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Lymphocyte Enhancer-binding Factor 1 (Lef1) Inhibits Terminal Differentiation of Osteoblasts

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Abstract Lef1 is a transcriptional regulator of the Wnt/β-catenin signaling cascade. Wnts directly augment bone formation and osteoblast differentiation from mesenchymal stem cells by receptor-mediated pathways involving Lrp5 and Frizzled. We previously reported that Lef1 represses Runx2-dependent activation of the late osteoblast differentiation gene, osteocalcin. Lef1 is expressed in preosteoblasts but is undetectable in fully differentiated osteoblasts. To determine if downregulation of Lef1 is necessary for osteoblast maturation, we constitutively overexpressed Lef1 in MC3T3-E1 preosteoblasts. Lef1-overexpressing cells produced alkaline phosphatase (ALP) and osteocalcin later, and at lower levels than control cells. Moreover, the extracellular matrices of Lef1-overexpressing cell cultures never mineralized. To further examine the role of Lef1 in osteoblasts, we suppressed Lef1 expression in MC3T3-E1 cells by RNA interference. Transient expression of a Lef1 shRNA efficiently reduced murine Lef1 levels and transcriptional activity. Stable suppression of Lef1 in MC3T3 preosteoblasts did not affect proliferation or Runx2 levels; however, ALP production and matrix mineralization were accelerated by 3–4 days. Gene chip analyses identified 14 genes that are differentially regulated in Lef1-suppressed cells. These data outline a role for Lef1 in delaying osteoblast maturation and suggest that Lef1 controls the expression of multiple genes in osteoblasts. J. Cell. Biochem. 97: 969–983, 2006.

Key words: Wnt; Runx2; matrix mineralization; collagens

The skeleton is a dynamic tissue that is constantly being remodeled to the extent that it is regenerated approximately every decade [Frost, 1973]. To maintain a healthy skeleton, homeostasis between matrix-forming osteoblasts and bone-resorbing osteoclasts must be achieved. Disruptions in the balance between anabolic and catabolic processes cause bone loss or overgrowth, either of which can decrease overall skeletal strength. Wnts are secreted glycoproteins and enhancers of skeletal mass and strength [Westendorf et al., 2004]. Activation of canonical Wnt signaling pathway components stimulates osteoblast differentiation from mesenchymal stem cells, enhances proliferation, and expansion of lineage-committed preosteoblasts and stimulates mature osteoblasts to secrete osteoprotegerin, an inhibitor of bone resorption [Bradbury et al., 1994; Rawadi et al., 2003; Bodine et al., 2004; De Boer et al., 2004; Bennett et al., 2005; Day et al., 2005; Glass et al., 2005; Hill et al., 2005]. Distinct and heritable point mutations in the Wnt co-receptor, Lrp5, that amplify or impair Wnt signaling cause high bone mass disorders or a juvenile osteoporosis-like syndrome, respectively [Gong et al., 2001; Boyden et al., 2002; Little et al., 2002a].

Lef1 is one of four high mobility group DNA binding factors that are nuclear effectors of the canonical Wnt signaling pathway. Wnts engage the Frizzled/Lrp5 receptor complex and initiate

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a cascade of events that stabilize and promote the nuclear translocation of the β-catenin, which serves a transcriptional co-factor for Lef1 and the related Tcf proteins (Tcf1, Tcf3, and Tcf4). Wnts and β-catenin promote context-dependent activation or repression of Lef1/Tcf target genes to induce cell proliferation or lineage differentiation [Dierick and Bejsovec, 1999; Wright et al., 1999; van de Wetering et al., 2002; Staal et al., 2004; Lowry et al., 2005]. As such, Wnts, Lrp5, Lef1, and Tcfs are highly expressed in regenerating tissues, including the colon, lymphatic tissues, hair follicles, and sites of bone remodeling [Travis et al., 1991; Waterman et al., 1991; van de Wetering et al., 1991; Zhou et al., 1995; Porfiri et al., 1997; Korinek et al., 1998; Barker et al., 1999; Roose and Clevers, 1999; Reya et al., 2000; Merrill et al., 2001]. Moreover, constitutive activation of the canonical Wnt pathways is a characteristic of many tumors (reviewed in Giles et al. [2003]). One cellular mechanism of silencing or dampening the canonical Wnt signaling pathway is the downregulation of β-catenin-responsive Lef1 and/or Tcf proteins. Lef1 expression decreases as cells stop proliferating and is undetectable in most terminally differentiated cells, including lymphocytes, endometrial cells, lung, and primary calvarial osteoblasts [Hattori et al., 1996; Kato et al., 2002; Waterman, 2004; De Langhe et al., 2005; Saegusa et al., 2005].

Lef1 has distinct roles during skeletal development, but unlike the transcription factors Runx2 and osterix, is not essential for osteoblast differentiation. *Lef1*-deficient and *Lef1*-mutant animals are smaller than wild-type littermates and exhibit numerous skeletal malformations, but have osteoblasts and mineralized skeletal structures [van Genderen et al., 1994; Galceran et al., 2004]. In situ studies of developing embryos detected *Lef1* in tail prevertebrae, osteogenic cells of the hipbone and mesenchymal cells around the cochlea [Oosterwegel et al., 1993]. By comparison, *Tcf1* is more readily detected in pre-cartilaginous cells of cranial-facial bones [Oosterwegel et al., 1993]. Together, these data suggest that Lef1 may regulate bone formation at distinct anatomical sites within the skeleton and that its effectiveness ceases upon terminal differentiation.

We previously showed that Lef1 is expressed in the pre-osteoblast cell line, MC3T3-E1. Lef1 blocked Runx2-dependent activation of the osteocalcin promoter in undifferentiated cells [Kahler and Westendorf, 2003]. Lef1 bound a consensus sequence in proximal promoter of the osteocalcin gene, which is a marker of osteoblast terminal differentiation. Mutation of the Lef1/Tcf consensus site enhanced basal levels of osteocalcin promoter activity. These data suggested that Lef1 might inhibit osteoblast maturation, at least in part by blocking Runx2-dependent gene expression. We tested this hypothesis by constitutively expressing high levels of Lef1 or by suppressing Lef1 expression in MC3T3-E1 osteoblasts. Our results demonstrate that Lef1 expression levels are indirectly correlated with osteoblast maturation. Alterations in Lef1 expression do not affect Runx2 expression or osteoblast proliferation, but they have profound inverse effects on the expression of osteoblast maturation genes and matrix mineralization. These data identify Lef1 as a negative regulator of osteoblast maturation and bone formation.

MATERIALS AND METHODS

Plasmids

The pSHAG and pSHAG-Firefly luciferase (Ffl) vectors were obtained from Dr. Gregory Hannon (Cold Spring Harbor Laboratories) [Paddison et al., 2002]. The pSHAG Lef1 shRNA constructs were designed with shRNA Retriever (http://katahdin.cshl.org:9331/homepage/siRNA/RNAi.cgi?type=shRNA). Oligonucleotides were annealed and subcloned into the BamHI/BseRI sites on the pSHAG vector [Paddison et al., 2002]. The sequences for the oligonucleotides are as follows, the target sequences are underlined: Lef1 shRNA #1 Forward, 5′-GATCATCTCATCGGTGGCGCAGAGTTCCGAAGCTTGGGAATTCTGCGCCGCCGATGAGGTGGTCCCCTTTTTT-3′; Lef1 shRNA #1 Reverse, 5′-GATCAAAAAAGGGGACCACCTCATCGGCGGCGCAGAATTCCCAAGCTTCGGAACTCTGCGCCACCGATGAGATGATCCG-3′; Lef1 shRNA #2 Forward, 5′-ATGTAGGCAGCTGTCATTCTGGGACCTGGAAGCTTGCGGGTCCTAGGATGACAGCTGCCTATATCTGTTTTTT-3′; Lef1 shRNA #2 Reverse, 5′-GATCAAAAAACAGATATAGGCAGCTGTCATCCTAGGACCCGCAAGCTTCCAGGTCCCAGAATGACAGCTGCCTACATCG-3′. Lef1 and Ffl shRNA templates were transferred into pSIN-MSCV-puro-HpaI-Gateway [Schroeder et al., 2004] with the Gateway cloning system (Invitrogen). The stable Lef1 expression vector,

pMSCV-Lef1-IRES-GFP, was produced by inserting Lef1 cDNA (sans the HA tag) from pCMV5B-Lef1-HA into the pMSCV-IRES-GFP vector. Dr. Liliana Attisano kindly provided pCMV5B-Lef1-HA.

Cell Culture and Transfection

MC3T3 cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 200 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Prior to transfection, MC3T3 cells were seeded at a density of 1.25×10^5 cells/well in six-well plates and allowed to adhere overnight. MC3T3 cells were transiently transfected with mOG2-luc (300 ng well), pRL-TK (50 ng/well), and various expression plasmids as indicated (pCMV5B-Lef1-HA, 300 ng/well; pCMV5-Runx2 (MASNS); 300 ng/well, pSHAG-Lef1-shRNA #2, 1.5 μg/well) using LipofectAMINE (Invitrogen), as directed by the manufacturer. Transcription of luciferase reporter constructs was measured using the Dual-Luciferase Reporter Assay System kit (Promega) according to the manufacturer's instructions. To induce differentiation, confluent MC3T3-E1 cells were transferred to differentiation medium (α-MEM supplemented with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml ascorbic acid, and 10 mM β-glycerolphosphate), which was replaced every 3 days. COS cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS, 200 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. COS cells were transfected using DEAE dextran as previously described [Kahler and Westendorf, 2003].

Primary Osteoblast Collection

Calvaria from 1–2-day old mice were dissected, rinsed three times with Hank's Buffered Saline Solution (HBSS) and placed in digestion medium (2 mg/ml collagenase type II, 0.25% trypsin in serum-free α-MEM) at 37°C with gentle shaking. The digestion medium was replaced three times after 20, 40, and 90 min. After the third digestion, primary osteoblasts were collected from the medium by centrifugation at 1,200 rpm for 5 min (Beckman GPR Centrifuge, bucket rotor). Cells were resuspended in MEM with 10% FBS, 1% non-essential amino acids, 200 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin and seeded into tissue culture plates.

Creation of Stable Cell Lines

To produce shRNA retroviruses, 293T cells were cotransfected with 20 μg pSIN-MSCV-puro-Lef1 shRNA or pSIN-MSCV-puro-Ffl, and 20 μg pCL2 using calcium phosphate precipitation. Virus-containing supernatants were harvested after 48 and 72 h, and added to MC3T3-E1 or MC3T3 clone 14 cells (kindly provided by Dr. Rajaram Gopalakrishnan) in the presence of 8 μg/ml polybrene. The transduction was repeated 24 h later. Transduced cells were selected with 10 μg/ml puromycin over a period of at least 5 days. To produce Lef1 retroviruses, 293T cells were cotransfected with 15 μg pMSCV-Lef1-IRES-GFP or pMSCV-IRES-GFP control vector, 10 μg pCMV-gag-pol, and 5 μg pMDG (VSV-G) using calcium phosphate precipitation. The pCMV-gag-pol and pMDG vectors were kindly provided by Dr. Nikunj Somia. Virus-containing supernatants were harvested after 48 and 72 h, and added to MC3T3-E1 cells as described above. Transduced cells were selected after two passages by flow cytometric sorting of GFP-positive cells.

RNA Blotting

RNA was harvested using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (15 μg) was resolved on a 1% agarose MOPS gel and transferred to a nylon membrane (Hybond-N+, Amersham Biosciences). The membrane was UV crosslinked before being hybridized with sequence-specific ^{32}P-labeled probes in Rapid-hyb hybridization buffer (Amersham Biosciences). cDNA probes were labeled using Rediprime II Random Prime Labeling System (Amersham Biosciences) according to the manufacturer's protocol. Plasmids containing cDNA probes for osteocalcin, bone sialoprotein, and osteopontin were kindly provided by Dr. Gerard Karsenty. Dr. Laura Mauro provided the probe for cyclophilin.

RT-PCR

RT-PCR was performed by reverse transcribing 1 μg RNA using the Invitrogen Superscript First-Strand Synthesis System for RT-PCR. Targets were amplified from the cDNA, 1 μl of a 1:10 dilution, using the sequence-specific primers: Lef1 forward 5′-CCAACTTTCCG-

GAGGAGGC-3′, Lef1 reverse 5′-GTAGGAGGGTCCCTTGTTGTAC-3′, bone sialoprotein forward 5′-GAAACGGTTTCCAGTCCAG-3′, bone sialoprotein reverse 5′-CTGCATCTCCAGCCTTCTT-3′, osteocalcin forward 5′-CTCTGTCTCTCTGACCTCACAG-3′, osteocalcin reverse 5′-GGAGCTGCTGTGACATCCATAC-3′, Tem1 forward 5′-GCTGGGAACAAGAGCTCAAC-3′, Tem1 reverse 5′-GTCCTGGAGTCCTGGTGTGT-3′, Ppp1r3C forward 5′-AGTACATGCACACCGGACAA-3′, Ppp1r3C reverse 5′-GTGGGGACTGACACCAGAGT-3′, Ril/Pdlim4 forward 5′-CTCCATATGGTCAGCCACCT-3′, Ril/Pdlim4 reverse 5′-CTGCTGGCTCCCTTAACATC-3′, Col3a1 forward 5′-GTCCACGAGGTGACAAAGGT-3′, Col3a1 reverse 5′-CATCTTTTCCAGGAGGTCCA-3′, Col11a1 forward 5′-CTGGTCATCCTGGGAAAGAA-3′, Col11a1 reverse 5′-TTGAATCCTGGAAAGCCATC-3′, actin forward 5′-AAGGAAGGCTGGAAAAGAGC-3′, actin reverse 5′-GCTACAGCTTCACCACCACA-3′. Standard RT-PCRs were performed in a Bio Rad iCycler. All quantitative PCRs were done in a Roche Light Cycler. Amplification of targets was performed on cDNA using the SYBR Green Taq ReadyMix, Capillary Formulation (Sigma).

Cell Proliferation

Cells were seeded at a density of 5×10^4 cells or 1×10^5 per well in 12-well plates. At each time point, triplicate wells were trypsinized and cells quantified by hemacytometer enumeration.

Immunoblotting

Cells were washed twice with PBS and lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.25% NaDOC, protease inhibitors). Lysates were sonicated and insoluble material was removed by centrifugation. Equal amounts of protein lysates were resolved by SDS–10% PAGE. Proteins were transferred to PVDF membrane (Immobilon-P, Millipore). Membranes were blotted with the indicated antibody diluted 1:1000 in 5% non-fat dried milk in TBST (TBS + 0.04% Tween-20), except for the polyclonal Lef1 antibody (kindly provided by Dr. Marian Waterman), which was diluted 1:4,000 in 3% BSA and TBST. The other antibodies used are: anti-Lef1 (clone REMB1, Oncogene), anti-Runx2 (kindly provided by Dr. Scott Hiebert), anti-Cdk4 (Santa Cruz), anti-Bcl2 (Santa Cruz), anti-actin (Santa Cruz).

Alkaline Phosphatase and Mineralization Assays

To qualitatively detect alkaline phosphatase (ALP) production and activity, cells in 12-well plates were washed with PBS and 500 μl of an NBT/BCIP solution (337.5 μg/ml nitro blue tetrazolium and 175 μg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) was added to each well and incubated for 10 min at 37°C. Cells were then fixed with 70% ethanol for 1 h. For qualitative measurements of ALP activity, cells were washed three times in PBS, harvested in ALP lysis buffer (0.2% NP-40, 1 mM $MgCl_2$), sonicated, pelleted at 3,000 rpm at 4°C, and stored at −20°C. Thawed lysates (50 μl) were added to 200 μl AMP buffer (0.6 M 2-amino-2-methyl-1-propanol, 2.4 mM $MgCl_2$, 9.6 mM *p*-nitrophenyl phosphate) and incubated at 37°C for 30 min. The reactions were stopped with 75 μl 2 N NaOH and analyzed on a plate spectrophotometer at 410 nm. ALP activity was normalized to protein content (Bio Rad D_C Protein Assay). Mineralization was qualitatively measured with alizarin red staining. Briefly, cells were washed with PBS, fixed with 70% ethanol for 1 h, incubated with alizarin red for 10 min, washed six times with water, and developed with PBS for 15 min. Calcium deposition was quantitatively measured by incorporation of ^{45}Ca into the extracellular matrix. Briefly, $^{45}CaCl_2$ was added to differentiation media to a concentration of 0.5 μCi/ml at day 15. Cells and extracellular matrices were harvested 24 h later by scraping in PBS. NaOH was added to a final concentration of 0.2 N and reactions were incubated overnight at 4°C. $^{45}CaCl_2$ levels were measured by liquid scintillation counting.

Gene Chip Analysis

RNAs from MC3T3-L2 and MC3T3-Ffl cells were collected using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. RNA was processed according to the Affymetrix Eukaryotic Sample and Array Processing protocol [Affymetrix, 2004]. cRNA quality was confirmed with the Affymetrix GeneChip Eukaryotic Hybridization Control Kit. Analyses were performed with three MC3T3-Ffl control samples and two MC3T3-L2 samples, which were hybridized to the Affymetrix Mouse Expression Array 430A chips. Data distribution was analyzed and normalized using Expressionist

software (Genedata). Genes that were upregulated or downregulated more than twofold, significantly changed with a P-value of ≤ 0.005, and had a raw expression value of at least 100 in all samples were chosen for further analysis.

RESULTS

Lef1 Expression Declines During Osteoblast Differentiation

We previously reported that Lef1 is expressed in MC3T3 preosteoblasts and abrogates Runx2 activation of the osteocalcin promoter in these cells [Kahler and Westendorf, 2003]. To define the biological role of Lef1 in osteoblasts, we first characterized the expression of Lef1 in primary osteoblasts and MC3T3-E1 cells (Fig. 1A). Lef1 was readily detectable in primary osteoblasts and in MC3T3 cells. Similar expression patterns were observed for Lef1-related proteins Tcf1, Tcf3, and Tcf4 (data not shown). *Lef1* encodes multiple protein products via alternative splicing and use of multiple promoters. Full-length Lef1 is approximately 54 kDa and contains binding domains for both β-catenin and DNA. Immunoblotting revealed that Lef1 is expressed in proliferating MC3T3 cells, but it declines to undetectable levels after 9–12 days of differentiation (Fig. 1B). β-actin levels were unchanged. These data indicate that a fully active form of Lef1 is expressed in osteoblasts, but its expression declines during terminal differentiation.

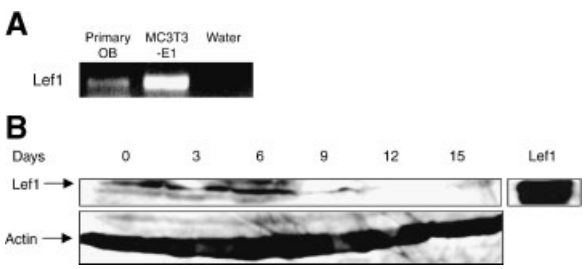

Fig. 1. Lef1 expression declines as MC3T3 cells differentiate. **A**: RNAs from MC3T3-E1 and primary calvarial osteoblasts were subjected to RT-PCR with a Lef1 primer set. PCR products were resolved on a 1% agarose gel. **B**: MC3T3-E1 cells were incubated in osteogenic medium for the indicated number of days. Total cell lysates (150 μg) were resolved by SDS–PAGE and immunoblotted with Lef1 antibodies. The positive control in the last lane is a lysate (10 μg) from COS cells transfected with pCMV5B-Lef1-HA. The blot was also incubated with actin antibodies to control for loading of cellular protein.

Lef1 Overexpression Stalls Osteoblast Differentiation

The natural downregulation of Lef1 during MC3T3 cell differentiation suggested that Lef1 may actively inhibit osteoblast maturation. To test this hypothesis, we produced MC3T3 cells that constitutively overexpress Lef1 and GFP (MC3T3-Lef1:GFP) or GFP alone (MC3T3-GFP). The mouse Lef1 cDNA was cloned into an MSCV vector under the control of the 5′ LTR followed by an internal ribosomal entry site (IRES) and the GFP reporter gene (Fig. 2A). The control plasmid contained only the IRES and GFP (Fig. 2A). Lef1 overexpression was confirmed by quantitative PCR, which indicated greater than 140-fold increase in Lef1 transcripts (Fig. 2B). Lef1 protein levels were also elevated as determined by electrophoretic mobility shift and immunoblot assays (data not shown). Lef1:GFP did not alter the proliferation rates of MC3T3 cells (Fig. 2C) or Runx2 levels (Fig. 3A). When induced to differentiate, MC3T3-Lef1:GFP cells produced significantly less bone sialoprotein (Fig. 3B), ALP (Fig. 3C,D), and osteocalcin (Fig. 3F), as compared to control cells. Furthermore, while MC3T3-GFP cultures mineralized normally as determined by alizarin red staining, MC3T3-Lef1:GFP cultures did not mineralize by day 29 (Fig. 3E). These results indicate that the forced constitutive expression of Lef1 delays osteoblast terminal differentiation and prevents matrix mineralization.

Lef1 Suppression Does Not Affect Osteoblast Proliferation

Because the MC3T3-Lef1:GFP cells express extremely high levels of Lef1 that are more than 100-fold greater than control cells, we felt it prudent to examine osteoblasts that do not express Lef1 to more thoroughly define the role of Lef1 during osteoblast maturation. As we were unable to obtain *Lef1*-deficient mice, we used RNA interference to reduce the expression of Lef1 in MC3T3 preosteoblasts. Short hairpin RNA (shRNA) templates targeting distinct regions of Lef1 were subcloned into the pSHAG vector (Figs. 4A and 5A) [Paddison et al., 2002]. Transient transfection of the shRNAs into COS cells with Lef1 expression vectors identified shRNA #2 as the best inhibitor of Lef1 expression while shRNA #1 had little, if any, effect in this assay (Fig. 4B). When used in a transcription assay, shRNA #2 abrogated Lef1

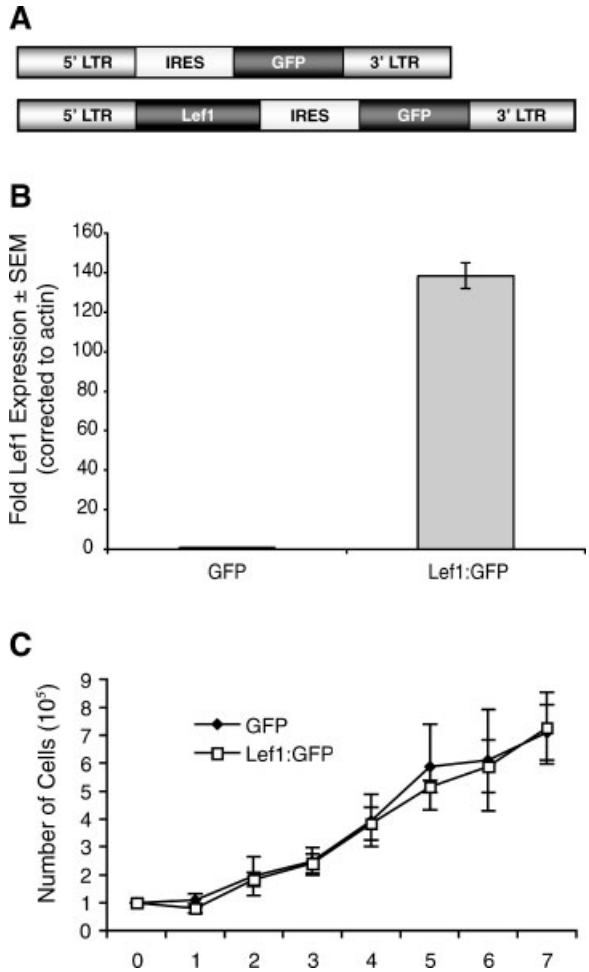

Fig. 2. Lef1 overexpression in MC3T3 cells. **A**: This schematic illustrates the organization of the retroviral vectors used to overexpress GFP (**top**) or Lef1 and GFP (**bottom**). Note that GFP is expressed concurrently with Lef1 in Lef1:GFP cells via a single transcript with an internal ribosomal entry site (IRES), but Lef1 and GFP proteins are not fused. LTR, long terminal repeat; IRES, internal ribosomal entry site; GFP, green fluorescent protein. **B**: Lef1 expression is elevated more than 140-fold in Lef1:GFP cells as compared to GFP cells as determined by quantitative real time RT-PCR. **C**: Overexpression of Lef1 does not affect cell proliferation as determined by daily direct cell counting.

suppression of Runx2-mediated activation of the osteocalcin promoter (Fig. 4C).

In our hands, transient transfection protocols introduce plasmids into approximately 30% of MC3T3 cells at best. We anticipated that the role of Lef1 in osteoblasts would be difficult to ascertain at this level, so we produced cell lines stably expressing Lef1 or control shRNAs (Fig. 5A) [Schroeder et al., 2004]. Pools of stably expressing shRNA cell lines expressed 50%–75% less Lef1 mRNA (Fig. 5B), as compared to either wild-type cells or control cells stably expressing a shRNA targeting firefly luciferase. MC3T3 subclones expressing shRNAs #1 and #2 were named L1 and L2, respectively. It is interesting to note that Lef1 shRNA #1 was nearly as effective at reducing Lef1 levels as shRNA #2 when expressed stably. This may be due to targeting the Lef1 mRNA over a longer period of time (as opposed to a period of 48 h for transient transfectants, (Fig. 4B,C)) thereby allowing the less efficient shRNA to reduce a relatively stable pool of Lef1 mRNA over time. Tcf3 and Tcf4 were unaffected by stable expression of Lef1 shRNAs, as determined by quantitative real time RT-PCR (data not shown).

To eliminate the possibility that Lef1 suppression affects proliferation and thereby has an indirect effect on MC3T3 maturation and terminal differentiation, we performed several experiments to characterize the proliferative status of Lef1-suppressed cells. The non-transduced, Ffl shRNA- and Lef1 shRNA-expressing cells expanded at equal rates over 3 days. Another measure of proliferation, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) production, also showed no differences between Ffl shRNA control cells and Lef1-suppressed cells (data not shown). Immunoblot analyses of lysates from wild type, Ffl shRNA-expressing and Lef1-suppressed cells indicated that cell cycle-related proteins are expressed at similar levels (Cdk4 and Bcl-2, Fig. 6B and data not shown). Taken together, these data dispel the possibility that Lef1 suppression would affect terminal differentiation indirectly through affecting cell proliferation.

Stable Reduction of Lef1 Expression Accelerates Osteoblast Differentiation

We hypothesized that Lef1 inhibited osteoblast differentiation because it repressed several late osteoblast genes (Fig. 3 and Kahler and Westendorf [2003]). If this was correct, then we would anticipate that Lef1-suppressed cells would express differentiation markers at earlier time points than control cells. To test this, we placed the MC3T3 cell lines stably expressing Lef1 or control shRNAs into medium containing ascorbic acid and β-glycerol phosphate. RNA blotting indicated that Lef1 suppression accelerated the expression of osteocalcin by approximately 4 days and resulted in higher expression levels at later time points (Fig. 6A, top panel). Osteopontin expression also peaked

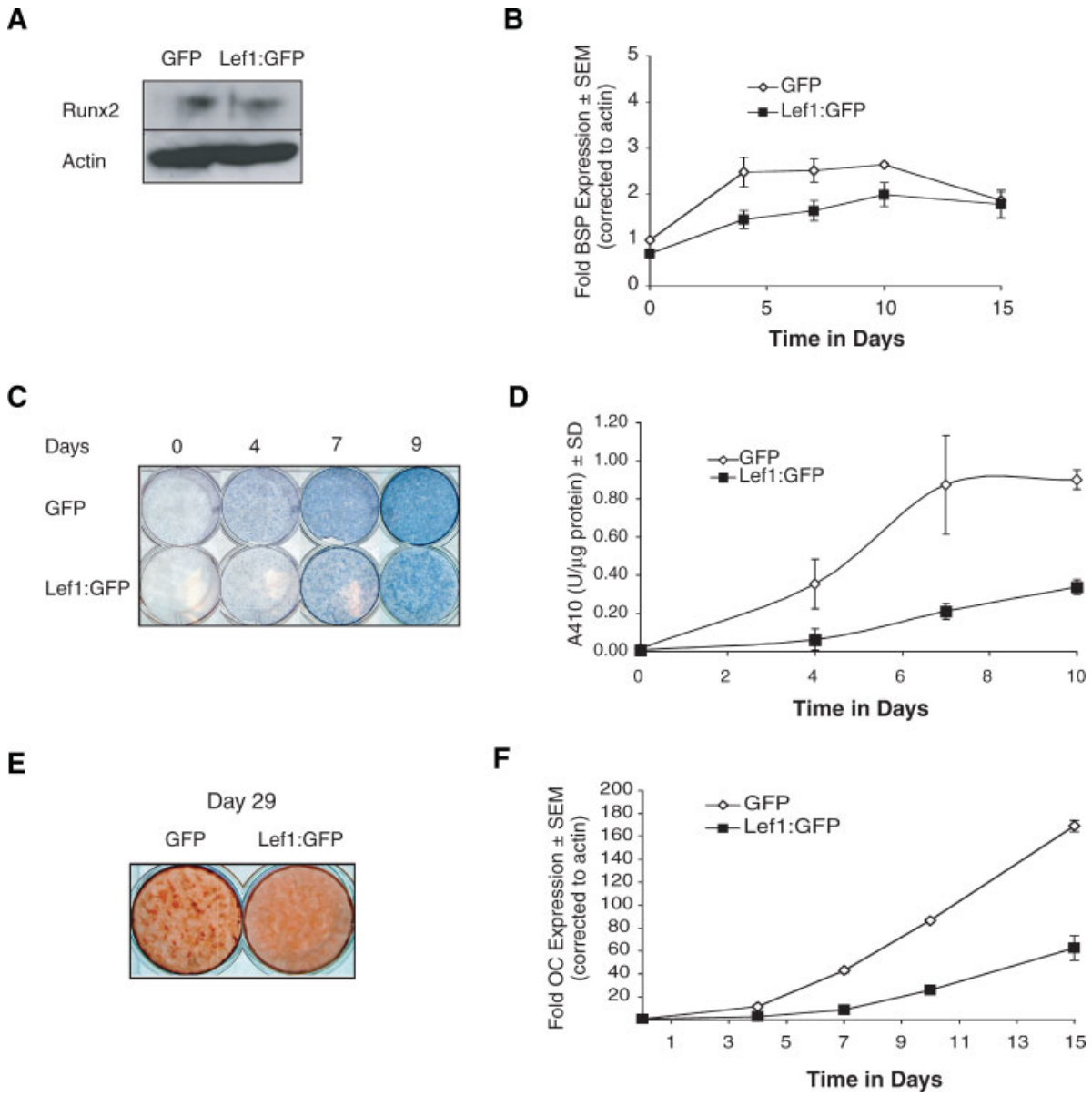

Fig. 3. Lef1 overexpression stalls osteoblast differentiation. **A**: Lef1 overexpression does not affect Runx2 expression as detected by immunoblotting. **B**: Quantitative real time RT-PCR reveals that expression of the osteoblast marker, bone sialoprotein, is reduced during differentiation in Lef1:GFP cells compared to GFP cells. **C** & **D**: Alkaline phosphatase (ALP) activity is qualitatively (C) and quantitatively (D) lower in Lef1:GFP cells than in GFP cells. **E**: While mineralization is apparent by alizarin stain in GFP cells at day 29 of differentiation, there are no discernable mineralized nodules in Lef1:GFP cells. **F**: Osteocalcin expression measured by quantitative real time RT-PCR is delayed in Lef1:GFP cells.

3 days earlier in Lef1-suppressed cells than in control cells (Fig. 6A, middle panel). In addition, Lef1-suppressed cells produced more ALP activity (Fig. 6C,D), and cultures mineralized earlier and to greater levels than control cells (Fig. 6E,F). Runx2 levels did not differ from either the parental MC3T3 or the control Ffl cells (Fig. 6B). Similar results were observed with three separate MC3T3-E1 or MC3T3-clone 14 transduction pools. These data suggest that Lef1 suppresses osteoblast differentiation by inhibiting the expression of differentiation markers but not by affecting Runx2 levels or altering proliferation rates of lineage-committed lines.

Lef1 Suppression Changes the Osteoblast Gene Expression Profile

To gain a better understanding of the mechanism(s) by which Lef1 influences osteoblast

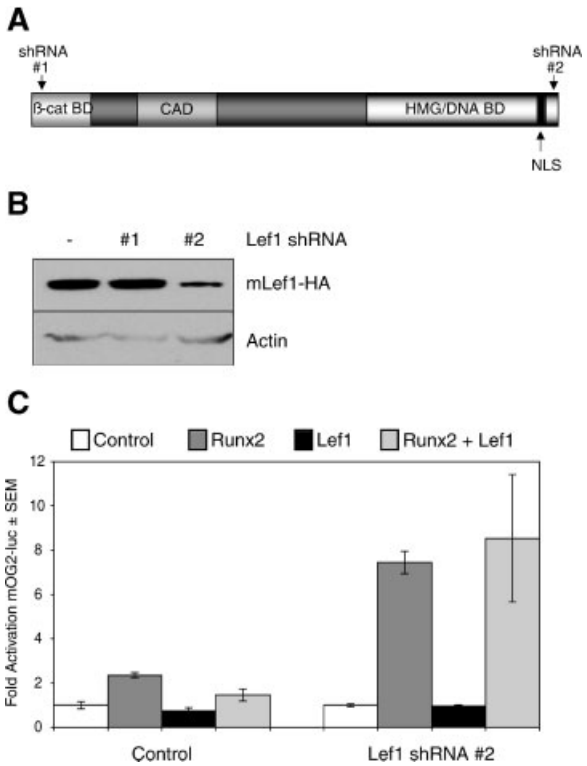

Fig. 4. Lef1 shRNA #2 efficiently reduces Lef1 expression. **A**: This schematic illustrates the relative location of the regions on the Lef1 cDNA that are predicted to hybridize to Lef1 shRNAs. **B**: COS cells were transiently transfected with pCMV5B-Lef1-HA and pSHAG-Lef1 shRNAs #1 or #2. Lysates were resolved by SDS–PAGE and immunoblotted with anti-Lef1 and anti-β-actin. **C**: Lef1 shRNA #2 relieves Lef1-mediated repression of Runx2-dependent activation of the osteocalcin promoter in MC3T3 cells. MC3T3 cells were transfected with mOG2-luc, pRL-TK, and empty vector (pCMV5), Runx2 (pCMV5-Runx2 (MASNS)) and/or Lef1 (pCMV5B-Lef1-HA) and with or without pSHAG Lef1 shRNA #2 as indicated. Transfection efficiency was normalized to renilla expression and to the CMV5 control in each group. β-cat BD: β-catenin binding domain; CAD, context-dependent activation domain; HMG/DNA BD, high mobility group/DNA binding domain; NLS, nuclear localization signal.

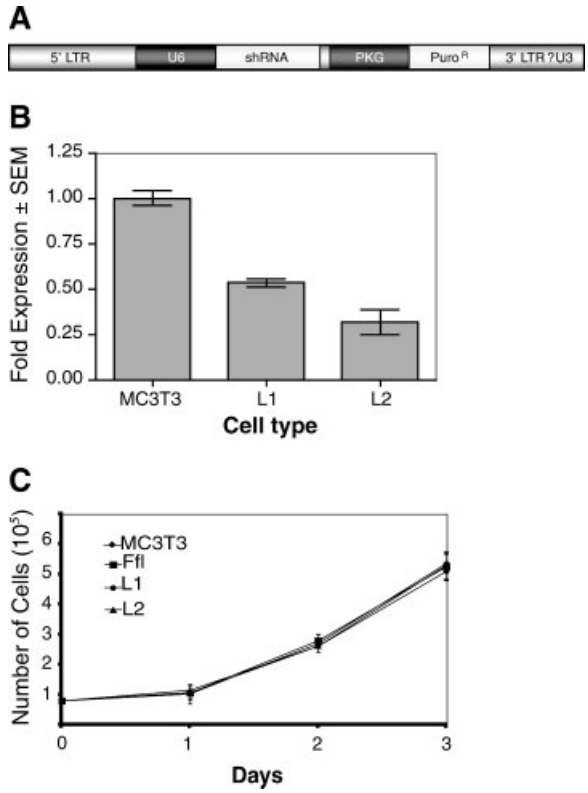

Fig. 5. Stable expression of Lef1 shRNA does not affect MC3T3 proliferation. **A**: This illustration shows the organization of the retroviral shRNA expression plasmid. The U3 section of the 3′LTR was deleted to produce a self-inactivating virus and reduce the interference of the 5′LTR on the U6 promoter [Schroeder et al., 2004]. **B**: Quantitative real time RT-PCR analysis revealed that Lef1 expression is reduced in MC3T3 cells stably expressing either Lef1 shRNA #1 (L1) or #2 (L2) compared to wild-type MC3T3 cells. **C**: Proliferation of MC3T3-L1 and -L2 cells is similar to both wild-type MC3T3 or MC3T3-Ffl cells. Proliferation was determined by directly counting cell numbers. Cells are derived from the MC3T3-E1 clone 14 cell line. These results are similar to ones from cells derived from the parental MC3T3-E1 line. U6: U6 promoter; PKG: murine phosphoglycerate kinase promoter; Puro^R: puromycin resistance gene.

maturation, we analyzed gene expression profiles of Lef1-suppressed cells with an Affymetrix Gene Chip. We identified 14 genes, including two ESTs, that were differentially regulated by at least twofold with a significance of $P = 0.005$ and a raw expression value greater than 100 in all samples (Table I). Twelve of the 14 genes on this list were downregulated in Lef1-suppressed cells. Quantitative real time RT-PCR confirmed the changes in expression for reversion induced LIM (Ril), protein phosphatase1 regulatory subunit 3C (Pp1rs3C), procollagen type XI alpha 1 (Col11a1), tumor endothelial marker 1 precursor (Tem1), and procollagen type III alpha 1 (Col3a1) (Table I). The majority of these genes encode extracellular matrix proteins. These data suggest that Lef1 regulates osteoblast differentiation by controlling the expression of extracellular matrix proteins that may participate in tissue mineralization and terminal osteoblast differentiation.

DISCUSSION

Lef1 is a DNA binding factor that bends promoters and enhancers to organize transcriptional complexes and regulate gene expression [Giese et al., 1991, 1992, 1995, 1997]. Lef1 and

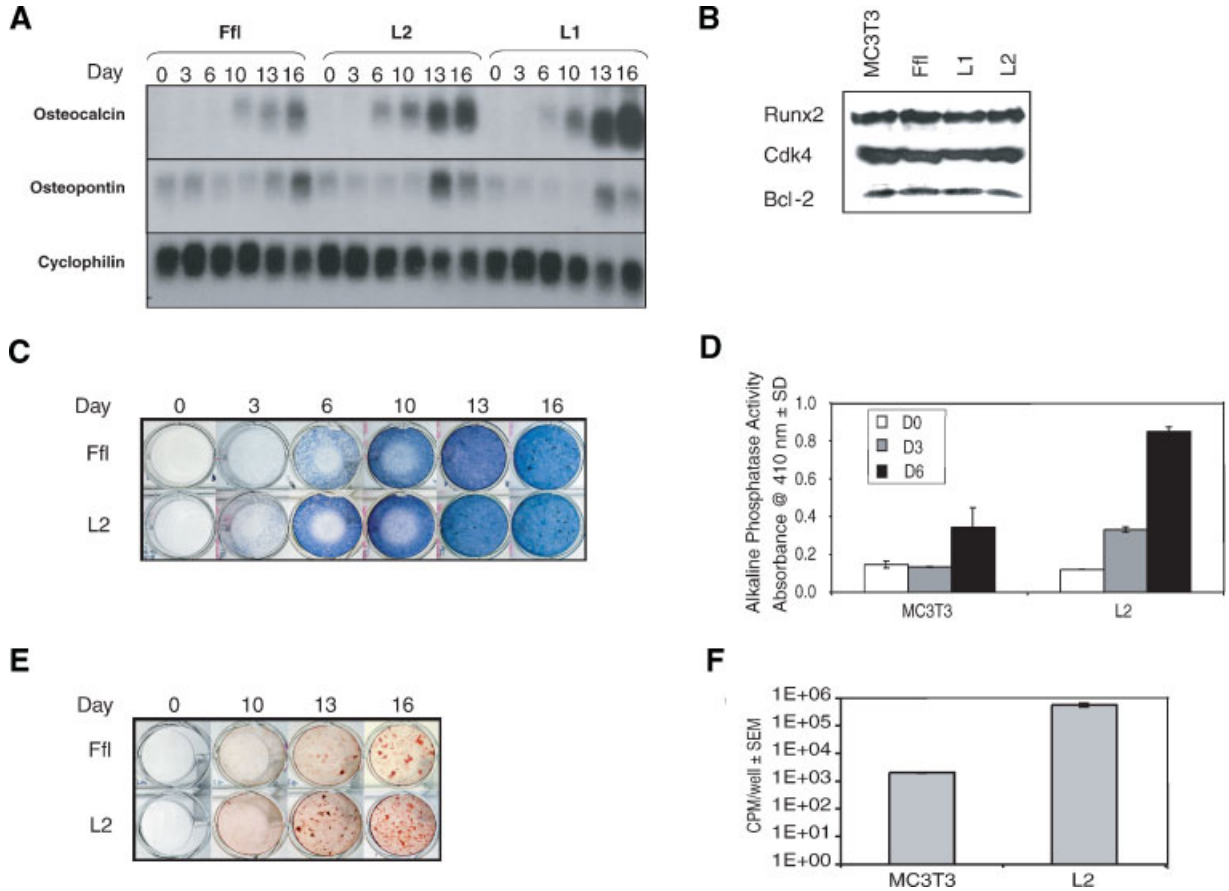

Fig. 6. Lef1 suppression accelerates osteoblast differentiation. **A**: The MC3T3-shRNA cell lines were incubated for the indicated number of days in osteogenic medium. Total RNA was isolated, resolved by electrophoresis, and analyzed for osteocalcin, osteopontin, bone sialoprotein, and cyclophilin transcript levels by RNA blotting. **B**: Protein lysates were made from the indicated cells lines and immunoblotted with antibodies for Runx2, Cdk4, and Bcl-2. **C** & **D**: ALP activity was qualitatively (C) and quantitatively (D) assessed in the indicated MC3T3 cells after culturing them in osteogenic medium for the indicated lengths of time. **E** & **F**: Mineralization of MC3T3 cell matrices was qualitatively assessed by alizarin red staining (E) and quantitatively measured by calcium incorporation over a 24-h period between days 15 and 16 (F).

related Tcfs also interact with Wnt-activated β-catenin and proteins integral to other cell signaling cascades (e.g., BMPs/TGF-activated SMADs, activated Notch intracellular domains, steroid receptors) (reviewed in Westendorf et al. [2004]). We previously demonstrated that Lef1 binds Runx2 and prevents Runx2-dependent activation of the osteocalcin promoter [Kahler and Westendorf, 2003]. Because the canonical Wnt signaling pathway and Runx2 have crucial roles in osteoblast differentiation and bone formation [Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997; Gong et al., 2001; Patel and Karsenty, 2002; Little et al., 2002b; Westendorf et al., 2004], we analyzed the consequences of altering Lef1 levels in osseous cells. Lef1 was present in primary calvarial osteoblasts and in the MC3T3-E1 preosteoblast cell line; however, Lef1 levels decreased to undetectable levels in MC3T3-E1 cells that were cultured in osteogenic medium for more than 9 days. This led to the hypothesis that Lef1 is naturally downregulated during osteoblast terminal differentiation. If this were true, then altering Lef1 levels in osteoblasts should affect lineage maturation. Indeed, expression of Lef1 from a retroviral LTR elevated Lef1 levels by more than 140-fold and blocked osteoblast terminal differentiation as measured by the production of ALP, osteocalcin and bone sialoprotein expression, and by calcium incorporation into the matrix. These proteins are activated by Runx2 and are coupled to mineralization of the bone matrix, a requirement for osteoblast differentiation. Suppressing Lef1 levels produced a complimentary phenotype,

TABLE I. Lef1 Suppression Changes the Gene Expression Profile of MC3T3 Cells

Gene	Accession number	Fold change (gene chip)	Fold change (RT-PCR)
Extracellular matrix			
Procollagen, type XI, alpha 1	BB836814	−6.7	−4.8
Collagenous repeat-containing sequence	NM_030888	−5.9	ND
Asporin	AF316825	−4.0	ND
Fibulin 5	NM_011812	−2.6	ND
Procollagen, type III, alpha 1	AW550625	−2.1	−3.2
Growth factor/growth factor associated			
Protein phosphatase 1, regulatory (inhibitor) subunit 3C	BQ176864	−9.1	−2.1
Interleukin 18 binding protein	AF110803	−6.7	ND
Platelet derived growth factor, B polypeptide	BC023427	3.0	ND
Cell surface			
Tumor endothelial marker precursor	NM_054042	−2.1	−2.0
Annexin A8	BC013271	2.1	ND
Cytoskeleton			
Reversion induced LIM	NM_019417	−20.0	−2.5
Transcription factor			
Myocyte enhancer factor 2C	AI595932	−2.9	ND
ESTs			
Expressed sequence AA673245	BG067392	−4.0	ND
RIKEN cDNA 2310016F22 gene	BC020489	−3.2	ND

These genes were differentially expressed twofold or more at a significance of $P = 0.005$. Values are expressed as fold change over control MC3T3-Ffl cells. Values for RT-PCR were obtained using quantitative real time RT-PCR.

as MC3T3 cells expressing Lef1-specific short hairpin RNAs differentiated at a faster rate than control cells. Thus, matrix mineralization and the expression of osteoblast marker genes (e.g., ALP, osteocalcin, and bone sialoprotein) were accelerated by 3–4 days. These data demonstrate that Lef1 plays important and crucial roles in osteoblast maturation.

One intriguing result of this study is that neither overexpression nor suppression of Lef1 affected the proliferation of MC3T3 preosteoblasts or Runx2 expression. This was initially unexpected because canonical Wnt signaling via the Frizzled/Lrp5 receptor complex, and β-catenin co-activator is directly associated with increased osteoblast numbers and high bone mass [Babij et al., 2003; Boland et al., 2004]. Furthermore, Wnt signaling activates Runx2 expression [Gaur et al., 2005] and Runx2 attenuates osteoblast proliferation [Pratap et al., 2003; Galindo et al., 2005]. However, recent data indicate that canonical Wnts promote proliferation of mesenchymal stem cells and their differentiation into the osteogenic lineage, while suppressing chondrogenesis and adipogenesis [Ross et al., 2000; Bennett et al., 2005; Day et al., 2005; Hill et al., 2005; Kennell and Macdougald, 2005]. Furthermore, while Wnts promote mesenchymal progenitor cell differentiation into osteoblasts, they suppress terminal differentiation of osteoblast lineage-committed cells [Boland et al., 2004]. Thus, the lack of changes in MC3T3 cell proliferation rates in response to altering Lef1 levels may be explained by the fact that these cells are pre-committed to the osteoblast lineage.

Our results support the concept that canonical Wnt signaling is suppressed in mature osteoblasts. Other data showing increased expression of Dkk1 and other Wnt antagonists in mature osteoblasts agree with this model [Kalajzic et al., 2005; Vaes et al., 2005]. Interestingly, the silencing of Wnt pathways in mature osteocytes may be reversible. In mature skeletons, Lef/Tcf transcriptional activity is present in osteocytes and is stimulated by mechanical loading [Hens et al., 2005]. Clearly, more studies are needed to determine how canonical Wnt signaling pathway components contribute to bone mass in developing and adult skeletons.

Genetic models will be important tools to identify the contributions of all Wnt pathway components, especially the functional redundant molecules like Lef1 and the Tcf proteins. As reviewed elsewhere, all Lef1/Tcf proteins have unique roles during mouse embryonic development [Westendorf et al., 2004]. Our findings, together with recent studies, indicate that pluripotent mesenchymal and osteogenic cells (such as the MC3T3 cell line) exhibit differential responses to Lef1 and Tcf1. Gaur et al. [2005] show that Tcf1 stimulates Runx2 expression and osteogenic genes at early stages of osteoblast differentiation, consistent with the bone promoting potential of canonical Wnt

signaling. Considering that cellular levels of Lef1 and Tcf1 are related to the cellular phenotype and stage of osteoblast maturation, the distinct activities of Lef1 and Tcf1 in osteogenic lineage cells may support the onset of osteoblastogenesis and control progression of differentiation. The effects of *Lef1*-deficiency on osteoblasts have not yet been reported; however, two *Lef1*-deficient mouse lines have mineralized skeletons that are smaller than normal and have patterning defects [van Genderen et al., 1994; Galceran et al., 2000]. *Tcf1*-deficiency caused no overt changes in skeletal size or structure, but histological analyses revealed low bone mass in 1-month old mice [Glass et al., 2005]. Similar to β-catenin overexpression, this is due to an indirect effect on osteoclasts caused by decreased OPG production. Our Lef1-suppressed MC3T3 cells also express approximately 80% less OPG than control cells (data not shown). These results indicate that altering Lef1 levels may affect bone formation via direct and indirect mechanisms.

To gain insight into the mechanism by which Lef1 may regulate osteoblast differentiation, we analyzed the gene expression profiles of Lef1-suppressed MC3T3 cells. Because Lef1-depletion accelerated the appearance of all osteoblast marker genes, including the early marker ALP, we hypothesized that stable Lef1 suppression genetically reprogrammed the MC3T3 cells and made them more susceptible to osteogenic stimuli. We identified 14 genes that were differentially regulated in Lef1-suppressed undifferentiated MC3T3 cells by more than twofold with a *P*-value of 0.005 and a minimal absolute expression level of 100. The majority ($n = 12$) of these genes were expressed at lower levels in Lef1-depleted cells. Within this group were genes that contribute to the extracellular matrix, growth factor signaling pathways, the cytoskeleton, and lineage-restriction through transcriptional regulation. Interestingly, several of these genes are known to regulate bone physiology. Allelic variations in Ril are overrepresented in osteoporosis [Omasu et al., 2003]. Mutations in Col11a1 cause Marshall and Stickler Syndromes, which are characterized by ophthalmologic abnormalities, craniofacial abnormalities, and other skeletal abnormalities including bony overgrowths of the calvarium [Li et al., 1995; Snead et al., 1996; Griffith et al., 1998; Wilkin et al., 1998; Martin et al., 1999; Snead and Yates, 1999]. A third example is collagenous repeat-containing sequence (Cors-26), which is expressed in mesenchymal cells but not in mature chondrocytes or osteoblasts [Maeda et al., 2001]. Fourth, asporin is highly expressed in developing intramembranous bones [Henry et al., 2001] and osteoarthritic joints [Kizawa et al., 2005]. Other extracellular matrix genes identified by our gene expression profile, including fibulin-5 and Col3a1, may also play a role in osteoblast maturation by regulating collagen fibril structure or growth factor signaling [Liu et al., 1997; Yanagisawa et al., 2002]. Finally, Mef2c, a transcription factor that maintains a mature muscle cell phenotype is downregulated. Although MC3T3 cells are calvarial-derived and committed to the osteoblast lineage, it is not surprising that they contain transcription factors for other lineages. Nor is it unexpected that the expression of genes, such as Mef2c, is further suppressed as osteoblast maturation proceeds. Studies are ongoing to determine if the genes identified by expression profiling are direct targets of Lef1 regulation.

Only annexin A8 and PDGF-B were present at significantly higher levels in Lef1-suppressed preosteoblasts. Annexin A8 is expressed in areas of endochondral ossification [White et al., 2002]. Other annexins (2, 5, and 8) are known to participate in osteoblastic and chondrocytic mineralization (reviewed in Balcerzak et al. [2003]); thus, its increased expression in Lef1-depleted cells is consistent with observed phenotypes. In contrast, the consequences of increased platelet derived growth factor (PDGF)-B levels are unclear. PDGF-B induces DNA synthesis and migration of osteoblastic cells [Hauschka et al., 1988; Canalis et al., 1989, 1992; Seyedin, 1989; Hughes et al., 1992]. Although PDGF levels were increased threefold, we did not detect significant changes in MC3T3 proliferation. These data suggest that increases in PDGF-B in the face of Lef1-depletion are not sufficient to accelerate proliferation in the MC3T3 cells. Additional experiments are needed to fully understand the relationship between Lef1 and PDGF-BB.

In summary, this study demonstrates that Lef1 has crucial roles in regulating osteoblast maturation. By using a lineage-committed cell line, we show that constitutive Lef1 expression is not conducive to the expression of osteoblast maturation genes or matrix mineralization.

These results are consistent with our previous data showing that Lef1 suppressed Runx2-dependent activation of the osteocalcin promoter [Kahler and Westendorf, 2003]. Lef1 is detectable in preosteoblasts, but in comparison to other Tcfs, it is expressed at low levels. We show that suppression Lef1 levels by RNA interference accelerates osteoblast maturation, perhaps in part by relieving repression of Runx2 activity. Interestingly, MC3T3 osteoblasts have a natural mechanism of downregulating Lef1 during osteogenic maturation. The Lef1 promoter is responsive to Wnts [Hovanes et al., 2001; Filali et al., 2002], so perhaps negative feedback to the canonical Wnt pathway also affects Lef1 expression.

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