CCAAT/Enhancer-binding Protein α (C/EBPα) is Important for Osteoclast Differentiation and Activity

Joel Jules, Wei Chen, Xu Feng, and Yi-Ping Li

From the Department of Pathology, University of Alabama, Birmingham, Alabama 35294

CCAAT/enhancer-binding protein (C/EBPα) can appoint mouse bone marrow (MBM) cells to the osteoclast (OC) lineage for osteoclastogenesis. However, whether C/EBPα is also involved in OC differentiation and activity is unknown. Here we demonstrated that C/EBPα overexpression in MBM cells can promote OC differentiation and strongly induces the expression of the OC genes encoding the nuclear factor of activated T-cells, c1 (NFATc1), cathepsin K (Cstk), and tartrate-resistant acid phosphatase 5 (TRAP) with receptor activator of NF-κB ligand-evoked OC lineage priming. Furthermore, while investigating the specific stage of OC differentiation that is regulated by C/EBPα, our gene overexpression studies revealed that, although C/EBPα plays a stronger role in the early stage of OC differentiation, it is also involved in the later stage. Accordingly, C/EBPα knockdown drastically inhibits osteoclastogenesis and markedly abrogates the expression of NFATc1, Cstk, and TRAP during OC differentiation. Consistently, C/EBPα silencing revealed that, although lack of C/EBPα affects all stages of OC differentiation, it has more impact on the early stage. Importantly, we showed that ectopic expression of rat C/EBPα restores osteoclastogenesis in C/EBPα-depleted MBM cells. Furthermore, our subsequent functional assays showed that C/EBPα exhibits a dispensable role on actin ring formation by mature OCs but is critically involved in bone resorption by stimulating extracellular acidification and regulating cell survival. We revealed that C/EBPα is important for receptor activator of NF-κB ligand-induced Akt activation, which is crucial for OC survival. Collectively, these results indicate that C/EBPα functions throughout osteoclastogenesis as well as in OC function. This study provides additional understanding of the roles of C/EBPα in OC biology.

C/EBPα is a transcription factor of the C/EBP family of transcription factors, and members of this family share a conserved

\* This work was supported by National Institutes of Health Grants R01-AR-044741, R01DE023813, and AR-055307 (to Y. P. L.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

\[1\] To whom correspondence may be addressed: Dept. of Pathology, University of Alabama, SHEL 810, 1825 University Blvd., Birmingham, AL 35294-2182. Tel: 205-975-2607; Fax: 205-975-4919; E-mail: wechen@uab.edu.

\[2\] To whom correspondence may be addressed: Dept. of Pathology, University of Alabama, 1825 University Blvd., Shelby Bldg. 810, Birmingham, AL 35294-2182. Tel: 205-975-2605; Fax: 205-975-4919; E-mail: ypli@uab.edu.

\[3\] The abbreviations used are: C/EBPα, CCAAT/Enhancer-binding protein α; OC, osteoclast; RANKL, receptor activator of NF-κB ligand; MBM, mouse bone marrow; TRAP, tartrate-resistant acid phosphatase 5; AO, acridine orange; qPCR, quantitative PCR; Hprt, hypoxanthine-guanine phosphoribosyl transferase; TAD, transcription activation domain; SEM, scanning electron microscopy.
Role of C/EBPα Is Osteoclast Differentiation and Function

M-CSF (20 ng/ml) for 48 h. Some cells were submitted to osteoclasisogenesis assays as indicated in individual experiments. Other cells were infected with virus in the presence of M-CSF (10 ng/ml) and Polybrene for 24 h before being submitted to osteoclasisogenesis assays (23). For rescue osteoclasisogenesis assays, MBM cells were first infected with a lentivirus for 24 h to deplete C/EBPα and then infected with a retrovirus encoding rat C/EBPα for 24 h to rescue C/EBPα expression as described previously (23, 28). At the end of the osteoclasisogenesis assays, all cells were stained for TRAP activity using a leukocyte acid phosphatase kit (catalog no. 387-A, Sigma) according to the instructions of the manufacturer. Assay quantification was carried by counting or accessing the size of the multinucleated TRAP-positive cells (more than three nuclei) in representative areas. The experiments involving mice were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Actin Ring and Acidine Orange (AO) Staining—MBM cells (5 × 10^4 cells/well) were treated as indicated in individual experiments. Actin ring staining was performed as described previously (23, 29). Briefly, cells were fixed with 4% paraformaldehyde for 10 min and then treated with 0.1% Triton X-100 in PBS for 10 min before staining with Alexa Fluor 488 phallolidin in PBS for 20 min. AO staining was also performed as described previously (29). Cells were incubated with 5 μg/ml AO solution in α-minimal essential medium at 37 °C for 15 min and then washed with PBS. Cells were imaged using a light-emitting diode fluorescence microscope (DM3000, Leica), and a representative image from each assay is shown.

In Vitro Bone Resorption Assays—Bone resorption analysis was carried as described previously (30, 31). Briefly, MBM cells (5 × 10^4 cells/well) seeded on bovine cortical bone slices were treated as indicated in individual experiments. Bone slices were then collected, and cells were removed with 0.25 M ammonium hydroxide and mechanical agitation. Bone resorption pits were imaged by a FEI Co. Quanta™ 650 FEG SEM (30) at the University of Alabama at Birmingham School of Engineering or stained with hematoxylin as described previously (31). A representative area from each assay was shown. Data were quantified by measuring the percent resorbed areas in three random areas using ImageJ software from the National Institutes of Health.

Western Blotting Analysis—Western blotting analysis was performed as described previously (32). Briefly, protein lysate was prepared and submitted to gel electrophoresis. Membranes were washed, and enhanced chemiluminescence detection was carried using Luminata Forte HRP substrate from Millipore. Membranes were visualized using a C-DiGit® blot scanner and Image Studio software from Li-Cor.

Quantitative Real-time PCR (qPCR) Analysis—qPCR analysis was carried out as described previously (11). Briefly, MBM cells were treated as indicated in individual experiments, and total RNA was collected using TRIzol reagent (Life Technologies). 1 μg of total RNA was used for cDNA synthesis by reverse transcription using SuperScript® VIVO™ Master Mix (Life Technologies) according to the instructions of the manufacturer. qPCR reactions were carried using Fast SYBR® Green
Master Mix reagent (Life Technologies). PCR conditions and primer sequences are available upon request. Hypoxanthine-guanine phosphoribosyl transferase (Hprt) was used as an endogenous control for normalization.

Statistical Analysis—Data are reported as mean ± S.D. Statistical significance was assessed using Student’s t test. \( p > 0.05 \) was considered significant.

Results

C/EBP\(\alpha\) Overexpression Can Mediate OC Differentiation and Strongly Induce Gene Expression with RANKL-evoked Lineage Priming—We have recently shown that forced expression of C/EBP\(\alpha\) can reprogram MBM cells into OC-like cells and thereby primes these cells into the OC lineage for osteoclastogenesis in the absence of RANKL (11). To further examine the role of C/EBP\(\alpha\) in OCs, we first wanted to confirm this previous finding by using the 293GPG retroviral system for gene expression (24). As expected, C/EBP\(\alpha\) overexpression in MBM cells could initiate osteoclastogenesis by generating TRAP-positive mononucleated cells independently of RANKL compared with a GFP control (supplemental Fig. 1A). Various studies have demonstrated that RANKL mediates OC formation by inducing the expression of various genes, including NFATc1, Cstk, and TRAP (21, 33, 34). To confirm the ability of C/EBP\(\alpha\) to mediate OC lineage priming, we examined its ability to induce the expression of the aforementioned OC genes independently of RANKL. C/EBP\(\alpha\) overexpression in MBM cells significantly up-regulated the expression of NFATc1, TRAP, and Cstk without RANKL stimulation (supplemental Fig. 1B). These results replicate our previous finding and thus confirm that C/EBP\(\alpha\) can mediate OC lineage commitment in the absence of RANKL.

Next we focused on OC differentiation to investigate whether C/EBP\(\alpha\) is also required beyond the lineage priming stage of osteoclastogenesis. It was reported that, although treatment of MBM cells with permissive levels of RANKL is unable to promote OC differentiation, these RANKL doses are sufficient to commit MBM cells into the OC lineage for TNF-\(\alpha\)- and IL-1-mediated OC differentiation (23, 35–37). Hence, we utilized this strategy to examine the role of C/EBP\(\alpha\) in OC differentiation. The permissive levels of RANKL required for OC lineage commitment is about one-tenth of the optimal RANKL dosage (10 ng/ml) utilized in standard in vitro osteoclastogenesis assays involving M-CSF and RANKL (11). Hence, we overexpressed C/EBP\(\alpha\) in the presence of 1 ng/ml RANKL to promote OC differentiation with RANKL-induced lineage commitment (Fig. 1). MBM cells overexpressing C/EBP\(\alpha\) or expressing the GFP control, as confirmed by Western blotting analysis (Fig. 1A), were submitted to osteoclastogenesis assays (Fig. 1B). Although MBM cells expressing GFP or overexpressing C/EBP\(\alpha\) formed numerous OCs with M-CSF and 10 ng/ml RANKL, the C/EBP\(\alpha\) overexpressers generated significantly more OCs than the GFP controls (Fig. 1C). Interestingly, only the cells overexpressing C/EBP\(\alpha\), but not the GFP expressers, could promote OC differentiation with permissive RANKL doses, indicating that C/EBP\(\alpha\) overexpression can stimulate OC differentiation (Fig. 1, B and C). Furthermore, our data showed that C/EBP\(\alpha\) overexpression did not induce a significant increase in OC size compared with the control cells (Fig. 1, D and E). To elucidate the molecular basis of the role of C/EBP\(\alpha\) in OC differentiation, we examined the ability of permissive RANKL levels to induce the expression of the aforementioned OC genes in the C/EBP\(\alpha\) overexpressers. Data showed that permissive RANKL dosages drastically up-regulated the expression of NFATc1, Cstk, and TRAP in MBM cells overexpressing C/EBP\(\alpha\) compared with the GFP controls during OC differentiation (Fig. 1F).

Given this new finding regarding the role of C/EBP\(\alpha\) in OC differentiation (Fig. 1), we then sought to isolate the specific stage of OC differentiation that is regulated by C/EBP\(\alpha\) (Fig. 2). Standard osteoclastogenesis assays require the stimulation of OC precursors with M-CSF and RANKL for 4–5 days (11, 23, 27, 36), so we infected MBM cells with a virus encoding the C/EBP\(\alpha\) gene or GFP control in different stages of osteoclastogenesis to investigate the effects of C/EBP\(\alpha\) overexpression in OC differentiation (Fig. 2A). Previous studies showed that treatment of MBM cells with M-CSF and RANKL for 12–24 h can commit these cells to the OC lineage for osteoclastogenesis (38, 39), so we overexpressed the C/EBP\(\alpha\) gene on day 1, 2, or 3 of osteoclastogenesis to investigate its role in OC differentiation. C/EBP\(\alpha\) overexpression on day 1, 2, or 3 of osteoclastogenesis, as confirmed by Western blotting analysis (Fig. 2B), could induce OC differentiation with permissive RANKL dosages (Fig. 2, C, bottom row versus center row, and D) compared with GFP controls. However, we noticed that C/EBP\(\alpha\) played a stronger role in the early stage (day 1) than later stage (day 2 or 3) of osteoclastogenesis, as substantiated by OC numbers (Fig. 2C). Consistently, culture of the C/EBP\(\alpha\) overexpressers with M-CSF and 10 ng/ml RANKL generated more OCs from cells in which C/EBP\(\alpha\) was overexpressed in the early stage of osteoclastogenesis than from cells in which C/EBP\(\alpha\) was overexpressed in the later stage (Fig. 2, C, bottom row, and E). In line with our previous data (11), we found that C/EBP\(\alpha\) overexpression on day 0 of osteoclastogenesis gave rise to more OCs than its overexpression on either day 1, 2, or 3 (data not shown), further confirming the role of C/EBP\(\alpha\) in OC lineage priming in addition to its role in OC differentiation. However, the stronger effect of C/EBP\(\alpha\) overexpression in the early stage of OC differentiation compared with the later stage may result from the different duration of C/EBP\(\alpha\) overexpression in these osteoclastogenesis assays. Therefore, in a modified experiment, we cultured all cells for 4 days after C/EBP\(\alpha\) overexpression in the different stages of OC differentiation (Fig. 2F). Our results confirmed that C/EBP\(\alpha\) overexpression in the early stage of osteoclastogenesis exhibited a stronger role in OC differentiation compared with GFP controls (Fig. 2, G–I). Moreover, C/EBP\(\alpha\) overexpressers treated with M-CSF and 10 ng/ml RANKL generated more OCs than the GFP control cells (Fig. 2, E and I). Taken together, these results show that C/EBP\(\alpha\) can promote OC differentiation by strongly inducing gene expression from RANKL-primed MBM cells.

C/EBP\(\alpha\) Silencing Inhibits OC Differentiation and Markedly Abrogates Gene Expression—To further investigate the role of C/EBP\(\alpha\) in OC differentiation, we utilized a loss-of-function strategy to examine the impact of C/EBP\(\alpha\) silencing on osteoclastogenesis (supplemental Fig. 2 and Fig. 3). We were able to...
knock down the C/EBPα gene using three of five C/EBPα shRNA constructs (C/EBPα shRNA 1, 3, and 5) that were purchased from Sigma (supplemental Fig. 2, A and B). The ability of MBM cells expressing C/EBPα shRNA 1, 3, or 5 to promote osteoclastogenesis was drastically inhibited compared with scramble controls (supplemental Fig. 2, C and D). Notably, MBM cells expressing C/EBPα shRNA 2 or 4, which were unable to suppress C/EBPα expression (supplemental Fig. 2, A and B), failed to inhibit osteoclastogenesis (supplemental Fig. 2, C and D), confirming the specificity of C/EBPα shRNA constructs 1, 3, and 5 in inhibiting osteoclastogenesis. Because C/EBPα shRNA constructs 1 and 5 can drastically inhibit osteoclastogenesis (supplemental Fig. 2C), these two shRNA constructs were chosen to further examine the role of C/EBPα in OC differentiation (Fig. 3, A–D). Similar to C/EBPα overexpression in different stages of OC differentiation (Fig. 2), we infected MBM cells with a virus encoding C/EBPα shRNAs or the scramble control on day 1, 2, or 3 of osteoclastogenesis (Fig. 3) to examine the impact of C/EBPα silencing on OC differentiation. We found that C/EBPα silencing in the early stage (day 1) of osteoclastogenesis had a stronger inhibitory effect on OC formation than the later stage (day 2 or 3) (Fig. 3, B–D), confirming that C/EBPα plays a stronger role in the early stage of osteoclastogenesis. Nevertheless, the stronger effect of C/EBPα silencing in the early stage of osteoclastogenesis compared with the later stage may result from the different duration of C/EBPα silencing in these osteoclastogenesis assays. Therefore, in a modified experiment, we cultured all cells for 4 days after C/EBPα silencing in the different stages of OC differentiation (Fig. 3E). Our results further confirmed that C/EBPα silencing...
FIGURE 2. C/EBPα overexpression plays a stronger role in the early stage of OC differentiation but is also important for the later stage. A, schematic of the research strategy for B–E. B, MBM cells were infected with a retrovirus encoding the GFP control (GFP) or FLAG-C/EBPα (C/EBPα) on day 1, 2, or 3 of osteoclastogenesis in the presence of M-CSF alone (10 ng/ml) for 24 h. The cultures were then continued for 5 days with M-CSF alone. Gene expression was examined by Western blotting using β-actin as a loading control. C, the same set of assays as in A was repeated, but cells were infected with a retrovirus for 24 h while undergoing treatment with M-CSF (10 ng/ml) plus RANKL (1 ng/ml) or M-CSF (10 ng/ml) plus RANKL (10 ng/ml) for 5 days. D and E, quantification of the assays in C for M-CSF + RANKL (1 ng/ml, D) or M-CSF + RANKL (10 ng/ml, E) in at least three independent experiments. F, schematic of the research strategy for G–I. G, MBM cells were infected with a retrovirus encoding the GFP control or C/EBPα on day 1, 2, or 3 of osteoclastogenesis with M-CSF (10 ng/ml) and RANKL (1 ng/ml) or M-CSF (10 ng/ml) plus RANKL (10 ng/ml) for 24 h. Following the infection, all cells were cultured with M-CSF (10 ng/ml) and RANKL (1 ng/ml) or M-CSF (10 ng/ml) plus RANKL (10 ng/ml) for 4 days. The cultures in C and G were stained for TRAP activity. H–I, quantification of the assays in G for M-CSF + RANKL (1 ng/ml, H) or M-CSF + RANKL (10 ng/ml, I) from at least three independent experiments. The numbers in parentheses show concentration in nanograms per milliliter. Error bars show averages ± S.D. *, p < 0.05; NS, not significant. Scale bars = 200 μm.
in the early stage of osteoclastogenesis exhibited a stronger role in OC differentiation compared with the scramble control (Fig. 3, F and G). Furthermore, our gene expression analysis showed that C/EBPα depletion drastically abrogated the expression of NFATc1, Cstk, and TRAP during OC differentiation (Fig. 3H).

The results show that C/EBPα silencing impedes OC differentiation by blunting gene expression.

Ectopic Expression of Rat C/EBPα Can Restore Osteoclastogenesis in C/EBPα-depleted MBM Cells—The C/EBPα gene is highly homologous between mouse and rat and has two natural
**Role of C/EBPα Is Osteoclast Differentiation and Function**

**FIGURE 4.** Ectopic expression of rat C/EBPα restores osteoclastogenesis in C/EBPα-depleted MBM cells. A, MBM cells were first infected with a virus encoding a scramble shRNA control (Scr-sh) or C/EBPα shRNA construct 5 (C/EBPα-sh 5) for 24 h and then infected with a virus encoding the GFP control (GFP) or FLAG-rat p30C/EBPα (rat C/EBPα) for 24 h. Doubly infected cells were then treated with M-CSF (10 ng/ml) for 3 days before being submitted to Western blotting analysis using β-actin as a loading control. B, the same set of assays as in A was repeated, but after the double infection, cells were treated with M-CSF (10 ng/ml) plus RANKL (1 ng/ml) or M-CSF (10 ng/ml) plus RANKL (10 ng/ml) for 4 days. Cultures were then stained for TRAP activity. Scale bars = 200 μm. C and D, quantification of the assays in B for M-CSF + RANKL (1 ng/ml, C) or M-CSF + RANKL (10 ng/ml, D) are shown from three independent experiments. The numbers in parentheses show concentration in nanograms per milliliter. Error bars show mean ± S.D. *, p < 0.05; NS, not significant.

isoforms: the fully translated protein (p42) and a shorter protein (p30) that lacks the first 117 amino acids at the N terminus (40). To confirm the role of C/EBPα in OC differentiation, we rescued C/EBPα expression after shRNA-induced gene silencing to investigate its impact on OC differentiation (Fig. 4). Because the region targeted by C/EBPα shRNA construct 5 is located around nucleotides 98–130 of the 5′ end of C/EBPα cDNA, we used rat p30C/EBPα cDNA, which lacks this region, to rescue C/EBPα expression after gene silencing (Fig. 4A). Although C/EBPα depletion followed by GFP expression failed to promote osteoclastogenesis with 10 ng/ml RANKL, ectopic expression of rat C/EBPα in C/EBPα-depleted cells submitted to the same treatment could restore osteoclastogenesis (Fig. 4, B and D). As expected, expression of GFP or rat C/EBPα in MBM cells expressing scramble shRNA controls could mediate osteoclastogenesis with 10 ng/ml RANKL (Fig. 4, B–D). Importantly, only MBM cells doubly expressing either C/EBPα shRNA and rat C/EBPα or scramble shRNA and rat C/EBPα could mediate osteoclastogenesis with permissive RANKL doses but not MBM cells expressing C/EBPα shRNA and GFP control or scramble shRNA and the GFP control (Fig. 4, B and C). These results confirm that C/EBPα is critical for OC differentiation.

**C/EBPα Is Important for OC Activity**—Upon finding that C/EBPα is also involved in OC differentiation aside from its previously identified role in OC lineage commitment (11), we investigated whether C/EBPα is still required beyond OC differentiation by examining its role in OC function. Toward this end, MBM cells were first differentiated into pre-OCs and then infected with a retrovirus to overexpress the C/EBPα gene (Fig. 5A), which was then continued with RANKL stimulation to form mature OCs for examination of actin ring formation, a critical feature of mature OCs, and extracellular acidification, critical for OC activity (Fig. 5B) and to promote bone resorption by mature OCs (Fig. 5, C and D, and supplemental Fig. 3). As negative controls, GFP expressers or C/EBPα overexpressers cultured with M-CSF alone remained mononucleated and did not form any actin ring (Fig. 5B, columns 1 versus 3, top row). Interestingly, C/EBPα overexpressers cultured with M-CSF alone showed extracellular acidification by mononucleated cells as compared with GFP controls (Fig. 5B, columns 2 versus 4, top row). As expected, GFP expressers or C/EBPα overexpressers treated with 10 ng/ml RANKL formed numerous OCs assessed by actin ring formation (Fig. 5B, column 1 versus column 3, bottom row) and extracellular acidification (41, 42) (Fig. 5B, column 2 versus column 4, bottom row). Notably, although
C/EBPα overexpressers treated with permissive RANKL doses formed many Ocs, as substantiated by actin ring formation (Fig. 5B, column 3, center row) and extracellular acidification (Fig. 5B, column 4, center row), GFP expressers submitted to the same treatment remained mononucleated and formed no actin ring (Fig. 5B, column 1, center row) but showed little extracellular acidification by mononucleated cells (Fig. 5B, column 2, center row). These results indicate that C/EBPα overexpression does not influence actin formation but can stimulate extracellular acidification in mature Ocs. Consistent with the role of
C/EBPα in extracellular acidification, we found that C/EBPα overexpression significantly promoted bone resorption with 10 ng/ml RANKL, as assessed by SEM analysis (Fig. 5, C, bottom row, and D) or hematoxylin staining of the bone resorption pits (supplemental Fig. 3). Moreover, although GFP expressers treated with permissive RANKL doses barely induced bone resorption, C/EBPα overexpressers submitted to this treatment strongly induced bone resorption (Fig. 5, C, center row, and D, and supplemental Fig. 3).

To confirm this finding, we turned to our loss-of-function strategy to examine the effect of C/EBPα depletion on OC activity (Fig. 6 and supplemental Fig. 4). MBM cells were differentiated into pre-OCs and then infected with a virus to ablate C/EBPα expression (Fig. 6A), which was then continued with RANKL stimulation to generate mature OCs for examination of actin ring formation and extracellular acidification (Fig. 6B) or to promote bone resorption by mature OCs (Fig. 6, C and D, and supplemental Fig. 4). C/EBPα silencing gave rise to fewer OCs than scramble controls but did not affect actin ring formation (Fig. 6B, left column). Fascinatingly, C/EBPα silencing markedly affected extracellular acidification by mature OCs, as assessed by AO staining (Fig. 6B). Moreover, C/EBPα depletion drastically abrogated bone resorption by OCs, as assessed by SEM analysis (Fig. 6, C and D) and hematoxylin staining of the bone resorption pits (supplemental Fig. 4). Taken together, these findings indicate that, although C/EBPα is dispensable for the actin ring, it is crucially involved in extracellular acidification and bone resorption by mature OCs.

C/EBPα Can Regulate Cell Survival—Given the drastic impact of C/EBPα silencing on osteoclastic bone resorption (Fig. 6 and supplemental Fig. 4), we reasoned that C/EBPα...
Role of C/EBPα Is Osteoclast Differentiation and Function

might also regulate cell survival aside from its role in extracellular acidification. This is consistent with the notion that an increase in OC survival by C/EBPα may also promote OC activity independently or in concert with an increase in extracellular acidification. In addressing this notion, MBM cells were first differentiated into pre-OCs and then infected with a virus to overexpress C/EBPα, which was followed by osteoclastogenic treatments to complete a 4-, 6-, and 8-day culture for examination of OC survival by assessing OC numbers (Fig. 7, A–C). We found that C/EBPα overexpression significantly enhanced OC survival from culture with M-CSF and 10 ng/ml RANKL after 4 days of culture (Fig. 7A) but only slightly enhanced OC survival after 6 or 8 days of culture (Fig. 7, B and C) compared with GFP controls. Interestingly, we noticed that, although C/EBPα overexpression generated few OCs with permissive RANKL doses that survived through 8 days of culture, GFP controls formed no OCs, further confirming the role of C/EBPα in OC differentiation (Fig. 7, A–C). Numerous studies have reported that RANKL can promote OC survival by activating the Akt signaling pathway in OC precursors (43–47). Hence, we examined the ability of C/EBPα overexpression to activate this pathway in MBM cells. However, the ability of C/EBPα overexpression to stimulate RANKL-induced Akt activation in MBM cells overexpressing the C/EBPα gene was similar to that of GFP controls (Fig. 7D), indicating that C/EBPα overexpression does not modulate RANKL-induced Akt activation to promote cell survival.

Finally, we repeated these survival experiments using the loss-of-function strategy by silencing the C/EBPα gene (Fig. 8). Fascinatingly, C/EBPα depletion drastically attenuated OC survival after 4, 6, and 8 days of culture, as assessed by a decrease in OC numbers (Fig. 8A). Consistently, C/EBPα ablation suppressed Akt activation by RANKL in MBM cells (Fig. 8B). Collectively, the data show that C/EBPα is critical for OC activity by also regulating cell survival.

Discussion

We have previously reported that C/EBPα is essential for OC lineage commitment, but its roles in terminal osteoclastogenesis and OC activity remain unresolved. This study was aimed at addressing these critical issues.

C/EBPα Can Mediate OC Differentiation in Precommitted MBM Cells—The C/EBPα protein was initially identified based on its ability to interact with gene promoters (1) and has two natural isoforms. p42C/EBPα has two transcription activation domains (TAD1 and TAD2) at the N terminus and a basic leucine zipper domain at the C terminus. p30C/EBPα lacks the first 117 amino acids of the N terminus, which include the TAD1 region (48). Although the TAD1 and TAD2 domains can recruit co-activators and remodeling complex, the basic leucine zipper domain is important for protein interaction and DNA binding for gene expression (40). In addressing the role of C/EBPα in OC differentiation, we first utilized a gain-of-function strategy to overexpress p42C/EBPα in MBM cells in the presence of permissive RANKL levels to promote OC differentiation. We adopted this strategy to investigate whether C/EBPα is required beyond the lineage priming of osteoclastogenesis. We showed that C/EBPα overexpression can induce OC differentiation. In deciphering the specific stage of osteoclastogenesis that is regulated by C/EBPα, we showed that C/EBPα functions throughout osteoclastogenesis but plays a more critical role in the early stage. This finding is consistent with our previous report that further supports the role of C/EBPα in lineage commitment while also elucidating its novel role in OC differentiation (11). In addressing the molecular basis of the role of C/EBPα overexpression in OC differentiation, we showed that C/EBPα overexpression can drastically promote the expression of OC genes with permissive levels of RANKL.

C/EBPα Silencing Abrogates OC Differentiation—Moreover, our C/EBPα silencing studies not only reinforce our findings that C/EBPα functions throughout OC differentiation but also supports that C/EBPα is more critical for the early stage of OC differentiation. In confirming the molecular basis of the requirement of C/EBPα in OC differentiation, C/EBPα ablation drastically abrogates the expression of the OC gene during differentiation. Finally, we confirmed the role of C/EBPα in OC differentiation by rescuing osteoclastogenesis in C/EBPα-depleted cells via ectopic expression of rat p30C/EBPα. We noted that rat p30C/EBPα only partially mediates osteoclastogenesis compared with mouse p42C/EBPα (data not shown). The inability of rat p30C/EBPα to fully mediate osteoclastogenesis in these assays stems from the lack of the TAD1 region (49, 50). Nonetheless, rat p30C/EBPα retains the TAD2 domain, which can compensate for the loss of the TAD1 domain by functioning with the basic leucine zipper region to promote cell survival (40). Notably, the ability of rat p30C/EBPα to restore osteoclastogenesis in our assays is consistent with another study reporting that p30C/EBPα can also promote adipocyte differentiation (48).

C/EBPα Promotes OC Activity by Stimulating Extracellular Acidification and Regulating Cell Survival—We showed that, although C/EBPα plays no overt role in actin ring formation, C/EBPα is critical for extracellular acidification (29), indicating that C/EBPα is crucial for osteoclastic bone resorption. Although our gene expression analyses suggest that the ability of C/EBPα to activate OC genes may account for its role in bone resorption, we suspected that C/EBPα might also promote OC function by other mechanisms. Hence, we have previously reported that C/EBPα does not play any roles in the activation of the NF-κB and p38 signaling pathways in OC precursors, which are important for OC survival (51–54). We thus focused on investigating whether C/EBPα could activate the Akt signaling pathway in OC precursors, which has also been reported to be a strong regulator of OC survival (54–57). We revealed that C/EBPα can induce Akt activation to promote OC survival (58, 59). Importantly, this finding also suggests that a threshold level of C/EBPα may be required for effective Akt activation and that a drastic increase in C/EBPα expression may not substantially enhance Akt activation by RANKL. Hence, C/EBPα can modulate OC activity by inducing gene expression, stimulating extracellular acidification, and regulating cell survival. However, it remains to be investigated how C/EBPα can stimulate extracellular acidification in OCs. We speculate that C/EBPα may modulate the
FIGURE 7. Analysis of the role of C/EBPα overexpression in OC survival. A–C, C/EBPα overexpression slightly enhances OC survival. MBM cells were first treated with M-CSF (10 ng/ml) and RANKL (10 ng/ml) for 2 days (d) before infection with a retrovirus encoding the GFP control (GFP) or FLAG-C/EBPα (C/EBPα) for 1 day in the presence of M-CSF (10 ng/ml) alone, M-CSF (10 ng/ml) plus RANKL (1 ng/ml), or M-CSF (10 ng/ml) plus RANKL (10 ng/ml). The cultures were then continued to complete a 4-day (A), 6-day (B), and 8-day culture (C). The cultures were stained for TRAP activity. Quantification is shown in the right panels from at least three independent experiments. Scale bars = 200 μm. D, C/EBPα overexpression does not enhance RANKL-induced Akt activation in MBM cells. MBM cells expressing GFP or overexpressing C/EBPα were treated with RANKL (10 ng/ml) as indicated. The activation of the Akt pathway was assessed by Western blotting as phosphorylation of Akt using total Akt as a loading control in three independent experiments. The numbers in parentheses show concentration in nanograms per milliliter. Error bars show mean ± S.D. *, p < 0.05; NS, not significant.
expression of specific genes, such as carbonic anhydrase II, vacuolar H\textsuperscript{+}-ATPase and chloride channel CIC-7, which are known to play roles in OC acidification.

In summary, our data reveal that C/EBP\(\alpha\) is critical for OC differentiation and activity aside from its previously reported role in OC lineage priming (11). It is likely that C/EBP\(\alpha\) func-
Role of C/EBPα Is Osteoclast Differentiation and Function

tions in a complex with different proteins at different stages of osteoclastogenesis to exert its actions. We anticipate that identification of the partners with which C/EBPα interacts will be crucial to enhance our understanding of its roles in OCs. Therefore, future studies are warranted to elucidate the molecular mechanism(s) through which C/EBPα functions, potentially in concert with other factors, in OCs.

Author Contributions—J. J., W. C., and Y. P. L. designed the study, carried out the experiments, analyzed the data, and prepared the manuscript. X. F. provided the pMX-puro and pMX-GFP retroviral constructs as well as the 293GPG retroviral packaging cells and analyzed the data. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgments—We thank the University of Alabama at Birmingham Center for Metabolic Bone Disease (P30 AR046031) for assistance. We also appreciate the support of the SEM Core at the University of Alabama at Birmingham School of Engineering.

References
1. Landschulz, W. H., Johnson, P. F., Adashi, E. Y., Graves, B. J., and McKnight, S. L. (1988) Isolation of a recombinant copy of the gene encoding C/EBP. Genes Dev. 2, 786–800
2. Nerlov, C. (2007) The C/EBP family of transcription factors: a paradigm for interaction between gene expression and proliferation control. Trends Cell Biol. 17, 318–324
3. Ramji, D. P., and Foka, P. (2002) CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem. J. 365, 561–575
4. Zhang, P., Iwasaki-Arai, J., Iwasaki, H., Fenyus, M. L., Dayaram, T., Owens, B. M., Shigematsu, H., Levantini, E., Huettner, C. S., Lekstrom-Himes, J. A., Akashi, K., and Tenen, D. G. (2004) Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBPα. Immunity 21, 853–863
5. Mueller, B. U., and Pabst, T. (2006) C/EBPα and the pathophysiology of acute myeloid leukemia. Curr. Opin. Hematol. 13, 7–14
6. Zhang, D. E., Zhang, P., Wang, N. D., Hetherington, C. J., Darlington, G. J., and Tenen, D. G. (1997) Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein-α-deficient mice. Proc. Natl. Acad. Sci. U.S.A. 94, 569–574
7. Wang, N. D., Finegold, M. J., Bradley, A., Ou, C. N., Abdelsayed, S. V., Wilde, M. D., Taylor, L. R., Wilson, D. R., and Darlington, G. J. (1995) Impaired energy homeostasis in C/EBPα knockout mice. Science 269, 1108–1112
8. Ye, M., Zhang, H., Amabile, G., Yang, H., Staber, P. B., Zhang, P., Levantini, E., Alberich-Jordà, M., Zhang, J., Kawasaki, A., and Tenen, D. G. (2013) C/EBPα controls acquisition and maintenance of adult haematopoietic stem cell quiescence. Nat. Cell Biol. 15, 385–394
9. Porse, B. T., Bryder, D., Theilgaard-Mønch, K., Hasemann, M. S., Anderson, K., Damgaard, I., Jacobsen, S. E., and Nerlov, C. (2005) Loss of C/EBPα cell cycle control increases myeloid progenitor proliferation and transforms the neutrophil granulocyte lineage. J. Exp. Med. 202, 85–96
10. Roe, J. S., and Vakoc, C. R. (2014) C/EBPα: critical at the origin of leukemic transformation. J. Exp. Med. 211, 1–4
11. Chen, W., Zhu, G., Hao, L., Wu, M., Ci, H., and Li, Y. P. (2013) C/EBPα regulates osteoclast lineage commitment. Proc. Natl. Acad. Sci. U.S.A. 110, 7294–7299
12. Feng, X., and McDonald, J. M. (2011) Disorders of bone remodeling. Annu. Rev. Pathol. 6, 121–145
13. Souza, P. P., and Lerner, U. H. (2013) The role of cytokines in inflammatory bone loss. Immunol. Infect. 42, 555–622
14. Boyle, W. J., Simonet, W. S., and Lacey, D. L. (2003) Osteoclast differentiation and activation. Nature 423, 337–342
15. Teitelbaum, S. L. (2000) Bone resorption by osteoclasts. Science 289, 1504–1508
16. Horowitz, M. C., Xi, Y., Wilson, K., and Kacena, M. A. (2001) Control of osteoclastogenesis and bone resorption by members of the TNF family of receptors and ligands. Cytokine Growth Factor Rev. 12, 9–18
17. Asagiri, M., and Takayanagi, H. (2007) The molecular understanding of osteoclast differentiation. Bone 40, 251–264
18. Karsenty, G., and Wagner, E. F. (2002) Reaching a genetic and molecular understanding of skeletal development. Dev. Cell 2, 389–406
19. Li, Y. P., Alexander, M., Wucherpfennig, A. L., Yelick, P., Chen, W., and Stashenko, P. (1995) Cloning and complete coding sequence of a novel human cathepsin expressed in giant cells of osteoclastomas. J. Bone Miner Res. 10, 1197–1202
20. Chen, W., Yang, S., Abe, Y., Li, M., Wang, Y., Shao, J., Li, E., and Li, Y. P. (2007) Novel pycnodysostosis mouse model uncovers cathepsin K function as a potential regulator of osteoclast apoptosis and senescence. Hum. Mol. Genet. 16, 410–423
21. Takayanagi, H. (2007) The role of NFAT in osteoclast formation. Ann. N.Y. Acad. Sci. 1116, 227–237
22. Onishi, M., Nosaka, T., Misawa, K., Mui, A. L., Gorman, D., McMahon, M., Miyajima, A., and Kitamura, T. (1998) Identification and characterization of a constitutively active STAT5 mutant that promotes cell proliferation. Mol. Cell. Biol. 18, 3871–3879
23. Jules, J., Shi, Z., Liu, J., Xu, D., Wang, S., and Feng, X. (2010) Receptor activator of NF-κB (RANK) cytoplasmic IVVYS535–538 motif plays an essential role in tumor necrosis factor-α (TNF)-mediated osteoclastogenesis. J. Biol. Chem. 285, 37427–37435
24. Ory, D. S., Neugeboren, B. A., and Mulligan, R. C. (1996) A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. Proc. Natl. Acad. Sci. U.S.A. 93, 11400–11406
25. Sakoda, T., Kasahara, N., Kedes, L., and Ohyanagi, M. (2007) Calcium phosphate coprecipitation greatly enhances transduction of cardiac myocytes and vascular smooth muscle cells by lentivirus vectors. Exp. Clin. Cardiol. 12, 133–138
26. Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. 36, 59–74
27. Yang, S., and Li, Y. P. (2007) RGS10-null mutation impairs osteoclast differentiation resulting from the loss of [Ca2+]i oscillation regulation. Genes Dev. 21, 1803–1816
28. Jules, J., Wang, S., Shi, Z., Liu, J., Wei, S., and Feng, X. (2015) The IVVY motif and tumor necrosis factor receptor-associated factor (TRAF) sites in the cytoplasmic domain of the receptor activator of nuclear factor κB (RANK) cooperate to induce osteoclastogenesis. J. Biol. Chem. 290, 23738–23750
29. Yang, D. Q., Feng, S., Chen, W., Zhao, H., Paulson, C., and Li, Y. P. (2012) V-ATPase subunit ATP6AP1 (Ac45) regulates osteoclast differentiation, extracellular acidification, lysosomal trafficking, and protease exocytosis in osteoclast-mediated bone resorption. J. Bone Miner. Res. 27, 1695–1707
30. Yang, S., Hao, L., McConnell, M., Zhou, X., Wang, M., Zhang, Y., Mountz, J. D., Reddy, M., Eleazer, P. D., Li, Y. P., and Chen, W. (2013) Inhibition of Rgs10 expression prevents immune cell infiltration in bacteria-induced inflammatory lesions and osteoclast-mediated bone destruction. Bone Res. 1, 267–281
31. Tamura, T., Takahashi, N., Akatsu, T., Sasaki, T., Udagawa, N., Tanaka, S., and Suda, T. (1993) New resorption assay with mouse osteoclast-like multinucleated cells formed in vitro. J. Bone Miner. Res. 8, 953–960
32. Chen, W., Ma, I., Zhu, G., Jules, J., Wu, M., McConnell, M., Tian, F., Paulson, C., Zhou, X., Wang, L., and Li, Y. P. (2014) Cbfβ deletion in mice recapitulates cleidocranial dysplasia and reveals multiple functions of Cbfβ required for skeletal development. Proc. Natl. Acad. Sci. U.S.A. 111, 8482–8487
33. Cappellen, D., Luong-Nguyen, N. H., Bongiovanni, S., Grenet, O., Wanke, C., and Susa, M. (2002) Transcriptional program of mouse osteoclast differentiation governed by the macrophage colony-stimulating factor and the ligand for the receptor activator of NFκB. J. Bone Miner Res. 17, 9482–9500
34. Takayanagi, H., Kim, S., Koga, T., Nishina, H., Ishikawa, M., Yoshida, H., Saiura, A., Isobe, M., Yokochi, T., Inoue, J., Wagner, E. F., Mak, T. W.,
Role of C/EBPα Is Osteoclast Differentiation and Function

Kodama, T., and Taniguchi, T. (2002) Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev. Cell 3, 889–901

35. Lam, J., Takeshita, S., Barker, J. E., Kanagawa, O., Ross, F. P., and Teitelbaum, S. L. (2000) TNF-α induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. J. Clin. Invest. 106, 1481–1488

36. Wei, S., Kitaura, H., Zhou, P., Ross, F. P., and Teitelbaum, S. L. (2005) IL-1 receptor activator of nuclear factor κB signaling for interleukin 1-mediated osteoclastogenesis. J. Biol. Chem. 280, 15728–15738

37. Taguchi, Y., Gohda, J., Koga, T., Takayanagi, H., and Inoue, J. (2009) A unique domain in RANK is required for Gab2 and PLCγ2 binding to establish osteoclastogenic signals. Genes Cells 14, 1331–1345

38. Xu, D., Wang, S., Liu, W., Liu, J., and Feng, X. (2006) A novel receptor activator of NF-κB (RANK) cytoplasmic motif plays an essential role in osteoclastogenesis by committing macrophages to the osteoclast lineage. J. Biol. Chem. 281, 4678–4690

39. Reckzeh, K., and Cammenza, J. (2010) Molecular mechanisms underlying deregulation of C/EBPα in acute myeloid leukemia. Int. J. Hematol. 91, 557–568

40. Li, Y. P., Chen, W., Liang, Y., Li, E., and Stashenko, P. (1999) Atp6i-deficient mice exhibit severe osteopetrosis due to loss of osteoclast-mediated extracellular acidification. Nat. Genet. 23, 447–451

41. Li, Y. P., Chen, W., and Stashenko, P. (1996) Molecular cloning and characterization of a putative novel human osteoclast-specific 116-kDa vacuolar proton pump subunit. Biochem. Biophys. Res. Commun. 218, 813–821

42. Liu, W., Wang, S., Wei, S., Sun, L., and Feng, X. (2005) Receptor activator of NF-κB (RANK) cytoplasmic motif, 369PFQEP373, plays a predominant role in osteoclast survival in part by activating Akt/PKB and its downstream effector AKT/FOXO4. J. Biol. Chem. 280, 43064–43072

43. Fuller, K., Wong, B., Fox, S., Choi, Y., and Chambers, T. J. (1998) TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. J. Exp. Med. 188, 997–1001

44. Wong, B. R., Besser, D., Kim, N., Arron, J. R., Vologodksaia, M., Hanafusa, H., and Choi, Y. (1999) TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. Mol. Cell 4, 1041–1049

45. Murakami, T., Yamamoto, M., Ono, K., Nishikawa, M., Nagata, N., Motoyoshi, K., and Akatsu, T. (1998) Transforming growth factor-β1 increases mRNA levels of osteoclastogenesis inhibitory factor in osteoblastic/stromal cells and inhibits the survival of murine osteoclast-like cells.

Biochem. Biophys. Res. Commun. 252, 747–752

46. Zhao, Q., Shao, J., Chen, W., and Li, Y. P. (2007) Osteoclast differentiation and gene regulation. Front Biosci. 12, 2519–2529

47. Lin, F. T., MacDougald, O. A., Diehl, A. M., and Lane, M. D. (1993) A 30-kDa alternative translation product of the CCAAT/enhancer binding protein α message: transcrptional activator lacking antimitotic activity. Proc. Natl. Acad. Sci. U.S.A. 90, 9606–9610

48. Nerlov, C., and Ziff, E. B. (1995) CCAAT/enhancer binding protein α amino acid motifs with dual TBP and TFIIB binding ability co-operate to activate transcription in both yeast and mammalian cells. EMBO J. 14, 4318–4328

49. Wang, H., Iakova, P., Wilde, M., Welm, A., Goode, T., Roesler, W. J., and Timchenko, N. A. (2001) C/EBPα arrests cell proliferation through direct inhibition of Cdk2 and Cdk4. Mol. Cell 8, 817–828

50. Gao, B., Chen, W., Hao, L., Zhu, G., Feng, S., Ci, H., Zhou, X., Stashenko, P., and Li, Y. P. (2013) Inhibiting peripapical lesions through AAV-RNAi silencing of cathepsin K. J. Dent. Res. 92, 180–186

51. Boyce, B. F., Xiu, Y., Li, J., Xing, L., and Yao, Z. (2015) NF-κB-mediated regulation of osteoclastogenesis. Endocrinol. Metab. (Seoul) 30, 35–44

52. Tai, T. W., Su, F. C., Chen, C. Y., Jou, I. M., and Lin, C. F. (2014) Activation of p38 MAPK-regulated Bel-4x signaling increases survival against zole-dronic acid-induced apoptosis in osteoclast precursors. Bone 67, 166–174

53. Gingery, A., Bradley, E. W., Pederson, L., Ruan, M., Horwood, N. J., and Oursler, M. J. (2008) TGFB-β coordinately activates TAK1/MEK/AKT/NFκB and SMAD pathways to promote osteoclast survival. Exp. Cell Res. 314, 2725–2738

54. Cicic, M., Vrabel, A., Sturchio, C., Pederson, L., Hauke, R. J., Subramaniam, M., Spelsberg, T. C., and Oursler, M. J. (2011) TGFB-β inducible early gene 1 regulates osteoclast differentiation and survival by mediating the NFATc1, AKT, and MEK/ERK signaling pathways. PloS ONE 6, e17522

55. Kwak, H. B., Sun, H. M., Ha, H., Lee, J. H., Kim, H. N., and Lee, Z. H. (2008) AG490, a Jak2-specific inhibitor, induces osteoclast survival by activating the Akt and ERK signaling pathways. Mol. Cells 26, 436–442

56. Hu, J. P., Nishishita, K., Sakai, E., Yoshida, H., Kato, Y., Tsukuba, T., and Okamoto, K. (2008) Berberine inhibits RANKL-induced osteoclast formation and survival through suppressing the NFκB and Akt pathways. Eur. J. Pharmacol. 580, 70–79

57. Qu, X., Zhai, Z., Liu, X., Li, H., Ouyang, Z., Wu, C., Liu, G., Fan, Q., Tang, T., Qin, A., and Dai, K. (2014) Dioscin inhibits osteoclast differentiation and bone resorption though down-regulating the Akt signaling cascades. Biochem. Biophys. Res. Commun. 443, 658–665

58. Tu, Q., Zhang, J., Dong, L. Q., Saunders, E., Luo, E., Tang, J., and Chen, J. (2011) Adiponectin inhibits osteoclastogenesis and bone resorption via APPL1-mediated suppression of Akt1. J. Biol. Chem. 286, 12542–12553