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The Paired-box Homeodomain Transcription Factor Pax6 Binds to the Upstream Region of the *TRAP* Gene Promoter and Suppresses Receptor Activator of NF-κB Ligand (RANKL)-induced Osteoclast Differentiation*

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Background: Negative regulation of osteoclast differentiation is critical for suppression of pathological bone destruction.
Results: Pax6 is induced by RANKL in osteoclasts and attenuates osteoclast differentiation via blocking *TRAP* gene expression.
Conclusion: Pax6 functions together with its co-receptor to suppress *TRAP* gene expression and osteoclastogenesis.
Significance: This study provides a new aspect for investigating the molecular targets linked to physiological bone resorption.

Osteoclast formation is regulated by balancing between the receptor activator of nuclear factor-κB ligand (RANKL) expressed in osteoblasts and extracellular negative regulatory cytokines such as interferon-γ (IFN-γ) and interferon-β (IFN-β), which can suppress excessive bone destruction. However, relatively little is known about intrinsic negative regulatory factors in RANKL-mediated osteoclast differentiation. Here, we show the paired-box homeodomain transcription factor Pax6 acts as a negative regulator of RANKL-mediated osteoclast differentiation. Electrophoretic mobility shift and reporter assays found that Pax6 binds endogenously to the proximal region of the tartrate acid phosphatase *(TRAP)* gene promoter and suppresses nuclear factor of activated T cells c1 (NFATc1)-induced *TRAP* gene expression. Introduction of Pax6 retrovirally into bone marrow macrophages attenuates RANKL-induced osteoclast formation. Moreover, we found that the Groucho family member co-repressor Grg6 contributes to Pax6-mediated suppression of the *TRAP* gene expression induced by NFATc1. These results suggest that Pax6 interferes with RANKL-mediated osteoclast differentiation together with Grg6. Our results demonstrate that the Pax6 pathway constitutes a new aspect of the negative regulatory circuit of RANKL-RANK signaling in osteoclastogenesis and that the augmentation of Pax6 might therefore represent a novel target to block pathological bone resorption.

Bone remodeling plays a pivotal role in calcium homeostasis, controlled by osteoblasts and osteoclasts, and the osteoclasts differentiate from hematopoietic myeloid precursors of the macrophage/monocyte lineage under the control of the osteoblasts. Osteoclast precursors expressing the receptor activator of nuclear factor-κB (RANK)[2] and c-Fms (receptor of macrophage colony-stimulating factor, M-CSF) differentiate into functional multinucleated cells in the presence of RANKL and M-CSF supplied by osteoblastic cells (1–4). Thus, the RANKL-RANK signaling pathway is essential for osteoclast differentiation (5–7). During osteoclastogenesis, stimulation of RANK triggers induction of osteoclast-specific genes, including cathepsin K, osteoclast-associated receptor (OSCAR), and tartrate acid phosphatase (TRAP), which determine the fate of the osteoclast differentiation and function. The expression of these

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[2] The abbreviations used are: RANK, receptor activator of NF-κB; RANKL, receptor activator of NF-κB ligand; TRAP, tartrate-resistant acid phosphatase; OCL, osteoclasts; Pax, paired box homeodomains; MITF, microphthalmia transcription factor; NFATc1, nuclear factor of activated T cells c1; M-CSF, macrophage colony-stimulation factor; WCE, whole cell extract; BMM, bone marrow macrophage; qRT-PCR, quantitative RT-PCR; TA, proline/serine/threonine-rich transactivator.

genes is in turn regulated by limited sets of transcriptional factors such as PU.1 (8, 9), c-Fos (10, 11), NF-κB (12, 13), and microphthalmia transcription factor (MITF) (14) in osteoclast precursors, downstream of RANKL-RANK signaling. We elucidated that the interaction of MITF with either PU.1 or PU.1-interacting protein (Pip) allows efficient induction of the characteristic osteoclast-specific gene, *TRAP*, in a synergistic manner, at the downstream of the p38 MAPK pathway (15, 16). A master transcriptional regulator nuclear factor of activated T cells c1 (NFATc1) is induced by RANKL stimulation, which then activates the *TRAP* gene promoter, synergistically with c-Fos (17). Moreover, the activation of NFATc1 robustly undergoes gene induction of cathepsin K, essential for bone resorption, in concert with PU.1 and MITF, providing a gradual transcriptional regulatory model during osteoclastogenesis (18). Thus, the inducibility of osteoclast-specific genes appears to be selectively controlled by the combination of RANKL-induced and constitutively expressed transcription factors in osteoclast precursors.

Paired-box homeodomain (*Pax*) genes encode a family of transcription factors that are important regulators of development in a wide variety of tissues across divergent species (19, 20). Among them, Pax2/3/7/8 are well defined as obligate contributors to neural development with genetic animal models (21–24). Mice lacking Pax5, which acts as both a transcriptional activator and a transcriptional repressor (25), show severe osteopenia, with a deficit of their bone mass and an increase in osteoclast number. Interestingly, expression of Pax5 was not detected in either osteoblasts or osteoclasts, indicating indirect regulatory mechanisms by Pax5 in bone remodeling. Loss of Pax6 causes small eyes and embryonic or perinatal lethality with forebrain defects (26). Recently, it has been unveiled that Pax6 acts as a repressor for the down-regulations of *Fgf15* and *Dkk* gene expressions cooperatively with MITF (27). In bone remodeling, however, the roles of Pax6 remain to be fully defined, although MITF mutant mice show defects in retinal development and osteoclastogenesis (14). One of the molecular mechanisms by which a family of Pax transcription factors function as a repressor can be understood by altering the structure of transcriptional machinery assembled with a Grouch (Grg)-related co-repressor (28). Indeed, Grg/TLE (Groucho/transducin-like enhancer of split) family protein functions as a modifying factor for the regulation of bone development when it interacts with Runx2, a crucial factor for osteoblasts *in vivo* (29). In humans with Darier disease, Aniridia, and multiple bone cysts, a possible link of double mutations between *PAX6* and *ATP2A2* could contribute in part to calcium homeostasis through bone resorption in the skeletal phenotype (30).

Balancing the molecular mechanisms for the activation of osteoclastogenesis are a number of inhibitory mechanisms required to maintain osteoclast number and bone resorptive activity at a level appropriate for bone repair and calcium homeostasis. Osteoprotegerin, which was initially identified as a novel secreted member of the tumor necrosis factor (TNF) receptor family and acts as a decoy receptor for RANKL function, thus attenuates osteoclast differentiation and function (31). In addition, IFN-γ can interfere with RANKL-mediated osteoclast differentiation, and this mechanism is critical for the suppression of pathological bone resorption associated with inflammation (32). Furthermore, RANKL induces the *IFN-β* gene in osteoclast precursor cells, and this induction constitutes a critical aspect of the negative feedback regulation of RANKL signaling that allows suppression of excessive osteoclastogenesis (33). Inhibitors of differentiation/DNA binding (Id) are repressors, which can also modulate RANKL-mediated osteoclastogenesis by inhibiting MITF binding to the promoter of *OSCAR* (34). In osteoclast precursors, the protein inhibitor of activated STAT3 (PIAS3) attenuates the transcriptional activity of MITF and the expression of the *NFATc1* gene as a repressor (35). Overexpression of PIAS3, however, cannot completely block TRAP-positive osteoclast differentiation, suggesting a possible involvement of another modulator(s) in the process of suppressing osteoclast differentiation.

In this study, we report that the expression of Pax6 is selectively increased in osteoclasts upon exposure of murine BMM cells to RANKL. Retroviral introduction and reporter assays show that Pax6 attenuates primary osteoclast differentiation and promoter activity of the NFATc1-mediated activation of *TRAP* gene by binding to the *TRAP* gene enhancer in cooperation with a co-repressor, Grg6. These results demonstrate that Pax6 may be a negative regulator in RANKL-induced osteoclastogenesis and indicate a potential target for the control of pathological bone resorption, such as osteoporosis and rheumatoid arthritis.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant soluble RANKL was purchased from PeproTech EC Ltd. (London, UK). Recombinant human M-CSF was kindly provided by Morinaga Milk Industry. Co., Ltd. (Tokyo, Japan). Monoclonal antibody against FLAG (M2) was purchased from Sigma. Polyclonal antibodies against Pax6 were purchased from Chemicon International, Inc. (Temecula, CA). Anti-nuclear factor of activated T cells c1 (NFATc1) monoclonal antibody was purchased from BD Biosciences. Polyclonal antibody against Grg6 was a kind gift from Thomas A. Look (Harvard University) (36). Polyclonal antibody against p38 MAPK as a negative control was purchased from Cell Signaling Technology, Inc. (Beverly, MA). Reverse transcription-polymerase chain reaction (RT-PCR) kits were obtained from Invitrogen.

Cell Culture—BMM cells were prepared as described previously (18). Briefly, femurs and tibias were removed from 12- to 16-week-old C5B/B6N mice, and cells were flushed out of the bone marrow cavity with α-modified minimum Eagle's medium (Sigma) containing 10% fetal bovine serum (FBS; Sigma) and 50 μg/ml penicillin/streptomycin from Nacalai Tesque Co., Ltd. (Kyoto, Japan). After lysing erythrocytes in lysing buffer (17 mM Tris, pH 7.65, 0.75% NH_4Cl), cells were seeded at 1.5×10^6 cells/well (0.5 ml) in 24-well plates in the presence of M-CSF (40 ng/ml). After a 3-day incubation, nonadherent cells were removed from the culture by pipetting and washing with phosphate-buffered saline (PBS). Adherent cells were further incubated in the presence of M-CSF (40 ng/ml) and soluble RANKL (100 ng/ml) for 5–7 days. The culture medium was replaced every 3 days with fresh complete medium. Then cell extracted mRNA and protein were subjected to RT-PCR and immunoblot analysis, respectively. The

HEK293 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% FBS (INC Biomedical, Inc., OH) and 50 μg/ml penicillin/streptomycin.

Preparation of RNA and Quantitative RT-PCR (qRT-PCR)—Total RNA for cDNA synthesis was isolated from murine bone marrow cells and cultured cells as described previously (18). RNA was reverse-transcribed using Superscript reverse transcriptase, 1 mM dNTPs, 500 ng of oligonucleotide (dT) primers (Invitrogen) or ReverTra Ace® quantitative PCR master mix (Toyobo Co., Ltd.). qRT-PCR assays were carried out using the following primer pairs; *Pax1*, 5′-GGCATCCGGACGTTTATGGAG-3′ (sense) and 5′-GAGGCTGGAAGCCGACTGAG-3′ (antisense); *Pax2*, 5′-CATCAAATCAGAACAGGGGAATG-3′ (sense) and 5′-AACCAGGTAGAGTGGTGCTCGTC-3′ (antisense); *Pax3*, 5′-ACACTGTGCCCTCAGTGAGTTC-3′ (sense) and 5′-CTCTTCAGCGGTAAATCAGGTTC-3′ (antisense); *Pax4*, 5′-GCTTTGTACCCAGGACAAGGC-3′ (sense) and 5′-ACGCTGAAACTCTTTCTCCAGTG-3′ (antisense); *Pax5*, 5′-AGCTTCCAGTCACAGCATAGTGTC-3′ (sense) and 5′-CCAGCTGCTGCTGTGTGAAC-3′ (antisense); *Pax6*, 5′-CAGTCACAGCGGAGTGAATC-3′ (sense) and 5′-CGCTTCAGCTGAAGTCGCAT-3′ (antisense); *Pax7*, 5′-GGCACAGAGGGACCAAGCTC-3′ (sense) and 5′-GCAGCCGGTTACTGAAC-3′ (antisense); *Pax8*, 5′-GCAGCTATGCCTCTTCTGCTA-3′ (sense) and 5′-GCTGTAGGCATTGCCAGAAT-3′ (antisense); *Pax9*, 5′-AGCAGGAAGCCAAGTACGG-3′ (sense) and 5′-TGGATGCTGAGACGAAACTG-3′ (antisense); *Grg1*, 5′-CATCCCATTTCTGTCTCAGGAAC-3′ (sense) and 5′-CACTAGACAGCGCGAGAAGG-3′ (antisense); *Grg2*, 5′-TCCCCAGAAGCAAAGACATAGC-3′ (sense) and 5′-GACAAGGTCAGGGGACTCCTC-3′ (antisense); *Grg3*, 5′-AAGGATGAGAAGAACCACCATGAAC-3′ (sense) and 5′-TCTTGTCCCCATCGCTATCG-3′ (antisense); *Grg4*, 5′-CTTTCCTGTCCCAAGAGCACC-3′ (sense) and 5′-ATGTCCATGTGATAAATGCTGAGC-3′ (antisense); *Grg6*, 5′-AGGAGTTCAGCAGCGTCGTC-3′ (sense) and 5′-AGACACATGACATGGCCAACTG-3′ (antisense). qRT-PCR was performed using Light Cycler 480 (Roche Applied Science). *Pax6* mRNA levels were normalized against those of *β-actin* mRNA (37).

Western Blot Analysis—Immunoblot analyses and immunoprecipitations were performed as described (18). In brief, cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM PMSF). Whole cell extracts (WCEs) were prepared by centrifugation at 10,000 × *g* for 15 min at 4 °C after sonicating cells four times for 5 s. WCEs (30 μg) were electrophoresed on a 9 or 12% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane. Immunoblot detection was performed with the corresponding antibodies using an ECL detection kit (Amersham Biosciences).

Plasmid Construction—The pcDNA3 *NFATc1* plasmid was kindly provided by Dr. M. A. Brown (Emory University School of Medicine). pRC-CMV *Pax6* and pBat12-*Pax4* expression plasmids were kindly provided by Dr. Yi-Hong Zhou (University of Arkansas) and Dr. Michael German (University of California, San Francisco), respectively. The MITF expression plasmid, pcDNA FLAG-*Mitf*, was constructed as described previously (16). pMX-puro vector and pMX-*GFP* vector were kindly provided by Dr. Kitamura (University of Tokyo). pRC-CMV *Pax6* was digested with HindIII and XbaI and cloned into pBluescript KS(−) vector, and following digestion with XbaI and HindIIIa, it was cloned into the pMX-puro vector. pMX-*Grg6* was created by amplification of *Grg6* by PCR and inserted into the pMX vector. pBrit-*Pax3* and *Pax7* were kindly provided by Drs. Zhengou Wu (Hong Kong University of Science and Technology) and Michael A. Rundnicki (University of Ottawa). The deletion mutant of Pax6 lacking the C-terminal TA domain (Δ344) was amplified by PCR using *Pfu* Turbo DNA polymerase (Stratagene, CA) and was cloned into the pRC-CMV vector. A genomic clone encoding the 5′-flanking region of the *TRAP* gene promoter TBX6 was a kind gift from Dr. G. D. Roodman (University of Texas, Health Science Center). The fragments of the region from −1492 to +1 of the *TRAP* gene promoter was amplified by PCR and cloned into the pGL3 basic vector containing the luciferase reporter gene as the p-1492-TRAP Luc (Promega, Madison, WI) as described previously (16).

Transfection and Luciferase Assay—For transfection of reporter plasmids, HEK293 cells were plated into 24-well plates at a density of 2.5×10^5 cells/well the day before transfection. A total of 3 μg of plasmid DNA was mixed with $CaCl_2$ and HEPES-buffered saline and transfected into the cells. After 48 h of transfection, the cells were washed three times with PBS and then lysed in reporter lysis buffer (Promega; Madison, WI). The luciferase activity was then measured with a luciferase assay system (Promega) according to the manufacturer's instructions. The luciferase activity was measured in triplicate, averaged, and then normalized against the β-galactosidase or *Renilla* luciferase (derived from pRL-TK vector) activity to correct for transfection efficiency. The β-galactosidase activity was measured using *o*-nitrophenyl-β-D-galactopyranoside as a substrate. Mutagenesis of PA1 and PA2 sites of the p-1294TRAP-Luc plasmid was carried out using PrimeSATAR MAX and DpnI (TAKARA Shuzo Co., Ltd.). The oligonucleotides (and their complementary strands) used for making mutations in the PA1 and PA2 sites are 5′-CTTCGAGAAACTTTTCCCTACACAGTCTGGCCTGGGTGC-3′ and 5′-GAGTTTATAGGAATGCTCGAGGAGACCAGGCTCAGCG-3′, respectively.

Immunofluorescence Staining—Authentic osteoclasts (OCLs) on coverslips were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature and then washed in PBS. The coverslips were permeabilized in 0.1% Triton X-100 for 5 min, immersed in 5% normal goat serum for 30 min, incubated in the primary antibodies against NFATc1 or Pax6 for 1 h, washed in PBS, incubated in the appropriate fluorescent-conjugated secondary antibodies, and washed. OCLs were subjected to fluorescent microscopic analysis (Axioplan2 Imaging MOT; Zeiss and BIOREVO BZ-9000; KEYENCE).

Electrophoretic Mobility Shift Analysis (EMSA)—Nuclear extracts were prepared from BMM and OCL based on the standard protocol as described previously (38). Oligonucleotide probes were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. DNA binding assays were performed by incubating 30 μg of nuclear extracts or WCEs and ^{32}P-labeled double-stranded oligonucleotides for 30 min at 30 °C. For the competition assay, a

10-fold excess of unlabeled double-stranded oligonucleotide was added in the reaction mixture. The samples were then resolved on a 4% polyacrylamide gel run in TBE buffer.

ChIP Assay—The ChIP assay was carried out using the agarose ChIP kit (Thermo Scientific, Rockford, IL). Briefly, mouse BMM and OCL were treated with 1% formaldehyde for 10 min. The cross-linked chromatin was prepared following the manufacturer's instructions prior to immunoprecipitation with Pax6 antibody (Millipore, Temecula, CA), RNA polymerase II antibody, or normal rabbit IgG antibody (Thermo Scientific) at 4 °C 2 h. After reversal of cross-linking, genomic DNA associated with the immunoprecipitated chromatin was used as a template for amplification by qRT-PCR. For PCR amplification of specific regions of the prospective *Pax6* genomic locus, the following primer sets were used: a region containing the Pax6-binding PA1 site, sense, CATCAGACCCTGGCTGACTG, and antisense, GACTTCCCCAGAACTGGAAAT; a region containing PA2 site, sense, AGGCTGGCCTTGAACTTCTG, and antisense, CCTGGTCCAAGCAAAGAAAGAC.

Retroviral Infection—To generate retrovirus stock, retroviral vectors (pMX, pMX-*Pax6*, pMX-*Grg6*, and pBrit-*Pax7*) were transfected into the packaging cell line Plat-E (provided kindly by Dr. Kitamura) using $CaCl_2$. Supernatant was collected from the virus culture media 48 h after transfection and filtered through a 0.45-μm filter. BMM cells were incubated with virus supernatant for 12 h in the presence of Polybrene (8 μl/ml). After removing the virus supernatant, BMM cells were incubated in the presence of M-CSF (40 ng/ml) and RANKL (100 ng/ml) for 5 or 7 days.

TRAP Assay—Osteoclast formation was measured by quantifying TRAP-positive cells, as described (34). In brief, adherent cells were fixed with 10% formaldehyde in PBS for 3 min. After the well surface was air-dried, fixed cells were incubated at room temperature in an acetate buffer (0.1 M sodium acetate, pH 5.0) containing 0.01% naphthol AS-MX phosphate (Sigma) in the presence of 50 mM sodium tartrate. Osteoclast-like TRAP-positive cells in each well were scored by counting the number of TRAP-positive multinucleated cells containing three or more nuclei.

RESULTS

Pax6 Expression during RANKL-induced Osteoclastogenesis—The Pax family has been implicated in a critical role for neural development and includes the gene networks through the mutual transcriptional regulation (21–24). It has been reported that Pax5-deficient mice exhibit osteopenia (25), a pathological condition in which osteoclasts are likely to be involved (39). Although Pax5 was detected only in the B cell lineage, and not in bone marrow macrophages nor in osteoclasts (40), the report nonetheless suggests that Pax5 or its related Pax family members may play a role in bone metabolism. In fact, the findings of a mutation in PAX6 in a human case with Darier disease, aniridia, and bone cysts implicate a possible role for PAX6 in bone remodeling with ATPA2 (30). As a first step toward exploring roles for the Pax family genes (*Pax1* to *Pax9*) during RANKL-induced osteoclast differentiation, we investigated their mRNA expression by qRT-PCR (*Pax1* to *Pax9*) analysis. Murine BMM cells were allowed to differentiate into mononuclear or multinucleated TRAP-positive osteoclasts in the presence of RANKL and M-CSF for 5 days, and total RNA was isolated for quantitative PCR analysis. As shown in Fig. 1*A*, among the nine Pax family members, only *Pax6* and *Pax7* mRNAs were selectively expressed in BMM at low levels, and then Pax6, but not Pax7, increased its expression by RANKL treatment, suggesting a role for Pax6 in osteoclastogenesis. This prompted us to conduct a time course analysis of the mRNA expression level during osteoclastogenesis. Upon RANKL stimulation of BMM, the expression of *Pax6* mRNA peaked at day 5 (~3-fold increase) and then returned to the original level (Fig. 1*C*). Similarly, the level of Pax6 protein expression increased slightly following RANKL treatment (Fig. 1, *A*, *upper left panel,* and *D*). Although a master regulator of osteoclasts, NFATc1, was undetectable at day 0, it was robustly up-regulated on day 2 until day 5 in response to RANKL (Fig. 1*E*). These results indicate that expression of Pax6 and NFATc1 is regulated by RANKL, and its efficacy for triggering expression of both factors changes during osteoclastogenesis.

Direct Binding of Pax6 to the PA1 and PA2 Sites of the TRAP Gene Promoter—The *TRAP* gene is a well known marker of osteoclast formation. Its expression is regulated during osteoclastogenesis by a number of transcription factors, including NFAT, PU.1, and MITF, whose binding sites are present in the upstream region (from −1492 to −1) of the *TRAP* gene (Fig. 2*A*) (16, 41). To determine whether Pax6 can potentially regulate *TRAP* gene expression, we first searched for potential Pax6-binding sites from −1492 to −1 of the *TRAP* gene and found two DNA sequences that closely match the consensus sequence of Pax6 (5′-ANNTTCACGCWTSANTKMNY-3′) (6) and (5′-RNGMANTSAWGCGKRMM-3′). These two sites are located from −637 to −618 (5′-TTCACGGCTCAGCTCG-3′) and from −117 to −101 (5′-AGGCATGCACCGTGAG-3′) in the upstream region of the *TRAP* gene, which we refer to hereafter as PA1 and PA2, respectively (Fig. 2*A*).

To further investigate whether endogenous Pax6 in BMM and OCL could bind to these probes, we used whole cell extracts derived from BMM treated with M-CSF for 3 days or from cells treated for an additional 1, 3, 5, and 7 days with RANKL and M-CSF. As shown in Fig. 2*B*, specific binding of Pax6 to the PA1 probe was detected at day 3–7 but not at day 0 and 1 (Fig. 2*B*, *white arrowhead*). Binding to the PA2 site was difficult to confirm, presumably because the sequence of PA2 is less similar to the consensus Pax6 site than that of PA1. The binding to the PA1 probe was specific because the complex was competed out by a 10-fold excess of the PA1-containing oligonucleotide but not by the oligonucleotide containing a mutated Pax6-binding PA1 sequence (Fig. 2*C*, *lanes 2–4*). Moreover, an antibody against Pax6, but not a control IgG, significantly reduced the DNA binding activity (Fig. 2*C*, *lanes 5* and *6*), indicating that the complex contained Pax6 (Fig. 2*C, lanes 5* and *6*). These data show that Pax6 binds to the PA1 site in the upstream region of the *TRAP* promoter, suggesting a role for Pax6 in regulating *TRAP* gene transcription. Moreover, these results prompted us to determine whether endogenous Pax6 specifically binds to the *TRAP* gene promoter in osteoclasts. To address this, a ChIP assay was carried out using specific primer sets to amplify either the PA1 or PA2 sites in the proximal

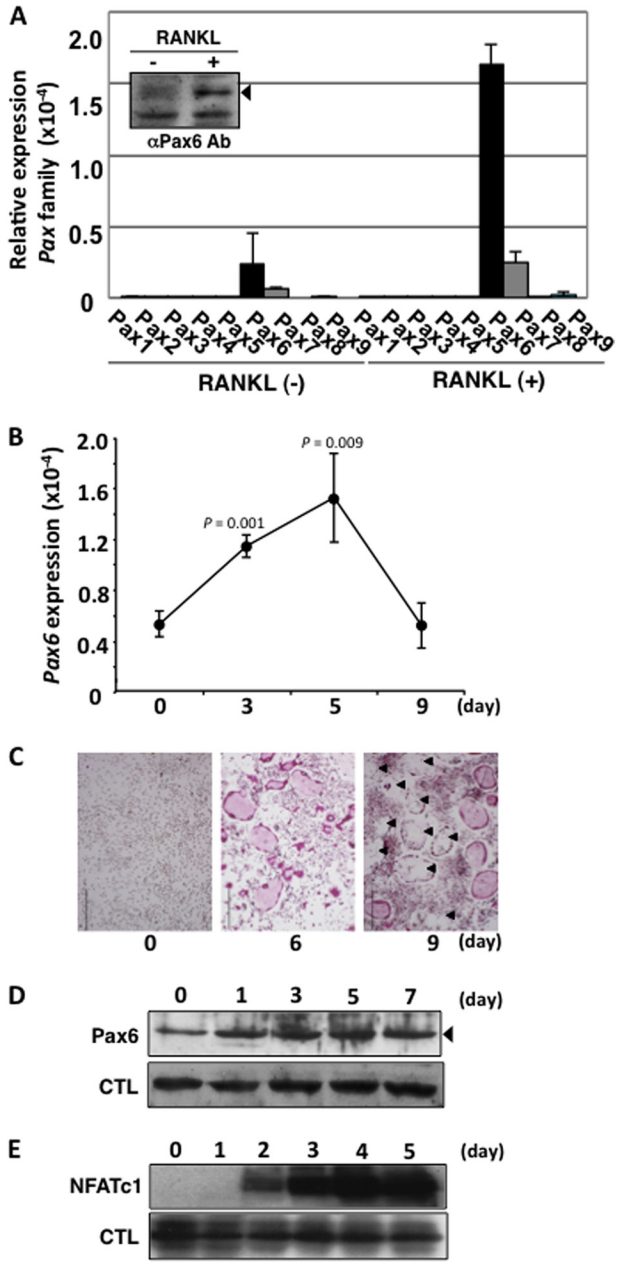

FIGURE 1. **Pax6 expression in BMM and OCL.** *A,* mouse BMM were incubated for 5 days in the presence of M-CSF (40 ng/ml) and RANKL (100 ng/ml). After incubation, total RNA was extracted, and the expression of *Pax1/2/3/4/5/6/7/8/9* genes was examined by qRT-PCR. As a control, expression of the *β-actin* gene was also examined for normalization. *Left upper panel* represents immunoblot analysis of WCE prepared from BMM and OCL using anti-Pax6 antibody. The *arrowhead* indicates Pax6. *B,* BMM were treated with RANKL (100 ng/ml) for 7 days. Total RNA was extracted every other day, and the expression of Pax6 and *TRAP* gene was examined by quantitative RT-PCR using primer sets for Pax1 to Pax6. Data shown are means of triplicate reactions ± S.D. normalized against the expression of *β-actin* mRNA. *C,* BMM were treated with RANKL (100 ng/ml) for 6 and 9 days, and then TRAP staining was performed. *Arrowheads* represent traces of apoptotic OCL that are distinct from TRAP-positive live OCL stained *red*. *D,* WCE from BMM and OCL, prepared every other day, were subjected to immunoblot analysis using a specific antibody against Pax6. Each lane used the same quantity of WCE indicated by similar intensities using a control (*CTL*) antibody. *E,* immunoblot analysis with a specific antibody against NFATc1 in BMM and osteoclasts in response to RANKL.

region of the *TRAP* gene promoter from intrinsic genomic DNA-bound Pax6 or RNA polymerase II immunoprecipitated with each specific antibody. The results show that regions associated with Pax6-bound PA1 and PA2 sites were remarkably enriched in osteoclasts relative to BMM (Fig. 2*D*). By comparison, and demonstrating the specificity of these results, the amount of PA1 and PA2 sites bound to RNA polymerase II was decreased in osteoclasts relative to BMM (Fig. 2*D*). Thus, a critical implication of these results is that endogenous Pax6, specifically recruited to the proximal regions of the intrinsic *TRAP* gene locus, could control RNA polymerase II-dependent transcriptional machinery in the nucleosome during osteoclast differentiation.

Pax6 Suppresses TRAP Gene Enhancer Activity—Pax6 has been shown to play important roles during eye formation, brain morphogenesis, and α-cell development in the pancreas, largely by acting as a transcriptional activator (20, 26, 42–44). To determine the functional role of Pax6 in *TRAP* gene regulation, we performed a reporter gene assay. As described previously (16), we created a reporter plasmid, p-1492-TRAP Luc, harboring the luciferase gene fused to the *TRAP* gene promoter region, and we then transfected it into HEK293 cells in combination with NFATc1 and Pax6 expression vectors. Consistent with the previous report (16), NFATc1 that functions as a master regulator induced by RANKL increased the luciferase activity of p-1492-TRAP Luc introduced into HEK293 cells by ~10-fold (Fig. 3*A*). However, when *Pax6* was co-transfected into the cells, the level of NFATc1-mediated *TRAP* gene expression decreased in a Pax6-dependent manner (Fig. 3*A*), indicating that Pax6 does not activate but attenuates the expression of the *TRAP* gene by binding to its upstream region.

As reported previously (45), Pax6 has a paired domain at the N terminus and a paired-like homeodomain in the middle, both of which are required for DNA binding. The C-terminal domain (TA), rich in proline, serine, and threonine, is essential for transcriptional activation by Pax6 (Fig. 3*B*). To further address the negative role of Pax6 in *TRAP* gene expression, we tested the deletion mutant of Pax6, termed as Δ344, that lacks the C-terminal TA domain responsible for the transcriptional activity (Fig. 3*B*). Although wild type Pax6 and Δ344 were expressed at similar levels (Fig. 3*C*), Δ344 showed a much reduced ability to attenuate transcription of the *TRAP* gene as compared with its wild type counterpart (Fig. 3*D*), suggesting that the TA domain of Pax6 is necessary for repressing NFATc1-mediated *TRAP* gene expression. Next, to investigate the specificity of Pax6 on the attenuation of the *TRAP* gene promoter, we tested the potential involvement of the additional Pax family members Pax3, Pax4, and Pax7. Among these, Pax6 was uniquely able to significantly attenuate NFATc1-mediated *TRAP* gene activation (Fig. 3*E*). To test if the PA1 and PA2 sites are critical for the functional specificity of Pax6, we generated mutant versions of the *TRAP-Luc* reporter construct in which these sites are mutated (Fig. 2*A*). We repeated the luciferase assay with these mutant constructs, and the results indicate that mutation of the PA1 site disrupts Pax6-dependent attenuation of NFATc1-mediated luciferase activity although mutation of the PA2 site had no effect (Fig. 3*F*). These data suggest that Pax6 regulates *TRAP* gene expression predominantly via the PA1 site rather than PA2. Consistent with the antagonistic roles of Pax6 and NFATc1 in regulating *TRAP* gene expression, immunofluorescence analyses showed that Pax6 and NFATc1 are co-local-

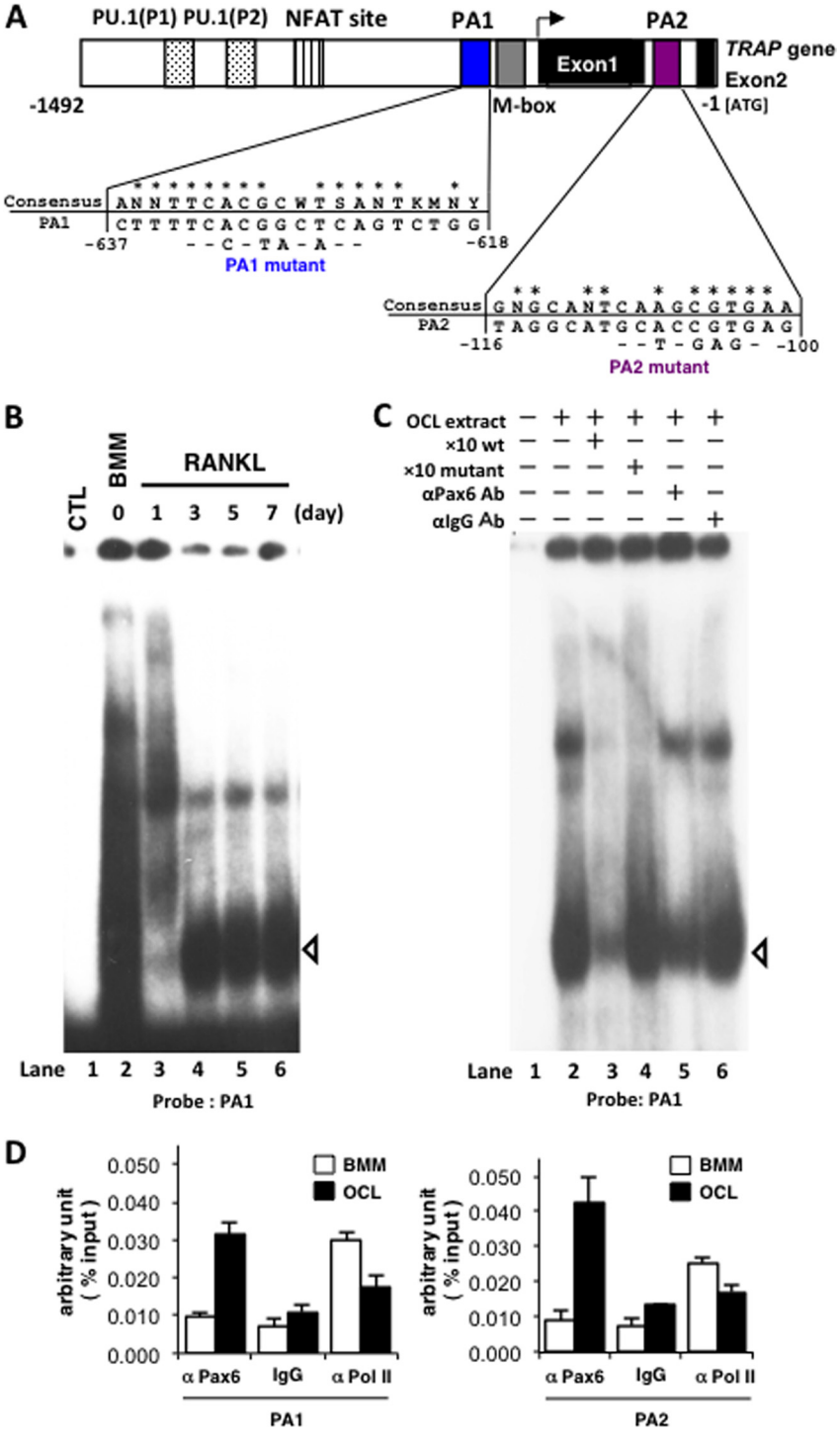

FIGURE 2. **Specific binding of Pax6 to the promoter element of the *TRAP* gene.** *A,* schematic representation of the putative Pax6-binding sites (PA1 and PA2) from the mouse *TRAP* gene promoter. *N*, NFATc1-binding site; *P1*, *P2*, PU.1-binding sites; *M-box*, MITF-binding site. *Asterisks* indicate where the PA1 and PA2 sequence matches the consensus sequence. *B,* cell extracts of BMM and osteoclasts, which were prepared by treatment with RANKL, were subjected to EMSA using probes representing the Pax6-binding site (*PA1*) from the mouse *TRAP* gene. The *open arrowhead* indicates specific DNA binding activities detected along with osteoclastogenesis (*lanes 4–6*). Control (*CTL*) shows negative control in the absence of cell extracts. *C,* WCE of osteoclasts prepared by treatment with RANKL was subjected to EMSA by using a probe representing the Pax6-binding site PA1. For competition analysis, a 10-fold excess of unlabeled wild type (*lane 3*) or mutant (*lane 4*) Pax6 site oligonucleotide was used as described under "Experimental Procedures." Cell extracts were mixed with anti-Pax6 Ab (*lane 5*) or anti-IgG Ab (*lane 6*) prior to loading samples into the gel. The *open arrowhead* indicates specific DNA binding activities of Pax6 (*lanes 2, 4,* and *6*) and a significantly reduced DNA binding activity by an antibody against Pax6 (*lane 5*). *D,* chromatin immunoprecipitation of endogenous Pax6 binding the PA1 and PA2 sites of the *TRAP* promoter. Intrinsic genomic DNA fragments purified from BMM and OCL with antibodies targeting Pax6 or RNA polymerase II (*Pol II*) were amplified by qRT-PCR using specific primer sets designed for the amplification of the PA1 and PA2 regions, respectively. Normal rabbit IgG antibody was also included as a negative control for the immunoprecipitation. Data shown are means of triplicate reactions ± S.D. normalized against the input genomic DNA amplification.

ized in the nuclei of RANKL-induced primary osteoclasts (Fig. 3*G*). Taken together, these results indicate that Pax6 counteracts with NFATc1 and attenuates *TRAP* gene expression in osteoclasts, probably through its C-terminal region.

Pax6 Cooperates with Co-repressor Grg6 to Suppress the Promoter Activity of TRAP—In addition to their roles as a transcriptional activator, recent studies show that the Pax transcription factors function as a transcriptional repressor as well. Their repressive activity is mediated by Groucho/transducin-like enhancer of split (Gro/TLE) family of transcriptional co-repressors, for example, a transcriptional co-repressor of the family, Grg4, is recruited by Pax5 or Pax3 to suppress B cell-specific genes (46) or the *dopachrome tautomerase* gene (47), respectively. In addition, Pax2 is shown to recruit Groucho co-repressors (48). These reports prompted us to investigate whether Groucho proteins could interact with Pax6 as co-repressors during osteoclastogenesis. First, we used qRT-PCR to analyze the expression of Groucho (Grg)/TLE family members in BMM and OCLs and found that some of them are indeed expressed during osteoclast differentiation. Among the Grg family members, we found that only *Grg6* mRNA was significantly increased during RANKL-induced osteoclast differentiation (Fig. 4, *A* and *B*), in a manner similar to that of Pax6 (Fig. 1*B*). Conversely, the expression levels of other Grg family members, Grg2 and Grg3, were decreased after stimulation with RANKL. The levels of *Grg1* and *Grg4* mRNA were largely indistinguishable between BMM and osteoclasts derived from RANKL-treated BMM (Fig. 4*A*).

Next, to determine the functional relationship among NFATc1, Pax6, and/or Grg in the induction of the *TRAP* gene, we performed a reporter gene assay by using p-1492-TRAP Luc. We chose to use Grg6 because it was expressed more highly in developing osteoclasts and because it has been reported that Grg4 does not potentiate attenuation in combination with Pax6 (49). As shown in Fig. 4*C*, co-transfection with Grg6 caused a 40% reduction in the *TRAP* gene activity stimulated by NFATc1 as compared with a 60% reduction by Pax6. Furthermore, introduction of Grg6 appeared to show an additive effect with Pax6 because simultaneous introduction of Pax6 and Grg6 decreased NFATc1-mediated induction of the *TRAP* gene by up to 85%, indicating that both Pax6 and Grg6 contribute to attenuate NFATc1-mediated transcription of the *TRAP* gene. In contrast, no such remarkable attenuation was observed when Grg6 was overexpressed in combination with Pax3, -4, or -7. It is interesting to note that Grg6 attenuated transcription in the absence of Pax6, although Grg6 has no DNA-binding motif and cannot bind DNA elements by itself. Although the attenuation could be due to the recruitment of Grg6 by the endogenous Pax6 to the *TRAP* gene promoter, it is more likely that Grg6 is recruited to the *TRAP* gene by NFATc1 as well. Moreover, similar effects by Pax6-Grg6 were also observed on the attenuation of the cathepsin K gene promoter activated by NFATc1 (Fig. 4*D*). The evidence that the expression of the *TRAP* gene is induced not only by NFATc1 but by MITF and PU.1 (15, 16, 18) led us to test whether Pax6 can attenuate the promoter activity of the *TRAP* gene activated by MITF and PU.1. However, no attenuation of the MITF-PU.1-mediated luciferase activity by Pax6 or Pax7 was observed (Fig. 4*E*). These results suggest that Pax6 specifically modulates gene expression of osteoclast markers induced by master regulators, including NFATc1 but not MITF and PU.1.

Grg6 Binds to Pax6 and NFATc1 Transcription Factors—Grg proteins lack DNA binding activity of their own but are recruited by a number of different DNA-binding transcription factors (50). Consistent with this finding, the Pax family members have been reported to bind Grg proteins to cooperatively displace a complex of other stimulating factors and result in attenuation of transcription (47). To determine how these factors interact with each other, expression plasmids encoding NFATc1, Pax6, and Grg6 were transfected into HEK293 cells in various combinations. We found that immunoprecipitates with anti-NFATc1 antibody contained detectable Grg6 protein (Fig. 5, *A* and *B, middle panel*), and interaction of Grg6 with Pax6 was clearly evident (Fig. 5*B*, *upper panel*). In contrast, direct association of Pax6 with NFATc1 was undetectable (Fig. 5*B*, *bottom panel*), suggesting that Pax6 interacts with NFATc1 indirectly by utilizing the transcriptional co-repressor Grg6 as an intermediate. Taken together, these results suggest that Grg6 plays a suppressive role by binding the master regulator NFATc1 and by inhibiting its positive function as a regulator of osteoclastogenesis.

Pax6 Attenuates Osteoclast Differentiation—To further investigate the importance of Pax6 expression by osteoclast precursors for osteoclast differentiation, we introduced Pax6 retrovirally into murine BMM prior to induction of osteoclast differentiation with RANKL treatment. As shown in Fig. 6*A*, TRAP-positive multinucleated cell formation was markedly attenuated by overexpression of Pax6, and the size of the resulting cells was much smaller than that of control osteoclasts. Statistical analysis showed that TRAP-positive osteoclast differentiation decreased significantly by about 50% compared with that of the control (Fig. 6*B*). Moreover, the endogenous expression of *TRAP* and cathepsin K in osteoclasts was remarkably attenuated when either Pax6 or Grg6 was introduced into these cells, and this inhibitory effect was enhanced when Pax6 and Grg6 were overexpressed together. Importantly, the effect on the regulation of osteoclast marker genes observed by combining Pax6 with Grg6 cannot be reproduced by combining Pax7 and Grg6 (Fig. 6*C*). These results suggest strongly that Pax6 functions together with Grg6 as a specific negative regulator of RANKL-induced osteoclast differentiation.

DISCUSSION

The *TRAP* gene, along with cathepsin K, calcitonin receptor, and *OSCAR*, is a well known differentiation marker of osteoclasts. Mice lacking the *TRAP* gene, for instance, have defective bone resorption and mild osteopetrosis, underscoring its essential role in osteoclast function (51). Consequently, studies on its transcriptional regulation have provided insights into not only its gene regulation but also osteoclastogenesis in general. Based on our continued efforts as well as by others, it has been shown that the upstream regulatory elements of the *TRAP* gene consist of several transcription factor-binding sites, including NFATc1, PU.1, MITF, and Pip, which are functionally involved in the regulation of RANKL-induced *TRAP* gene expression and promote osteoclast differentiation and also regulate their

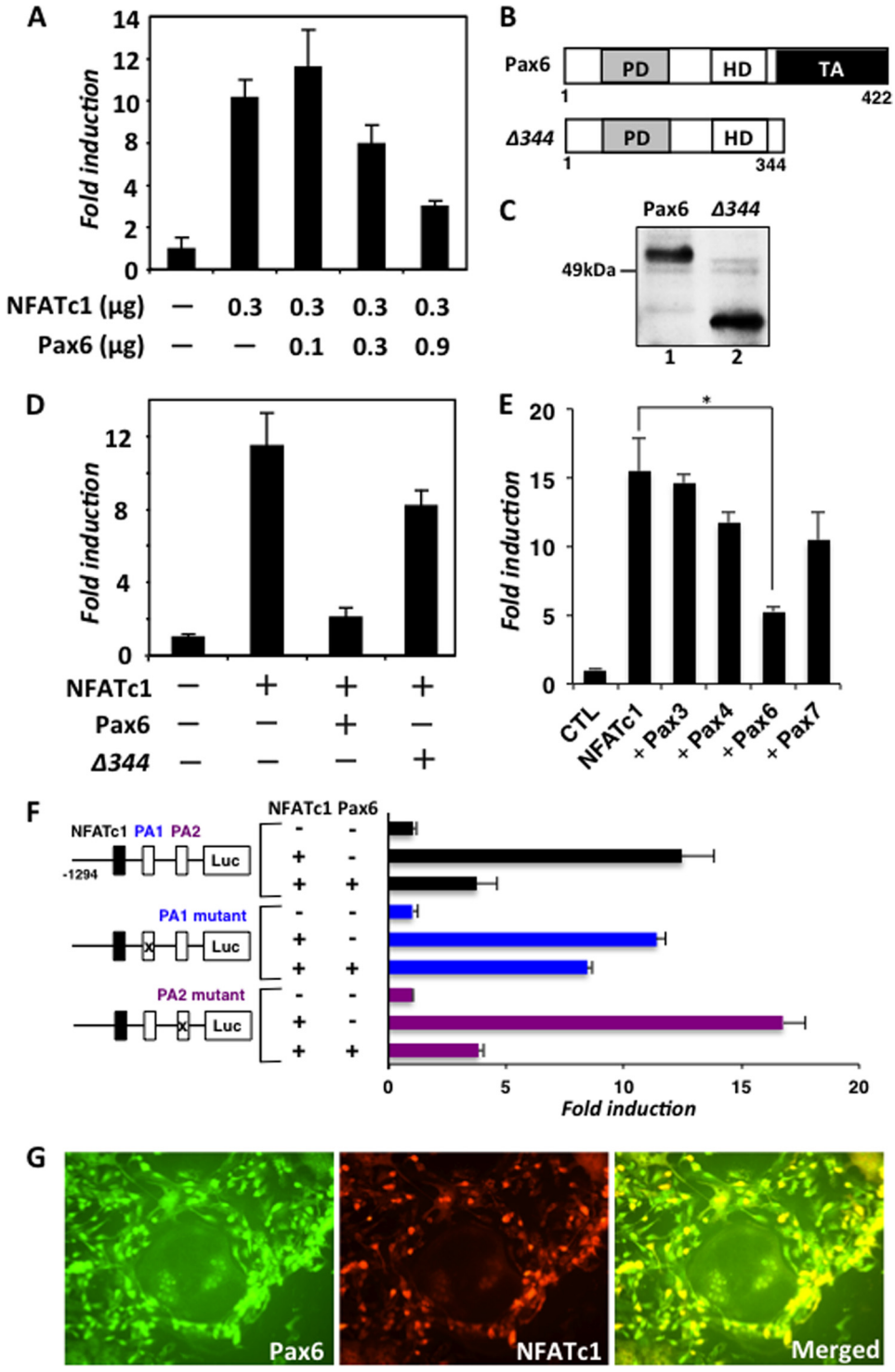

FIGURE 3. **Attenuation of promoter activity of *TRAP* gene by Pax6.** *A,* HEK293 cells were co-transfected with 1.5 μg of TRAP-Luc and expression vectors encoding NFATc1 (0.3 μg) and Pax6 (0.1, 0.3, and 0.9 μg), as indicated. After 48 h, the luciferase activity was measured as described and normalized against the β-galactosidase activity of a co-transfected internal control plasmid. *B,* schematic diagrams of the full-length human Pax6 (1–422 amino acids) and mutant Pax6 (1–344 amino acids; referred to as Δ344) that lacks the proline/serine/threonine-rich transactivator (*TA*) domain. The *numbers below* the diagram refer to the amino acid numbers. *PD,* paired domain; *HD,* homeodomain. *C,* immunoblot analysis of wild type Pax6 and mutant Pax6 (Δ344) with a polyclonal antibody against Pax6. *D,* HEK293 cells were co-transfected with expression vectors encoding NFATc1, Pax6, or Pax6-Δ344, with or without an empty vector as a control. After 48 h, the luciferase activity was measured and normalized to the β-galactosidase activity of a co-transfected internal control plasmid. *E,* NFATc1-mediated *TRAP* promoter in HEK293 cells transfected with expression vectors encoding the Pax family members *Pax3*, *Pax4*, *Pax6,* and *Pax7* as indicated. *Asterisk* indicates statistically significance differences from NFATc1-mediated luciferase activity and specifically attenuated activity by Pax6 (*, $p < 0.05$). *CTL,* control. *F,* HEK293 cells were transfected with wild type, PA1 mutant, or PA2 mutant (Fig. 2*A*), p-1294TRAP-Luc vector, and expression vectors encoding *NFATc1* and Pax6 as indicated. *G,* immunocytochemistry of OCL with antibodies against Pax6 and NFATc1. After treatment of BMM with RANKL in the presence of M-CSF for 5 days, cells were fixed and then stained with either Pax6 or NFATc1 antibody, respectively, followed by anti-rabbit antibody conjugated with Alexa-488 or anti-mouse antibody conjugated with Cy3, respectively, for fluorescent microscopic analysis.

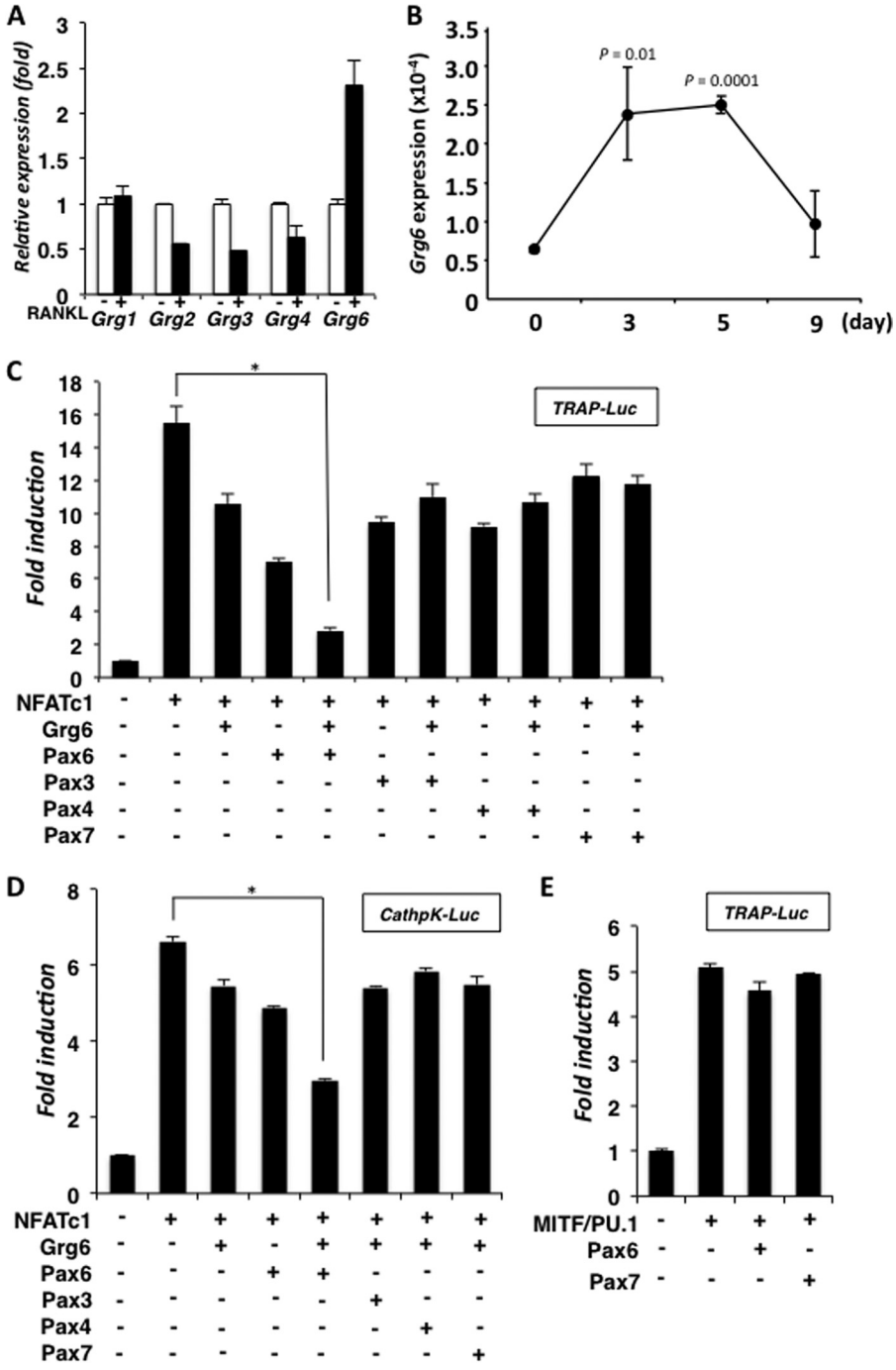

FIGURE 4. **Selective attenuation of the promoter activity of the *TRAP* gene by Pax6 together with Grg6.** *A,* BMM were incubated with M-CSF (40 ng/ml) for 3 days in the presence or absence of RANKL (100 ng/ml). The expression levels of the Groucho (Grg) related family, Grg1 to Grg6, were determined by qRT-PCR. *B,* time course expression of *Grg6* mRNA was examined by qRT-PCR as indicated at day 0, 3, 5, and 9 after treatment with RANKL and normalized against that of *β-actin*. *C,* HEK293 cells were co-transfected with 1.5 μg of TRAP-Luc plasmid and 0.3 μg of plasmid encoding either *NFATc1, Grg6, Pax6, Pax3, Pax4,* and *Pax7*. After 48 h, the luciferase activity was measured as described and normalized against the *Renilla* luciferase activity of a co-transfected pRL-TK internal control plasmid. Data shown are means of triplicate reactions ± S.E. The *asterisk* indicates statistically significant differences between the NFATc1-mediated luciferase activity and the activity following co-transfection of Pax6 and Grg6 (*, $p < 0.05$). *D,* representative luciferase activity driven by cathepsin K promoter (*CathpK-Luc*) regulated by Pax6 and Grg6. HEK293 cells were transfected with 1.5 μg of p-1108CathK-Luc plasmid and 0.3 μg of plasmid encoding either *NFATc1, Grg6, Pax6, Pax3, Pax4,* or *Pax7* with pRL-TK as an internal control plasmid. Data shown are means of triplicate reactions ± S.E. The *asterisk* indicates statistically significant difference between the NFATc1-mediated luciferase activity and the activity reduced by co-transfection with Pax6 and Grg6 (*, $p < 0.05$). *E,* Pax6 and Pax7 cannot attenuate the *TRAP* promoter activity by MITF and PU.1. HEK293 cells were transfected with 1.5 μg of *TRAP-Luc* plasmid and 0.3 μg of plasmid encoding *NFATc1, Grg6, Pax6, Pax3, Pax4,* and/or *Pax7* with pRL-TK as an internal control plasmid. Data shown are means of triplicate reactions ± S.E.

function (16, 17, 52). Although the molecular mechanisms for positive factors that promote osteoclastogenesis have been elucidated in considerable detail, the mechanisms for its negative regulation are poorly understood. In this study we show that the Pax6 is up-regulated in BMM-stimulated NFATc1-enhanced activity of the *TRAP* gene. To our knowledge, this study represents the first observation of the possible involvement of Pax6 in the attenuation of *TRAP* gene expression and osteoclast differentiation.

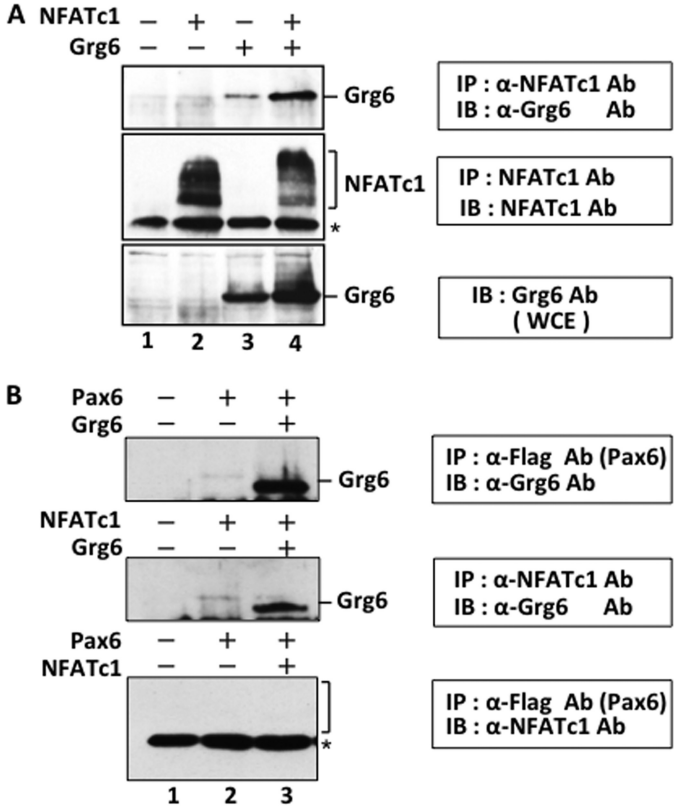

FIGURE 5. **Interaction of Pax6 with NFATc1 mediated by co-repressor Grg6.** *A,* WCE from HEK293 cells transfected with pcDNA3-Grg6 together with pcDNA3.1NFATc1 or empty vector were incubated with anti-NFATc1 antibody. Immunoprecipitates (*IP*) of NFATc1-bound complexes were analyzed by 9% SDS-PAGE prior to immunoblotting (*IB*) with anti-Grg6 antibody (*upper panel*) or anti-NFATc1 (*middle panel*). As a control, WCEs were subjected to immunoblot analysis with anti-Grg6 antibody (*bottom panel*). *B,* WCE from cells transfected with pcDNA3-Grg6 (3 μg), together with pRC-CMV-Pax6, were incubated with anti-FLAG (M2) or anti-NFATc1 antibody. Immunoprecipitates of Pax6- or NFATc1-bound complexes were analyzed by immunoblotting with anti-Grg6 antibody (*upper panel*) or anti-Grg6 antibody (*middle panel*). Immunoprecipitates of Pax6-bound complexes were subjected to immunoblot analysis with anti-NFATc1 antibody (*bottom panel*).

The *Pax* gene family belongs to a group of transcription factors implicated as regulators in pattern formation during embryogenesis and many organogeneses (20, 53). It has been shown that Pax6 acts as transcriptional activator critical for retinal and pancreatic endocrine cell development and the central nervous system (26, 42, 43). In addition to these roles, we found Pax6 is induced during osteoclast differentiation and attenuates transcription of the *TRAP* gene in RANKL-induced osteoclasts (Figs. 3, *A* and *D,* and 6). Similar to other Pax family members (47–49, 54–56), Pax6 recruits a member of the Groucho (Grg) co-repressor family, which allows Pax transcription factors to act as a repressor. This repressive function of Pax6 can be at least in part defined by the alterations of the transcriptional complex assembled with co-repressor Grg6 induced by RANKL stimulation (Fig. 4, *A* and *B*). Although the Grg family proteins do not bind DNA directly (57, 58), Grg6 binds Pax6 and serves as a key mediator between Pax6 and NFATc1, resulting in the cooperative attenuation for the gene induction of the *TRAP* gene by forming a repressor complex of Pax6-Grg6 (Figs. 4*C*, 5, *A* and *B,* and 6*C*), presumably stabilized by DNA binding of Pax6 to the PA1 site of the *TRAP* gene promoter (Fig. 2, *C* and *D*). In addition, the combinatorial action of Pax6-Grg6 together with MITF might define the cell fate specification and serve as bone remodeling based on evidence that Pax6 interacts with MITF (46).

The functional significance of Pax6-Grg6 during osteoclastogenesis remains to be determined and requires further studies. However, our results suggest that one role for Pax6 and Grg6 may be to attenuate excessive activity of RANKL-stimulated osteoclasts. First, the expression of both Pax6 and Grg6 was up-regulated through days 3–5 and then down-regulated on day 9 (Figs. 1*B* and 4, *A* and *B*). Second, RANKL-induced osteoclast differentiation proceeds unhindered even when Pax6 and Grg6 are induced as if their expression is part of obligate processes of osteoclastogenesis. Third, overexpression of Pax6 by retrovirus-mediated gene transfer reduces the formation of large multinucleated osteoclasts from BMMs, yet it allows formation of smaller osteoclasts. Fourth, the reduction of osteoclastogenesis by Pax6 is strikingly supported by the results of the maximal attenuation of the *TRAP* gene promoter activity by both Pax6 and Grg6 (Figs. 4*C* and 6*C*). Thus, Pax6 and Grg6 may be involved in reining in the activity of osteoclasts at an appropriate level, especially when osteoclast activities are heightened by RANKL. According to these observations and together with others, we propose a model for the Pax6-Grg6 modulatory complex controlling the NFATc1-mediated transcriptional activity of *TRAP* and cathepsin K genes at the stage of terminal differentiation of osteoclasts rather than that of earlier osteoclast precursors (Fig. 7).

Previous studies showed that *null* mutant mice deficient for Pax6 exhibit the absence of ocular structures and glucagon-producing α-cells in the pancreas, which results in perinatal lethality. There is, however, no report of bone abnormalities in these mice (26, 44), raising the question as to whether Pax6 plays a role in bone remodeling. However, a recent case report implicated a possible role for Pax6 in bone remodeling. A human disease with bone cyst and abnormalities of skeletal development, caused by increasing osteoclast and bone resorption, might be implicated with a possible link between bone cyst formation and *PAX6* mutation (30). Thus, it is possible that Pax6 mutant mice show only minor abnormalities in bones, which may escape detection because of their premature death. Alternatively, the loss of Pax6 may have been compensated by Pax7, which is also expressed in osteoclasts (Fig. 1*A*). In any case, it is interesting to test a conditional knock-out of Pax6 in osteoclasts, especially under pathological conditions such as bone fracture and inflammation, where appropriate response of osteoclasts is needed.

In addition to Pax6, previous mouse models suggested roles for other Pax transcription factors in bone metabolism. Pax5-deficient mice exhibit early onset osteopenia, with increased osteoclast precursors and delayed osteoblast maturation (40), and Pax5 can modulate bone resorption. Consistent with our results (Fig. 1*A*), however, no expression of Pax5 in osteoclasts and osteoblasts is detected, suggesting an indirect regulatory system (40). Given that bone metabolism is regulated not only by factors intrinsic to osteoblasts and osteoclasts but also by numerous extrinsic factors, the phenotypes observed in Pax5

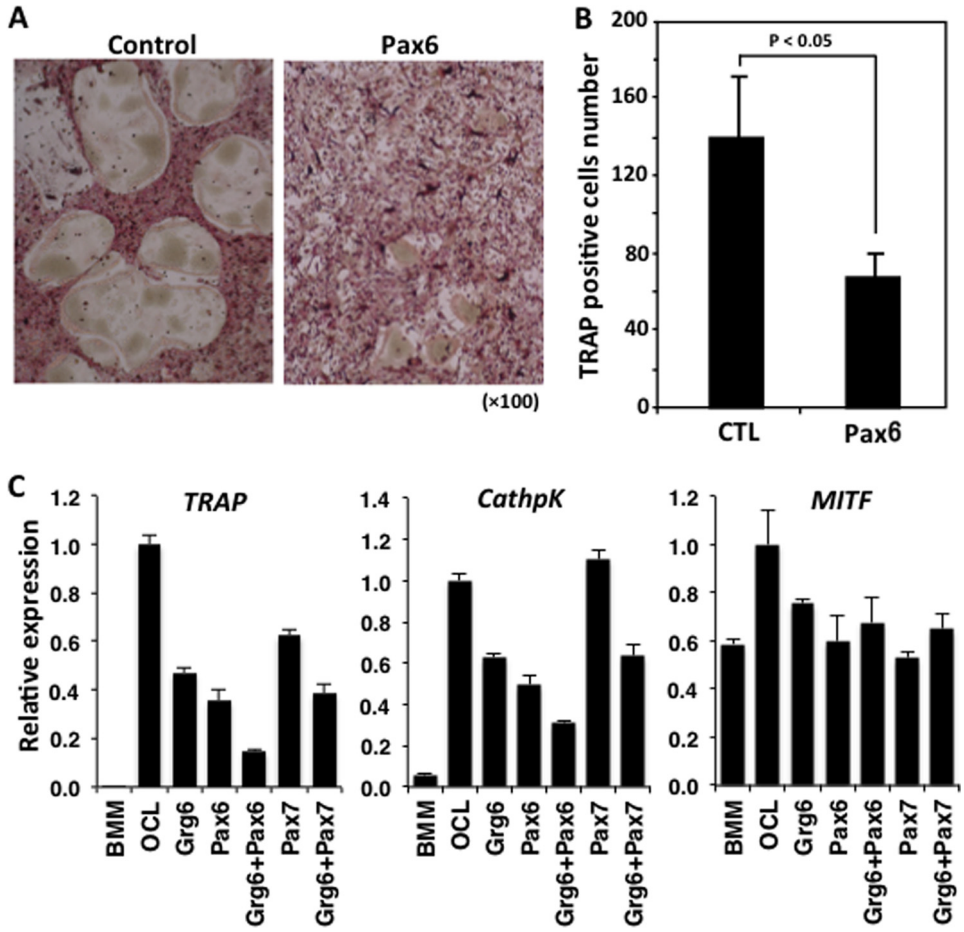

FIGURE 6. **Specific attenuation of osteoclastogenesis and endogenous osteoclast marker genes by overexpression of Pax6.** BMM were infected with pMX-GFP (*control*) or Pax6 retrovirus and cultured for 5 days in the presence of M-CSF and RANKL. *A,* cultured cells were fixed and stained for TRAP. *B,* TRAP-positive multinucleated cells were counted. Data shown are means ± S.D. of quadruplicate wells. *C,* BMM were infected with retrovirus of pMX-GFP (control (*CTL*); OCL), Grg6, Pax6, or Pax7 and cultured for 7 days in the presence of M-CSF and RANKL. Endogenous expression levels of the endogenous osteoclast lineage markers *TRAP*, *CathpK,* and *MITF* were measured by qRT-PCR using cDNA prepared from each total RNA as a template. Data shown are means of triplicate reactions ± S.E. and normalized against that of mouse *Hprt* as an internal control.

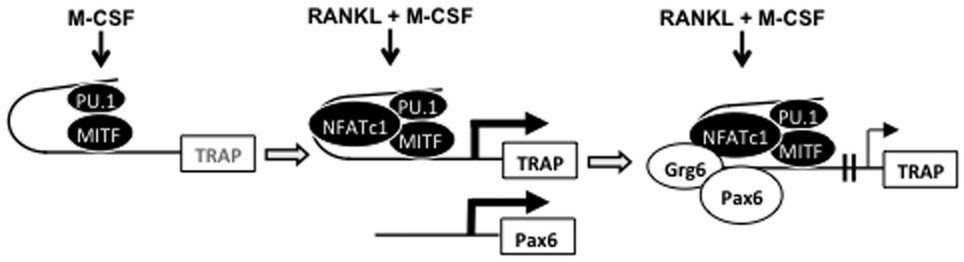

FIGURE 7. **Model of transcriptional regulation of *TRAP* gene expression.** Both RANKL and M-CSF, which are essential for osteoclast differentiation, may induce shared biological activities by activating a common set of transcription factors. Transcription factors, MITF and PU.1, critical for osteoclastogenesis are expressed in osteoclast precursors, and then RANKL stimulation invokes the expression of the master regulator NFATc1, Pax6, and Grg6 during osteoclast development. At the late stage of the differentiation, a combinatory complex of Pax6-Grg6 functions cooperatively to provide negative feedback regulation for osteoclast differentiation and resorptive processes.

mutant mice may be caused by regulatory mechanisms extrinsic to osteoblasts and osteoclasts. Pax1- and Pax7-deficient mice demonstrate a lack or malformation of ribs and maxilla, respectively (23, 60), and mice deficient for Pax3 and Pax9 exhibit limb abnormalities (61, 62). Because Pax1, Pax3, and Pax9 are not expressed in osteoclasts (Fig. 1*A*), their phenotypes are unlikely to be due to the osteoclast abnormalities. However, we cannot rule out the possibility that Pax7 may be involved in the regulation of osteoclastogenesis because Pax7 was weakly detected in BMM and osteoclasts. However, it is not likely that the co-repressor Grg6 contributes to Pax7-mediated attenuation of expression of osteoclast marker genes (Figs. 4, *C* and *D,* and 6*C*). Further analyses will explore the possible involvement of other *Pax* genes in osteoclastogenesis.

The importance of negative regulatory factors in osteoclastogenesis is underscored by the presence of several such factors that prevent excessive loss of bone mass. Inhibitors of differentiation/DNA binding (Ids) genes, helix-loop-helix transcription factors, negatively regulate RANKL-induced osteoclast differentiation through inhibiting MITF of bind-

ing to the osteoclast-specific gene promoter (34). Moreover, the protein inhibitor of activated STAT3 (PIAS3) also binds MITF as a target and MITF-mediated gene expression of osteoclast-specific markers cathepsin K and NFATc1 induced by RANKL (35). These proteins are abundantly expressed in committed osteoclast precursors and act as suppressive modulators in osteoclastogenesis. These factors show decreased expression after RANKL stimulation, indicating that they largely play a suppressive role in the earlier stages of osteoclastogenesis compared with that of Pax6. Thus, osteoclast differentiation is strictly controlled at the different stages by intracellular transcription factors Id, PIAS3, and Pax6. As other negative influences on osteoclast function by extracellular signaling pathways, osteoprotegerin, the decoy receptor for RANKL, plays an essential role to attenuate osteoclast differentiation and function to maintain bone homeostasis (63). IFN-β attenuates osteoclast differentiation by interfering with the RANKL-induced expression of c-Fos (33). In pathological conditions, T cell production of IFN-γ strongly suppresses osteoclastogenesis by interfering with the RANK-RANKL signaling pathway through degradation of tumor necrosis factor receptor-associated factor 6 (TRAF6) (32). IL-4 also attenuates osteoclast differentiation through a STAT6-dependent mechanism by regulating RANK expression (59, 64). Therefore, our findings provide an insight into the self-limiting system that attenuates pathological bone resorption by invoking limited sets of negative regulators of intracellular transcription factors at the distinct stages during osteoclastogenesis.

In conclusion, our results suggest that Pax6 is a negative regulator of RANKL-mediated osteoclast formation and that Pax6 at least exerts a negative effect on the *TRAP* gene promoter activated by the master regulator NFATc1. A possible partnership with Grg6 in the regulation of *TRAP* gene expression is also elucidated by our results. Additional studies of the detailed mechanism of transcription factor gene regulation will allow for a clearer understanding of the true nature of Pax6 and its functions in osteoclast differentiation and also offer therapeutic approaches to bone diseases caused by excessive osteoclastogenesis, such as autoimmune arthritis and osteoporosis.

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