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Induction and Activation of the Transcription Factor NFATc1 (NFAT2) Integrate RANKL Signaling in Terminal Differentiation of Osteoclasts

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Summary

Signaling by RANKL is essential for terminal differentiation of monocytes/macrophages into osteoclasts. The TRAF6 and c-Fos signaling pathways both play important roles downstream of RANKL. We show here that RANKL selectively induces *NFATc1* expression via these two pathways. RANKL also evokes Ca^{2+} oscillations that lead to calcineurin-mediated activation of NFATc1, and therefore triggers a sustained NFATc1-dependent transcriptional program during osteoclast differentiation. We also show that *NFATc1*-deficient embryonic stem cells fail to differentiate into osteoclasts in response to RANKL stimulation, and that ectopic expression of NFATc1 causes precursor cells to undergo efficient differentiation without RANKL signaling. Thus, NFATc1 may represent a master switch for regulating terminal differentiation of osteoclasts, functioning downstream of RANKL.

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Introduction

Cell differentiation is an aspect central to the development and homeostatic maintenance of the multicellular organism, and much has been done to understand the mechanisms underlying this process in many biological systems. Skeletal development depends on harmonious differentiation of constituent cells, including chondrocytes, osteoblasts, and osteoclasts; the defect of each lineage causes various diseases manifesting skeletal malformation (reviewed in Karsenty and Wagner, 2002; Teitelbaum, 2000). One typical example in maintaining the homeostasis of the skeletal system is bone remodeling, in which bone is constantly renewed by the balanced action of osteoblastic bone formation and osteoclastic bone resorption (reviewed in Manolagas, 2000). In fact, tipping this balance in favor of the latter leads to pathological bone resorption, as seen in autoimmune arthritis, periodontitis, postmenopausal osteoporosis, Paget's disease, and bone tumors (Rodan and Martin, 2000; Takayanagi et al., 2000a). Thus, the investigation of the regulatory mechanism of osteoclast differentiation is important in the understanding of the physiology and pathology of the skeletal system.

Bone marrow-derived monocyte/macrophage precursor cells (BMMs) of hematopoietic origin develop into osteoclasts through cell-cell signaling with mesenchymal cells including osteoblasts (reviewed in Suda et al., 1999; Chambers, 2000). The essential signaling molecules for osteoclast differentiation include RANKL (receptor activator of NF-κB ligand) and M-CSF (macrophage colony stimulating factor). RANKL is expressed on osteoblasts and induces the signaling essential for precursor cells to differentiate into osteoclasts (Yasuda et al., 1998; Lacey et al., 1998; Kong et al., 1999; Theill et al., 2002), whereas M-CSF, secreted by osteoblasts, provides the survival signal to these cells (Yoshida et al., 1990; Lagasse and Weissman, 1997).

The signaling mechanism of RANKL has been extensively studied. Binding of RANKL to its receptor RANK, expressed in BMMs, results in the recruitment of TRAF family proteins such as TRAF6, which is linked to the NF-κB and Jun N-terminal kinase (JNK) pathways (Wong

et al., 1998; Naito et al., 1999; Kobayashi et al., 2001). Additionally, RANKL induces the expression of c-Fos by an as yet unknown mechanism (Matsuo et al., 2000; Wagner and Karsenty, 2001). In addition, negative regulators of RANKL signaling have been reported, which include osteoprotegerin (OPG), a soluble "decoy" receptor for RANKL (Simonet et al., 1997), and interferon (IFN)-β, a negative regulator of c-Fos expression (Takayanagi et al., 2002). The essential role of these molecules in maintaining bone homeostasis is fully underscored by gene disruption studies (reviewed in Karsenty and Wagner, 2002; Alliston and Derynck, 2002).

Cell differentiation often depends on extracellular stimuli that transmit signals to the cell interior so as to induce the necessary gene transcription program(s). In each developmental stage, transcription factors coordinate to induce target genes essential for the survival and maturation of the lineage. During the differentiation process of hematopoietic stem cells to osteoclasts with bone-resorbing activity, transcription factors such as PU.1, c-Fos, NF-κB, and MITF play a critical and specific role (Teitelbaum, 2000). Briefly, PU.1 is critical for the early "determination" phase of bone marrow progenitor cells to myeloid cells that respond to M-CSF (Tondravi et al., 1997), and M-CSF-activated MITF is important for Bcl-2 induction and survival of this lineage (Weilbaecher et al., 2001; McGill et al., 2002). Both NF-κB and c-Fos function downstream of RANKL signaling, and there is genetic evidence that these factors play an essential function in the developmental stage from monocyte/macrophage precursors to mature osteoclasts (Grigoriadis et al., 1994; Franzoso et al., 1997).

To date, little is known about how RANKL, but not other cytokines that activate similar pathways, specifically induces terminal differentiation of osteoclasts through a specific transcriptional program. Several lines of evidence suggest hitherto unrecognized, critical events downstream of the TRAF6 and c-Fos pathways. First, the defect in osteoclast differentiation of *TRAF6*-deficient cells cannot be fully rescued by expressing a TRAF6 mutant, by which NF-κB activation still occurs (Kobayashi et al., 2001). Although the essential role of NF-κB in osteoclastogenesis has been well documented by gene disruption studies, this observation suggests the presence of an additional function(s) of TRAF6. Second, a similar defect in cells lacking c-Fos was shown to be rescued by the overexpression of one of its target genes, *Fra-1*, but a recent conditional gene targeting study revealed that *Fra-1* is not required for osteoclastogenesis (R. Eferl and E.F.W., unpublished data). Finally, the TRAF6 and c-Fos pathways, as well as other RANKL-activated molecules such as ERK, p38, and Akt, are also activated by various other cytokines, such as IL-1 (Cao et al., 1996; Reddy et al., 1997; Ninomiya-Tsuji et al., 1999), which are not capable of inducing osteoclast differentiation. These observations in toto suggest that RANKL signaling activates an as yet unknown pathway(s) that specifically invokes the transcriptional program leading the cells to undergo terminal differentiation.

To gain insight into the mechanism of the RANKL-specific induction of the osteoclast differentiation program, we took a genome-wide screening approach to identify genes specifically induced by RANKL, but not by IL-1. In particular, we focused on transcription factors that may induce the conjectured gene transcription program during osteoclast differentiation. In this screening, we found that *NFATc1*, also referred to as *NFAT2* or *NFATc* (Rao et al., 1997), a member of the NFAT (nuclear factor of activated T cells) family of transcription factor genes, is the most strongly induced transcription factor gene following RANKL stimulation. Transcription factors of the NFAT family, originally discovered in the context of the activation of the immune system (Shaw et al., 1988), are also involved in the function and development of diverse cells in other biological systems, including cardiovascular and musculoskeletal systems in vertebrates, where they are under control of the Ca^{2+}-regulated phosphatase, calcineurin (Berridge et al., 2000; Crabtree and Olson, 2002). However, their involvement in bone remodeling, wherein RANKL-induced osteoclast differentiation plays a central role, is as yet unknown.

We provide evidence that NFATc1 expression is dependent on both the TRAF6 and c-Fos pathways. We also show that RANKL stimulation, but not IL-1 stimulation, results in the induction of Ca^{2+} oscillation, which would contribute to the sustained activation of NFATc1 via a calcineurin-dependent mechanism. While the activated NFATc1 induces a number of genes involved in cell differentiation, NFATc1 also acts on its own gene to amplify the NFATc1-mediated transcriptional program. We provide data indicating the essential role of the *NFATc1* gene using a newly developed system for osteoclast differentiation in embryonic stem (ES) cell culture. We further demonstrate that ectopic expression of NFATc1 causes the BMMs to undergo efficient osteoclast differentiation in the absence of RANKL stimulation. Finally, we provide evidence that NFATc1 cooperates with c-Fos on the promoter of osteoclast-specific genes. Collectively, our results show that NFATc1 plays an integral role in the RANKL-induced transcriptional program during the terminal differentiation of osteoclasts. We will discuss our findings in the light of the intricate signaling network during RANKL-specific and NFATc1-dependent osteoclast differentiation.

Results

Identification of mRNAs Selectively Induced by RANKL

To identify genes selectively activated by RANKL, we performed a genome-wide screening of mRNAs in the BMMs with or without RANKL stimulation, using an oligonucleotide array (Affymetrix GeneChip). When BMMs, obtained by culturing bone marrow cells in the presence of M-CSF for 48 hr, were stimulated by recombinant RANKL in the presence of M-CSF for 72 hr, as many as 30% of the cells undergo differentiation into osteoclasts with bone-resorbing activity (Takayanagi et al., 2000b). Total RNA was extracted 2, 24, and 72 hr after RANKL stimulation of BMMs. At the same time points, total RNA was also isolated from mock-stimulated BMMs or BMMs stimulated by IL-1 instead of RANKL, in order to compare mRNA expression profiles. As shown in Figure 1A, mRNAs known to be strongly expressed in osteoclasts, that is, those of *tartrate-resistant acid phosphatase* (*TRAP*), *calcitonin receptor*, *cathepsin K*, *carbonic anhydrase II* (*CAII*), *and matrix metalloprotease* (*MMP*)-*9*, were specifically induced by RANKL, confirming the validity of our screening protocol.

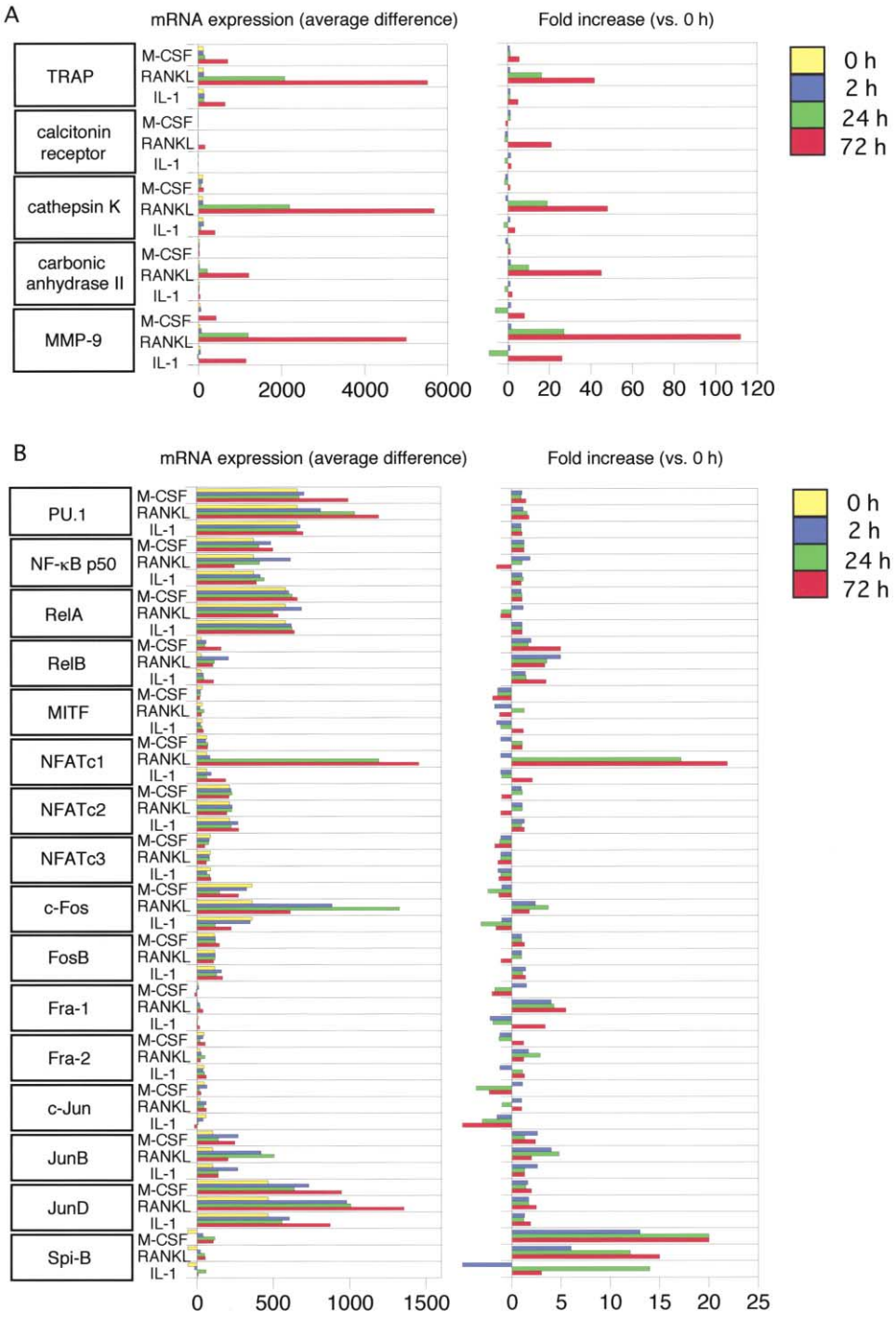

Figure 1. Genome-Wide Screening of RANKL-Inducible Genes by GeneChip

(A) Evaluation of the screening system by the expression profiles of the genes that are known to be highly expressed in osteoclasts. As summarized here, these genes were specifically and strongly induced by RANKL. The left panel shows mRNA expression level (indicated as average difference) and the right panel shows the fold increases in the mRNA expression level compared with that in unstimulated cells. GeneChip analysis was repeated several times, and yielded similar results; a representative set of data is shown.

(B) A list of the genes encoding transcription factors that are highly induced by RANKL or are suggested to act downstream of the RANKL signaling pathway. The level and the fold induction of the mRNA expression were most notable for *NFATc1*.

To gain insight into the transcriptional program induced by RANKL, we focused on the induction of genes encoding transcription factors. As shown in Figure 1B, the most notable gene was *NFATc1*. In fact, NFATc1 mRNA was not induced by M-CSF alone or by additional stimulation by IL-1, and such induction was not observed in mRNAs for the other NFAT members. mRNAs for the other transcription factors such as c-Fos and Fra-1, which are involved in osteoclast differentiation, are also selectively induced by RANKL, albeit at much

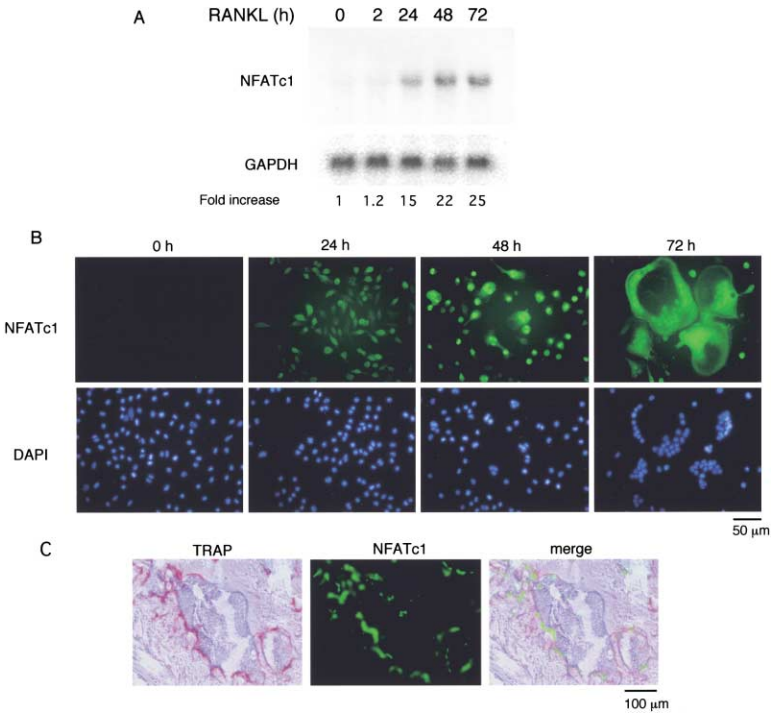

Figure 2. Expression of NFATc1 mRNA and Protein during and after Osteoclast Differentiation

(A) RNA blotting analysis of NFATc1 mRNA. Total RNA was extracted from BMMs stimulated with RANKL/M-CSF for the indicated periods. Five micrograms of total RNA was applied to each lane.

(B) Immunostaining of NFATc1 protein in BMMs stimulated with RANKL/M-CSF. NFATc1 is initially induced in the cytoplasm, and gradually translocalizes into the nucleus. Note the marked NFATc1 expression in multinucleated osteoclasts. Nuclei are stained by DAPI in the lower panel.

(C) In vivo expression of NFATc1 in osteoclasts. Serial sections of proximal tibia from 8-week-old C57BL/6 mice were stained by TRAP and hematoxylin or immunostained by the anti-NFATc1 antibody followed by the Alexa 488-conjugated secondary antibody.

lower levels (Figure 1B). Consistent with this result, RNA blotting analysis revealed that robust induction of NFATc1 mRNA occurred in BMMs, culminating 48 hr after RANKL stimulation. At this time point, mRNA expression was more than 20-fold of the basal level, and was sustained thereafter (Figure 2A).

Expression of NFATc1 and Its Localization during Osteoclast Differentiation

We next examined the expression and cellular localization of the NFATc1 protein in RANKL-stimulated BMMs undergoing terminal differentiation into multinucleated osteoclasts. As shown in Figure 2B, immunofluorescence staining revealed that the NFATc1 protein expression level increased continuously, followed by its nuclear translocation, during the RANKL-induced differentiation of BMMs to osteoclasts. In fact, the expression of NFATc1 is dominant in the cytoplasm at 24 hr after RANKL stimulation, but its nuclear translocation becomes apparent at 48 hr, when cellular fusion characteristic of osteoclasts initiates (Figure 2B). At this time point, cells positive for TRAP, diagnostic for osteoclasts, can already be observed (data not shown). At 72 hr after RANKL stimulation, essentially all the nuclei of the multinucleated cells (MNCs) fully expressing the TRAP marker became strongly positive for NFATc1 (Figure 2B). Thus, there is a close correlation between the kinetics of RANKL-induced increase in NFATc1 expression level in nuclei and differentiation of BMMs to osteoclasts in vitro. Consistently, the in vivo histological examination of the bone tissue also revealed that NFATc1 is specifically expressed in TRAP-positive cells, characteristic of osteoclasts (Figure 2C). As expected, NFATc1-positive cells also expressed cathepsin K or MMP-9 (data not shown). On the other hand, expression of other NFAT members such as NFATc2 and NFATc3 was undetectable in these cells (see Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/3/6/889/DC1), further suggesting the role of NFATc1 in vivo.

Requirement of the TRAF6 and c-Fos Pathways in RANKL-Induced NFATc1 Expression

In view of the previous results demonstrating that the TRAF6 and c-Fos signaling pathways are both critical for osteoclast differentiation, it is an interesting issue whether NFATc1 induction is linked to either or both of these pathways. We therefore examined the RANKL-induced expression of NFATc1 mRNA and protein in osteoclast precursor cells derived from mice lacking TRAF6 or c-Fos (*TRAF6*$^{-/-}$ or *Fos*$^{-/-}$ mice). As shown in Figure 3A, the induction of NFATc1 mRNA by RANKL was abolished in the cells lacking c-Fos, and it also decreased significantly (to approximately one-fourth) in the cells lacking TRAF6. As expected, the NFATc1 protein was not detectable in the *Fos*$^{-/-}$ cells after RANKL stimulation, as revealed by immunofluorescence staining. Notably, the NFATc1 protein was also barely detectable in the *TRAF6*$^{-/-}$ cells (Figure 3B). Although the detailed mechanisms underlying this process need further clarification, these results indicate that the induction of NFATc1 by RANKL is dependent on the two signaling pathways bifurcated from RANK.

Induction of Ca^{2+} Signaling in RANKL-Stimulated Cells

The activities of transcription factors of the NFAT family are regulated by the calcium (Ca^{2+})/calmodulin-dependent calcineurin, a serine/threonine phosphatase, which allows nuclear translocation of these transcription factors (Rao et al., 1997; Berridge et al., 2000; Crabtree

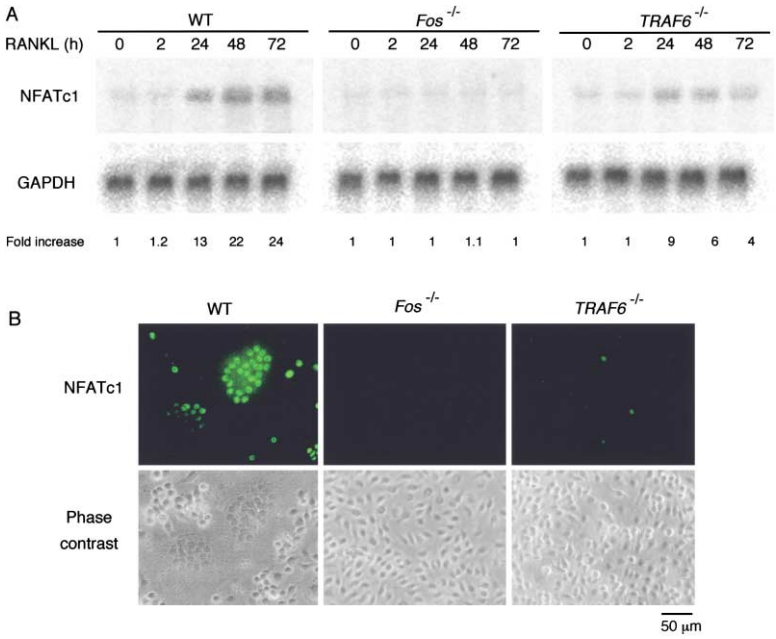

Figure 3. NFATc1 Expression Is Dependent on the TRAF6 and c-Fos Pathways

(A) Expression of NFATc1 mRNA in *Fos*$^{-/-}$ and *TRAF6*$^{-/-}$ cells stimulated with RANKL and M-CSF (RNA blotting analysis). NFATc1 mRNA was not induced in *Fos*$^{-/-}$ cells, and the induction was significantly decreased in *TRAF6*$^{-/-}$ cells.

(B) Expression of NFATc1 protein in *Fos*$^{-/-}$ and *TRAF6*$^{-/-}$ cells stimulated with RANKL and M-CSF. M-CSF-treated precursor cells derived from splenocytes were further stimulated with RANKL/M-CSF for 3 days, and were immunostained with the anti-NFATc1 antibody. Multinucleated osteoclasts were formed only in WT precursor cells, and these multinucleated cells strongly express NFATc1. NFATc1 protein expression was absent or very weak in the RANKL-stimulated *Fos*$^{-/-}$ and *TRAF6*$^{-/-}$ cells, respectively.

and Olson, 2002). Since NFATc1 induced by RANKL undergoes efficient nuclear translocation (Figure 2B), one may envisage the Ca^{2+}-dependent activation of calcineurin by RANKL. Interestingly, a sustained Ca^{2+} oscillation was observed in BMMs stimulated by RANKL, which was not observed in IL-1-stimulated BMMs (Figure 4A). Neither Ca^{2+} oscillation nor a Ca^{2+} spike was induced immediately after RANKL stimulation. Rather, the RANKL-induced Ca^{2+} oscillation initiated as late as 24 hr after RANKL stimulation, that is, when NFATc1 induction becomes notable; the oscillation was sustained thereafter, provided that RANKL was present (data not shown; see the legend for Figure 4A). It may be worth noting that Ca^{2+} ionophores such as PMA and ionomycin, instead of RANKL, caused neither NFATc1 induction nor osteoclast differentiation in the presence of M-CSF alone or in combination with IL-1 (data not shown). This observation suggests that sustained Ca^{2+} oscillation, rather than transient activation of a Ca^{2+} spike, is necessary for the sustained NFATc1 activation during osteoclastogenesis.

Activation of NFATc1 and Autoamplification of NFATc1-Mediated Transcription

The above results showing the induction of Ca^{2+} oscillation by RANKL stimulation suggest its critical involvement during the terminal differentiation of osteoclasts, wherein NFATc1 would be constantly activated through the Ca^{2+}-dependent calcineurin pathway. This notion is supported by the following two observations. First, BAPTA-AM, a selective Ca^{2+} chelator, strongly inhibited RANKL-induced osteoclastogenesis as well as induction of NFATc1 (Figure 4B). Second, FK506 and cyclosporin A (CsA), the specific inhibitors of calcineurin (Liu et al., 1991), also inhibited RANKL-induced osteoclast differentiation from BMMs in a dose-dependent manner (Figure 4C). The same inhibitory effect was observed in RAW 264.7 cells, a macrophage cell line that differentiates into osteoclasts in response to RANKL alone (data not shown). Interestingly, FK506 not only inhibited the nuclear translocation of NFATc1, but also suppressed the increase in NFATc1 expression levels (Figure 4D). RNA blotting analysis shows that induction of NFATc1 mRNA was also inhibited by FK506 (Figure 4E). Our results suggest in toto that RANKL stimulation induces the calcineurin-dependent autoamplification of NFATc1 expression, through NFATc1-dependent *NFATc1* gene transcription, so as to sustain the NFATc1-mediated gene transcription program during the terminal differentiation of osteoclasts (see also below).

Essential Role of NFATc1 in RANKL-Induced Osteoclast Differentiation

The above observations strongly suggest that NFATc1, which is induced and activated by RANKL, is essential for osteoclast differentiation. In order to address this issue further, we examined whether a loss-of-function mutation in *NFATc1* alleles affects the differentiation of osteoclasts. Since mice carrying such a mutation are lethal at day 14.5 of gestation (de la Pompa et al., 1998; Ranger et al., 1998), we developed a new, feeder cell-independent culture system of ES cells for an osteoclast formation assay. In this system, ES cells first differentiate into monocyte/macrophage progenitor cells in the presence of M-CSF, and these cells then undergo osteoclast differentiation by RANKL stimulation (Figure 5A). To maintain the microenvironments suitable for hematopoietic cell development, we performed a three-dimensional culture by formation of an embryoid body (EB; Hidaka et al., 1999). After a 2 day stimulation of the EBs by M-CSF, the development of monocyte/macrophage progenitor cells was observed, and these cells were enzymatically dispersed and seeded into new culture dishes. When these cells were stimulated by RANKL in

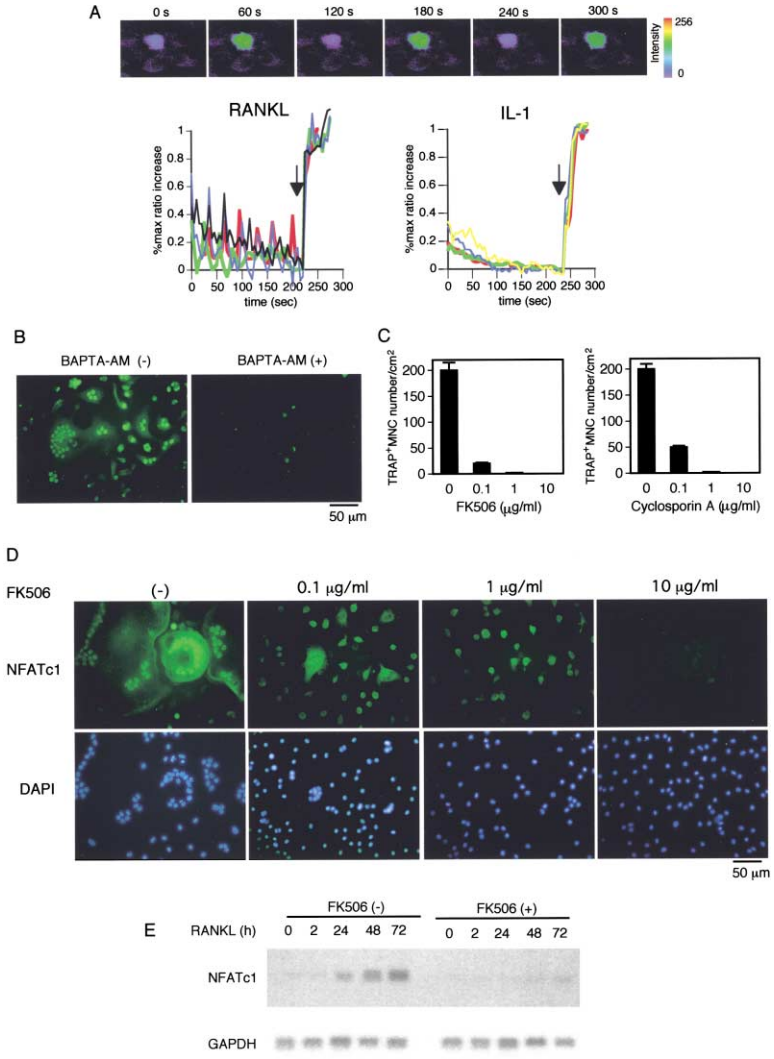

Figure 4. Regulation of NFATc1 by Calcineurin and Ca^{2+} Signaling

(A) Ca^{2+} oscillation observed in RANKL-treated BMMs. BMMs were incubated with RANKL or IL-1 in the presence of M-CSF for 72 hr. Oscillations were observed about 24–72 hr after RANKL stimulation. The upper panel shows an example of successive pseudocolored Ca^{2+} images of a cell treated with RANKL for 24 hr. Note that the cell loaded with fluo-4 shows oscillation with a frequency of about a 2 min interval. The lower panel shows traces of $[Ca^{2+}]i$ change in single cells treated with RANKL (left) or IL-1 (right) for 72 hr. $[Ca^{2+}]i$ change in cells coloaded with fluo-4 and Fura Red were estimated as the ratio of fluorescence intensity of the fluo-4 to Fura Red, and the percent maximum ratio increase from the baseline was plotted with an interval of 5 s. Maximum ratio increase was obtained with the addition of 10 μM ionomycin at the end of each experiment, indicated by an arrow. Each color indicates a different cell in the same field, and $[Ca^{2+}]i$ in only RANKL-treated cells are oscillating.

(B) The calcium chelator, BAPTA-AM, suppresses RANKL-induced osteoclastogenesis in BMMs. Note that *NFATc1* induction is also severely suppressed.

(C) Suppressive effects of calcineurin inhibitors, FK506 and cyclosporin A, on RANKL-induced osteoclastogenesis in BMMs. Formation of TRAP-positive multinucleated cells ($TRAP^+$ MNCs) was inhibited dose dependently.

(D) FK506 inhibits RANKL-induced nuclear translocalization and enhanced expression of NFATc1 in a dose-dependent manner. Nuclear localization of NFATc1 was strongly inhibited by as low as 0.1 μg/ml, accompanied with significant reduction in the osteoclast number as shown in (C).

(E) Inhibition of RANKL-activated induction of NFATc1 mRNA by FK506 (10 μg/ml); RNA blotting analysis. See text and Experimental Procedures for details.

the presence of M-CSF for 4 days, differentiation of osteoclasts was observed, with an efficiency of about 50% compared to that of BMMs (data not shown), based on the TRAP-positive and multinucleated phenotypes and the bone-resorbing activity on dentine slices (Figure 5B).

We then subjected *NFATc1*$^{-/-}$ ES cell lines, generated from *NFATc1*$^{+/-}$ ES cells by selection at a high G418 concentration (Yoshida et al., 1998), to the osteoclast differentiation assay. The M-CSF-dependent generation of monocyte/macrophage precursor cells positive for CD11b (Mac-1) or nonspecific esterase (NSE) was observed in *NFATc1*$^{-/-}$ ES cells at a level similar to that in the case of parental *NFATc1*$^{+/-}$ ES cells (Figure 5C). However, these mutant cells failed to differentiate further into osteoclasts by RANKL stimulation (Figure 5B). The expression levels of c-Fms (M-CSF receptor) and RANK on *NFATc1*$^{-/-}$ precursor cells were comparable to that on *NFATc1*$^{+/-}$ precursor cells (data not shown). These results collectively suggest that the *NFATc1* gene is indispensable, and hence cannot be replaced by other *NFAT* gene members at the stage when monocyte/macrophage precursor cells, developed from ES cells, undergo further differentiation into osteoclasts following RANKL stimulation.

Induction of Osteoclast Differentiation by Ectopic Expression of NFATc1

In order to further investigate the role of NFATc1, we also examined the effect of an ectopic NFATc1 expression in BMMs. We constructed a retrovirus vector, pMX-NFATc1-IRES-EGFP, which is engineered to express both NFATc1 and green fluorescence protein (GFP). Then, BMMs were infected with the NFATc1-expressing virus (denoted as pMX-NFATc1 in Figure 6) or other viruses expressing either c-Fos (pMX-c-*fos*-IRES-EGFP) or c-Jun (pMX-c-*jun*-IRES-EGFP), or with a control virus (pMX-IRES-EGFP) (see Experimental Procedures for details). Surprisingly, the expression of NFATc1 in BMMs caused efficient induction of osteoclast differentiation even without RANKL stimulation (Figures 6A and 6B). Actually, as many as about 70% of the cells positive for

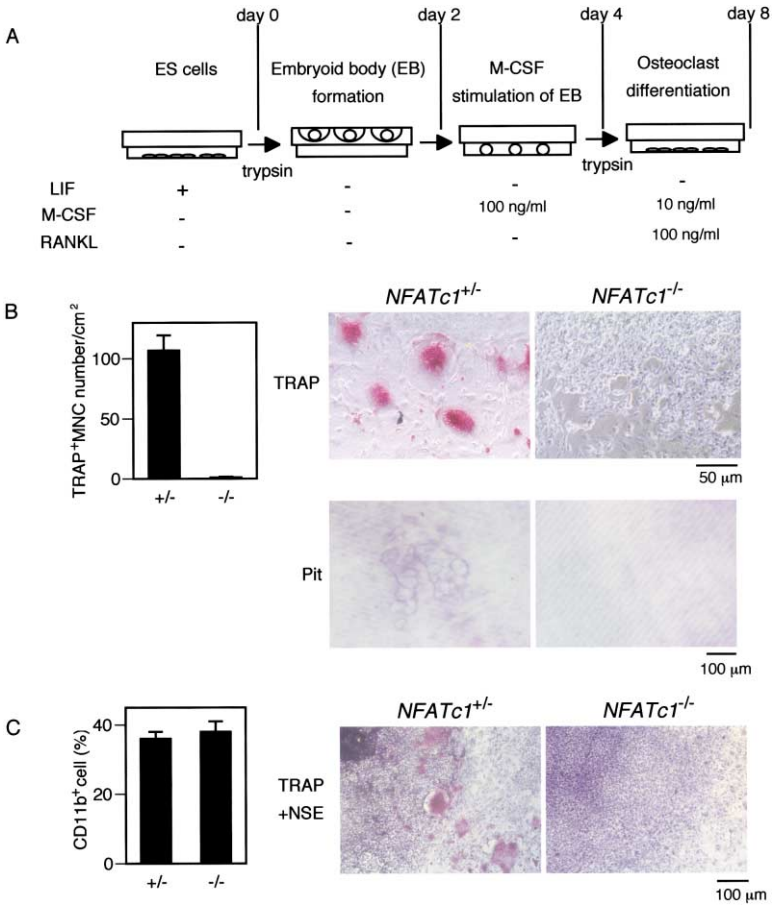

Figure 5. Requirement of NFATc1 for ES Cells Undergoing Osteoclast Differentiation

(A) A schematic illustration of the ES cell culture system for generating osteoclasts. Under LIF-depleted conditions, embryoid bodies (EBs) were formed by the hanging drop method. These EBs were stimulated by 100 ng/ml M-CSF for 2 days. The EBs were then treated with trypsin to obtain single cells, which were reseeded and cultured for 4 days in the presence of 100 ng/ml RANKL and 10 ng/ml M-CSF.

(B) ES cells lacking NFATc1 are defective in osteoclast formation. Formation of $TRAP^+$ MNCs in *NFATc1*$^{+/-}$ ES cell cultures on day 8 (left), and the absence of $TRAP^+$ MNCs in *NFATc1*$^{-/-}$ ES cell cultures (right) are shown. *NFATc1*$^{+/-}$ ES cells underwent differentiation into $TRAP^+$ cells, which are multinucleated (upper) and have bone-resorbing activity on dentine slices (lower). As expected, these MNCs highly express NFATc1 (not shown).

(C) Differentiation of *NFATc1*$^{+/-}$ and *NFATc1*$^{-/-}$ ES cells into monocyte/macrophage precursor cells. $CD11b$ is equally expressed on the cells obtained after M-CSF stimulation in EBs on day 4 (left). Nonspecific esterase $(NSE)^+$ colonies are also equally formed in both cultures (right, day 8).

GFP underwent differentiation into $TRAP^+$ cells, which have bone-resorbing activity (Figure 6B; data not shown). On the other hand, only 7% and 1% of the GFP-positive cells underwent differentiation into $TRAP^+$ cells by c-Fos and c-Jun expression, respectively (Figure 6A). As expected from the data in Figure 3 showing the requirement of both c-Fos and TRAF6 pathways for efficient NFATc1 expression, NFATc1 expression induced by c-Fos-expressing virus remained very low (Figure 6C).

Thus, the ectopic NFATc1 expression causes efficient osteoclast differentiation even in the absence of RANKL. Interestingly, this NFATc1-driven osteoclast differentiation is partially resistant to FK506 (Figure 6D; left panel). In addition, the thus expressed NFATc1 is detected in the nuclei of those cells that underwent differentiation despite the presence of FK-506 (Figure 6D; right panel). These observations are distinct from those of the RANKL-stimulated cells, in which FK506 completely inhibits these events at the same concentration (Figure 4C). Furthermore, in the BMMs expressing the virus-encoded NFATc1, induction of NFATc1 mRNA from the endogenous *NFATc1* gene is detected (Figure 6E), indicating that the ectopic NFATc1 expression results in the NFATc1-dependent *NFATc1* gene induction.

Transcriptional Targets and Partners of NFATc1

Genes such as *TRAP*, *calcitonin receptor*, *cathepsin K*, *CAII*, and *MMP-9*, which are specifically induced during the terminal differentiation of osteoclasts, contain multiple NFAT and AP-1 binding sites (Reddy et al., 1995; Anusaksathien et al., 2001; Motyckova et al., 2001; David et al., 2001). NFAT is known to cooperate with AP-1 by forming a complex (Chen et al., 1998; Macián et al., 2000, 2001). Since NFATc1 and c-Fos, one of the major components of AP-1, are both induced by RANKL, we reasoned that NFATc1 and c-Fos might cooperate with each other on these osteoclast-specific promoters. We then examined activation of the *TRAP* promoter, which is characterized for its NFAT and AP-1 binding sites (Reddy et al., 1995) by a transient assay in 293T cells. pTRAP-luc, in which the *TRAP* promoter is linked to the luciferase reporter gene, was cotransfected with either an *NFATc1* cDNA expression vector (Sherman et al., 1999), *Fos* cDNA expression vector, or both.

As shown in Figure 6F (lower panel), expression of NFATc1 caused a significant induction of luciferase activity. Although c-Fos alone had little effect on this promoter, coexpression of NFATc1 and c-Fos had a synergistic effect on luciferase expression. To investigate the critical role of the interaction between these transcription factors, we constructed a cDNA expression vector for an NFATc1 mutant (NFATc1RL) that would affect its interaction with c-Fos (see Chen et al., 1998; Macián et al., 2000). Coimmunoprecipitation experiments revealed that the interaction of NFATc1 with c-Fos is markedly enhanced by coexpressing the two cDNAs, but such

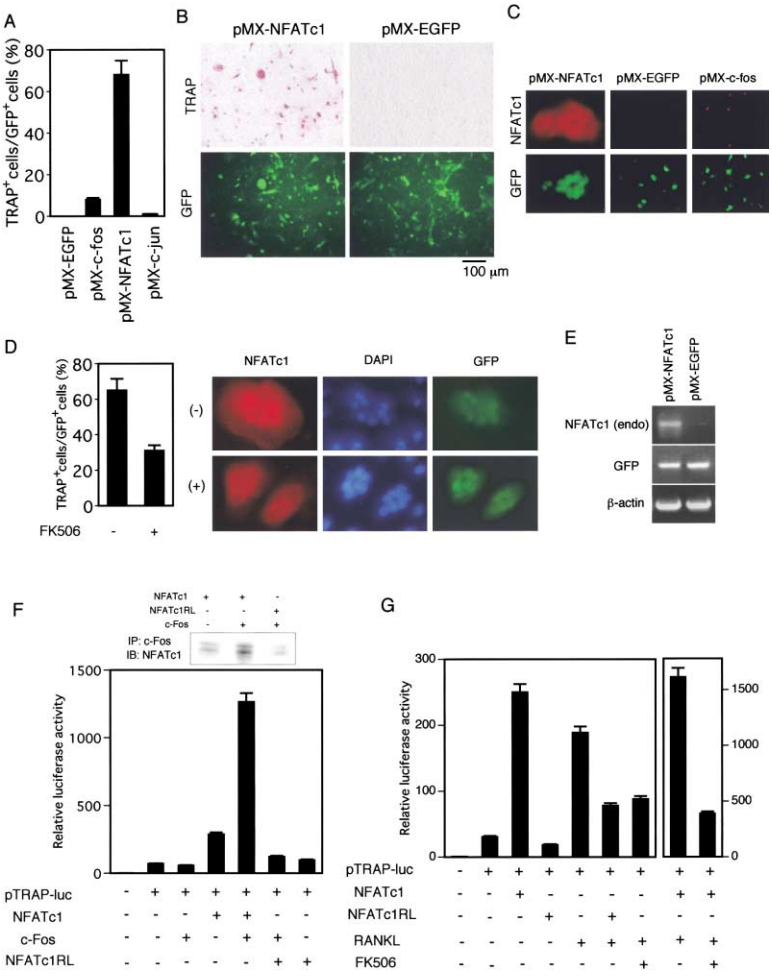

Figure 6. Induction of Osteoclastogenesis and Promoter Activation by NFATc1

(A) Retroviral expression of NFATc1 induces osteoclasts in the absence of RANKL. Induction efficiency is indicated as a ratio of $TRAP^+$ cell number to the number of transfected cells positive for GFP. These $TRAP^+$ cells have bone resorption activity on dentine slices (not shown).

(B) TRAP staining of BMMs transfected with either pMX-NFATc1 or pMX-EGFP. Note that the $TRAP^+$ cells are multinucleated in pMX-NFATc1-transfected cells (see also [C] and [D], upper right panel).

(C) NFATc1 expression in BMMs transfected with pMX-NFATc1, pMX-EGFP, or pMX-c-*fos*. Note that transfection with pMX-NFATc1 results in NFATc1 expression at a level much higher than that with pMX-c-*fos*. See text for details.

(D) Effect of FK506 (1 μg/ml) on the osteoclastogenesis induced by ectopic NFATc1 expression and cellular localization of the NFATc1 protein. FK506 partially inhibited the retrovirus-mediated osteoclastogenesis (left). Alexa 594-stained NFATc1 is localized in the nuclei (stained with DAPI) as well as in the cytoplasm in NFATc1 virus-infected cells. Even in the presence of FK506, retrovirus-induced NFATc1 is still detectable in the nuclei of osteoclasts, albeit at lower levels (right).

(E) Induction of endogenous *NFATc1* gene by virally expressed NFATc1. Total RNA was extracted from BMMs 5 days after transfection with NFATc1 or EGFP virus. RT-PCR primers were designed to detect the mRNA transcript from the endogenous *NFATc1* gene, but not the virus-derived cDNA. In this figure, endo denotes endogenously expressed mRNA .

(F) NFATc1 cooperates with c-Fos in the activation of the *TRAP* promoter. Coexpression of the wild-type NFATc1 and c-Fos results in a synergistic activation of the *TRAP* promoter in 293T cells (lower panel). On the other hand, such activation was not seen with an NFATc1 mutant defective in forming a complex with c-Fos (NFATc1RL) (see upper panel).

(G) Activation of the *TRAP* promoter by RANKL in RAW 264.7 cells. RANKL stimulation causes activation of the promoter, and this activation is inhibited by FK506 or by coexpression of the NFATc1RL mutant (left). Furthermore, synergistic activation of the promoter is observed when the cells were cotransfected with the reporter gene and NFATc1-expressing vector, and then stimulated with RANKL (right). This activation is also inhibited by FK-506.

enhancement was not observed with the NFATc1 mutant, suggesting that this mutant has a defect in forming a complex with c-Fos (Figure 6F, upper panel). As shown in Figure 6F, this NFATc1 mutant was less effective in the activation of the promoter compared to the wild-type NFATc1 and, more importantly, coexpression of c-Fos had virtually no effect. Using the same reporter construct, we found that RANKL stimulation caused the activation of this promoter in RAW 264.7 cells. This activation is inhibited by NFATc1RL and by FK506, indicating that NFATc1 induced by RANKL is indeed transcriptionally active (Figure 6G, left panel). Furthermore, when the cells were cotransfected with the reporter gene and NFATc1-expressing vector and then stimulated with RANKL, the promoter was synergistically activated, and this activation was also inhibited by FK-506 (Figure 6G, right panel). Similar results were observed with the *calcitonin receptor* promoter, which also contains NFAT and AP-1 binding sites (data not shown). These results also lend support to the notion that RANKL-induced NFATc1 cooperates with the similarly induced c-Fos, perhaps in the context of AP-1, in order to activate the transcription of osteoclast-specific genes.

Discussion

Induction of *NFATc1* by the RANKL/RANK-Associated Signaling Pathways

RANKL signaling is known to be essential for osteoclast differentiation, at the stage after the commitment of the bone marrow progenitor cells into the myelomonocytic lineage (Arai et al., 1999). However, it remains elusive how RANKL, in the face of similar functions of other cytokines, specifically transmits the differentiation signal(s) to determine the fate of cells. In this study, we demonstrated that RANKL, but not IL-1 or M-CSF, induces NFATc1 expression in primary BMMs, and that this induction was impaired in both *TRAF6*$^{-/-}$ and *Fos*$^{-/-}$ cells. Previous gene disruption studies revealed the essential role of c-Fos and TRAF6-dependent NF-κB pathways, bifurcating from the RANKL receptor, RANK, and it was thought that these pathways act in concert to

evoke the downstream transcriptional program(s) (reviewed in Karsenty and Wagner, 2002).

It is interesting that both AP-1 and NF-κB binding sites are present within the promoter region of the *NFATc1* gene (Zhou et al., 2002). It is therefore possible that the initial induction of the gene is mediated by these two transcription factors, which is then followed by NFATc1-mediated autoamplification of gene induction. This may explain, at least in part, why gene transcription is dependent on both c-Fos and TRAF6. In addition, in view of very weak expression of the NFATc1 protein in the RANKL-stimulated *TRAF6*$^{-/-}$ cells (Figure 3B), it is possible that the TRAF6 pathway is also involved in the regulation of NFATc1 protein expression. On the contrary, the expression of TRAF6 and c-Fos was normal in *NFATc1*-deficient monocyte/macrophage precursor cells (data not shown). Collectively, these results indicate that efficient NFATc1 induction is a result of integration of the two signaling pathways bifurcated from RANK, that is, that the *NFATc1* gene is the convergent target of the two pathways.

Induction of Ca^{2+} Signal by RANKL and NFATc1 Activation

Our study also demonstrates a pathway uniquely linked to RANKL signaling, that is, induction of Ca^{2+} oscillation, which is not observed when the cells were stimulated by IL-1 (Figure 4A). In view of the strong inhibitory effects of FK506 and CsA, as well as BAPTA-AM, on NFATc1 expression, *NFATc1* gene induction is apparently dependent on Ca^{2+}-dependent calcineurin activation. It has been reported that in T cells, NFATc2 induces the *NFATc1* gene, the promoter of which contains the NFAT binding site (Zhou et al., 2002). Our results indicate that this autoamplification mechanism of the *NFATc1* gene, accompanied by Ca^{2+} oscillation, also operates in the BMMs. This would lead to the robust induction of the NFATc1-mediated transcriptional program, which is specifically conferred to RANKL signaling. It has been reported that Ca^{2+} oscillation is observed shortly after TNF-α stimulation in neutrophils (Schumann et al., 1993). However, Ca^{2+} oscillation was minimal, if at all, in the TNF-α-treated BMMs, and the induction of NFATc1 by TNF-α was much lower than that by RANKL (see Supplemental Figure S2). This may explain, at least in part, why TNF-α cannot fully induce osteoclast differentiation, but acts in synergy with RANKL in osteoclast differentiation (Lam et al., 2000). Interestingly, other *NFAT* family genes also contain NFAT binding sites within their promoters, but these genes are not induced in RANKL-stimulated BMMs (Figure 1A; and H.T., unpublished observation).

It is as yet unknown how RANKL induces Ca^{2+} oscillation in the cell. The best-known mechanism is the activation of phospholipase C (PLC), followed by the release of inositol 3-phosphate (IP_3), which triggers intracellular Ca^{2+} release and extracellular Ca^{2+} influx (Berridge et al., 2000). This mechanism may operate in both the immune and cardiovascular systems, in which distinct extracellular stimuli induce the PLC cascade. We currently have no evidence that RANKL directly induces PLC activation (H.T., unpublished observation). The oscillation initiates as late as 24 hr following the ligand stimulation, preceded by induction and activation of c-Fos and NF-κB, respectively. Therefore, it is possible that RANKL induction of Ca^{2+} oscillation may be triggered indirectly by a molecule(s) that is induced by RANKL. In support of this possibility, we have preliminary results that the supernatant of RANKL-stimulated BMMs has a stimulatory effect on osteoclastogenesis and Ca^{2+} signaling, which was not suppressed by OPG (S.K. and H.T., unpublished data). Further study will be required to elucidate the detailed mechanism of the RANKL-induced Ca^{2+} oscillation, which was previously found in fertilized eggs and other cells (Berridge et al., 2000; Crabtree and Olson, 2002).

However, whatever the mechanism, it is clear that the Ca^{2+}/calcineurin pathway is an essential pathway associated with RANKL signaling. The sustained activation of NFATc1, mediated by a low-level Ca^{2+} oscillation, may be necessary during the entire differentiation process. Indeed, continuous RANKL stimulation is necessary until the BMMs reach the final stage of differentiation (data not shown). It has been reported that differential activation of transcription factors is contingent on the calcium signaling pattern; NFATs are activated by a low, but sustained Ca^{2+} plateau (Dolmetsch et al., 1997), and that Ca^{2+} oscillations reduce the effective Ca^{2+} threshold for activation of transcription factors (Dolmetsch et al., 1998). Our findings are congruent with these reports in that sustained Ca^{2+} oscillation ensures the NFATc1-mediated transcriptional program in RANKL-stimulated BMMs.

Critical Function of NFATc1 in the Osteoclast Differentiation Program

Using *NFATc1*-deficient ES cells, we demonstrated that the *NFATc1* gene is essential for RANKL-induced osteoclast differentiation. Consistently, NFATc1, but not NFATc2 or NFATc3, is expressed by osteoclasts in vivo (Figure 2C; Supplemental Figure S1). Although further studies are necessary to rigorously assess the in vivo mechanism, these results point to the indispensable, selective role of NFATc1 in bone metabolism, and strongly suggest that a similar mechanism is also operational in vivo. Since *NFATc1* induction is abolished if FK506 and RANKL are added at the same time, the calcineurin-mediated NFATc1 activation is critical for autoamplification of the *NFATc1* gene at an early phase (Figure 4E). In addition, when FK506 is added to BMMs 48 hr after RANKL stimulation, osteoclastogenesis was also inhibited completely (data not shown) despite the presence of NFATc1, indicating the importance of sustained NFATc1 activation throughout the differentiation process. After completion of this study, a report was published pointing to the role of NFATc1 induction by RANKL in the differentiation of RAW 264.7 cells into $TRAP^+$ multinucleated cells (Ishida et al., 2002). Thus, their observations are consistent with our results, obtained with primary BMMs.

From the above viewpoint, it is interesting that BMMs undergo osteoclast differentiation in the absence of RANKL, when NFATc1 is expressed ectopically in these cells. It is well documented that the cellular localization of NFAT family members is under tight control at multiple levels (Macián et al., 2001; Crabtree and Olson, 2002). We infer that a high NFATc1 expression level partially overrides this negative regulatory machinery. In fact,

NFATc1 is detected in the nuclei of these cells, suggesting an equilibrium shift of NFATc1 localization between the nucleus and the cytoplasm in these cells (Figure 6D). Our results showing that FK506 inhibits only partially the cell differentiation caused by the ectopically expressed NFATc1 support this view. Alternatively, we cannot rigorously rule out the possibility that the retrovirus-driven *NFATc1* cDNA expression results in the production of a calcineurin-independent form of NFATc1, which may be similar to the previously constructed mutant (Timmerman et al., 1996). On the other hand, partial suppression of this differentiation by FK506 also suggests that, unlike other cells such as resting T cells, these BMMs retain certain level of calcineurin activities, possibly through cellular stimulation by other molecules in the cell culture.

Whatever the detailed mechanism underlying the above process, our results indicate that once the NFATc1 expression level reaches above the threshold, the ectopically expressed NFATc1 also induces NFATc1 from the endogenous gene (Figure 6E). Thus, NFATc1 may represent a master regulator of RANKL-induced osteoclast differentiation, switching on the transcriptional program for the terminal differentiation of osteoclasts, wherein the autoamplification of NFATc1 intimately participates in sustaining the program.

This study further supports the contention of Crabtree and colleagues that members of the NFAT family evolved to meet the specialized needs of vertebrate development (Crabtree and Olson, 2002). RANKL signaling is also known to be essential in the development of the mammary gland and immune system, or organization of lymph nodes (Fata et al., 2000; Kong et al., 1999). Although the detailed mechanism of this RANKL-mediated organogenesis is still unknown, the RANKL-associated NFATc1 pathway described here may also be involved in these processes.

Cooperation of NFATc1 and Other Transcription Factors

Using a transient assay, we provide evidence that the formation of a complex between NFATc1 and c-Fos, presumably in the context of AP-1, is critical for the activation of the genes involved in osteoclastogenesis. Therefore, we infer that a mechanism similar to that of the immune and other systems (Chen et al., 1998; Macián et al., 2000, 2001) is also operational during osteoclast differentiation. This cooperation between NFATc1 and c-Fos may also be critical for the autoamplification of the *NFATc1* gene (see Figure 3A). Consistently, we also observed a synergistic effect of retrovirus vectors carrying *NFATc1* and *Fos* on osteoclastogenesis, suggesting that this cooperation indeed contributes to cell differentiation (data not shown). It is worth noting that the target genes described above are continuously expressed in mature osteoclasts; therefore, it is likely that these transcription factors remain active after the differentiation of BMMs into bone-resorbing osteoclasts.

How do these observations reconcile with the result that retrovirus-mediated expression of NFATc1 by itself efficiently induces osteoclast differentiation? We considered the following explanation: the components of AP-1 are expressed above threshold levels, so as to cooperate with NFATc1 in the cells cultured in the presence of M-CSF. In this regard, c-Fos is detected to a certain extent in those cells even before RANKL stimulation (Figure 1B; see also Takayanagi et al., 2002). It is known that AP-1 proteins are induced by various intracellular signaling molecules such as Ras and MAP kinases, both of which are activated by M-CSF (Bourette and Rohrschneider, 2000). Thus, the level of ectopically expressed NFATc1, directed by the virus-encoded cDNA, may be the major limiting factor for initiation of the differentiation program by the NFATc1:AP1 complex.

Clearly, NFATc1 needs the AP-1 component under physiological conditions, such as RANKL-induced differentiation of BMMs into osteoclasts. It remains to be clarified whether NFATc1 also needs the cooperation of other transcription factors, such as NF-κB and GATA family members, which also interact with NFAT family members (Macián et al., 2001).

Therapeutic Implications

The essential role of NFATc1 induction and activation by RANKL in osteoclast differentiation described in this study may offer an auspicious target for therapy in bone diseases caused by excessive bone resorption. Systemic effects of FK506 and CsA on bone mass is not always advantageous (Cvetkovic et al., 1994; Leidig-Bruckner et al., 2001), and this may be caused by their effect on the other cell types such as osteoblasts or T cells (Buchinsky et al., 1996). Therefore, these calcineurin inhibitors may be beneficial if we can develop the methods that would allow the delivery of these drugs selectively to the osteoclast lineage. If one could identify a specific molecule(s) involved in RANKL-induced Ca^{2+} oscillation, an alternative therapeutic target(s) of bone diseases may become available. In addition, targeting NFATc1 activity by inhibiting the interaction of NFATc1 and AP-1 may be an interesting strategy.

Experimental Procedures

In Vitro Osteoclastogenesis

Nonadherent bone marrow cells derived from C57BL/6 mice were seeded (5×10^4 cells per well in a 24-well plate, 1.5–2.0 $\times 10^7$ cells in a 10 cm dish) and cultured in α-MEM (GIBCO-BRL) with 10% FBS (Sigma) containing 10 ng/ml M-CSF (Genzyme). After 2 days, adherent cells were used as BMMs after washing out the nonadherent cells including lymphocytes. Monocyte/macrophage progenitor cells of *Fos*$^{-/-}$ and *TRAF6*$^{-/-}$ mice were derived from the spleen and similarly cultured with 10 ng/ml M-CSF for 2 days. These osteoclast precursor cells were further cultured in the presence of 100 ng/ml soluble RANKL (Peprotech) and 10 ng/ml M-CSF to generate osteoclasts; we used these reagents at these concentrations unless otherwise indicated. Calcineurin inhibitors, FK506 (Calbiochem) or cyclosporin A (Sigma), were added at the same time with RANKL. Three days later, TRAP$^+$ multinucleated (>3 nuclei) cells were counted. All data are expressed as mean ± s. e. ($n = 6$). TRAP$^+$ MNCs were characterized by examining the bone-resorbing activity on dentine slices and the expression of calcitonin receptors as described previously (Takayanagi et al., 2000b).

GeneChip Analysis

BMMs were stimulated for 2, 24, and 72 hr with M-CSF alone, RANKL with M-CSF, or IL-1 (10 ng/ml) with M-CSF. Total RNA was extracted from BMMs using a Sepasol RNA extraction kit (Nakarai). Total RNA (15 μg) was utilized for cDNA synthesis by reverse transcription followed by synthesis of biotinylated cRNA through in vitro transcription. After cRNA fragmentation, hybridization with mouse U74Av2

GeneChip (Affymetrix) displaying probes for 12,000 mouse genes/ESTs was performed according to the manufacturer's protocol. Chips were washed, stained with SA-PE, and analyzed using an Affymetrix GeneChip scanner and accompanying gene expression software.

RNA Blotting Analysis and Immunofluorescence Staining

BMMs were stimulated with RANKL in the presence of M-CSF for the indicated periods. Five micrograms of total RNA isolated with Sepasol was loaded in each lane. The blots were hybridized with a 230 bp EcoRI and SmaI fragment of mouse *NFATc1*.α cDNA (GenBank accession number AF087434) labeled with [α-^{32}P]dCTP. For immunostaining, cells were fixed in 4% paraformaldehyde for 20 min and treated with 0.2% Triton X for 5 min. Cells were sequentially incubated in 5% BSA/PBS for 30 min, 2 μg/ml mouse anti-NFATc1 monoclonal antibody (7A6; Santa Cruz) in PBST for 60 min, and then 4 μg/ml Alexa 488 or 594-labeled anti-mouse IgG antibody (Molecular Probe) for 60 min.

BAPTA-AM Treatment

For chelating intracellular Ca^{2+}, cells were incubated in the presence of 15 μM BAPTA-AM and 0.04% pluronic F127 for 60 min in serum-free α-MEM. Cells were then washed twice with α-MEM and incubated in α-MEM/FBS with RANKL/M-CSF for an osteoclast formation assay as described above. This BAPTA-AM treatment was performed once a day throughout the RANKL-induced osteoclastogenesis.

Ca^{2+} Measurement

BMMs were incubated with RANKL or IL-1 (10 ng/ml) in the presence of M-CSF for 72 hr. For Ca^{2+} measurement, cells were incubated in the presence of 5 μM fluo-4 AM, 5 μM Fura Red AM, and 0.05% pluronic F127 for 30 min in serum-free DMEM. Cells were then washed twice with DMEM and postincubated in the presence of DMEM with 10% FBS and 10 ng/ml M-CSF for 20 min. Cells loaded with these dyes were washed three times with Hank's balanced salt solution and mounted on the inverted stage of a confocal microscope (Leica). Cells were excited at 488 nm, and emissions at 505–530 nm for fluo-4 and 600–680 nm for Fura Red were acquired simultaneously at 5 s intervals. To estimate intracellular Ca^{2+} concentration in single cells, the ratio of the fluorescence intensity of the fluo-4 to Fura Red was calculated. The increase in the ratio from the basal level was then divided by the maximum ratio increase obtained by adding 10 μM ionomycin, and expressed as the percent maximum ratio increase.

Osteoclast Differentiation from ES Cells

Feeder cell-independent ES cell lines were maintained on a gelatin-coated dish in DMEM (GIBCO) containing 15% FBS, 1×10^{-4} M 2-mercaptoethanol, 1000 U/ml leukemia inhibitory factor (LIF), 50 U/ml streptomycin, and 50 mg/ml penicillin. We used wild-type ES line E14K (*NFATc1*$^{+/+}$), a heterozygous mutant clone NFDK34 (*NFATc1*$^{+/-}$), and two homozygous mutant clones (*NFATc1*$^{-/-}$), NFDK10 and NFDK17, which generated identical results. For their differentiation into osteoclasts, ES cells were trypsinized and plated in hanging drops at 3×10^3 ES cells per 25 μl drop in an inverted bacterial petri dish. Embryoid bodies (EBs) were collected from the hanging drops on day 2, and transferred into a petri dish. These EBs were cultured in α-MEM/10% FBS containing high concentrations of M-CSF (100 ng/ml) for 2 days. The EBs were treated with 0.25% trypsin/EDTA/PBS in order to harvest single cells, which were examined by flow cytometry for the expression of CD11b, c-Fms, and RANK using specific antibodies (Santa Cruz; day 4). Harvested single cells were reseeded in culture dishes and cultured in α-MEM/10% FBS with RANKL (100 ng/ml) and M-CSF (10 ng/ml). After 4 days, TRAP$^+$ MNCs were efficiently formed in the culture (day 8). These TRAP$^+$ MNCs were characterized and found to fulfill the criteria of osteoclasts, including bone-resorbing activity on dentine slices.

Retroviral Gene Transfer

A retroviral vector, pMX-NFATc1-IRES-EGFP, was constructed by inserting a 2.7 kb EcoRI fragment of the mouse full-length *NFATc1* cDNA into the same site of pMX-IRES-EGFP (a gift from T. Kitamura). The other retrovirus vectors (pMX-c-*fos*-IRES-EGFP, pMX-c-*jun*-IRES-EGFP) have been previously described (Takayanagi et al., 2002). Retrovirus packaging was performed by cotransfection of these pMX vectors and pPAMpsi2 (a gift from C.L. Sawyers) into 293T cells. Two days after inoculation, BMMs were assayed for infection efficiency, and the rest of the cells were further cultured with M-CSF in the presence or absence of RANKL for the osteoclast formation assay. After 3 days, osteoclastogenesis was evaluated by TRAP staining and bone resorption assay. Detection of fluorescence was performed before the staining for TRAP, whose autofluorescence interferes with others.

RT-PCR Analyses

To evaluate the induction of mRNA of the endogenous *NFATc1* gene, one of the PCR primers was designed to hybridize with the sequence in the 5′UTR of *NFATc1* gene, which is absent in virus-encoded *NFATc1* cDNA. The primers for endogenous NFATc1 mRNA are 5′-TGCTCCTCCTCCTGCTGCTC-3′ and 5′-CGTCTTCCACCTCCACGTCG-3′. Those for EGFP (5′-ATCCTGGTCGAGCTGGACG-3′ and 5′-GGCCATGATATAGACGTTGTGGC-3′) were used to determine that the retrovirus-derived EGFP mRNAs are equally expressed.

Luciferase Reporter Gene Assay and Coimmunoprecipitation Assay

The reporter plasmid, pTRAP-luc, was constructed by inserting a 1.85 kb KpnI-BglII fragment of the mouse *TRAP* promoter into the same sites of the PicaGene luciferase reporter plasmid, basic vector 2 (Toyo Ink). The mouse *calcitonin receptor* promoter region specific to osteoclasts (P3 promoter) was amplified by PCR using sense 5′-AAGATCTCAACACAACTCTTAACTGGACC-3′ and antisense 5′-AAGCTTAATGTAAATGTAGATATAGC-3′ primers. Another reporter plasmid, pCTR-luc, was constructed by inserting a 461 bp PCR fragment into the BglII-HindIII sites of the basic vector 2. We designed an NFATc1 mutant based on the sequence homology with NFATc2 mutants, which cannot interact with AP-1 (Macián et al., 2000). An NFATc1 mutant (NFATc1RL) was produced by introducing mutations (R485A/L486A) using site-directed mutagenesis (Sawano and Miyawaki, 2000). The primer was 5′-TTGGGACGGCTGACGACGCCGCGCTGAGGCCTCACGCCTTCTACCAGG-3′. We transfected 293T cells and RAW 264.7 cells using FuGene 6 (Roche) and SuperFect (QIAGEN), respectively, and luciferase activity was assayed 24 hr after transfection. RANKL was added to the culture of RAW 264.7 cells 3 hr after transfection. All data are expressed as mean $\pm$ s.e. (n = 6). At the same time point, whole-cell lysate was extracted from 293T cells. Immunoprecipitates using anti-c-Fos antibody (Santa Cruz) and protein G sepharose (Amersham) were separated with SDS-PAGE, followed by immunoblotting with anti-NFATc1 antibody.

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