Transcription factor decoy against NFATc1 in human primary osteoblasts

LETIZIA PENOLAZZI[1], GINA LISIGNOLI[2], ELISABETTA LAMBERTINI[1], ELENA TORREGGIANI[1], CRISTINA MANFERDINI[2], ANDREA LOLLI[1], RENATA VECCHIATINI[1], FRANCESCA CIARDO[1], ELENA GABUSI[2], ANDREA FACCHINI[2], ROBERTO GAMBARI[1] and ROBERTA PIVA[1]

[1]Dipartimento di Biochimica e Biologia Molecolare, Sezione di Biologia Molecolare, Università degli Studi di Ferrara, Ferrara; [2]Struttura Complessa Laboratorio di Immunoreumatologia e Rigenerazione Tissutale, Istituto Ortopedico Rizzoli, Bologna, Italy

Abstract. The present study describes, for the first time, the removal of the nuclear factor of activated T cells cytoplasmic 1 (NFATc1) by a decoy approach in human primary osteoblasts (hOBs). hOBs with different NFATc1 expression levels were used. The functionality of endogenous NFAT proteins in our experimental model was analyzed by monitoring the transcriptional activity on a luciferase reporter construct driven by three copies of an NFAT response element (pNFAT-TA-luc). Cell treatment with the decoy against NFATc1 resulted in a significant increase in the expression of osteoblastic markers, including ERα and ColXV. On the contrary, the expression of Runx2, which is known to not be transcriptionally regulated by NFATc1, was not altered, indicating the specificity of the decoy effect. To our knowledge, this is the first time that transcription factor decoy has been successful in hOBs to allow the investigation of the role of NFATc1 in an experimental model that, compared to the use of cell lines, more closely resembles an *in vivo* model. In addition, by using chromatin immunoprecipitation we found that *in vivo* NFATc1 is recruited on the ColXV gene promoter. The specific role of NFATc1 in osteoblast differentiation is not well understood, however, our findings reinforce the action of NFATc1 in the transcriptional program of osteoblasts, also supporting the therapeutic potential for the proper manipulation of NFATc1-mediated events in different bone disorders. At the same time, our data add important information on the regulation of the expression of ColXV, which only recently has been proposed as an osteoblastic marker.

Correspondence to: Professor Roberta Piva, Dipartimento di Biochimica e Biologia Molecolare, Sezione di Biologia Molecolare, Via Fossato di Mortara 74, I-44121 Ferrara, Italy
E-mail: piv@unife.it

Introduction

Modulation of the activities of bone-forming osteoblasts and bone-resorbing osteoclasts is one of the major therapeutic objectives in a variety of bone tissue diseases (1,2). As a therapeutic target for modulation of bone remodeling, the nuclear factor of activated T cells (NFAT) has recently received considerable attention, even if many reports describe the importance of the NFAT-mediated signaling in the control of both bone resorption and bone formation with contradictory findings (3-8).

NFAT proteins comprise a family of transcription factors (NFAT 1-5) that, after calcium/calcineurin-dependent dephosphorylation, are activated and regulate the expression of many genes involved in a wide range of cellular processes (9-11). NFAT proteins are capable of forming strong cooperative complexes with related transcription factors such as NF-κB proteins, and unrelated binding partners such as GATA, Maf, Oct, PPAR-γ, and members of the AP-1 family on composite cis elements (1,12-15).

To gain insight into the role of NFAT proteins in the bone, and in the search for novel NFAT target genes in osteoblasts, we analyzed the effect of the inhibition of NFATc1 function, through a specific decoy approach, in human primary osteoblasts (hOBs). The transcription factor decoy (TFD) strategy is a kind of gene silencing approach aimed at eliminating or attenuating the activity of a specific transcription factor (16-23). TFD is based on the cellular transfection of double-stranded DNA oligonucleotides (ODNs) mimicking the *cis*-element that is recognized by the transcription factor, and transcription factor binding on endogenous target genes is quickly and effectively blocked. Therefore, the decoy approach is a powerful tool for interfering with the activity of a specific transcription factor and for modulating transcription of its target genes. To our knowledge, this is the first time that TFD has been successfully used in hOBs to investigate the role of NFATc1 in an experimental model which, compared to the use of cell lines, more closely resembles an *in vivo* model.

Our previous study identified NFATc1 as negative regulator of the estrogen receptor α (ERα) gene transcription in

the SaOS-2 osteosarcoma cell line (24). Considering that ERα plays an important positive role in the control of proliferation, differentiation, and survival of osteoblasts (25), our previous data (24) led us to more thoroughly investigate the effect of NFATc1 inhibition in hOBs, an experimental model more easily associated with an *in vivo* context.

Materials and methods

Isolation and culture of osteoblasts. Bone tissues were harvested from ten elderly patients undergoing total knee replacement for osteoarthritis (mean age 70±9). The study was approved by the local Ethics Committee and informed consent was obtained from each patient.

Trabecular bone was obtained from the inner portion of the tibial plateau. Bone chips were removed from the tibial plateau, collected in a V-glass tube containing 1.5 ml of α-MEM supplemented with 15% FBS, 25 mg/ml ascorbic acid and penicillin G (Sigma, St. Louis, MO, USA) (referred as α-MEM medium).

Transient transfections and the luciferase assay. Transient transfections were performed on cells grown to 60% confluency, in 24-well plates with α-MEM medium plus 10% FBS, using Lipofectamine reagent (Invitrogen Corp., Carlsbad, CA, USA), according to the manufacturer's instructions. Cells were transfected with a total of 1 μg/ml of ODN-decoy complexed with 3 μg of Lipofectamine 24 h after the seeding (first administration); on day 4, the cells were further transfected with the same DNA amount until day 7, when the cells were harvested.

For transfection experiments with pNFAT-TA-luc or empty vectors, the cells were plated in 24-well plates, maintained in α-MEM medium with 10% FBS, in the presence of different amounts of $CaCl_2$ (0.5, 1.3, 2.6 mM) and in the presence of 0.5 μM ionomycin and 100 μg/ml PMA (Sigma). After 24 h the cells were transiently transfected using Lipofectamine reagent (Invitrogen Corp.) with 5 μg of plasmid vector. After overnight incubation, the medium was removed and fresh medium was added. After another 24 h, the cells were harvested, lysed, and assayed for luciferase activity. The luciferase activity was normalized by the total protein amount and by β-galactosidase values resulting from co-transfection of 0.25 μg of pCMVSport β-gal (Invitrogen Corp.). β-galactosidase activity was measured by using the Beta-Glo Assay System (Promega, WI, USA).

Chromatin immunoprecipitation (ChIP) assay. The ChIP assay was carried out as previously described (26) using the standard protocol supplied by Upstate Biotechnology (Lake Placid, NY) with their ChIP assay reagents. The cells were cross-linked with 1% formaldehyde for 10 min at 37˚C, washed in ice-cold PBS, and suspended in SDS lysis buffer for 10 min on ice. Samples were sonicated, diluted 10-fold in dilution buffer supplemented with protease inhibitors, and precleared with 80 μl of DNA-coated protein A-agarose; the supernatant was used directly for immunoprecipitation with 10 μg of anti-NFATc1, (H-110) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), overnight at 4˚C. Immunocomplexes were mixed with 80 μl of DNA-coated protein A-agarose

followed by incubation for 1 h at 4˚C. Beads were collected and sequentially washed 3 times with 1 ml each of the following buffers, low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), LiCl wash buffer (0.25 mM LiCl, 1% Igepal CA630, 1% deoxycholic acid, 1 mM EDTA,10 mM Tris pH 8.1), and TE buffer. The immunocomplexes were eluted two times by adding a 250 μl aliquot of a freshly prepared solution of 1% SDS, 0.1 M $NaHCO_3$ and the cross-linking reactions were reversed by incubation at 65˚C for 4 h. The samples were then digested with proteinase K (10 mg/ml) at 45˚C for 2 h and DNA was purified by Qiaquick Spin Columns (Qiagen Inc., Germantown, MD). For PCR analysis, aliquots of chromatin before immunoprecipitation were saved (Input). PCR was performed to analyze the presence of DNA precipitated by the NFATc1-specific antibody, and by using specific primers (Table I) to amplify fragments of the collagen type XV (ColXV) gene promoter. Each PCR reaction was performed with 5 μl of the bound DNA fraction or 2 μl of the Input. The PCR was performed as follows, pre-incubation at 95˚C for 5 min, 30 cycles of 1 min denaturation at 95˚C, 1 min annealing at 52˚C and 1 min at 72˚C, with one final incubation at 72˚C for 5 min. A no-antibody control was included in each experiment.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts from Jurkat cells were prepared as previously described (27). Double-stranded oligonucleotides were end-labeled with [γ-^{32}P]-ATP and T4 polynucleotide kinase (GE Healthcare, Germany). For the gel shift assay 10 μg of nuclear proteins were pre-incubated for 5 min at room temperature with 1.2 μg of non-specific competitor DNA poly(dI-dC)-(dI-dC) (Pharmacia Corp., Bridgewater, NJ) and then 10,000 cpm of labeled oligonucleotides were added and incubated for another 30 min at room temperature. For the competition experiments a 50- and 100-fold excess of unlabeled oligonucleotides was incubated with the nuclear extracts for 15 min at room temperature and then incubated with the appropriate ^{32}P-labeled DNA probe. For supershift experiments, nuclear extracts were pre-incubated for 15 min at room temperature with 4 μg of the antibody against NFATc1 prior to their 30 min incubation with the labelled oligonucleotides at room temperature. Reactions were run on 6% polyacrylamide gels and electrophoresed at 150 V for 2 h in 0.5X TBE buffer. Gels were then dried and exposed to film at -80˚C.

Immunocytochemistry. Cells grown in chamber slides were fixed in 4% PFA for 20 min at room temperature and then hydrated with TBS 1% BSA for 5 min at room temperature. The slides were incubated with the monoclonal antibodies: anti-human-Runx2, anti-osteocalcin (OC; R&D Systems, Minneapolis, MN), anti-bone sialoprotein (BSP; Fisher Scientific, Pittsburg, PA, USA), anti-collagen type 1 (Col1A1; obtained from DSHB, Department of Biological Sciences, Iowa City, IA), anti-NFATc1 (Santa Cruz Biotechnology) and anti-ERα (Upstate Biotechnology), for 1 h at room temperature. The slides were washed three times with TBS 1% BSA and then sequentially incubated with multilinker biotinylated secondary antibody and alkaline phosphatase-conjugated

Table I. PCR primers used for the chromatin immunoprecipitation assay (ChIP) on the ColXV gene.

	Primer sequences	Product size (bp)
Forward F1	5'-TCCACTCCTGGGCATTCAAG-3'	314
Reverse R1	5'-GACGGAATACATTGGACGCT-3'	
Forward F2	5'-TTACTGGCAGGCAGCATGGCT-3'	310
Reverse R2	5'-CACAAACTAAGTCAAGGTGAGAT-3'	
Forward F3	5'-CACAGGGAATGTGAACAGATT-3'	370
Reverse R3	5'-AGAACGGGAACCAGACTAAA-3'	
Forward F4	5'-CATGTTCTTCTCCCCATATATC-3'	296
Reverse R4	5'-AACTCTGAAGACCTAAGAAAAACT-3'	

streptavidin (BioGenex kit, San Ramon, CA, USA) or goat anti-rabbit HRP (DakoCytomation), at room temperature for 20 min. The slides were developed using fast red as a substrate or a peroxidise reaction using 3,3'-diaminobenzidine as a substrate, counterstained with haematoxylin, mounted with glycerol jelly and and observed using a Leitz microscope. Negative and isotype matched controls were performed.

Mineralization assay. The extent of mineralized matrix in the plates was determined by Alizarin Red S staining (ARS; Sigma) in the cells cultured for up to 21 days in osteogenic medium consisting of DMEM, high-glucose, supplemented with 10% FBS, 10 mM β-glycerophosphate, 0.1 μM dexamethasone, and 50 mM ascorbate (Sigma). In the committed cells, the osteogenic medium was changed every 3 days. The cells were then fixed in 70% ethanol for 1 h at room temperature, washed with PBS, stained with 40 mM ARS (pH 4.2) for 10 min at room temperature, washed five times with deionized water, and incubated in PBS for 15 min to eliminate non-specific staining. The stained matrix was observed at different magnifications using a Leitz microscope.

Real-time quantitative RT-PCR. For RT-PCR analysis, total-RNA was isolated from hOBs using a total-RNA isolation system (RNeasy Plus Micro kit, Qiagen, Italy). Total-RNA (2 μg) was reverse transcribed with the ImProm-II RT System (Promega). The mRNA of the target genes was quantified by real-time PCR using the ABI Prism 7700 system and TaqMan probes for Runx-2, ERα and ColXV (Applied Biosystems, CA, USA). PCR was carried out in a final volume of 25 μl after a 10 min pre-incubation at 95°C (denaturation), 1 min at 60°C (annealing/elongation). The mRNA levels were corrected for GAPDH mRNA levels (reference gene) and normalized to a calibrator sample (control cells).

Calcium measurements. Basal levels of the cytoplasmic Ca^{2+} concentration were measured in Fura-2-AM-loaded cells as previously described (28). Cells were grown on 24 mm coverslips in α-MEM medium supplemented with 1% FBS and loaded with 4-AM Fura-2-AM (added to KRB/ Ca^{2+} solution + 1% FBS) at 37°C for 30 min. The coverslip was washed and transferred to the thermostated stage of a Zeiss Axiovert 200 inverted microscope, equipped with a Sutter filterwheel and 340/380 excitation filters. The sample was excited at 340 and 380 nm using a random access monochromator (Photon Technology International, NJ, USA) and emission was detected using a 505/30 emission filter. Images were acquired every 3 sec by the BFT512 camera (Princeton Instruments, AZ, USA). Image acquisition was controlled by the MetaFluor 5.0 software (Universal Imaging Corp., PA, USA). Calibration was performed using the MetaFluor and the MetaFluor Analyst software, according to the equation $[Ca^{2+}] = K_d \times (Sf2/Sb2) \times (R - R_{min})/(R_{max} - R)$, where $K_d = 224$ nM. R_{max} and R_{min} were determined at the end of each experiment in KRB/Ca^{2+}/1 μM ionomycin, and KRB/1 mM EGTA/ ionomycin, respectively.

Statistical analysis. Data are presented as the mean ± SEM from at least three independent experiments, where indicated. Statistical analysis was performed by one-way analysis of variance followed by the Student's t-test. A P-value <0.05 was considered statistically significant.

Results

Characterization of primary osteoblast cultures. hOBs were generated from bone chips removed from the tibial plateau as previously described (29) and characterized for the typical osteogenic markers. In Fig. 1A the analysis of a representative sample of the cells that we used is shown. The cells were highly positive for Runx2, Col1A1, BSP and OC, and exhibited an evident extracellular matrix mineralization after 21 days of culture under osteogenic conditions (ARS). In addition, the cells were positive for ERα, a transcription factor strictly associated with osteoblast differentiation, and for NFATc1, the target of our decoy treatment, as demonstrated by immunocytochemical analysis and quantitative RT-PCR (Fig. 1B).

NFATc1 binds to the decoy ODN molecule. We used the EMSA to analyze NFATc1 binding to the decoy ODN, which contains one NFAT motif (24). Proteins from the nuclear extract of Jurkat, a highly NFATc1 positive human T-leukemia cell line, efficiently bound to the decoy ODN probe, giving two major retarded complexes (Fig. 2). The labeled retarded complexes were found to be specifically out-competed by increasing concentrations of cold excess of decoy ODN, while they were unaffected by a 100-fold excess of an unrelated
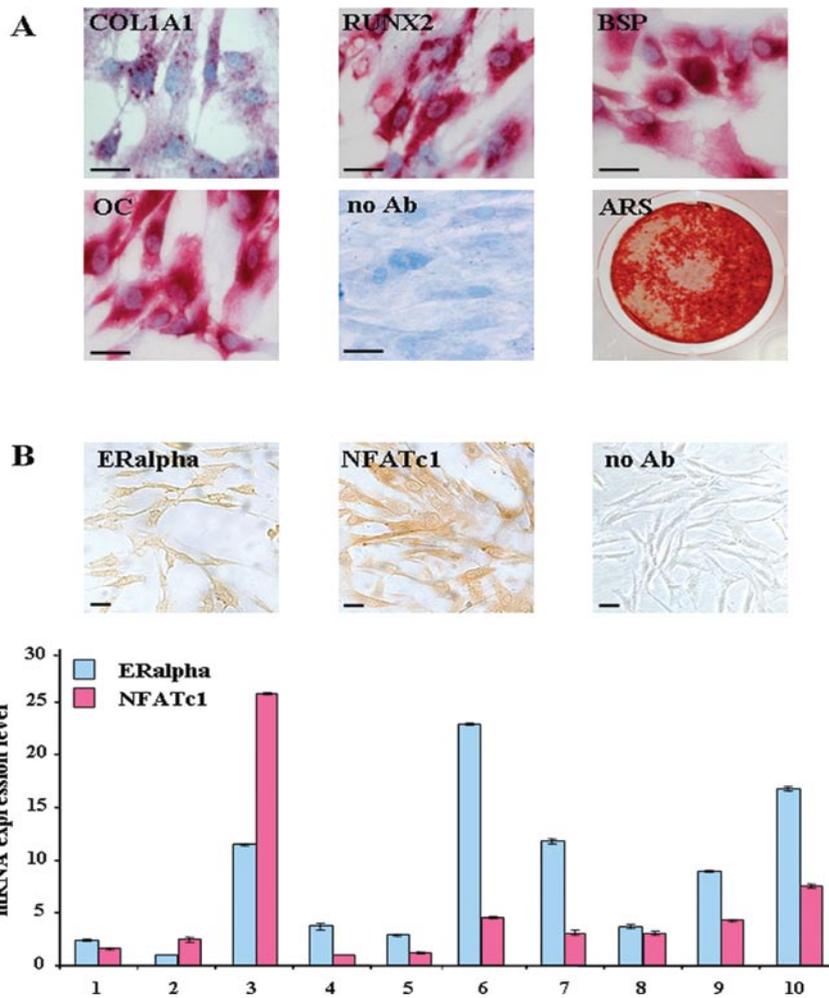
Figure 1. Phenotypical characterization of hOBs. (A) Ten hOB samples were subjected to immunocytochemical analysis for collagen type 1 (Col1A1), Runx2, bone sialoprotein (BSP) and osteocalcin (OC). Mineral matrix deposition was analyzed by Alizarin Red staining (ARS). The staining shows the local expression of the markers analyzed in a representative sample (x40 magnification; bar denotes 20 μm). (B) The same samples were analyzed for ERα and NFATc1 by immunocytochemistry [a representative sample and a negative control (no Ab) are shown, x20 magnification; bar denotes, 20 μm], and quantitative RT-PCR. The mRNA expression levels were normalized on the basis of GAPDH expression and the ΔΔCt method was used to compare gene expression data; the means and standard error of the means (SEM) were calculated.
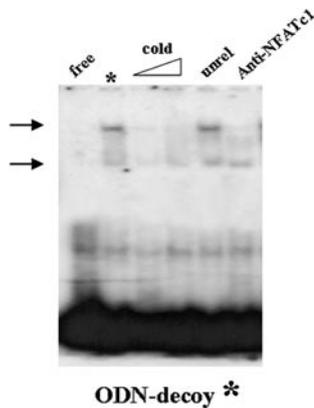
Figure 2. A gel shift experiment was performed with nuclear extracts from Jurkat cells in the presence of ^{32}P-labeled ODN-decoy oligonucleotide against NFATc1. Protein DNA complexes were resolved from the free probe on a non-denaturing 6% polyacrylamide gel. A representative gel is shown. The ^{32}P-labeled oligonucleotide was incubated alone (free), in combination with 10 μg of nuclear extracts (*), and in the presence of 50- and 100-fold molar excess of specific unlabeled oligonucleotide (cold), or unrelated oligonucleotide (unrel). An antibody against NFATc1 (4 μg) was also incorporated into the binding reaction (anti-NFATc1). The main retarded complexes are indicated by arrows.

oligonucleotide, demonstrating the specificity of these DNA protein interactions. The upper band was abolished when an anti-NFATc1 antibody was added, confirming that NFATc1 was present in this retarded complex.

Analysis of NFAT activity. The functionality of the endogenous NFAT proteins in our experimental model were analyzed by monitoring the transcriptional activity on a luciferase reporter construct driven by three copies of an NFAT response element (pNFAT-TA-luc). As shown in Fig. 3A, NFAT-driven luciferase activity in all transfected hOBs cultured in the presence of ionomycin and PMA, was remarkable and positively correlated with calcium levels, demonstrating the ability of endogenous-activated NFAT to bind the specific *cis*-acting elements in these cells. The presence of NFAT-driven luciferase activity was also found in hOBs cultured in medium without ionomycin and PMA in the absence of exogenous calcium (Fig. 3B), suggesting that the intracellular calcium may be sufficient to activate NFAT proteins. The calcium mobilization ability of hOBs was demonstrated through accurate measurements of
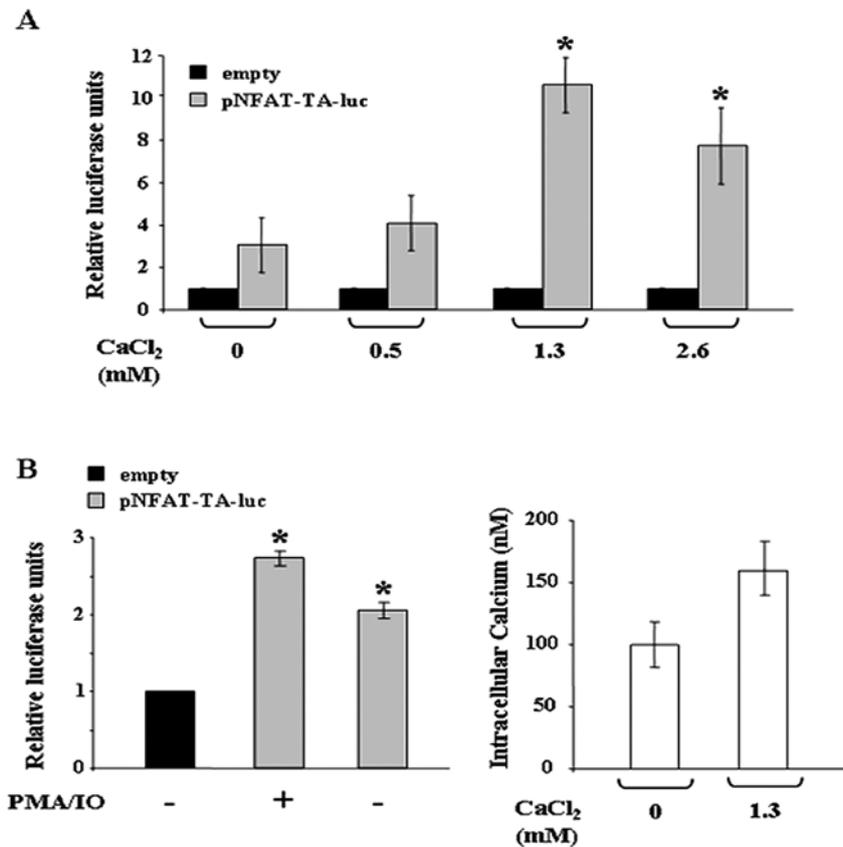
Figure 3. NFAT reporter gene assay. (A) Five different hOB samples were transiently transfected with pNFAT-TA-luc or empty vector in four different culture medium conditions, 0, 0.5, 1.3 and 2.6 mM $CaCl_2$, in the presence of 0.5 μM ionomycin and 100 μg/ml PMA. The results were normalized to the protein concentration and β-galactosidase activity for the transfection efficiency and represent the average of five different experiments performed in duplicate. The data are expressed as the mean ± SEM and indicated as fold-inductions over the empty vector. *P<0.05 vs. 0 mM. (B) The same protocol was applied for hOBs cultured in α-MEM 10% FBS, in the presence (+) or in the absence (-) of 0.5 μM ionomycin (IO) and 100 μg/ml PMA treatment before pNFAT-TA-luc transient transfection. *P<0.05 vs. empty vector. The basal intracellular Ca^{2+} concentrations (nM) in hOBs was measured (diagram at the right). Cells were grown in the absence (0) and in the presence of 1.3 mM $CaCl_2$ medium on coverslips, then loaded with Fura-2-AM and analyzed as reported in Materials and methods. The average and the SEM of all recorded data are reported.

intracellular calcium release in the absence or presence of exogenous calcium in Fura-2-AM-loaded cells (Fig. 3B).

The effect of the decoy against NFATc1. The cells were plated at 60% confluence, and after 24 h a first administration of NFATc1 decoy ODN or scrambled ODN was performed. A second administration was applied on day 4 before cell harvesting on day 7 for the molecular analysis (scheme at the top of Fig. 4).

On the basis of the results reported in Fig. 3, we performed decoy experiments in hOBs cultured in α-MEM medium and left untreated with ionomycin and PMA. This allowed the estimation of the decoy effect at basal conditions in the absence of the calcium mobilization induced by the phorbol ester, PMA, and the ionomycin calcium ionophore.

We first examined the efficiency of hOBs treatment with the NFATc1 decoy ODN by analyzing the expression of the ERα gene by quantitative RT-PCR. In fact, we previously demonstrated that in different cell lines this decoy treatment induced a significative increase of ERα gene expression because NFATc1 acts as negative regulator of ERα gene transcription. As shown in Fig. 4, decoy against NFATc1, but not scrambled oligonucleotide, increased the expression of ERα confirming the specific role of NFATc1. We then

turned our attention to the expression pattern of Runx2, the master regulator of osteoblastogenesis that is not transcriptionally regulated by NFATc1 (5), and of ColXV, a recently investigated novel marker of osteoblast differentiation whose expression is regulated by exogenous calcium addition (30). The data are reported in Fig. 4. As expected, the functional inactivation of NFATc1 did not affect Runx2 expression indicating the specificity of the decoy effect. On the contrary, a significant increase of ColXV expression was found in the decoy-treated cells. Using a program for predicting transcription factor binding sites (TFSEARCH, www.cbrc.jp/research/db/TFSEARCH.html), we found that 5' regulatory regions of all three genes analyzed contain potential binding sites (5'-GGAAA-3'), for NFATc1. However, only ERα and ColXV but not Runx2 seem to be critical downstream targets of NFATc1.

NFATc1 interacts in vivo with the promoter of the ColXV gene. We then focused our attention on the poorly studied ColXV gene promoter by investigating *in vivo* NFATc1 recruitment by ChIP binding assays (Fig. 5). To this aim, we employed four hOB samples that were treated with PMA/ionomycin to ensure full NFAT induction. hOBs were exposed to formaldehyde to cross-link proteins and
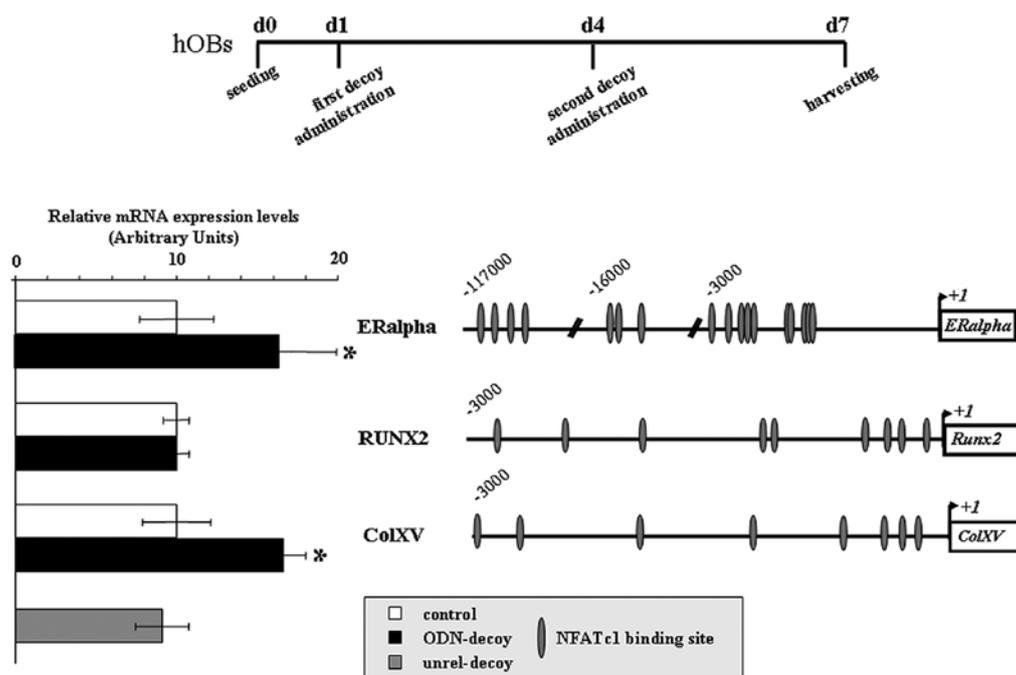
Figure 4. Analysis of ERα, Runx2 and ColXV expression by quantitative RT-PCR after decoy against NFATc1. Ten hOB samples were cultured up to 7 days with 1 µg/ml of ODN-decoy administered in two different steps, as indicated in the scheme above. The expression levels were normalized on the basis of GAPDH expression and results of the experiments are reported as relative mRNA expression levels over the control cells. Arbitrary units are used for the attribution of the fold inductions. The ΔΔCt method was used to compare gene expression data; means ± standard error of the mean (SEM) was calculated. The effect of unrelated oligonucleotides was also reported. *P<0.05. The localization of the potential binding sites for NFATc1 in the 5' regulatory regions of human ERα, Runx2 and ColXV genes are reported.
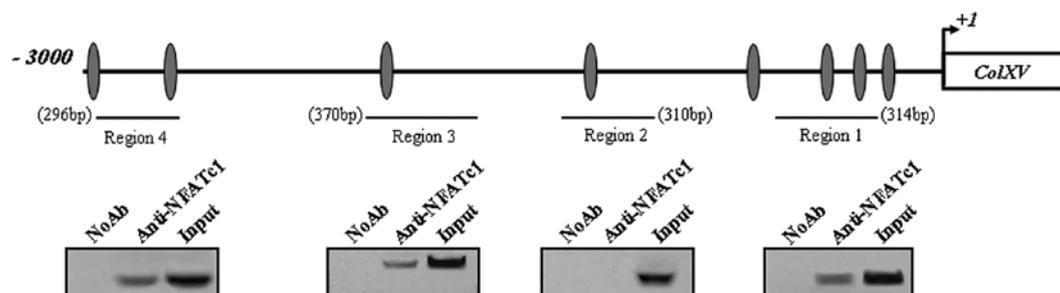
Figure 5. NFATc1 binds the human ColXV promoter *in vivo*. The ColXV promoter region under investigation is reported (+1 to -3000). The positions of the putative NFATc1 consensus binding sites are enclosed by gray ovals. Recruitment of the NFATc1 transcription factor to the human ColXV promoter was demonstrated by *in vivo* ChIP binding assays. Soluble chromatin was prepared from hOBs and immunoprecipitated with the specific antibody against NFATc1. The association of the transcription factor on the regions 1-4 of the human ColXV promoter was analyzed by PCR using primer pairs spanning the reported regions of ColXV promoter. The specific molecular weights of PCR fragments are shown in parentheses. No Ab, negative control; Input, a positive control using the starting material (0.2%) prior to the immunoprecipitation. Representative agarose gels are shown.

DNA, and sonicated to fragment the chromatin. A specific antibody against NFATc1 was used to immunoprecipitate the protein DNA complexes. The presence of the promoter-specific DNA region before immunoprecipitation was confirmed by PCR (Input). After immunoprecipitation, DNA was extracted from the beads and used as a template to generate specific PCR products spanning the putative NFATc1 binding sites from -3000 bp to +1 bp in the promoter of the ColXV gene. NFATc1 recruitment was assessed at the different promoter regions by using specific sets of primers (Table I). As shown in Fig. 5, NFATc1 occupancy was detected at the regions 1, 3 and 4, but not at region 2. This demonstrated that ColXV is a specific NFATc1 target gene in human osteoblasts.

Discussion

To our knowledge, the results of the present study establish, for the first time, that removal of the NFATc1 transcription factor by a decoy approach in hOBs induces the expression of ERα and ColXV that are positively correlated with osteoblastic differentiation. These findings have a double meaning: on the one hand, they reinforce the role of this factor in the transcriptional program of osteoblasts, on the other they contribute important information on ColXV which only recently has been proposed as an osteoblastic marker (30). In addition, the present study in agreement with several published reports (17,18,21-23,31-33) supports the potential utility of the decoy strategy to treat specific diseases. It is

well known that synthetic double-stranded ODN as decoy cis elements block the binding of nuclear factors to promoter regions of targeted genes, resulting in the modulation of gene expression. The interest in this approach is supported by studies correlating the altered activity of transcription factors with specific diseases (34,35).

NFATc1 is referred to as the key transcription factor in osteoclastogenesis (36); however, several lines of evidence indicate that it regulates bone mass by functioning not only in osteoclasts but also in osteoblasts (6,7). In fact, it has recently been shown that NFATc1 contributes to the regulation of osteoblastic specific genes including Osterix (8) and Fra-2 (37), modulates the activity of ligands for osteoclast-associated receptors such as OSCAR (38) primarily produced by osteoblasts, and is involved in the transcriptional regulation of the ERα gene in the SaOS-2 osteosarcoma cell line (24).

Here we have demonstrated that hOBs efficiently respond to NFATc1 decoy treatment in terms of an increase in ERα gene expression. This evidence may be correlated with an increased potential for cell sensitivity to estrogens and consequently an improvement in osteoblast differentiation. Actually, the positive effect of estrogen on bone metabolism is well known (39). These considerations substantiate the interest in NFATc1 as a promising therapeutic target that could be employed for the development of anabolic agents for the treatment of bone loss. This conclusion is supported by the evidence that decoy-treated hOBs showed a significant increase in ColXV mRNA levels. Interestingly, the expression of ColXV, was recently demonstrated in human osteoblasts both *in vitro* and *in vivo* (30).

In addition, a direct involvement of this molecule in the mineralization process of mesenchymal stem cells induced to osteogenesis and in bone tissue remodelling has been suggested (30). 5'-Regulatory sequences of the ColXV gene promoter have been only partially characterized (40) and, until now, it is known that its expression is modulated by exogenous calcium induction. Our results demonstrate, for the first time, a specific *in vivo* interaction of NFATc1 with specific sequences of the ColXV gene promoter suggesting a direct involvement of NFATc1 during osteoblastogenesis. Therefore, even if further studies are required to investigate the molecular mechanism of action of NFATc1, the novelty of our findings provide insight into the regulation of the ColXV gene transcription, ascribing to NFATc1 a role of negative transcription factor.

Regarding the therapeutic potential of the decoy experimental approach for bone diseases, it is worth noting that NFATc1 is utilized in both bone-forming and bone-resorbing cells. Therefore, the decoy against NFATc1 in the bone microenviroment may be effective for treating bone diseases exerting positive and negative action on osteoblasts and osteoclasts, respectively. Based on these considerations, the decoy against NFATc1, working upstream of the transcription machinery, may prove to be a gene-targeted tool for treating skeletal disorders caused by excessive bone resorption, including osteoporosis and destructive bone metastasis. In addition, considering that NFATc1 cooperates with other transcription factors, the here described approach may help to shed light on upstream signal-transduction networks that may lead to altered transcription programs in bone cells. In particular, after translocation to the nucleus and DNA binding, NFATc1 protein facilitates the binding of AP-1 to NFAT-AP-1 binding sites whereupon the AP-1 components can mediate the induction of transcription (13). As AP-1 family members play a key role in the regulation of bone-specific genes and consequently in osteoblast differentiation (41), the interaction with NFATc1 is particularly relevant in the bone microenvironment. In this regard, it will be useful to further investigate the recruitment of the AP-1 family members at the ColXV gene promoter by ChIP analysis.

In conclusion, to the best of our knowledge this is the first time that TFD has been successful in hOBs allowing the investigation of the role of NFATc1 in an experimental model that compared to cell lines more closely resembles an *in vivo* model. In addition, our results provide new insight into the regulation of osteoblastic markers and strengthen the therapeutic potential of the decoy approach to treat skeletal disorders by properly manipulating NFATc1-mediated cellular events.

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