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Protein Inhibitor of Activated STAT 3 Modulates Osteoclastogenesis by Down-Regulation of NFATc1 and Osteoclast-Associated Receptor

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Protein inhibitor of activated STAT3 (PIAS3) has been shown to regulate the activity of various transcription factors. In this study, we show that the overexpression of PIAS3 in bone marrow-derived monocyte/macrophage lineage cells attenuates osteoclast formation and down-regulates the expression of NFATc1 and osteoclast-associated receptor (OSCAR), which are important modulators in osteoclastogenesis. PIAS3 has been shown to associate with histone deacetylase 1 as well as with transcription factors, including the microphthalmia transcription factor, NFATc1, and c-Fos. Moreover, overexpression of PIAS3 inhibits the transactivation of target genes such as NFATc1 and OSCAR. This inhibitory effect of PIAS3 is possibly mediated by histone deacetylase 1 recruitment to the promoter regions of NFATc1 and OSCAR. Furthermore, silencing of PIAS3 by RNA interference in osteoclast precursors enhances osteoclast formation as well as gene expression of NFATc1 and OSCAR. Taken together, our results reveal that PIAS3 acts as a modulator in osteoclastogenesis. The Journal of Immunology, 2007, 178: 5588–5594.

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4 Abbreviations used in this paper: RANKL; also called TRANCE, OPG, and ODF) induces osteoclast formation from monocyte/macrophage precursors of hemopoietic origin (1–3). The binding of RANKL to its receptor, receptor activator of nuclear factor-κB (RANK), activates NF-κB, JNK, p38, ERK, and AKT, which are important for the differentiation, activation, and survival of osteoclasts (4, 5). RANKL activates and/or induces the expression of transcription factors known to be important for osteoclastogenesis in vitro and in vivo including c-Fos, microphthalmia transcription factor (Mitf), PU.1, and NFATc1 (5–7). Co-stimulatory signals mediated by ITAM-harboring adaptors, including DNAX-activating protein 12 (DAP12) and FcγR, cooperate with RANKL for osteoclastogenesis, and their activation enhances the induction of NFATc1 via calcium signaling (8–10). Osteoclast-associated receptor (OSCAR) is a member of the Ig-like surface receptor family and plays an important role as a costimulatory receptor for osteoclast differentiation by activating NFATc1 through association with the FeγR-γ-chain (8, 11–13). In addition, NFATc1 synergistically induces OSCAR gene expression with Mitf and PU.1 (14, 15). Therefore, a positive feedback circuit involving RANKL, NFATc1, and OSCAR appears to be important for the efficient differentiation of osteoclasts (14, 16).

The mammalian protein inhibitor of activated STAT (PIAS) family consists of PIAS1, PIAS3, PIASx, and PIASy, and the PIAS proteins were initially identified as negative regulators of STAT signaling (17, 18). Later, a role for PIAS in regulating the activity of transcription factors, including NF-κB, the SMA- and MAD-related proteins (SMADs), and tumor suppressor p53 has been proposed (19, 20). Although PIAS proteins act chiefly as repressors of transcription, they have been shown to be positive regulators of transcription as well (19). Recent studies have demonstrated that PIAS proteins contain a small ubiquitin-like modifier (SUMO) with ligase activity (19, 21). SUMO modification proceeds by a three-step enzyme shuttle analogous to ubiquitination (22). SUMO modification appears to play an important role in targeting proteins to specific subnuclear structures, stabilizing target proteins, and regulating the transcriptional activity of substrate proteins (22). However, it has also been suggested that PIAS proteins induce transcriptional repression independently of SUMO ligase activity by recruiting other corepressors such as histone deacetylases (HDACs) to the promoter region of their target genes (20, 23).

Mitf, a basic helix-loop-helix zipper protein, plays a key regulatory role in several cell types such as mast cells, melanocytes, and osteoclasts. Mitf regulates the expression of various genes, including mast cell protease 6, OSCAR, and cathepsin K (15, 24). The mice with mutations at the mi locus develop severe osteoporosis due to defective osteoclasts (25, 26), suggesting a key role for Mitf in osteoclastogenesis. Recently, PIAS3 has been shown to suppress Mitf transcriptional activity by blocking its DNA binding domain, leading to the intriguing possibility that PIAS3 may play a key role in the regulation of bone remodeling (27). It has been...
shown that PIAS proteins regulate cell cycle, apoptosis, and immune responses in many cell types, but the role of PIAS3 in osteoclast differentiation has yet to be determined.

We report here that overexpression of PIAS3 in osteoclast precursor inhibits osteoclast formation and attenuates the expression of NFATc1 and OSCAR during osteoclastogenesis. This inhibitory effect of PIAS3 is mediated by the recruitment of HDAC1, a corepressor protein, to the promoter regions of NFATc1 and OSCAR. Moreover, the silencing of PIAS3 by RNA interference induces the expression of NFATc1 and OSCAR as well as enhances osteoclast differentiation. Thus, this study shows that PIAS3 may play an important role in RANKL-mediated osteoclastogenesis.

Materials and Methods

Reagents and Plasmids

All cell culture media and supplements were obtained from Invitrogen Life Technologies. Soluble recombinant mouse RANKL and human M-CSF were purchased from PeproTech. Mouse IL-4, IFN-α, IFN-β, IFN-γ, and GM-CSF were purchased from R&D Systems. Full-length PIAS3 was amplified by RT-PCR and subcloned into Flag-pcDNA3, hemagglutinin (HA)-pcDNA3, or the pMX-IRES-EGFP vector. Flag-Mitf, HA-NFATc1, and HA-c-Fos were constructed as previously described (14). The OSCAR luciferase (Luc) vector (OSCAR-Luc) and NFATc1 luciferase vector (NFATc1-Luc) were also previously described (14, 28).

Osteoclast formation

Murine osteoclasts were prepared from bone marrow cells as previously described (15, 29). In brief, bone marrow cells were obtained from female 6- to 8-wk-old ICR mice. Bone marrow cells were cultured in α-MEM containing 10% FBS with M-CSF (30 ng/ml) for 3 days, and attached cells were used as osteoclast precursors (bone marrow-derived macrophage-like cells (BMMS)). To generate osteoclasts, BMMS were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 3 days. To generate osteoclasts from coculture with osteoblasts and BMMS, primary osteoblasts were prepared from the calvariae of newborn mice as previously described (14). Primary osteoblasts and transduced BMMS with control or a PIAS3 retrovirus were cocultured for 6 days in the presence of 1,25(OH)2D3 (10−9 M) and then lysed in extraction buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and 0.01% protease inhibitor mixture). Immunoprecipitated samples with the indicated Abs or whole cell lysates were subjected to SDS-PAGE and Western blotting. Primary Abs used included c-Fos (Calbiochem), NFATc1 (BD Pharmingen), OSCAR (12), actin, Flag (Sigma-Aldrich), and HA (Roche). HRP-conjugated secondary Abs (Amersham Biosciences) were probed and developed with ECL solution (Amersham Biosciences). Signals were detected and analyzed by LAS3000 luminescent image analyzer (Fuji Photo Film).

Transfection and luciferase assay

For transfection of reporter plasmids, 293T cells were plated on 24-well plates at a density of 3 × 104 cells/well 1 day before transfection. Plasmid DNA was mixed with FuGENE 6 (Roche) and transfected into the cells following the manufacturer’s protocol. Consistent amounts of total transfected DNA were achieved by the addition of empty vector DNA when necessary. After 48 h of transfection, the cells were washed twice with PBS and then lysed in reporter lysis buffer (Promega). Luciferase activity was measured with a luciferase assay system (Promega) according to the manufacturer’s instructions. Luciferase activity was measured in triplicate, averaged, and then normalized with β-galactosidase activity using o-nitrophenyl-β-D-galactopyranoside (Sigma-Aldrich) as a substrate.

Chromatin immunoprecipitation (ChIP) assay

A ChIP assay was performed with a ChIP kit (Upstate Biotechnologies) according to the manufacturer’s instructions using Abs against HDAC-1 and control IgG (Santa Cruz Biotechnology). The precipitated DNA was subjected to PCR amplification with primers specific for the promoter region of NFATc1 containing an AP1 binding site or for the OSCAR promoter region containing Mitf and NFATc1 binding sites. The following primers were used for PCR: NFATc1 sense (5′-CCGGGACGCCCATGC AATCTGTGTAATT-3′) and NFATc1 antisense (5′-CCGGGGTCGCC CGGAAAGAACTGCTTT-3′) and OSCAR sense (5′-GAAGCTATCGACTGTTTE-3′) and OSCAR antisense (5′-CCGGGAC GCCGAGAAAGATGTT-3′).

Generation of small interference RNA (siRNA) by in vitro transcription and transfection

siRNA of PIAS3 was synthesized with a Silencer siRNA construction kit (Ambion) according to the manufacturer’s instructions. Briefly, two 29-mer DNA template oligonucleotides with 21 and 8 nt complementary to the T7 promoter primer (underlined below) were synthesized. The sequences used for PIAS3 inhibition are as follows: 5′-AAAGAGAGGCCATCA GAGGTCCTTCTGCTC-3′ (sense), and the sense and antisense siRNA templates were transcribed by T7 RNA polymerase and the resulting RNA transcripts were hybridized to create dsRNA. The leader sequences of dsRNA were digested with a single-strand specific RNase. The resulting siRNA was purified and used for transfection. GAPDH DNA templates supplied with the kit were used to make control siRNA. siRNAs were transfected into BMMS using the X-tremeGENE siRNA transfection reagent (Roche) according to the manufacturer’s recommendations. After 48 h, cell were used for RT-PCR and RANKL-induced osteoclastogenesis.

Statistical analysis

Data represent the means and the SD of triplicate samples. Statistical significance was determined using one-way ANOVA followed by the Student 𝑡 test.

Results

The transcriptional regulation of PIAS3 by inhibitory factors in osteoclast precursors

There is accumulating evidence to suggest that cytokines and growth factors regulate RANKL-mediated osteoclastogenesis. For example, TNF-α, IL-1, and IL-6 stimulate osteoclast formation, whereas IFN-γ, IL-4, and GM-CSF can suppress RANKL-induced osteoclast differentiation. To examine whether the expression level of PIAS family members could be affected by various factors, we performed real-time PCR. When we treated BMMS with stimulatory factors such as TNF-α, IL-1, and IL-6 for 24 h, Western blot analysis

Cells from transfected 293T, BMMS, or osteoclasts were harvested after washing with ice-cold PBS and then lysed in extraction buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and 0.01% protease inhibitor mixture). Immunoprecipitated samples with the indicated Abs or whole cell lysates were subjected to SDS-PAGE and Western blotting. Primary Abs used included c-Fos (Calbiochem), NFATc1 (BD Pharmingen), OSCAR (12), actin, Flag (Sigma-Aldrich), and HA (Roche). HRP-conjugated secondary Abs (Amersham Biosciences) were probed and developed with ECL solution (Amersham Biosciences). Signals were detected and analyzed by LAS3000 luminescent image analyzer (Fuji Photo Film).
TNF-α significantly down-regulated the expression of PIASx, PIAS1, and PIAS3, whereas IL-1 and IL-6 showed a marginal effect on the expression of four PIAS family members (Fig. 1A). However, the expression of PIAS3 in BMMs was significantly up-regulated by treatment with IL-4, IFN-α, IFN-β, and IFN-γ, but the expression of other PIAS family members such as PIASx, PIASy, and PIAS1 was not strongly affected by these inhibitory factors (Fig. 1B). These results suggest that PIAS3 may play a role in inhibitory cytokine-mediated inhibition of osteoclast differentiation. This prompted us to examine whether the down-regulation of PIAS3 can rescue the inhibition of RANKL-induced osteoclastogenesis mediated by these inhibitory cytokines. Consistent with previous results (31, 32), RANKL-mediated osteoclastogenesis was strongly inhibited by IL-4 and IFNs (Fig. 1C). The down-regulation of PIAS3 (data not shown) by the transfection of PIAS3-specific siRNA into BMMs significantly reduced the inhibitory effect of IL-4 and IFNs on RANKL-induced osteoclastogenesis compared with the GFP-specific siRNA (control). These results suggest that inhibitory cytokines such as IL-4 and IFNs may regulate osteoclastogenesis, in part, through the up-regulation of PIAS3 gene expression.

**Overexpression of PIAS3 inhibits osteoclast formation**

To examine the role of PIAS3 in osteoclastogenesis mediated by RANKL, we retrovirally overexpressed PIAS3 in BMMs and confirmed the expression of PIAS3 (Fig. 2F). Transduced BMMs were cultured with different concentrations of RANKL in the presence of M-CSF, and cultured cells were fixed and stained for TRAP, TRAP⁺ MNCs having more than three nuclei (B) or 10 nuclei (C) were counted as osteoclasts. D, Primary osteoblasts and BMMs transduced with control or PIAS3 retrovirus were cocultured for 6 days in the presence of 1,25(OH)₂D₃ and PGE₂. Cultured cells were fixed and stained for TRAP, TRAP⁺ MNCs having more than three nuclei were counted as osteoclasts. E, The expression of PIAS3 was confirmed by Western blot analysis. The data represent the means and the SD of triplicates. #, p < 0.05; *, p < 0.005; **, p < 0.0005 vs control vector.
the formation of bigger TRAP+ MNCs containing > 10 nuclei was suppressed by the overexpression of PIAS3 (Fig. 2C). When BMMs transduced with the PIAS3 retrovirus and primary osteoblasts were cocultured in the presence of M-CSF and RANKL, real-time PCR (A) and Western blot analysis (B and C) were performed to assess the expression of the indicated genes. B and C. The relative amounts of the indicated genes were shown under the lanes. The data represent the means and the SD of triplicates. #, p < 0.05; *, p < 0.005; **, p < 0.0005 vs control vector.

FIGURE 3. Overexpression of PIAS3 attenuates the expression of NFATc1 and OSCAR during osteoclastogenesis. BMMs transduced with control (pMX-IRES-EGFP) or the PIAS3 retrovirus were cultured for the indicated times in the presence of M-CSF and RANKL. Real-time PCR (A) and Western blot analysis (B and C) were performed to assess the expression of the indicated genes. B and C. The relative amounts of the indicated genes were shown under the lanes. The data represent the means and the SD of triplicates. #, p < 0.05; *, p < 0.005; **, p < 0.0005 vs control vector.

Overexpression of PIAS3 attenuates the expression of NFATc1 and OSCAR during osteoclastogenesis

RANKL induces osteoclast formation by regulating transcription factors such as Mitf, PU.1, and NFATc1, all of which can affect OSCAR gene expression (5, 14, 15). Because the overexpression of PIAS3 inhibited osteoclastogenesis, we investigated the effect of the overexpression of PIAS3 on the expression of osteoclast-specific transcription factors important for RANKL-induced osteoclastogenesis. The expression of NFATc1 and OSCAR, key modulators of late phase of osteoclastogenesis, was significantly attenuated by the overexpression of PIAS3, whereas the overexpression of PIAS3 did not affect the expression of Mitf and PU.1 during osteoclastogenesis (Fig. 3A). The down-regulation of NFATc1 and OSCAR by PIAS3 overexpression was confirmed by Western blotting (Fig. 3B). Because AP-1 transcription factors are important for NFATc1 up-regulation (33) and we have shown that NFATc1 expression is down-regulated by the overexpression of PIAS3, we investigated the effect of PIAS3 overexpression on c-Fos expression. RANKL induces c-Fos expression in BMMs, and this induction during osteoclastogenesis was not affected by PIAS3 overexpression (Fig. 3C). These results indicate that the overexpression of PIAS3 attenuates osteoclast differentiation through the down-regulation of two key modulators, NFATc1 and OSCAR, in late stage osteoclastogenesis, but RANKL-mediated c-Fos expression is not affected by PIAS3 overexpression.

Silencing of PIAS3 by RNA interference enhances osteoclastogenesis

To investigate the physiological role of PIAS3 in osteoclastogenesis, we performed a loss-of-function experiment using siRNA. The expression of PIAS3 was reduced by PIAS3-specific siRNA, not by control GFP-specific siRNA (data not shown). The silencing of PIAS3 in BMMs resulted in a significant increase of RANKL-mediated osteoclastogenesis (Fig. 4, A–C). Because the overexpression of PIAS3 affected the gene expression of NFATc1 and OSCAR, we examined the expression level of those genes in BMMs and osteoclasts by real-time PCR. As expected, the silencing of PIAS3 strongly increased the expression of NFATc1 and OSCAR in osteoclasts as compared with control (Fig. 4D). These results indicate that PIAS3 plays a negative role in RANKL-mediated osteoclastogenesis.

The regulation of transcription factors by PIAS3

Because the overexpression of PIAS3 in BMMs down-regulated RANKL-mediated OSCAR gene expression (Fig. 3, A and B), we tested whether the overexpression of PIAS3 could inhibit transactivation of the OSCAR promoter by transcription factors. When 293T cells were cotransfected with a luciferase reporter plasmid containing the 1.7-kb OSCAR promoter together with Mitf, Mitf induced OSCAR promoter activity as compared with control (Fig. 5A). The induction of OSCAR promoter activity by Mitf was decreased in a dose-dependent manner by PIAS3. Similarly as Mitf, the induction of OSCAR promoter activity by NFATc1 was also
attenuated by PIAS3 overexpression (Fig. 5B). Moreover, the synergistic induction of OSCAR promoter activity by Mitf and NFATc1 was strongly abolished by PIAS3 (Fig. 5C). These results demonstrate that PIAS3 inhibits the transactivation of OSCAR by Mitf and NFATc1.

Because PIAS3 reduced the expression of NFATc1 and OSCAR mediated by c-Fos, Mitf, and NFATc1, we tested whether PIAS3 could bind directly to those transcription factors by immunoprecipitation. 293T cells were cotransfected with c-Fos together with Flag-PIAS3, and cell lysates were immunoprecipitated with an anti-PIAS3 Ab. Immunoprecipitated samples were subjected to SDS-PAGE and Western blotting by anti-Flag or anti-HA Ab. We show that PIAS3 can interact directly with Mitf, c-Fos, and NFATc1, respectively (Fig. 6, A–C).

Because PIAS3 can recruit other coregulators such as HDAC to repress transcription (20), we investigated whether PIAS3 directly interacts with HDAC in vitro. Consistent with a previous report (34), we show PIAS3 association with HDAC1 (Fig. 6D). These results prompted us to investigate whether PIAS3 recruits HDAC1 on the promoter regions of NFATc1 and OSCAR during osteoclastogenesis. To test this hypothesis, we performed a ChIP assay using BMMs and bone marrow-derived osteoclast cells. The crosslinked protein/chromosome complex from BMMs and osteoclasts was precipitated with anti-HDAC1 or control Ab. The precipitated DNA was subjected to PCR amplification with primers specific for the NFATc1 promoter region containing an AP1-binding site. Consistent with the induced expression of NFATc1 during RANKL-mediated osteoclastogenesis (Fig. 3A), the recruitment of HDAC1 at the NFATc1 promoter region was decreased in osteoclasts as compared with BMMs (Fig. 6E). However, the overexpression of PIAS3 increased the recruitment of HDAC1 to the NFATc1 promoter region in BMMs as well as osteoclasts. In addition, when a ChIP assay was performed with primers specific for

FIGURE 5. Transactivation of NFATc1 and OSCAR promoter constructs by transcription factors. A and B, The OSCAR reporter was cotransfected into 293T cells with Mitf (A) or NFATc1 (B) and various amounts of the PIAS3 expression plasmid. C, 293T cells were cotransfected with OSCAR reporter plasmid and the indicated plasmids expressing Mitf (100 ng), NFATc1 (150 ng), or PIAS3 (50 ng). D, The 6.2-kb mouse NFATc1 promoter Luc reporter was cotransfected into 293T cells with c-Fos and various amounts of PIAS3 expression plasmid. Results are representative of two independent sets of similar experiments. Data represent the means and the SD of triplicate samples.

PIAS3 interacts with transcription factors and induces epigenetic regulation of NFATc1 and OSCAR

Because PIAS3 inhibits the transactivation of NFATc1 and OSCAR mediated by c-Fos, Mitf, and NFATc1, we tested whether PIAS3 could bind directly to those transcription factors by immunoprecipitation. 293T cells were cotransfected with Mitf, NFATc1, or c-Fos together with Flag-PIAS3, and cell lysates were immunoprecipitated with an anti-PIAS3 Ab. Immunoprecipitated samples were subjected to SDS-PAGE and Western blotting by anti-Flag or anti-HA Ab. We show that PIAS3 can interact directly with Mitf, c-Fos, and NFATc1, respectively (Fig. 6, A–C).

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FIGURE 4. Down-regulation of PIAS3 in BMMs enhances osteoclastogenesis. A, GFP or PIAS3 siRNA (Si) was transfected into BMMs. A, BMMs transfected with GFP or PIAS3 siRNA were cultured for 3 days in the presence of M-CSF and various concentrations of RANKL. Cultured cells were fixed and stained for TRAP. B and C, TRAP⁺ MNCs having more than three nuclei (B) or 10 nuclei (C) were counted as osteoclasts. D, BMMs transfected with GFP or PIAS3 siRNA were cultured for 2 days with M-CSF in the absence (BMM) or presence (OC) of RANKL. Total RNA was harvested from cultured cells and real-time PCR was performed to assess the expression of the indicated genes. #, $p < 0.05; *, $p < 0.005; **, $p < 0.0005$ vs GFP siRNA.

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can affect RANKL-induced osteoclastogenesis (31, 32, 35, 36). Many inhibitory factors, including IL-4, IL-12, IFNs, and GM-CSF, have different roles in regulating RANKL-mediated osteoclastogenesis. Among them, it has been shown that PIAS3 may play an important role in regulating RANKL-mediated osteoclastogenesis.

**Discussion**

We demonstrated that inhibitory cytokines such as IL-4 and IFNs induce PIAS3 expression in BMMs and that silencing PIAS3 by RNA interference reduces the inhibitory effect of these cytokines on RANKL-induced osteoclastogenesis, suggesting that inhibitory cytokines may regulate RANKL-mediated osteoclastogenesis, in part, via up-regulation of PIAS3 gene expression. In addition, we investigated the role of PIAS3 in RANKL-mediated osteoclastogenesis by retroviral overexpression of PIAS3 in BMMs and the reduction of PIAS3 expression using RNA interference. We demonstrated that PIAS3 regulates osteoclastogenesis and the expression of NFATc1 and OSCAR, which are important molecules for osteoclast differentiation, and that this regulation is mediated by HDAC1 recruitment to the promoter regions of NFATc1 and OSCAR. Thus, our data suggest that PIAS3 may play an important role in regulating RANKL-mediated osteoclastogenesis.

Various cytokines and hormones are involved in osteoclast differentiation and function. Among them, it has been shown that many inhibitory factors, including IL-4, IL-12, IFNs, and GM-CSF, can affect RANKL-induced osteoclastogenesis (31, 32, 35, 36). Although previous results showed that these inhibitory factors can affect RANKL-induced signaling pathways, we have demonstrated here that inhibitory factors, including IL-4 and IFNs, can also block RANKL-mediated osteoclastogenesis, in part, through the up-regulation of PIAS3.

Levy et al. showed that PIAS3 interacts with Mitf and represses Mitf-mediated transcriptional activation by blocking its DNA-binding activity (27). Interestingly, the interaction between Mitf and PIAS3 is controlled by serine phosphorylation of Mitf, which inhibits the PIAS3-Mitf interaction. In the nucleus of resting cells, unphosphorylated Mitf is inactivated by association with PIAS3. Activation of the gp130 receptor or c-Kit receptor in melanoma and mast cells induces Mitf phosphorylation and results in the release of PIAS3, which then binds STAT3 (37, 38). This indicates that the phosphorylation and dephosphorylation of Mitf is a key step in the regulation of interaction between Mitf, PIAS3, and STAT3. Similar to the Kit-induced activation of Mitf in melanocytes, the treatment of osteoclasts with RANKL results in p38 MAPK-mediated phosphorylation of Mitf with a resulting increase in transcriptional activation at the osteoclast-specific TRAP promoter (39). In a previous study we observed that the RANKL/MKK6/p38 signaling cascade induces Mitf transactivation of OSCAR (15). Because Mitf and PIAS3 are both expressed in osteoclast precursors (BMMs), PIAS3 may interact with Mitf and block Mitf binding to its target genes, including TRAP and OSCAR. During osteoclastogenesis, the phosphorylation of Mitf by RANKL stimulation may result in the release of PIAS3 from Mitf and, in turn, activated Mitf could bind to the E-box region of the OSCAR promoter, eliciting synergistic activation of OSCAR by cooperation with NFATc1. However, when PIAS3 was overexpressed the level of HDAC1 recruited to the OSCAR promoter region was dramatically increased in osteoclasts, possibly due to the increased recruitment of HDAC1 by interaction between NFATc1 and overexpressed PIAS3. We also observed that the recruitment of HDAC1 to the NFATc1 promoter region containing an AP-1 binding site was strongly increased by the overexpression of PIAS3, thereby resulting in the attenuation of NFATc1 expression. Therefore, we have demonstrated that the overexpression of PIAS3 down-regulates the expression of NFATc1 and OSCAR in part through the increased recruitment of HDAC1, a corepressor, to their promoter regions.

PIAS3 proteins can regulate transcription either positively or negatively. Four mechanisms have been proposed for PIAS-mediated transcription repression: 1) PIAS proteins block the DNA-binding activity of a transcription factor; 2) PIAS proteins function as transcriptional repressors by recruiting HDAC to inhibit transcription; 3) PIAS3 proteins promote sumoylation of the transcription factor, which inhibits its transcriptional activity; and 4) PIAS3 proteins inhibit transcription by sequestering transcription factors in distant subnuclear structures (20). Although we showed that PIAS3 may function as a repressor by recruiting HDAC1, we cannot rule out the possibility that these other mechanism(s) might also be involved in PIAS3-mediated gene repression of NFATc1 and OSCAR. It is worth noting that transcription factors such as Mitf and NFATc1 have some potential sites for sumoylation by PIAS3 proteins. Therefore, further study will be required to elucidate whether other mechanism(s) may be involved in PIAS3-mediated gene regulation during osteoclastogenesis.

In conclusion, our data show that PIAS3 is a potential negative regulator of osteoclastogenesis by various cytokines. We show that PIAS3 acts as a negative regulator in RANKL-induced osteoclastogenesis by down-regulating NFATc1 and OSCAR, in part, through recruiting the HDAC1 corepressor. Recently, we showed that Ids, helix-loop-helix transcription factors, have an inhibitory
effect on osteoclast differentiation as well (40). Hence, our work reveals an additional layer of negative regulation in RANKL-mediated osteoclastogenesis. However, further elucidation of the detailed mechanism of transcription factor regulation by PIAS3 should provide additional therapeutic approaches to various bone diseases.

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Disclosures

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