Contribution of Nuclear Factor of Activated T Cells c1 to the Transcriptional Control of Immunoreceptor Osteoclast-associated Receptor but Not Triggering Receptor Expressed by Myeloid Cells-2 during Osteoclastogenesis*

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Bone homeostasis depends on the coordination of osteoclastic bone resorption and osteoblastic bone formation. Receptor activator of NF-κB ligand (RANKL) induces osteoclast differentiation through activating a transcriptional program mediated by the key transcription factor nuclear factor of activated T cells (NFAT) c1. Immunoreceptors, including osteoclast-associated receptor (OSCAR) and triggering receptor expressed by myeloid cells (TREM)-2, constitute the co-stimulatory signals required for RANKL-mediated activation of calcium signaling, which leads to the activation of NFATc1. However, it remains unknown whether the expression of immunoreceptors are under the control of NFATc1. Here we demonstrate that the expression of OSCAR, but not that of TREM-2, is up-regulated during osteoclastogenesis and markedly suppressed by the calcineurin inhibitor FK506, suggesting that OSCAR is transcriptionally regulated by NFATc1. NFATc1 expression results in the activation of the OSCAR promoter, which was found to be further enhanced by co-expression of PU.1 and microphthalmia-associated transcription factor (MITF). We further provide evidence that NFATc1 specifically regulates OSCAR by chromatin immunoprecipitation assay and quantification of OSCAR and TREM-2 mRNA in NFATc1−/− cells. Thus, OSCAR but not TREM-2 is involved in the positive feedback loop of the immunoreceptor-NFATc1 pathway during osteoclastogenesis. Although several immunoreceptors have been identified as co-stimulatory molecules for RANKL, the expression and function are differentially regulated. These mechanisms, possibly together with the delicate regulation of their ligands on osteoblasts, may provide the requisite machinery for the modulation of osteoclastogenesis in the maintenance of bone homeostasis.

Bone is continuously remodeled by a series of cellular actions of bone-resorbing osteoclasts and bone-forming osteoblasts. Osteoclasts are the primary cells that resorb bone, and our knowledge concerning the mechanisms of osteoclast differentiation has been increasing rapidly (1–4). The finding that bone marrow monocyte/macrophage lineage cells (BMMs)2 can differentiate into osteoclasts in vitro when co-cultured with osteoblasts (5) suggested that osteoblast-derived factors induce osteoclast differentiation. Receptor activator of NF-κB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) are provided by osteoblasts and essential for osteoclastogenesis (1–4). In addition, we discovered recently that the immunoreceptor-mediated calcium signals are also critical for osteoclastogenesis (6).

In the osteoclast differentiation program, transcription factor nuclear factor of activated T cells (NFAT) c1 is induced significantly by RANKL (7). NFATc1 is involved in the transcriptional regulation of osteoclast-specific genes, such as ACP5 (encoding tartrate-resistant acid phosphatase (TRAP)) (7), calcitonin receptor (7, 8), and cathepsin K (9). Moreover, NFATc1-deficient embryonic stem cells failed to differentiate into osteoclasts in vitro, and ectopic expression of NFATc1 caused induction of osteoclast differentiation even in the absence of RANKL (7). Thus, NFATc1 can be referred to as the master transcription factor for osteoclastogenesis.

The activity of NFATc1 is controlled by the phosphatase calcineurin, which plays a critical role in the coupling of calcium signals with cellular responses (10–13). Once activated by an increase of the intracellular calcium concentration, calcineurin induces the translocation of NFATc1 into the nucleus, where it activates the transcription of specific target genes. RANKL-induced osteoclastogenesis and NFATc1 induction are strongly inhibited by the calcineurin inhibitor FK506, and this shows the importance of calcium signaling during osteoclast differentiation (7). However, it is not likely that RANKL, whose receptor RANK belongs to the tumor necrosis family receptor family (14, 15), mediates
Transcriptional Control of OSCAR by NFATc1

such calcium signaling directly. Thus we focused on the immunoreceptor tyrosine-based activation motif (ITAM), which is necessary for the transduction of the calcium signal in immune cells (16, 17), and found that ITAM-mediated calcium signaling is also indispensable to osteoclastogenesis (6).

ITAMs are present in the cytoplasmic domains of several transmembrane adaptor molecules, including FcRγ (Fc receptor common γ subunit) and DAP12 (DNAX-activating protein 12), and serve as signal transducers for immunoreceptors (18–21). We and others have reported that the combined deficiency of DAP12 and FcRγ in mice results in severe osteopetrosis, and the osteoclast precursor cells derived from these mice do not differentiate into osteoclasts in vitro (6, 22). Moreover, we demonstrated that immunoreceptor osteoclast-associated receptor (OSCAR) (23) and paired immunoglobulin-like receptor-A (24) associate with FcRγ in vitro to activate a NFATc1. Using reporter gene assay and chromatin immunoprecipitation, we reported that the combined deficiency of DAP12 and FcRγ in mice results in severe osteopetrosis, and the osteoclast precursor cells derived from these mice do not differentiate into osteoclasts in vitro (6, 22). However, little is known about how these receptors are regulated during osteoclastogenesis and whether they are conversely regulated by NFATc1.

In this report, we focused on OSCAR and TREM-2, which are representative receptors associated with FcRγ and DAP12, respectively, and investigated the transcriptional regulation of OSCAR and TREM-2 by NFATc1. Using reporter gene assay and chromatin immunoprecipitation, we showed that NFATc1 selectively activates the OSCAR promoter. Marked inhibition of the RANKL-induced OSCAR expression in NFATc1−/− cells clearly demonstrates the importance of NFATc1 in the transcriptional control of OSCAR in osteoclast differentiation, which will be discussed in the context of positive feedback regulation of osteoclastogenic signaling.

EXPERIMENTAL PROCEDURES

*Mice—*C57BL/6 mice (6- to 10-week-old) were purchased from CLEA Japan. NFATc1−/− deficient mice (27) were kindly provided by T. W. Mak. All mice were kept under specific pathogen-free conditions. All animal experiments were performed with the approval of the institutional committee and conformed to the recognized guidelines and laws.

*In Vitro Osteoclastogenesis—*The methods of in vitro osteoclast differentiation have been described previously (5, 28, 29). Briefly, mouse bone marrow cells were cultured in α-minimal essential medium (Invitrogen) with 10% fetal bovine serum (Sigma) containing 10 ng ml−1 M-CSF (Genzyme). After 2 days, adherent cells were used as BMMs. They were further cultured in the presence of 50 ng ml−1 soluble RANKL (PeptroTech) and 10 ng ml−1 M-CSF. If indicated, FK506 (provided by Fujisawa Pharmaceutical) was added at a concentration of 10 μg ml−1. The day of RANKL addition was designated as day 0 throughout this report. NFATc1−/− and NFATc1+/− mouse fetal liver cells derived from E13.5 embryos were obtained by crossing NFATc1−/− parental mice and were cultured in the same way as the bone marrow cells described above. Co-culture of osteoblasts derived from mouse calvaria cells and bone marrow cells was performed in the presence of 10−8 M 1,25-(OH)2 vitamin D3 (Wako) and 10−6 M prostaglandin E2 (PGE2) (Wako) (5). 20 μg ml−1 Fc fusion protein or human IgG (Jackson ImmunoResearch Laboratories) was added. TRAP-positive multinucleated cells, which have more than three nuclei, were counted.

*Generation of Fc Fusion Proteins—*DNA fragments encoding the extracellular domains of OSCAR and TREM-2 were amplified by Fast-Start High Fidelity PCR System (Roche Applied Science) using cDNA of RANKL-stimulated mouse BMMs as a template. The following sense and antisense primers were used for PCR: OSCAR, 5′-AGCTTAGT-GCCACCATGTTCCGTGT-3′ (sense), 5′-CTCAGGTTTCTCCGT-GGTATAGTCAC-3′ (antisense); TREM-2, 5′-AAGCTTGAAGGTTGGT-GCCATTGGACCTC-3′ (sense), 5′-CTCAGGAGGGTGGGGAAGGGAG-3′ (antisense). The sequence of the amplified fragments was confirmed by DNA sequencing. Then the fragments were fused to the cDNA corresponding to the Fc portion of human IgG1a (kindly provided by L. Lanier) and inserted into the HindIII/XhoI site of pcDNA3.1 (Invitrogen). After transient transfection into COS7 cells with the plasmids, culture supernatants were collected and affinity-purified with the MAbTrap Kit (Amersham Biosciences). Purified Fc fusion proteins were precipitated with protein A-Sepharose (Amersham Biosciences) and separated with SDS-PAGE, followed by immunoblotting with anti-human IgG antibody (Serotec). The same membranes were stripped and subjected to immunoblotting with anti-mouse OSCAR (G-T Research) or anti-mouse TREM-2 (R&D Systems) antibodies, respectively. For analysis of glycosylation, precipitates were treated overnight with N-glycosidase F (Wako) according to the manufacturer's protocol.

*Flow Cytometric Analysis with Fc Fusion Proteins—*Single cell suspensions of osteoblasts, obtained by detaching cells with cell dissociation buffer (Invitrogen), were preincubated with anti-mouse CD16/CD32 (Fcγ receptor) monoclonal antibody (BD Biosciences) for 15 min on ice to block nonspecific staining. Cells were then mixed with a saturating concentration of Fc fusion proteins or control human IgG (BD Biosciences) for 30 min, followed by incubation with phycoerythrin-conjugated anti-human IgG (BD Biosciences) for 15 min on ice. Stained cells were analyzed by FACScan using CellQuest software (BD Biosciences).

*RNA Isolation and Quantitative RT-PCR—*Total RNA was extracted using Sepasol (Nacalai Tesque) from cultured cells. 1 μg of RNA was treated with DNase I and reverse-transcribed with the SuperScript First-Strand Synthesis System (Invitrogen) according to the manufacturer's protocol. The following sense and antisense primers were used for mouse OSCAR, TREM-2, and GAPDH: OSCAR, 5′-TGCGGGTT-TGCACCTTCTCA-3′ (sense), 5′-GACCTGTTACAGAGGTTCCAC-GA-3′ (antisense); TREM-2, 5′-TCCAGACGCTCCCAACACCA-3′ (sense), 5′-TCTGAGCAAGGTTGTCACTGCG-3′ (antisense); GAPDH, 5′-ACCAGACTCCATGCGATC-3′ (sense), 5′-TCCACCACCTGGTTGTGTA-3′ (antisense). Quantitative RT-PCR was performed with a LightCycler (Roche Applied Science) using SYBR Green (TOYOBO). The values were normalized with those of GAPDH.

*Luciferase Assay—*The transcription initiation site of mouse TREM-2 promoter was determined with the 5′ rapid amplification of cDNA ends (5′ RACE) system (Invitrogen). The mouse OSCAR promoter was reported previously (30). The 1.7-kb fragment of the OSCAR promoter and 1.5-kb fragment of the TREM-2 promoter regions were amplified by PCR using the following primers: OSCAR promoter, 5′-AGCGGTGA-GGACAGTAAATGTCAGA-3′ (sense), 5′-CTCGAGCGGTTGGG-CTTAGGAAAAAGGT-3′ (antisense); TREM-2 promoter, 5′-AGCCTGGAAGGTCCTGGTGGATGCCGG-3′ (sense), 5′-CTCGAGCGGTTGGG-CTTAGGAAAAAGGT-3′ (antisense). The reporter plasmids were constructed by amplifying the fragment of Fc fusion proteins or control human IgG (BD Biosciences) or TREM-2 promoter into the NotI/XhoI site of the PicaGene luciferase reporter plasmid (Toyo Ink). The reporter plasmids of mouse TRAP and calcitonin receptor promoters, and the expression vectors of NFATc1 were described previously (7). Expression vectors of PU.1 (31) and microphthalmia-associated transcription factor (MITF) (32) were kindly provided by H. Singh and M. Matsumoto, respectively. Transfection of HEK293T cells and measurement of luciferase activity were performed as previously described (7) using the Dual-Luciferase Reporter Assay System (Promega).
FIGURE 1. OSCAR and TREM-2 are cooperatively involved in osteoclastogenesis. A, characterization of Fc fusion proteins by Western blotting. COS7 cells were transiently transfected with the plasmids for OSCAR-Fc and TREM-2-Fc, and the culture supernatants were analyzed by immunoblotting with anti-human IgG antibody (lanes 1, 2, 5, and 6). Each membrane was re-immunoblotted with anti-mouse OSCAR (lanes 3 and 4) and anti-mouse TREM-2 (lanes 7 and 8) antibodies, respectively. Each sample was treated with N-glycopeptidase F (lanes 2, 4, 6, and 8). B, flow cytometric analysis of putative OSCAR and TREM-2 ligands on osteoblasts. Osteoblasts derived from mouse calvarial cells were cultured for 6 days and stained with OSCAR-Fc (left panel), TREM-2-Fc (right panel), or control human IgG. The dotted line with the open curve indicates the cells cultured in the presence of 1,25-(OH)2 vitamin D3 and PGE2, and the solid bold line with the open curve indicates unstimulated cells. The solid line with the filled curve indicates unstimulated cells stained with IgG (negative control). C, inhibition of osteoclastogenesis by the Fc fusion proteins. Mouse calvarial cells and BMMs were co-cultured in the presence of 1,25-(OH)2 vitamin D3 and PGE2. The fusion proteins (alone or in combination) or control human IgG was added to the co-culture. TRAP-positive multinucleated cells (TRAP+ MNC) were counted on day 7. Bars represent the mean ± S.E. (n = 3). *, p < 0.05.
Transcriptional Control of OSCAR by NFATc1

Chromatin Immunoprecipitation—Mouse BMMs cultured with or without RANKL stimulation were harvested at days 0–2, and a chromatin immunoprecipitation assay was performed using ChIP Assay Kit (Upstate) according to the manufacturer’s protocol. The cross-linked chromatin was immunoprecipitated with the antibody against NFATc1 (Santa Cruz Biotechnology) or control IgG (BD Biosciences). The following primers specific for the OSCAR and TREM-2 promoters were used: OSCAR promoter, 5′-GCCTTCTTGTAGGACGGA-3′ (sense), 5′-GAAAGCAACCAAGG-3′ (antisense); TREM-2 promoter, 5′-AGGGTTTGT-3′ (sense), 5′-GCGAAAAGGATGACCAAGCCA-G-3′ (antisense).

RNA Interference—For transfection of small interfering RNA (siRNA), mouse BMMs were plated on 24-well plates at a density of 2.5 × 10⁵ cells per well. Transfection of siRNA was performed on day 1 using TransIT-TKO Transfection Reagent (Mirus Bio) according to the manufacturer’s protocol. siRNA specific for green fluorescent protein (GFP) was used as a control, and Cy3-labeled nonspecific siRNA (B-Bridge International) was used to estimate the transfection efficiency. siRNAs for NFATc1 and GFP were synthesized by Proligo LLC. Quantitative RT-PCR was performed with samples harvested on day 3. The values of mRNA expression were normalized with those of GAPDH.

Statistical Analysis—Throughout this report, statistical analysis was performed using Student’s t test (*, p < 0.05), and all data are expressed as the mean ± S.E. (n = 3).

RESULTS

OSCAR and TREM-2 Are Cooperatively Involved in Osteoclastogenesis—Chimeric proteins of the Fc portion of immunoglobulin and the ligand-binding domain of a certain receptor are often used for detecting putative ligands of the receptor and for blocking signals mediated by the ligand (21, 23). First, we constructed expression plasmids for soluble OSCAR-Fc and TREM-2-Fc chimeric proteins by fusing the extracellular domain of each immunoreceptor to the Fc portion of human IgG1. Culture supernatants were collected after transfection of the plasmids into COS7 cells, and the expression of the soluble proteins was confirmed by Western blotting (Fig. 1A). OSCAR-Fc was detected as a ~57-kDa band (Fig. 1A, lanes 1 and 3), and it migrated to ~50 kDa after N-glycopeptidase F treatment (Fig. 1A, lanes 2 and 4), indicating OSCAR-Fc is secreted as a glycosylated form. This core size is in agreement with the predicted molecular mass of OSCAR-Fc. TREM-2-Fc was detected as a ~55-kDa molecule (Fig. 1A, lanes 5 and 7) and migrated to ~50 kDa after N-glycopeptidase F treatment (Fig. 1A, lanes 6 and 8).

To examine the expression of putative ligands of OSCAR and TREM-2, osteoblasts derived from mouse calvaria were cultured with or without the osteotropic factors 1,25-(OH)₂ vitamin D₃ and PGE₂ for 6 days and stained with either control human IgG, OSCAR-Fc, or TREM-2-Fc (10 μg ml⁻¹ each) were added, osteoclast differentiation was further suppressed. These results indicate OSCAR and TREM-2 are cooperatively involved in osteoclastogenesis.

Expression of OSCAR and TREM-2 during Osteoclast Differentiation—To examine the expression of OSCAR and TREM-2 on osteoclast pre-
process of osteoclast differentiation, appears not to be regulated by TREM-2, the expression of which is relatively constant throughout the course of osteoclast differentiation and was strongly reduced by the calcineurin inhibitor FK506 (Fig. 2). The expression of TREM-2 protein was not significantly affected by either RANKL or FK506, displaying a similar expression pattern to that of OSCAR (30). Thus, we examined whether NFATc1 synergistically acts with MITF and PU.1. Co-transfection of NFATc1, PU.1, and MITF expression vectors caused an increase of ~300-fold in the promoter activity of OSCAR. In contrast, the promoter activity of TREM-2 was not significantly changed either by NFATc1 alone or by co-expression with MITF and PU.1 (Fig. 4B).

To determine whether the transcriptional complex consisting of NFATc1, PU.1, and MITF is involved in the regulation of other osteoclast-specific genes (1–4), we analyzed the TRAP and calcitonin receptor promoters using a luciferase assay (Fig. 4, C and D). The activity of the TRAP promoter was synergistically increased by co-transfection of NFATc1, PU.1, and MITF expression vectors (Fig. 4C). However, the activity of the calcitonin receptor promoter was controlled differently (Fig. 4D). Overexpression of NFATc1, as well as that of either PU.1 or MITF, led to a significant increase in the activity of calcitonin receptor promoter, but co-transfection of NFATc1 expression vector with either the MITF or PU.1 expression vector did not enhance the luciferase activity synergistically. Rather, NFATc1 antagonized the PU.1-induced transcriptional control of OSCAR by NFATc1

The OSCAR promoter, but Not the TREM-2 promoter has multiple NFAT binding sites. A, sequences of the putative promoter region of mouse TREM-2. The transcription initiation site was determined by 5’ RACE and designated as +1. The TREM-2 promoter contains one MITF and two PU.1 consensus binding sites. The binding sites for other transcription factors, including GATA, MyoD, and AML-1, are also shown. B, schematic diagrams of the OSCAR and TREM-2 promoters. The consensus binding sites of NFAT, PU.1, and MITF found in the mouse OSCAR and TREM-2 promoters are indicated. Unlike the TREM-2 promoter, the OSCAR promoter contains multiple NFAT binding sites.

Transcriptional Control of OSCAR by NFATc1

The OSCAR promoter, but Not the TREM-2 promoter, Has Multiple NFAT Binding Sites—Previous analysis of the mouse OSCAR promoter revealed that the transcription factors MITF and PU.1 are important for the regulation of the OSCAR gene expression (30). However, the contribution of NFATc1, which is critical for the differentiation of osteoclasts, has not been previously investigated on the transcription of either OSCAR or TREM-2 promoter. To study the transcriptional regulation of OSCAR and TREM-2 genes by NFATc1, we analyzed the putative promoter regions of mouse OSCAR and TREM-2 using a program for predicting transcription factor binding sites (TFSEARCH, www.cbrc.jp/research/db/TFSEARCH.html) (Fig. 3, A and B). The transcription initiation site of TREM-2 was determined by 5’ RACE. As expected from the expression patterns during osteoclast differentiation (Fig. 2), the TREM-2 promoter contained no putative NFAT binding site, whereas multiple putative NFAT binding sites were found in the OSCAR promoter. However, the TREM-2 promoter contained one MITF and two PU.1 binding sites (Fig. 3, A and B).

NFATc1 Synergistically Enhances the Activity of the OSCAR Promoter in Combination with PU.1 and MITF—To examine the transcriptional regulation of OSCAR and TREM-2 by NFATc1, luciferase reporter plasmids containing the mouse OSCAR promoter or TREM-2 promoter were constructed and transfected into HEK293T cells. As shown in Fig. 4A, overexpression of NFATc1 alone increased the promoter activity of OSCAR by 16-fold. Previous study had shown that the combination of MITF and PU.1 synergistically activates the promoter activity of OSCAR (30). Thus, we examined whether NFATc1 synergistically acts with MITF and PU.1. Co-transfection of NFATc1, PU.1, and MITF expression vectors caused an increase of ~300-fold in the promoter activity of OSCAR. In contrast, the promoter activity of TREM-2 was not significantly changed either by NFATc1 alone or by co-expression with MITF and PU.1 (Fig. 4B).

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NFATc1 Is Specifically Involved in OSCAR Induction during Osteoclastogenesis—We performed chromatin immunoprecipitation to detect the recruitment of NFATc1 to the OSCAR promoter during osteoclast differentiation. In the presence of M-CSF, mouse BMMs were cultured with or without RANKL and collected at days 0–2. Fig. 5 shows that during RANKL-induced osteoclast differentiation, NFATc1 was recruited to the OSCAR promoter, but not to the TREM-2 promoter, suggesting that OSCAR is the direct transcriptional target gene of NFATc1.

Next, we performed a gene silencing experiment using siRNA and investigated whether knockdown of NFATc1 influences OSCAR and TREM-2 expression. By observing the uptake of Cy3-labeled nonspecific siRNA, transfection efficiency into primary BMMs was estimated to be over 70% (Fig. 6A, left panel). The transfection of siRNA specific for NFATc1 significantly suppressed the expression of the target gene NFATc1 at the mRNA level, compared with the expression level of NFATc1 in cells treated with the same concentration of siRNA specific for GFP (Fig. 6A, right panel). The expression of OSCAR was decreased significantly (Fig. 6B) due to the knockdown of NFATc1. In contrast, TREM-2 mRNA expression level was not influenced by the knockdown of NFATc1 (Fig. 6B).

To provide further evidence that NFATc1 is essential for the induction of OSCAR by RANKL, osteoclast precursor cells derived from NFATc1<sup>+/−</sup> and NFATc1<sup>−/−</sup> cells were cultured with RANKL and M-CSF, and samples harvested at days 0–2 were used for quantitative RT-PCR analysis (Fig. 6C). The expression of OSCAR was barely detectable in NFATc1<sup>−/−</sup> cells compared with the significant induction in NFATc1<sup>+/−</sup> cells. On the other hand, the expression of TREM-2 showed no significant difference between the NFATc1<sup>+/−</sup> and NFATc1<sup>−/−</sup> cells. These results demonstrate that NFATc1 is selectively involved in the induction of OSCAR, and the expressions of OSCAR and TREM-2 are regulated by distinct mechanisms.

**DISCUSSION**

Immunoglobulin-like receptors are a novel class of receptors that are critically involved in the regulation of bone homeostasis (4, 6, 33–35). Initially identified and well studied in the immune system, they have been thought to be important regulators of the immunological processes such as the recognition of self and non-self as well as the initiation of antibody-mediated signaling events (19, 35). Immunoglobulin-like receptors are often composed of activating receptors that associate with ITAM-containing adaptors and inhibitory receptors that contain immunoreceptor tyrosine-based inhibition motif (ITIM) in their cytoplasmic region (17, 36). We have recently shown that the ITAM-harboring adaptors, FcRγ and DAP12, are essential for osteoclast differentiation and determined that the immunoglobulin-like receptors OSCAR and TREM-2 associate with FcRγ and DAP12, respectively (6).

We investigated the immunoreceptors in the context of osteoclastogenesis and discovered that OSCAR and TREM-2 are cooperatively involved in osteoclastogenesis. When both OSCAR-Fc and TREM-2-Fc activation. Thus, the complex containing NFATc1, PU.1, and MITF synergistically contributes to the regulation of OSCAR and TRAP, but not the calcitonin receptor, suggesting that osteoclast-specific genes are regulated by distinct transcriptional complexes involving NFATc1 (see "Discussion").
were added to the co-cultures of BMMs and osteoblasts, the combined inhibitory effect of OSCAR-Fc and TREM-2-Fc was greater than the inhibitory effect of either OSCAR-Fc or TREM-2-Fc alone, although the same amount of Fc fusion protein was added. These results suggest a cooperation of DAP12- and FcRγ/H9253-mediated signals during osteoclastogenesis, which is consistent with the previous results that both FcRγ and DAP12 activate calcium signaling through ITAM in the osteoclast lineage (6).

It is interesting that the expression patterns of OSCAR and TREM-2 are quite different from each other, despite their shared function. OSCAR is induced in the late phase of osteoclast differentiation, whereas TREM-2 expression is relatively constant and not induced by RANKL. It is also worth noting that the expression of the putative ligands for OSCAR and TREM-2 is differentially regulated. In the co-culture system of osteoclast formation, osteotropic factors such as vitamin D₃ and PGE₂ are often used to enhance osteoclast differentiation by inducing RANKL on osteoblasts (5, 37). Flow cytometric analysis of putative OSCAR and TREM-2 ligands using the Fc fusion proteins showed that the OSCAR ligands are induced on osteoblasts stimulated with vitamin D₃ and PGE₂, whereas the TREM-2 ligands are expressed both on stimulated and non-stimulated osteoblasts. These results suggest that OSCAR ligands are inducibly expressed on osteoblasts when RANKL is induced, but TREM-2 ligands are constitutively expressed on osteoblasts regardless of RANKL expression. Taken together with the differential expression of receptors, these results suggest that OSCAR signaling becomes pronounced in the course of osteoclast differentiation, while TREM-2-mediated signaling can be transmitted even at an earlier stage of osteoclast differentiation.

What is the mechanism underlying the differential expression of OSCAR and TREM-2 during osteoclastogenesis? Because NFATc1 plays an important role in the transcriptional activation of osteoclast-specific genes (7–9), we infer that NFATc1 is selectively involved in the transcriptional control of OSCAR. As expected, multiple putative NFAT binding sites are observed in the OSCAR promoter, but there is
no putative NFAT binding site in the TREM-2 promoter. Luciferase assay demonstrated that the OSCAR promoter, but not the TREM-2 promoter, was strongly regulated by NFATc1. PU.1 and MITF have been implicated in the transcriptional regulation of OSCAR (30), and we showed that NFATc1 clearly synergizes with both PU.1 and MITF on the OSCAR promoter. These results suggest that NFATc1, PU.1, and MITF form a complex to cooperatively activate the transcription of OSCAR. Because we have reported that NFATc1, PU.1, and MITF synergistically activate the promoter of cathepsin K, another osteoclast-specific gene (9), it is conceivable that the complex containing NFATc1, PU.1, and MITF may be universally involved in the transcription of osteoclast-specific genes. Therefore, we investigated two other osteoclast marker genes, the promoters of which are regulated by NFATc1 (7, 8). Interestingly, the TRAP promoter is similarly regulated by the synergistic cooperation of NFATc1, PU.1, and MITF, but the calciitonin receptor promoter was not regulated by the same mechanism. Although a number of osteoclast-specific genes are regulated by NFATc1, each gene requires distinct components of the transcriptional machinery, which may ensure the specific spatiotemporal expression.

As we observed the effect of NFATc1 on the activity of the OSCAR promoter in transient overexpression experiments, we further examined the involvement of NFATc1 in the transcriptional control of OSCAR in primary cells. In fact, chromatin immunoprecipitation assay revealed that NFATc1 is recruited to the promoter of OSCAR in the osteoclast precursor cells stimulated with RANKL. Furthermore, the mRNA of OSCAR was barely induced in NFATc1−/− cells, whereas the induction of TREM-2 was not affected (Fig. 6C). This provides the first genetic evidence that NFATc1 is necessary for OSCAR induction, but not for TREM-2 induction.

Because NFATc1 is activated downstream of OSCAR-mediated calcium signaling (6), an interesting positive feedback loop presents itself: NFATc1 induction leads to the transcriptional activation of OSCAR, and calcium signaling, which is mediated by the ITAM of OSCAR-NFATc1. Calcium signaling (6), an interesting positive feedback loop presents itself: not for lacking FcR. An inhibitory effect of OSCAR-Fc and TREM-2-Fc suggests that these Fc fusion proteins may potentially exert therapeutic effects on bone diseases caused by enhanced osteoclast differentiation.

In conclusion, we have shown here that the immunoreceptor OSCAR, but not TREM-2, is transcriptionally regulated by NFATc1. Determining how to regulate the distinct regulatory mechanisms of the specific immunoreceptors expressed on osteoclasts, and their ligands on osteoblasts, has important therapeutic potential in a variety of bone diseases. In this regard, the identification and characterization of the ligands of these receptors are urgent challenges demanding resolution.

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REFERENCES
1. Karsey, G., and Wagner, E. F. (2002) Dev. Cell 2, 389–406
2. Teitelbaum, S. L., and Ross, F. P. (2003) Nat. Rev. Genet. 4, 638–649
3. Boyle, W. J., Simontet, W. S., and Lacey, D. L. (2003) Nature 423, 337–342
4. Takayanagi, H. (2005) J. Mol. Med. 83, 170–179
5. Takahashi, N., Akatsu, T., Udagawa, N., Sasaki, T., Yamaguchi, A., Moseley, J. M., Martin, T. J., and Suda, T. (1988) Endocrinology 123, 2600–2602
6. Koga, T., Iruui, M., Inoue, K., Kim, S., Suenatsu, A., Kohayashi, E., Iwata, T., Ohnishi, H., Matozaki, T., Kodama, T., Taniguchi, T., Takayanagi, H., and Takai, T. (2004) Nature 428, 758–763
7. Takayanagi, H., Kim, S., Koga, T., Nishina, H., Ishikii, M., Yoshida, H., Saiura, A., Isobe, M., Yokochi, T., Inoue, J., Wagner, E. F., Mak, T. W., Kodama, T., and Taniguchi, T. (2002) Dev. Cell 3, 889–901
8. Matsuo, K., Galson, D. L., Zhao, C., Peng, L., Laplace, C., Wang, K. Z., Bachler, M. A., Amano, H., Aharutuni, H., Ishikawa, H., and Wagner, E. F. (2004) J. Biol. Chem. 279, 26475–26480
9. Matsumoto, M., Kogawa, M., Wada, S., Takayanagi, H., Tsuchimoto, M., Katayama, S., Hisatake, T., and Nogi, Y. (2004) J. Biol. Chem. 279, 45969–45979
10. Klee, C. B., Ren, H., and Wang, X. (1998) J. Biol. Chem. 273, 13367–13370
11. Crabtree, G. R., and Olson, E. N. (2002) Cell 109 (suppl.) 567–579
12. Rao, A., Luo, C., and Hogan, P. G. (1997) Annu. Rev. Immunol. 15, 707–747
13. Berridge, M. J., Lipp, P., and Bootman, M. D. (2000) Nat. Rev. Mol. Cell. Biol. 1, 11–21
14. Wong, B. R., Rho, J., Arrom, J., Robinson, E., Orlinick, J., Chao, M., Kalachakov, S., Cayani, E., Bartlett, F. S., 3rd, Frankel, W. N., Lee, S. Y., and Choi, Y. (1997) J. Biol. Chem. 272, 25190–25194
15. Anderson, D. M., Maraskovsky, E., Billingsley, W. L., Dougall, W. C., Tometsko, M. E., Ross, E. R., Teepe, M. C., Dubose, R. F., Cosman, D., and Galibert, L. (1997) Nature 390, 175–179
16. Lanier, L. L., Corliss, B. C., Wu, J., Leong, C., and Phillips, J. H. (1998) Nature 391, 703–707
17. Cambier, J. C. (1995) J. Immunol. 155, 3281–3285
18. Horejsi, V., Zhang, W., and Schraven, B. (2004) Nat. Rev. Immunol. 4, 603–616
19. Lanier, L. L., and Bakker, A. B. (2000) Immunol. Today 21, 611–614
20. Ohtsuka, M., Arase, H., Takeuchi, A., Yamakasi, S., Shina, R., Suenaga, T., Sakurai, D., Yokosuka, T., Arase, N., Ishiwada, M., Kitamura, T., Moriya, H., and Saito, T. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8216–8219
21. Shiratori, I., Ogasawara, K., Saito, T., Lanier, L. L., and Arase, H. (2004) J. Exp. Med. 199, 525–533
22. Mocsai, A., Humphrey, M. B., Van Zijlfe, J. A., Yu, H., Burghardt, A., Spusta, S. C., Mayumdar, S., Lanier, L. L., Lowell, C. A., and Nakamura, M. C. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 6158–6163
23. Kim, N., Takami, M., Rho, J., Josein, R., and Choi, Y. (2002) J. Exp. Med. 195, 201–209
24. Kubagawa, H., Burrows, P. D., and Cooper, M. D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5261–5266
25. Daws, M. R., Lanier, L. L., Seaman, W. E., and Ryan, J. C. (2001) Eur. J. Immunol. 31, 783–791
26. Dietrich, J., Cella, M., Seiffert, M., Buhring, H. J., and Colonna, M. (2000) J. Immunol. 164, 9–12
27. dela Pompa, J. L., Timmerman, L. A., Takimoto, H., Yoshida, H., Elia, A. J., Samper, E., Potter, J., Wakeham, A., Marengere, L., Langille, B. R., Crabtree, G. R., and Mak, T. W. (1998) Nature 392, 182–186
28. Takayanagi, H., Kim, S., Matsuo, K., Suzuki, H., Suzuki, T., Sato, K., Yokochi, T., Oda, H., Nakamura, K., Iida, N., Wagner, E. F., and Taniguchi, T. (2002) Nature 416, 744–749
29. Takayanagi, H., Ogasawara, K., Hida, S., Chiba, T., Murata, S., Sato, K., Takaoka, A., Yokochi, T., Oda, H., Tanaka, K., Nakamura, K., and Taniguchi, T. (2000) Nature 408, 600–605
30. So, H., Rho, J., Jeong, D., Park, R., Fisher, D. E., Ostrowski, M. C., Choi, Y., and Kim, N. (2003) J. Biol. Chem. 278, 24209–24216
31. DeKoter, R. P., Lee, H. J., and Singh, H. (2002) Immunity 16, 297–309
32. Matsumoto, M., Hisatake, K., Nogi, Y., and Tsujimoto, M. (2001) J. Biol. Chem. 276, 33086–33092
33. Paloneva, J., Mandelin, J., Kiialainen, A., Bohling, T., Prudlo, J., Hakola, P., Haltia, M., Konttinen, Y. T., Peltonen, L. (2003) J. Exp. Med. 198, 669–675
34. Cella, M., Buonsanti, C., Strader, C., Kondo, T., Salmaggi, A., and Colonna, M. (2003) J. Exp. Med. 198, 645–651
35. Takai, T. (2002) Nat. Rev. Immunol. 2, 580–592
36. Long, E. O. (1999) Annu. Rev. Immunol. 17, 875–904
37. Karsenty, G. (1999) Genes Dev. 13, 3037–3051
38. Eissner, G., Kolch, W., and Scheurich, P. (2004) Cytokine Growth Factor Rev. 15, 353–366