The Transcription Factor T-box 3 Regulates Colony-stimulating Factor 1-dependent Jun Dimerization Protein 2 Expression and Plays an Important Role in Osteoclastogenesis*

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Background: Colony-stimulating factor 1 (CSF1) and Jun dimerization protein 2 (JDP2) regulate osteoclastogenesis, but a molecular interaction between the two has not been reported.

Results: CSF1 up-regulates JDP2 by inducing T-box 3 (Tbx3) binding to the JDP2 promoter, and suppressing Tbx3 expression impairs osteoclastogenesis.

Conclusion: Tbx3 is a target for CSF1 in osteoclasts.

Significance: Novel molecular targets for CSF1 in modulating osteoclastogenesis are described.

Colony-stimulating factor 1 (CSF1) is known to promote osteoclast progenitor survival, but its roles in osteoclast differentiation and mature osteoclast function are less well understood. In a microarray screen, Jun dimerization protein 2 (JDP2) was identified as significantly induced by CSF1. Recent reports indicate that JDP2 is required for normal osteoclastogenesis and skeletal metabolism. Because there are no reports on the transcriptional regulation of this gene, the DNA sequence from −2612 to +682 bp (relative to the transcription start site) of the JDP2 gene was cloned, and promoter activity was analyzed. The T box-binding element (TBE) between −191 and −141 bp was identified as the cis-element responsible for CSF1-dependent JDP2 expression. Using degenerate PCR, Tbx3 was identified as the major isoform binding the TBE. Overexpression of Tbx3 induced JDP2 promoter activity, whereas suppressing Tbx3 expression substantially attenuated CSF1-induced transcription. Suppressing Tbx3 in osteoclast precursors reduced JDP2 expression and significantly impaired RANKL/CSF1-induced osteoclastogenesis. A MEK1/2-specific inhibitor was found to block CSF1-induced JDP2 expression. Consistent with these data, JDP2−/− mice were found to have increased bone mass. In summary, CSF1 up-regulates JDP2 expression by inducing Tbx3 binding to the JDP2 promoter. The downstream signaling cascade from activated c-Fms involves the MEK1/2-ERK1/2 pathway. Tbx3 plays an important role in osteoclastogenesis at least in part by regulating CSF1-dependent expression of JDP2.

Osteoclasts are multinucleated giant cells, which have the unique capacity to resorb bone. They are derived from a hematopoietic progenitor in the myeloid lineage by cytokine-driven proliferation and differentiation. Colony-stimulating factor 1 (CSF1) and receptor activator of NF-κB ligand (RANKL) produced by osteoblasts and osteocytes are the two key cytokines required for osteoclast differentiation and function (1–3). CSF1 is a glycoprotein growth factor that specifically regulates the survival, proliferation, and differentiation of monocyte-macrophage lineage cells through a cell surface receptor selectively expressed on these cell types, c-Fms. A critical role of CSF1 in osteoclast biology was established by a series of experiments in osteopetrotic (op/op) mice, which lack CSF1 activity in vivo because of a thymidine insert in the coding region of the CSF1 gene that introduces a premature stop codon (1, 2). These animals are devoid of osteoclasts, and osteoclast differentiation is impaired in co-cultures of osteoblasts obtained from op/op mice and normal spleen cells, which was restored by adding recombinant CSF1 (4). Although the role of CSF1 in regulating the proliferation of macrophage lineage cells has been extensively studied, its role in osteoclasts is not fully understood. It is generally agreed that CSF1 maintains the survival of osteoclast precursors and mature osteoclasts and induces RANK expression in preosteoclasts (5, 6). RANKL is thought to have a central role in mediating osteoclast differentiation, but less is known about the contribution of CSF1 to this process.

Several transcription factors play important roles in osteoclast differentiation and function, including NF-κB, PU.1, NFAT, and the AP1 family members, c-Jun and Fos. The regulation of these factors by RANKL has been studied, but whether CSF1 regulates the expression of transcription factors important for osteoclast differentiation is unclear, although we have previously reported that CSF1 transcriptionally increases c-Fos expression in mature osteoclasts (7). Jun dimerization protein 2 (JDP2), a member of the AP-1 transcription factor family, was first identified as a binding partner of c-Jun in a yeast two-hybrid screen (8). JDP2 was later characterized as a histone chaperone and appears to play important roles in cellular differentiation and senescence (9, 10). A role for JDP2 in osteoclast differentiation and bone homeostasis has recently been
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reported. Mice with targeted deletion of the JDP2 gene have high bone mass with evidence for impaired osteoclast function in vivo (11). Suppressing JDP2 expression in RAW264.7 cells has been reported to abrogate RANKL-induced osteoclast formation (12). Despite these data, there are no studies exploring the regulatory elements of the JDP2 gene.

The T-box (Tbx) family of transcription factors is composed of at least 22 family members. They have diverse roles in development, comprise 0.1% of the genome, and are identified in a wide variety of species ranging from ctenophores to mammals. T-box proteins range in size from 50 to 73 kDa and include a 180-amino acid T-box DNA binding domain encoded by at least five exons and a dual function repressor/activator domain (13). The T-box domain is highly conserved among T-box proteins and is distinct from other DNA binding motifs. The binding sites for the T-box domain are often referred to as T-box-binding elements (TBE), with "GGTGTGA" considered the consensus sequence. One family member, Tbx3, plays critical roles in a variety of developmental processes, such as maintenance of stem cells, cell fate determination, and organogenesis, including the development of the skeleton. Mutations in Tbx3 are the genetic basis for the human ulnar-mammary syndrome, characterized by defective apocrine gland, limb, hair, tooth, and mammary development. However, the molecular targets of Tbx3 in bone are incompletely understood, and its role in osteoclastogenesis has never been reported. The current work identifies a novel pathway entrained by activated c-Fms in which Tbx3 transcriptionally regulates the expression of JDP2 gene, a known regulator of the immune and skeletal systems.

EXPERIMENTAL PROCEDURES

Animals—Mice in which exon 2 of the JDP2 gene is interrupted by the Neo gene (11) were kindly provided by Dr. Kazunari Yokoyama (Graduate Institute of Medicine, Kaohsiung Medical University, Taiwan). JDP2 knock-out mice (JDP2−/−) were generated by crossing male and female heterozygous for the disrupted allele (JDP2/H11001/H11002). Mouse tail DNA extraction and PCR genotyping were performed using the REDExtract-N-Rohrschneider. pZen cells were routinely grown in MEM supplemented with 10% FBS (Atlanta Biologicals). RAW 264.7 cells were passaged by scraping with a plastic policeman and replating. The LentiX 293T packaging cell line was purchased from Clontech (Mountain View, CA) and grown in DMEM (high glucose; Invitrogen) supplemented with 10% tetracycline-free FBS (Clontech), 1% penicillin/streptomycin, and 10 mM sodium pyruvate (Invitrogen).

Antibodies, Growth Factors, and Inhibitors—Antibodies specific for mouse Brachyury (catalog no. sc-177743), Tbx1 (catalog no. sc-17877), Tbx2 (catalog no. sc-17880), Tbx3 (catalog no. sc-31657), and β-actin (catalog no. sc-47778) were purchased from Santa Cruz Biotechnology, Inc. Recombinant mouse RANKL and CSF1 were from R&D Systems, Inc. (Minneapolis, MN). The MEK1/2 inhibitor, U0126, and the PI3K inhibitor, wortmannin, were purchased from Cell Signaling (Danvers, MA). The p38 inhibitor, SB203580, was purchased from Sigma-Aldrich.

Peritoneal Macrophage Preparation and Microarray Analysis—C57/B6J mice were given 0.2 ml of 10% thioglycolate medium (FTG) by intraperitoneal injection. One week later, mice were sacrificed, followed by intraperitoneal lavage with 15 ml of PBS to collect macrophages. Macrophages were plated at a density of 1.5 × 106/well in 6-well plates and cultured in α-MEM (Sigma-Aldrich) supplemented with 10% FBS (Atlanta Biologicals). At 80% confluence, cells were treated with 100 ng/ml CSF1 for 12 h, and RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). Microarray analysis was performed using Affymetrix Mouse Genome 430 2.0 Arrays (Affymetrix, Santa Clara, CA). Data were analyzed by GeneSpring 6.2 software (Agilent Technologies, Englewood, CO). The microarray data have been deposited into the Gene Expression Omnibus (GEO; accession number GSE46390).

Osteoclast-like Cells (OCLs) Generated from Co-cultures—OCLs were generated in vitro by co-culturing murine osteoblasts and bone marrow cells on 10-cm tissue culture plates as reported previously (15). Briefly, primary murine osteoblasts were obtained by serial collagenase/dispose digestion of neonatal CD1 mouse calvariae and plated at an initial density of 2.5 × 104 cells/cm2 for co-culture. Bone marrow cells were prepared by flushing the marrow from the tibiae and femurs of 7-week-old CD1 mice. Nucleated marrow cells were plated at an initial density of 1.5 × 105 cells/cm2. Co-cultures were grown in α-MEM with 10% FBS, 1% penicillin/streptomycin, 1% γ-glutammine, 20 mM HEPES, pH 7.36, containing 10−8 M 1,25-dihydroxyvitamin D3 and 10−6 M prostaglandin E2, with a medium change every other day for 6 days. Contaminating mononuclear cells were removed by treating with 5 mM EDTA for 10 min. Approximately 90% of the cellular material derived from these purified cultures is from OCLs. Purified OCLs were directly lysed with TRIzol® (Invitrogen) for RNA extraction.

Osteoclastogenesis Assay—Preparation of bone marrow cells and the osteoclastogenesis assay were described previously (16). Briefly, unfractionated bone marrow cells were isolated from 8-week-old male CD-1 mice and centrifuged for 10 min at 1000 × g. The cell pellet was resuspended, and the cells were plated overnight in 10 ml of α-MEM containing 10% FBS and 50 ng/ml CSF1 in 10-cm dishes. Non-adherent cells were then collected and layered on 10 ml of Ficoll-Paque (GE Healthcare) and centrifuged for 20 min at 707 × g. Cells at the interface were

3 The abbreviations used are: MEM, minimum essential medium; OCL, osteoclast-like cell; qPCR, quantitative PCR; TBE, T-box-binding element.
collected and washed twice with ice-cold PBS and plated in 24-well plates at a density of $2.5 \times 10^5$/well in α-MEM containing 10% FBS, 100 ng/ml CSF1, 100 ng/ml RANKL. Cells were maintained at 37 °C in a humidified incubator with a medium change every other day. To quantify osteoclast numbers, cells were fixed at day 6 of culture, and TRAP staining was performed using the acid phosphatase, leukocyte (TRAP) kit (Sigma-Aldrich).

**Plasmid Constructs of Jdp2 Promoter Reporters**—A 3297-bp DNA fragment of the Jdp2 gene from 2612 bp upstream of the transcription start site to 685 bp downstream of the transcription start site (−2612 to +685) was generated by PCR and cloned into the pGL3 basic vector (Promega, Madison, WI) using KpnI and BglII restriction enzymes (New England Biolabs, Ipswich, MA). A series of deletion constructs (−689/+685; −10/+685; −557/+54; −281/+54; −191/+54, and −141/+54) were also generated. The construct Δ−557/+54 was created by removing the −261/+52 region of the −557/+54 construct using the ApaI restriction enzyme (New England Biolabs, Ipswich, MA). All constructs were confirmed by sequencing.

**Cloning of Murine Tbx3 and Jdp2**—The complete coding sequences for murine Tbx3 and Jdp2 were amplified by RT-PCR using cDNA prepared from co-cultured OCLs. The primers were as follows: for Tbx3, 5′-GTGGATGACCCCTTCCCATGA-3′ (forward) and 5′-CCCTCCTGAGAAGACAAACTGAA-3′ (reverse); for Jdp2, 5′-GGCCACTCTCTCTGCTCATGAT-3′ (forward) and 5′-TCACCTTGCTGCTCTCTTT-3′ (reverse). RT-PCR was performed using PrimeSTAR HS DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan). A 2322-bp Tbx3 fragment was cloned into pcDNA4c HisMax vector (Invitrogen), whereas a 1662-bp Jdp2 fragment was cloned into the pLVX-IRES-mCherry vector (Clontech) for lentiviral production.

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Englewood, CO). Mutant Sp1 binding sites were generated in the −191/+54 and −141/+54 constructs by introducing 3-bp substitutions into the wild type sequence as follows: GGAGGGTT to mutant GGAGAGTCT. The Brachyury binding site in the −261/+54 fragment of the Jdp2 promoter was generated by introducing 2- and 3-nucleotide substitutions into the wild type sequence as follows: AGTTGGGTGTAG to AGTGAGGAGAGAGG and AGAGGGAGAGAGG, respectively. The mutations were confirmed by sequence analysis.

**Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)**—Nuclear extracts from pZen cells and bone marrow cells were prepared by using the NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific, Rockford, IL). Protein concentration was measured using a BCA protein assay kit (Thermo Scientific), and equal amounts of nuclear extract protein were used in each binding reaction. The wild type 50-bp fragment derived from the Jdp2 promoter and the T-box consensus fragment were 5′-end biotin-labeled. The sequences for all of the probes used in EMSA and supershift experiments are listed in Table 1. EMSA was performed using the LightShift chemiluminescent EMSA kit (Thermo Scientific) following the manufacturer’s instructions. Binding reactions were performed in a 20-μl volume with 6 μg of nuclear extract, 0.2 μg/μl poly(dl-dc), and 50 ng/μl BSA. In competition assays, a 100-fold excess of unlabeled probe was added to the reaction. For supershift assays, 2 μg of antibody was added in each reaction and preincubated without labeled probe for 30 min on ice. Labeled probe was then added, and the reaction mixture was incubated at room temperature for an additional 30 min before loading onto the gel.

**Chromatin Immunoprecipitation (ChIP) Assay**—pZen cells were cultured under reduced serum conditions for 6 h (α-MEM containing 1% FBS) and then treated with 100 ng/ml CSF1 overnight. Cells were fixed by adding 37% formaldehyde to the culture medium to achieve a final concentration of 1%. ChIP assays were performed using the ChIP assay kit (Millipore, Lake Placid, NY). Chromatin was sheared using the Misonix 3000 sonicator for six rounds (10-s pulse and 30-s stop) at a power setting of 6. The target transcription factor-chromatin complex was precipitated with the anti-Tbx3 antibody (Santa Cruz Biotechnology, Inc.). Real-time PCR was performed using the Power SYBR® Green Master Mix (Applied Biosystems, Foster City, CA) and a Bio-Rad MyiQ2 detection system. All qPCRs were performed in duplicate. Cycling conditions were 95 °C for 20 s and 60 °C for 1 min for 40 cycles. All test $C_T$ values were normalized by the input $C_T$ value, and data are presented as -fold enrichment in CSF1-treated cells over non-treated cells. Primers used in the ChIP assay are listed in Table 2.

**RNA Isolation, qPCR, and Degenerate PCR**—Total cellular RNA from pZen cells and bone marrow cells was isolated using the RNasea RNA extraction kit (Qiagen, Valencia, CA), following the company’s instructions. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). Taqman probes and primer sets for mouse JDP2 (Mm00473044), Tbx3 (Mm01195726), cathepsin K (CTSK, Mm00484039), calcitonin receptor (CTR, Mm00801463), and β-glucuronidase (GusB, Mm00446953) were purchased from Applied Biosystems (Foster City, CA), and real-time PCR was performed using the iQ Supermix (Bio-Rad) and a Bio-Rad MyiQ2 detection system. All qPCRs were performed in duplicate. Cycling conditions were as follows: 95 °C for 20 s and 60 °C for 1 min for 40 cycles. The relative expression level of each transcript was determined using the comparative $C_T$ method for relative quantitation using GusB as an endogenous reference.

The identification of isoform-specific T-box transcripts was accomplished by PCR amplification using pZen cell and OCL cDNA as template and two degenerate primers: 5′-TACA-

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**Table 1**

| Name | Sequence |
|------|----------|
| 50-bp fragment | 5′-AGGTCTAGTGGTTAGACGCGGAGACGCCTCCACCCCC-3′ |
| BRACwT | 5′-TAAGTACACCTTACGTTGTTAAGAAATTCT-3′ |
| BRACmt | 5′-TAAGTACACCTTACGTTGTTAAGAAATTCT-3′ |
| RunXwT | 5′-CGATATTATGGTTGATATCCAG-3′ |
| RunXmt | 5′-CGATATTATTGGTTGATACAC-3′ |
| KLwT | 5′-TAAGTACACCTTACGTTGTTAAGAAATTCT-3′ |
| KLmT | 5′-TAAGTACACCTTACGTTGTTAAGAAATTCT-3′ |
| 1–35 bp | 5′-AGGTCTAGTGGTTAGACGCGGAGACGCCTCCACCCCC-3′ |
| 35mt1 | 5′-AGGTCTAGTGGTTAGACGCGGAGACGCCTCCACCCCC-3′ |
| 35mt2 | 5′-AGGTCTAGTGGTTAGACGCGGAGACGCCTCCACCCCC-3′ |
| 3Amt | 5′-AGGTCTAGTGGTTAGACGCGGAGACGCCTCCACCCCC-3′ |

* The BRACwT sequence was also used as the labeled T-box consensus probe in supershift assays.
GATCTAGATAYATHCAYCCIGAYWSICC-3' (forward) and 5'-GACAGATCTAGATYTGRTAIGCITACIGCC-3' (reverse) (17). PCR was performed with a reaction mixture containing 250 mM deoxynucleotides, 40 ng/µl primers, 5% dimethyl sulfoxide, and 1 unit of Taq polymerase (Roche Applied Science). Cycling parameters were 2 min at 94 °C, 2 min at 55 °C, and 1 min at 68 °C for 36 cycles. A Bio-Rad iCycler thermocycler was used. PCR products were gel-purified and cloned into the pCR2.1 TOPO/TA vector (Invitrogen) and used to transform One Shot TOP10 competent Escherichia coli (Invitrogen). 15 clones were selected for each cDNA and sequenced. The veracity of the sequences was confirmed by online BLAST analysis.

**Immunoblotting**—pZen cells were cultured under reduced serum conditions overnight (a-MEM containing 1% FBS), pretreated with 25 µM U0126 for 1 h, and treated with 100 ng/ml CSF1 for 12 h. Equal amounts of clarified cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose paper (Trans-Blot Transfer Medium, Bio-Rad). After SDS-PAGE and trans-blotting, nitrocellulose membranes were probed with an anti-Tbx3 antibody or an anti-β-actin antibody. The blots were developed using HRP-conjugated secondary antibodies (Promega) followed by enhanced chemiluminescence detection (ECL detection kit, Amersham Biosciences).

**DNA Transfection, siRNA Knockdown, and Luciferase Assay**—pZen cells were plated 1 day prior to transfection in 24-well plates at a density of 4–8 × 10⁴ cells/well. The Tbx3 expression vector and luciferase constructs were transfected using Extreme Gene HP reagent (Roche Applied Science) at a ratio of 1:3. Twenty-four hours after transfection, cells were stimulated with or without 100 ng/ml CSF1 in α-MEM containing 1% FBS for another 24 h before harvesting for the luciferase assay. On-Target siRNAs specific for Tbx3 and non-targeting control were purchased from Dharmacon (Lafayette, CO). A total of four different siRNAs were tested for knockdown efficiency by qPCR, and the most effective siRNA was determined. For transfection of siRNAs, Lipofectamine 2000 reagent (Invitrogen) was used. The siRNA and Lipofectamine were mixed according to the company’s protocol and aliquotted into each well of the pGL4.73 vector (Promega) as the internal control. Data are presented as -fold increase over basal (cells transfected only with pGL3 basic vector).

**Table 2**

| Name       | Forward primer | Reverse primer | Amplified region (primers included) |
|------------|----------------|----------------|------------------------------------|
| Primer 1   | GCTGAGATCTAGATYTGRTAIGCITACIGC | CTAATAACGAGGCCCTGAGTG | -194/⁻⁴¹ |
| Primer 2   | AAAACACGCTCGAGAATCT | AGTGACGAGAACCCCTCTCC | -579/⁻⁴⁴ |
| Primer 3   | ACCCTGCTGTCCTGAGAAGG | ACATAGACGACAGTGCTCTCAT | -1139/⁻¹⁰⁰⁶ |
| Primer 4   | AGGGAAATAAAGACCCCTTAGTGC | GTGGTCCTTAAAGGTGTCCTCTCT | -1867/⁻¹⁴²¹ |
| Primer 5   | TCTCAGGACTGTCGACTACATT | AAACGACGACAGTGCTCTGAGA | -2416/⁻²⁹⁹⁴ |
| Control primer | AAACCCCTGACGCTGAGCTCT | AAGGCTATCA | Unrelated gene |

Tbx3 regulates CSF1-dependent JDP2 expression

**Tbx3 shRNA Construct, Lentivirus Production, and Transduction of Bone Marrow Cells**—An shRNA sequence specific for mouse Tbx3 (targeting sequence, 5'-GCTGACGACTGTCGATATA-3') and a scrambled control (5'-GTCCAGAGTTGAGCTATCA-3') were synthesized by the Kech Biotechnology Resource Center at Yale. The shRNA sequence was verified by sequencing. The synthesized forward and reverse sequences were annealed, gel-purified, and inserted between the BamHI and EcoRI sites in the plLVX-shRNA2 vector (Clontech). The Tbx3-specific shRNA vector and the JDP2 overexpression vector (described under “Cloning of Murine Tbx3 and Jdp2”) were transfected into the Lentix 293T packaging cell line (Clontech) using Xfect transfection reagent (Clontech) per the manufacturer’s instructions. Medium was changed after 48 h, and supernatant containing lentiviral particles was harvested after an additional 48, 72, and 96 h post-transfection. Viral supernatants were concentrated 100 times and purified by ultracentrifugation through a sucrose cushion. Viral titers were determined by a Lentix p24 rapid titer ELISA kit (Clontech). For transduction, 1 × 10⁵ murine bone marrow cells were plated in each well of a 24-well plate and incubated for 2 days in the presence of 30 ng/ml CSF1. The same amount of virus was then added to each well and incubated for 24 h with 8 µg/ml Polybrene. After 24 h of exposure to the lentivirus, the medium was replaced with normal growth medium containing 30 ng/ml CSF1, and the cells were allowed to recover for 48 h. Cells were then treated with 100 ng/ml CSF1 and 100 ng/ml RANKL for 7 days, with a medium change every other day. TRAP staining was performed at day 7 of culture. Fluorescent images of the OCLs formed were taken using an Olympus fluorescent phase-contrast microscope.

**Bone Density Measurements**—In vivo bone density measurements were performed by dual-energy x-ray absorptiometry using a PIxImus densitometer (Lunar, Madison, WI). Anesthetized mice (ketamine at 100 mg/kg body weight and xylazine at 10 mg/kg body weight, given intraperitoneally) were placed in the prone position, and scans were performed with a 1.270-mm diameter collimator, 0.762-mm line spacing, 0.380-mm point resolution, and an acquisition time of 5 min. The spine window is a rectangle spanning a length of the spine from T1 to the beginning of the sacrum. The femur window encompasses the entire right femur of each mouse. The coefficient of variation for total body bone mineral density is 1.5%. Microcomputed tomography was performed as reported previously (16). Femurs and the L3 lumbar vertebra were stripped of soft tissue, stored in 70% EtOH at 4 °C, and scanned using a Scanco μCT-35 instrument (Scanco, Bruttisellen, Switzerland). Volumetric regions for trabecular analyses, selected within the endosteal borders of the distal femoral metaphysis to include...
the secondary spongiosa located 1 mm from the growth plate and extending 1 mm proximally, were scanned at 12 μm resolution. Cortical morphometry was quantified and averaged volumetrically through 50 serial cross-sections (600 μm) extending distally from the diaphyseal midpoint between the proximal and distal growth plates. For vertebrae, the entire trabecular region was analyzed. No cortical analyses were performed on vertebrae. We used a customized thresholding technique (Scanco, Bruttisellen, Switzerland) that provided the best segmentation of the bone tissue. Data included bone volume/total volume fraction, trabecular number, trabecular thickness, trabecular spacing, and trabecular connectivity density. Cortical thickness averaged for both cortices was also quantified.

Biomechanical Testing—All femurs were loaded to failure in four-point bending. All whole bone tests were conducted by loading the femur in the anterior to posterior direction, such that the posterior quadrant was subjected to tensile loads. The widths of the lower and upper supports of the four-point bending apparatus were 7 and 3 mm, respectively. Tests were conducted with a deflection rate of 0.05 mm/s using a servohydraulic testing machine (Instron model 8874, Instron Corp., Norwood, MA). The load and midspan deflection were acquired directly at a sampling frequency of 200 Hz. Load deflection curves were analyzed for stiffness, maximum load, and work to fracture. Yield is defined as a 10% reduction in the secant stiffness (load range normalized for deflection range) relative to the initial tangent stiffness. Postyield deflection, which is defined as the deflection at failure minus the deflection at yield, was also measured. Femurs were tested at room temperature and kept moist with phosphate-buffered saline (PBS).

RESULTS

CSF1 Induces JDP2 Expression in Osteoclasts and Plays an Important Role in Osteoclastogenesis—A microarray screen using RNA isolated from peritoneal macrophages treated with or without CSF1 for 12 h identified 483 genes whose expression was at least 2-fold induced by CSF1 treatment. There were 51 genes with a signal strength of least 600 in RAW counts. This list included several genes with known roles in macrophage and hematopoietic progenitor cells, including proline-serine-threonine phosphatase-interacting protein 1 (Pstpip1) and kit ligand (Kit). Jdp2 was also identified as a gene regulated by CSF1 and was chosen for further study because of its importance in skeletal homeostasis (11).

To confirm that CSF1 induced Jdp2 expression in osteoclast precursors and mature osteoclasts, qPCR analysis was undertaken using RNA from RAW264.7 cells, bone marrow-derived preosteoclasts, and OCLs treated with either vehicle or CSF1. Jdp2 was induced 3.6 ± 1.5-fold in RAW264.7 cells, 4.5 ± 1.3-fold in preosteoclasts, and 2.2 ± 0.2-fold in OCLs in response to CSF1 (Fig. 1A). To further confirm the role of JDP2 in osteoclast differentiation, murine JDP2 was overexpressed in bone marrow macrophages using a lentiviral vector system. As shown in Fig. 1, B and C (right), osteoclastogenesis was significantly enhanced with JDP2 overexpression as compared with cells infected with empty vector (571 ± 38 versus 143 ± 24 cells/well, JDP2 overexpression versus empty vector, p < 0.05). JDP2 overexpression was confirmed by qPCR (Fig. 1C, left). These results were in accordance with a previous report indicating that JDP2 plays an important role in osteoclastogenesis (12).

The CSF1-responsive Cis-element Is Located between −191 and −141 bp in the Jdp2 Gene—Although JDP2 is critical to a variety of cellular functions, its regulatory elements have not been studied. In order to localize CSF1-responsive cis-elements in the Jdp2 promoter, deletion constructs were created, and luciferase activity was assayed in the presence and absence of CSF1. In the absence of CSF1, the sequence −261 to +54 was shown to be required for basal promoter activity (Fig. 1D). The response to CSF1 was diminished when the 50-bp sequence −191 to −141 was deleted (Fig. 1E). A database search identified a putative Sp1 site at −70/−79 bp, and it is known that CSF1 activates Sp1-dependent transcription (18). However, when this Sp1 site was mutated, CSF1 responsiveness was unaffected.

The Brachyury/T Binding Element Is Responsible for CSF1-induced Jdp2 Promoter Activity—To identify other possible CSF1-responsive cis-elements, the 50-bp sequence −191 to −141 was used as a labeled probe in gel shift assays. The 50-bp sequence was also divided into three fragments: −191 to −172 (fragment 1–20 bp), −176 to −157 (fragment 16–35 bp), and −161 to −140 (fragment 31–50 bp). These fragments were used as unlabeled probes in competition assays. Nuclear extracts prepared from pZen cells treated with or without CSF1 were used in EMSAs using these probes. The intensity of several shifted bands was increased by CSF1 treatment. The binding both with and without CSF1 could be effectively competed by the 50-bp unlabeled probe (Fig. 2A, lanes labeled 50). One band in particular (Fig. 2A, black arrow) was consistently and most robustly induced by CSF1 treatment and was the focus of additional experiments. The full-length unlabeled 50-bp fragment completely competed away this band, whereas a shorter 31–50 bp fragment exhibited almost no competition (Fig. 2, A and B). By contrast, the 1–20 bp fragment was nearly as effective a competitor as the full-length 50-bp probe. Fragment 16–35 bp evidenced only modest competitive activity with large variability in the response (Fig. 2B). Based on these data, it appeared that the first 35 bp very likely harbored the CSF1-responsive cis-element. This sequence was interrogated for putative transcription factor binding sites using two online databases (JASPAR and Genomatix) (19, 20), and binding sequences for the transcription factors Kruppel-like factors, Runx, and Brachyury/T were identified. Consensus sequences for each of these three binding sites were generated along with mutant variants and used as unlabeled probes in a competition assay. Neither the Kruppel-like factors nor Runx sequences showed competition, whereas the Brachyury/T consensus sequence successfully competed the target band, which was abolished by mutating this sequence (Fig. 2, C–E).

A canonical Brachyury/T element is present in the 1–21 bp fragment from bp 9 to 18 (TGCGTGTGAAGC; see Fig. 2F, top line). Mutations were made in this sequence using a 1–35 bp fragment, and these mutant oligonucleotides were used as unlabeled probes in competition assays (Fig. 2F). When the GTG-TGTGA sequence was replaced by CCTCCTCC (35mt1), the 1–35 bp fragment lost most of its competitive ability, whereas mutating only 2 to 9 bp of the 1–35 bp parent fragment did little to affect its competitive ability (35mt2; Fig. 2G). The sequence...
CCTCCTCC was used to replace both the Brachyury/T element (in 35mt1) and, as a control, the 2 to 9 bp of the 1–35 bp fragment (in 35mt2) because that sequence is contained in the 31–50 bp fragment, which we had already established had no competitive activity (Fig. 2, A and B). Point mutations in the core sequence (3Amt; Fig. 2F) also showed reduced competition (Fig. 2H). To examine the biological relevance of CSF1-induced binding to the Brachyury/T site, the 3Amt and 2Amt mutations (Fig. 2F, fourth and fifth lines) were introduced into the −261 to +54 promoter construct and analyzed in a luciferase assay. Both mutations completely abrogated luciferase activity (Fig. 2F). These data suggest that Brachyury/T element mediates CSF1-induced JDP2 promoter activity and that the TGTG motif is the core sequence.

TBX3 Binds to the Brachyury/T Element in the Jdp2 Promoter—Because the Brachyury/T element is functionally important, it is possible that it is conserved among different species. BLAST results showed that the Brachyury/T element in the Jdp2 gene is highly conserved among different mammalian species, including humans, mice, and cows (Fig. 3A). The Brachyury/T element, which is also known as the T-box-binding element (TBE), serves as the binding site for Brachyury and the T-box family of proteins (21). The T-box proteins are so named because they contain a highly conserved DNA binding protein motif called the T-box domain. Although the canonical TBE is composed of a palindromic sequence, which includes two T-half sites, it has been reported that only one half of the palindrome is sufficient for Brachyury and T-box transcription factor functional binding (21). To determine if and which of these two families of proteins was binding to the TBE, supershift EMSAs were conducted. A Brachyury-specific antibody was unable to supershift the relevant band (Fig. 3B), suggesting that a T-box family member could be binding there. There are 22 T-box family members expressed in vertebrates, and their

Figure 1. CSF1-induced JDP2 expression requires the −191/−141 bp sequence in the Jdp2 promoter. A, to confirm the relevance of the microarray screen to the actions of CSF1 in osteoclasts, qPCR was undertaken using RNA prepared from mature OCLs (left), RAW264.7 cells (RAW; middle), or bone marrow-derived preosteoclasts (BM-preOc; right) treated with 5 nM CSF1 for 6 h. Data are fold induction in Jdp2 transcript expression compared with untreated cells (2). Bone marrow cells were infected with lentiviral vectors expressing either JDP2 (JDP2) or empty vector (EV) and then induced to differentiate to mature OCLs with RANKL (100 ng/ml) and CSF1 (100 ng/ml) for 6 days. Cells were fixed and stained for TRAP. Cultures overexpressing JDP2 generated more OCLs than those expressing empty vector. C, overexpression of JDP2 in bone marrow cells was confirmed by qPCR (left). On the right is shown a quantification of the number of TRAP-positive multinucleated cells generated from bone marrow cells expressing either JDP2 (JDP2) or empty vector (EV). Data are presented as mean ± S.E. (error bars) (*, p < 0.05; ***, p < 0.001). D, basal promoter activity of a 3297-bp fragment of the Jdp2 gene (−2612 to +685 relative to the transcription start site) and a series of 5′-deletions as shown. Activity was assessed in a pGL3 vector expressing luciferase. E, further 5′-deletions of the 300-bp fragment −261 to +54 bp, including two constructs in which the Sp1 site was mutated. Basal and CSF1-induced (100 ng/ml) promoter activity was measured. The data summarize the results from three experiments. Data are presented as mean ± S.E.
expression pattern and function have not been well studied. To investigate which T-box proteins are expressed in pZen cells and OCLs, degenerate PCR primers targeting the highly conserved regions of the T-box domain (see “Experimental Procedures”) were used in reactions with cDNAs prepared from these two cell types. Analysis of the PCR products by agarose gel electrophoresis revealed a single band of ~250 bp, consistent with the predicted size of the T-box domain sequence (Fig. 3C). The PCR products were cloned into the pCR2.1 vector, and 15 independent clones were sequenced for each cell type. All 15
clones generated from OCL cDNA contained the same sequence, corresponding to the *Tbx3* gene. Clones generated from pZen cDNA contained sequences encoding *Tbx1*, -2, and -3. To determine which member binds to the 50-bp probe in pZen cells, *Tbx1*-, *Tbx2*-, and *Tbx3*-specific antibodies were used in an EMSA supershift. The target band disappeared only
with the addition of the Tbx3-specific antibody (Fig. 3D, left). The same results were obtained when the consensus palindromic T-box sequence was used as a labeled probe in the supershift assay (Fig. 3D, right). The same findings were also obtained using nuclear extracts from preosteoclasts (Fig. 3E).

To confirm that Tbx3 was binding to the target JDP2 promoter region in a CSF1-dependent manner, a ChIP assay was performed using DNA from pZen cells. Primers were designed to scan the promoter region from −2612 to +685 bp. Primer 1 (targeting the TBE) and primer 2 (targeting the sequence 300 bp upstream of TBE) showed significant increases in Tbx3 binding with CSF1 treatment (Fig. 4F). The sequence amplified by primer 2 did not have a TBE but demonstrated results similar to those obtained with the fragment amplified by Primer 1, probably because this sequence is sufficiently close to the TBE to be on the same chromatin fragment pulled down with the TBE. These results confirm CSF1-induced Tbx3 binding to the TBE in the JDP2 promoter.

Different isoforms of Tbx3 have been identified, including a splice variant, Tbx3+2a, which contains 20 extra amino acids.
encoded by exon 2a inserted into the T-box domain (22). The insertion of exon 2a into the highly conserved T-box domain suggests that this splice variant may have altered DNA-binding properties and a different function. BLAST analysis for our screened Tbx3 clones did not identify the presence of exon 2a. To further confirm this, Tbx3+2a-specific primers were designed, but no amplicons were observed using either the pZen or preosteoclast cDNA as a template. Thus, it is likely that this splice variant is not expressed in these cells.

**Tbx3 Regulates CSF1-dependent JDP2 Expression**

Suppression of Tbx3 was associated with suppression of JDP2 expression at day 4 of differentiation. The levels of Tbx3 and Jdp2 transcript expression were reduced by 59 and 38%, respectively (Fig. 5A). The results were confirmed by Western blot analysis (Fig. 5B). TRAP staining at day 7 of culture demonstrated significantly fewer mature OCLs in cells infected with the Tbx3-specific shRNA compared with those infected with a scrambled shRNA (242 ± 27 versus 98 ± 15 cells/well; scrambled versus Tbx3 shRNA, p < 0.05; Fig. 5, C and E). The lentiviral vector also co-expressed the green fluorescent protein ZeGreen, and the scrambled shRNA-transduced cells showed abundant numbers of green OCLs when viewed using fluorescent microscopy, whereas no green multinucleated OCLs were observed in the cultures in which Tbx3 had been suppressed (Fig. 5D). The few non-fluorescent OCLs observed in the Tbx3 knockdown group probably arose from preosteoclasts that were not infected with the lentiviral vector because the efficiency of the knockdown was less than 100% (Fig. 5A, left). Those cells infected with the virus probably were prevented from differentiating into OCLs. These data support an important role for Tbx3 in osteoclast differentiation and are consistent with the conclusion that one role of CSF1 in osteoclastogenesis is to induce Jdp2 expression via this transcription factor. Moreover, Tbx3 itself may be a critical factor for osteoclastogenesis because cells in which its expression was suppressed could not differentiate to mature osteoclasts.

**CSF1 Transcriptional Activation of Jdp2 Requires ERK1/2**—It has been reported that p38 mitogen-activated protein kinase (MAPK) can mediate RANKL-induced JDP2 expression (12). It is known that p38 MAPK is a downstream target of CSF1 and that the cytokine can also activate PI3K and ERK1/2 (25–27). To analyze the relative contributions of these downstream targets from activated c-Fms to JDP2 expression, the effects of inhibitors specific for PI3K, MEK1/2, the upstream kinase required for ERK1/2 activation (18, 26), and p38 were evaluated in JDP2 promoter luciferase assays. The MEK1/2 inhibitor U0126 specifically blocked CSF1-induced JDP2 expression with little effect on basal promoter activity (Fig. 6A). The PI3K (wortmannin) and p38 (SB203580) inhibitors had little effect on promoter activity. Western blot analysis demonstrated that U0126 but not SB203580 blocked CSF1 induction of JDP2 expression (Fig. 6B).

Because Tbx3 is an important cellular target for CSF1, the effect of the cytokine on the expression level of Tbx3 was evaluated. CSF1 up-regulated Tbx3 expression in pZen cells as assessed by qPCR. This induction was blocked by the MEK1/2 inhibitor, U0126, whereas a p38 inhibitor showed little effect (Fig. 6C). CSF1 treatment also increased Tbx3 protein levels in pZen cells, an effect that was also blocked by U0126 (Fig. 6D). To confirm the role of MEK1/2-ERK1/2-dependent Tbx3 expression in CSF1-induced JDP2 transactivation, Tbx3 was overexpressed in the presence of a MEK1/2 inhibitor, which partially rescued the CSF1 responsiveness of JDP2 promoter activity (Fig. 6E). These data are consistent with the idea that the MEK1/2-ERK1/2 pathway is downstream from c-Fms in a pathway that contributes to Tbx3-induced JDP2 promoter activation.
Mice Have Increased Bone Density—JDP2<sup>−/−</sup> mice had significantly higher bone density than heterozygous littermates when analyzed by DXA (Fig. 7A). Bone density at the femur and spine and total bone density were increased by 12.8, 9.4, and 8.1%, respectively, compared with heterozygous controls (p < 0.0001 for each site). Consistent with these data, microcomputed tomography analyses revealed that trabecular bone volume (bone volume/total volume fraction; BV/TV) both in the spine and femur was higher in the knock-out animals (Fig. 7, B and C). Both in the spine and femur, trabecular number was significantly greater and trabecular spacing was significantly reduced in the knock-out animals when compared with heterozygous animals. Mean femur cortical bone density was not different in JDP2 knock-out and heterozygous mice (data not shown).

The biomechanical properties of femurs isolated from JDP2<sup>−/−</sup> were evaluated by a four-point bending assay. Femurs from JDP2 knock-out mice had reduced stiffness, maximum load, and total work and increased postyield deflection compared with controls, although these changes were not statistically significant (Table 3).

**DISCUSSION**

JDP2 is a DNA-binding protein that forms homodimers or heterodimers with c-Jun, ATF2, and C/EBPβ (8, 28). Although JDP2 represses AP-1-mediated transcriptional activation, it can also act as a co-activator in certain cell types (28–30). Its role in differentiation is also context-specific. JDP2 suppresses differentiation in embryonal F9 cells by regulating histone acetylation (10). However, JDP2 enhances myogenic differentiation in C2C12 cells (28). JDP2 has also been reported to be a key mediator of RANKL-induced osteoclast differentiation because suppression of JDP2 expression attenuates RANKL-induced osteoclastogenesis in RAW264.7 cells. The current study demonstrates that in addition to RANKL, CSF1 induces JDP2 expression in both osteoclast precursors and mature OCLs via a novel cell signaling pathway. We found that the expression of JDP2 was significantly up-regulated by day 3 of an in vitro osteoclastogenesis assay, which suggests that JDP2 is required at an early stage of osteoclast differentiation. Because both RANKL and CSF1 regulate JDP2 expression and because both of these cytokines are important for osteoclastogenesis, this redun-
Dancy in regulation suggests a particularly important role for JDP2 in this process. Despite its pleiotropic actions, the transcriptional control of JDP2 has not, to our knowledge, previously been reported. We found that the Jdp2 gene was responsible for basal promoter activity. This study further demonstrates that the T-box protein family member Tbx3 binds to the TBE in this region and mediates CSF1 responsiveness. As noted in the Introduction, the T-box transcription factors all contain a DNA-binding domain composed of ~180 amino acids, the T-box domain. The T-box DNA-binding sequence, TBE, was first defined as the binding sequence for Brachyury. Brachyury binds this palindromic sequence as a dimer, with each monomer of Brachyury binding half of the sequence, or T-half-site (5'-AGGTGTGAATT-3'). Extensive studies have subsequently demonstrated that all T-box proteins tested are capable

**FIGURE 6. CSF1 induces Tbx3 expression through a MEK1/2-ERK1/2 pathway.** A, pZen cells transfected with the −261/+54 bp Jdp2 promoter/luciferase expression construct were pretreated with the indicated inhibitors for 1 h followed by CSF1 treatment for 24 h in the continued presence of the inhibitors. The MEK1/2 inhibitor U0126 significantly inhibited CSF1-induced promoter activity. B, Western blot analyses demonstrated that the CSF1-induced up-regulation of JDP2 protein expression in pZen cells was suppressed by the MEK1/2 inhibitor U0126 but not the p38 inhibitor SB203580. Cells were pretreated with the indicated inhibitors for 1 h followed by CSF1 treatment for 24 h in the continued presence of the inhibitors. C, CSF1-induced Tbx3 transcript expression in pZen cells was blocked by MAPK1/2 inhibitor U0126 (25 μM) but not the p38 inhibitor SB203580 (20 μM). Cells were treated with 100 ng/ml CSF1 for 6 h. The graph summarizes the results of three experiments. D, CSF1 (100 ng/ml) treatment of pZen cells for 12 h increased the cellular levels of Tbx3, which was blocked by U0126 (25 μM). E, overexpression of Tbx3 in pZen cells partially rescues the inhibitory effect of U0126 (25 μM) on CSF1-induced Jdp2 transcription. Error bars, S.E.
of binding the T-half-site as monomers, and specifically a half-site can bind Tbx1, -2, and -3 (31, 32). Interestingly, Tbx3 has been shown to preferentially bind as a monomer (32). We have identified a T-half-site in the promoter region of \( Jdp2 \) with a TGTTG motif as the core sequence. This T-half-site, shown in the black box in Fig. 3A, is highly conserved among different vertebrate species. Mutating the TBE in the JDP2 promoter markedly attenuated CSF1 induction, although it had little effect on basal promoter activity.

We identified Tbx3 as the only family member binding the TBE in the JDP2 promoter. In OCLs, only Tbx3 is expressed. In pZen cells, Tbx1, -2, and -3 are all expressed, but only Tbx3 binds to the TBE, as demonstrated by the supershift assay. The results of supershift assays using the 50-bp labeled probe derived from the JDP2 promoter and the consensus T-box labeled probe both demonstrated that TBX3 was binding at this site. The ChIP assay further confirmed that TBX3 binding was significantly increased by CSF1. The fact that a single protein/DNA band was observed by EMSA when the consensus T-box labeled probe was used, suggests that there is only one major protein involved in binding at that site. Consistent with an important role for Tbx3 in mediating induction of JDP2, its expression preceded that of JDP2 by 24 h in the osteoclastogenesis assay. Despite most reports indicating that Tbx3 is a transcriptional repressor, these data demonstrate that Tbx3 can function to activate transcription, which is perhaps not totally unexpected because Tbx3 contains both repression and activation domains.

To begin to define components of the signaling pathway from activated c-Fms to Tbx3 and JDP2, we used inhibitors for key components of several known signaling pathways downstream from that receptor. The MEK1/2-ERK1/2 pathway has been previously reported to be downstream from activated c-Fms. In bone marrow macrophages, CSF1 activates MEK/ERK pathway signaling, which plays an important role in cyto-
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kine-induced DNA synthesis. In contrast, inhibiting p38 does not affect CSF1-stimulated DNA synthesis (33). In addition, ERK activity appears to control CSF1-induced cyclin D1 and c-myc transcript expression (34). In the current study, blocking the MEK/ERK pathway had little effect on basal JDP2 promoter activity but did inhibit the response to CSF1, indicating that this pathway is required for the actions of CSF1 on JDP2. In contrast, inhibiting PI3K and p38 had little effect on promoter activity. Further, as shown in Fig. 6, CSF1-induced expression of Tbx3 was also dependent on the MEK/ERK pathway. Relatively little is known about the transcriptional regulation of Tbx3 expression, although it has been reported that Wnt signaling and protein kinase C activation increase its expression (35, 36). Although it does not appear that p38 is downstream from c-Fms in the signaling cascade to the JDP2 promoter, it is known that p38 can phosphorylate serine 692 on Tbx3, which facilitates its nuclear translocation and transcriptional activity (37). How ERK1/2, which are also serine/threonine kinases, alter Tbx3 activity is at present not known.

We were unable to totally eliminate CSF1-induced JDP2 promoter activation in either pZen cells or preosteoclasts by suppressing Tbx3. However, CSF1 responsiveness was completely abrogated by mutating the TBE cis-element in the JDP2 promoter. One likely explanation is that the efficiency of the Tbx3 knockdown was less than complete because, as Fig. 5D demonstrates, not all cells were successfully infected with our lentivirus. Alternatively, it is possible that in the absence of Tbx3, other T-box family members are binding to this element. This seems less likely because we were unable to detect any other T-box genes by PCR in osteoclasts, and although pZen cells express Tbx1 and Tbx2 as well as Tbx3, there was no residual signal remaining in the supershift assays using nuclear extracts from either cell type when the anti-Tbx3 antibody was used.

Although a role for T-box transcription factors in osteoclastogenesis has not been reported previously, a role for this family of proteins in other aspects of skeletal physiology has been described. Heterozygous loss of function mutations in human Tbx3 are the cause of the ulnar mammary syndrome, an autosomal disorder characterized by malformations of the ulnar side of the upper limb and hypoplasia of the genitals and mammary/apocrine glands. An activator form of Tbx3 was shown to affect skeletogenesis by shifting limb development to more caudal regions (38). A whole genome linkage scan also suggested that Tbx3 may be a regulator of skeletal mass (39). Mutations in Brachyury and Tbx6 result in human congenital vertebral malformations (40). Mutation of Tbx5 leads to Holt-Oram syndrome, which is characterized by bilateral forelimb deformities and congenital heart defects (41). Although there appear to be no prior reports of a role of Tbx3 in osteoclasts, widely divergent effects have been reported in osteoblasts. Tbx3 has been reported to negatively regulate osteoblast differentiation by inhibiting expression of Runx2 and osterix (42). It has also been reported that Tbx3 may negatively regulate human mesenchymal stem cell differentiation to osteoblasts (43). In addition, Runx2 showed reduced activity and an aberrant cytoplasmic location when Tbx3 was overexpressed in C3H10T1/2 cells (44). However, there is also evidence that Tbx3 promotes osteoblast proliferation (45).

Our in vitro studies demonstrate that JDP2 is an important downstream target of activated c-Fms in osteoclasts. Earlier work by Kawaida et al. (12) as well as the work presented here establish a critical role for JDP2 in osteoclastogenesis in vitro. We also found that JDP2 knock-out mice have increased trabecular bone mass. While the current studies were being conducted, Maruyama et al. (11) reported similar findings and concluded that the basis for the increase in bone mass was impaired osteoclastogenesis. Whether the genetic absence of JDP2 also results in impaired function of mature osteoclasts requires further investigation, but we found that JDP2 expression was high in mature osteoclasts. Because we have reported that CSF1 subserves a variety of important functions in mature osteoclasts (7, 15, 46), it is possible that one function of this cytokine is to maintain expression of JDP2 in mature osteoclasts.

Despite the increase in bone mass, biomechanical testing suggested that the bones of JDP2−/− mice may be more fragile compared with heterozygous mice. Whether this results from a reduced rate of skeletal turnover due to impaired osteoclast function is unclear. This has not been previously reported in other murine models of impaired osteoclast function. However, it is noteworthy that in some patients treated with long term bisphosphonates, it has been suggested that suppressed bone turnover due to impaired osteoclast function increases bone fragility and increases the risk of fracture (47, 48).

The current study demonstrates that Tbx3 plays an important role in osteoclastogenesis. During osteoclastogenesis, Tbx3 expression increased quickly and preceded expression of JDP2, an important regulator of skeletal metabolism. Tbx3 expression levels declined from a peak at day 3 but remained at a relatively high level in mature osteoclasts (Fig. 4D). This suggests that Tbx3 and JDP2 are involved in the early commitment of hematopoietic precursors to the osteoclast lineage and remain functionally important in mature osteoclasts.

REFERENCES

1. Yoshida, H., Hayashi, S., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T., and Shultz, L. D. (1990) The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature 345, 442–444
2. Wiktor-Jedrzejczak, W., Bartocci, A., Ferrante, A. W., Jr., Ahmed-Ansari, A., Sell, K. W., Pollard, J. W., and Stanley, E. R. (1990) Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. Proc. Natl. Acad. Sci. U.S.A. 87, 4828–4832
3. Lacey, D. L., Timms, E., Tan, H. L., Kelley, M. J., Dunstan, C. R., Burgess, T., Elliott, R., Colombiero, A., Elliott, G., Scully, S., Hsu, H., Sullivan, J., Hawkins, N., Davy, E., Capparelli, C., Eli, A., Qian, Y. X., Kaufman, S., Sarosi, I., Shalhoub, V., Senaldi, G., Guo, J., Delaney, J., and Boyle, W. J. (1998) Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93, 165–176
4. Takahashi, N., Udagawa, N., Akatsu, T., Tanaka, H., Isoagi, Y., and Suda, T. (1991) Deficiency of osteoclasts in osteopetrotic mice is due to a defect in the local microenvironment provided by osteoblastic cells. Endocrinology 128, 1792–1796
5. Jimi, E., Shuto, T., and Koga, T. (1995) Macrophage colony-stimulating factor and interleukin-1 α maintain the survival of osteoclast-like cells. Endocrinology 136, 808–811
6. Lagasse, E., and Weissman, I. L. (1997) Enforced expression of Bcl-2 in monocytes rescues macrophages and partially reverses osteopetrosis in op/op mice. Cell 89, 1021–1031
Tbx3 Regulates CSF1-dependent JDP2 Expression

26. Gobert Gossé, S., Bourgin, C., Liu, W. Q., Garbay, C., and Mouchiroud, G. (2005) M-CSF stimulated differentiation requires persistent MEK activity and MAPK phosphorylationdependent of Grb2-Sos association and phosphatidylinositol 3-kinase activity. Cell. Signal. 17, 1352–1362

27. Foltz, I. N., Lee, J. C., Young, P. R., and Schrader, J. W. (1997) Hemopoietic growth factors with the exception of interleukin-4 activate the p38 mitogen-activated protein kinase pathway. J. Biol. Chem. 272, 3296–3301

28. Ostrovsky, O., Bengal, E., and Aronheim, A. (2002) Induction of terminal differentiation by the c-Jun dimerization protein JDP2 in C2 myoblasts and rhabdomyosarcoma cells. J. Biol. Chem. 277, 40043–40054

29. Wardell, S. E., Boonyaratankornkit, V., Adelman, J. S., Aronheim, A., and Edwards, D. P. (2002) Jun dimerization protein 2 functions as a progesterone receptor N-terminal domain coactivator. Mol. Cell. Biol. 22, 5451–5466

30. Hwang, H. C., Martins, C. P., Bronkhorst, Y., Randel, E., Berns, A., Ferro, M., and Clurman, B. E. (2002) Identification of oncogenes collaborating with p27kip1 loss by insertional mutagenesis and high-throughput insertion site analysis. Proc. Natl. Acad. Sci. U.S.A. 99, 11293–11298

31. Sinha, S., Abraham, S., Gronostajski, R. M., and Campbell, C. E. (2000) Differential DNA binding and transcription modulation by three T-box proteins, T. TXB1 and TXB2. Gene 258, 15–29

32. Coll, M., Seidman, J. G., and Müller, C. W. (2002) Structure of the DNA-binding domain T-box of human Tbx3, a transcription factor responsible for ulnar-mammary syndrome. Structure 10, 343–356

33. Jaworski, A., Wilson, N. I., Christy, E., Byrne, R., and Hamilton, J. A. (1999) Roles of the mitogen-activated protein kinase family in macrophage responses to colony stimulating factor-1 addition and withdrawal. J. Biol. Chem. 274, 15127–15133

34. Aziz, N., Cherwinski, H., and McMahon, M. (1999) Complementation of defective colony-stimulating factor 1 receptor signaling and mitogenesis by Raf and v-Src. Mol. Cell. Biol. 19, 1101–1115

35. Renard, C. A., Labalette, C., Armengol, C., Cougot, D., Wei, Y., Cairo, S., Pineau, P., Neuveut, C., de Reyniès, A., Dejean, A., Perret, C., and Buendia, M. A. (2007) Tbx3 is a downstream target of the Wnt/β-catenin pathway and a critical mediator of β-catenin survival functions in liver cancer. Cancer Res. 67, 901–910

36. Mowla, S., Pinnock, R., Leamer, Y. D., Godding, C. R., and Prince, S. (2011) PMA-induced up-regulation of Tbx3 is mediated by AP-1 and contributes to breast cancer cell migration. Biochem. J. 433, 145–153

37. Yano, T., Yamazaki, Y., Adachi, M., Okawa, K., Fort, P., Uji, M., and Tsukita, S. (2011) Tαa up-regulates E-cadherin transcription by binding to the Trio RhoGEF and inhibiting Rac signaling. J. Cell Biol. 193, 319–332

38. Rallis, G. P., Solberg, B., and Logan, M. P. (2005) Tbx3 can alter limb position along the rostrocaudal axis of the developing embryo. Development 132, 1961–1970

39. Deng, H. W., Xu, F. H., Huang, Q. Y., Shen, H., Deng, H., Conway, T., Luo, Y. J., Liu, Y. Z., Li, J. Z., Zhang, H. T., Davies, K. M., and Recker, R. R. (2002) A whole-genome linkage scan suggests several genomic regions potentially containing quantitative trait Loci for osteoporosis. J. Clin. Endocrinol. Metab. 87, 5151–5159

40. Ghebranious, N., Blank, R. D., Raggio, C. L., Moorman, A. F. (2008) A gain-of-function TBX5 mutation is associated with atypical Holt-Oram syndrome and paroxysmal atrial fibrillation. Circ. Res. 102, 1433–1442

41. Govoni, K. E., Linares, G. R., Chen, S. T., Pourteymoor, S., and Mohan, S. (2009) T-box 3 negatively regulates osteoblast differentiation by inhibiting expression of osterix and runx2. J. Cell. Biochem. 106, 482–490

42. Zhao, Y., and Ding, S. (2007) A high-throughput siRNA library screen identifies osteogenic suppressors in human mesenchymal stem cells. Proc. Natl. Acad. Sci. U.S.A. 104, 9673–9678

43. Deepak, V., Zhang, Z., Meng, L., Zeng, X., and Liu, W. (2011) Reduced activity and cytoplasmic localization of Runx2 is observed in Chs101/2
Tbx3 Regulates CSF1-dependent JDP2 Expression

cells overexpressing Tbx3. Cell Biochem. Funct. 29, 348–350
45. Govoni, K. E., Lee, S. K., Chadwick, R. B., Yu, H., Kasukawa, Y., Baylink, D. J., and Mohan, S. (2006) Whole genome microarray analysis of growth hormone-induced gene expression in bone. T-box3, a novel transcription factor, regulates osteoblast proliferation. Am. J. Physiol. Endocrinol. Metab. 291, E128–E136
46. Bouyer, P., Sakai, H., Itokawa, T., Kawano, T., Fulton, C. M., Boron, W. F., and Insogna, K. L. (2007) Colony-stimulating factor-1 increases osteoclast intracellular pH and promotes survival via the electroneutral Na/HCO3 cotransporter NBCn1. Endocrinology 148, 831–840
47. Gedmintas, L., Solomon, D. H., and Kim, S. C. (2013) Bisphosphonates and risk of subtrochanteric, femoral shaft, and atypical femur fracture. A systematic review and meta-analysis. J. Bone Miner. Res. 28, 1729–1737
48. Odvina, C. V., Zerwekh, J. E., Rao, D. S., Maalouf, N., Gottschalk, F. A., and Pak, C. Y. (2005) Severely suppressed bone turnover. A potential complication of alendronate therapy. J. Clin. Endocrinol. Metab. 90, 1294–1301