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NFATc1 Mediates HDAC-Dependent Transcriptional Repression of Osteocalcin Expression During Osteoblast Differentiation

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Abstract

We previously reported that the *in vivo* and *in vitro* suppression of Nuclear Factor of Activated T Cells (NFAT) signaling increases osteoblast differentiation and bone formation. To investigate the mechanism by which NFATc1 regulates osteoblast differentiation, we established an osteoblast cell line that overexpresses a constitutively active NFATc1 (ca-NFATc1). The activation of NFATc1 significantly inhibits osteoblast differentiation and function, demonstrated by inhibition of alkaline phosphatase activity and mineralization as well as a decrease in gene expression of early and late markers of osteoblast differentiation such as osterix and osteocalcin, respectively. By focusing on the specific role of NFATc1 during late differentiation, we discovered that the inhibition of osteocalcin gene expression by NFATc1 was associated with a repression of the osteocalcin promoter activity, and a decrease in TCF/LEF transactivation. Also, overexpression of NFATc1 completely blocked the decrease in total histone deacetylase (HDAC) activity during osteoblast differentiation and prevented the hyperacetylation of histones H3 and H4. Mechanistically, we show by Chromatin Immunoprecipitation (ChIP) assay that the overexpression of NFATc1 sustains the binding of HDAC3 on the proximal region of the osteocalcin promoter, resulting in complete hypoacetylation of histones H3 and H4 when compared to GFP-expressing osteoblasts. In contrast, the inhibition of NFATc1 nuclear translocation either by cyclosporin or by using primary mouse osteoblasts with deleted calcineurin b1 prevents HDAC3 from associating with the proximal regulatory site of the osteocalcin promoter. These preliminary results suggest that NFATc1 acts as a transcriptional co-repressor of osteocalcin promoter possibly in an HDAC-dependent manner.

Keywords

NFATc1; HDAC3; osteoblast; osteocalcin; TCF/LEF

Introduction

The Nuclear Factor of Activated T Cells (NFAT) is a family of transcription factors that is composed of five proteins related to the Rel/NFκB family (NFAT 1-5) and are best known for

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their role in T lymphocyte activation [1]. In resting cells, NFAT proteins are highly phosphorylated and reside in the cytoplasm. Upon stimulation and the activation of the phosphatase calcineurin (Cn), NFAT proteins are dephosphorylated and translocate to the nucleus where they regulate the transcription of NFAT-dependent genes [2]. We previously reported that the pharmacological inhibition of Cn/NFAT signaling by low concentrations of cyclosporine increases osteoblast differentiation *in vitro* and bone mass *in vivo* [3]. Recently, we demonstrated that the conditional disruption of calcineurin B1 and NFATc1 signaling in osteoblasts increases osteoblast differentiation and bone formation in an *in vivo* animal model [2].

Osteoblasts are the bone forming cells. In culture, as *in vivo*, osteoblasts form bone-like mineralized nodules by undergoing three stages of development: proliferation, extracellular matrix maturation, and mineralization [4,5]. During each stage of development, specific subsets of genes are sequentially expressed or repressed. For example, osteocalcin, the most abundant noncollagenous protein in bone, is a well known marker of osteoblast differentiation [6,7]. In osteoblasts, the mouse osteocalcin protein is encoded by two genes, OG1 and OG2. The promoters of both OG1 and OG2 contain two classical proximal and distal regions [8,9]. The initiation of transcription of the osteocalcin gene starts at the proximal region (-0.2~0 kbp) which has Runx2 and TCF/LEF binding sites, and is known to be responsible for basal tissue-specific transcription [10,11]. After initiation, transcriptional activity is enhanced by the distal region (-0.8~0.4 kbp), which contains a 1,25(OH)₂D₃ response element (VDRE) as well as Runx2 and AP-1 binding sites [10,12]. Functional and physical interaction between these transcription factors and the two regions of the osteocalcin promoter are thought to be responsible for the regulation of osteocalcin expression during osteoblast differentiation.

TCF/LEF's transcriptional activity is known to be modulated by forming complexes with co-activators and co-repressors on the target gene promoters [13-15]. Upon the activation of the canonical Wnt signaling, β -catenin is dephosphorylated, leading to the interaction with TCF/LEF to convert them into transcriptional activators of several genes such as Runx2 in pre-osteoblasts [13,14,16]. This interaction has been shown to be critical in preventing osteoblasts from differentiating into chondrocytes [17,18]. In contrast, in the absence of Wnt signaling, TCF/LEF forms complexes with co-repressors, such as TLE (Gro/TLE family of corepressor) and histone deacetylases (HDACs) [15]. This is supported by reports demonstrating that the stimulation of HDAC1 by glucocorticoids suppresses the transcriptional activity of LEF/TCF, ultimately inhibiting osteoblast differentiation [19].

HDACs are enzymes that catalyze the removal of acetyl groups from lysine residues in histones and non-histone proteins. HDAC proteins have been shown to be critical regulators of functional cellular events, such as cell cycle control, apoptosis, and differentiation in several biological systems including osteoblasts [20]. It has been shown that several HDAC isoforms (HDACs 1-5) are expressed in MC3T3-E1 osteoblast cell line and primary calvarial osteoblasts [21-23]. Furthermore, the acetylation of histones H3 and H4 and the inhibition of the activation of HDACs 1 and 3 has been shown to induce chromatin remodeling events that mediate the transcriptional induction of osteocalcin gene expression during osteoblast differentiation [24]. Moreover, several known negative regulators of bone formation such as transforming growth factor- β (TGF- β) have been shown to repress osteoblast differentiation by recruiting HDACs 4 and 5 on the osteocalcin promoter [22].

Here, we examine a novel mechanism by which NFATc1 negatively regulates osteocalcin expression during the late stage of osteoblast differentiation. We demonstrate that ectopic expression of constitutively active NFATc1 (ca-NFATc1) inhibits osteoblast differentiation in MC3T3-E1 osteoblasts, and reduces both the gene expression and the promoter activity of osteocalcin. Furthermore, we show that ca-NFATc1 forms a repressor complex with several

proteins including HDAC3 at the first 236bp of the osteocalcin regulatory region, resulting in the repression of osteocalcin gene expression. These results suggest that NFATc1 acts as a transcriptional co-repressor of osteocalcin promoter in an HDAC-dependent manner.

Materials and Methods

Cell culture and differentiation

MC3T3-E1 preosteoblast cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in Minimum Essential Medium Eagle, Alpha modification (α -MEM, Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), 100 units/ml penicillin G and 100 μ g/ml streptomycin (Invitrogen, Carlsbad, CA) at 37°C with 5% CO₂. Osteoblastic induction was performed by supplementing medium with 10 mM β -glycerophosphate and 250 μ M ascorbic acid-2-phosphate [2,3]. Cyclosporin A was purchased from Calbiochem (San Diego, CA).

Isolation of primary osteoblasts and infection with adenovirus -GFP and -Cre

Primary osteoblasts were isolated from tibiae and femora of CnB1^{f/f} mice as previously described [2]. Cells were maintained in α -MEM containing 10% FBS, 100 units/ml penicillin G and 100 μ g/ml streptomycin at 37°C with 5% CO₂. Adenovirus-GFP and -Cre were purchased from Vector Biolabs (Philadelphia, PA). At confluency, cells were infected for 48 h with viruses at a multiplicity of infection of 100 [2].

Retrovirus production and infection

We used retroviral expression vectors that express either GFP (pMSCV-GFP) or constitutively active NFATc1 (pMSCV-NFATc1) [3,25]. Retroviruses were produced by cotransfecting pMSCV vectors with pVSV-G into BOSC23 cells using Lipofectamine (Invitrogen). Twenty-four hours after transfection, the media was replaced, and retroviral supernatant was collected. For infection, 2×10^4 cells/cm² MC3T3-E1 cells were plated onto 6-well plates. The culture media were replaced with 500 μ l of retroviral supernatant with 8 μ g/ml polybrene (Sigma) and cells were incubated for 2 h at 37 °C in 5% CO₂. Retroviral supernatant was then removed and cells were cultured in regular growth medium [3,25].

Alkaline phosphatase and mineral deposition assay

Cultured MC3T3-E1 osteoblasts (GFP and ca-NFATc1) were washed with cold PBS, fixed in 2% paraformaldehyde/PBS for 10 min and then incubated at 37 °C with freshly prepared alkaline phosphatase substrate solution (100 mM Tris-Maleate buffer (pH 8.4), 2.8% N,N dimethyl formamide (v/v), 1 mg/ml Fast Red TR and 0.5 mg/ml naphthol AS-MX phosphate). The reaction was terminated after 30 min by removal of the substrate solution. Mineralization was assessed by von Kossa staining of the cultures (2 min in 3% w/v AgNO₃) as previously described [2,3]. Calcium contents were measured using a calcium detection kit (Arsenazo III; Sigma) [26].

Immunofluorescence

MC3T3-E1 osteoblasts (GFP and ca-NFATc1) were cultured on coverslips for 24 h. Cells were then washed with cold PBS and fixed with 2% paraformaldehyde. Samples were then blocked for 1 h in Fc receptor blocker (Innovex Biosciences, Richmond, CA). NFATc1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted in Fc blocker solution and applied to the samples for overnight incubation at 4°C. Fluorescein isothiocyanate-conjugated secondary antibody (Molecular Probes, Eugene, OR) was then used for 45 min. A nonspecific IgG was used for negative controls. Microscopic fields were examined using $\times 100$ objectives of a Nikon

Eclipse 90i fluorescence microscope and photographs were taken using a monochrome Nikon digital camera [2,27].

RNA Extraction and RT-PCR

Total RNA was extracted by the TRIzol method as recommended by the manufacturer (Invitrogen). One μg of RNA was reversed-transcribed using M-MLV reverse transcriptase, and the equivalent of 10 ng was used for TaqMan real-time quantitative RT-PCRs as previously described [2,3]. The expression of actin was used for normalization of gene expression values. The sequences for the specific primers used in this study were followed; Runx2, forward 5'-AAATGCCTCCGCTGTTATGAA-3', reverse 5'-GCTCCGGCCCACAAATCT-3', osterix, forward 5'-TGAGGAAGAAGCCCATTAC-3', reverse 5'-ACTTCTTCTCCCGGGTGTG-3' and osteocalcin, forward 5'-CCG GGA GCA GTG TGA GCT TA-3', reverse 5'-AGG CGG TCT TCA AGC CAT ACT-3'.

Transient transfections and luciferase reporter assays

MC3T3-E1 osteoblasts (GFP and ca-NFATc1) were plated at a density of 2×10^4 cells/cm² in 6-well plates. Twenty-four hours after plating, cells were transfected with 1 μg of NFAT luciferase plasmid (Clontech, Palo Alto, CA), rat osteocalcin promoter luciferase construct (pOC-1050 and pOC-285) which was generously provided by Dr. Amjad Javed, University of Alabama at Birmingham, Birmingham, AL [28] or TOP flash (Upstate Biotechnology, Lake Placid, NY) and 0.2 μg CMV- β -galactosidase reporter construct (as a control) using LipofectAMINE (Invitrogen) according to the manufacturer's instruction. Forty-eight hours post-transfection, cells were lysed and reporter activity was measured using a luciferase (Promega, Madison, WI) or β -galactosidase (Clontech) assay system [3].

Nuclear Protein Extraction

MC3T3-E1 osteoblasts (GFP and ca-NFATc1) were washed with chilled PBS and centrifuged at $800 \times g$ for 5 min at 4 °C. Nuclei were then isolated by detergent lysis of the cells with a Nonidet P40 lysis buffer containing 10 mM Tris, 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P40, and 0.56 M sucrose. Nuclei were then treated with a hypotonic solution containing 10 mM HEPES, 1.5 mM MgCl₂, and 10 mM KCl followed by a 30-min incubation at 4 °C in an extraction buffer containing 20 mM HEPES, 20% glycerol, 600 mM KCl, 1.5 mM MgCl₂, and 0.2 mM EDTA. Nuclei were finally centrifuged at $14,000 \times g$ for 30 min at 4 °C, and the supernatant protein concentration was measured using the Bio-Rad DC protein assay. All solutions in this procedure contained a mixture of protease and phosphatase inhibitors [2,3].

Western Blot Analysis

Nuclear extracts were loaded (20 μg /lane) onto a mini-SDS-PAGE system. Following electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane, Immobilon-P (Millipore Co., Milford, MA), using a Bio-Rad wet transfer system. Membranes were then blocked with Tris-buffered saline-Blotto B (Santa Cruz Biotechnology) for 1 h at room temperature and subsequently incubated overnight with antibodies directed against NFATc1, HDAC1, HDAC2, HDAC3, HDAC4, β -catenin, lamin B1, histone 3, histone 4 (Santa Cruz Biotechnology), acetylated histone 3 and acetylated histone 4 (Upstate Biotechnology, Lake Placid, NY). Signals were detected using a horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence detection kit (ECL; Amersham Biosciences, Pittsburgh, PA) [2,3].

HDAC activity

MC3T3-E1 osteoblasts (GFP and ca-NFATc1) were cultured and nuclear extraction was performed as described above. HDAC activity was measured using an HDAC activity assay

kit according to the manufacturer's instructions (Abcam, Cambridge, MA). Briefly, cells were treated with buffer A (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT and 0.05% NP-40) and incubated on ice for 10 minutes followed by centrifugation for 10 min at 3,000 rpm at 4°C. The pellets were resuspended with buffer B (5 mM HEPES, 1.5mM MgCl₂, 0.2 m EDTA, 0.5 mM DTT and 26% glycerol) and 300 mM NaCl and homogenized with 20 strokes in a Dounce homogenizer. After incubating for 30 min on ice, extracts were centrifuged at 24,000 g for 20 minutes at 4°C. HDAC activity was measured using an HDAC activity kit, as recommended by the manufacturer [29].

Immunoprecipitation

MC3T3-E1 osteoblasts (GFP and ca-NFATc1) were cultured and nuclear extraction was performed as described above. Nuclear proteins were precleared with protein G-agarose beads (Upstate Biotechnology) for 1 hr and precipitated with 4 µg of NFATc1 and normal IgG antibodies as a control (Santa Cruz Biotechnology), followed by the addition of protein G-agarose beads. Immunoprecipitates were washed 4 times with PBS and eluted with protein sample buffer. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting analysis using antibodies against NFATc1 and HDAC3 (Santa Cruz Biotechnology) [27].

Chromatin Immunoprecipitation (ChIP) assay

MC3T3-E1 osteoblasts (GFP and ca-NFATc1) were cultured as described above. To cross-link DNA-protein complex, cells were fixed with 1% formaldehyde at room temperature for 10 min. Nuclei from cross-linked cells were resuspended in Tris-EDTA buffer and sonicated (Fisher Sonic dismembrator, Model 500). The soluble chromatin was adjusted into RIPA buffer (0.1% sodium dodecyl sulfate, 1% Triton X-100, 0.1% sodium deoxycholate, 140 mM NaCl) and immunocleared with 2 µg of salmon sperm DNA/Protein A agarose beads (Upstate Biotechnology) for 1 hr at 4°C. Immunoprecipitation was performed with antibodies for NFATc1, HDAC1, HDAC2, HDAC3, HDAC4, acetylated histone 3, acetylated histone 4 and normal rabbit or mouse IgG overnight at 4°C, followed by adding salmon sperm DNA/protein A agarose for 1 hr. Immunoprecipitates were sequentially washed with the following buffers: once with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1) and 150 mM NaCl), once with high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1) and 500 mM NaCl), once with LiCl buffer (0.25% LiCl, 1% NP-40, 1% Na-Deoxycholate, 1 mM EDTA and 10 mM Tris-HCl (pH 8.1)) and twice with Tris-EDTA buffer. Cross-linking was reversed by heating with 0.2 M NaCl at 65°C overnight. DNA was precipitated with Phenol/Chloroform and the DNA template was amplified using primers targeting specific binding site of TCF/LEF in the osteocalcin promoter: 5'-GCC CCT CAG GGA AGA GGT CTT-3' (-236 bp to -216 bp) and 5'-CTG CAC CCT CCA GCG TCC AG-3' (-20 bp to -1 bp from the transcription start site), 3'UTR primer; 5'-GAT CCC ATA TCA GCC AGC AC-3' and 5'-GAC TGC CCT GGA TCA CAA GT-3'. The product was separated by 1.5 % agarose gel electrophoresis [30].

Statistical analysis

All statistical analyses were performed using the Microsoft Excel data analysis program for Student's *t*-test analysis. Experiments were independently repeated at least three times, each repeated in triplicate unless otherwise stated. Values are expressed as the mean ± SE.

Results

We have previously reported that the Cn/NFAT signaling pathway negatively regulates osteoblast differentiation and bone formation [2,3]. To investigate the mechanism by which NFATc1 regulates osteoblast differentiation, we established an MC3T3-E1 osteoblast cell line that overexpresses a constitutively active NFATc1 (ca-NFATc1) using retroviral expression

system. These constructs were a generous gift from Dr. Neil Clipstone, Northwestern University, Chicago, IL [25]. Transduction efficiency was approximately 95% (data not shown). In order to confirm that ca-NFATc1 is indeed expressed and biologically active in osteoblasts, we examined the cellular localization of NFATc1 by immunofluorescence, in GFP and ca-NFATc1 osteoblasts. As expected, unstimulated MC3T3-E1 GFP cells expressed a basal level of NFATc1 located in the cytoplasm (Fig. 1A). However, the levels of nuclear NFATc1 are dramatically increased in ca-NFATc1 despite the unstimulated state of the cells (Fig. 1A). To further confirm the successful expression of ca-NFATc1 in osteoblasts, GFP and ca-NFATc1 MC3T3-E1 preosteoblasts were cultured for 4 days followed by nuclear protein extraction. Here we show that the levels of active (dephosphorylated) nuclear NFATc1 protein in ca-NFATc1 cells are dramatically increased when compared to GFP cells (Fig. 1B). As expected, the mobility and migration of the fully dephosphorylated ca-NFATc1 transgene is different from the partially dephosphorylated endogenous NFATc1. This is because the ca-NFAT transgene has all of the 21 serine residues substituted with alanine, which makes ca-NFATc1 completely dephosphorylated, when compared to the partially dephosphorylated endogenous NFATc1 [31,32]. Finally, in order to determine whether the increase of the nuclear NFATc1 in ca-NFATc1 osteoblasts is functionally active, we transfected GFP and ca-NFATc1 MC3T3-E1 osteoblasts with pNFAT-TA-luciferase for 48 hours. Cells were then lysed and luciferase activity measured. Our results demonstrate that the overexpression of ca-NFATc1 causes a 600% increase in NFAT transactivation when compared to GFP expressing osteoblasts (Fig. 1C). Together, this confirms that our newly established MC3T3-E1 osteoblast cell line expresses a constitutively and functionally active NFATc1.

To examine the biological effects of the overexpression of NFATc1 on osteoblast differentiation, we cultured GFP and ca-NFATc1 MC3T3-E1 cells for 14 days or 21 days in the presence of β -glycerophosphate and ascorbic acid-2-phosphate. At the end of the study, cultured cells were fixed and stained for alkaline phosphatase activity (ALP) (Red) or for mineralization by von Kossa (black). As shown in Figures 2A and 2B, the overexpression of ca-NFATc1 in osteoblasts results in a dramatic decrease in alkaline phosphatase activity and mineralization, as compared to GFP expression cells (Fig 2A, 2B). Furthermore, the overexpression of ca-NFATc1 in osteoblasts results in a 90% decrease in the amount of matrix-deposited calcium when compared to GFP control osteoblasts (Fig 2C). Although the overexpression of ca-NFATc1 decreases osteoblast differentiation, the proliferation of osteoblasts with constitutively active NFATc1, 48 h after culturing in serum free medium, was 25% more than that of the GFP-expressing cells, as determined by MTT assay (data not shown). These findings confirm our previously published results that NFATc1 negatively regulates osteoblast differentiation [2, 3]. To investigate the regulatory mechanism by which NFATc1 controls osteoblast differentiation, we examined the role of NFATc1 on the expression of early and late gene markers of osteoblast differentiation. We cultured GFP, and ca-NFATc1 MC3T3-E1 osteoblasts cells for 4 days in maintenance media (Proliferation) or for 11 days in osteogenic media (Differentiation). At the end of the study cells were harvested and RNA was extracted. Real-time RT-PCR was performed for *Runx2* and *osterix* (*Osx*) as early markers of osteoblast differentiation, and *osteocalcin* as a later marker. Surprisingly, overexpression of NFATc1 did not affect *Runx2* gene expression (Fig. 2D). However, it caused a 52% decrease in *Osx* gene expression when compared to GFP-control osteoblasts (Fig. 2E). Moreover, the increase in the osteocalcin gene expression during the osteoblastic differentiation of GFP MC3T3-E1 was 85% decreased in ca-NFATc1 MC3T3-E1 osteoblasts (Fig. 2F). These results demonstrate that the activation of NFATc1 suppresses osteoblast function and inhibits the expression of early and late osteoblast gene markers, independent of *Runx2*.

The expression of osteocalcin, the late marker of osteoblast differentiation, is known to be controlled at the transcriptional level by the coordinated activation of basal tissue specific or enhancer molecules which regulate the osteocalcin promoter [33]. This promoter has been

shown to consist of two critical transcriptionally active regions, a proximal promoter region (-200bp and -70bp) and a distal promoter region (-600bp to -400bp) (Fig. 3A) [10]. In order to investigate the role of NFATc1 in the regulation of osteocalcin promoter activity, we transfected GFP and ca-NFATc1 MC3T3-E1 osteoblasts with osteocalcin-luciferase plasmids containing either the full-length osteocalcin promoter (1050 base pairs, pOC-1050) or the initial 285 base pairs of the osteocalcin promoter (-285bp to +1bp, pOC-285). Cells were then lysed and luciferase activity measured. Here we show that the expression of ca-NFATc1 results in a 70% decrease in the activation of both the full length and the proximal region of the osteocalcin promoter (Fig. 3B and C respectively). These results suggest that the NFATc1-mediated inhibition of osteocalcin gene expression is due to the repression of the osteocalcin promoter activity, specifically its proximal region.

The proximal region of the osteocalcin promoter has been shown to be regulated by binding sites for Runx2 and TCF/LEF. To examine whether NFATc1 regulates the transactivation of Runx2 or TCF/LEF, we transfected GFP and ca-NFATc1 MC3T3-E1 osteoblasts with a p6OSE2-luciferase construct for Runx2 transactivation or a TOP flash construct for TCF transactivation, and luciferase activity was measured. Our data demonstrate that the overexpression of ca-NFATc1 does not affect Runx2 transactivation, whereas it significantly (80%) inhibits TCF/LEF activity (Fig. 3D and 3E). These data suggest that NFATc1 regulates the osteocalcin promoter activity mainly by affecting the TCF transcriptional activity.

We next examined whether NFATc1-mediated down-regulation of TCF/LEF transcriptional activity results in changes in the levels of some of the known TCF/LEF activators or repressors during osteoblast differentiation. TCF/LEF transcriptional activity has been shown to be modulated by the formation of complexes on the promoters of target gene with co-activators such as β -catenin or co-repressors such as HDACs [13-15]. GFP and ca-NFATc1 MC3T3-E1 osteoblasts were cultured for 4 days in maintenance media (Pro) or for 11 days in osteogenic media (Diff). At the end of the study, cells were harvested and nuclear proteins were collected. Despite the NFATc1-mediated down-regulation of TCF/LEF transcriptional activity, there was no change in the total protein levels of the TCF/LEF co-activators (β -catenin) or co-repressors (HDACs 1-4) during the proliferation and differentiation of osteoblasts when compared to GFP-expressing cells (Fig. 4A). However, the expected decrease in the levels of histone acetylation during osteoblast differentiation was completely blocked by the overexpression of ca-NFATc1. Our results demonstrate that during osteoblast differentiation the total HDAC enzymatic activity in ca-NFATc1 expressing osteoblasts did not decrease as compared to the 40% decrease in the GFP expressing control cells (Fig. 4B). The ability of ca-NFATc1 to maintain elevated levels of HDAC activity was further confirmed by examining the ratio of acetylated histone proteins to total histone protein levels during osteoblast differentiation. Similarly, we demonstrate by western blotting that the acetylation of both H3 and H4 histones are increased by 450% during the osteoblastic differentiation of GFP-MC3T3-E1 osteoblasts, while the overexpression of ca-NFATc1 completely blocked histone acetylation in osteoblasts (Fig. 4C).

It has been previously shown that enzymatic activation of HDAC and its function to deacetylate histones can be regulated by the direct interaction and the association of HDAC with other proteins [34]. For example, it was shown that HDAC1 in other biological systems directly interacts with NFATc2 and binds to the promoter of cyclin-dependent kinase 4 (*cdk4*) which ultimately leads to the repression of *cdk4* gene expression [35]. Therefore, in order to investigate whether the blocking of HDAC enzymatic activity in response to the overexpression of ca-NFATc1 is responsible for the decrease in TCF/LEF transactivation of the osteocalcin promoter, we performed a chromatin immunoprecipitation assay (ChIP) using specific primers that recognize the first 236bp of the osteocalcin promoter that contains TCF/LEF consensus sequence (Fig. 5A). GFP and ca-NFATc1 MC3T3-E1 osteoblasts were cultured for 4 days in maintenance media (Pro) or for 11 days in osteogenic media (Diff). At the end of the study,

cells were fixed with formaldehyde. Nuclei from cross-linked cells were sonicated and then immunoprecipitated using antibodies against acetylated histone H3, H4, HDACs 1-4, NFATc1, TCF, Runx2 and normal rabbit or mouse IgG as a negative control. Immunoprecipitated DNA fragments were then amplified by PCR using specific primers for the first 236 bp of the osteocalcin regulatory region that contains TCF/LEF consensus sequence, as well as primers for 3'UTR as a control region of the OC gene not containing regulatory sites. As expected, the binding of acetylated histones H3 and H4 to the proximal osteocalcin regulatory region increased 3.5 fold in mature differentiated osteoblasts, when compared to proliferating osteoblasts (Fig. 5B). Moreover, the binding of HDAC3 to this region was decreased 70% during osteoblast differentiation. All of this was accompanied by a total loss of NFATc1 binding to the proximal osteocalcin regulatory region in differentiated osteoblasts (Fig. 5B). In contrast, the overexpression of ca-NFATc1 in osteogenically induced osteoblasts not only maintained elevated levels of NFATc1 protein binding to osteocalcin promoter but also sustained the presence of high levels of HDAC3 and prevented the acetylation of both histones H3 and H4 (Fig. 5B). This is accompanied by insignificant changes in the binding of Runx2 and TCF to the proximal osteocalcin regulatory region.

In order to demonstrate that NFATc1 and HDAC3 proteins physically interact, GFP and ca-NFATc1 MC3T3-E1 osteoblasts were cultured in osteogenic media for 11 days. At the end of the study, nuclear protein fraction was extracted and immunoprecipitated with specific antibody against NFATc1. As expected, our data reveal that in control GFP-expressing osteoblasts, NFATc1 protein minimally interacts with HDAC3 in differentiating osteoblasts. However, this binding dramatically increases in response to the elevated levels of NFATc1 as a result of the overexpression of ca-NFATc1 (Fig. 5C).

Finally, in order to further demonstrate the ability of NFATc1 in recruiting HDAC3 to the proximal 236bp of the osteocalcin regulatory region of the promoter, we inhibited calcineurin/NFAT signaling in MC3T3-E1 cells using low dose cyclosporine during proliferation and differentiation [3]. Also, we deleted calcineurin b1 gene in primary osteoblasts which were harvested from calcineurin *flox/flox* mice using adeno-*cre* virus [2]. As expected, the treatment with cyclosporine (Fig. 6A) as well as the deletion of calcineurin b1 (Cre⁺ cells) (Fig. 6B) in osteoblasts completely inhibits the recruitment of both NFATc1 and HDAC3 to the proximal regulatory region of the osteocalcin promoter.

Taken together, our preliminary data demonstrate that NFATc1 acts as a transcriptional co-repressor of the first 236bp of the regulatory region of the osteocalcin promoter. It mediates its suppressive action by forming an inhibitory protein complex that includes, NFATc1, TCF, Runx2 and HDAC3. This ultimately leads to a suppression of osteocalcin proximal promoter activation and gene expression.

Discussion

We have recently reported that the *in vivo* and *in vitro* pharmacologic or genetic suppression of calcineurin and NFAT signaling increases osteoblast differentiation and bone formation [2,3]. However, the mechanisms by which NFAT signaling regulates osteoblastic gene expression are not well elucidated. The present study demonstrates that the activation of NFATc1 dramatically inhibits osteoblast differentiation and function. We also discovered that NFATc1 mediates an HDAC-dependent transcriptional repression of osteocalcin gene expression during osteoblast differentiation.

We generated an osteoblastic cell line, by overexpressing constitutively active NFATc1 in MC3T3-E1 osteoblasts. The newly established osteoblast cell line was confirmed to express a constitutively and functionally active NFATc1 by showing the nuclear localization of NFATc1

protein and the basal increase in NFAT transactivation. The proliferation of osteoblasts with constitutively active NFATc1, 48 h after culturing in serum free medium, was 25% more than that of the GFP-expressing cells, as determined by MTT assay (data not shown). However, ca-NFATc1 expressing osteoblasts failed to differentiate into functional osteoblasts that are capable of secreting alkaline phosphatase and mineralizing the extracellular matrix. This is consistent with our previously published findings demonstrating that the inhibition or deletion of calcineurin/NFAT signaling increases osteoblast differentiation [2,3]. Although NFATc1 is thought to be a transcriptional activator of many genes such as IL-2, IFN- γ and TNF- α [36], its role in the inhibition of gene expression has also been reported [35]. For example, NFATc2 was reported to play a unique role in the negative regulation of the expression of several genes such as *cdk4* [35]. Similarly, we discovered that the constitutive activation of NFATc1 inhibits the expression of early and late markers of osteoblast differentiation, *Osx* and *osteocalcin*.

Osteoblast differentiation has been shown to be regulated by two critical transcription factors; Runx2 and osterix [37-39]. Our data demonstrate that the inhibition of osteoblast differentiation in response to the overexpression of NFATc1 is independent of Runx2. These data are consistent with several previously published reports confirming that Runx2 does not mediate NFATc1 action in osteoblasts [2,40,41]. Furthermore, although canonical Wnt/ β -catenin signaling has been shown to stimulate Runx2 gene expression in the process of osteoblast differentiation [16,17], other studies have shown that β -catenin is not essential for the initial activation of Runx2 [11,42]. These findings do not exclude the possibility that Wnt signaling could be regulating the levels or duration of Runx2 expression during osteoblast development and differentiation. Our data support the argument that NFATc1 is inhibiting TCF/LEF activation without affecting Runx2 expression. Interestingly, we also discovered that the inhibition of osteoblast differentiation by NFATc1 is associated with a significant decrease in the expression of *Osx*, independent of Runx2. This is consistent with other studies which demonstrate that the *Osx* expression is β -catenin dependent [42]. Also, the analysis of Runx2 expression in *Osx* null mice indicates that *Osx* activation lies downstream of *Runx2*; thus *Runx2*, although required *in vivo* for *Osx* activation, is not sufficient in the absence of β -catenin [39,42]. Finally, BMP-2 has been shown to activate *Osx* in *Runx2* mutant cells; thus, the molecular hierarchies and interactions underlying *Osx* activation are unclear, although its crucial role in the specification of all osteoblasts has been clearly demonstrated [39,42,43].

The gene expression of osteocalcin has been shown to be transcriptionally regulated by the interactions of several transcription factors and regulatory proteins which compete to bind at specific regulatory elements on the osteocalcin promoter [44,45]. Our data confirm that the ca-NFATc1 in osteoblasts equally decrease the transcriptional activation of the full length and proximal region of the osteocalcin promoter. Although the osteocalcin promoter possesses several NFAT DNA binding motifs that can potentially allow NFATc1 to directly regulate the activation of the promoter, all of these NFAT binding sites exist only in distal regions (-370, -420, -700, -880) which is far from the start of the proximal region of osteocalcin promoter (-285). This suggests that NFATc1 is able to suppress osteocalcin expression by controlling the activation of proximal regions of the osteocalcin promoter, independent of direct NFATc1 binding to its motif. This region of the promoter has been previously shown to be critical for the regulation of the osteocalcin promoter. For example, TGF- β was shown to negatively regulate osteoblast differentiation and osteocalcin expression by inhibiting the activation of Runx2 and/or TCF/LEF at the proximal regions of the osteocalcin promoter [22]. Similarly, we discovered that ca-NFATc1 causes an 80% decrease in TCF/LEF transactivation, which has had previously been thought to be able to control osteocalcin expression [22]. However, our data demonstrate that ca-NFATc1 suppresses osteoblast differentiation and osteocalcin expression independent of Runx-2 which is known to be essential for osteoblast differentiation, bone formation and the expression of several osteoblastic genes including osteocalcin. Also,

it has previously been shown, similar to our findings, that osteoblast differentiation can be inhibited independent of Runx-2 [46].

The transcriptional activity of TCF/LEF has been shown to be tightly regulated by the binding of different co-activators or co-repressors on the TCF/LEF binding sites during osteoblast differentiation [13,15]. For example, it has been shown that TCF/LEF transcriptional activity is modulated by the formation of complexes on the promoters of target gene with co-activators such as β -catenin or co-repressors such as HDACs [13-15]. Our results show that the overexpression of ca-NFATc1 does not result in differences in the levels of the TCF/LEF co-activators (β -catenin) or co-repressors (HDACs 1-4) during the proliferation and differentiation when compared to GFP-expressing osteoblasts. However, the expected decrease in the levels of histone acetylation during osteoblast differentiation was completely blocked by the overexpression of ca-NFATc1. These results suggest that the activation of NFATc1 in osteoblasts decreases the total HDAC enzymatic activity without affecting nuclear protein levels of HDACs. Our results are supported by several studies which describe the contribution of NFAT in silencing a subset of a large number of genes by the recruitment of different HDAC family members. It has been previously shown that the enzymatic activation of HDAC and its function to deacetylate histones can be regulated by the direct interaction and the association of HDAC with other proteins [34]. Furthermore, a study of *cdk4* gene regulation found that NFATc2, by direct interaction with HDAC1, recruits and stabilizes HDAC on *cdk4* promoter and suppresses its transcription [35]. Similarly, our data demonstrate that the overexpression of NFATc1 in osteoblasts not only increases the interaction between NFATc1 and HDAC3, but also inhibits the acetylation of both histones H3 and H4. This ultimately is a critical factor that may be involved in the NFAT-mediated suppression of osteoblast differentiation. This is consistent with previous reports describing the role of acetylated histone H4, and to a lesser extent acetylated histone H3, in the regulation of osteocalcin expression during osteoblast differentiation [47].

Furthermore, it has been previously reported that HDACs function as a negative regulator of osteoblast differentiation and osteocalcin expression [21-23] and that total HDAC activity is decreased in differentiated osteoblasts [23]. Similarly, we demonstrated that total HDAC activity is significantly decreased in differentiated control GFP MC3T3-E1 osteoblasts when compared with ca-NFATc1 expressing osteoblasts. Although, a mechanism for this action was previously proposed in which HDAC3 interacts with Runx2 and results in an inhibition of osteoblast differentiation [21]. Our data demonstrate that, independent of Runx-2, ca-NFATc1 inhibits HDAC enzymatic activity, TCF/LEF transactivation, and osteoblast differentiation. It is presently unclear how NFATc1 is able to regulate the activation of HDAC which binds TCF/LEF protein complex and yet spares the HDAC/Runx2 protein complex. It is possible that NFATc1 also interacts and binds to an unidentified protein which is present in the protein complex that includes HDAC/TCF/LEF, but not the HDAC/Runx2. This is a testable hypothesis, and we are currently working to answer this question.

Our ChIP assay data confirm that the binding of acetylated histones H3 and H4 at the proximal osteocalcin promoter is increased in mature, differentiated osteoblasts when compared to proliferating osteoblasts. Moreover, the binding of different HDACs, especially HDAC3, to the regulatory region of the osteocalcin promoter was decreased during osteoblast differentiation. This is consistent with several previously published reports which demonstrate that the acetylation of histones H3 and H4 in osteoblasts is critical for the expression of osteoblastic genes such as osteocalcin, and osteoblast differentiation [23,47]. In contrast, the overexpression of ca-NFATc1 in osteogenically induced osteoblasts not only maintained elevated levels of NFATc1 protein binding to the first 236bp of the osteocalcin regulatory region, but also sustained the presence of high levels of HDAC3 and prevented the acetylation of both histones H3 and H4. These findings were further confirmed by demonstrating the

complete inhibition of the recruitment of both NFATc1 and HDAC3 to this region of the osteocalcin promoter after the pharmacological or genetic inhibition of NFATc1 nuclear translocation.

In summary, our results describe a novel mechanism in which NFATc1 interacts with HDAC3 to form the transcriptional repressor complex at the proximal element of the osteocalcin promoter, thus inhibiting osteocalcin gene expression and finally decreasing osteoblast differentiation.

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Abbreviations

NFAT	Nuclear factor of activated T cells
HDAC	Histone deacetylase
OC	osteocalcin
ALP	alkaline phosphatase
TCF/LEF	T-cell factor/lymphoid enhancer-binding factor

Figure 1. Generation of MC3T3-E1 osteoblastic cell line overexpressing constitutively active NFATc1

MC3T3-E1 cells were transduced with GFP-empty vector (GFP) or constitutively active NFATc1 (ca-NFATc1) retrovirus. **(A)** Cells were immunostained with NFATc1 antibody followed by fluorescein isothiocyanate-conjugated secondary antibody. A nonspecific IgG was used for negative controls (insets). Microscopic fields were examined using $\times 100$ objectives. **(B)** Nuclear proteins were used for immunoblotting with the antibodies against NFATc1 and Lamin B1. **(C)** Cells were transfected with pTA-NFAT luciferase construct. Forty eight hours after transfection, luciferase activity was measured. Data are expressed relative to CMV- β -

galactosidase, and values represent the mean $\pm$ SE of 3 separate experiments each repeated in triplicate; *, $p \leq 0.01$.

Figure 2. NFATc1 negatively regulates osteoblast differentiation

GFP and ca-NFATc1 MC3T3-E1 osteoblasts were cultured for 14 days (**A**) or 21 days (**B** and **C**) in the presence of β -glycerophosphate and ascorbic acid-2-phosphate to induce osteoblast differentiation. Cells were stained for ALP activity (red) (**A**) or for mineralization by von Kossa (Brown) (**B**). Calcium deposition in the matrix was measured using a calcium detection kit (**C**). GFP, and ca-NFATc1 MC3T3-E1 osteoblasts were cultured for 4 days in proliferation medium (Pro) or 11 days in differentiation medium (Diff). RNA was extracted and Real time RT-PCR was performed for *Runx2* (**D**), *osterix* (**E**) *osteocalcin* (**F**) and actin. Values, obtained from three independent experiments, represent the mean $\pm$ SE of mRNA expression relative to actin expression; **, $p \leq 0.05$, *, $p \leq 0.01$ compared to control.

Figure 3. NFATc1 decreases osteocalcin promoter activity

Schematic illustration of the osteocalcin promoter showing the relative positions of binding sites for the indicated transcription factors (**A**). GFP, and ca-NFATc1 MC3T3-E1 osteoblasts were transiently transfected with rat osteocalcin luciferase constructs, including full length osteocalcin promoter “OC-1050” (**B**) or proximal region (-285 to +1) “OC-285” (**C**), Runx2 luciferase construct (**D**) and TOP flash luciferase construct (**E**). Forty eight hours after transfection, luciferase activity was measured. Data are expressed relative to internal control (CMV- β -galactosidase) and values represent the mean $\pm$ SE of 3 separate experiments, each repeated in a triplicate; *, $p \leq 0.01$.

Figure 4. NFATc1 maintains elevated levels of HDAC activity and inhibits the acetylation of histones during osteoblast differentiation

GFP and ca-NFATc1 MC3T3-E1 osteoblasts were cultured for 4 days in proliferation medium (Pro) or 11 days in differentiation medium (Diff). (A) Nuclear proteins were used for immunoblotting with several antibodies. Data shown are representative of three separate experiments. (B) Nuclear proteins were collected on day 0, 3 and 11 after osteoblastic induction. Total histone deacetylase enzymatic activity was measured. Values were obtained from three independent experiments, each performed in triplicate and represent the mean $\pm$ SE of HDAC activity per 100 μ g concentration of protein; *, $p < 0.05$. (C) Nuclear extracts were used for immunoblotting with antibodies against total and acetylated histones 3 and 4. Shown

is a representative of three separate experiments (left panel). Quantitative data were obtained as a ratio of acetylated histones/total histones by image analysis software (Image J:NIH). Values, obtained from three independent experiments, represent the mean $\pm$ SE (right panel).

Figure 5. NFATc1 forms a transcriptional repressor complex through the interaction with HDAC3 on the proximal regulatory region of the osteocalcin promoter

Schematic illustration of the osteocalcin proximal promoter showing the location of TCF and Runx2 sites and primers used in this study (A). GFP and ca-NFATc1 MC3T3-E1 osteoblasts were cultured for 4 days in proliferation medium (Pro) or 11 days in differentiation medium (Diff). Cellular DNA fragments were immunoprecipitated with antibodies against different proteins and normal rabbit or normal mouse IgG as controls. Immunoprecipitated DNA fragments were amplified by PCR using primers that encompass -236 bp to -1 bp or 3'UTR site of the osteocalcin promoter. Quantitative data of protein binding to this site during osteoblast differentiation was obtained by image analysis software (Image J:NIH) and compared to that

of proliferating GFP cells; set to 1.0 as a basal level. Values, obtained from three independent experiments, represent the mean of fold change (**B**). Nuclear proteins were immunoprecipitated with anti-NFATc1 antibody (IP:NFATc1) or normal mouse IgG as a control (IP:mIgG). Immunoblots were developed using antibodies against NFATc1 or HDAC3. Shown is a representative of three separate experiments (**C**).

Figure 6. Inhibition of calcineurin abrogates the recruitment of NFATc1 and HDAC3 to the proximal regulatory region of the osteocalcin promoter

MC3T3-E1 cells were treated with cyclosporine (500 nM) for 4 days in proliferation medium (Pro) or for 11 days in differentiation medium (Diff) (**A**). Primary osteoblasts from CnB1^{f/f} mice were transduced with adenovirus-GFP (Cre⁻) or (Cre⁺) (**B**). Cellular DNA fragments were immunoprecipitated with antibodies NFATc1, HDAC3 and normal IgG. Immunoprecipitated DNA fragments were amplified by PCR using primers which encompass -236 bp to -1 bp or 3'UTR site of the osteocalcin promoter. Quantitative data of protein binding to this site during osteoblast differentiation were obtained by image analysis software (Image J:NIH) and compared to proliferating GFP cells; set to 1.0 as a basal level. Values, obtained from three independent experiments, represent the mean of fold change.