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E Proteins regulate osteoclast maturation and survival

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Abstract

Osteoclasts are bone specific polykaryons derived from myeloid precursors under the stimulation of MCSF and RANKL. E proteins are basic helix-loop-helix (bHLH) transcription factors that modulate lymphoid versus myeloid cell fate decisions. To study the role of E proteins in osteoclasts, myeloid specific E protein gain-of-function transgenic mice were generated. These mice have high bone mass due to decreased osteoclast numbers and increased osteoclast apoptosis leading to overall reductions in resorptive capacity. The molecular mechanism of decreased osteoclast numbers and resorption is due, in part, to elevated expression of CD38, a regulator of intracellular calcium pools with known anti-osteoclastogenic properties, which increases sensitivity to apoptosis. *In vivo*, exogenous RANKL stimulation can overcome this inhibition to drive osteoclastogenesis and bone loss. *In vitro* derived ET2 osteoclasts are more spread and more numerous with increases in RANK, TREM2, and NFATc1 compared to wild type. However, their resorptive capacity does not increase accordingly. Thus, E proteins participate in osteoclast maturation and survival in homeostatic bone remodeling.

Keywords

Osteoclast; E proteins; CD38; transcription factor

Introduction

Osteoclasts are large, multinucleated cells responsible for bone resorption and arise from myeloid precursors differentiated with macrophage colony stimulating factor (MCSF) and receptor activator of NF- κ B ligand (RANKL). Stimulation leads to activation of several transcription factors, including PU.1, NF- κ B, NFATc1, AP-1, and Mitf, that regulate genes for osteoclast proliferation, survival, fusion, motility, and resorption¹. PU.1 and NF- κ B determine myeloid progenitor commitment and mice deficient in either factor fail to produce

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Conflict of Interest

All authors state they have no conflicts of interest.

sufficient numbers of osteoclasts leading to severe osteopetrosis²⁻⁸. AP-1 transcription factors, c-fos and FOS-related antigens (FRA1 and 2), are vital for preosteoclast commitment and survival⁹⁻¹². RANKL strongly stimulates the master osteoclastogenic transcription factor NFATc1, which is required for osteoclastogenesis¹³⁻¹⁶.

E proteins are ubiquitously expressed class A basic helix-loop-helix (bHLH) transcription regulators that regulate cell proliferation, survival, and tumorigenesis¹⁷. Via their HLH domains, E proteins form homodimers or heterodimers with other classes of bHLH's and bind to E box (CANNTG) DNA sequences through their basic domain¹⁸⁻²⁰. The E proteins include E47, E12, HEB, and E2-2, and can activate or repress transcription by binding the promoter or enhancer region of a gene. E protein activity is regulated by inhibitors including Id proteins (Inhibitor of DNA binding or Inhibiting Differentiation) which lack a basic binding domain or by TAL1 (T-cell acute lymphocytic leukemia protein 1) which lacks appropriate activation domains. Inhibitors sequester E proteins through high affinity interactions thus disrupting transcriptional activation^{21,22}. The balance of Id and E proteins are key in determining the myeloid versus lymphoid fate²³.

Work investigating the role of E proteins and Ids in bone is relatively limited; however, a role for both has been established in osteoblasts. Two E box binding sites are in the promoter of osteocalcin, a mature osteoblast gene product important for bone mineralization and E47 participates in pre-osteoblast differentiation by inducing cell cycle arrest required prior to differentiation^{24,25}. However, when coupled with the HLH factor Twist-1, E47 represses bone morphogenic protein (BMP) induced-osteoblastogenesis²⁶. The E47 inhibitor, Id1, acts as an antagonist to Twist-1 in a positive feedback loop and thereby regulates BMP expression²⁶. Furthermore, measurements of bone formation rates in Id1/Id3 heterozygous knockout mice showed a 50% reduction in calvarial suture width and suppressed proliferation and mineralization of primary osteoblasts *in vitro*²⁷. Thus, when the balance is biased towards E47 activity over that of Id, suppression of osteoblast differentiation and mineralization occurs.

The regulatory role of Id proteins in osteoclasts, however, is quite different. Upon RANKL stimulation, Id1-3 proteins are markedly downregulated during osteoclastogenesis and overexpression of Id1-3 inhibits the expression of tartrate resistant acid phosphatase (TRAP) as well as multinucleation, two hallmarks of osteoclasts²⁸. Additionally, Id1^{-/-} mice have severe osteoporosis due to increased osteoclast numbers and function²⁹. While these data suggest that the balance of E proteins and Ids in myeloid cells will contribute to bone homeostasis, no studies have yet identified a role for E proteins in osteoclasts. Based on these previous findings, the goal of this study was to identify a role for E proteins within osteoclast differentiation and bone remodeling *in vivo*.

Materials and Methods

Animals

A E47 and Tal1 hybrid protein, ET2, which is capable of dimerizing with endogenous E proteins to bind E boxes and activate transcription, was created as described³⁰. Mice carrying the ROSA26-stop-ET2-IRES-GFP locus were previously reported by Cochrane et al²³ and crossed with LysM-Cre mice from Jackson Laboratory. As controls, ROSA26-stop-YFP mice were bred to LysM-Cre mice. All mice were on the C57BL/6J background and cohorts of age and gender match mice were used in the experiments. Studies were performed with approval of the animal care committee at the Oklahoma Medical Research Foundation.

Primary Bone Marrow Macrophage Isolation

Bone marrow cells, aspirated from tibias and femurs, were cultured in α -MEM (Gibco) supplemented with 10% fetal bovine serum (Atlantic Biologics), 1% glutamine Pen-strep (Irvine Scientific), and 20ng/ml MCSF (Sigma). At 24hours, non-adherent cells were moved to new plates for further proliferation and 48hours later were collected and sorted for GFP or YFP expression by BD Biosciences Influx cell sorter, and used for further studies.

Antibodies and Reagents

Alexa647-conjugated anti-CD11b, Alexa647-conjugated anti-F4/80, PE-conjugated rat IgG2a, Alexa647-conjugated rat IgG2a antibodies (eBioscience); Purified rat IgG, anti-NFATc1, and anti-CD38 antibodies (Santa Cruz Biotechnology, Inc.); Purified anti-mouse CD3 molecular complex, Purified anti-mouse CD8a, Purified anti-mouse CD19, PE-conjugated anti-CD11b, Biotin anti-mouse NK-1.1, PE-conjugated anti-CD38 antibody, APC streptavidin (BD Pharmingen); Biotin conjugated anti-BrdU antibody, Biotin conjugated mouse IgG1 κ (Biolegend); PE-conjugated or PE-Cy7-conjugated Annexin V antibody (eBioscience).

Molecular Biology

RNA was harvested utilizing TRIzol reagent (Invitrogen) according to the manufacturer's instructions. 0.5 μ g DNA-free RNA was reverse transcribed by iScriptTM cDNA Synthesis kit (BioRad). CD38 transcript levels were determined with TaqMan[®] Gene Expression Assay by real-time PCR (ABI 7500 Fast Thermocycler, Applied Biosystems). Relative quantities of mRNA were normalized to ribosomal protein gene L19 or internal control GAPDH. Samples were assayed in triplicate. Results are expressed as relative transcripts of ET2 compared to expression in YFP.

Osteoclast Assays

Cells were plated at 50,000 cells/well in 96-well dishes and treated with RANKL (50 ng/ml) and MCSF (20 ng/ml) for 5 days with media changed every 2 days. After 5 days in culture, cells were fixed, stained for tartrate resistant acid phosphatase (TRAP) (Sigma), and multinucleated (>3 nuclei), TRAP positive cells counted. Actin rings were stained with Texas-Red phalloidin (Invitrogen Molecular Probes) and visualized by fluorescent microscopy. For resorption, 1×10^5 macrophages were seeded on BD BioCoat Osteologic Discs (BD Biosciences) and treated with RANKL 50 ng/ml and MCSF 20 ng/ml for 5–10days. Media was changed every 3 days. Resorption pits were analyzed via light microscopy and digital images recorded. The resorption area was determined by Metamorph Analysis software (Molecular Devices).

Micro-CT

The right proximal tibias were scanned by micro-CT (μ CT-20, Scanco Medical, Bassersdorf, Switzerland) according to our published protocol³¹.

RANKL In vivo study

Mice were injected intraperitoneally with 25 μ g of RANKL (Shenandoah Biotechnology) for 7 days. After 8 days, serum was collected for assays with Mouse Osteocalcin EIA kit (Biomedical Technologies Inc.) or in RatLaps EIA C-terminal telopeptides (Immunodiagnostic Systems Ltd.) and bone marrow harvested for osteoclast assays.

Protein Isolation and Western Blot

Cells were with ice-cold radioimmunoprecipitation assay (RIPA) buffer³². Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Millipore) by Western Blotting. After incubation with appropriate antibodies, visualization occurred by enhanced chemiluminescence (ECL) system (Pierce).

Flow Cytometry

Cells were blocked with 10% FBS in PBS, washed, and stained with conjugated primary antibody or unconjugated primary antibody followed by a conjugated secondary antibody. Cells were analyzed by flow cytometry using a FACScalibur (BD Biosciences) and analysis was performed with Summit 4.3 software (Dako Colorado, Inc.).

Histology

Mouse femurs were excised, fixed in 70% ethanol, and embedded in methylmethacrylate (MMA). Longitudinal sections (5 μ m thick) were cut at the 50% plane using a Leica 2265 microtome. Sections were stained with Goldner's Trichrome for the static measurements, and additional sections were cut at 10 μ m, and left unstained for dynamic (fluorescent) measurements.

Histomorphometry

A region of interest was selected that is exactly 250 μ m distal to the growth plate, and extending 1 mm downward (thereby avoiding the primary spongiosa) through the metaphysis of the femur. Standard bone histomorphometry was performed by the methods of Parfitt et al using Bioquant Image Analysis software (R & M Biometrics, Nashville, TN)³³. Histology and Histomorphometry was performed by University of Alabama at Birmingham, Center for Metabolic Bone Disease--Histomorphometry and Molecular Analysis Core Laboratory.

SOLiD Sequencing

RNA quality was evaluated with an Agilent Bioanalyzer 2100 using the Nano RNA Chip. Whole transcriptome libraries were built using SOLiD's Total-RNA Sequencing kit per the manufacturer's protocol (Applied Biosystems, LLC). A Whole Transcriptome Analysis approach was applied for the purpose of assessing gene expression, exon/intron usage, as well as splicing across the entire transcriptome using the Geospizac Genesifter Analysis Edition pipeline (release version 3.6). Expression values for annotated genes were calculated by taking raw mapped reads to exon and splicing events and dividing by total number of mapped million reads in a sample.

MTT Assay

Cells were grown with or without 20 ng/ml MCSF for indicated times. Numbers of viable cells were then quantified by adding 10 μ l MTT 5 mg/ml (3-(4, 5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide, Sigma Aldrich) to media during the last 4 hours of stimulation. MTT was incubated for 4 hours at 37°C until the reaction was stopped with addition of 150 μ l 0.4 N HCl in isopropanol. Absorbance was measured at 570 nm

Apoptosis Assay

Apoptosis was detected by AnnexinV PE Apoptosis Detection kit (eBiosciences) and by Cell Death Detection Elisa kit (Roche Diagnostics Corp.) according to manufacturer's instruction. Briefly, for Annexin V and 7AAD viability staining, cells were cultured in the presence of 20 ng/ml MCSF with or without 50 ng/ml RANKL for 24–96 hours. For the Cell

Death Detection kit, cells were cultured in 96 well plates in the presence of 20 ng/ml MCSF with or without 50 ng/ml RANKL for 36 hours. In the induced apoptosis assay, cells were given MCSF and RANKL for 36 hours then serum starved for 36 hours. Absorbance was read at 405 nm.

Immunofluorescence and TUNEL staining

Bones were fixed in 70% ethanol then paraffin embedded. After heat fixing bone slices to slides, staining was performed. TUNEL staining was performed with ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (S7101, Milipore, Temecula, CA) per manufacturer's instruction.

shRNA construction and Lentivirus infection

To generate constitutive miR-shRNA expressing entry vector, the tet responsive promoter (TT) was excised from pEN_TTRmiRc2 (addgene) to create pEN_RmiRc2. Sequences (CD38#1 TGGAAAGTGATTTCCTCAAAA, #2 GGAGATACTGAGTACATTCAA, #3 TGGATCTTAGATCACCTGTA) generated from open biosystems were cloned into pEN_RmiRc2. To transfer sequences into lentiviral destination vectors, each entry vector and CMV PURO DEST (gift from Dr. Eric Campeau) were incubated with Clonase 2 (Invitrogen) per company instructions. To infect cells, lentivirus was generated by co-transfecting the packaging plasmids pMD2.G (addgene), pMDL/RRE g/p (addgene), pRSV-Rev (addgene) along with the miR-shRNA expressing lentiviral vector into 293T cells using the calcium phosphate precipitation method. 48 h and 72 h post-transfection supernatant was harvested and filtered with a 0.45 micron filter then concentrated using ultracentrifugation. BMMs were infected by centrifugation in the presence of 8µg/ml of polybrene (sigma)

Statistical Analysis

Student *t* tests were conducted by GraphPad Prism version 4 and 5. Differences were considered significant if $p < 0.05$. Data are presented as mean (+SEM) for each experiment shown.

Results

E protein gain-of-function Increases Bone Mass

To determine the role of E proteins in bone remodeling, we analyzed an E protein gain-of-function transgenic mouse model, ET2³⁰. ET2 is composed of the NH₂-terminus of E47, containing the transactivation domains, fused to the basic helix loop helix (bHLH) and the C-terminus of Tal1 (Figure 1A). The HLH domain mediates dimerization of endogenous TAL1 with E proteins, and *in vitro* and *in vivo* studies have confirmed preferred binding proclivity of E47 for TAL1³⁴. ET2 functions as a dominant E protein gain-of-function by preferentially binding to endogenous E proteins thereby preventing E protein inhibitors, TAL1 and Ids, from binding. The ET2 chimeric protein, when dimerized with endogenous E47, is capable of activating transcription of target genes known to be regulated by E47 homodimers³⁰. The ET2 sequence, followed by an internal ribosome entry site (IRES) and the sequence for green fluorescent protein (GFP), was inserted in the ROSA26 locus and downstream of a floxed transcriptional stop sequence. As a result, ET2 and GFP expression from the ROSA26 promoter is inducible by Cre-mediated recombination and ET2 expression is indicated by the co-expression of GFP. Myeloid-specific expression was achieved by crossing ROSA26-ET2 mice with LysM-Cre transgenic mice, where expression of the Cre gene is driven by the promoter of the M-lysozyme gene (Figure S1A). As a negative control, ROSA26-YFP mice, where YFP sequence was inserted in place of ET2 and GFP, were crossed to LysM-Cre mice and used in parallel. ET2 expression is shown in

bone marrow macrophages (BMM) isolated from ET2 mice but is not expressed in YFP BMM. Furthermore, ET2 expression is restricted to myeloid lineage cells (Figure S1B–C).

In vivo bone micro-architecture of 3 month old female mice was evaluated by microcomputed tomography (microCT) and revealed a significant increase in the bone volume/tissue volume (BV/TV) in the trabecular rich proximal tibia of ET2 mice compared to YFP control mice (Figure 1B). The increased bone mass persisted in aged female mice analyzed at five months of age (data not shown). Male mice also had increased bone mass at 8 weeks (BV/TV YFP 0.1664 ± 0.0052 , ET2 0.2393 ± 0.02477 ($p < 0.0109$)), and at 5 months (YFP 0.1428 ± 0.01433 , ET2 0.2618 ± 0.04162 ($p < 0.0450$)). Trabecular number increased while trabecular spacing decreased consistent with increased bone mass (Figure 1C). Although, the trabecular thickness and connection density were increased in ET2 mice, these values did not reach statistical significance. Cortical bone had no significant changes in bone mass or bone mineral density. (Figure 1D).

Histomorphometric analysis confirmed increased bone mass with significantly increased trabecular number (Figure 2A and B), increased trabecular size, and decreased trabecular spacing (YFP 26.43 ± 2.331 ET2 29.91 ± 2.007 $p = 0.3250$, YFP 625 ± 49.07 ET2 386.1 ± 18.40 $p < 0.0001$, respectively). Quantification of the cellular compartments revealed similar osteoclasts and osteoblasts numbers per bone surface in ET2 and control mice (Figure 2B). Osteoclast surface to bone surface and osteoclast perimeter (OcS) were increased but failed to reach statistical significance in ET2 compared to YFP control mice (Figure 2B). To determine osteoblast activity *in vivo*, we performed dynamic labeling of bone by serial injections of calcein to label newly formed bone. As seen in Figure 2C, double calcein labeled bone was similar between ET2 and control mice and was quantified as the mineral apposition rate (MAR) and bone formation rate (BFR). Both MAR and BFR were similar between both genotypes. Furthermore, serum osteocalcin, an indicator of osteoblastic activity, measurements were similar between genotypes (Figure 2D). Serum C-terminal telopeptides of type I collagen, an indicator of *in vivo* bone resorption, was similar between genotypes (YFP 7.364 ± 1.046 , ET2 7.679 ± 0.4795 ($p = 0.7728$)). These data confirm the increased bone mass of ET2 mice and further rule out a significant contribution from osteoblasts.

E protein gain-of-function decreases osteoclast numbers in vitro

Previous studies have revealed a role for E47 and Id proteins in myeloid versus lymphoid cell fate decisions where E47 skews towards the lymphoid lineage²³. As osteoclasts derive from myeloid precursors, we postulated that inappropriate ET2 expression might alter osteoclast cell fate decisions and differentiation leading to the high bone mass phenotype seen in the ET2 mice. To test this hypothesis, bone marrow derived macrophages from ET2 and control mice were differentiated under osteoclastogenic conditions with RANKL and MCSF. Significantly fewer osteoclasts were derived from ET2 mice compared to control mice (Figure 3A). However, there was a striking difference in ET2 osteoclast morphology versus controls, with ET2 osteoclasts being much larger, more spread, and with more nuclei. By dividing the osteoclasts into groups based on number of nuclei (3–10, 11–15, or over 15 nuclei), we determined that the reduction in ET2 osteoclast numbers was due to fewer osteoclasts with less than 15 nuclei, whereas there was a similar number of larger osteoclasts (Figure 3E). Additionally, ET2 osteoclasts were significantly more spread than control osteoclasts independent of numbers of nuclei (Figure 3F). The histomorphometry data showed a trend towards increased osteoclast surface and perimeter *in vivo* but number of osteoclasts was unchanged compared to YFP. We postulated that ET2 osteoclasts might be more nucleated or spread *in vivo*. Therefore we performed bone TRAP staining in conjunction with DAPI to stain nuclei. As seen in figure 3G, ET2 osteoclasts were highly nucleated compared to YFP osteoclasts *in vivo*.

E protein gain-of-function alters osteoclast spreading but not resorptive capacity

The number of nuclei has been correlated with resorptive capacity³⁵, therefore we suspected that the highly nucleated ET2 osteoclasts would have enhanced resorption. Surprisingly, ET2 osteoclasts had non-significant increased resorptive capacity as control osteoclasts (Figure 3B). To correct for the decreased numbers of ET2 osteoclasts, the resorptive capacity of individual osteoclasts (Figure 3C) was measured and revealed that resorption per osteoclast was similar to control osteoclasts. Despite the highly spread phenotype of the ET2 osteoclasts, it was possible that their decreased resorptive capacity could result from abnormal actin ring formation³⁶. However, Tx-Red phalloidin staining revealed normal actin ring structures in both ET2 and control osteoclasts (Figure 3D). Thus, ET2-derived osteoclasts are limited in number, highly spread, and have similar resorption capacity as control osteoclasts *in vitro*.

E protein gain-of-function increases osteoclast apoptosis

Fewer osteoclasts could be the result of decreased precursors, decreased proliferation or increased apoptosis. To evaluate the precursor pool, the number of CD11b+ myeloid cells from freshly isolated bone marrow from ET2 (GFP+) and control (YFP+) mice was determined. Flow cytometric analysis revealed similar percentages of YFP and GFP positive cells (Figure S2A) suggesting there was not a skewing in myeloid cell numbers, as marked by GFP or YFP expression, because of transgene expression. We also found no difference in CD11b+ and YFP or GFP double positive cells indicating similar preosteoclasts pool. Therefore, the decrease in osteoclast numbers was not due to fewer precursors.

To investigate proliferation, MTT and BrdU proliferation assays were performed on sorted GFP+ ET2 or YFP+ control cells. Preosteoclast proliferation in the presence of MCSF over 72 hours of stimulation was similar between ET2 and control cells (Figure S2). To assess for apoptosis, BMMs were cultured in the presence of 20ng/ml MCSF with or without 50ng/ml RANKL for 36 hours and assessed DNA fragmentation was quantified (Figure 4A). ET2 cells had increased apoptosis compared to control cells in the presence of RANKL. We further defined the levels of apoptosis by flow cytometry analysis of Annexin V staining (Figure 4B). Preosteoclasts stimulated with MCSF or a combination of MCSF and RANKL for 24, 48, 72, or 96 hours showed increased Annexin V staining in ET2 cells compared to controls (Figure 4B). ET2 cells treated with MCSF or MCSF plus RANKL were significantly more apoptotic than controls. Therefore, ET2 preosteoclasts have increased apoptosis. We then determined if E protein gain-of-function altered mature osteoclast survival by inducing apoptosis with the withdrawal of MCSF and RANKL. There was a significant increase in DNA fragmentation in ET2 cells after 36 hours of cytokine withdrawal compared to control cells suggesting E protein gain of function causes enhanced apoptosis of mature osteoclasts (Figure 4C). To confirm increased apoptosis *in vivo*, we analyzed TUNEL staining of osteoclasts on fixed bone slices. ET2 mice had a significant increase in TUNEL+ cells per bone surface compared to YFP (Figure 4D and E). Taken together, these data suggest E protein gain-of-function leads increased preosteoclasts and mature osteoclast apoptosis *in vivo*.

E protein gain-of-function leads to alterations in key osteoclast genes

Little is known about the endogenous expression of E proteins in osteoclasts; therefore, we determined the expression level of endogenous E2A gene during osteoclastogenesis. E2A is constitutively expressed in osteoclast precursors during differentiation with RANKL (Figure S3A). To determine how the expression of ET2 could alter gene transcription during osteoclastogenesis, SOLiD sequencing of RNA from osteoclast precursors, treated with RANKL for 24 hours, from ET2 and control mice was completed. Based on pair wise analysis of mapped genes, we found differences many osteoclast-specific genes (Figure 5A

and Figure S4). While the levels of E2A and Tcf3, encoding the isoforms of E47/E12, was unchanged in ET2 cells, several of the Id genes were upregulated over two fold compared to controls cells (Figure S4C). The increase in Id expression is specific to ET2 cells as previous work has shown RANKL drives down Id expression²⁸. Therefore, enhanced E protein transcriptional activity likely drives Id expression via the known E47 binding sites within the promoters.

Multiple genes important for osteoclast differentiation were upregulated by ET2 expression including triggering receptor expressed on myeloid cells 2 (TREM2), microphthalmia-associated transcription factor (Mitf), and RANK (Figure 5A and S4). Genes known to be critical for osteoclast resorptive capacity were modestly decreased including phospholipase C γ 2 and matrix metalloproteinase 9 (Figure S4B and D). Interestingly, many downstream calcium regulators like calcium/calmodulin-dependent protein kinase IV (CAMK4), phospholipase C γ 2 (Plcg1/2), and cyclic AMP-responsive element-binding protein 1 (CREB1) were downregulated (Figure S4D). Given the increases in RANK and TREM2 expression, we determined the effect of ET2 on RANKL-induced production of NFATc1. In response to RANKL, ET2 cells produced significantly more NFATc1 compared to controls (Figure S3B). Thus, ET2 expression increases RANK and TREM2 expression, leading to more rapid induction of NFATc1 and increased osteoclast complexity as measured by size and number of nuclei.

E protein gain-of-function increases expression of CD38

The most highly upregulated gene in ET2 preosteoclasts was CD38, an ectoenzyme important in transmembrane signaling and cell adhesion. CD38 has known anti-osteoclastogenic activity on mature osteoclasts *in vitro*, and CD38-deficient mice are osteoporotic with increased osteoclasts^{37,38}. CD38 was increased four-fold in ET2 preosteoclasts compared to controls (Figure 5A). Importantly, recent studies have shown that the CD38 promoter has a functional E-box and is regulated in part by E2A³⁹.

To confirm increased CD38 expression in ET2 cells during osteoclast differentiation, we took several approaches. Quantitative PCR (qPCR) of ET2 and control preosteoclasts stimulated with RANKL for up to 5 days was performed. CD38 expression was highest in the presence of MCSF, decreased in the presence of RANKL, and increased again when mature osteoclasts were formed at 5 days (Figure 5B). To verify that CD38 protein expression was increased, we evaluated CD38 surface expression by flow cytometry. Bone marrow-derived preosteoclasts from ET2 or control mice were stained with anti-CD38 antibody on the day of harvest (D0) and 3 days (D3) after MCSF induced proliferation. A significantly higher percentage of CD38-positive cells was detected in ET2-derived cells (55%) compared to controls (45%) on D0 (Figure 5C). The increased CD38 expression on ET2 BMMs (40% versus 25%) persisted after 3 days of MCSF stimulation (Figure 5C). RANKL significantly reduced the expression of CD38 in control and ET2 cells, but ET2 preosteoclasts maintained a significantly higher percentage of CD38 positive cells (Figure 5C). To confirm that CD38 expression was enhanced on preosteoclasts, we additionally stained with CD11b, a marker of preosteoclasts. ET2 BMMs treated with or without RANKL, had a greater percentage of cells that were CD38+CD11b+ (Figure 5D). Thus, E proteins regulate CD38 expression in preosteoclasts, and RANKL regulates CD38 expression during normal osteoclastogenesis. In the presence of E protein-gain-of-function, CD38 expression remains significantly elevated in mature osteoclasts.

CD38 knockdown restores E protein gain-of-function cell survival

CD38 is a known regulator of intracellular calcium. Since we found ET2 cells to have increased apoptosis rates in the presence of RANKL, we hypothesized that inappropriate

CD38 expression contributed to the enhanced apoptosis rates seen in ET2 preosteoclasts. To test this hypothesis, we used lentivirus knockdown of CD38 in ET2 cells and measured apoptosis. Compared to scrambled shRNA transduced cells, CD38 shRNA decreased CD38 expression by 30–40% as determined by qPCR (Figure 5E) and immunoblot (data not shown). Quantification of Annexin V by flow cytometry revealed that ET2 cells, with this modest CD38 knock down, had normalization of apoptosis rates (Figure 5E). These results suggest that E protein induced increases in CD38 expression lead to enhanced RANKL-induced apoptosis and that restoration of low CD38 levels can normalize apoptosis.

In vivo Induced Bone Remodeling Occurs in E protein Gain-of-Function Mice

The increased bone mass of ET2 mice suggests that the gain-of-function of E proteins leads to increased osteoclast apoptosis, resulting in an overall increase in bone mass *in vivo*. We postulated that exogenous RANKL might further exacerbate the ET2 phenotype by driving osteoclast apoptosis. To test this hypothesis, ET2 and control mice were treated with daily injections of RANKL (25 µg/mouse) for 7 days. Serial microCT analysis of the proximal tibia before and after the RANKL injections was performed. Similar to control mice, RANKL treated ET2 mice showed a significant decrease (70%) in BV/TV indicating that increased RANKL could stimulate the E protein gain-of-function osteoclastic bone resorption *in vivo* (Figure 6A). *In vitro* derived osteoclasts from the RANKL treated mice showed a significant increase in TRAP⁺ osteoclasts in ET2-derived cultures compared to control (Figure 6B). Similar to basal conditions, the ET2-derived osteoclasts were highly spread (Figure 6B) but had non-significant increases in overall resorption (Figure 6C). However, analysis of pit area per osteoclast revealed a significant increase in osteoclast resorption of individual ET2 osteoclasts compared to YFP osteoclasts (Figure 6D). TRAP staining *in vivo* showed increased osteoclast numbers in ET2 bone slices compared to controls with a significant increase in osteoclast numbers per bone surface (Figure 6E). Thus, exogenous RANKL treatment can effectively recruit a larger pool of preosteoclasts in the presence of E protein gain-of-function. Taken together, this model shows that E protein gain-of-function can promote osteoclastogenesis under strong osteoclastogenic conditions. RANKL treated ET2 mice had significantly more *in vitro* derived osteoclasts post-treatment than RANKL treated control mice (Figure 6B). Therefore, we conclude that under basal conditions, excessive E protein function induces osteoclast apoptosis leading to fewer osteoclasts. However, in the presence of higher levels of RANKL *in vivo*, E protein gain-of-function promotes osteoclast generation likely by the upregulation of RANK and TREM2 expression on preosteoclasts driving NFATc1.

Discussion

We have defined a complex role for E protein transcription factors in osteoclast progenitors using a myeloid specific conditional knock-in mouse expressing ET2, a constitutively active binding partner for E proteins. This work has elucidated several novel findings. First, elevated E protein function in myeloid cells induces a high bone mass phenotype secondary to decreased osteoclast numbers. Secondly, the molecular mechanism of high bone mass is due to increased sensitivity of ET2-derived osteoclasts to apoptosis and correlates with significantly upregulated ectoenzyme CD38. Reduction of CD38 in ET2 cells normalizes apoptosis rates. Finally, we have linked E protein activity to the expression of several key pro-osteoclastogenic genes including RANK and TREM2 which leads to increased size and multinucleation of *in vitro* derived osteoclasts from ET2 mice and a modest increase in size and multinucleation *in vivo*. These findings have revealed a previously unknown role of E proteins in osteoclast regulation.

We were surprised to find that E protein gain-of-function mice have increased bone mass because loss of Id proteins, the negative regulator of E proteins, leads to increased

osteoclastogenesis and osteoporosis²⁹. This discrepancy could be explained in several different ways. First in the osteoclast, E proteins may not be the only major targets of Id1, but instead Id1 may regulate other bHLH proteins, such as Mitf. Alternatively, E protein gain-of-function may prevent additional negative regulators, such as Tal1, from functioning. Indeed, Tal1 was recently shown to repress osteoclast fusion by counteracting the activation of PU.1 and MITF at DC-STAMP, receptor required for osteoclast fusion⁴⁰. The difference in models could also account for the difference in phenotypes. The global knockout utilized by Chan et al. not only affects the osteoclast intrinsically, but could also be affecting additional factors in the microenvironment of osteoclast precursors and mature osteoclasts, apart from stem cell factor (SCF) identified as one reason for their bone phenotype. Taken together, our data suggest a fine balance in Id and E protein expression and their interactions is required for functional regulation of osteoclasts.

The balance of Ids and E proteins on a genetic and protein level has proven important in determining cell fate, proliferation, and survival¹⁷. In T cells, E proteins are highly active until pre-TCR signaling, at which time E protein activity is downregulated thus allowing the cells to continue on to the double positive (DP) stage. If E protein expression is lost at the DP stage, massive apoptosis is induced⁴¹. Thus, the overall balance of Id proteins provide critical regulation of lymphoid cellular commitment likely by allowing for differential transcriptional activation of binding partners such as E proteins. Similarly, our data suggests that E proteins serve to inhibit osteoclast commitment and regulate apoptosis possibly by driving CD38 expression in osteoclast precursors (Figure 7A). After RANKL stimulation (Figure 7B), high E protein levels promote osteoclast multinucleation by increasing RANK and TREM2 and leading to more NFATc1 induction. In wild type cells stimulated with RANKL, CD38 expression is decreased allowing for the preosteoclasts to progress towards a mature, functional osteoclast with a normal life span. However, in the presence of increased E protein activity, CD38 expression remains inappropriately elevated even in the presence of RANKL, thus increasing preosteoclast and mature osteoclast apoptosis, leading to overall decreased osteoclast numbers (Figure 7C).

We also observed a startling change in ET2-derived osteoclast size and multinucleation with RANKL stimulation *in vitro*. Based on our SOLiD Sequencing data, several genes critical to osteoclastogenesis, including RANK and TREM2, are significantly upregulated. TREM2 coupled to DAP12 in conjunction with RANK signaling is known to positively regulate osteoclast size and complexity^{31,42}. Thus, inappropriate E protein activation leads to significant upregulation of pro-osteoclastogenic receptors that can drive larger more complex osteoclasts. Analogous to the dual role of E proteins and CD38 in lymphoid cell commitment and maturation, E proteins appear to play an inhibitory role in early osteoclast commitment but to promote maturation after RANKL-induced osteoclastogenesis.

Our data show that excessive E protein activity leads to increased RNA and protein expression of CD38, an ADP-ribosylcyclase. CD38 is a major regulator of intracellular calcium release due to its enzymatic activity on nicotinamide adenine dinucleotide (NAD⁺) to cyclic ADP-ribose (cADPr) which can then act on ryanodine receptors (RyR) located on the endoplasmic reticulum, thus releasing calcium⁴³. CD38 plays roles in apoptosis, cell activation, differentiation, and proliferation⁴⁴. Two groups have previously shown that E proteins regulate the CD38 promoter and that CD38 is anti-osteoclastogenic in part due to its intracellular calcium effects^{38,39}. Calcium regulation is imperative for proper osteoclast formation and function. Zaidi et al. showed previously that CD38 induces a burst of intracellular calcium and that in turn signals the osteoclast to retract from bone thus inhibiting bone resorption in mature osteoclasts^{37,45,46}. Therefore, we speculate that E protein gain-of-function inhibits early osteoclast precursor commitment in part through the

upregulation of CD38 and that sustained CD38 subsequently leads to inappropriate intracellular calcium regulation leading to inhibition of resorption.

During osteoclastogenesis, NFATc1 induction requires sustained calcium oscillations in response to RANKL stimulation. Development of calcium oscillations requires immunoreceptor tyrosine- based activation motif proteins DAP12 and/or FcR γ in addition to RANKL. In osteoclasts, RANK is co-stimulated by TREM2 coupled to DAP12, and activation of DAP12 induces Syk activation and subsequent PLC γ activation leading to calcium oscillations in an IP3-dependent manner ultimately driving NFATc1^{32,47,48}.

Our data suggests a regulatory role of E proteins and CD38 in early osteoclast commitment and survival, as fewer osteoclasts develop in bone marrow macrophages expressing higher CD38 levels than wild type macrophages. This data is consistent with previous work showing 1) CD38 expressed on various stages of myeloid cell commitment can drive increased apoptosis and 2) TNF α stimulation of osteoclast precursor cells, prior to RANKL stimulation, leads to increased CD38 expression and inhibition of osteoclastogenesis⁴⁹. Thus, CD38 expression, as regulated by E proteins, may act to drive inflammatory macrophage commitment and inhibit osteoclastogenesis by raising the threshold for RANKL induced osteoclastogenesis. Indeed, our *in vivo* data supports this conclusion, as we see decreased osteoclast numbers during homeostatic conditions but when RANKL is significantly elevated, the higher threshold can be overcome and drive excessive osteoclastogenesis.

In our ET2 mouse model, there is a net increase in E protein transcriptional activation that drives the E protein/Id balance towards E protein function. The M-lysozyme promoter, which is activated early in myeloid precursors, allows for myeloid specific expression of our transgene. Therefore we can study E protein gain-of-function in a myeloid cell specific manner from the earliest myeloid precursor to the mature bone resorbing osteoclast. However, there are several limitations to this model. First, the inappropriate functional activation of E protein may alter other myeloid populations such as the development of M1 or M2 macrophages or myeloid suppressor cells that could impact osteoclastogenesis. Secondly, ET2 binding to E proteins prevents other normal binding partners (E47, E12, E2-2, and HEB) and inhibitors (Id proteins or Tal1) from associating with the E proteins thus leaving these binding partners to bind aberrantly to other partners and/or to induce or repress inappropriate gene transcription. As shown in Figure S2A, E protein gain-of-function increases Id1-3 expression by up to 2 fold. Although unlikely, we cannot rule out that the phenotype of the ET2 mice is secondary to increased Id protein expression in myeloid cells. Despite these limitations, the E protein gain-of-function ET2 mouse model has revealed a novel role for E proteins in the regulation of osteoclast differentiation and function.

In summary, we have shown for the first time that E protein transcriptional activity regulates osteoclastogenesis and maturation *in vivo* and *in vitro*. E protein gain-of-function mice have high bone mass due to decreased osteoclast numbers and increased osteoclast apoptosis leading to overall reductions in resorptive capacity. The molecular mechanism of decreased osteoclast numbers is due, in part, to E protein driven expression of CD38 leading to increased apoptosis of osteoclast precursors, preosteoclasts, and mature osteoclasts. *In vivo*, exogenous RANKL can overcome this threshold to drive exuberant osteoclastogenesis. Thus, E proteins likely regulate early osteoclast commitment, preosteoclast and mature osteoclast survival, and promote larger more complex osteoclasts during maturation by increasing the expression of TREM2 and RANK to drive NFATc1 production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Authors' roles: CLL, MBH, WLB, and XHS designed this research. CLL acquired the data. CLL, MBH, WLB, and XHS analyzed and interpreted the data. Y.Z. and X.H.S. provided all of the mice used in the study. X.H.S. initiated the study and provided scientific inputs.

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Figure 1. ET2 mice have increased bone BV/TV

(A) The amino-terminus of E47, containing the transactivation domain, was ligated to the basic helix loop helix (black box) and carboxy-terminus (white and vertical striped boxes) of Tal1 to construct ET2. (B) Proximal tibias of 12 week old female mice were scanned by microCT, (top) tibias were reconstructed and (bottom) BV/TV quantified. (C) Analysis of bone microarchitecture: Connection density (Conn. Dens.), Trabecular thickness (Tb. Th.), Trabecular Spacing (Tb. Sp.), and Trabecular Number (Tb. N.). (D) Reconstruction of cortical mid-tibial shafts. YFP n=10, ET2 n=9. ^a<0.005, ^b<0.008

Figure 2. Increased bone mass of ET2 mice is not due to increased osteoblast function *in vivo*
 (A) Trichrome-stained sections of tibia from YFP and ET2 mice showing increased bone (blue). (B) Histomorphometric analysis was performed on trichrome-stained sections to determine bone volume over tissue volume (BV/TV), trabecular number, number of OB (N. OB/BS) or OC (N. OC/BS) per bone surface, osteoclast surface per bone surface (OcS/BS), and osteoclast perimeter (OcS). (C) Representative sections of calcein double labeling taken from proximal tibias. (D) Histomorphometric analysis of dynamic indices: mineral apposition rate (MAR) and bone formation rate (BFR). (E) Serum osteocalcin concentrations were measured. ^a<0.01, ^b<0.0001

Figure 3. ET2 derived osteoclasts are reduced in number and are highly spread

(A) TRAP-stained osteoclasts grown in the presence of MCSF and RANKL (10 $\times$). (A, Right) Quantification of TRAP⁺ osteoclast-like cells/well. (B) Representative images of resorption trails formed on BD Biocoat (white areas) (20 $\times$). (B, right) Analysis of area resorbed per well and (C) average pit area per osteoclast as measured by Metamorph analysis software. (D) Actin rings are visualized by Texas Red-phalloidin staining (10 $\times$). (E) Complexity, as indicated by size and number of nuclei, is quantified by comparing the number of osteoclasts per field containing small (3–10 nuclei), medium (11–15), and large (>15 nuclei) number of nuclei. (F) Pixel area of small, medium, and large osteoclasts per field. (G) Representative images of DAPI, TRAP, and overlay images from tibia histological sections (40 $\times$). ^a <0.0001 , ^b <0.0005 , ^c <0.05

Figure 4. E protein gain- of- function preosteoclasts have normal proliferation but increased apoptosis

(A) BMMs were cultured in the presence of 20ng/ml MCSF with or without 50ng/ml RANKL for 36 hours and assessed for apoptosis by DNA fragmentation. (B) BMMs were cultured with or without 50ng/ml RANKL for up to 96 hours and assessed for apoptosis by Annexin V staining. Statistics are comparing the same timepoints for each genotype. (C) BMMs were cultured with 50ng/ml RANKL for 36 hours followed by RANKL withdrawal for an additional 36 hours. Apoptosis was measured by DNA Fragmentation. Experiments were repeated two times. (D) Quantification of TUNEL positive cells per bone surface. (E) Representative images of tibia stained with TUNEL with insets showing higher magnification. $a < 0.05$, $b < 0.001$, $c < 0.001$

Figure 5. SOLiD sequencing comparing ET2 and YFP control preosteoclast shows differential gene expression in osteoclast-related genes

Total RNA was isolated from bone marrow macrophage (BMM) cells and stimulated 24hrs with MCSF and RANKL. (A) Heat map of differentially expressed preosteoclast genes including receptors and cytokines. (B) Quantitative PCR of CD38 from BMM stimulated 0, 24, 72, and 120hrs with MCSF and RANKL. (C) Flow cytometry data showing the percent CD38 and YFP (control cells) or GFP (ET2 cells). BMM was isolated on D0 and allowed to proliferate for 3 days in 20 ng/ml MCSF. After 3 days of proliferation cells were stimulated for 24 or 48 hours with 50 ng/ml RANKL. Cells were stained with anti-CD38 at 3 days, 3 days + 24hrs RANKL, or 3 days + 48hrs RANKL. Cells were sorted based on CD38+ and GFP/YFP fluorescence. (D) Cells from (C) were additionally stained for CD11b. (E) Lentiviral shRNA was used to knockdown CD38 expression in BMMs. Knockdown was confirmed by qPCR (right). After infection, BMMs were incubated with 50ng/ml RANKL for 36hrs then assayed for apoptosis by Annexin V staining by flow cytometry. $a < 0.05$, $b < 0.0007$, $c < 0.0005$.

Figure 6. RANKL treatment drives bone loss in ET2 mice and increase osteoclastogenesis *in vitro* Mice were injected with 25 μg RANKL for 7 days. (A) *In vivo* microCT measurements of proximal tibia pre- and post- RANKL injection. (B) Trap staining of *in vitro* derived osteoclast from RANKL treated YFP and ET2 mice. (C) BD biocoat resorption pits from *in vitro* derived osteoclasts from RANKL treated mice. (D) Quantification of pit area per osteoclast. (E) Representation images of TRAP stained osteoclasts along the bone surface of tibia and quantification of TRAP+ OC per bone surface (right). ^a<0.05, ^b<0.008, ^c<0.0001

Figure 7. Model for role of E protein gain-of-function during osteoclastogenesis

(A) In ET2 osteoclast precursors, ET2 binds endogenous E proteins (represented by E47) and drives increased expression of CD38 mRNA and protein. These preosteoclasts are more sensitive to apoptosis. (B) ET2 increases RANK and TREM2 expression leading to increased RANKL induced NFATc1 production and osteoclast differentiation. Increases in TREM2 and DAP12 induce increased osteoclast spreading but osteoclasts are more sensitive to apoptosis due to increased CD38 expression leading to fewer overall osteoclasts. (C) Once the mature osteoclast binds to bone, the cell undergoes intense metabolic activity to sustain bone resorption. NAD⁺ is exported out of the cell where it binds to the increased CD38. NAD⁺-CD38 complex is endocytosed where NAD⁺ is converted into cADPR which binds Ryanodine Receptors (RyRs) on the endoplasmic reticulum leading to release of intracellular calcium. Increases in intracellular calcium leads to osteoclast cytoskeletal retraction thereby limiting resorption. ET2-derived mature osteoclasts remain sensitive to apoptosis.