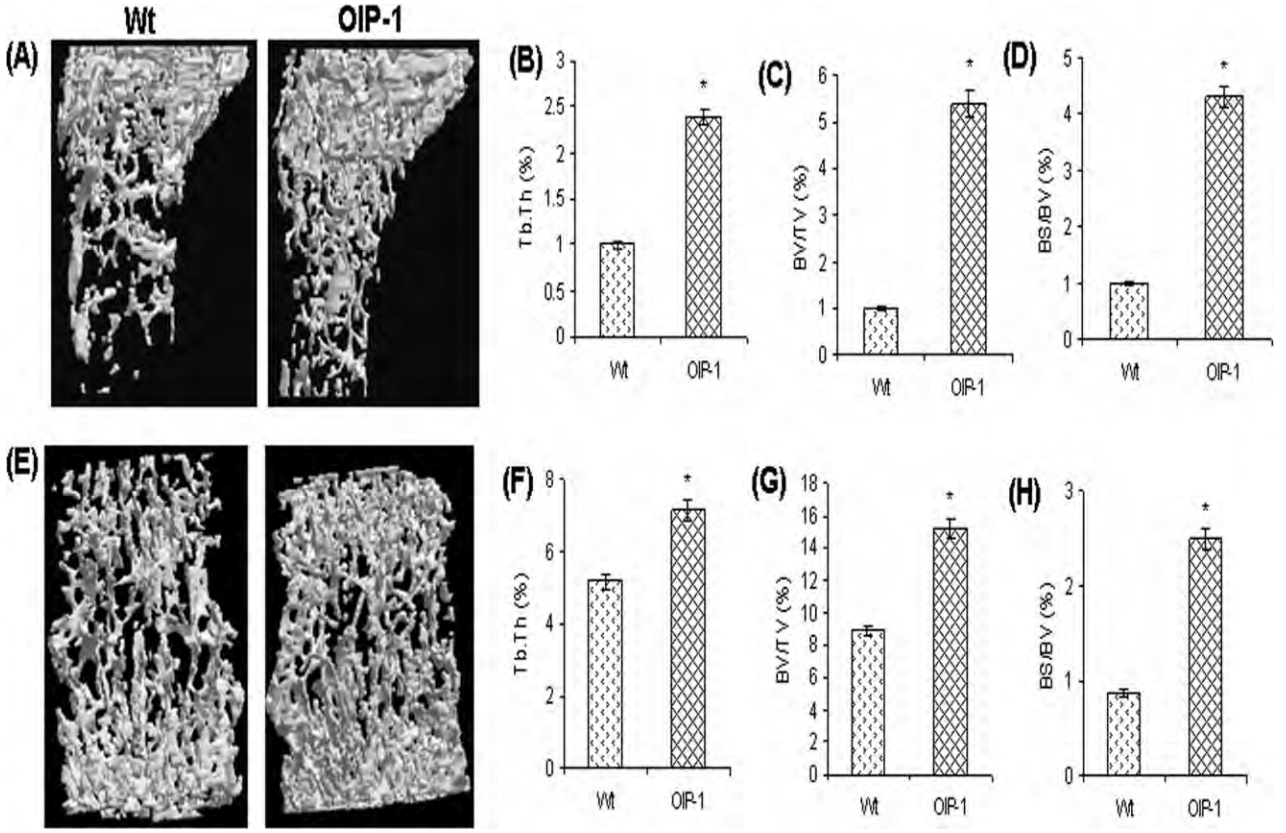

Figure 2. μCT analysis of bones from 4 week old wild-type (Wt) and OIP-1 mice. (A) μCT images showed increased bone mass in the humeral region of OIP-1 mice compared to wild-type littermates. (B) Quantification of trabecular thickness (TbTh). (C) trabecular bone volume (bone volume per tissue volume, BV/TV) and (D) bone surface/volume (BS/BV) in the humeral region of wild-type and OIP-1 mice. (E) μCT images of the lumber vertebrae from OIP-1 mice compared to wild-type littermates. (F) Quantification of trabecular thickness (TbTh). (G) trabecular bone volume (BV/TV) and (H) bone surface/volume (BS/BV) in the vertebral region of wild-type and OIP-1 mice. Values represent mean ± SD ($n = 5$; $p < 0.05$)

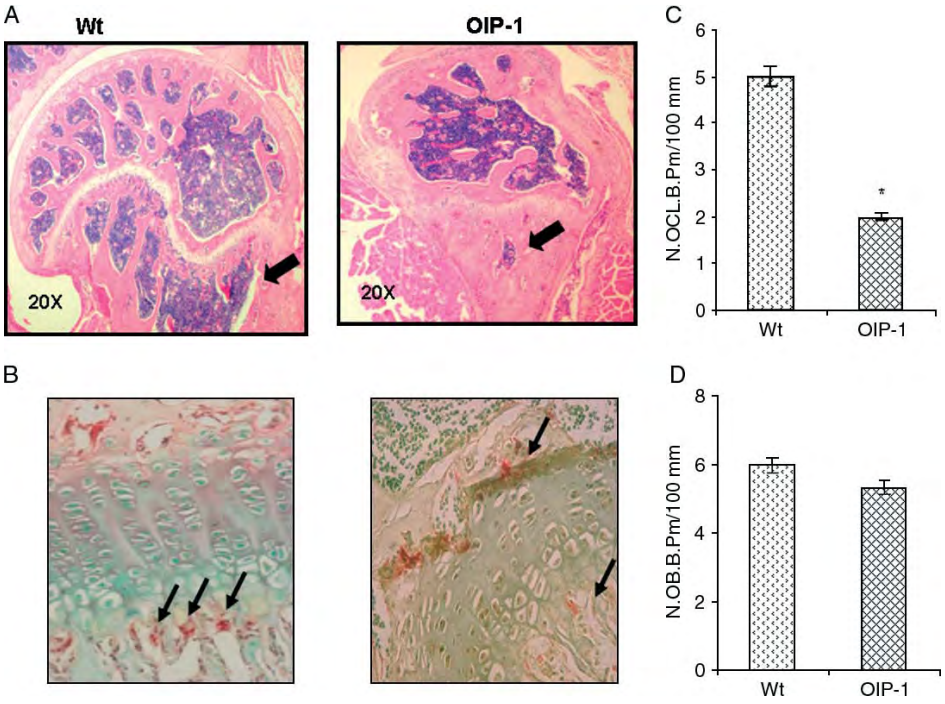

Figure 3. Histological and immunohistochemical analysis of humeri from wild-type (Wt) and OIP-1 mice. (A) Haematoxylin and eosin staining of the growth zone of the proximal end of the humerus of OIP-1 and Wt mice. OIP-1 mice show the osteopetrotic-like bone phenotype with inadequate formation of marrow spaces (as shown by an arrow). Original magnification: ×20. (B) Histochemical staining for TRAP-positive OCLs present at the growth plate region of humeri from Wt and OIP-1 mice (4 weeks old). The TRAP(+) OCLs are indicated by arrows. Original magnification: ×200. (C) Histomorphometric analysis of the number of osteoclasts/bone perimeter (N.OCL/B.Pm/100 mm) and (D) the number of osteoblast/bone perimeter (N.OB/B.Pm/100 mm) in humeri of Wt and OIP-1 mice; values represent mean ± SD ($n = 5$; $p < 0.05$)

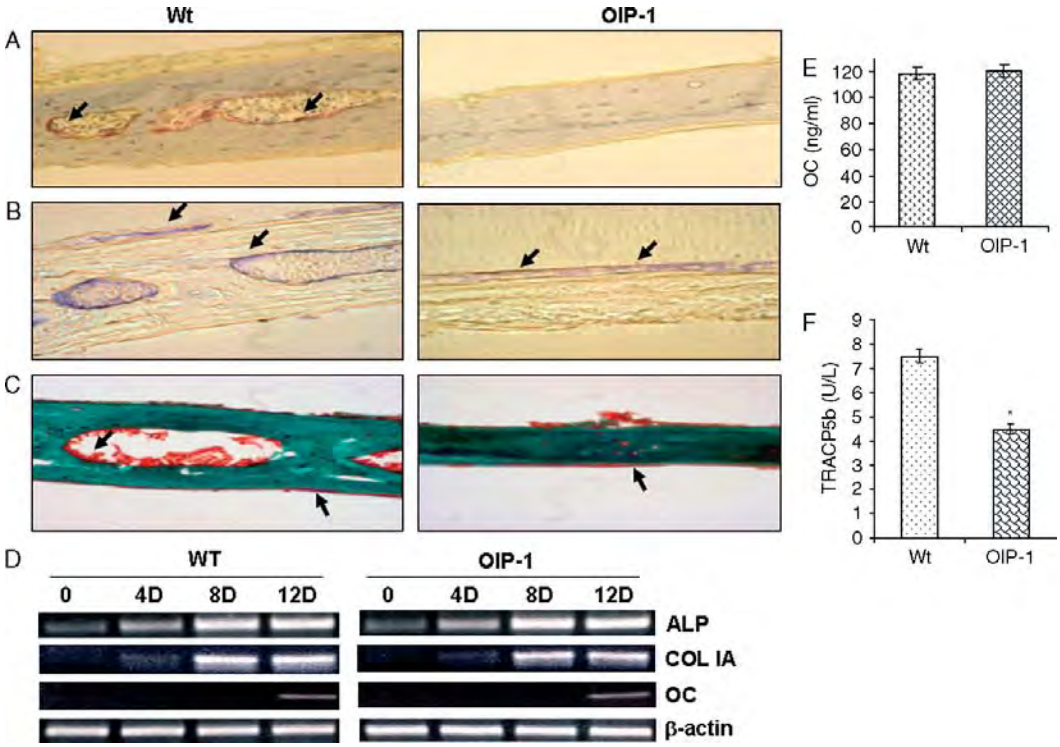

Figure 4. Histological analysis of wild-type (Wt) and OIP-1 transgenic mouse calvaria. (A) Wt mice show TRAP(+) OCLs on the endosteal bone surface of marrow cavities, as indicated by arrows. OIP-1 mouse calvaria showed absence of TRAP(+) OCLs and underdeveloped bone marrow spaces ($n = 10$). (B) Calvaria stained for alkaline phosphatase activity. (C) Goldner's trichrome staining shows osteoid seams on periosteal and endosteal bone surfaces. Original magnification: ×20. (D) Semi-quantitative RT-PCR analysis of osteoblast marker genes, alkaline phosphatase (ALP), collagen type IA (COL IA), and osteocalcin (OC) expression levels in Wt and OIP-1 mouse bone marrow-derived osteoblast cultures. Total RNA isolated from the Wt and OIP-1 mouse bone marrow-derived stromal/pre-osteoblast cells cultured with osteogenic medium for the period indicated (0–12 days) was subjected to RT-PCR analysis for osteoblast differentiation markers as described in the Materials and methods section. ELISA analysis of serum (E) osteocalcin and (F) TRACP5b levels in OIP-1 and Wt mice as noted in the Materials and methods section

on the periosteal and endosteal bone surfaces of both OIP-1 and wild-type mice (Figure 4C).

RT-PCR analysis of the total RNA isolated from osteoblast cells derived from the OIP-1 mouse bone marrow cultures did not show a significant difference in the expression levels of osteoblast differentiation marker genes such as alkaline phosphatase, type I collagen, and osteocalcin gene expression compared with wild-type littermates (Figure 4D). Furthermore, ELISA analysis demonstrated no significant change in the serum osteocalcin levels in OIP-1 mice (Figure 4E). However, the osteoclast activity marker, serum TRAcP5b, was significantly reduced in the OIP-1 transgenic mice, compared with wild-type littermates (Figure 4F). Collectively, these results indicate an osteopetrotic bone phenotype in OIP-1 mice due to inhibition of OCL formation/activity *in vivo*, and that OIP-1 does not modulate bone formation.

Inhibition of osteoclastogenesis in OIP-1 mouse bone marrow cultures

We next performed *ex vivo* experiments using bone marrow cells isolated from the OIP-1 and control mice to assess OIP-1 inhibition of OCL formation. Non-adherent bone marrow cells derived from the wild-type and OIP-1 mice were cultured in the presence of hGM-CSF (10 ng/ml) in methyl cellulose (1.2%) to form CFU-GM colonies. As shown in Figure 5A, OIP-1 transgenic mouse line 5 and 13-derived cells showed a significant decrease in the number of CFU-GM colonies, by 35% and 41% respectively, compared with control mice. We further examined the capacity for OCL differentiation and bone resorption by OIP-1 transgenic mouse-derived bone marrow cells. OIP-1 mouse and wild-type mouse derived non-adherent bone marrow cells were stimulated with various concentrations of RANKL (5–100 ng/ml) with M-CSF (10 ng/ml), and the number of TRAP-positive multinucleated OCLs formed in these cultures was scored. As shown in Figure 5B, the number of OCLs formed in OIP-1 No 5 and OIP-1 No 13 mouse bone marrow cultures was significantly decreased, by 39% and 42% respectively, compared with control mice in response to RANKL (100 ng/ml) treatment. Immunocytochemical staining further confirmed OIP-1 transgene expression in OCLs formed in OIP-1 mouse bone marrow cultures (Figure 5C). As is evident from the osteopetrotic bone phenotype in OIP-1 mice, it is likely that OIP-1 inhibits the bone resorption capacity of OCLs in these mice. We therefore further examined the bone resorption capacity of OCLs formed in OIP-1 mouse bone marrow cultures. As shown in Figures 5D and 5E, OCLs formed in OIP-1 No 5 and OIP-1 No 13 mouse bone marrow cultures demonstrated a significant decrease (39%) in resorption area on dentine slices compared with wild-type mice when cultured in the presence of M-CSF (10 ng/ml) and RANKL (100 ng/ml). These data indicate that targeted overexpression of OIP-1 to the OCL lineage results in significant inhibition of osteoclastogenesis and bone resorption activity *in vivo*.

RANK receptor signalling in pre-osteoclast cells from OIP-1 mice

RANKL–RANK signalling plays a critical role in OCL differentiation and bone resorption activity. We have also demonstrated that OIP-1 c-peptide treatment of RAW 264.7 mouse macrophage cells inhibits OCL formation through the suppression of molecules associated with RANKL–RANK signalling during OCL differentiation [14]. We therefore further examined the status of RANK receptor signalling molecules such as TRAF-2, ERK, JNK, c-Fos, and NFATc1 which are responsive to RANKL stimulation of OIP-1 mouse bone marrow cultures [16]. Non-adherent bone marrow cells derived from the wild-type and OIP-1 transgenic mice were stimulated with M-CSF (10 ng/ml) and RANKL (100 ng/ml) for 48 h. Western blot analysis of total cell lysates obtained from these pre-osteoclast cells identified no significant changes in the levels of RANK receptor expression. In contrast, RANK adaptor protein TRAF-2 expression, but not TRAF-6, was significantly decreased (ten-fold) in OIP-1 transgenic mouse-derived cells compared with wild-type mice. OIP-1 mouse-derived pre-osteoclasts also demonstrated a three-fold decrease in the levels of c-Fos and NFATc1 (Figure 6A).

We further examined the activation status of extracellular signal-regulated kinases (ERK) and c-Jun N-terminal kinase (JNK) molecules in response to RANKL stimulation of OIP-1 mouse bone marrow cells. As shown in Figure 6B, OIP-1 mouse-derived pre-osteoclast cells showed a time-dependent inhibition of ERK phosphorylation in response to RANKL stimulation, compared with wild-type mice. Similarly, JNK activation was significantly decreased in OIP-1 mouse pre-osteoclast cells compared with wild-type mice (Figure 6C). Taken together, OIP-1 inhibits OCL formation and bone resorption activity through the suppression of RANK receptor signalling molecules. These results further support the osteopetrotic bone phenotype in OIP-1 transgenic mice.

Discussion

We have previously reported on OIP-1/hSca as an autocrine/paracrine inhibitor of OCL formation and bone resorption activity [3,6]. Expression of OIP-1 mRNA in early and more committed OCL cell lineages and osteoblasts [17] suggested that OIP-1/hSca may play an important role in OCL formation. Immune cell products such as interferon (IFN)-γ are potent inhibitors of OCL formation. We have previously shown that IFN-γ stimulates OIP-1/hSca expression in OCL precursor cells [14]. It is more likely, therefore, that OIP-1 is an important physiological regulator of osteoclast development and bone resorption activity *in vivo*. Previously it has been shown

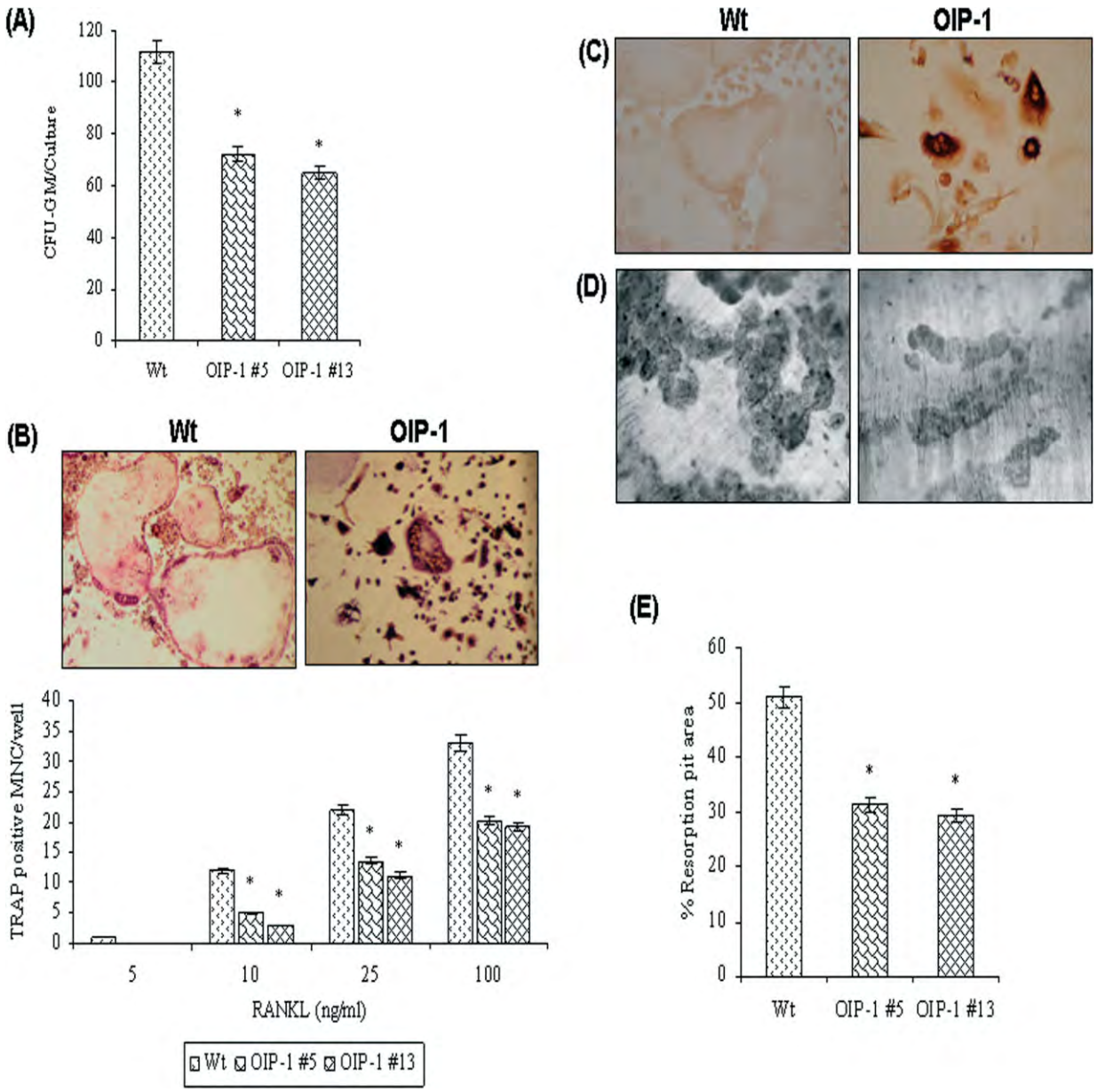

Figure 5. Inhibition of osteoclastogenesis in OIP-1 mouse bone marrow cultures. (A) CFU-GM formation in OIP-1 mouse bone marrow cultures. Wild-type (Wt) and OIP-1 mouse derived non-adherent bone marrow cells (4×10^5/ml) were cultured with hGM-CSF (10 ng/ml) in 1.2% methyl cellulose to form CFU-GM colonies. At the end of a 7 day culture period, CFU-GM colonies (aggregates >50 cells) formed in these cultures were scored using a light microscope as described earlier [7]. (B) Wild-type (Wt) and OIP-1 transgenic mouse bone marrow derived non-adherent cells were stimulated with RANKL (5–100 ng/ml) and M-CSF (10 ng/ml) for 5 days and the TRAP (+) multinucleated OCLs formed in these cultures were scored. The results represent quadruplicate cultures of three independent experiments ($p < 0.05$). (C) Immune staining for OIP-1/hSca expression in OCL formed in Wt and OIP-1 mouse bone marrow cultures was performed using rat anti-mouse TSA-1/Sca-2 antibody at a concentration of 1 μg/ml. Photomicrographs were taken at magnification ×20. (D) Wt and OIP-1 mouse bone marrow derived non-adherent cells (1×10^6) were cultured to form OCL as described on dentine slices for 10 days. The cells were removed by treating the disc with 1 M NaOH and stained with 0.1% toluidine blue. Resorption lacunae formed on dentine slices were identified by light microscopy. (E) The percentage of resorbed area on dentine was quantified as described in the methods. The results represent quadruplicate cultures of three independent experiments ($p < 0.05$)

that c-Src, a proto-oncogene deficiency, causes severe osteopetrosis in mice which lacks tooth eruption. The OCL development is normal in Src-deficient mice; however, the OCLs cannot form ruffled borders to resorb bone [18]. In contrast, OIP-1 mice show normal tooth eruption and no significant difference in body size. However, μCT analysis indicated an osteopetrosis bone phenotype in OIP-1 mice. Also, DEXA analysis showed a significant increase in BMD and BMC in the humeri from OIP-1 mice. Recently it has been reported that mice lacking the immunomodulatory adapter proteins DAP12 and Fc receptor gamma chain (FcRγ) exhibit severe osteopetrosis; however, they develop teeth, distinguishing their phenotype from $Src^{-/-}$ and RANKL-deficient mice [19].

Histological analysis indicated inadequate formation of marrow spaces in calvaria and humeri of OIP-1 mice. Histomorphometric analysis of humeri and

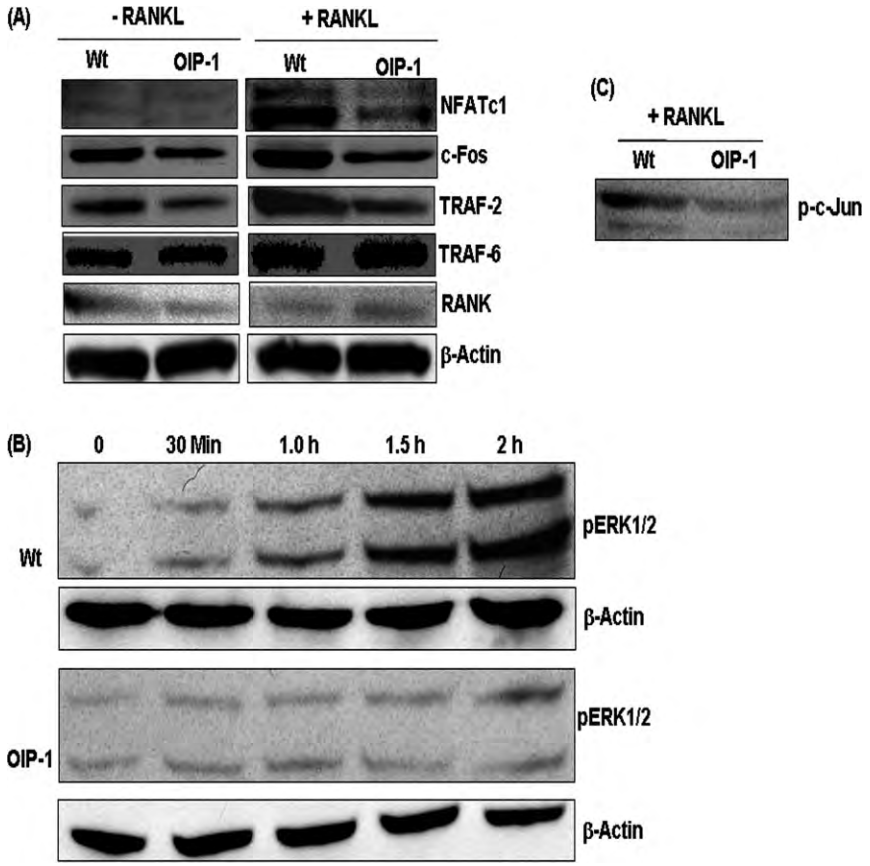

Figure 6. Western blot analysis of RANK receptor associated signaling molecules during OCL differentiation. (A) Wild-type (Wt) and OIP-1 mouse derived bone marrow cells were cultured in the presence of 10 ng/ml M-CSF for 24 h. The non-adherent cells were treated with M-CSF (10 ng/ml) and stimulated with or without RANKL (100 ng/ml) for 2 days. Total cell lysates prepared from the preosteoclast cells were subjected to Western blot analysis for TRAF2, TRAF6, c-Fos and NFATc1. β-actin expression levels were also analyzed to normalize the protein loading onto the gels in all the samples. (B) The preosteoclast cells were incubated with RANKL (100 ng/ml) for an indicated period and expression of pERK was determined. (C) JNK activity in the preosteoclast cells derived from Wt and OIP-1 mice

calvaria further demonstrated a significant decrease in OCL development. In contrast, femurs from OIP-1 mice had no significant change in the numbers of TRAP-positive osteoclasts; however, they demonstrated a significant change in bone volume (data not shown). These results indicate OIP-1-specific inhibition of osteoclast formation/activity *in vivo*. Furthermore, our results that the number of osteoblasts per bone perimeter area was not affected in OIP-1 mice compared with control mice and that there was a lack of significant differences in the levels of serum osteocalcin and osteoblast-specific gene expression in OIP-1 mice compared with wild-type mice suggest that bone formation is not affected in these mice.

Mouse bone marrow culture studies *ex vivo* further suggested that the osteopetrotic bone phenotype is due to a significant decrease in OCL number and activity compared with control mice. These results are consistent using bone marrow cells derived from two of the founder lines established with comparable levels of OIP-1 expression. It is possible that genomic integration of the OIP-1 transgene may influence the severity of the osteopetrotic bone phenotype in these mice. We have further confirmed OIP-1-specific inhibition of OCL differentiation of RAW cells *in vitro* through constitutive overexpression (data not shown). Our results are therefore consistent with an osteopetrotic phenotype due to inhibition of osteoclastogenesis and bone resorption activity in OIP-1 mice.

Earlier we demonstrated that OIP-1 significantly decreased RANKL-induced JNK activity in RAW 264.7 cells. Furthermore, OIP-1 decreased TRAF-2 expression in pre-osteoclast cells, but had no significant effect on TRAF-6 and RANK expression in these cells [14]. These data suggest that targeted overexpression of OIP-1 to the OCL lineage *in vivo* results in inhibition of OCL differentiation through suppression of TRAF-2 and JNK activity. Although TRAF-2 is essential for TNF-α-induced osteoclastogenesis and TRAF-5 functions in both RANKL and TNF-α-induced osteoclastogenesis [20,21], our results indicate that OIP-1 suppresses RANK receptor signalling through TRAF-2, but not TRAF-6, to inhibit OCL differentiation. It is possible that TRAF-2 interaction with other signalling molecules may play an important role in downstream signalling that is necessary to activate JNK activity during OCL differentiation. ERK has been implicated in cellular proliferation, migration, and survival [22]. Thus, inhibition of ERK activation in OIP-1 mouse-derived pre-osteoclast

cells further supports our results indicating a significant decrease in the growth of early OCL precursors, CFU-GM. NFATc1 is a critical transcription factor that modulates OCL-specific genes such as the calcitonin receptor and TRAP expression during OCL differentiation [23–25]. OIP-1 mouse-derived pre-osteoclast cells demonstrated a significant decrease in the level of NFATc1 expression in response to RANKL stimulation compared with wild-type mice. It is therefore possible that NFATc1 may play a critical role in OIP-1 function as a downstream effector to suppress gene expression during OCL differentiation.

In summary, OIP-1/hSca represents a novel physiological inhibitor of osteoclast development and bone resorption *in vivo*. Thus, OIP-1 may have therapeutic utility for bone disease with high bone turnover, such as osteoporosis and Paget's disease of bone.

Acknowledgements

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Osteoclast Inhibitory Peptide-1 (OIP-1) Inhibits Measles Virus Nucleocapsid Protein Stimulated Osteoclast Formation/Activity

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Abstract Paget's disease (PD) of bone is characterized by increased activity of large abnormal osteoclasts (OCLs) which contain paramyxoviral nuclear and cytoplasmic inclusions. MVNP gene expression has been shown to induce pagetic phenotype in OCLs. We previously characterized the osteoclast inhibitory peptide-1 (OIP-1/hSca) which inhibits OCL formation/bone resorption. OIP-1 is a glycophosphatidylinositol (GPI)-linked membrane protein containing a 79 amino acid extra cellular peptide and a 32 amino acid carboxy terminal GPI-linked peptide (c-peptide) which is critical for OCL inhibition. In this study, we demonstrate that OIP-1 c-peptide significantly decreased (43%) osteoclast differentiation of peripheral blood mononuclear cells from patients with PD. Also, OIP-1 treatment to normal human bone marrow mononuclear cells transduced with the MVNP inhibited (41%) osteoclast precursor (CFU-GM) growth in methyl-cellulose cultures. We further tested if OIP-1 overexpression in the OCL lineage in transgenic mice inhibits MVNP stimulated OCL formation. MVNP transduction and RANKL stimulation of OIP-1 mouse bone marrow cells showed a significant decrease (43%) in OCL formation and inhibition (38%) of bone resorption area compared to wild-type mice. Western blot analysis identified that OIP-1 decreased (3.5-fold) MVNP induced TRAF2 expression during OCL differentiation. MVNP or OIP-1 expression did not affect TRAF6 levels. Furthermore, OIP-1 expression resulted in a significant inhibition of MVNP stimulated ASK1, Rac1, c-Fos, p-JNK, and NFATc1 expression during OCL differentiation. These results suggest that OIP-1 inhibits MVNP induced pagetic OCL formation/activity through suppression of RANK signaling. Thus, OIP-1 may have therapeutic utility against excess bone resorption in patients with PD. J. Cell. Biochem. 104: 1500–1508, 2008.

Key words: Paget's disease of bone; osteoclast; measles virus nucleocapsid; OIP-1; RANK ligand

Paget's disease (PD) of bone is characterized by markedly increased osteoclast (OCL) formation and bone resorption followed by excessive new bone formation. Osteoclasts in PD are increased both in number and size, contain paramyxoviral nuclear inclusions, and contain more nuclei per cell than normal OCL. Furthermore, marrow culture studies have identified several abnormalities in OCL formation in PD [Roodman and Windle, 2005]. OCL precursors and circulating peripheral blood cells from Paget's patients express measles virus nucleocapsid (MVNP) transcripts [Reddy et al., 1996]. Also, we previously reported that MVNP gene transduction to normal human OCL precursors results in formation of OCLs with pagetic phenotype [Kurihara et al., 2000]. Further, measles virus (MV) infection of OCL precursors from CD46 transgenic mice form OCL with a pagetic phenotype in vitro [Reddy et al., 2001]. However, viral etiology of PD remains controversial as others have failed to detect paramyxoviral transcripts [Helfrich et al., 2000; Ooi et al., 2000]. Bisphosphonates have offered an advanced therapy for PD of bone. Recent data

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indicate that a single infusion of zoledronic acid produces high therapeutic response in PD than daily treatment with risedronate [Reid et al., 2005]. Although complications associated with bisphosphonate therapy such as osteonecrosis of the jaw (ONJ) is reported rarely in patients with PD, risk factors and underlying mechanisms of ONJ are still unclear [Bilezikian, 2006].

We have previously identified and characterized a novel inhibitor of OCL formation and bone resorption termed osteoclast inhibitory peptide-1 (OIP-1/hSca) [Koide et al., 2002, 2003]. More recently, we have shown that targeted overexpression of OIP-1 in the OCL lineage develops an osteopetrosis bone phenotype in mice [Shanmugarajan et al., 2007]. OIP-1/hSca is also termed as retinoic acid-induced gene expression (RIG-E) or human thymic shared antigen (TSA-1/Sca-2) is a Ly-6 gene related differentiation antigen expressed on immature thymocytes and thymic epithelial cells [Mao et al., 1996]. OIP-1/hSca is a glycophosphatidylinositol (GPI) linked membrane protein (16 kDa) containing a 79 amino acid extracellular peptide and a 32 amino acid carboxy terminal GPI linked peptide (c-peptide). We have further identified that the OIP-1 c-peptide region is critical for OCL inhibitory activity [Koide et al., 2002]. It has been suggested that GPI-anchored proteins are membrane bound proteins that can be shed from the cell surface in membrane bound vesicles or cleaved and released by the action of phospholipase C or other proteolytic enzymes. These proteins have been reported to act as cell activators, cell communicators, and signaling molecules in hematopoietic cells [Nosjean et al., 1997]. In addition, GPI linked proteins transmit signals to the cell interior by interacting with non-receptor type tyrosine kinases $p56^{lck}$ and 59^{fyn} [Stefanova et al., 1991]. Moreover, Ly-6A (Sca-1) knock-out mice demonstrated a decreased bone mineral density and bone mineral content [Bonyadi et al., 2003] implicating an essential role for LY-6 gene family in normal bone remodeling. In this study, we determined the mechanism of OIP-1 inhibition of MVNP stimulated pagetic OCL formation and bone resorption activity in vitro.

MATERIALS AND METHODS

MVNP Expression in Bone Marrow Cells

We have previously developed a retroviral plasmid construct, pILXAN#1 that transcribes MVNP mRNA expression under the control of 5′ LTR viral promoter elements. The recombinant plasmid construct was transfected into the PT67 amphotropic packaging cell line using the LipofectAMINE (Invitrogen Corp., CA). Stable clonal cell lines producing MVNP recombinant retrovirus at high titer (1×10^6 virus particles/mL) were established by selecting for resistance to neomycin (600 μg/mL). Similarly, a control retrovirus producer cell line was established by transfecting the cells with the pLXSN empty vector (EV). Producer cell lines were maintained in DMEM containing 10% FCS (Gibco BRL, MD), 100 U/mL each of streptomycin and penicillin, 4 mM L-glutamine, and high glucose (4.5 g/L). Bone marrow cells were transduced with EV or MVNP retroviral supernatants (20%) from the producer cell lines in 4 μg/mL of polybrene for 24 h at 37°C in a 5% CO_2 incubator as described earlier [Kurihara et al., 2000].

OCL Precursor (CFU-GM) Culture

Normal human ($n = 6$) bone marrow derived non-adherent mononuclear cells were cultured in methyl-cellulose to form CFU-GM as described previously [Koide et al., 2002]. Briefly, the EV or MVNP transduced non-adherent bone marrow cells (4×10^5/mL) were cultured in Methocult H4230 (Stem cell technologies, Inc., WA) and incubated for 7 days. Cultures were scored for colonies (aggregates > 50 cells) using an Olympus dissecting microscope (Olympus Optical Co., Tokyo, Japan) at 100× magnification. The results are reported as the mean ± SD for triplicate cultures.

Peripheral Blood Mononuclear Cell Culture Assay for OCL Differentiation

Peripheral blood mononuclear cells (PBMC) derived from patients with PD and normal subjects ($n = 6$) were isolated as described [Susa et al., 2004]. All human samples were obtained following the IRB approved protocol at the Medical University of South Carolina. Briefly, 15 mL of whole blood was mixed with 15 mL of warm (37°C) α-MEM, layered over 15 mL of Ficoll-Paque (Sigma, MO) and centrifuged ($1500 \times g$, 30 min) at room temperature. The cell layer on top of the Ficoll-Paque was collected, resuspended in 10 mL of α-MEM and centrifuged. The mononuclear cells collected were plated in 96-well plates at 6×10^5 cells per well in 0.2 mL of medium (α-MEM, pH 7.4,

containing 10% FCS) supplemented with 10 ng/mL hM-CSF, 100 ng/mL hRANKL and 1 μM dexamethasone. The cells were re-fed twice weekly by semi-depletion (half of the medium withdrawn and replaced with fresh medium). At the end of culture period (17 days) the cells were fixed with 2% glutaraldehyde in PBS and stained for tartrate resistant acid phosphatase (TRAP) activity using a histochemical kit (Sigma). TRAP positive multinucleated cells (MNC) containing three or more nuclei were scored as osteoclast (OCL) cells under a microscope.

OIP-1 Mouse Bone Marrow Culture Assay for OCL Formation/Bone Resorption

We have recently developed transgenic mice targeted with OIP-1 expression in cells of OCL lineage [Shanmugarajan et al., 2007]. Bone marrow cells isolated from wild-type (Wt) and OIP-1 transgenic mice were centrifuged at 1,500 rpm. The cells were resuspended in α-MEM containing 10% FCS and cultured in the presence of 10 ng/mL mM-CSF for 12 h. The non-adherent cells (1.5×10^6/mL) transduced with EV or MVNP were treated with 10 ng/mL mM-CSF for 24 h at 37°C in a 5% CO_2 incubator. The cells were resuspended with fresh α-MEM containing 10% FCS and cultured for 5 days in the presence of 10 ng/mL mM-CSF and 100 ng/mL mRANKL (R&D systems, MN). At the end of the culture period, the cells were fixed with 2% glutaraldehyde in PBS for 20 min and stained for TRAP activity. TRAP positive multinucleated OCLs containing three or more nuclei were scored under a microscope.

To determine the bone resorption activity, the non-adherent bone marrow cells (1.5×10^6/mL) from Wt and OIP-1 mice were transduced with EV or MVNP as described above. The cells were resuspended with fresh α-MEM containing 10% FCS and cultured for 10 days on dentine slices in the presence of 10 ng/mL mM-CSF and 100 ng/mL mRANKL. The cells were removed from the dentine slices using 1 M NaOH, and digital images of the dentine were taken using an Olympus microscope. The areas of resorption lacunae on the digital images were quantified using a computerized image analysis (Adobe Photoshop and Scion MicroImaging version beta 4.2). The percentage of the resorbed area was calculated relative to the total dentine area. All experiments were performed using OIP-1 mice (3–4-week-old) with appropriate littermate controls following the IACUC approved procedure at the Medical University of South Carolina, Charleston.

Western Blot Analysis of MVNP Expression and RANK Signaling Molecules

Bone marrow derived non-adherent cells (1.5×10^6/mL) isolated from Wt and OIP-1 mice were transduced with EV or MVNP. The cells were resuspended with fresh α-MEM containing 10% FCS for 48 h in the presence of 10 ng/mL mM-CSF and 100 ng/mL mRANKL. Total cell lysates prepared from the preosteoclast cells were subjected to Western blot analysis for expression of MVNP using rat anti-mouse MVNP antibody (Abcam Inc, MA) and RANK receptor signaling molecules using antibodies against RANK, TRAF2, ASK1, Rac1, c-Fos, NFATc1. β-Actin expression levels in all the samples were used as loading control (Santa Cruz Inc., CA).

Statistical Analysis

Results are presented as mean ± SD for three independent experiments and were compared by Student's *t*-test. Results were considered significantly different for $P < 0.05$.

RESULTS

OIP-1 Inhibition of OCL Differentiation in Pagetic PBMC Cultures

We have previously characterized OIP-1 inhibition of OCL differentiation and bone resorption in vitro in normal bone marrow cultures and further shown that OIP-1 c-peptide region is critical for OCL inhibitory activity [Koide et al., 2002]. We therefore tested the potential of OIP-1 to inhibit OCL differentiation of PBMC derived from patients with PD. As shown in Figure 1, PBMC derived from patients with PD resulted in significant increase (46%) in TRAP positive MNC compared to normal subjects. OIP-1 c-peptide treatment to pagetic PBMC inhibited 26% and 43% OCL formation at 10 and 100 ng/mL concentrations, respectively. OIP-1 c-peptide treatment thus demonstrated a significant decrease in the enhanced rate of OCL differentiation in pagetic cultures compared to normal PBMC cultures. These results indicate that OIP-1 is an efficient inhibitor of pagetic OCL differentiation in vitro.

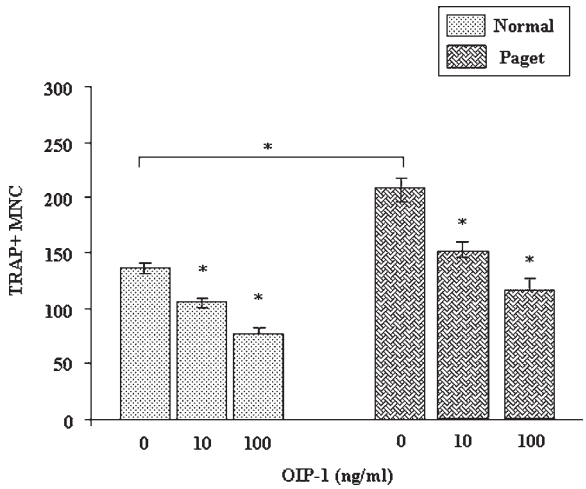

Fig. 1. OIP-1 inhibits osteoclast formation in PBMC cultures from patients with PD. PBMC derived from normal and PD subjects (n = 6) were cultured to form OCL in the presence of 10 ng/mL hM-CSF, 1 μm dexamethasone and 100 ng/mL hRANKL with or without OIP-1 cpeptide at different concentrations (0–100 ng/mL) as described in the methods. The results represent quadruplicate cultures of five independent experiments and data shown as mean ± SD, ($P < 0.05$).

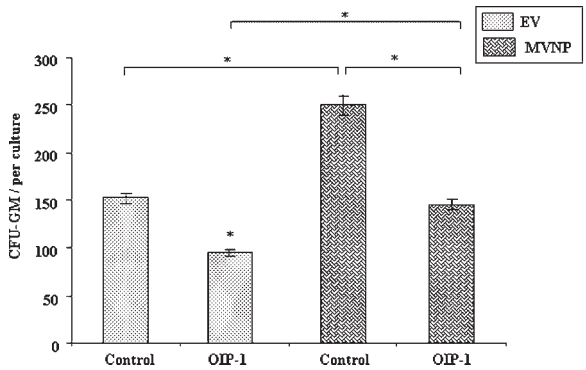

Fig. 2. OIP-1 inhibits MVNP stimulated CFU-GM formation in human bone marrow cultures. Non-adherent human (n = 6) bone marrow cells (4×10^5/mL) transduced with MVNP or empty vector (EV) were cultured with GM-CSF (10 ng/ml) in the presence or absence of OIP-1 (100 ng/ml) in methylcellulose to form CFU-GM colonies. At the end of a 7-day culture period, CFU-GM colonies formed in these cultures were scored using a light microscope. The results represent quadruplicate cultures of five independent experiments and data shown as mean ± SD, ($P < 0.05$).

OIP-1 Inhibition of MVNP Stimulated CFU-GM Growth

Previously it has been shown that the number of early OCL precursors, CFU-GM, was increased significantly in marrow aspirates from patients with PD compared to normal subjects [Demulder et al., 1993]. Furthermore, it has been demonstrated that MVNP gene expression in normal OCL precursors stimulates OCL formation and induces pagetic phenotype [Kurihara et al., 2006]. Therefore, we next examined if OIP-1 inhibit MVNP stimulation of OCL precursor growth in methyl-cellulose cultures. Normal human bone marrow derived non-adherent cells were transduced with EV or MVNP gene expression retroviral vectors and cultured in methylcellulose with GM-CSF (10 ng/mL) to form CFU-GM colonies as described in methods. As shown in Figure 2, MVNP transduced marrow mononuclear cells demonstrated a significant increase (38%) in the numbers of CFU-GM colony formation compared to EV transduced cells. OIP-1 treatment to MVNP transduced cells showed a significant decrease (41%) in CFU-GM colony formation. These results indicate OIP-1 suppression of MVNP stimulated OCL precursor growth in vitro.

OIP-1 Inhibition of MVNP Stimulated OCL Differentiation/Bone Resorption

We recently developed mice targeted with OIP-1 expression in cells of the OCL lineage and characterized inhibition of osteoclastogenesis and bone resorption activity in vivo [Shanmugarajan et al., 2007]. Therefore, we further investigated if OIP-1 expression in the OCL lineage inhibits MVNP stimulated OCL formation in bone marrow cultures. The non-adherent bone marrow cells from Wt and OIP-1 mice were transduced with EV or MVNP retroviral vectors and cultured with 10 ng/mL mM-CSF, 100 ng/mL mRANKL for 2 days. Western blot analysis of total cell lysates obtained from the preosteoclast cells showed no significant difference in the levels of MVNP expression indicating the normalized transduction efficiency (Fig. 3A). MVNP transduction and RANKL stimulation of bone marrow cells derived from OIP-1 mice showed a significant decrease in TRAP positive MNC formation compared to Wt mice (Fig. 3B). Quantification of these results showed that MVNP transduction of Wt mouse bone marrow cells resulted in a 33% increase in OCL formation compared to EV transduced cells. In contrast MVNP enhanced rate of OCL formation is significantly decreased in OIP-1 mouse bone marrow cultures compared to Wt mouse bone marrow cultures (Fig. 3C).

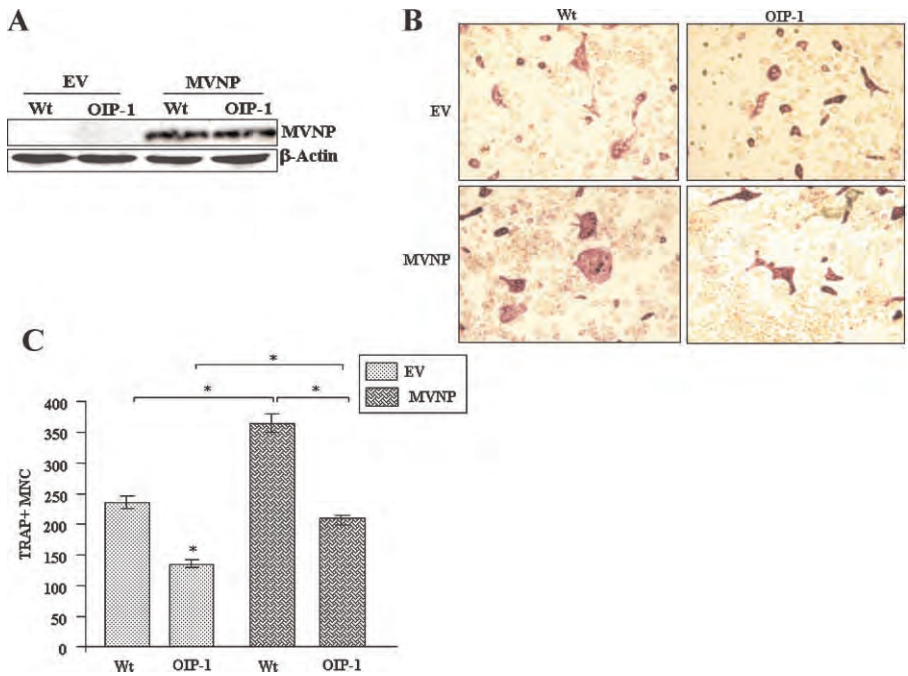

Fig. 3. Osteoclast formation in wild-type (Wt) and OIP-1 transgenic mouse bone marrow cultures transduced with MVNP or empty vector (EV). (**A**) The non-adherent bone marrow cells from Wt and OIP-1 mice were transduced with EV or MVNP retroviral vectors as described in the methods and cultured with 10 ng/mL mM-CSF, 100 ng/mL mRANKL for 2 days. Total cell lysates were prepared from these preosteoclast cells and MVNP expression was confirmed by Western blot analysis using rabbit anti-MVNP antibody. (**B**) Bone marrow cells from Wt and OIP-1 transgenic mice were transduced with MVNP or EV and cultured for OCL formation in the presence of 10 ng/mL mM-CSF and mRANKL (100 ng/mL) for 4 days. At the end of the culture period the cells were fixed and stained for TRAP activity. (**C**) The TRAP (+) multinucleated cells (MNC) formed were scored under a light microscope. The results represent quadruplicate cultures of three independent experiments and data shown as mean ± SD, ($P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

We further examined the bone resorption capacity of OCLs formed in Wt and OIP-1 mice bone marrow cultures transduced with EV or MVNP. MVNP expression in OCL derived from Wt mice showed a significant increase (34%) in resorption area on dentine slices compared to EV transduced cells. In contrast, OCL formed in OIP-1 mouse bone marrow cultures transduced with EV demonstrated a significant decrease (35%) in resorption lacunae on dentine compared to Wt mice. Furthermore, OIP-1 mouse bone marrow cultures transduced with MVNP resulted in a significant inhibition (38%) of OCL bone resorption activity compared with MVNP transduced Wt mouse bone marrow cultures (Fig. 4A and B). These results suggest that OIP-1 inhibit MVNP stimulated OCL formation/bone resorption activity in vitro.

Inhibition of MVNP Stimulated RANK Signaling During OCL Differentiation

RANK-RANKL signal transduction pathway is critical for OCL differentiation, activation, and survival [Takayanagi, 2007]. We previously identified that OIP-1 inhibits OCL formation through RANK receptor signaling mechanism in normal OCL precursor cells [Koide et al., 2003]. We therefore examined the expression levels of RANK receptor signaling molecules such as TRAF2, c-Fos and NFATc1 in response to MVNP expression and RANKL stimulation. Western blot analysis of total cell lysates obtained from the Wt and OIP-1 mice derived preosteoclast cells transduced with EV or MVNP and stimulated with RANKL revealed that OIP-1 expression did not affect the RANK receptor expression in these cells. However, RANK expression was moderately increased in MVNP transduced Wt and OIP-1 mouse derived preosteoclast cells. Further, MVNP stimulated RANK adaptor protein TRAF2 expression (3.5-fold) in preosteoclast cells from Wt mice; however, in contrast TRAF6 expression levels were not affected. Furthermore, MVNP expression did not stimulate TRAF2 expression in OIP-1 mouse derived preosteoclast cells. In addition, c-Fos and NFATc1 transcription factor expression levels were

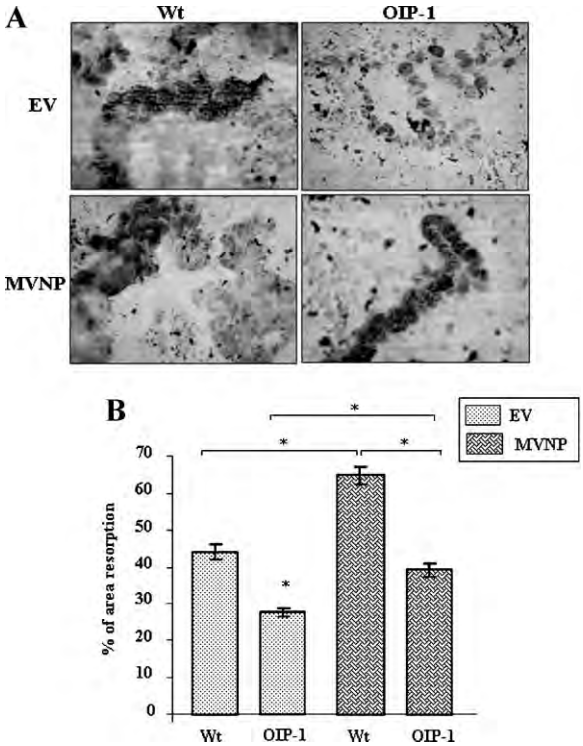

Fig. 4. OIP-1 inhibits MVNP stimulated OCL bone resorption activity. (**A**) Wild-type (Wt) and OIP-1 mouse bone marrow derived non-adherent cells (1×10^6) transduced with MVNP and EV were cultured on dentine slices for 10 days in the presence of 10 ng/mL mM-CSF and 100 ng/mL mRANKL. At the end of the culture period, the cells were removed and stained with toludine blue. (**B**) The percentage of the mineralized surface area resorbed was quantified as described in the methods. The results represent quadruplicate cultures of three independent experiments and data shown as mean ± SD, ($P < 0.05$).

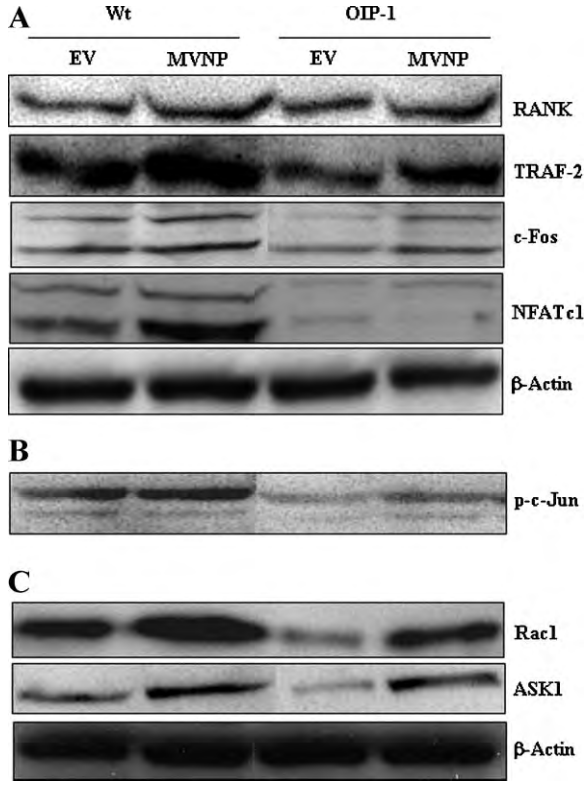

Fig. 5. Western blot analysis of RANK receptor signaling molecules in MVNP transduced OIP-1 mice derived preosteoclast cells. Bone marrow cells from Wt and OIP-1 mouse were transduced with MVNP or EV and cultured in the presence of 10 ng/mL mMCSF and 100 ng/mL mRANKL for 2 days and the total cell lysates prepared were subjected to Western blot analysis for RANK receptor signaling molecules.

significantly decreased in OIP-1 transgenic mice derived preosteoclast cells (Fig. 5A).

We further examined the c-Jun N-terminal kinase (JNK) activation in response to MVNP stimulation in OIP-1 mouse bone marrow cells. As shown in Figure 5B, OIP-1 mouse derived preosteoclast cells demonstrated suppression of JNK phosphorylation compared to Wt mice. However, MVNP expression did not stimulate JNK phosphorylation in OIP-1 mouse bone marrow cells in response RANKL stimulation. In contrast, MVNP expression in Wt mouse bone marrow derived preosteoclasts showed a 2.5-fold increase in JNK phosphorylation compared to EV transduced cells. In addition, the expression of JNK activators such as Rac1 and ASK1 expression levels were suppressed in OIP-1 preosteoclast cells compared to Wt mice. Further, MVNP expression in Wt preosteoclasts induced 3.6 and 3.2-fold increase in Rac1 and ASK1 expression, respectively. However, there is a significant decrease in MVNP stimulation of Rac1 and ASK1 expression in OIP-1 preosteoclast cells (Fig. 5C). Taken together, these results indicate that OIP-1 inhibits OCL formation and bone resorption activity through suppression of RANK signaling in MVNP stimulated OCL differentiation.

DISCUSSION

Ultrastrutural and immunocytochemical studies identified expression of paramyxoviral nucleocapsid antigens in pagetic OCL [Roodman and Windle, 2005]. Bone marrow culture techniques further identified several abnormalities in OCL and precursors cells from patients with PD. The number of early OCL precursors, CFU-GM, was increased significantly in marrow aspirates from patients with PD compared to normal subjects. Also, it has been shown that OCL formed more rapidly with increased

numbers (10–100-fold) of nuclei per OCL in marrow cultures from patients with PD compared to normal subjects [Demulder et al., 1993]. Although recurrent mutations occur in the UBA domain of Sequestosome 1 (SQSTM1/p62) in patients with PD [Laurin et al., 2002; Layfield et al., 2004], recent evidence indicated that mutant p62 expression is not sufficient to induce pagetic phenotype in OCL, but may cause a predisposition to the development of PD through enhanced sensitivity of OCL precursors to osteoclastogenic cytokines [Kurihara et al., 2007]. Lack of skeletal abnormalities in p62 deficient mice [Duran et al., 2004] further suggests a genetic defect may favor the environmental factors such as MV infection to have a potential role in abnormal OCL development in PD. However, the molecular basis for the abnormalities associated with OCLs and the role of paramyxoviral infection in patients with PD is unclear.

In this study, our results indicating that OIP-1 is an inhibitor of MVNP stimulated OCL progenitor growth and differentiation implicate a potential therapeutic utility for high bone turnover associated with PD. Pagetic OCL produce increased levels of IL-6 and express high levels of IL-6 receptors compared to normal subjects. Coculture studies of OCL precursors and cells from the marrow microenvironment of patients with PD and normals have demonstrated that the marrow microenvironment is more osteoclastogenic than normal [Reddy, 2006]. However, we show that OIP-1 transgenic mouse bone marrow cells targeted with OIP-1 expression in cells of the OCL lineage did not respond to MVNP stimulation of OCL formation/bone resorption activity. Therefore, OIP-1 expression in OCL may have a direct effect on signaling pathways and gene expression critical for OCL differentiation. Previously it has been shown that the levels of IL-6, c-Fos, Bcl 2 anti-apoptotic gene mRNA expression in pagetic OCLs are elevated [Brandwood et al., 2003; Roodman and Windle, 2005]. Therefore, gene array profiling studies using OIP-1 mice derived OCL further provide insights into MVNP altered gene expression and pagetic phenotype in OCL.

RANKL is a critical OCL differentiation factor that is expressed on marrow stromal and osteoblast cells in response to several osteotropic factors. It has been shown that marrow stromal cells from pagetic lesions have increased RANKL expression and that OCL precursors from patients with PD are hyperresponsive to RANKL [Menaa et al., 2000; Neale et al., 2002]. Further, enhanced levels of TAF_{II}-17 expression is responsible for the hypersensitivity of pagetic OCL precursors to 1,25-dihydroxyvitamin D_3 [Kurihara et al., 2004]. Gene expression profiling studies indicated that IFN-γ and associated signaling molecules are upregulated in pagetic PBMC and in contrast TNF-α is downregulated [Nagy et al., 2007]. Although IFN-γ is a direct inhibitor of OCL differentiation, recent evidence indicates that IFN-γ stimulates osteoclastogenesis through enhanced levels RANKL levels in T-lymphocytes in vivo [Kotake et al., 2005; Gao et al., 2007]. Therefore, OIP-1 inhibition of RANK receptor signaling in OCL precursor cells counteracts osteoclastogenic factors such as IL-6, RANKL, M-CSF that are elevated in PD [Neale et al., 2002; Reddy, 2006].

The RANK-RANKL signaling promotes the binding of TNF receptor associated factor (TRAF) family proteins such as TRAF-6 to RANK receptor, resulting in activation of NF-κB and Jun N-terminal kinase (JNK) pathways [Takayanagi, 2007]. Previously, it has been shown that MVNP enhance OCL formation through enhanced levels of NF-κB and JNK levels [Kurihara et al., 2000]. However, we have demonstrated that the OIP-1 signaling mechanism is independent of NF-κB activation and involves suppression of p-c-*Jun* kinase to inhibit OCL formation [Koide et al., 2003]. Also, consistent to our previous results, OIP-1 mice derived preosteoclast cells demonstrated a significant decrease in TRAF-2 expression but had no change in the levels of TRAF-6 (data not shown) and RANK expression in these cells. Therefore, OIP-1 inhibition of p-c-*Jun* kinase activity is mediated by TRAF-2. Transcription factors such as c-Fos and NFATc1 which modulate target gene expression are critical for OCL development and bone resorption activity [Ikeda et al., 2004]. We show that MVNP expression significantly elevated NFATc1 expression, however, in contrast no significant change in c-Fos expression occurred in OCL precursor cells derived from Wt mice. c-Fos expression levels are moderately decreased in OIP-1 mouse preosteoclast cells compared to Wt mice. Interestingly, MVNP expression did not stimulate NFATc1 expression in OIP-1 mouse derived preosteoclasts indicating that OIP-1 is a

potent inhibitor of the RANK receptor signaling pathway critical for OCL differentiation. This is consistent with our recent report that mice targeted with OIP-1 overexpression in cells of OCL lineage develop an osteopetrosis bone phenotype due to inhibition of OCL formation activity in vivo [Shanmugarajan et al., 2007]. It has been reported that ASK1 is activated by TRAF2, TRAF5, and TRAF6 overexpression and mediates TRAF2 induced JNK activation [Nishitoh et al., 1998]. Similarly, Rac has been shown to activate JNK and p38 MAPK pathways [Caron and Hall, 1998]. In the present study, we show that MVNP expression significantly increased Rac1 and ASK1 signaling molecules in Wt mouse derived preosteoclast cells. However, OIP-1 mouse derived preosteoclast cells demonstrated suppression of Rac1, ASK1, and JNK activity further suggesting that OIP-1 inhibits MVNP stimulated OCL formation through suppression of TRAF2 dependent JNK activity. Thus, OIP-1 may have therapeutic utility against excess bone resorption activity in patients with PD.

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