Activating transcription factor 4 regulates osteoclast differentiation in mice

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Activating transcription factor 4 (ATF4) is a critical transcription factor for osteoblast (OBL) function and bone formation; however, a direct role in osteoclasts (OCLs) has not been established. Here, we targeted expression of ATF4 to the OCL lineage using the Trap promoter or through deletion of Atf4 in mice. OCL differentiation was drastically decreased in Atf4−/− bone marrow monocyte (BMM) cultures and bones. Coculture of Atf4−/− BMMs with WT OBLs or a high concentration of RANKL failed to restore the OCL differentiation defect. Conversely, Trap-Atf4−tg mice displayed severe osteopetrosis with dramatically increased osteoclastogenesis and bone resorption.

We further showed that ATF4 was an upstream activator of the critical transcription factor Nfatc1 and was critical for RANKL activation of multiple MAPK pathways in OCL progenitors. Furthermore, ATF4 was crucial for M-CSF induction of RANK expression on BMMs, and lack of ATF4 caused a shift in OCL precursors to macrophages. Finally, ATF4 was largely modulated by M-CSF signaling and the PI3K/AKT pathways in BMMs. These results demonstrate that ATF4 plays a direct role in regulating OCL differentiation and suggest that it may be a therapeutic target for treating bone diseases associated with increased OCL activity.

Introduction

Skeletal integrity requires a delicate balance between bone-forming osteoblasts (OBLs) and bone-resorbing osteoclasts (OCLs). Abnormal osteoclastogenesis results in bone destruction, such as osteoporosis, metastatic osteolytic lesions, Paget disease of bone, and rheumatoid arthritis. In contrast, reduced osteoclastogenesis causes osteoporosis, a disorder characterized by significantly increased skeletal mass and lack of a marrow space. Osteopetrosis is usually observed in animals or humans in which genes encoding cytokines, receptors, and signal transduction and transcription factors critical for OCL differentiation — such as RANKL; its receptor, RANK; the M-CSF receptor CSF1R (also known as c-Fms); TNF receptor-associated factor 6 (TRAF6); Src; PU.1, encoded by Spp1; Tp50/p52 NF-κB subunits; c-Fos; or v-ATPase V0 subunit — are deleted or mutated (1–10). Defining the molecular mechanisms underlying osteoclastogenesis is essential to advance the understanding of the molecular basis for the pathogenesis of bone diseases with altered OCL activity. This knowledge will be important for the prevention and treatment of these diseases.

OCLs originate from cells in the monocyte/macrophage lineage (11). OCL formation and maturation are tightly regulated by OBL/stromal cell/hypertrophic chondrocyte-derived factors such as M-CSF, RANKL, and osteoprotegerin (OPG), a soluble decoy receptor that blocks RANKL binding to RANK and thereby inhibits OCL differentiation (12–15). M-CSF binds to its receptor, CSF1R, on early macrophage lineage cells and activates the Rank gene to generate OCL progenitors (16). The PI3K/AKT signaling pathways, which are strongly activated by M-CSF, play a critical role in activating OCL differentiation and bone resorption in normal and diseased states (17–24). However, little is known about the downstream molecular events that result from M-CSF–PI3K/AKT signaling and their relationship to osteoclastogenesis.

RANKL, a member of the TNF superfamily, binds to RANK on OCL precursors and recruits TRAF6, resulting in the activation of multiple signaling pathways including IKK complexes (IKKα, IKKβ, IKKγ, and NIK-IKKα) and MAPKs (Erk1/2, p38, and JNK) (2, 3, 25), which leads to activation of critical transcription factors such as NF-κB and c-Fos. RANKL activation of NF-κB and c-Fos results in induction of initial expression of the key transcription factor NFATc1 (also designated as NFAT2 or NFATc), which is activated by the Ca2+/calmodulin-regulated phosphatase calcineurin (3, 26–29). Eventually, calcium signaling occurs and activates the existing NFATc1, which triggers NFATc1 autoamplification (3, 30) required for further OCL differentiation. Inactivation of the Nfatc1 gene in vivo is embryonic lethal due to a heart valve defect (31, 32). However, the lack of rescue of osteopetrosis in OCL-deficient c-Fos−/− mice by adoptive transfer of Nfatc1−/− (as opposed to Nfatc1−/−) hematopoietic stem cells provided evidence that NFATc1 is essential for osteoclastogenesis in vivo (33). Accordingly, transgenic overexpression of a constitutively active form of NFATc1 (caNFATc1) results in a skeletal phenotype of osteopenia associated with increased osteoclastogenesis and bone resorption (34). Importantly, overexpression of NFATc1 activates osteoclastogenesis in the absence of RANKL (29, 35, 36). In addition, ectopic NFATc1 can restore osteoclastogenesis in both the p50/p52 double knockout (37) and the c-Fos knockout mouse (38). These results suggest that the major OCL differentiation signals converge on NFATc1. However, the molecular mechanisms that control the expression of this key factor are not completely understood.

Critical roles for ATF4 in OBLs and bone formation are well established. ATF4 favors bone formation by promoting OBL-specific gene expression, amino acid import and the synthesis of type I collagen, and the expression of osteopontin and osteocalcin (39, 40). ATF4 also inhibits OCL differentiation (12–15). M-CSF binds to its receptor, CSF1R, on early macrophage lineage cells and activates the Rank gene to generate OCL progenitors (16). The PI3K/AKT signaling pathways, which are strongly activated by M-CSF, play a critical role in activating OCL differentiation and bone resorption in normal and diseased states (17–24). However, little is known about the downstream molecular events that result from M-CSF–PI3K/AKT signaling and their relationship to osteoclastogenesis.

In summary, the major OCL differentiation signals converge on NFATc1, which triggers NFATc1 autoamplification (3, 30) required for further OCL differentiation. Inactivation of the Nfatc1 gene in vivo is embryonic lethal due to a heart valve defect (31, 32). However, the lack of rescue of osteopetrosis in OCL-deficient c-Fos−/− mice by adoptive transfer of Nfatc1−/− (as opposed to Nfatc1−/−) hematopoietic stem cells provided evidence that NFATc1 is essential for osteoclastogenesis in vivo (33). Accordingly, transgenic overexpression of a constitutively active form of NFATc1 (caNFATc1) results in a skeletal phenotype of osteopenia associated with increased osteoclastogenesis and bone resorption (34). Importantly, overexpression of NFATc1 activates osteoclastogenesis in the absence of RANKL (29, 35, 36). In addition, ectopic NFATc1 can restore osteoclastogenesis in both the p50/p52 double knockout (37) and the c-Fos knockout mouse (38). These results suggest that the major OCL differentiation signals converge on NFATc1. However, the molecular mechanisms that control the expression of this key factor are not completely understood.

Critical roles for ATF4 in OBLs and bone formation are well established. ATF4 favors bone formation by promoting OBL-specific gene expression, amino acid import and the synthesis of type I collagen and the expression of osteopontin and osteocalcin (39, 40).
collagen, and proliferation and survival of OBLs (39, 40). Furthermore, our most recent work showed that ATF4 is critical for osteoblastic responses to parathyroid hormone (PTH) to increase bone formation (41). Elefteriou and coworkers showed that ATF4 mediates β-adrenergic induction of Rankl mRNA expression via direct binding to the upstream OSE1 site in the Rankl promoter in OBLs (42). Work from the same group further showed that OBL-targeted expression of ATF4 increased osteoblastic Rankl expression and thereby OCL differentiation (43). Although these studies clearly demonstrate that increased OBL expression of ATF4 enhances OCL differentiation via RANKL production in OBLs, to our knowledge, the possibility of an OCL-intrinsic direct role for ATF4 in regulating OCL differentiation has not previously been addressed.

Using biochemical, cellular, and genetic approaches, the present study demonstrates that ATF4 is an osteoclastic transcription factor, which we believe to be novel, that is essential for OCL differentiation. We showed that both in vitro and in vivo OCL differentiation was severely impaired by lack of ATF4 in a cell-autonomous manner and increased by OCL-targeted transgenic ATF4 expression. Our results revealed that, mechanistically, ATF4 functioned as a direct upstream activator of the gene encoding the critical transcription factor NFATc1. Further, ATF4 modulated RANKL activation of MAPK pathways, a key molecular event in OCL differentiation. Additionally, we demonstrated that ATF4 was critical for M-CSF induction of RANK expression, a key step to generate OCL progenitors, and the level of ATF4 protein was largely modulated by M-CSF and the PI3K/AKT pathways in BMMs. Therefore, these results indicate that ATF4 has important OCL-intrinsic functions both upstream and downstream of RANKL signaling during OCL differentiation.

Results

Inactivation of the Atf4 gene severely impairs OCL differentiation in vitro and in vivo in a cell-autonomous manner. We first tested whether ATF4, a previously known OBL-enriched transcription factor (39), is expressed in OCL-like cells. As shown in Supplemental Figure 1 (supplemental material available online with this article; doi:10.1172/JCI42106DS1), the levels of ATF4 protein in primary mouse BMMs and RAW264.7 cells (a mouse monocyte/macrophage cell line) were comparable to those in OBL-like cells (mouse
MC-4 preosteoblastic cells, primary mouse bone marrow stromal cells [BMSCs], and rat UMR106-01 osteoblastic cells). As shown in Figure 1A, ATF4 protein was phosphorylated in primary mouse BMMs (note loss of upper bands with phosphatase treatment). Furthermore, a strong ATF4 signal was detected in the cytoplasm of the large multinuclear OCLs by immunohistochemical (IHC) staining using a specific ATF4 antibody (Figure 1B).

To determine whether ATF4 is required for osteoclastogenesis in vivo, the tibiae of 4-week-old WT and Atf4–/– mice were decalcified, and histological sections were stained for the OCL enzyme tartrate-resistant acid phosphatase (TRAP). We found that TRAP activity throughout the tibiae, including both the metaphyseal and the epiphyseal regions, was dramatically reduced in Atf4–/– compared with WT mice (Figure 1C). We next measured the effect of ATF4 deficiency on OCL differentiation in both primary and secondary spongiosa. OCL surface/bone surface (Oc.S/BS) and OCL number/bone perimeter (OcNb/BPm) were reduced similarly in both primary and secondary spongiosa in Atf4–/– tibiae relative to WT tibiae (Figure 1D and Table 1). Given the dramatic decreases in Oc.S/BS and OcNb/BPm in the Atf4–/– bone, we next determined whether ATF4 is intrinsically required in BMMs for OCL differentiation by assessing whether OCL differentiation was normal upon addition of exogenous RANKL to Atf4–/– compared with WT BMM cultures in vitro by measuring the number of TRAP+ multinucleated cells (MNCs; defined as having 3 or more nuclei per cell) generated by each. We found that TRAP+ MNCs in BMM cultures from Atf4–/– mice were dramatically reduced compared with those from WT mice (Figure 1E and F). Furthermore, the number of nuclei per MNC was decreased by 75% in Atf4–/– versus WT BMM cultures (Figure 1G), and the MNCs that formed in Atf4–/– BMM cultures were much smaller than those formed in WT cultures. Similar results were obtained in purified CD11b+ BMM cultures (Supplemental Figure 2, A and B). The ability to form TRAP+ MNCs was almost completely lost in BMM cultures from 15-month-old Atf4–/– mice (Supplemental Figure 2, C and D), which suggests that ATF4 is even more important for OCL differentiation in old animals. Furthermore, the resorption pit area on dentin slices was dramatically reduced in Atf4–/– versus WT BMM cultures (Figure 1, H and I). Although MNC number and size were both dramatically reduced in Atf4–/– BMM cultures relative to WT cultures, the ratio of total resorption pit area to total TRAP+ MNC area per slice was not significantly different in WT and Atf4–/– BMM cultures (WT, 0.46 ± 0.11; Atf4–/–, 0.56 ± 0.05), which suggests that the bone-resorbing activity of Atf4–/– TRAP+ MNCs is not impaired. Time-course experiments showed that the percentage of TRAP+ mononuclear OCLs was dramatically reduced in Atf4–/– versus WT BMM cultures in the first 4 days in OCL differentiation media. At day 5, this difference disappeared (Figure 1I), suggestive of a substantial delay rather than absolute decrease in early OCL differentiation. However, we noted that although the number of Atf4–/– and WT TRAP+ mononuclear cells was equivalent at day 5, the Atf4–/– cells did not go on to efficiently form MNCs at day 9 as described above (Figure 1, E–G).

To further investigate the delayed early OCL differentiation, we assessed the in vitro formation of CFU-GMs, which are

**Table 1**

Bone histomorphometry in WT and Atf4–/– tibiae

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<th>Primary spongiosa</th>
<th>WT</th>
<th>Atf4–/–</th>
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<td>OcNb/BPm</td>
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Oc.S/BS and OcNb/BPm in primary and secondary spongiosa of tibiae in Figure 1C were measured as described in Methods. *P < 0.01 versus WT.

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**Figure 2**

ATF4 deficiency impairs OCL differentiation in a cell-autonomous manner. (A and B) CFU-GM assay. 2 × 10^4 BMMs from WT and Atf4–/– mice (6 per group) were cultured in methylcellulose semisolid medium in 35-mm dishes in the presence of 1.0 ng/ml recombinant human GM-CSF for 10 days. The number of CFU-GM colonies was counted under an inverted microscope. Experiments were repeated 2 times. (C and D) OCL-OBL coculture. Primary calvarial OBLs from 3-day-old WT mice were cocultured with WT or Atf4–/– BMMs as described in Methods. (E and F) Effects of increased RANKL. Primary BMMs (E) and purified CD11b+ BMMs (F) from both genotypes were differentiated in the presence of increasing concentrations of RANKL for 7 days, and the number of TRAP+ MNCs per well was counted. *P < 0.01 versus WT. Original magnification, ×40 (A); ×200 (C).
known to contain the earliest OCL precursors (44), and found that it was severely compromised in Atf4−/− BMMs (Figure 2, A and B). We next examined whether coculture with WT OBLs rescues the OCL formation of Atf4−/− BMMs (Figure 2, C and D). As expected, cocultures of WT OBLs with WT BMMs significantly induced the formation of TRAP+ MNCs. Although WT OBLs did induce some TRAP+ mononuclear OCLs in coculture with Atf4−/− BMMs, there were almost no TRAP+ MNCs observed. Furthermore, primary calvarial OBLs from 3-day-old Atf4−/− mice failed to induce TRAP+ MNC formation when cocultured with either WT or Atf4−/− BMMs (H. Cao and G. Xiao, unpublished observation), in support of the notion that osteoblastic ATF4 plays a role in OCL differentiation, probably via upregulation of RANKL expression (42). In addition, high concentrations of RANKL (up to 200 ng/ml) did not restore the OCL differentiation defect in BMMs or purified CD11b+ BMMs of Atf4−/− mice (Figure 2, E and F). Collectively, these findings suggest that ATF4 deficiency impairs osteoclastogenesis in a cell-autonomous manner.

OCL-targeted overexpression of ATF4 dramatically increases OCL differentiation and bone resorption and results in a severe osteopenic phenotype. To further examine the OCL-intrinsic role of ATF4 in regulating osteoclastogenesis in vivo, we developed transgenic mice in which the Atf4 transgene is driven by an 1,846-bp mouse Trap promoter that selectively expresses ATF4 in OCLs (referred to herein as Trap-Atf4-tg mice; Figure 3A). This promoter has been successfully used to target OCL expression of many transgenes (45–47).
Transgenic mRNA was highly expressed in RANKL-differentiated BMMs, but was minimal in undifferentiated BMMs, calvarial OBLs, or BMSCs (Figure 3A). As shown in Figure 3B, the level of ATF4 protein was dramatically increased in RANKL-differentiated BMM cultures from Trap-Atf4-tg mice compared with BMM from the control littersmates. Transgenic ATF4 dramatically increased the protein levels of NFATc1/A, but not of PU.1 or CSFR1, in differentiated BMM cultures. The levels of OCL differentiation marker gene mRNAs (Trap, Rank, Cat K, and Mmp9) were all dramatically elevated in differentiated BMM cultures from Trap-Atf4-tg mice compared with those from WT mice. In contrast, like the proteins, the levels of Spi1 and Csfr1 mRNAs were not increased by transgenic ATF4 (Figure 3C). Using BMMs from 3 different transgenic lines, we found that OCL-targeted overexpression of ATF4 dramatically increased the number of TRAP+ MNCs in vitro (WT, 56 ± 11; TRAP+ MNCs/well; Trap-Atf4-tg, 270 ± 17 TRAP+ MNCs/well; P < 0.01; Figure 3D). We found a similar effect of transgenic ATF4 expression in vivo. TRAP activity was markedly increased in Trap-Atf4-tg compared with WT tibiae (Figure 3E). Oc.S/BS and Oc.Nb/BPm in both primary and secondary spongiosa of tibiae were dramatically increased in differentiated BMM cultures from Trap-Atf4-tg mice compared with those from WT mice (P < 0.01; Figure 3F and Table 2). The serum level of C-telopeptide (CTX), an indicator of in vivo OCL activity, was elevated 2.4-fold in Trap-Atf4-tg mice relative to WT mice (WT, 19.5 ± 3.7; Trap-Atf4-tg, 46 ± 3.3; P < 0.01). Quantitative μCT analysis of femur histomorphometric parameters showed that Trap-Atf4-tg mice had a significant reduction in bone volume/tissue volume (BV/TV) and trabecular number (Tb.N), as well as a marked increase in trabecular space (Tb.Sp), compared with WT littermates (P < 0.01, all comparisons; Figure 3G and Table 3). Results from these experiments demonstrated that osteoclastic ATF4 overexpression increased OCL differentiation and bone resorption, resulting in a severe osteopenic phenotype.

ATF4 is an upstream activator of the Nfatc1 gene. We next examined whether deletion of Atf4 reduces expression of Nfatc1, a master regulator of OCL differentiation. Results from quantitative real-time RT-PCR analysis showed that the level of Nfatc1 mRNA relative to Gapdh mRNA was greatly decreased in Atf4−/− BMM cultures compared with WT cells (Figure 4A and Supplemental Figure 3). The expression of Cat K, a well-known NFATc1 downstream target gene, was almost abolished in Atf4−/− cells compared with WT control cells. Western blot analysis confirmed that NFATc1/A protein, the major isoform of NFATc1 expressed in OCLs, was drastically reduced by ATF4 deficiency (Figure 4B). In contrast, the levels of TRAF6 and c-Fos, both critical factors for OCL differentiation and NFATc1 induction (7, 8, 48, 49), were not decreased by ATF4 deficiency. Likewise, the mRNA and protein levels of PU.1 and CSF1R, both critical factors for early OCL lineage commitment and development, were not reduced by the lack of ATF4 (Figure 4, A and B), in accordance with their observed lack of increase by overexpression of ATF4 (Figure 3, B and C). As expected, the level of Atf4 mRNA was minimal in Atf4−/− BMM cultures (Figure 4A). IHC staining of differentiated BMM cultures using a specific antibody for NFATc1 showed a strong signal in WT cultures that was drastically reduced in Atf4−/− cultures, although there were some mononuclear OCLs that were NFATc1+ in Atf4−/− cultures (Figure 4C). Similarly, in vivo, a strong NFATc1 signal was identified on all surfaces of trabeculae throughout WT tibiae, where OCLs are usually located (Figure 4D). In contrast, the majority of the trabecular surfaces of Atf4−/− tibiae were negative for NFATc1.

Adenoviral ATF4 overexpression in BMMs increased the level of NFATc1 protein in a dose-dependent manner (Figure 4E). ATF4 activated −847/+66 Nfatc1 P1 promoter–pGL3-luciferase reporter activity in a dose-dependent manner, but failed to stimulate a 2.8-kb mouse Runx2 promoter (Figure 4F). In contrast, the Nfatc1 P1 promoter was not activated by Runx2 (Supplemental Figure 4). As shown in Figure 4G, introduction of a 4-bp substitution mutation to the known API binding site located at −644/−637 (from TGGACTTC to TGCGAACA) decreased ATF4 activation by 50% without affecting basal promoter activity, which indicates that this site is critical for ATF4 regulation. ChIP assays showed that RANKL induced ATF4 interaction with a chromatin fragment of the proximal Nfatc1 promoter in RAW264.7 OCL-like cells (Figure 4H). Consistent with results from previous studies (33, 38), both c-Fos and NFATc1 itself were also recruited to the same region of the Nfatc1 promoter in a RANKL-dependent manner. These data demonstrate that ATF4 is a critical upstream activator of the Nfatc1 gene and indicate that ATF4 not only regulates the number of OCL progenitors, but also has a direct role in activating genes downstream of RANK signaling.

We next examined whether NFATc1 can rescue the defective OCL differentiation of Atf4−/− BMMs. WT and Atf4−/− BMMs were infected with increasing amounts of retrovirus expressing a constitutively active form of NFATc1 (50) and differentiated for 7 days, followed by TRAP staining. Although caNFAT1−/− cells only increased the number of TRAP+ MNCs in Atf4−/− BMM cultures (Figure 4I), surprisingly, at even the highest dose, it only slightly increased the number of nuclei per MNC (WT plus empty virus, 24 ± 3.8; WT plus NFATc1 virus, 32.6 ± 5.5; P < 0.01; KO plus empty virus, 3.3 ± 4.2; KO plus NFATc1 virus, 4.8 ± 0.71; P < 0.01). ATF4 modulates RANKL activation of MAPKs, but not IκBα pathways, in OCL progenitors. Because activation of the MAPK and IκBα/NF-κB pathways by RANKL is crucial for NFATc1 expression and OCL differentiation, we next examined whether ATF4 deficiency affects

| Table 2 |
| Bone histomorphometry in WT and Trap-Atf4-tg tibiae |

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<td>Oc.Nb/BPm</td>
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Oc.S/BS and Oc.Nb/BPm in both primary and secondary spongiosa of tibiae were measured as described in Methods. *P < 0.01 versus WT."
RANKL activation of these important pathways in OCL progenitors. As shown in Figure 5, RANKL rapidly induced the phosphorylation of Erk1/2 in a time-dependent manner, which was delayed and reduced in Atf4−/− cells. ATF4 deficiency also slightly reduced the basal phosphorylation level of Erk1/2. Lack of ATF4 similarly compromised RANKL activation of p38 and JNK without markedly affecting their basal levels. In contrast, no difference was seen in RANKL-induced phosphorylation of IκBα in cells of the 2 genotypes. Collectively, lack of ATF4 reduced the ability of RANKL to activate the Erk1/2, p38, and JNK MAPK pathways in OCL progenitors.

We next determined whether ATF4 deficiency affects M-CSF-dependent signaling in BMMs. As shown in Supplemental Figure S5, M-CSF rapidly stimulated the phosphorylation of AKT and JNK in WT cells, which was slightly reduced in Atf4−/− cells. The levels of both phosphorylated and total Src were similar in WT and Atf4−/− cells with or without M-CSF. In contrast to RANKL, M-CSF similarly activated Erk1/2 and p38 in WT and Atf4−/− cells. Thus, ATF4 deficiency did not dramatically impact M-CSF signaling in BMMs.

Levels of ATF4 protein in BMMs are modulated by M-CSF and PI3K/AKT, and ATF4 is required for M-CSF induction of RANK expression. To determine whether ATF4 is regulated by M-CSF in early OCL differentiation, BMMs were cultured in the presence and absence of 30 ng/ml M-CSF for 0, 6, 12, 24, and 48 hours, followed by Western blot for ATF4. The results showed that the level of ATF4 protein was dramatically reduced in the absence of M-CSF in a time-dependent manner. However, this reduction was completely prevented by M-CSF. M-CSF did not alter the level of Atf4 mRNA, which suggests that a posttranscriptional mechanism is involved in this regulation.

To define the signaling pathways through which M-CSF regulates ATF4, BMMs were treated with and without inhibitors or activators for various pathways in the presence of M-CSF for 24 hours. As shown in Figure 6C, LY294002, a specific inhibitor of the PI3K/AKT pathway, dramatically reduced total and phosphorylated ATF4. In contrast, the p38 inhibitor SB203580, the Erk1/2 inhibitor U0126, the PKA inhibitor H89, the PKC inhibitor GF109203X, and the PKA activator FSK did not markedly decrease or increase ATF4 or

Figure 4
ATF4 regulates NFATc1 expression in BMM cultures and bones. (A and B) Total RNAs and protein lysates from differentiated WT and Atf4−/− BMMs were used for real-time RT/PCR analysis (A) and Western blot (B). (C and D) Differentiated BMMs and tibial sections were subjected to IHC staining for NFATc1. (E) WT BMMs were infected with increasing amounts of pRluc plasmids containing a NFATc1 luciferase reporter and pRL-SV40 with the indicated amounts of ATF4 expression plasmid. After 24 hours, cells were harvested for the dual luciferase assay. *P < 0.01 versus 0 μg ATF4. (G) COS-7 cells transfected with 0.8-kb NFATc1-luc (WT) or the same plasmid containing a 4-bp substitution mutation (MT) in the putative ATF4-binding site and pRL-SV40 with or without ATF4 expression plasmid. *P < 0.05 versus β-gal; #P < 0.05, WT versus MT ATF4/β-gal. (H) ChIP assay. A schematic representation of the relevant region of the mouse NFATc1 promoter is shown. P1 and P2 indicate PCR primers used to analyze ChIP DNAs. RAW264.7 cells were treated with or without 50 ng/ml RANKL for 24 hours. ChIP assays were performed using antibodies against ATF4, c-Fos, or NFATc1. (I) WT and Atf4−/− BMMs were cultured and infected with increasing amounts of retrovirus expressing cαNFATc1, and switched to differentiation medium for 7 days. The number of TRAP+ MNCs per well was counted. *P < 0.01 versus WT. Original magnification, ×100.
alter its phosphorylation. Importantly, exposure to LY294002 for only 24 hours prior to the addition of differentiation media inhibited in vitro OCL differentiation in a dose-dependent manner (Figure 6D). The concentrations of the inhibitors or activators used in this study are in the ranges previously reported to selectively affect the relevant pathways (51–54), or were as suggested by the respective manufacturers. We found no evidence of cell toxicity using these experimental conditions. Interestingly, the decrease in ATF4 in COS-7 cells induced by LY294002 was completely abolished by cycloheximide (CHX) and dramatically reduced by the proteasome inhibitor MG115 (Figure 6, E and F). These results suggest that regulation of ATF4 by LY294002 involves de novo protein biosynthesis as well as modulation of protein stability.

M-CSF–induced RANK expression is essential for generating OCL progenitors. We next evaluated whether ATF4 is required for this regulation. To this end, highly purified CD11b+ BMMs were cultured in M-CSF–containing medium for 72 hours and stained with an anti-RANK antibody or control IgG. As shown in Figure 6, G and H, the RANK signal was dramatically decreased in Atf4–/– relative to WT cells, as measured by both IHC and Western blot using specific antibodies. M-CSF time-dependently induced Rank mRNA expression in WT BMM cultures (Figure 6I), consistent with results from a previous study (16). However, this induction was dramatically reduced in Atf4–/– cells. Taken together, these results suggest that ATF4 regulates early OCL differentiation at least in part by facilitating M-CSF induction of the Rank gene.

ATF4 deficiency results in increased CD11b+ cells in bone marrow and spleen and reduced CD3 CD45R c-kit+CD11bhi cells in bone marrow. Because OCLs and macrophages share the same precursor, we next determined whether lack of ATF4 affects monocyte/macrophage precursors by measuring the CD11b+ cell population in splenocytes and bone marrow from WT and Atf4–/– mice. In 5 independent experiments, the percentage of CD11b+ cells was dramatically increased in Atf4–/– splenocytes compared with WT cells, as measured by flow cytometry (WT, 3.04% ± 0.64%; KO, 9.6% ± 2.1%; P < 0.01; Figure 7A). This increase in CD11b+ cells was specific, because the percentages of both T lymphocytes (CD3+; Figure 7A) and dendritic cells (CD11c+; Supplemental Figure 6, A and B) were not increased by ATF4 deficiency (WT, 27.63% ± 1.9% CD3+; 1.59% CD11c+; KO, 21.04% ± 4.1% CD3+; 1.52% CD11c+; P > 0.05). Likewise, in bone marrow, the number of CD11b+ cells was similarly increased in Atf4–/– mice (WT, 41.5% ± 0.08%; KO, 63.6% ± 3.4%; P < 0.01), but the number of CD3+ cells (WT, 10.4% ± 0.2%; KO, 0.8% ± 0.06%; P > 0.05) and CD11c+ cells (Supplemental Figure 6C) was not. At the same time, the percentage of CD11bhi cells, the osteoclastogenic population in the bone marrow, was dramatically reduced in Atf4–/– marrow and spleen. Since the CD3 CD45R CD11bhi c-kit+CD115hi population (approximately 2% of fresh murine bone marrow preparations) contains the highest in vitro osteoclastogenic activity (55), we next determined whether ATF4 inactivation affects this OCL precursor population in bone marrow cells and splenocytes. In 5 independent experiments, the percentage of this cell population was slightly but significantly reduced in bone marrow cells by ATF4 deficiency (WT, 2.3% ± 0.11%; KO, 2.0% ± 0.02%; P = 0.01; Figure 7A). However, the percentage of this cell population in splenocytes was much lower than in bone marrow (approximately 0.25%) and was not significantly changed by ATF4 deficiency. It should be noted that although Atf4–/– bones are smaller and thinner than WT bones, total nucleated bone marrow cells per bone were not reduced in Atf4–/– compared with WT mice (Supplementary Figure 6D). ATF4 deficiency did not alter the proliferation and survival of CD11b+ BMMs (Figure 7, B–E). Therefore, the lack of
ATF4 causes a lineage shift between OCLs and macrophages, resulting in an increase in macrophages. Interestingly, a similar increase in macrophages was observed in mice lacking c-Fos, whose deficiency also reduces NFATc1 and OCL differentiation (7–9).

Discussion

The results of our present study establish, for the first time to our knowledge, that ATF4, a transcription factor previously shown to be important in OBLs, also plays a direct and critical role in regulating OCL differentiation both in vitro and in bones. Drastically reduced OCL differentiation in BMM cultures from Atf4–/– animals was not rescued by coculture with WT OBLs or high concentrations of RANKL, which suggests that ATF4 plays an intrinsic role in OCLs that is indispensable for RANKL-induced OCL differentiation. Dramatic reduction in the formation of CFU-GMs in Atf4–/– BMM cultures suggests that ATF4 deficiency impairs the formation of OCL precursors. To examine the in vivo actions of ATF4 in OCLs, this study used the mouse Trap promoter to drive expression of ATF4 in OCLs. Using this approach, ATF4 was shown to stimulate expression of NFATc1 and other OCL-specific genes and OCL differentiation in BMM cultures. Of particular significance, Trap-Atf4-tg animals displayed a striking in vivo effect on OCL differentiation and bone resorption, resulting in a severe osteopenic phenotype. Because the Trap promoter is not active in OBLs, we were able to discriminate between the effects of ATF4 in OCLs and those in OBLs or BMSCs (i.e., via ATF4-dependent production of RANKL). Furthermore, as shown in Figure 1C and Figure 3E, TRAP activity in hypertrophic chondrocytes close to primary spongiosa was very weak, which suggests that the Trap promoter is not active in these cells. Therefore, Atf4 transgene expression driven by this promoter should be low in the hypertrophic chondrocytes; consequently, the potential contribution of the Atf4 transgene expression in these cells to the observed bone phenotype in Trap-Atf4-tg mice (i.e., osteopenia, increased OCL differentiation, and bone resorption) should be minimal.
One striking result in the present study, which we believe to be novel, is the finding that ATF4 is a direct upstream activator of the Nfatc1 gene, a master regulator of OCL differentiation. Levels of NFATc1 were drastically reduced in Atf4−/− OCLs and bones. Conversely, OCL-specific expression of ATF4 in transgenic mice greatly increased the expression of NFATc1 and its downstream target genes as well as OCL differentiation. ATF4 activated Nfatc1 gene transcription via interaction with the P1 promoter. Interestingly, a previously known AP1 binding site located at −644/−637 was critical for ATF4 activation of the Nfatc1 P1 promoter, which suggests that ATF4 directly binds to this site and/or functions via interactions with AP1 factors. Future study will differentiate among these possibilities. Of particular significance, ATF4 interaction with the P1 promoter was stimulated by RANKL. In addition to its direct regulation, ATF4 may also indirectly increase NFATc1 expression by promoting RANKL-RANK signaling. This notion is supported by our findings that (a) M-CSF induction of RANK, the receptor for RANKL in OCL precursors, was severely impaired in Atf4−/− BMMs; and (b) RANKL activation of the Erk1/2, JNK, and p38 MAPK pathways in OCL progenitors, which is crucial for the subsequent expression of NFATc1, was greatly compromised by the lack of ATF4. Although ATF4 is essential for its expression,
retroviral transduction of NFATc1 cDNA into BMM only partially rescued the OCL differentiation defect in Atf4−/− BMMs, cultures, as large OCLs were still not formed. Possible explanations include: (a) the magnitude and duration of NFATc1 expression was inappropriate for full rescue; and (b) other OCL differentiation-related genes and/or factors are also regulated by ATF4.

Our results established that ATF4 is a critical downstream target of M-CSF–PI3K/AKT signaling in early OCL differentiation. The level of ATF4 protein was largely dependent upon the presence of M-CSF and the PI3K/AKT pathway in BMMs. M-CSF upregulated ATF4 at least in part by stabilizing its protein, which involves the PI3K/AKT pathway. Strikingly, short-term pharmacologic inhibition of the PI3K/AKT pathway dramatically reduced the level of ATF4 protein in undifferentiated BMMs and subsequent OCL differentiation. These results strongly suggest that the M-CSF–PI3K/AKT–ATF4 axis identified in the present study plays a crucial role in regulating early OCL differentiation. PI3K/AKT signaling has been implicated in OCL activation and bone resorption in neurofibromatosis type I (NF1), a congenital disorder resulting from loss of function of the tumor suppressor gene NF1, which encodes neurofibromin, a GTPase-activating protein for Ras. NF1 patients have a significantly higher incidence of osteoporosis and osteopenia (21–23). Recent studies showed that increased OCL activity and osteoporosis is caused by increased AKT signaling in OCLs in murine and human NF1 haploinsufficiency (18, 19). It would be interesting to test whether ATF4 plays a role in the AKT-induced OCL differentiation and bone resorption in NF1 patients. Notably, mice selectively lacking NF1 in OBLs displayed increased OCL activity, probably via upregulation of ATF4 in OBLs, and thereby exhibited increased RANKL expression (43).

Results from the present study demonstrated that ATF4 is not required for early OCL lineage commitment and development. First, CSFRI and PU.1 expression, which is required for the generation of the common progenitors for both macrophages and OCLs, was not altered in Atf4−/− BMMs or bones. Second, M-CSF activation of AKT and MAPKs was not markedly different in WT and Atf4−/− BMMs. Third, both proliferation and survival of highly purified CD11b+ BMMs of the 2 genotypes were similar. Finally, the number of macrophages was increased in Atf4−/− animals. Therefore, ATF4 deficiency impairs OCL, but not macrophage, differentiation, and the OCL differentiation defect in Atf4−/− mice occurs later than in Spi1- or Csf1r-deficient mice.

Although ATF4 is crucial for OCL differentiation, Atf4−/− mice did not display an osteopetrotic phenotype, which is often observed in mice and humans in which genes encoding key OCL-regulating factors — such as CSFRI, RANK, RANKL, TRAF6, Src, PU.1, NF-κB (p50/p52 double knockout), and C-Fos — are inactivated or mutated (1–9). Lack of osteopetrosis in Atf4−/− mice occurs later than in Spi1- or Csf1r-deficient mice. In vitro OCL assays and serum CTX assay. Nonadherent BMMs were isolated from total bone marrow cells cultured on tissue culture dishes for 48 hours. CD11b+ or CD11c+ cells were isolated from BMMs using the CD11b or CD11c microbeads and the LS columns according to the manufacturer’s instructions (Miltenyi Biotec Inc.). For differentiation, cells were first cultured in proliferation medium (6×MEM containing 1% FBS and 10 ng/ml human recombinant M-CSF) for 3 days, then switched to differentiation medium (proliferation medium plus 50 ng/ml human recombinant RANKL) for 4–9 days. The TRAP+ MNCs were scored using an inverted microscope. For Pit assay, BMMs (5 × 105 cells/well) were seeded on dentin slices in 24-well plates in proliferation medium for 3 days and switched to differentiation medium for 9 days. Bone resorption pits were stained with hematoxylin solution. Pit area versus total bone area and pit area versus TRAP+ MNC area of each dentin slice were measured using Image Pro Plus 6.2 software (Media Cybernetics Inc.). Coclureulture experiments were performed as previously described (64). Briefly, primary calvarial OBLs (3.2 × 104 cells/well) were seeded in 24-well plates and cultured in α-MEM containing 10% FBS and 50 ng/ml ascorbic acid. BMMs (4 × 104 cells/well) were seeded on top of the OBLs. The medium was

Methods

Reagents. Tissue culture media and FBS were obtained from Thermo Scientific HyClone. LY294002, SB209580, U0126, H89, FSK, GF109203X, and DMSO were purchased from Sigma-Aldrich. Calf intestinal phosphatase was purchased from Promega. All other chemicals were of analytical grade.

Atf4-deficient and Trap-Atf4-tg mice. Breeding pairs of Atf4-heterozygous mice (Swiss black), as described previously (40, 53), were used to generate WT Atf4+−, heterozygous Atf4−/+, and homozygous mutant Atf4−/− mice for this study. 4- to 8-week-old mice were sacrificed for BMMs. Mice selectively expressing ATF4 in OCLs were developed at the Transgenic & Chimeric Animal Care and Use Committee of the VA Pittsburgh Healthcare System, where this study was conducted.

In vitro OCL assays and serum CTX assay. Nonadherent BMMs were isolated from total bone marrow cells cultured on tissue culture dishes for 48 hours. CD11b+ or CD11c+ cells were isolated from BMMs using the CD11b or CD11c microbeads and the LS columns according to the manufacturer’s instructions (Miltenyi Biotec Inc.). For differentiation, cells were first cultured in proliferation medium (6×MEM containing 1% FBS and 10 ng/ml human recombinant M-CSF) for 3 days, then switched to differentiation medium (proliferation medium plus 50 ng/ml human recombinant RANKL) for 4–9 days. The TRAP+ MNCs were scored using an inverted microscope. For Pit assay, BMMs (5 × 105 cells/well) were seeded on dentin slices in 24-well plates in proliferation medium for 3 days and switched to differentiation medium for 9 days. Bone resorption pits were stained with hematoxylin solution. Pit area versus total bone area and pit area versus TRAP+ MNC area of each dentin slice were measured using Image Pro Plus 6.2 software (Media Cybernetics Inc.). Coclureulture experiments were performed as previously described (64). Briefly, primary calvarial OBLs (3.2 × 104 cells/well) were seeded in 24-well plates and cultured in α-MEM containing 10% FBS and 50 ng/ml ascorbic acid. BMMs (4 × 104 cells/well) were seeded on top of the OBLs. The medium was
supplemented with 10 ng/ml M-CSF and 10⁻⁴ M 1,25 dihydroxyvitamin D₃. OCLs were identified by TRAP staining and counted. Serum levels of CTX, degradation products from type I collagen during osteoclastic bone resorption, were measured using the RatLaps ELA Kit according to the manufacturer’s instruction (Immunodiagnostic Systems Limited).

**Histological evaluation, bone histomorphometry, and IHC** WT and Atf4⁻/⁻ mice were euthanized, and tibiae were fixed in 10% formalin for 4–24 hours, decalcified in 10% EDTA (pH 7.4) for 10–14 days, and embedded in paraffin. Sections of tibiae from WT and Atf4⁻/⁻ mice were used for TRAP staining as described previously (65). Bone histomorphometry, such as Oc.S/BS and Oc.Nb/Bpm, in both primary and secondary spongiosa of tibiae was measured using Image Pro Plus 6.2 software (Media Cybernetics Inc.) as previously described (43, 66). Cells cultured in 8-well culture chambers (Nalgene Nunc), or 5-μm sections of tibiae, were subjected to IHC staining with antibodies against ATF4, NFATc1, or RANK (Santa Cruz Biotechnology) using the EnVision System-HRP (DAB) kit (Dako North America Inc.) as described previously (41). Fixed nondemineralized femurs were used for DNA sequencing. A 1-way ANOVA analysis was used, followed by the Tukey test. Results are expressed as mean ± SD. Differences with a P value less than 0.05 were considered statistically significant.

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