0026-895X/07/7106-1457–1462$20.00
MOLECULAR PHARMACOLOGY
Vol. 71, No. 6
34561/3214275
Mol Pharmacol 71:1457–1462, 2007
Printed in U.S.A.

ACCELERATED COMMUNICATION

Induction of Estrogen Receptor α Expression with Decoy Oligonucleotide Targeted to NFATc1 Binding Sites in Osteoblasts

Letizia Penolazzi, Margherita Zennaro, Elisabetta Lambertini, Elisa Tavanti, Elena Torreggiani, Roberto Gambari, and Roberta Piva

Department of Biochemistry and Molecular Biology, Molecular Biology Section, Ferrara University, Ferrara, Italy

Received January 29, 2007; accepted March 27, 2007

ABSTRACT

The nuclear factor of activated T cell cytoplasmic 1 (NFATc1) is a member of the NFAT family and is strictly implicated in the growth and development of bone. Most studies have focused on the effects of NFATc1 activation on osteoclastogenesis. On the contrary, the specific roles of NFAT in osteoblast differentiation are not well understood and, in some instances, reports of its role are contradictory. In the present study, we demonstrated that NFATc1 was involved in the transcriptional regulation of human estrogen receptor α (*ER*α) gene in SaOS-2 osteoblastic like cells. NFATc1 was specifically recruited "in vivo" at C and F distal promoters of ERα gene. In addition, it is here identified as the negative transcription factor removed by the RA4-3′decoy oligonucleotide able to induce ERα expression in osteoblasts. Ca^{2+}/calcineurin-NFAT-mediated signaling pathways and ERα-dependent signals are involved in diverse cellular reactions by regulating gene expression under both physiological and pathological conditions. Therefore, our data might be useful for proper manipulation of NFATc1- and ERα- mediated cellular reactions in different bone disorders, such as osteoporosis.

NFATc1 (also known as $NFAT_2$) belongs to the nuclear factor of activated T cells family that is composed of five proteins related to the Rel/NFB family (NFATc1-c4 and NFAT5) (Hogan et al., 2003). These proteins were initially identified as transcription factors that mediate Ca^{2+}/calmodulin- and calcineurin (Cn)-dependent transcription of many cytokines involved in T-cell activation (Rao et al., 1997). NFATc1 is referred to as the master transcription factor for differentiation of osteoclasts (OCs), the cells responsible for bone resorption (Takayanagi et al., 2002; Day et al., 2005; Kim et al., 2005; Asagiri and Takayanagi, 2007). Nevertheless, recent studies suggest that it also has an important role on the transcriptional program of osteoblasts (OBs), the bone-forming cells that play a central role also in modulating osteoclastogenesis (Koga et al., 2005; Zayzafoon, 2005; Winslow et al., 2006; Yeo et al., 2006). In fact, several lines of evidence indicate that NFATc1 is not only a transcriptional regulator of a number of osteoclast-specific genes, such as cathepsin K, calcitonin receptor, β_3 integrin, TRAP, and ACP5 (Ikeda et al., 2004; Matsumoto et al., 2004; Matsuo et al., 2004; Day et al., 2005; Kim et al., 2005; Crotti et al., 2006) but also contributes to the transcriptional control of genes expressed in OBs including Osterix (Koga et al., 2005) and Fra-2 (Zayzafoon, 2005) and modulates the activity of ligands for osteoclast-associated receptors such as OSCAR (Kim et al., 2005; Zayzafoon, 2006) primarily produced by osteoblasts.

This research was supported by grants from Ministero dell'Istruzione, dell'Università e della Ricerca Fondo per gli Investimenti della Ricerca di Base-2005, the Associazione Italiana Ricerca contro il Cancro (AIRC), and the Fondazione Cassa di Risparmio di Ferrara. E.L. is a recipient of a fellowship from the Fondazione Cassa di Risparmio di Cento.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.107.034561.

ABBREVIATIONS: NFAT, nuclear factor of activated T cells; Cn, calcineurin; OB, osteoblast; OC, osteoclast; ER, estrogen receptor; ERE, estrogen-responsive elements; ODN, oligodeoxynucleotide; PCR, polymerase chain reaction; ChIP, chromatin immunoprecipitation; PBS, phosphate-buffered saline; IP(3)K, inositol 1,4,5-trisphosphate 3-kinase; PMA, phorbol 12-myristate 13-acetate; RT-PCR, reverse transcriptase-polymerase chain reaction.

The activation of these target genes involved in osteoblast and osteoclast differentiation and function is also triggered by steroid hormones. In particular, it is finely regulated by estrogen, which is involved in more than one step during the bone remodeling cycle (Oursler, 1998; Manolagas et al., 2002), by binding specific intracellular receptors, ERα and ERβ, that act as transcription factors for estrogen responsive genes (Nilsson et al., 2001; Knox et al., 2006). In particular, ERα positively regulates OB differentiation and negatively affects osteoclastogenesis, inducing bone formation (Jessop et al., 2004; Stossi et al., 2004). Interestingly, the Ca^{2+}/calmodulin/Cn system that regulates NFAT is also involved in ERα function (Li et al., 2005). In particular, calmodulin has been shown to increase the affinity of ERα for the estrogen-responsive elements and to reduce ERα degradation (Li et al., 2006) thus modulating the levels of ERα that are very critical in the control of proliferation, differentiation, and survival of bone cells under both normal and pathological conditions (Deroo and Korach, 2006).

It is well known in osteopenic diseases, such as osteoporosis, that a failure to harmonize osteoclast and osteoblast functions is due to a down-regulation or complete loss of this receptor with a consequent reduced control of bone mass and architecture (Deroo and Korach, 2006; Lerner, 2006).

We have demonstrated that it is possible to obtain reactivation or increase of human ERα expression by a specific decoy approach that induces apoptosis in osteoclasts, but not in osteoblasts, which, conversely, increase their differentiation markers (Piva et al., 2005), in agreement with the opposite role of estrogen in these two cellular types (Oursler, 1998; Manolagas et al., 2002; Syed and Khosla, 2005).

The decoy oligodeoxynucleotide technology involves synthetic double-stranded ODN containing a cis-element with high affinity for a target transcription factor (TF) that is able to bind the TF after being introduced into target cells (Mann and Dzau, 2000; Gao et al., 2006). This attenuates the authentic *cis*-trans interactions, leading to removal of *trans* factors from the endogenous *cis* elements with subsequent specific modulation of gene expression. Therefore, the decoy approach is a powerful tool to modulate transcription of a target gene and directly study the transcriptional control of a gene of interest (Borgatti et al., 2005; Lambertini et al., 2005; Piva et al., 2005).

ERα increase was obtained by interfering with the activity of an unidentified negative transcription factor binding to a cis element belonging to promoter C of the ERα gene (Kos et al., 2001; Lambertini et al., 2005). It is noteworthy that the sequence of the oligonucleotide used as decoy molecule (named RA4-3′) contains a consensus sequence for NFAT (TGAAAA) (Piva et al., 2005). Considering our interest in understanding the molecular mechanisms by which ERα affects bone tissue-specific gene expression, we explore the relationship between NFAT and ERα, investigating whether NFATc1 is involved in mediating the decoy effect and consequently the transcriptional regulation of ERα gene.

Materials and Methods

Cell Cultures and Transient Transfections. SaOS-2 cells were maintained in α-minimal essential medium (Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (CELBIO EuroClone, Milan, Italy) in a humidified incubator at 37°C with a 5% of CO_2. For transfection experiments with pNFAT-TA-Luc or pGL2 basic-Vit3-ERE Luc plasmid vectors, the cells were plated in six-well plates, maintained in phenol-red free α-minimal essential medium + 10% charcoal stripped fetal bovine serum, and transiently transfected using Lipofectamine reagent (Invitrogen, Carlsbad, CA) with 5 μg of plasmid vector. After overnight incubation, the medium was removed and fresh medium was added. After a further 24 h, cells were harvested, lysed, and assayed for luciferase activity. The luciferase activity was normalized by total protein amount and by β-galactosidase values resulting from cotransfection of 0.25 mg of pCMV-Sport-βgal (Invitrogen). β-Galactosidase activity was measured at 420 nm using the NpGal (*O*-nitrophenyl β-D-galactopyranoside) substrate.

RT-PCR Analysis. For mRNA analysis, total cellular RNA was extracted using Total RNA Isolation System (Promega, Madison, WI). Two Micrograms of total RNA were reverse-transcribed with the ImProm-II RT System (Promega). mRNA for human ERα were quantified by real-time PCR using TaqMan probe: 5′-5-carboxyfluorescein-ATGATGAAAGGTGGGATACGAAAAG-5-carboxytetramethylrhodamine-3′ and the ABI Prism 7700 system (Applied Biosystems, Rotkreuz, Switzerland). After a 10-min preincubation at 95°C, runs corresponded to 40 cycles of 15 s at 95°C (denaturation) and 1 min at 60°C (annealing/elongation). The mRNA levels of target genes were corrected for glyceraldehyde-3-phosphate dehydrogenase mRNA levels.

Chromatin Immunoprecipitation Assay. The ChIP assay was carried out using the ChIP kit from Upstate Biotechnology, Inc. (Lake Placid, NY). The cells were cross-linked with 1% formaldehyde for 10 min at 37°C, washed in ice-cold PBS, and resuspended in SDS lysis buffer for 10 minutes 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 5 μg of anti-NFATc1 (Santa Cruz Biotechnology, Santa Cruz, CA) 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 five 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, and 150 mM NaCl), high-salt wash buffer (0.1% SDS,1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 500 mM NaCl), LiCl wash buffer (0.25 mM LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1 mM EDTA, and 10 mM Tris, pH 8.1) and Tris-EDTA buffer. The immunocomplexes were eluted two times by adding a 250-μl aliquot of a freshly prepared solution of 1% SDS and 0.1 M $NaHCO_3$, and the cross-linking reactions were reversed by incubation at 65°C for 4 h. Furthermore, the samples were digested with proteinase K (10 mg/ml) at 42°C for 1 h, DNA was recovered by phenol/chloroform extractions, ethanol was precipitated using 1 μl of 20 mg/ml glycogen as carrier and resuspended in sterile water. For PCR analysis, aliquots of chromatin before immunoprecipitation were saved (Input). Nested PCR was performed to analyze the presence of DNA precipitated by specific antibodies by using the following primers: forward (5′-TGAGATTTTCCAATCCTAGT-3′ and reverse (5′-ACTGTCTTCTTATGCTATAGAA-3′) to detect a DNA segment located at −117,254 and −117,040 within the F promoter; forward (5′-ACTGGTCTAGAGCATGGGTGG-3′) and reverse (5′-CCAGTATTGCAGTGTTTGAGCA-3′) to detect a DNA segment located at −3277 and −3117 within the C promoter; and forward (5′-GAGTTGTGCCTGGAGTGATG-3′) and reverse (5′-GGGCCTCCAACTTTAAGTACTG-3′) to detect a DNA segment located at −129 and −9 within the A promoter.

Each PCR reaction was performed with 10 μl of the bound DNA fraction or 2 μl of the input. The PCR was performed as follows: preincubation at 95°C for 10 min, 30 cycles of 1-min denaturation at 95°C, 1 min at the optimal annealing temperature for each primer pair, and 1 min at 72°C, with one final incubation at 72°C for 5 min. For Re-ChIP assay, the complexes were eluted from the primary immunoprecipitation by incubation with 10 mM dithiothreitol at

37°C 30 min and diluted 1:40 in buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, and 20 mM Tris-HCl, pH 8.1) followed by reimmunoprecipitation with a different relevant antibody. Subsequent steps of ChIP reimmunoprecipitations were as for the initial immunoprecipitations. No-antibody control was included in each experiment.

Western Blotting. For Western blotting analysis, the cells were washed twice with ice-cold PBS, and cell lysates were prepared as described previously (Dignam et al., 1983). Each sample (25 μg) was then electrophoresed on a 12% SDS-polyacrylamide gel. The proteins were then transferred onto an Immobilon-P polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA). After blocking with Dulbecco's PBS, 0.05% Tween 20, and 5% dried milk, the membrane was incubated with polyclonal anti-NFATc1 (H-110. 1:1000; Santa Cruz Biotechnology), monoclonal anti-ERα (clone 60C. 1:2000; Upstate Biotechnology, Inc., Lake Placid, NY), and polyclonal anti-IP(3)-Kinase (p85, 1:3000; Upstate Biotechnology Inc.). After washing with PBS-Tween, the membranes were incubated with peroxidase-conjugated anti-rabbit antibody (1:2000; DakoCytomation Denmark A/S, Glostrup, Denmark) in 5% nonfat milk. Immunocomplexes were detected using Supersignal West Femto Substrate (Pierce, Rockford, IL). Anti-IP(3)K was used to confirm equal protein loading.

Statistical Analysis. Data are presented as the mean ± S.E.M. from at least three independent experiments. 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 and Discussion

NFATc1 Bound to C and F Promoters of ERα Gene. We have demonstrated previously that ERα expression increases in osteoblasts by interfering with the activity of negative transcription factors using a decoy oligonucleotide (referred as RA4-3′) mimicking a region of distal promoter C of the ERα gene (Piva et al., 2005). This sequence contains the NFAT binding site 5′-TGAAAA-3′. We speculated, based on cross-competitions in electrophoretic mobility shift assays and RT-PCR analysis, that 5′-TGAAAA-3′ may participate in the transcriptional regulation of ERα gene interfering with the more distal F promoter, which is the only one active in bone. The experiments reported here were designed to verify this hypothesis. It is noteworthy that the homology search (Fig. 1) indicates a strong nucleotide similarity between the sequence used as decoy molecule and a region at −117,223/−117,175 belonging to the F promoter.

To investigate the binding of NFATc1 to the C and F promoters of ERα gene in vivo, we performed ChIP. To this aim, after NFAT activation with 0.5 μM ionomycin and 100 μg/ml PMA, SaOS-2 osteoblast-like cells were exposed to formaldehyde to cross-link proteins and DNA and sonicated to fragment the chromatin. After immunoprecipitation with NFATc1 antibody, DNA was extracted from the beads and the binding of NFATc1 protein to C and F promoters was then determined by PCR with two oligonucleotides pairs generating the 161- and 215-bp PCR products, respectively. As shown in the Fig. 1, A and B, both C and F promoters were able to recruit NFATc1, whereas A promoter, used as negative control, was not (Fig. 1C). Likewise, genomic DNA not subjected to immunoprecipitation was amplified (input). In contrast, no amplified product was obtained when the ERα-negative breast cancer MDA-MB-231 cells were used (data not shown). Likewise, no amplification was obtained in the samples collected with beads alone (−Ab). This result pro-

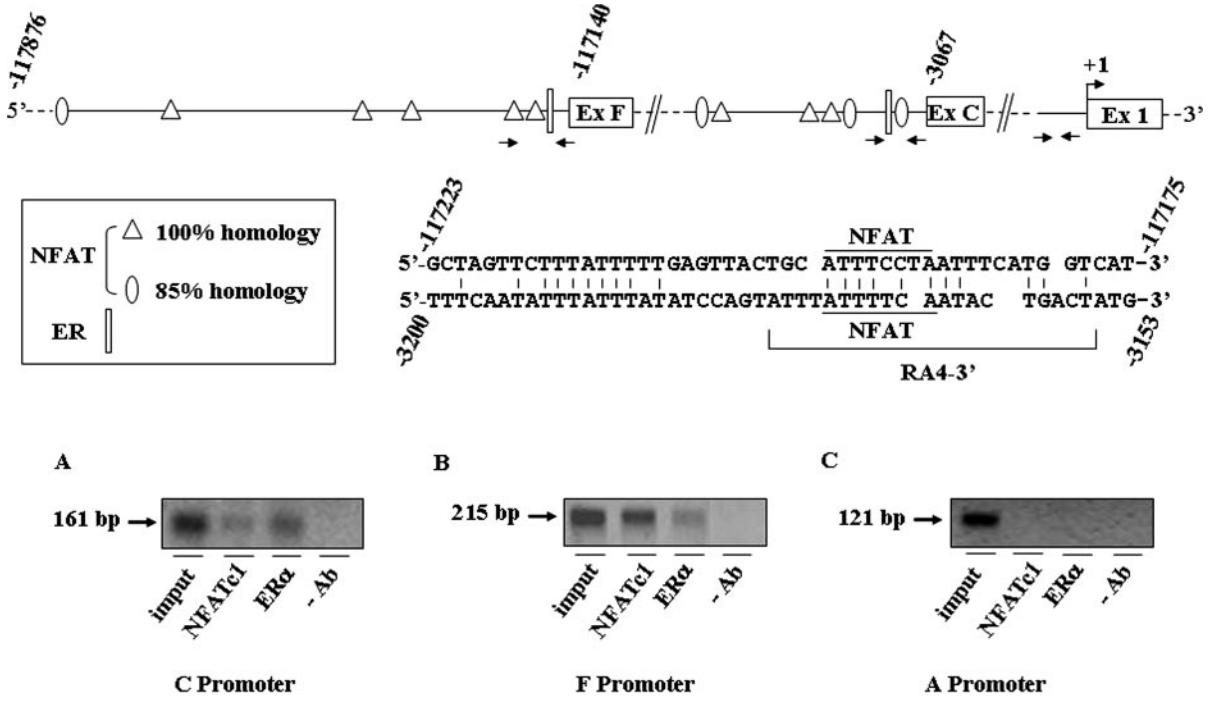

Fig. 1. Recruitment of NFATc1 to the C and F promoters in vivo. A–C, soluble chromatin was prepared from SaOS-2 cells and immunoprecipitated with NFATc1 and ERα antibodies. PCR was performed to detect the coprecipitated C, F, and A promoters using specific primers whose positions are indicated with arrows in the scheme at the top of the figure. Input are positive PCR controls performed on genomic DNA, −Ab are negative controls for ChIP. At the top, the location of human ERα gene regulatory regions under investigation is reported. The numbers are the nucleotide positions relative to the transcription initiation site (+1). The consensus binding sites of NFAT and ER found in the A, C, and F promoters using the Transfac database and the TF search (http://www.cbrc.jp/research/db/TFSEARCH.html) are indicated. The sequence homology between C and F promoters and the position of RA4-3′ oligonucleotide decoy molecule are also reported.

vides direct evidence that NFATc1 forms a complex with C and F promoters of ERα gene in vivo, suggesting that ERα is a direct target of the transcription factor NFATc1.

The same chromatin regions of C and F promoters recruit ERα protein as demonstrated by PCR products obtained after immunoprecipitation also with ERα antibody. This is in agreement with the presence of estrogen-responsive elements in these promoters (see the scheme at the top of Fig. 1) and previous data regarding autoregulation of ERα gene (Denger et al., 2001). In addition, considering that a member of the NFAT family, NFAT3, has recently been found to act as a novel ER coactivator in breast cancer cells (Zhang et al., 2007), the possibility that ERα is a binding partner for NFATc1 in osteoblasts will be investigated with further studies.

Activation of ERα Gene Expression by Transfection of the NFAT Consensus Decoy. To determine the role of NFATc1 in the above findings, SaOS-2 cells were transfected with the NFAT consensus oligonucleotide mimicking distal NFAT site in the human IL-2 gene promoter (Kaminuma et al., 2001), and its decoy activity was compared with RA4-3′ oligonucleotide. The decoy effect was evaluated on ERα gene expression by quantitative RT-PCR at days 1 and 6 with decoy administration every 48 h, in the presence of 17 β-estradiol to allow complete functionality of ERα protein. As shown in the left side of Fig. 2, the increase of ERα mRNA levels was obtained with both the decoy molecules and was time-dependent. The fact that the RA4-3′ decoy molecule retains an higher ability in increasing ERα expression may be explained with its perfect homology with the regulatory region inside the C promoter. In contrast, scramble oligonucleotide was completely unable to modify ERα mRNA expression. These results strongly indicate that the functional inactivation of NFATc1 due to its interaction with the excess of NFAT consensus or RA4-3′ oligonucleotides, instead of binding to the endogenous regulatory motifs of the genes, is associated with increase of ERα expression in SaOS-2 cells.

These data were confirmed by Western blot analysis. As reported in Fig. 2, the densitometric analysis revealed an increase in ERα protein levels in NFAT consensus and RA4-3′ 6-day, decoy-treated cells (1.92- and 1.86-fold increases, respectively), compared with control untreated cells. Therefore, we may conclude that NFATc1 is responsible for a negative control on human ERα gene expression.

Effect of RA4-3′ Decoy on NFAT-Mediated Transcriptional Activity. The possibility that NFATc1 is involved in the decoy effect was then analyzed monitoring the transcriptional activity on pNFAT-TA-Luc plasmid vector. pNFAT-TA-Luc contains three tandem copies of the NFAT-consensus sequence upstream of the minimal TA promotor. Significant induction of luciferase activity was observed in SaOS-2 cells transfected with pNFAT-TA-Luc (Fig. 3, left), demonstrating the binding ability of endogenous activated NFAT to the *cis*-acting enhancer element. For analyzing the regulatory relationship between NFATc1 and ERα, the pNFAT-TA-Luc–transfected cells were also treated with RA4-3′ decoy molecule. The decoy RA4-3′ ODN is able to block the effect of endogenous NFATc1 on the promoter activity of pNFAT-TA-Luc, whereas a scramble ODN had no effect, suggesting that NFATc1 is effectively sequestrated by RA4-3′ sequence and is required in mediating the decoy effect. Because the expression of ERα is potentiated after RA4-3′ treatment, the functional success of the decoy was monitored by assaying the transactivation of a transfected estrogen response element (ERE)-regulated reporter gene (Vit3-ERE) into the decoy-treated cells. In the cells exposed to decoy transfection, a significant increase of Luc activity was obtained, confirming that the induced ERα protein was able to bind the ERE sequences of the reporter vector mediating luciferase gene expression.

The nuclear content of NFATc1 and ERα was analyzed by Western blotting. As shown in Fig. 3, the levels of these two proteins are appreciable in our experimental model and, consistent with luciferase data, the activation of NFAT proteins with PMA and ionomycin (Hogan et al., 2003) increased the nuclear levels of NFATc1 but decreased endogenous ERα expression (a 50% decrease).

In contrast, no marked changes in relative luciferase activity were detected in MDA-MB-231 breast cancer cells under the same conditions. In fact, when MDA-MB-231 cells, originally ERα-negative, become ERα-positive after RA4-3′ decoy treatment (see the Western blot analysis), Vit3-ERE activity strongly increased (Lambertini et al., 2005), but the decoy effect seems not to be regulated by NFATc1, as dem-

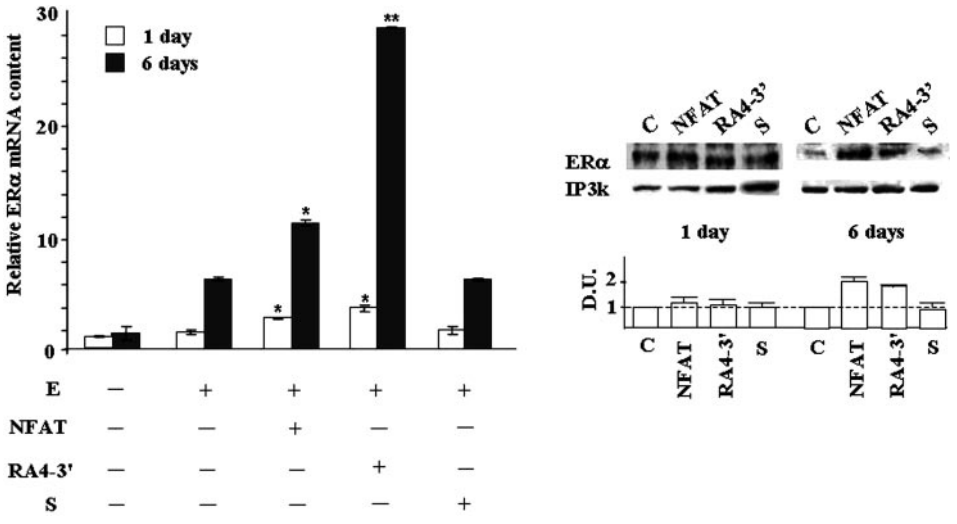

Fig. 2. Expression of ERα during decoy treatment. Analysis of mRNA and protein levels. SaOS-2 cells were cultured with (+) or without (−) 17-β-estradiol (E), in presence of 2.5 μg/ml unrelated scramble DNA (S), NFAT consensus, or RA4-3′ decoy oligonucleotides. Total RNA was collected at the indicated days, and quantitative TaqMan RT-PCR was performed. The values were normalized using glyceraldehyde-3-phosphate dehydrogenase as an internal control and results are representative of three independent experiments carried out in triplicate. S.E.M. was calculated. *, $P < 0.05$; **, $P < 0.01$ with respect to controls (+E). Western blot analysis with antibody against ERα is reported on the right. The cells treated as for RNA analysis were harvested and analyzed by Western blotting for the detection of ERα. The intensity of the bands was quantified by densitometric analysis of three different experiments and normalized using IP(3)K as a control. D.U. = ERα/IP(3)K densitometric units.

onstrated by the unchanged promoter activity of pNFAT-TA-Luc in these cells (Fig. 3). This indicates that even if NFATc1 is expressed in MDA-MB-231 cells, as results from Western blot analysis, nevertheless it is not involved in mediating the decoy effect in this cellular type. This is consistent with the effective specificity of the decoy treatment and with the recognition that the NFATc1 pathway affects bone cells rather than breast tissue (Stern, 2006).

Overall, our decoy approach in combination with ChIP and reporter gene assays open up new opportunities for the understanding the complex ERα gene regulation, providing the identification of NFATc1 as negative regulator of ERα gene transcription in SaOS-2 osteoblastic cells. Given the important role that estrogen plays in both bone health and disease (Oursler, 1998; Manolagas et al., 2002), the understanding of how ERα expression levels is regulated could be useful in addressing physiological and pathological conditions.

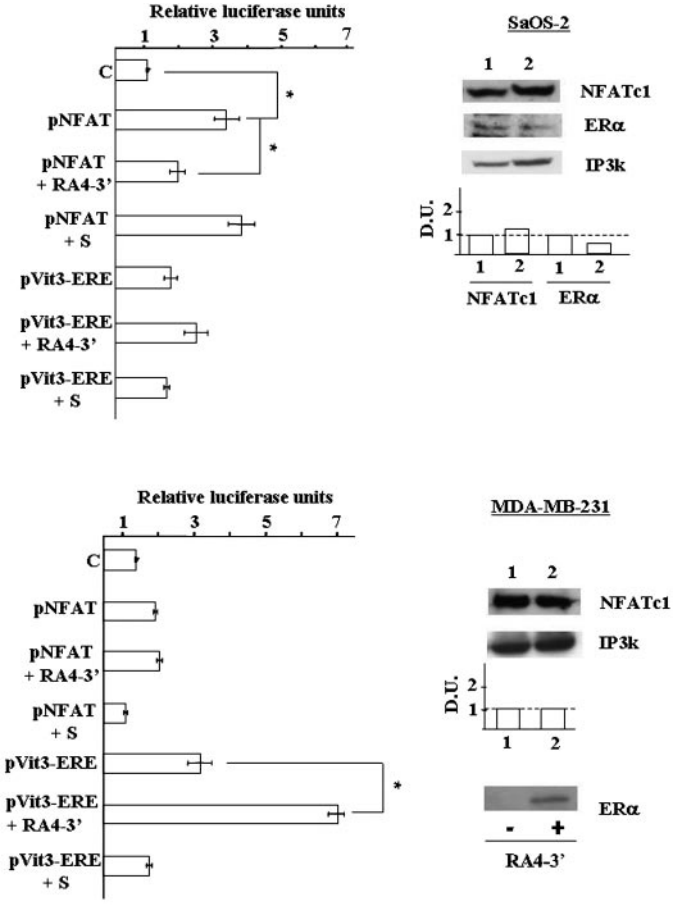

Fig. 3. NFAT reporter gene assay. SaOS-2 and MDA-MB-231 cells were transiently transfected with the pNFAT-TA-Luc or pVIT3-ERE-Luc reporter vectors. The cells were cultured in presence of 2.5 μg/ml unrelated scramble DNA (S) or RA4-3′ decoy oligonucleotides. Luciferase activity is expressed relative to control (C). The results were normalized with protein concentration and β-gal activity for transfection efficiency and represent the average of three independent experiments performed in duplicate. The data are expressed as the mean ± S.E. and indicated as -fold inductions over the promoter-less pGL3 Basic vector (C). *, $P < 0.05$. The ERα-positive SaOS-2 and the ERα-negative MDA-MB-231 cells were treated with 0.5 μM ionomycin and 100 μg/ml PMA (2) or remained untreated (1), harvested and analyzed by Western blotting for the detection of NFATc1 and ERα as indicated. Western blotting analysis with antibody against ERα in the RA4-3′treated (+) and untreated (−) MDA-MB-231 is also shown. The intensity of the bands was quantified by densitometric analysis and normalized using IP(3)K as a control. D.U. = NFATc1/IP(3)K or ERα/IP(3)K densitometric units.

In addition, because the specific roles of NFAT in osteoblast differentiation are not well understood and, in some instances, reports of its role are contradictory, our study may contribute to clarify the involvement of Cn/NFAT in regulating osteoblast differentiation through NFATc1-mediated regulation of ERα gene expression. It is noteworthy that the interaction of NFAT signaling and ERα expression may have a clinical implications, in particular for what concerns the process of osteoporosis (Raisz, 2005). In agreement with the demonstration that NFATc1 regulates bone mass by functioning in both osteoblasts and osteoclasts (Winslow et al., 2006), one hypothesis is that NFATc1 activation would attenuate the cellular response to estrogen exactly in the context of bone formation. In this way, NFATc1 alone or cooperating with other transcription factors (Takayanagi et al., 2002) ensures the decrease of ERα gene expression, a critical step in osteoclastogenesis (Syed and Khosla, 2005). Therefore, we believe that our findings may be taken into account also to characterize potential therapeutic targets that could be employed for development anabolic agents for the treatment of a variety of bone diseases caused by excessive bone resorption, including osteoporosis.

References

Asagiri M and Takayanagi H (2007) The molecular understanding of osteoclast differentiation. *Bone* **40:**251–264.

Borgatti M, Boyd DD, Lampronti I, Bianchi N, Fabbri E, Saviano M, Romanelli A, Pedone C, and Gambari R (2005) Decoy molecules based on PNA-DNA chimeras and targeting Sp1 transcription factors inhibit the activity of urokinase-type plasminogen activator receptor (uPAR) promoter. *Oncol Res* **15:**373–383.

Crotti TN, Flannery M, Walsh NC, Fleming JD, Goldring SR, and McHugh KP (2006) NFATc1 regulation of the human beta3 integrin promoter in osteoclast differentiation. *Gene* **372:**92–102.

Day CJ, Kim MS, Lopez CM, Nicholson GC, and Morrison NA (2005) NFAT expression in human osteoclasts. *J Cell Biochem* **95:**17–23.

Denger S, Reid G, Brand H, Kos M, and Gannon F (2001) Tissue-specific expression of human ERalpha and ERbeta in the male. *Mol Cell Endocrinol* **178:**155–160.

Deroo BJ and Korach KS (2006) Estrogen receptors and human disease. *J Clin Investigig* **116:**561–570.

Dignam JD, Lebovitz RM, and Roeder RG (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* **11:**1475–1489.

Gao H, Xiao J, Sun Q, Lin H, Bai Y, Yang L, Yang B, Wang H, and Wang Z (2006) A single decoy oligodeoxynucleotides targeting multiple oncoproteins produces strong anticancer effects. *Mol Pharmacol* **70:**1621–1629.

Hogan PG, Chen L, Nardone J, and Rao A (2003) Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev* **17:**2205–2232.

Ikeda F, Nishimura R, Matsubara T, Tanaka S, Inoue J, Reddy SV, Hata K, Yamashita K, Hiraga T, Watanabe T, et al. (2004) Critical roles of c-Jun signaling in regulation of NFAT family and RANKL-regulated osteoclast differentiation. *J Clin Investigig* **114:**475–484.

Jessop HL, Suswillo R, Rawlinson S, Zaman G, Lee K, Das-Gupta V, Pitsillides AA, and Lanyon LE (2004) Osteoblast-like cells from estrogen receptor alpha knockout mice have deficient responses to mechanical strain. *J Bone Miner Res* **19:**938–946.

Kaminuma O, Deckert M, Elly C, Liu YC, and Altman A (2001) Vav-Rac1-mediated activation of the c-Jun N-terminal kinase/c-Jun/AP-1 pathway plays a major role in stimulation of the distal NFAT site in the interleukin-2 gene promoter. *Mol Cell Biol* **21:**3126–3136.

Kim Y, Sato K, Asagiri M, Morita I, Soma K, and Takayanagi H (2005) Contribution of NFATc1 to the transcriptional control of immunoreceptor OSCAR but not TREM-2 during osteoclastogenesis. *J Biol Chem* **280:**32905–32913.

Knox AJ, Meegan MJ, and Lloyd DG (2006) Estrogen receptors: molecular interactions, virtual screening and future prospects. *Curr Top Med Chem* **6:**217–243.

Koga T, Matsui Y, Asagiri M, Kodama T, de Crombrugghe B, Nakashima K, and Takayanagi H (2005) NFAT and Osterix cooperatively regulate bone formation. *Nat Med* **11:**880–885.

Kos M, Reid G, Denger S, and Gannon F (2001) Minireview: Genomic organization of the human ER alpha gene promoter region. *Mol Endocrinol* **15:**2057–2063.

Lambertini E, Penolazzi L, Magaldi S, Giordano S, del Senno L, and Piva R (2005) Transcription factor decoy against promoter C of estrogen receptor alpha gene induces a functional ER alpha protein in breast cancer cells. *Breast Cancer Res Treat* **92:**125–132.

Lerner UH (2006) Bone remodeling in post-menopausal osteoporosis. *J Dent Res* **85:**584–595.

Li L, Li Z, and Sacks DB (2005) The transcriptional activity of estrogen receptor-alpha is dependent on Ca^{2+}/calmodulin. *J Biol Chem* **280:**13097–13104.

Li L, Li Z, Howley PM, and Sacks DB (2006) E6AP and calmodulin reciprocally regulate estrogen receptor stability. *J Biol Chem* **281:**1978–1985.

Mann MJ and Dzau VJ (2000) Therapeutic applications of transcription factor decoy oligonucleotides. *J Clin Investig* **106:**1071–1075.

Manolagas SC, Kousteni S, and Jilka RL (2002) Sex steroids and bone. *Recent Prog Horm Res* **57:**385–409.

Matsumoto M, Kogawa M, Wada S, Takayanagi H, Tsujimoto M, Katayama S, Hisatake K, and Nogi Y (2004) Essential role of p38 mitogen-activated protein kinase in cathepsin K gene expression during osteoclastogenesis through association of NFATc1 and PU. 1. *J Biol Chem* **279:**45969–45979.

Matsuo K, Galson DL, Zhao C, Peng L, Laplace C, Wang KZ, Bachler MA, Amano H, Aburatani H, Ishikawa H, et al. (2004) Nuclear factor of activated T-cells (NFAT) rescues osteoclastogenesis in precursors lacking c-Fos. *J Biol Chem* **279:**26475–26480.

Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, and Gustafsson J (2001) Mechanisms of estrogen action. *Physiol Rev* **81:**1535–1565.

Oursler MJ (1998) Estrogen regulation of gene expression in osteoblasts and osteoclasts. *Crit Rev Eukaryot Gene Expr* **8:**125–140.

Piva R, Penolazzi L, Lambertini E, Giordano S, and Gambari R (2005) Induction of apoptosis of human primary osteoclasts treated with a transcription factor decoy mimicking a promoter region of estrogen receptor alpha. *Apoptosis* **10:**1079–1094.

Raisz LG (2005) Pathogenesis of osteoporosis: concepts, conflicts, and prospects. *J Clin Investig* **115:**3318–3325.

Rao A, Luo C, and Hogan PG (1997) Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol* **15:**707–747.

Stern PH (2006) The Calcineurin-NFAT Pathway and Bone: Intriguing New Findings. *Mol Interv* **6:**193–196.

Stossi F, Barnett DH, Frasor J, Komm B, Lyttle CR, and Katzenellenbogen BS (2004) Transcriptional profiling of estrogen-regulated gene expression via estrogen receptor (ER)α or ERβ in human osteosarcoma cells: distinct and common target genes for these receptors. *Endocrinology* **145:**3473–3486.

Syed F and Khosla S (2005) Mechanisms of sex steroid effects on bone. *Biochem Biophys Res Commun* **328:**688–696.

Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, Saiura A, Isobe M, Yokochi T, Inoue J, et al. (2002) Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev Cell* **3:**889–901.

Winslow MM, Pan M, Starbuck M, Gallo EM, Deng L, Karsenty G, and Crabtree GR (2006) Calcineurin/NFAT signaling in osteoblasts regulates bone mass. *Dev Cell* **10:**771–782.

Yeo H, McDonald JM, and Zayzafoon M (2006) NFATc1: a novel anabolic therapeutic target for osteoporosis. *Ann N Y Acad Sci* **1068:**564–567.

Zayzafoon M (2005) Inhibition of NFAT increases osteoblast differentiation by increasing Fra-2 expression. *J Musculoskelet Neuronal Interact* **5:**347.

Zayzafoon M (2006) Calcium/calmodulin signaling controls osteoblast growth and differentiation. *J Cell Biochem* **97:**56–70.

Zhang H, Wang X, Li J, Zhu J, Xie X, Yuan B, Yang Z, Zeng M, Jiang Z, Li J, et al. (2007) Tissue type-specific modulation of ER transcriptional activity by NFAT3. *Biochem Biophys Res Commun* **353:**576–581.

Address correspondence to: Roberta Piva, Department of Biochemistry and Molecular Biology, Molecular Biology Section, Via Fossato di Mortara, 74, 44100 Ferrara, Italy. E-mail: piv@unife.it