Modulation of gene expression in human osteoblasts by targeting a distal promoter region of human estrogen receptor-α gene

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Abstract

Estrogen receptor (ER) α is expressed during osteoblast differentiation; however, both its functional role in bone metabolism and its involvement in osteoporotic pathogenesis caused by estrogen deficiency are not well understood. Loss of ERα gene expression could be one of the mechanisms leading to osteoporosis. Therefore, we investigated a possible modulation of ERα gene expression in a human osteoblastic cell line and in four primary osteoblast cultures by using a decoy strategy. Double stranded DNA molecules, mimicking a regulatory region of the ERα gene promoter (DNA-102) and acting as a 'silencer' in breast cancer cells, were introduced into osteoblasts as 'decoy' cis-elements to bind and functionally inactivate a putative negative transcription factor, and thus to induce ERα gene expression.

We found that the DNA-102 molecule was able to specifically bind osteoblast nuclear proteins.

Before decoy treatment, absence or variable low levels of ERα RNAs in the different cultures were detected. When the cells were transfected with the DNA-102 decoy, an increase in expression of ERα and osteoblastic markers, such as osteopontin, was observed, indicating a more differentiated osteoblastic phenotype both in the cell line and in primary cultures. These results showed that the DNA-102 sequence competes with endogenous specific negative transcription factors that may be critical for a decrease in or lack of ERα gene transcription. Therefore, osteoblastic transfection with the DNA-102 decoy molecule may be considered a tempting model in a putative therapeutic approach for those pathologies, such as osteoporosis, in which the decrease or loss of ERα expression plays a critical role in bone function.

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Introduction

Estrogens regulate a variety of metabolic processes through their specific nuclear receptors, which belong to a nuclear receptor superfamily (Carson-Jurica *et al.* 1990, Mangelsdorf *et al.* 1995) and act as ligand-dependent transcription factors (Green & Chambon 1988). Two specific estrogen receptors have been identified: ERα (Green *et al.* 1986, Auchus & Fuqua 1994) and, more recently, ERβ (Mosselman *et al.* 1996, Vidal *et al.* 1999). Both receptors exibit a specific tissue distribution and modulate activities of different estrogen responsive gene promoters in a different manner (Bord *et al.* 2001, Braidman *et al.* 2001).

The importance of estrogen and nuclear ERs to skeletal growth and bone metabolism is supported by a body of evidence (Eriksen *et al.* 1988, Bodine *et al.* 1998, Rickard *et al.* 1999, Compston 2001). Nevertheless, the network of interactions and molecular mechanisms is very complex

and the design of a unique model of estrogen action in bone is very difficult. Recent advances have defined potential sites of estrogen action within the bone microenvironment: these mainly include proliferation and differentiation of osteoprogenitor cells, activity of mature osteoblasts and osteoclasts, bone matrix synthesis and bone resorption, and interaction with co-regulatory factors (Rickard *et al.* 1999, Spelsberg *et al.* 1999).

Expression studies in skeletal cells both *in vitro* and *in vivo* have demonstrated that the concentration of ERα is higher than ERβ in bone and in osteoblasts at all stages of differentiation (Arts *et al.* 1997, Denger *et al.* 2001).

As for the clinical aspects, the positive effect of estrogens on bone homeostasis is well known. Estrogen replacement therapy reduces the incidence and severity of pathologies such as osteoporosis and cardiovascular disease in post-menopausal women (Pacifici 1996, Riggs 2000), even if long term estrogen treatment increases the risk of endometrial and breast cancers. At present, a number of studies

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aimed at understanding the wide spectrum of effects exerted by estrogen on the bone have described the development of drugs and therapeutic approaches for the treatment of osteopenic disorders (Windahl *et al.* 1999, Rodan & Martin 2000, Compston 2001), such as osteoporosis, tumor-associated osteolysis, rheumathoid arthritis, periodontal disease and orthopedic implant osteolysis.

Since the expression level of endogenous ERs is limiting for estrogen responses, it is tempting to speculate that a strategy able to modulate ERs gene expression may be a new tool for stimulating bone formation. An increase in gene expression could be fulfilled either by recruiting positive transcription factors or by reducing the action of negative factors. We focused our attention on ERα gene expression and we hypothesized that subtracting negative transcription factors able to bind ERα gene promoter(s), by using specific approaches or agents, could result in a reduction in their negative effect and an increase in ERα gene expression.

The concept of using nucleic acids to bind target proteins has been explored as a way of manipulating gene expression in living cells (Mann & Dzau 2000). This strategy involves the delivery of double-stranded DNA molecules termed 'decoys' which are able to squelch the activity of the target transcription factor (Piva & Gambari 1999). The competition for trans-acting factors between the endogenous cis-elements present on the target gene and the exogenously added decoy DNA molecule, containing a sequence identical to that of the specific cis-element, results in an inhibition or attenuation of the 'authentic' interaction of trans–factor(s) with its cis-element(s). Therefore, this approach represents a method for testing the biological involvement of genomic sequences in the regulation of gene expression and in the maintenance of a specific phenotype (Morishita *et al.* 1996, Sharma *et al.* 1996, Yamashita *et al.* 1998, Wang *et al.* 2000). Additionally, this approach can be considered a useful method for modulating the gene expression for potential therapeutic intervention (Morishita *et al.* 1995, Tomita *et al.* 1999, Mann & Dzau 2000).

In this study, the involvement of the ERα in bone cells was investigated by transfecting cultured human osteoblast cells with a decoy molecule against a distal promoter of the ERα gene. The decoy molecule that we propose is a synthetic double-stranded DNA belonging to the P3 distal promoter of the ERα gene ($-3258/-3157$, termed DNA-102), showing a high affinity for a putative negative transcription factor (nTF) found in ER–negative cells. In a previous study, we transfected this sequence into ER–negative breast cancer cells, and we obtained the reactivation of ERα gene transcription (Penolazzi *et al.* 2000).

The aim of this study was to investigate the ability of DNA-102 decoy to bind to nTF and to affect the induction of ERα gene expression in the TE85 osteosarcoma cell line and in human primary osteoblasts. The

analysis concerns ERα because levels of ERβ mRNA were undetectable in the primary osteoblast cultures analyzed.

We report that, in these osteoblast-like cells, the DNA-102 decoy increases both the ERα gene expression, in particular through the activity of upstream ERα gene promoters, and the expression of osteopontin (OPN) (Denhardt & Guo 1993) and osteonectin (ON) (Termine *et al.* 1981) that are typical markers of osteoblastic function and differentiation. By contrast, ERβ gene expression was not reactivated by decoy treatment.

Therefore, our experimental approach may contribute to the development of bone anabolic therapeutic molecules that would stimulate bone formation through the specific manipulation of gene expression.

Materials and Methods

Materials

Egg phosphatidyl choline was purchased from Lipid Products (Nutfield Nurseries, Surrey, UK). The cationic surfactant N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl-ammonium methyl-sulfate (DOTAP) was purchased from Sigma Chemical Co. (St. Louis, MO, USA)

As decoy molecule, a DNA fragment belonging to the 5′ region of the human (h) estrogen receptor gene, 102 bp in size (DNA-102), was generated by PCR using RA1 (5′-GCCATTGTTGACCTACAGGAG-3′) and RA4 (5′-TATTTATATCCAGTATTTATTTTCAATACT GACT-3′) primers. As control, a 150 bp plasmidic fragment (DNA-150) was used. pBLCAT8 ERCAT1 (Piva *et al.* 2000) and pGEX-2TK (Nilsson *et al.* 1985) recombinant plasmids were used as templates for DNA-102 and DNA-150 respectively. After amplification, DNAs were purified by an ultrafiltration procedure with the Microcon-30 system (Amicon, Inc, Beverly, MA, USA) as previously described (Penolazzi *et al.* 1997).

Liposome preparation

Cationic liposomes, composed of egg phosphatidyl choline (PC) and the cationic surfactant DOTAP (PC:DOTAP; 8:1 mol/mol), were prepared by reverse phase evaporation followed by three extrusion cycles through 200 nm pore size polycarbonate membranes. The extrusion step was performed in order to obtain unilamellar liposomes with a homogeneous size distribution, as confirmed by freeze-fracture electron microphotographs (Cortesi *et al.* 1996).

Cell culture and DNA transfection efficiency

Normal human spongy bone specimens were collected during surgical procedures. Patients were in good health, consistent with their age, and were not suffering from

autoimmune or metabolic diseases or malignancies. They were affected by arthritis of the hip and in the case of patients 3 and 4 clinical diagnosis of osteoporosis was made. For the surgical procedure we followed Hardinge's surgical approach to the hip. As regards the ethics of the experimental procedures on human subjects, informed consent was obtained from each patient after full explanation of the purpose and nature of all procedures used.

Bone specimens were cultured according to Maurizi *et al.* (1983). Primary cultures were grown in Falcon flasks containing Eagle's Minimum Essential Medium supplemented with 20% fetal bovine serum (Gibco, Gaithersburg, MD, USA) and antibiotics at 37 °C in an humidified atmosphere of 5% CO_2 (Sollazzo *et al.* 1997). Subcultures were obtained about 30 days later. The TE85 osteosarcoma cell line was grown in the same conditions.

Decoy DNA molecule (600 ng) was used to transfect cells at 60% confluence plated in 31-mm diameter plates. DNA was mixed with cationic liposome suspension (lipid:DNA ratio 10:1 w/w) in a final volume of 200 µl. After 30-min incubation at room temperature, 200 µl serum-free medium were added to the liposome/DNA complex and used to transfect one well. Twenty-four hours later, the transfection solution was replaced with complete medium that was not deprived of endogenous estrogenic activity. After transfection, cells were washed five times with PBS.

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was measured in confluent human osteoblastic cells by the hydrolysis of *p*-nitrophenylphosphate (PNPP) according to Ibbotson *et al.* (1986). Enzyme activity was expressed as U/mg protein. One unit was defined as the amount of enzyme which hydrolysed 1 µmol PNPP/minute. Cell protein was determined according to the Lowry method (Lowry *et al.* 1951). The effect of 1,25-dihydroxyvitamin D3 (1,25-$(OH)_2D_3$) on ALP activity was verified after incubation in medium containing 10 nM 1,25-$(OH)_2D_3$ for 48 h.

Analysis of gene transcription

Gene expression was detected by reverse transcription-polymerase chain reaction (RT-PCR) on total RNA (1–5 µg) from transfected cells.

The amplification reactions were performed using the SuperScript One-Step RT-PCR System (Life Technologies, GibcoBRL, Gaithersburg, MD, USA) and a Violet Thermal Cycler. The following primers and conditions were used. ERα: forward (F)=5′-CTATATG TGTCCAGCCACCAACC-3′ (exon 3), reverse (R)=5′-CTCTACACATTTTCCCTGGTTCCT-3′ (exon 6); 30 cycles: 60 s at 94 °C, 60 s at 57 °C and 60 s at 72 °C. ERβ: F=5′-ATCTTTGACATGCTCCTGGC-3′,

R=5′-ACGCTTCAGCTTGTGACCTC-3′; 30 cycles: 60 s at 94 °C, 60 s at 56 °C and 60 s at 72 °C. OPN: F=5′-CAGAATCTCCTAGCCCCACA-3′, R=5′-AA CTCCTCGCTTTCCATGTG-3′; 30 cycles: 60 s at 94 °C, 60 s at 51 °C and 60 s at 72 °C. ON: F=5′-GTATCTGTGGGAGCTAATCCT-3′, R=5′-AGAGT CGAAGGTCTTGTTGTC-3′; 30 cycles: 60 s at 94 °C, 60 s at 52 °C and 60 s at 72 °C. β-Actin: F=5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′, R=5′-CTAGAAGCATTTGCGGTGGACGATGGAG GG-3′; 20 cycles: 45 s at 94 °C, 45 s at 60 °C and 45 s at 72 °C.

PCR amplifications specific for different ERα transcripts were performed under the following conditions. FG/R2: FG=5′-TCGTCCTGGGAGCTGCACTT-3′, R2=5′-GATAATCGACGCCAGGGTGGCAGA-3′; 30 cycles: 60 s at 94 °C, 60 s at 53 °C and 60 s at 72 °C. FP/R1: FP=5′-AAGACGTTCTTGATCCAGC-3′, R1=5′-ACCAAAGCATCTGGGATG-3′; 30 cycles: 60 s at 94 °C, 60 s at 54 °C and 60 s at 72 °C. FH/R1: FH=5′-AGGAAGGAGTAAGCACAAAG-3′, R1=5′-ACCAAAGCATCTGGGATG-3′; 30 cycles: 60 s at 94 °C, 60 s at 48 °C and 60 s at 72 °C.

All amplifications were compared with a negative control (primers without RNA) and the levels of expression of the different genes were normalized against the β-actin mRNA content using a densitometric analysis. RT-PCR products were separated on agarose gel, electrophoresed and, for ER RNA analysis, were subsequently blotted onto nylon membrane using standard procedures (Penolazzi *et al.* 1998). Hybridizations were performed with the following ^{32}P-labeled probes: pOR15 (Green *et al.* 1986) for total ERα transcription analysis, pGHER1 (Ponglikitmongkol *et al.* 1988) for FG/R2, FP/R1 and FH/R1 PCR amplifications used to discriminate the activity of different promoters, and pSG5-hERβ (Ogawa *et al.* 1998) for ERβ mRNA.

Western blot analysis

Cell extracts from TE85 cells were separated by 10% SDS-PAGE, essentially according to Laemmli (1970), and proteins were then transferred to nitrocellulose membrane (Hybond C). After electroblotting, proteins were visualized using Ponceau S reagent (Sigma). The blots were blocked for 2 h at room temperature with 1 × phosphate-buffered saline containing 0·1% Tween 20 (PBST) and 3% BSA, incubated for 2 h with purified monoclonal antibody (290 ng/ml) to the human ERα (H222, diluted 1:1000), polyclonal antisera against the human bone OPN (LF-123, diluted 1:1000) and bovine bone osteonectin (BON-1, diluted 1:1000) in blocking solution. The blots were then washed three times with PBST for 30 min and incubated for 45 min with PBST containing alkaline phosphatase-conjugated goat anti-rat IgG antibody (Promega) diluted 1:4000, and washed three
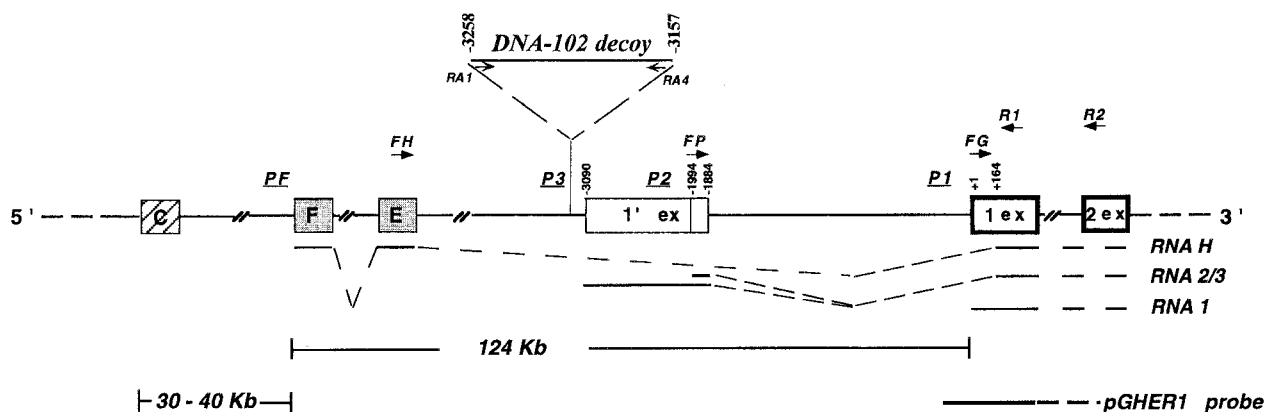
Figure 1 Schematic representation of the 5′ upstream region of the human estrogen receptor (ER)α gene and some of the corresponding transcripts so far characterized. The exons are indicated with boxes and the P1 canonical promoter, P2, P3 and PF distal promoters are shown. Exon usage and alternative splicing patterns for some of the different RNA isoforms are schematically represented. Nucleotide +1 represents the transcription start site of the canonical ERα RNA (RNA1). Upstream transcription start sites at −1994 and at −3090 for isoforms 2 and 3 respectively, as well as the splicing acceptor site position at +164 inside exon 1 are indicated. RNA H indicates the RNA transcript containing the E and F upstream exons. The location of the DNA-102 decoy molecule, primers employed for the RT-PCR experiments and pGHER1 probe are also shown.

times with PBST for 30 min. Immunoreactive proteins were visualized using ProtoBlot Western Blot AP Systems (Promega).

Electrophoretic mobility shift assay

RA4 (− 3190/ − 3157) radiolabeled oligonucleotides in-side DNA-102 were used as a probe in the incubation with nuclear extracts from TE85 and MCF7 cells. To prepare nuclear extracts, cultured cells were washed twice with PBS and collected with a scraper. The cytoplasmic membranes were ruptured mechanically using a Dounce B homogenizer, and nuclear proteins were obtained essentially as described by Dignam *et al.* (1983). Protein concentration was determined using a Bio-Rad (Hercules, CA, USA) protein assay. Nuclear extracts were incubated with 0·1 ng (6000 c.p.m.) labeled probes in 1 × binding buffer (10 mM Tris–HCl pH 7·5, 20 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 5 mM EDTA, 0·01% Triton X-100, 0·5% glycerol) containing 1·2 mg

poly(dI–dC).poly(dI–dC) (Sigma) for 30 min at room temperature. Specific competitors including unlabeled probes and nonspecific competitor (150 bp PCR product from pGEX-2TK plasmid) were added at different molar excesses. The DNA–protein complexes were separated from the uncomplexed DNA on 6% polyacrylamide gel in 0·25 × Tris–borate-EDTA by electrophoresis at 150 volts. Gels were dried and then exposed to X-ray film.

Results

DNA-102 molecule interacts with nuclear extracts from osteoblasts

In order to look for nuclear proteins that might bind to the DNA-102 sequence to be used as the decoy molecule, we tested the RA4 oligonucleotide in the electrophoretic mobility shift assay with nuclear extracts from the TE85 osteoblastic–like cell line. This oligonucleotide covers the sequence from −3190 to −3157, inside the DNA-102

Figure 2 (A) Gel mobility shift assay. RA4 (− 3190/ − 3157) radiolabeled oligonucleotide belonging to the ERα P3 promoter was used as a probe in the incubation with nuclear extracts from TE85 osteosarcoma and MCF7 breast cancer cells. Specific competitors including unlabeled probe and nonspecific competitor (150 bp PCR product from pGEX-2TK plasmid) were added at different molar excess. The retarded bands generated by specific interaction between oligonucleotides and nuclear factors are arrowed. (B) Photomicrographs of TE85 cells treated with the DNA-102 decoy molecule complexed with cationic liposomes (a), or left untreated (b). Original magnification: ×100. (C) Effect of DNA-102 decoy on modulation of ERα, ERβ and osteopontin (OPN) gene expression. Expression of ERα, ERβ, OPN and β-actin mRNA was detected by RT-PCR on total RNA from decoy-treated (T) or untreated (C) TE85 cells. All amplifications were compared with a negative control (primers without RNA) and the levels of ERα and OPN mRNA were normalized against the β-actin mRNA content using a densitometric analysis. In the case of ERα mRNA, the specificity of electrophoretic data was confirmed by hybridization with a ^{32}P-labeled ERα specific probe (pOR15) followed by autoradiography. For ERβ mRNA, the specific probe used was pSG5-hERβ (Ogawa *et al.* 1998). The specific RT-PCR products are arrowed. M, molecular weight marker (HaeIII restricted pBR322 DNA); (−), negative control. (D) Determination of ERα and OPN by immunoblot analysis. Twenty micrograms whole cell extracts from TE85 cells treated with the DNA-102 decoy molecule (TE85 T) or untreated (TE85 C) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filter; blots were probed with ERα-specific monoclonal antibody and OPN-specific polyclonal antibody respectively. MCF7 cellular extracts were used as a control.

A

B

C

ERα

ER β

β-actin   OPN

D

ERα   OPN
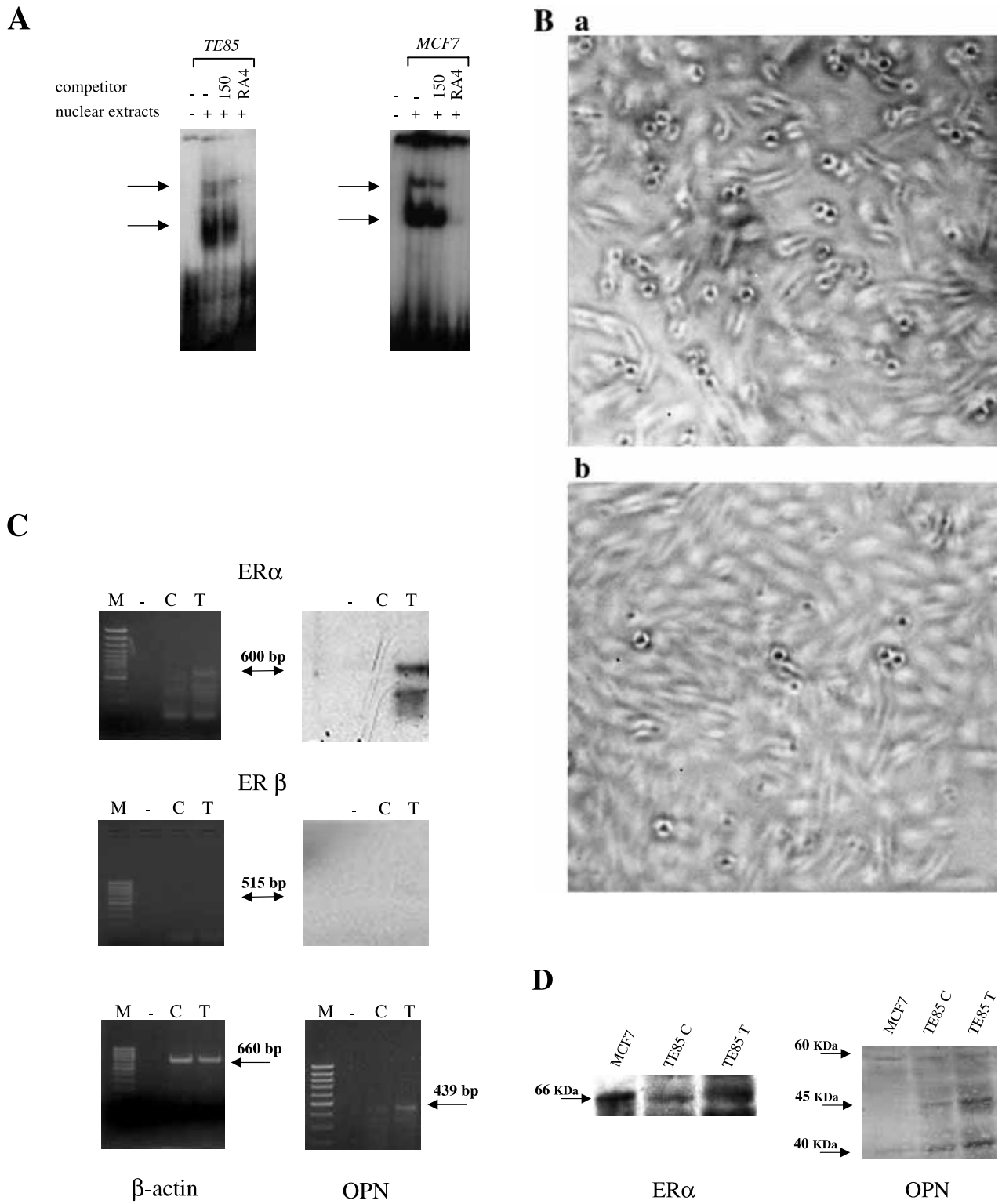
molecule (see Fig. 1), and it was previously characterized as the sequence mainly involved in DNA–protein interactions (Penolazzi *et al.* 2000).

As shown in Fig. 2A, two main labeled complexes were generated by the binding of nucleoproteins to the RA4 oligonucleotide, with a pattern which was very similar to that observed when nuclear extracts from MCF7 ER-positive breast cancer cells were used. The specific DNA–protein complexes were displaced by a 250-fold excess of unlabeled homologous competitor, but not by the unlabeled unrelated 150 bp double stranded (ds)-DNA, demonstrating the specificity of the observed protein–DNA interactions. Therefore, these findings suggest that the osteoblastic-like cells express nuclear protein(s), that are able to bind specifically to the DNA-102 sequence.

The decoy effect on gene expression in the TE85 cell line

The PCR product DNA-102, including the RA4 oligonucleotide, was used as decoy molecule because it retains a major nuclease resistance compared with the RA4 oligonucleotide. Transfection experiments were carried out in medium not deprived of endogenous estrogenic activity, in the presence of DNA-102 complexed with PC:DOTAP cationic liposomes (Cortesi *et al.* 1996). Cytotoxic effects were not observed: decoy-treated cells showed only slight changes in their morphology (see Fig. 2B); in addition, their ability to proliferate, in comparison with untreated cells, was only slightly decreased (data not shown).

Reverse transcription-PCR (RT-PCR) analysis was then performed in order to investigate whether the decoy treatment would affect gene expression in osteoblastic cells. Total RNA was isolated from treated and untreated cells, transfected in duplicate; subsequently, the same amount of RNA was reverse transcribed with random and ERα-specific oligonucleotides. The cDNA obtained was subjected to the PCR amplification in a first step for ERα gene using primers specific for exon 3 and exon 6, and then for the osteopontin (OPN) gene, to evaluate the effect of decoy DNA-102 on estrogen-related gene expression. The cDNA was also amplified with primers specific for a gene whose expression is not estrogen dependent, such as β-actin. In all the experiments, the β-actin PCR product was used as an internal control in order to obtain a semiquantitative comparison of the gene expression. A representative experiment is shown in Fig. 2C. The levels of PCR product derived from the ERα transcript were almost undetectable in the TE85 cell line, but when the cells were transfected with the DNA-102 decoy molecule, the PCR product was clearly evident, as seen from agarose gel analysis and confirmed by hybridization with ^{32}P-labeled pOR15 specific probe. Also, in the case of OPN mRNA, decoy treatment resulted in a positive effect. The mean values obtained by densitometric analysis of the band intensity in different

RT-PCR experiments, expressed as optical density (O.D.) arbitrary units, were 0·38 untreated cells/1·43 treated cells for ERα, 0·195 untreated cells/0·824 treated cells for OPN and 1·510 untreated cells/1·383 treated cells for β-actin. By contrast, ERβ mRNA was undetectable even after DNA-102 decoy treatment. When the cells were transfected with an unrelated plasmid 150 bp PCR product, the expression of ERα and β-actin genes was completely unaffected (data not shown), thus demonstrating that the effect of DNA-102 decoy can be considered specific. Therefore, these experiments showed that a specific gene expression may be positively regulated by DNA-102 decoy in the osteoblastic cell line analyzed.

Next, we analyzed the promoter usage in the TE85 cell line and the effect of the decoy on the upstream ERα transcripts, but, as expected from the very low level of ERα gene transcription, this analysis was not satisfactory.

An examination of protein levels of ERα and osteopontin by Western blot analysis was then carried out. As shown in Fig. 2D, these cells were found to express the marker of the osteoblast phenotype (OPN) typically in the three isoforms (60, 45 and 40 kDa) and the ERα protein. After the decoy treatment, an increase in ERα and OPN level was observed.

The decoy effect on gene expression in human primary osteoblasts

Next, we examined the ability of decoy DNA-102 to induce ERα gene expression in primary osteoblasts. These human bone-derived cells displayed specific osteoblast features such as the expression of high ALP activity that increased after 1,25-$(OH)_2D_3$ treatment (Beresdorf *et al.* 1986), as shown in Table 1.

Also, in the case of these cells, the transfection experiments were carried out in medium not deprived of endogenous estrogenic activity, with DNA-102 complexed with PC:DOTAP cationic liposomes. During the decoy treatment cytotoxic effects were very slight, as confirmed by the absence of significant cellular morphological changes (Fig. 3A).

The cDNAs obtained from untreated and decoy-treated cells were amplified by PCR, first using primers specific for exon 3 and exon 6 of the ERα gene to estimate its expression as a whole, and then using the appropriate primers to distinguish transcription at upstream or at main promoters (see Fig. 1 for the localization of the primers). When different RNA isoforms of a gene originate from upstream exonic sequences and from alternative splicing events, such as in the case of the ERα gene, it is possible to analyze the level of expression of a single isoform by RT-PCR choosing the forward primer inside the specific upstream exon used. The primers for the amplification of the only canonical ERα transcript were: the forward FG (+20/+39), inside exon 1 and located upstream of the splice site position at +164 that is employed as a splicing

Table 1 Alkaline phosphatase (ALP) activity (basal and following 1,25-$(OH)_2D_3$ stimulation) in human primary osteoblasts, expressed as U/mg protein × 10^3. Values are the means ± S.D. of three parallel measurements

	Sex	Age (years)	ALP basal	ALP 1,25-$(OH)_2D_3$
Patient no.				
1	Female	65	4·5 ± 0·17	5·4 ± 0·1
2	Male	64	15·07 ± 0·21	19·9 ± 0·96
3	Female	78	53 ± 1	64·07 ± 0·67
4	Female	80	11 ± 0·5	16 ± 0·53

acceptor site of the other ER RNA isoforms analyzed; the reverse R2 (+695/+718), inside exon 2. The forward primers used for the amplification of some possible upstream transcripts were: FP, inside exon 1 (Keaveney *et al.* 1992, Piva *et al.* 1993) and FH, inside exon E described by Flouriot *et al.* (1998) and corresponding to exon Hb described by Thompson *et al.* (1997). R1 primer (+251/+268) localized inside exon 1 of the ERα gene was used as reverse primer. To improve gene expression analysis, the levels of type 1 (FG/R2), type 2/3 (FP/R1) and type H (FH/R1) ERα mRNAs were estimated by RT-PCR followed by specific hybridization with upstream pGHER1 probe (Ponglikitmongkol *et al.* 1988) as shown in Fig. 3B, in which a representative experiment, corresponding to sample no. 1, is illustrated. The levels of ERβ, OPN and ON gene expression were also evaluated using specific primers (Fig. 3B). All RT-PCR products corresponding to endogenous and decoy-dependent gene expression levels of each sample were then subjected to densitometric analysis: the results are summarized in Table 2 as total ERα mRNA expression level, and in Table 3 as OPN and ON mRNA levels.

The highest level of ERα RNA was observed in the cell culture obtained from the youngest female patient, sample no. 1 (aged 65 years) (Table 2 and Fig. 3B). As regards promoter usage, the canonic P1 promoter was active only in sample 1 because only in this patient was the ERα mRNA 1 (FG/R2) detected (see Fig. 3B). The P2/P3 and PF distal promoters were preferentially used in the osteo-blasts from samples 2, 3 and 4 (see Table 2): in these cases the ERα mRNA 1 (FG/R2) was not detected, whereas the ERα mRNA type 2/3 (FP/R1) and especially the ERα mRNA type H (FH/R1) were detected.

The usage of each specific promoter did not change when the cells were transfected with DNA-102 decoy molecule, suggesting that the ERα gene regulatory regions, that are specifically used in bone cells, play a critical role in the committed differentiation of osteoblasts. After decoy treatment, however, the level of ERα gene transcription was increased in three of the four samples analyzed. The major increase in transcriptional levels was obtained for sample 1, whereas in sample 4, corresponding to the osteoblasts of the oldest patient (age 80 years), ERα gene transcription seemed to be unaffected by the decoy treatment (see Table 2).

As far as the expression of markers of osteoblastic differentiation was concerned, the DNA-102 decoy treatment resulted in a marked increase in OPN RNA expression in three of the four cultures (nos 1, 2 and 4), and in a slight increase in ON RNA expression levels (see Table 3). By contrast, ERβ mRNA levels were undetectable even after decoy treatment in all primary osteoblast cultures analyzed.

Discussion

Many studies employing Northern blot, RT-PCR and immunohistochemical analysis indicate that the level of estrogen receptors in different osteoblastic cells is very low, in spite of their being highly responsive to estrogens, and that there is a heterogeneity of ERα and ERβ expression among osteoblastic cells (Ikegami *et al.* 1993, 1994, Rao & Murray 2000, Bord *et al.* 2001, Compston 2001).

The questions remain as to what level of ERs expression is sufficient to sensitize osteoblasts to estrogen and if the possibility to modulate ERs gene expression may be a tool to stimulate bone formation. Our work is aimed, in particular, at identifying a method to induce an increase in ERα gene expression in ERα-deficient cells. This should confirm that bone-forming osteoblasts, that are physio-logical targets for estrogen action, can also be a good target for a therapeutic approach aimed at restoring or increasing ERα expression. In the study presented here, we have shown the positive modulation of ERα mRNA expression in the TE85 human osteosarcoma cell line and in three of four human primary osteoblastic cells by the transfection of decoy molecules (DNA-102) against a sequence of distal promoter (−3258/−3157) of the ERα gene, previously described as a silencer in breast cancer cells (Penolazzi *et al.* 2000). After decoy treatment the strongest increase in ERα gene transcription was observed in the TE85 osteo-sarcoma cell line. In primary osteoblasts, where the inves-tigation of upstream RNA levels was also performed, we demonstrated that expression of the ERα gene is mainly due to the activity of upstream promoters (P2/P3 and PF), in agreement with the observations of other authors (Grandien *et al.* 1995, Flouriot *et al.* 1998), and that, after decoy treatment, it increased in an appreciable manner. Even if the significance of the different ERα RNA isoforms and the cooperation of specific transcription
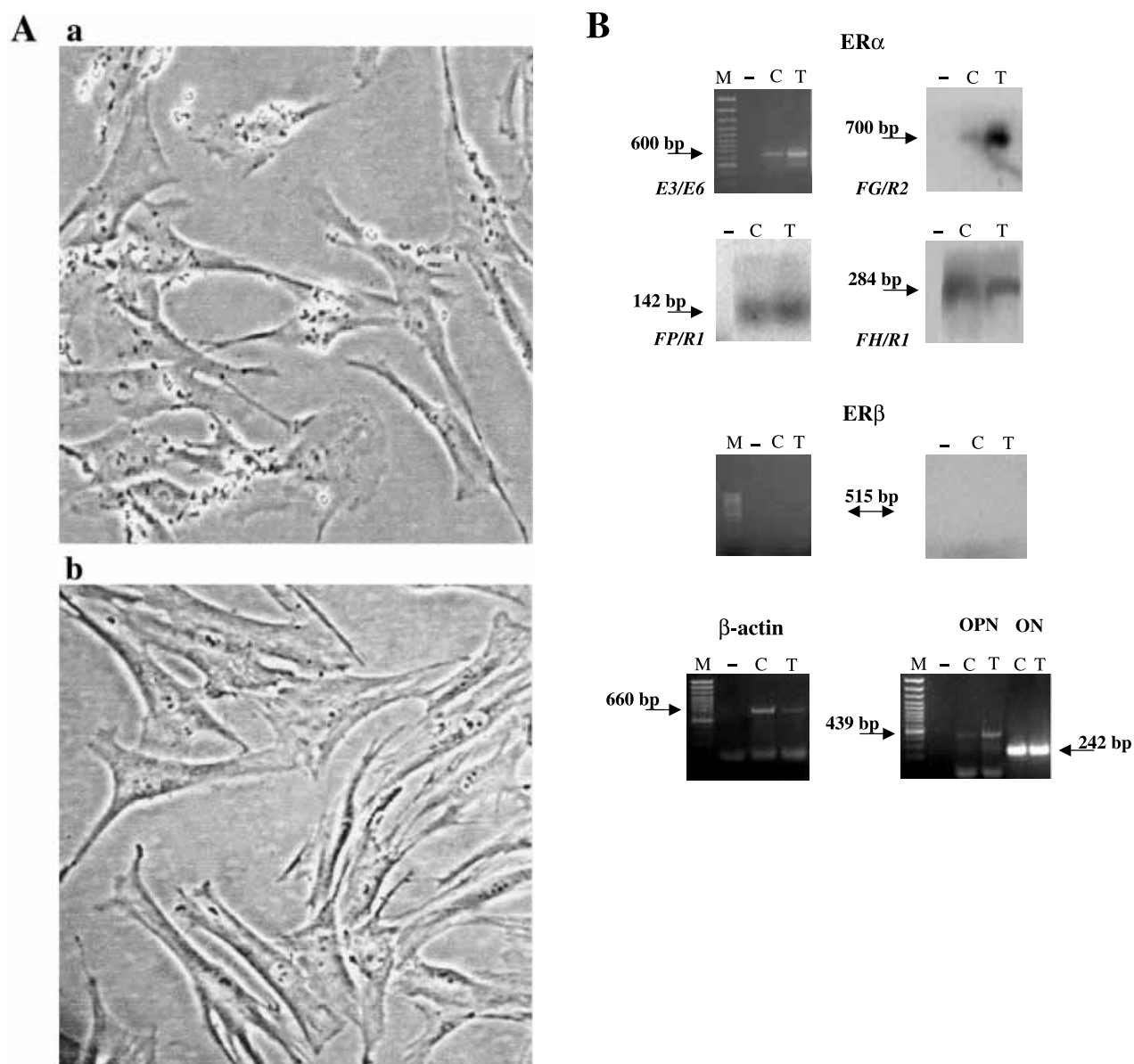
Figure 3 (A) Photomicrographs of primary osteoblasts treated with the DNA-102 decoy molecule complexed with cationic liposomes (a), or left untreated (b). Original magnification: × 100. (B) Effect of DNA-102 decoy on modulation of ERα, ERβ, OPN and osteonectin (ON) gene transcription. Osteoblastic cells isolated from osteopenic bone specimens obtained from four patients (3 women aged 65, 78 and 80 years, samples 1, 3 and 4 respectively; and one man aged 64, sample 2) were transfected with the DNA-102 decoy molecule (T) or were untreated (C). RT-PCR products of total ERα mRNA (E3/E6), ERα RNA 1 isoform (FG/R2), ERα RNA 2 and 3 isoforms (FP/R1) and ERα RNA H isoform (FH/R1) were separated in 1·8% agarose gel and stained with ethidium bromide. For the analysis of RNA isoforms, RT-PCR products were blotted onto nylon membrane and hybridized with the pGHER1 specific probe (Ponglikitmongkol *et al.* 1988). For ERβ mRNA, the specific probe used was pSG5-hERβ (Ogawa *et al.* 1998). A representative RT-PCR experiment corresponding to the expression of ERα, ERβ, OPN and ON mRNA from sample 1 is shown. All amplifications were compared with a negative control (primers without RNA), and the levels of ERα, OPN and ON mRNA were normalized against the β-actin mRNA content using a densitometric analysis. All RT-PCR products visible on agarose gel or the autoradiographic signals were then subjected to densitometry. The results are summarized in Tables 2 and 3. M, molecular weight marker.

factors for their expression awaits further investigation, it is likely that the complex promoter organization of ERα gene limits the cell species competent for the expression.

Our data suggest that the sequence extending from − 3258 to − 3157 may be considered critical for the lack of or decrease in ERα gene transcription in the bone

Table 2 The effect of DNA-102 decoy on estrogen receptor (ER)α gene transcription assessed by RT-PCR analysis. Total ERα mRNA results from the levels of type 1, type 2 and type H ERα mRNAs determined by semiquantitative RT-PCR as described in the experimental procedures. All samples (1–4), treated with DNA-102 (+) or untreated (−), were quantified in at least two independent experiments. The fold induction (Fold ind.) of gene transcription after decoy is also reported. The promoter utilization, after decoy treatment, is based on the expression levels of upstream ERα RNA isoforms (for P2/P3 and PF promoters) and canonical ERα transcript (for P1 promoter)

	Total ERα mRNA			Promoter usage		
	−	+	Fold ind.	P1	P2/P3	PF
Sample						
1	0·76	1·87	× 2·46	●	◉	◉
2	0·20	0·37	× 1·8	○	◉	●
3	0·37	0·46	× 1·2	○	◉	●
4	0·56	0·57	× 1·01	○	●	●

Solid circles, preferentially used; stippled circles, partially used; open circles, not used.

Table 3 The effect of DNA-102 decoy on osteopontin (OPN) and osteonectin (ON) gene transcription assessed by RT-PCR analysis. OPN and ON mRNA levels were determined by semiquantitative RT-PCR as described in the experimental procedures. All samples (1–4), treated with DNA-102 (+) or untreated (−), were quantified in at least two independent experiments. The fold induction (Fold ind.) of gene transcription is also reported

	OPN mRNA			ON mRNA		
	−	+	Fold ind.	−	+	Fold ind.
Sample						
1	0·28	1·0	× 3·5	0·6	0·6	× 1·0
2	0·33	0·96	× 2·9	0·75	0·85	× 1·13
3	0·17	0·1	−	0·85	1·0	× 1·17
4	0·12	0·2	× 1·6	0·33	0·42	× 1·3

and that, when exogenously transfected, could compete with endogenous specific negative transcription factors. This is also in agreement with the data obtained in breast cancer cell lines (Penolazzi *et al.* 2000) and with the concept that the ERα gene promoters are under different controls.

It is interesting that the decoy–induced positive effect could also be observed on the expression of a bone differentiation marker, such as OPN, both in the TE85 cell line and in primary osteoblasts. By contrast, the cells analyzed remained ERβ negative after DNA-102 decoy treatment. Taken together, these results suggest that the approach here described may be considered an effective method to improve the osteoblastic phenotype through a mechanism in which ERβ does not appear to be involved.

The mechanism by which DNA-102 decoy brought about an increase in or activation of ERα gene expression is not clear. However, the fact that this change in expression was previously observed also in breast cancer cells after the same decoy treatment, suggests that DNA-102 decoy may act through modulation of DNA–protein binding or protein–protein interactions, stabilizing factors or conformation modifiers specific for ERα gene, and subtracting specific negative transcription factor(s) − nTF - that binds the sequence of DNA-102. Therefore, we speculate that DNA-102 decoy molecule, through reducing the nTF binding to its putative sequence inside DNA-102, would prevent an inhibitory signaling pathway on ERα gene transcription, favoring the positive control of ERα on transcription of target genes and inducing a more differentiated bone phenotype. Our experiments demonstrating that the promoter usage did not change when the cells were transfected with DNA-102 decoy

molecule strengthen this hypothesis, suggesting that our decoy approach may intensify a committed osteoblastic phenotype.

Nevertheless, we cannot exclude the possibility that the DNA–102 decoy is able to induce osteoblastic differentiation independently of ERs and estrogen and that the increase in ERα and OPN expression may be a secondary event. Therefore, further investigations are required not only to quantitatively correlate the level of ERα gene transcription with ERα protein levels, but also to accurately analyze whether or not the ERα-mediated effect on osteoblastic differentiation, which is suggested here, is ligand dependent. Further investigation regarding the correlation between the increase in ERα gene expression and improvement in bone mass, in relation to specific clinical parameters, is also required to confirm the utility of the decoy approach here proposed. This may be of great significance for the development of new therapeutic strategies to improve bone mass in bone diseases such as postmenopausal osteoporosis which is characterized by a low bone mass and an increased risk of fracture (Rizzoli *et al.* 2001).

It is noteworthy that the study of regulatory mechanisms of ERα expression may contribute to a better understanding of the wide spectrum of effects of estrogen action in the bone microenvironment depending on the different ER isoforms (Rickard *et al.* 1999), the presence of the two orphan receptors that are closely related to the ERs, estrogen receptor-related receptors α and β (ERRα and ERRβ) (Vanacker *et al.* 1999), the balance between co-activators and co-repressors (Shibata *et al.* 1997), and the type of target DNAs (Rickard *et al.* 1999). In particular, because the interaction between ERα and ERβ is described (Bord *et al.* 2001, Compston 2001), it will be interesting to assess the DNA–102 decoy effect on osteoblast primary cultures that will express ERβ protein.

In addition, although estrogen appears to be the most important sex steroid involved in skeletal maturation and

mineralization (Rao & Murray 2000), osteoblast proliferation and differentiation are believed to be regulated by the combined effect of a key number of growth factors, cytokines and hormones that, alternatively, might mediate the effect of the DNA-102 decoy molecule.

In spite of the fact that there is limited information on the intermediate stages of the osteoblast differentiation pathway, by analyzing the effects of our decoy molecule on ERα, OPN and ON gene expression in primary cultures and identifing regulatory elements that maintain a specific bone phenotype via ER cell-specific gene expression, we can provide valuable information delineating the role of specific DNA–protein interactions on regulatory regions of the ERα gene in osteoblast differentiation.

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References

Arts J, Kuiper GG, Janssen JM, Gustafsson J-A, Lowik CW, Pols HAP & van Leeuwen JP 1997 Differential expression of estrogen receptor α and β mRNA during differentiation of human osteoblast SV-HFO cells. *Endocrinology* **138** 5067–5070.

Auchus RJ & Fuqua SAW 1994 Hormone-nuclear receptor interactions in health and disease. The estrogen receptor. *Bailiere's Clinical Endocrinology and Metabolism* **8** 433–449.

Beresdorf JN, Gallagher JA & Russell RG 1986 1,25-Dihydroxyvitamin D3 and human bone-derived cells *in vitro*: effects on alkaline phosphatase, type I collagen and proliferation. *Endocrinology* **119** 1776–1785.

Bodine PVN, Henderson RA, Green J, Aronow M, Owen T, Stein GS, Lian JB & Komm BS 1998 Estrogen receptor-α is developmentally regulated during osteoblast differentiation and contributes to selective estrogen responsiveness of gene expression. *Endocrinology* **139** 2048–2057.

Bord S, Horner A, Beavan S & Compston J 2001 Estrogen receptors alpha and beta are differentially expressed in developing human bone. *Journal of Clinical Endocrinology and Metabolism* **86** 2309–2314.

Braidman IP, Hainey L, Batra G, Selby PL, Saunders PT & Hoyland JA 2001 Localization of estrogen receptor beta protein expression in adult human bone. *Journal of Bone and Mineral Research* **16** 214–220.

Carson-Jurica MA, Schrader WT & O'Malley BW 1990 Steroid receptor family: structure and functions. *Endocrine Reviews* **11** 201–220.

Compston JE 2001 Sex steroids and bone. *Physiological Reviews* **81** 419–447.

Cortesi R, Esposito E, Menegatti E, Gambari R & Nastruzzi C 1996 Effect of cationic liposome composition on *in vitro* cytotoxicity and protective effect on carried DNA. *International Journal of Pharmacology* **139** 69–78.

Denger S, Reid G, Brand H, Kos M & Gannon F 2001 Tissue-specific expression of human ERα and ERβ in the male. *Molecular and Cellular Endocrinology* **178** 155–160.

Denhardt DT & Guo X 1993 Osteopontin: a protein of many functions. *FASEB Journal* **7** 1475–1482.

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

Eriksen EF, Colvard DS, Berg NJ, Graham ML, Mann KG, Spelsberg TC & Riggs BL 1988 Evidence of estrogen receptor in human osteoblast-like cells. *Science* **241** 84–86.

Flouriot G, Griffin C, Kenealy M, Sonntag-Buck V & Gannon F 1998 Differentially expressed mRNA isoforms of the human estrogen receptor-α gene are generated by alternative splicing and promoter usage. *Molecular Endocrinology* **12** 1938–1954.

Grandien K, Backdahl M, Ljunggren O, Gustafsson J-A & Berkenstam A 1995 Estrogen target tissue determines alternative promoter utilization of the human estrogen receptor gene in osteoblasts and tumor cell lines. *Endocrinology* **136** 2223–2229.

Green S & Chambon P 1988 Nuclear receptors enhance our understanding of transcriptional regulation. *Trends in Genetics* **4** 309–314.

Green S, Walter P, Kumar V, Krust A, Bonert J, Argos P & Chambon P 1986 Human estrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature* **320** 134–139.

Ibbotson KJ, Harrod J, Gowen M, D'Souza S, Smith DD, Winkler ME, Derynck R & Mundy GR 1986 Human recombinant transforming growth factor alpha stimulates bone resorption and inhibits formation *in vitro*. *PNAS* **83** 2228–2232.

Ikegami A, Inoue S, Hosoi T, Mizuno Y, Nakamura T, Ouchi Y & Orimo H 1993 Immunohistochemical detection and northern blot analysis of estrogen receptor in osteoblastic cells. *Journal of Bone and Mineral Research* **8** 1103–1109.

Ikegami A, Inoue S, Hosoi T, Kaneki M, Mizuno Y, Akedo Y, Ouchi Y & Orimo H 1994 Cell cycle-dependent expression of estrogen receptor and effect of estrogen on proliferation of synchronized human osteoblast-like osteosarcoma cells. *Endocrinology* **135** 782–789.

Keaveney M, Klug J & Gannon F 1992 Sequence analysis of the 5′ flanking region of the human estrogen receptor gene. *DNA Sequence Journal* **2** 347–358.

Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680–685.

Lowry OH, Rosebrough NJ, Farr AL & Randall RJ 1951 Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* **193** 265–275.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P & Evans RM 1995 The nuclear receptor superfamily: the second decade. *Cell* **83** 835–839.

Mann MJ & Dzau VJ 2000 Therapeutic applications of transcription factor decoy oligonucleotides. *Journal of Clinical Investigation* **106** 1071–1075.

Maurizi M, Binaglia L, Donti E, Ottaviani F, Paludetti G & Venti Donti G 1983 Morphological and functional characterization of human temporal-bone cell cultures. *Cell and Tissue Research* **229** 505–513.

Morishita R, Gibbons GH, Masatsugu H, Ellison KE, Nakajima M, Zhang L, Kaneda Y, Ogihara T & Dzau VJ 1995 A gene therapy strategy using a transcription factor decoy of the E2F binding site inhibits smooth muscle proliferation *in vivo*. *PNAS* **92** 5855–5859.

Morishita R, Higaki J, Tomita N, Aoki M, Moriguchi A, Tamura K, Murakami K, Kaneda Y & Ogihara T 1996 Role of transcriptional cis-elements, angiotensinogen gene-activating elements, of angiotensinogen gene in blood pressure regulation. *Hypertension* **27** 502–507.

Mosselman S, Polman J & Dijkema R 1996 ERβ: identification and characterization of a novel human estrogen receptor. *FEBS Letters* **392** 49–53.

Nilsson B, Abrahmsen L & Uhlen M 1985 Immobilization and purification of enzymes with staphylococcal protein A gene fusion vectors. *EMBO Journal* **4** 1075–1080.

Ogawa S, Inoue S, Watanabe T, Hiroi H, Orimo A, Hosoi T, Ouchi Y & Muramatsu M 1998 Complete primary structure of human estrogen receptor β (hERβ) and its heterodimerization with ERα *in vivo* and *in vitro*. *Biochemical and Biophysical Research Communications* **243** 122–126.

Pacifici R 1996 Estrogen, cytokines and pathogenesis of postmenopausal osteoporosis. *Journal of Bone and Mineral Research* **11** 1043–1051.

Penolazzi L, Facciolo MC, Aguiari GL, del Senno L & Piva R 1997 Direct transfection of PCR-generated DNA fragments into mammalian cells employing ethidium bromide indicator and ultrafiltration. *Analytical Biochemistry* **248** 190–193.

Penolazzi L, Lambertini E, Aguiari GL, del Senno L & Piva R 1998 Modulation of estrogen receptor gene expression in human MCF7 breast cancer cells: a decoy strategy with specific PCR-generated DNA fragments. *Breast Cancer Research and Treatment* **49** 227–235.

Penolazzi L, Lambertini E, Aguiari G, del Senno L & Piva R 2000 Cis element 'decoy' against the upstream promoter of the human estrogen receptor gene. *Biochimica et Biophysica Acta* **1492** 560–567.

Piva R & Gambari R 1999 New trends in molecular pharmacology: artificial modulation of gene expression by transcription modifiers. *Minerva Biotec* **11** 193–203.

Piva R, Bianchi N, Aguiari GL, Gambari R & del Senno L 1993 Sequencing of an RNA transcript of the human estrogen receptor gene: evidence for a new transcriptional event. *Journal of Steroid Biochemistry and Molecular Biology* **46** 531–538.

Piva R, del Senno L, Lambertini E, Penolazzi L & Nastruzzi C 2000 Modulation of estrogen receptor gene transcription in breast cancer cells by liposome delivered decoy molecules. *Journal of Steroid Biochemistry and Molecular Biology* **75** 121–128.

Ponglikitmongkol M, Green S & Chambon P 1988 Genomic organization of the human oestrogen receptor gene. *EMBO Journal* **7** 3385–3388.

Rao LG & Murray TM 2000 Studies of human osteoblasts *in vitro*: estrogen actions and interactions with other hormones at different stages of differentiation. *Drug Development Research* **49** 174–186.

Rickard DJ, Subramaniam M & Spelsberg TC 1999 Molecular and cellular mechanisms of estrogen action on the skeleton. *Journal of Cell Biochemistry* (Suppl) **32/33** 123–132.

Riggs BL 2000 The mechanisms of estrogen regulation of bone resorption. *Journal of Clinical Investigation* **106** 1203–1204.

Rizzoli R, Bonjour JP & Ferrari SL 2001 Osteoporosis, genetics and hormones. *Journal of Molecular Endocrinology* **26** 79–84.

Rodan GA & Martin TJ 2000 Therapeutic approaches to bone diseases. *Science* **289** 1508–1514.

Sharma HW, Perez JR, Higgins-Sochaski K, Hsiao R & Narayanan R 1996 Transcription factor decoy approach to decipher the role of NF-kappa B in oncogenesis. *Anticancer Research* **16** 61–69.

Shibata H, Spencer TE, Onate SA, Jenster G, Tsai SY, Tsai M-J & O'Malley BW 1997 Role of co-activators and co-repressors in the mechanisms of steroid/thyroid receptor action. *Recent Progress in Hormone Research* **52** 141–165.

Sollazzo V, Traina GC, DeMattei M, Pellati A, Pezzetti F & Caruso A 1997 Responses of human MG-63 osteosarcoma cell line and human osteoblast-like cells to pulsed electromagnetic fields. *Bioelectromagnetics* **18** 541–547.

Spelsberg TC, Subramaniam M, Riggs BL & Khosla S 1999 The actions and interactions of sex steroids and growth factors/cytokines on the skeleton. *Molecular Endocrinology* **13** 819–828.

Termine JD, Kleinman HK, Whitson SW, Conn KM, Mcgarvey ML & Martin GR 1981 Osteonectin, a bone-specific protein linking mineral to collagen. *Cell* **26** 99–105.

Thompson DA, McPherson LA, Carmeci C, de Coninck EC & Weigel RJ 1997 Identification of two estrogen receptor transcripts with novel 5′ exons isolated from a MCF7 cDNA library. *Journal of Steroid Biochemistry and Molecular Biology* **62** 143–153.

Tomita T, Takeuchi E, Tomita N, Morishita R, Kaneko M, Yamamoto K, Nakase T, Seki H, Kato K, Kaneda Y & Ochi T 1999 Suppressed severity of collagen-induced arthritis by *in vivo* transfection of nuclear factor κB decoy oligodeoxynucleotides as a gene therapy. *Arthritis and Rheumatism* **42** 2532–2542.

Vanacker JM, Pettersson K, Gustafsson JA & Laudet V 1999 Transcriptional targets shared by estrogen receptor-related receptors (ERRs) and estrogen receptor (ER) α, but not by ERβ. *EMBO Journal* **18** 4270–4279.

Vidal O, Kindblom L-G & Ohlsson C 1999 Expression and localization of estrogen receptor-beta in murine and human bone. *Journal of Bone and Mineral Research* **14** 923–929.

Wang LH, Yang XY, Kirken RA, Resau JH & Farrar WL 2000 Targeted disruption of Stat6 DNA binding activity by an oligonucleotide decoy blocks IL-4-driven TH2 cell response. *Gene Therapy* **95** 1249–1257.

Windahl SH, Vidal O, Andersson G, Gustafsson JA & Ohlsson C 1999 Increased cortical bone mineral content but unchanged trabecular bone mineral density in female ERβ -/- mice. *Journal of Clinical Investigation* **104** 895–901.

Yamashita J, Yoshimasa T, Arai H, Hiraoka J, Takaya K, Miyamoto Y, Ogawa Y, Itoh H & Nakao K 1998 Identification of cis-elements of the human endothelin-A receptor gene and inhibition of the gene expression by the decoy strategy. *Journal of Biological Chemistry* **273** 15993–15999.