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*J Immunol* 2015; 194:2878-2887; Prepublished online 18 February 2015;
doi: 10.4049/jimmunol.1402211
http://www.jimmunol.org/content/194/6/2878

Supplementary Material

http://www.jimmunol.org/content/suppl/2015/02/13/jimmunol.140221
1.DCSupplemental

References

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
FOXO1 Mediates RANKL-Induced Osteoclast Formation and Activity

Yu Wang,*† Guangyu Dong,† Hyeran Helen Jeon,‡ Mohamad Elazizi,† Lan B. La,† Alhassan Hameedaldeen,† E Xiao,†,‡,§ Chen Tian,† Sarah Alsadun,† Yongwon Choi,† and Dana T. Graves†

We have previously shown that the transcription factor FOXO1 is elevated in conditions with high levels of bone resorption. To investigate the role of FOXO1 in the formation of osteoclasts, we examined mice with lineage-specific deletion of FOXO1 in osteoclast precursors and by knockdown of FOXO1 with small interfering RNA. The receptor activator for NF-κB ligand (RANKL), a principal bone-resorbing factor, induced FOXO1 expression and nuclear localization 2 d after stimulation in bone marrow macrophages and RAW264.7 osteoclast precursors. RANKL-induced osteoclast formation and osteoclast activity was reduced in half in vivo and in vitro with lineage-specific FOXO1 deletion (LyzM.Cre'FOXO1<sup>L/L</sup>) compared with matched controls (LyzM.Cre'FOXO1<sup>m/m</sup>). Similar results were obtained by knockdown of FOXO1 in RAW264.7 cells. Moreover, FOXO1-mediated osteoclast formation was linked to regulation of NFATc1 nuclear localization and expression as well as a number of downstream factors, including dendritic cell–specific transmembrane protein, ATP6vod2, cathepsin K, and integrin α<sub>v</sub>. Lastly, FOXO1 deletion reduced M-CSF–induced RANK expression and migration of osteoclast precursors. In the present study, we provide evidence that FOXO1 plays a direct role in osteoclast formation by mediating the effect of RANKL on NFATc1 and several downstream effectors. This is likely to be significant because FOXO1 and RANKL are elevated in osteolytic conditions. The Journal of Immunology, 2015, 194: 2878–2887.

Osteoclasts play a critical role in bone development, normal remodeling, and pathologic conditions such as periodontitis, rheumatoid arthritis, and osteoporosis. Osteoclasts are generated from myeloid progenitors that are produced in bone marrow and released into the vasculature (1). The formation of osteoclasts is governed by ligands, particularly M-CSF, receptor activator for NF-κB ligand (RANKL), and TNF-α as well as several other factors that directly or indirectly contribute to their formation and activity (2). Osteoclast formation involves several steps, including differentiation of myeloid precursors to preosteoclasts, fusion of mononuclear preosteoclasts to multinucleated osteoclasts, and maturation and activation of osteoclasts to resorbing bone.

Several cytokines have been shown to play a key role in osteoclast formation. M-CSF stimulates progenitors to upregulate RANK expression, the receptor for RANKL (3, 4). RANKL signaling stimulates formation of single-cell TRAP<sup>+</sup> osteoclast precursors and induces cell fusion to form multinucleated TRAP<sup>+</sup> cells. M-CSF and RANKL stimulate several signaling intermediates and transcription factors such as DAP12, TRAF6, NF-κB components (relA, p50, p52), and potential downstream effector molecules needed for cell fusion, osteoclast activity, and survival, including CD9, dendritic cell–specific transmembrane protein (DC-STAMP), NFATc1, Atph6vod2, OC-STAMP, integrin α<sub>v</sub>β<sub>3</sub>, cathepsin K, and others (5, 6).

Cell fusion is a characteristic feature of osteoclasts. It involves cell migration, chemotaxis, intercellular adhesion, and fusion of membranes. DC-STAMP, a transmembrane protein, plays an important role in cell fusion (7). It plays a central role in cell fusion and is induced by two transcription factors, NFATc1 and c-Fos (8). NFATc1 is involved in a number of cellular functions in various cell types. RANKL induces NFATc1 in osteoclast precursors through activation of NF-κB, which is further enhanced by Ca<sup>2+</sup> oscillation regulated by Tmem64 (9). Overexpression of NFATc1 can stimulate osteoclast differentiation without RANKL signaling, suggesting that NFATc1 is a master switch that regulates osteoclast differentiation downstream of RANKL (8). Once formed, osteoclasts are polarized so that the cellular constituents needed for resorption are localized to the bone surface. This process involves the formation of actin rings and the integrin α<sub>v</sub>β<sub>3</sub> (10). Resorption of the bone surface requires the action of vacuolar ATPase (Atp6v1c1) proton pump located in the osteoclast cell membrane and the release of cathepsin K, a lysosomal cysteine protease that degrades bone matrix proteins, including collagen (11, 12).

FOXO1 is a transcription factor that has been linked to protection of osteoblasts from oxidative stress (13, 14). FOXO1 is a member of the forkhead transcription factors in the O-box subfamily. There are four members, FOXO-1, -3, -4, and -6 (15). FOXO1 regulates transcription of many different classes of genes depending on the cell type and nature of the stimulus (16). We have recently shown...
that FOXO1 mediates inflammatory gene expression in dendritic cells (17) and that FOXO1 is elevated in conditions with high levels of osteoclastogenesis (18, 19). Similarly FOXO1 mediates gene expression in macrophages (20). A recent report indicated that long-term combined deletion of FOXO1, -3, and -4 decreases physical bone mass by increasing osteoclast numbers and activity (21). Although that report examined the indirect effects of FOXO1 deletion associated with aging and focused on simultaneous deletion of three FOXO members in vivo, we took an alternative approach and examined the direct effect of FOXO1 in mediating short-term RANKL-stimulated osteoclastogenesis. The results indicate that FOXO1 is activated 2 d after RANKL stimulation in bone marrow macrophages (BMMs) and RAW264.7 cells. To determine whether FOXO1 mediates the effects of RANKL stimulation in vivo, a calvarial model was used (2, 6). FOXO1 deletion significantly reduced osteoclastogenesis and osteoclast activity stimulated by RANKL injection. In vitro experiments agreed well with in vivo experiments and demonstrated that FOXO1 deletion reduced osteoclastogenesis and osteoclast activity induced by RANKL in both BMMs derived from experimental mice and in RAW264.7 cells with FOXO1 knockdown by small interfering RNA (siRNA). Moreover, FOXO1 deletion reduced the expression of several proteins that are involved in osteoclast formation or function. These results point to FOXO1 having a role in mediating RANKL-stimulated osteoclast formation, which is distinct from its long-term effect associated with aging (21).

Materials and Methods

Reagents and mice

Abs were obtained from Santa Cruz Biotechnology (Dallas, Texas) unless noted otherwise. Mice that express Cre recombinase under control of the lysozyme M promoter (LyzM<sup>+</sup>Cre) were purchased from The Jackson Laboratory (Bar Harbor, ME). FOXO1<sup>−/−</sup> mice were provided by Dr. Ronald De Pinho (University of Pennsylvania. Soluble RANKL (3 μg/mouse, Invitrogen, Carlsbad, CA) or GeneTools siRNA transfection reagents (Signagn Laboratories, Rockville, MD). In some experiments, freshly isolated single-cell suspensions of bone marrow cells were obtained from bone marrow and centrifuged at 500 rpm for 5 min in a cytocentrifuge (Thermo Scientific, Rockford, IL). Attached cells were fixed with 4% paraformaldehyde, washed, and subjected to immunofluorescence with Ab specific for FOXO1 or control IgG. No signal was detected with matched control. Results were obtained by measuring mean fluorescence intensity or counting immunopositive cells.

In vitro formation of TRAP<sup>+</sup> multinucleated cells and osteoclast activity assays

BMMs were stimulated with 100 ng/ml RANKL and 30 ng/ml M-CSF or 100 ng/ml RANKL alone for 4 d and fixed with 4% paraformaldehyde, whereas RAW264.7 cells were stimulated with 100 ng/ml RANKL and then fixed with 4% paraformaldehyde. The number of multinucleated TRAP<sup>+</sup> (more than three nuclei) cells, average area, and average number of nuclei per multinucleated TRAP<sup>+</sup> cell were counted after staining for TRAP with a kit from Sigma-Aldrich or F-actin with FITC-labeled streptavidin and counterstained with DAPI. Images were acquired using a Nikon 90i fluorescent microscope, and image analysis was performed using NIS-Elements software (Nikon).

Treatment by inhibitors

RAW264.7 cells were stimulated with RANKL for 48 h. Inhibitors such as BML-257 (12.5 μM), triciribine (1 μM), SB203580 (10 μM), INK inhibitor I (10 μM), PD098059 (20 μM), N-acetyl-D-cysteine (5mM), 1,2-DN-nitroarginine-methylster (100 μM), sirtinol (10 μM), and DON (50 μM) were added for the last 24 h. Cells were then fixed in 100% methanol at −20°C for 10 min and incubated with primary Ab to FOXO1 or matched nonimmune primary Ab overnight at 4°C. Ab was then detected by a biotinylated secondary Ab and visualized using Alexa Fluor 546-conjugated streptavidin and counterstained with DAPI. Images were acquired using a Nikon 90i fluorescence microscope, and image analysis was performed using NIS-Elements software (Nikon, Melville, NY). Nuclear localization with standard immunofluorescence was confirmed with deconvolution immunofluorescence microscopy. Cells were imaged in five 1.7-μm stacks. The middle stack representing the center of the cell was then further analyzed for FOXO1 nuclear localization using NIS-Elements AR software (Nikon).

Quantitative real-time PCR

Total RNA was extracted and quantitative real-time PCR was performed with primers designed using the Universal ProbeLibrary Assay Design Center (Roche Applied Science) and labeled probes (Roche Applied Science). Each value was normalized to ribosomal protein L32 and represents the mean of three independent experiments.

Migration assay

Chemotaxis of BMMs and RAW264.7 cells was measured using transwells with polycarbonate filters (8-μm pores) (Corning, Corning, NY) without M-CSF and the bottom chamber was filled with medium. Cells on the top of the filter were removed and those that had migrated to the bottom chamber were counted after DAPI staining by fluorescence microscopy.
Western blot analysis
Cytoplasmic and nuclear extractions were prepared by using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific). The cytosolic and nuclear fractions were resolved by SDS-PAGE. Primary Ab against FOXO1 (Cell Signaling Technology, Boston, MA), laminin A (Sigma-Aldrich), and actin (Cell Signaling Technology) were used according to the manufacturers’ directions. Bands were visualized by biotinylated species-specific secondary Ab and chemiluminescence using a kit from Thermo Scientific. No signal was detected with matched control.

Immunofluorescence
For in vitro assays, cells were fixed in 100% methanol (10 min, −20°C) followed by incubation with primary Ab specific for FOXO1, CD11b, RANK, and the integrin αv, or matched control Ab. Ab was then detected by a biotinylated secondary Ab and visualized using Alexa Fluor 546-conjugated streptavidin and counterstained with DAPI. Images were acquired using a Nikon 90i fluorescence microscope, and image analysis was performed using NIS-Elements software (Nikon). No signal was detected with matched control. For most assays data are presented as the mean fluorescence intensity for each group. In some experiments nuclear localization was determined by colocalization of FOXO1 immunostaining and DAPI nuclear staining. For in vivo assays, paraffin sections were subjected to Ag retrieval in citrate buffer at 95°C. Sections were incubated with Ab specific to TRAP, FOXO1, or NFATc1. Abs were localized with biotinylated secondary Ab followed by avidin–biotin HRP complex (Vector Laboratories, Burlingame, CA). Abs were visualized using streptavidin–Alexa Fluor 546 (Invitrogen) for TRAP and fluorescein–avidin DCS (Vector Laboratories) for FOXO1 or NFATc1 and counterstained with DAPI. Tyramide signal amplification (PerkinElmer, Waltham, MA) was used to enhance the signal. Fluorescent immunostaining of TRAP+ and FOXO1+ or NFATc1+ cells were observed under ×20 magnification of images captured with a Nikon Eclipse 90i microscope (Nikon) and analyzed with NIS-Elements AR software (Nikon).

Luciferase reporter assay
RAW264.7 cells were transfected with FOXO1 WT, FOXO1 AAA construct or pcDNA empty vector alone, as we have described (27), by electroporation (Neon transfection system; Life Technologies) and cotransfected with an NFATc1–luciferase reporter (provided by Dr. Yihong Wan) (28). Luciferase activity was measured 2 d later using a Dual-Luciferase assay system (Promega) and reported as the firefly/Renilla luciferase ratio.

Results
FOXO1-specific deletion in osteoclast precursors
BMMs were isolated from experimental (LyzM.Cre<sup>L/L</sup>) and matched control (LyzM.Cre<sup>L/L</sup>) mice. LyzM-driven Cre recombinase reduced FOXO1 mRNA levels (Fig. 1A) by 95% and protein levels by 90% in BMMs (Fig. 1B). Most bone marrow cells and splenocytes directly isolated from experimental and control mice did not exhibit FOXO1 deletion as expected because most are not CD11c<sup>+</sup> (Fig. 1C, 1D, Supplemental Fig. 1).

FIGURE 1. LyzM deletes FOXO1 in BMMs. (A) FOXO1 mRNA levels were measured in BMMs by quantitative real-time PCR. (B) FOXO1 expression was measured by immunofluorescence as mean fluorescence intensity in BMMs. (C) Percentage FOXO1 immunopositive cells in single-cell suspension of bone marrow cells was measured by immunofluorescence. (D) FOXO1 expression was measured by immunofluorescence as mean fluorescence intensity in splenic cells. Each value represents the mean ± SEM of triplicate specimens. *p < 0.05 compared with matched Cre<sup>−</sup> group.

Statistical analysis
Most in vitro experiments were carried out three times to obtain mean and SE from independent experiments. In some cases experiments were carried out in triplicate to provide a mean and SEM, with experiments having been performed three times to confirm results. In vivo experiments were carried out with five to seven animals per group. Statistical significance was determined by one-way ANOVA with a Tukey post hoc test for comparisons between multiple groups at the p < 0.05 level. In some cases, significance between experimental and control groups was directly compared at a given time point by a Student t test to establish significance at the p < 0.05 level.

FIGURE 2. FOXO1 deletion reduces osteoclastogenesis in vivo. (A) Soluble RANKL (3 μg/injection) or vehicle (PBS) was injected at the midline of the calvaria of LyzM.Cre<sup>L/L</sup> and littermate control LyzM.Cre<sup>L/L</sup> mice daily for 5 consecutive days. After 12 d, calvarial bone was examined by μCT. (B and C) Pit number and total pit area per calvaria were analyzed with μCT. (D and E) TRAP<sup>+</sup> multinucleated bone-lining osteoclasts were identified by histostain in calvarial sutures or bone marrow cavities. (F) Osteoclasts were counted as TRAP<sup>+</sup> multinucleated cells lining bone in bone marrow cavity and detected by immunofluorescence. Each value is the mean ± SEM for n = 6 mice/group. *p < 0.05 compared with matched Cre<sup>−</sup> group.
FOXO1 deletion reduces osteoclastogenesis in vivo

Studies were carried out in which RANKL was injected over the calvarial bone of experimental and control mice. The formation of resorption pits was measured on the surface of calvarial bone in three-dimensional μCT images. The number of resorption pits decreased by 60% and total pit area was reduced by 64% in experimental mice compared with control mice \( (p < 0.05) \) (Fig. 2A–C). The number of osteoclasts was measured in the sutures and bone marrow cavities of calvarial bone by TRAP histostain (Fig. 2D, 2E) or by immunofluorescence with Ab to TRAP (Fig. 2F). RANKL stimulated a 3- to 10-fold increase in osteoclasts. RANKL-induced osteoclast formation was reduced by 67% in sutures of LyzM.Cre\(^+\)FOXO1\(^{L/L}\) mice compared with control LyzM.Cre\(^2\)FOXO1\(^{L/L}\) mice \( (p < 0.05) \) and was completely blocked in bone marrow cavities (Fig. 2D–F). There was no difference in osteoclast numbers in PBS-injected groups \( (p > 0.05) \). The effect of FOXO1 deletion on cortical bone architecture and cancellous bone volume, mineral density, number, thickness, and spacing of trabeculae associated with remodeling in young mice was examined (Supplemental Fig. 2A–E). No significant difference was observed between experimental and control mice \( (p > 0.1) \).

Loss of FOXO1 leads to reduced osteoclast formation and activity in vitro

BMMs from FOXO1 deleted or matched control mice were stimulated with RANKL in vitro to induce osteoclastogenesis (Fig. 3A, Supplemental Fig. 2F). Deletion of FOXO1 resulted in

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**FIGURE 3.** FOXO1 deletion or FOXO1 silencing reduces osteoclastogenesis and osteoclast activity in vitro. (A–D) BMMs from LyzM.Cre\(^+\)FOXO1\(^{L/L}\) and littermate control LyzM.Cre\(^2\)FOXO1\(^{L/L}\) mice were incubated with RANKL (100 ng/ml) and M-CSF (30 ng/ml). After 4 d cells were fixed and stained as indicated. (A) TRAP\(^+\) multinucleated osteoclasts (original magnification, \( \times 10 \)). (B) TRAP\(^+\) multinucleated osteoclasts with \( > 30 \) nuclei per osteoclast. (C) Mean area per osteoclast. (D) Mean number of nuclei per osteoclast. (E–H) RAW264.7 cells were transfected with FOXO1 siRNA or scrambled siRNA and incubated with RANKL (100 ng/ml). After 4 d cells were fixed and stained as indicated. (E, F) FOXO1 mRNA levels were measured in RAW264.7 cells by quantitative real-time PCR after siRNA transfection. (F) TRAP\(^+\) multinucleated osteoclasts with more than three nuclei per osteoclast (original magnification, \( \times 10 \)). (G) Mean area per osteoclast. (H) Mean number of nuclei per osteoclast. Each value represents the mean \( \pm \) SEM of three independent measurements. (I, J) BMMs from experimental and control mice were stimulated with RANKL (100 ng/ml) and M-CSF (30 ng/ml) or incubated with vehicle alone. Osteoclast activity was measured by release of europium-labeled collagen measured by florescence. (J) RAW264.7 cells were transfected with FOXO1 siRNA or scramble siRNA and were stimulated with RANKL (100 ng/ml) for 4 d. Osteoclast activity was measured as in (I). (K) RAW264.7 cells were transfected with siFOX1, siFOX3, or scrambled siRNA and then incubated with RANKL (100 ng/ml) for 4 d and fixed and stained for osteoclasts with TRAP histostain. (L) RAW264.7 cells were transfected with siFOX3 or scrambled siRNA and FOX3 mRNAs were measured by quantitative real-time PCR after 2 d. Each value represents the mean \( \pm \) SEM of three or four independent experiments. *\( p < 0.05 \) compared with matched Cre\(^-\) or scrambled siRNA group, \( p < 0.05 \) compared with matched Cre\(^-\) or scrambled siRNA group stimulated with RANKL and M-CSF or RANKL alone.
a 65% reduction in the number of osteoclasts formed (p < 0.05) (Fig. 3B). Additionally, there was a 58% reduction in the average area per osteoclast and a 59% reduction in the average number of nuclei per osteoclast (p < 0.05) (Fig. 3C, 3D). Experiments were also carried out with RAW264.7 cells transfected with FOXO1 siRNA or scrambled siRNA followed by RANKL stimulation (Supplemental Fig. 2G). FOXO1 siRNA reduced FOXO1 levels by 70% (Fig. 3E) and reduced the number of osteoclasts induced by RANKL in RAW264.7 cells by 49%, the average area per osteoclast by 27%, and the average nuclei per osteoclast by 35% (p < 0.05) (Fig. 3F–H). Scrambled siRNA had no effect (p > 0.05).

Osteoclast activity in vitro was measured by release of labeled collagen. RANKL stimulated a 16-fold increase in resorptive activity in osteoclasts from BMMs of control mice, which was blocked by FOXO1 deletion (p < 0.05) (Fig. 3I). FOXO1 knockdown by siRNA also blocked RANKL-induced resorptive activity from RAW264.7 cells (p < 0.05) (Fig. 3J).

To compare the role of FOXO1 and FOXO3, each was knocked down separately in RAW264.7 cells and then stimulated with RANKL. FOXO1 knockdown reduced osteoclast formation by 66%, and FOXO3 knockdown had no significant impact on osteoclastogenesis even though FOXO3 siRNA effectively reduced FOXO3 levels (Fig. 3K, 3L).

**FOXO1 is downregulated by RANKL through different pathways**

Both BMMs and RAW264.7 cells were examined for RANKL-induced FOXO1 by both quantitative real-time PCR and immunofluorescence (Fig. 4A–C). At the protein level little to no FOXO1 was detected in BMMs or RAW264.7 cells at baseline. RANKL stimulated a maximum 7.9-fold increase in FOXO1 in BMMs and a 4.5-fold increase in RAW264.7 cells (p < 0.05) (Fig. 4C–E). To confirm that FOXO1 expression was associated with osteoclastogenesis in vivo, double immunofluorescence was performed to identify FOXO1*, TRAP* multinucleated osteoclastic cells (Fig. 4F).

FOXO1 is active in the nucleus, and FOXO1 activation can be followed by nuclear localization. FOXO1 nuclear localization induced by RANKL could also be detected after 1 d and peaked with a 10-fold increase on day 2 in both BMMs and RAW264.7 cells by immunofluorescence (Fig. 5A, 5B). Interestingly, the pattern of FOXO1 nuclear localization differed from the expression pattern by declining substantially between days 2 and 4 with a 70% reduction in FOXO1 nuclear localization in BMMs and a 64% decline in RAW264.7 cells (p < 0.05) (Fig. 5A, 5B). Nuclear localization was confirmed by deconvolution immuno-
fluorescent microscopy, which examined FOXO1 colocalization with DAPI at the center of each cell. By this analysis, RANKL stimulated a 7.4-fold increase in FOXO1 nuclear localization in BMMs from WT mice and a 5.6-fold increase in RAW264.7 cells (p < 0.05) (Fig. 5C, 5D), consistent with results obtained by standard immunofluorescence microscopy (Fig. 5A, 5B). RANKL-induced FOXO1 nuclear localization was also examined by Western blot analysis. FOXO1 protein accumulated in the cytoplasm within 4 h of RANKL stimulation (Fig. 5E). Nuclear localization was strongly detected 2 d after RANKL stimulation (Fig. 5E), which was consistent with the immunofluorescence data.

To investigate RANKL-stimulated FOXO1 nuclear localization further, experiments were performed examining pathways that may mediate RANKL-induced FOXO1 nuclear localization. Inhibitors of p38 and JNK but not Erk MAPK inhibitors reduced FOXO1 nuclear localization by ~50%. A reactive oxygen species (ROS) inhibitor, sirtuin-1 inhibitor, and NO synthase (NOS) inhibitor also reduced FOXO1 nuclear localization by 39–65% (p < 0.05) (Fig. 5F). Thus, a number of known pathways stimulated by RANKL induce FOXO1 nuclear localization. However, an Akt inhibitor did not have a significant effect on RANKL-induced FOXO1 nuclear localization.

**Effect of FOXO1 on expression of target genes**

Several potential target genes were examined at the mRNA level to investigate the role of FOXO1 in mediating RANKL stimulation. This was done in BMMs obtained from experimental mice with FOXO1 deletion compared with matched controls. RANKL increased FOXO1 mRNA levels, which were blocked by Cre recombinase (Fig. 6A) (p < 0.05). Deletion of FOXO1 reduced by ~60% the increase in NFATc1 mRNA levels stimulated by RANKL in vitro (Fig. 6B, 6C, Supplemental Fig. 3A) and reduced RANKL-stimulated NFATc1 nuclear localization by 53% (p < 0.05) (Fig. 6D). In vivo RANKL simulated a 2.1-fold increase in NFATc1 TRAP+ multinucleated cells measured by double immunofluorescence staining. This was reduced 70% in LyzM.Cre+FOXO1L/L mice compared with LyzM.Cre+2FOXO1L/L mice (p < 0.05) (Fig. 6E). However, cotransfection of WT (FOXO1 WT) or a constitutively active FOXO1 (FOXO1 AAA) expression vector with NFATc1 reporter did not enhance NFATc1 transcriptional activity compared with cotransfection with empty vector (PcDNS) (Fig. 6F). However, deletion of FOXO1 reduced the capacity of RANKL to stimulate increased mRNA levels of factors that are involved in osteoclast formation and activity including ATP6v0d2.

**FIGURE 5.** RANKL stimulates FOXO1 nuclear localization. (A and B) BMMs were incubated with M-CSF (30 ng/ml) and RANKL (100 ng/ml) or RAW264.7 cells were incubated with RANKL (100 ng/ml) for 0-4 d. FOXO1 nuclear localization was determined by FOXO1 immunofluorescence and colocalization with DAPI nuclear stain. (C and D) Immunofluorescence was repeated and colocalization of FOXO1 and DAPI nuclear stain was determined by Z-stack analysis in the center plane of BMMs and RAW264.7 cells. (E) RAW264.7 cells were incubated with RANKL (100 ng/ml). Cells were fixed after 0, 4, and 48 h. Cytoplasmic and nuclear extracts were prepared, and FOXO1 cytoplasmic and nuclear levels were measured by immunoblot analysis. (F) RAW264.7 cells were stimulated with RANKL (100 ng/ml) for 48 h. Inhibitors of Akt, BML-257 (12.5 μM), tricirbine (1 μM); p38, SB203580 (10 μM); JNK inhibitor I (10 μM); ERK, PD098059 (20 μM); ROS, N-acetyl-l-cysteine (5 mM); NOS, L-NG-nitro-arginine-methylester (100 μM); sirtuin-1, sirtinol (10 μM); and phosphate-activated glutaminase, DON (50 μM) were added for the last 24 h. Cells were then fixed and FOXO1 was measured as described in (A). Each value represents mean ± SEM of three independent measurements. *p < 0.05 compared with BMMs on day 0 or with matched control group, †p < 0.05 compared with BMMs on day 2 after RANKL and M-CSF stimulation or with matched control group.
by 49%, cathepsin K by 63%, and DC-STAMP by 55% ($p < 0.05$) (Fig. 6G–I).

FOXO1 regulates RANK and osteoclast precursor migration

Osteoclast precursors (CD11b+ BMMs) are induced by M-CSF from myeloid progenitor cells (6). The effect of FOXO1 deletion on production of CD11b+ BMMs was examined in vitro by immunofluorescence. Neither the percentage CD11b+ bone marrow cells or the number of M-CSF–induced BMMs in vitro was affected by FOXO1 deletion, indicating that the effects of FOXO1 on osteoclastogenesis are downstream of BMM formation ($p < 0.05$) (Fig. 6G–I).

FOXO1 deletion resulted in a 73% decrease of integrin $\alpha_v$ at the protein level ($p < 0.05$) (Fig. 7F).

Discussion

The role of FOXO1 in regulating monocyctic cells has received relatively little attention. FOXO1 has been shown to modulate TLR expression and cytokine production in monocytes and dendritic cells, which share a common precursor with osteoclasts (17, 20). In the present study, we report that FOXO1 has an important role in mediating induced osteoclastogenesis. RANKL stimulated FOXO1 expression and FOXO1 nuclear localization in vivo and in vitro. FOXO1 deletion or knockdown reduced RANKL-stimulated osteoclast formation in vivo and reduced osteoclast formation from BMMs and RAW264.7 cells in vitro. Additionally, FOXO1 deletion in myeloid cells reduced M-CSF–induced RANK...
expression and migration of BMMs. However, FOXO1 deletion did not affect formation of BMMs in vivo or in vitro.

To investigate the role of FOXO1 on RANKL-stimulated osteoclastogenesis, mice that expressed lysozyme M promoter-driven Cre recombinase (33) were bred with floxed FOXO1 mice (34). This led to substantial deletion of FOXO1 in osteoclast precursor cells, BMMs. BMMs from experimental mice had a 95% reduction of FOXO1 mRNA and 90% reduction of FOXO1 at the protein level compared with control mice. This was not a general reduction of FOXO1 in hematopoietic cells because there was no significant difference in FOXO1 levels in bone marrow cells or splenocytes from experimental and control mice.

Several lines of evidence establish that RANKL stimulates FOXO1 in osteoclast precursors. RANKL induced a time-dependent increase in FOXO1 mRNA and protein expression in both BMMs and the osteoclast precursor cell line, RAW264.7, which increased 5- to 8-fold after stimulation. In vivo RANKL stimulated an increase in FOXO1 in osteoclasts as determined by the number of FOXO1+TRAP+ double-positive multinucleated cells. Interestingly, RANKL also stimulated an increase in FOXO1 nuclear localization, which peaked at day 2 and declined on day 4, although expression was still high on day 4. This pattern was observed in both BMMs and RAW264.7 cells. FOXO1 nuclear localization 2 d after RANKL stimulation was shown by both immunofluorescence and Western blot assays. Each of these assays was carried out with primary Abs to FOXO1 from different sources and gave consistent results. The time frame for induction of FOXO1 nuclear localization by RANKL is considerably slower than the 1–2 h induction that is observed for TNF stimulation. However, it is similar to the time frame of RANKL-induced NFATc1 (8). Interestingly, both the protein expression and nuclear localization of NFATc1 are continuously increased during RANKL-induced differentiation of BMMs to osteoclasts. Because FOXO1 activity is directly related to its nuclear localization, the functional significance of high FOXO1 expression but exclusion from the nucleus on day 4 requires further investigation. Pathways that are known to mediate RANKL-induced NFATc1 also were shown to be involved in FOXO1 nuclear localization (35–38). Thus, FOXO1 nuclear localization was significantly reduced by inhibitors of MAPK, ROS, NOS, and deacetylase. In contrast, Akt, which is known to negatively regulate FOXO1, did not have a significant effect on RANKL-induced FOXO1 nuclear localization 2 d after stimulation.

That FOXO1 had a significant effect in mediating RANKL stimulation was shown by significantly reduced osteoclast formation in vivo and reduced total resorption pit area in experimental mice compared with matched control mice. The effect of FOXO1 deletion on reducing RANKL-stimulated osteoclast formation from BMMs or RAW264.7 cells in vitro was similar to in vivo results. Additionally, the size and number of nuclei per osteo-
clast was significantly smaller when FOXO1 was deleted or silenced, suggesting that the amount of cell fusion was diminished. Osteoclast resorbing activity was also reduced when FOXO1 was deleted in BMMs or silenced in RAW264.7 cells in vitro. We also compared cancellous bone volume and several aspects of bone architecture in vertebrae of Lyz.M.Cre^+/FOXO1^+/− mice versus control littermates. However, there was no significant difference between the two groups, suggesting that FOXO1 deletion alone does not have a major impact in physiologic bone remodeling in young mice.

Several downstream targets were identified to investigate mechanisms through which FOXO1 may affect osteoclastogenesis. These studies investigated whether FOXO1 mediated the expression of gene targets that are induced by RANKL stimulation (1, 39). NFATc1, ATP6v0d2, and DC-STAMP expression and nuclear localization of NFATc1 were reduced by FOXO1 deletion. NFATc1 is a master regulator of osteoclastogenesis, transducing the effects of RANKL-induced fusion of precursor cells (1, 39). Thus, FOXO1 may impact cell fusion through downstream regulation of NFATc1, which in turn regulates DC-STAMP and ATP6v0d2 (7). FOXO1 also modulated mRNA levels of genes that are needed for osteoclast function, including cathepsin K and ATP6v0d2, which are responsible for dissolution of the mineral phase and proteolytic degradation of bone matrix proteins (6). The latter are consistent with our findings that FOXO1 deletion or knockdown reduced osteoclast activity stimulated by RANKL in vitro. Moreover, the linkage between NFATc1 and FOXO1 is supported by in vivo results that FOXO1 deletion reduces NFATc1 expression in osteoclasts following RANKL injection. However, a luciferase reporter assay suggests that NFATc1 promoter activity is not directly regulated by FOXO1, which may indicate that other mechanisms are involved.

FOXO1 deletion did not affect the formation of osteoclast precursors but did affect M-CSF–induced BMM migration and M-CSF–induced integrin and RANK expression. RANK, integrin αv, and BMM migration are important in the formation of osteoclasts from BMM osteoclast precursors (31, 32). Thus, FOXO1 may also influence the formation of osteoclasts by mediating M-CSF stimulation of BMMs. Bartell et al. (21) reported that RANKL reduced FOXO1 promoter activity at 16 h in vitro and that simultaneous FOXO1, -3, and -4 deletion in vivo increased osteoclastogenesis and bone resorption. Their report concluded that FOXO1, -3, and 4 acted to inhibit bone loss by inducing catabolic production, which acted to reduce formation of H2O2. In contrast, we focused on FOXO1 alone and downstream events that occur in a later time frame, 2 d after stimulation when osteoclast precursors fuse to form multinuclear cells. Our results demonstrate that FOXO1 mediates several events associated with RANKL-induced osteoclastogenesis in vivo and in vitro and also affects events downstream of M-CSF, including RANK and integrin αv expression as well as M-CSF–induced migration of BMMs. Thus, we have shown in the present study that under direct stimulation FOXO1 promotes M-CSF/RANKL–induced osteoclastogenesis, whereas the net long-term indirect effect of FOXO1, -3, and -4, as shown by Bartell et al. is to reduce osteoclastogenesis associated with aging by attenuating oxidative stress.

Acknowledgments

We thank Dr. Ronald DePinho for providing the FOXO1^−/− mice. We thank Sri Aravind Thaitikonda for assistance with genotyping and Sunitha Batchu for help in preparing this manuscript.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1: LyzM deletes FOXO1 in osteoclast precursors (bone marrow-derived macrophages) but does not affect other hematopoietic cells.

FOXO1-immunostained cells (red) were counterstained with DAPI (blue) in bone marrow cells and bone marrow macrophages.
Supplemental Figure 2: FOXO1 deletion in myloid cells has no effect on trabecular bone of vertebra but leads to reduced osteoclast formation from BMMs and RAW264.7 cells after RANKL stimulation in vitro.

(A-E) The vertebrae are harvested from LyzM.Cre⁺ FOXO1/L and LyzM.Cre⁻ FOXO1/L mice and then fixed. L4 lumbar vertebrae were scanned at 55 kVp, 145 μA. Images of two-dimensional cancellous bone slices were selected and used to generate three-dimensional reconstructions. Bone morphometric parameters were analyzed including bone volume over total volume (BV/TV*), trabecular number (Tb.N*), trabecular separation (Tb.Sp*), trabecular thickness (Tb.Th*), and bone mineral density (BMD). (F) BMMs from LyzM.cre⁺ FOXO1/L and littermate control LyzM.cre⁻ FOXO1/L mice were incubated with RANKL (100ng/ml) and M-CSF (30ng/ml). After 4 days, cells were fixed and stained for F-actin (green) and counterstained with DAPI (blue) (10x magnification). (G) RAW 264.7 cells were transfected with FOXO1 siRNA or scrambled siRNA and incubated with RANKL (100ng/ml). After 4 days, cells were fixed and stained as above (10x magnification).
Supplemental Figure 3: FOXO1 deletion reduces NFATc1 and RANK expression and NFATc1 nuclear localization

A. Bone marrow macrophages (BMMs) from LyzM.cre<sup>+</sup>FOXO1<sup>L/L</sup> and littermate control LyzM.cre<sup>-</sup>FOXO1<sup>L/L</sup> mice were incubated with RANKL (100ng/ml) and M-CSF (30ng/ml). After 0 or 2 days cells were fixed and NFATc1 (red) was detected by immunofluorescence and nuclei by DAPI counterstain (blue) (20x magnification). (B) BM were obtained from bone marrow and centrifuged in a cytocentrifuge, BMMs were obtained from BM stimulated with M-CSF (30ng/ml) for 3 days. Both BM and BMMs from LyzM.cre<sup>+</sup>FOXO1<sup>L/L</sup> and littermate control LyzM.cre<sup>-</sup>FOXO1<sup>L/L</sup> mice were fixed, RANK was stained as indicated and was visualized by immunofluorescence. RANK stained (red), DAPI counterstained (blue) cells (20x magnification).
Supplemental Figure 4: FOXO1 deletion has no effect on formation of bone marrow macrophages.

Bone marrow cells were obtained following cytocpin of single cell suspensions from bone marrow of LyzMcre⁺FOXO1⁺/⁻ and littermate control LyzMcre⁻FOXO1⁻/⁻ mice. Bone marrow macrophages were obtained by in vitro culture of bone marrow suspensions as described in Methods. Bone marrow macrophages were identified by immunofluorescence with antibody specific for CD11b (red) and DAPI counterstain (blue). No signal was detected with matched control antibody (data not shown).