Transcriptional activation of insulin-like growth factor binding protein 6 by 17β-estradiol in SaOS-2 cells

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Abstract

Osteoblasts can synthesize the insulin-like growth factors (IGFs) and the IGF-binding proteins (IGFBPs), which may either enhance or attenuate IGF-stimulated bone cell proliferation. Since estrogen induced osteoblastic differentiation and proliferation through an estrogen-responsive gene in target cells, we investigated the effects of estrogen on IGFBP-6 expression in the human osteoblastic-like cell line SaOS-2. Expressions of IGFBP-6 protein and mRNA increased 2.8 and 2-fold, respectively, in the presence of 17-β-estradiol (E2) (0.01 to 1 μM) and estrogen receptor (ER) in SaOS-2 cells. On the other hand, E2 induced a 2-fold increase in SaOS-2 cell proliferation. To identify genomic sequences associated with estrogen responsiveness, the 5'-promoter region (-44 to +118) of the IGFBP-6 gene was cloned into a chloramphenicol acetyltransferase (CAT) reporter vector. E2 induced a 3-fold increase in CAT activity in SaOS-2 cells transiently transfected with this construct. Identification of the estrogen-responsive element (ERE) [5'-CCTTCA CCTG-3'] (-9 to +1) in this IGFBP-6 gene promoter region was confirmed using electromobility shift assays and deletion analysis. This functional ERE was important for E2-induced trans-activation of the IGFBP-6 gene.

These results demonstrate that E2 exhibits a positive effect on IGFBP-6 gene transcription through estrogen-liganded ER binding to the functional ERE in the IGFBP-6 gene promoter in SaOS-2 cells.

Introduction

Insulin-like growth factors (IGFs) play an important role in the regulation of cell metabolism. The biological activity of IGFs is modulated by a family of seven high-affinity IGF binding proteins (IGFBP 1-7) and their receptors (Jones *et al.*, 1995; Oh *et al.*, 1996). Among them, IGFBP-6 has a 20- to 100-fold higher binding affinity for IGF-II than for IGF-I. IGFBP-6 is expressed in many cell types, such as fibroblasts, myoblasts, smooth muscle cells, keratinocytes, and osteoblasts (Yan *et al.*, 2001; Strohbach *et al.*, 2008). Because of its binding preference, IGFBP-6 might regulate IGF-II-induced cell proliferation, differentiation, and survival (Bach *et al.*, 1994; Dake *et al.*, 2002; Kim *et al.*, 2002; Gallicchio *et al.*, 2003; Fu *et al.*, 2007). Generally, IGFBPs play an IGF-dependent role in high affinity IGF binding. Some studies have suggested that IGFBP-6 has IGF-independent effects (Grellier *et al.*, 1998; Seurin *et al.*, 2002; Sueoka *et al.*, 2000). Among the IGFBPs, IGFBP-6 is unique in stimulation of osteosarcoma cell growth and has an anti-apoptotic effect (Schmid *et al.*, 1999). Furthermore, IGFBP-6 inhibits programmed cell death in non-small cell lung cancer cells (Sueoka *et al.*, 2000). Overexpression of IGFBP-6 inhibits both neuroblastoma growth *in vivo* (Bienvenu *et al.*, 2004) and proliferation of human bronchial epithelial cells (Sueoka *et al.*, 2000). Therefore, IGFBP-6 exerts differential effects on the regulation of cell metabolism in different cell lines.

As an important endocrine-hormone, estrogen is essential for regulation of the growth and differentiation of target cells. Estrogen receptor (ER), a member of the steroid-thyroid hormone receptor superfamily, mediates the action of estrogen by dependently binding ligands to estrogen-responsive elements (EREs) in the target gene promoter, thus directly regulating gene transcription (Evans
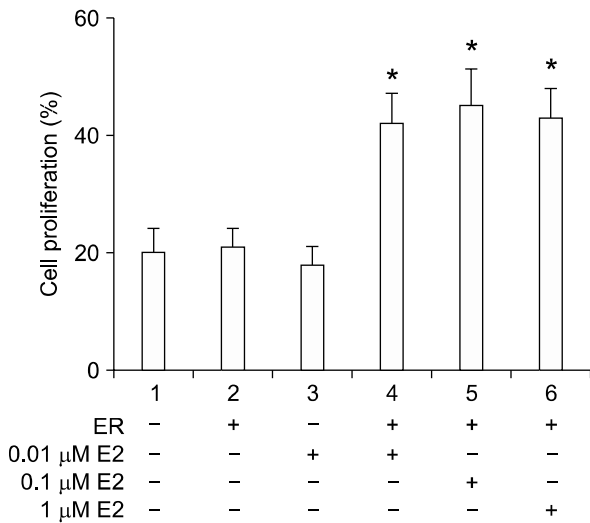
Figure 1. Estrogen affected SaOS-2 cell proliferation. To determine the effect of estrogen on osteoblastic cell growth *in vitro*, SaOS-2 cell proliferation was examined using MTT. E2 induced a 2-fold increase in cell proliferation in SaOS-2 cells co-treated with the wild-type human ERα expression vector pSG5HEO. *indicates a significant difference between results with and without E2 or ER ($P < 0.05$).

that estrogen and ER exert differential effects on the biological activity of IGFs. IGFBP-4 was up-regulated by estrogen in the ECC-1 cell line, and IGFBP-6 was down-regulated by progesterone in the PRAB-36 cell line (Gielen *et al.*, 2006). IGFBP-3 and IGFBP-5 were regulated by estrogen in an endometrial cancer cell line (Hondermarck *et al.*, 2003; Gielen *et al.*, 2006). Estrogen (0.01 μM estradiol) reduced the level of IGFBP-3, but induced increase in the level of IGFBP-6 1.5- to 2-fold in MCF-7 cells (Salahifar *et al.*, 2000; Brockdorff *et al.*, 2003). However, the mechanisms of estrogen regulation of IGF signaling are poorly understood. There are few identified EREs in the promoter regions of estrogen-regulated IGF genes.

Structural and functional analyses of the IGFBP-6 gene promoter have been performed (Hodzic *et al.*, 1997; Dailly *et al.*, 2001), and the 5'-promoter region contains retinoic acid response elements, CAAT boxes, CACCC boxes, NF-1, and activated protein-1 (AP-1)/AP-2 sites (Dailly *et al.*, 2001; Song *et al.*, 2001). However, the molecular mechanism of estrogen signaling through ER in IGFBP-6 transcription has not been determined. In order to understand the mechanisms of estrogen in IGF signaling, we previously investigated estrogen regulation of IGFBP-6 gene *in vivo* (Guo *et al.*, 2003, 2007). Herein, we present evidence that the human osteoblastic-like cell line SaOS-2 expresses IGFBP-6 and that estrogen increases the abundance of both IGFBP-6 mRNA and protein in the presence of ER. We first determined that
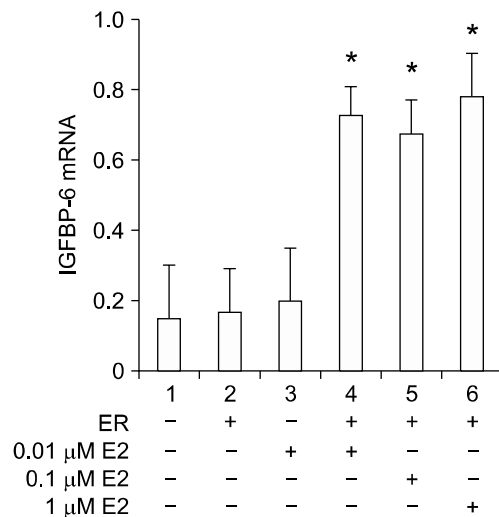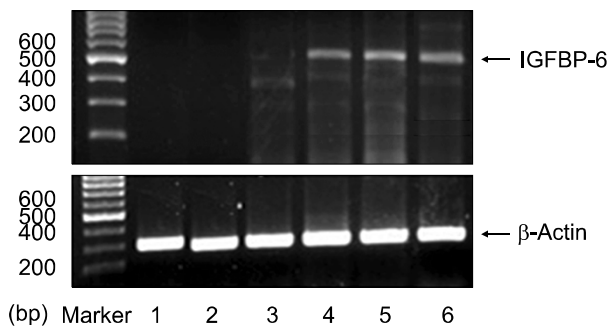
et al., 1988; Green *et al.*, 1988). ER has been found in female organs and in non-reproductive tissues of the central nervous system (Brown *et al.*, 1988; Simerly *et al.*, 1990), the cardiovascular system (Orimo *et al.*, 1993; Stumpf *et al.*, 1977), and the skeletal system (Eriksen *et al.*, 1988; Komm *et al.*, 1988). It has recently been suggested

Figure 2. Effect of E2 on up-regulation of IGFBP-6 expression in SaOS-2 cells. SaOS-2 cells were cotreated for 24 h with E2 (0.01, 0.1, and 1 μM) in SaOS-2 cells transfected with pSG5HEO. Total RNA and protein from fetal SaOS-2 cells were subjected to semi-quantitative RT-PCR. The band intensity from each sample was quantified using Fluorchem 2.0 software. The level of IGFBP-6 mRNA is presented as a multiple of the β-actin density. RT-PCR results indicated that the level of IGFBP-6 mRNA in SaOS-2 cells treated with 1 μM E2 and pSG5HEO was 2-fold higher than with 1 μM E2 or pSG5HEO alone. *indicates a significant difference between results with and without E2 and/or ER ($P < 0.05$).

estradiol exhibits a positive effect on IGFBP-6 gene transcription through a functional ERE in the IGFBP-6 gene promoter region. These results contribute to a better understanding of the trans-criptional estrogen regulation of IGFBP-6 gene.

Results

The effect of up-regulation of E2 on IGFBP-6 gene transcription

In order to determine whether transcription and translation of the IGFBP-6 gene are regulated by E2, we isolated total mRNA and protein from SaOS-2 cells previously treated with E2 and pSG5HEO (the ERα expression vector). As shown in Figures 2 and 3, E2 induced a significant increase in IGFBP-6 mRNA and protein levels based on RT-PCR and Western blot analytical
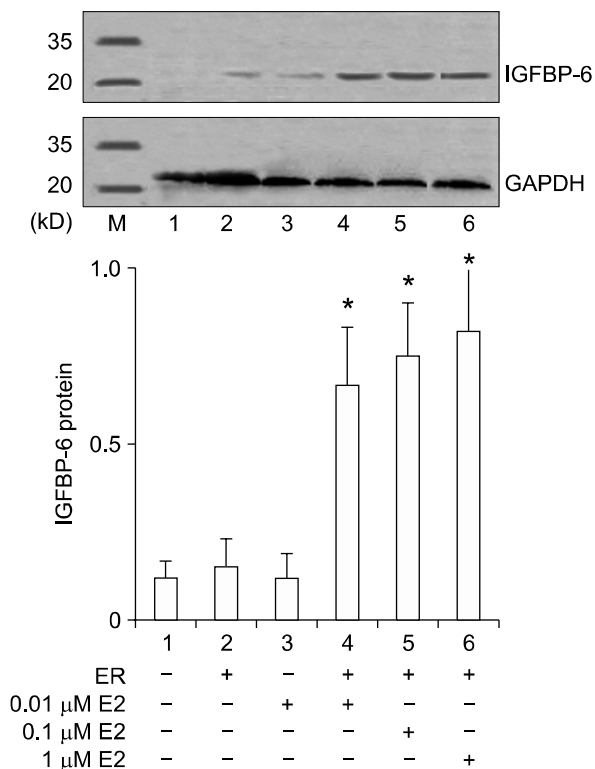

Figure 3. E2 regulation of IGFBP-6 protein level in SaOS-2 cells. SaOS-2 cells were cotreated for 24 h with E2 (0.01, 0.1, and 1 μM) in cells transfected with pSG5HEO. Western blot analysis was used to ex-amine the protein expression of IGFBP-6. The band intensity was quanti-fied using Fluorchem 2.0 software. The level of the IGFBP-6 protein is presented as a multiple of the GAPDH density. Western blot analysis in-dicated that the level of the IGFBP-6 protein in SaOS-2 cells treated with E2 (0.01, 0.1, 1 μM) and pSG5HEO was 2.8- to 3.5-fold higher than with E2 or pSG5HEO alone. *indicates a significant difference between re-sults with and without E2 and/or ER ($P < 0.05$).

results. As seen in Figure 2, 1 μM E2 caused a significant 2-fold decrease in the IGFBP-6 mRNA level. As shown in Figure 3, 1 μM E2 resulted in a significant 2.8-fold increase in the IGFBP-6 protein level in SaOS-2 cells, compared with cells un-treated with E2. These results indicate that IGFBP-6 gene expression is regulated by E2 at the trans-criptional level *in vitro*. However, at doses of 0.01-1 μM, E2 did not have a dose-dependent effect on IGFBP-6 mRNA expression (Figure 2). Co-treat-ment with E2 (0.01-1 μM) and ERα caused a significant increase in cell proliferation in SaOS-2 cells compared to treatment with E2 or ERα alone (Figure 1).

Identification of the ERE located in the IGFBP-6 gene promoter

Sequence analysis was carried out to search for EREs in the 1.8 kb region of the human IGFBP-6 gene promoter (GenBank access numbers AF-297519). We did not find EREs in the IGFBP-6 gene promoter using TESS, but the two putative EREs in the IGFBP-6 gene promoter were identified. The putative ERE sequences were 5'-CCTTCACCTG-3' (-9 to +1) and 5'-AACCCTGACC-3' (+105 to +115). To determine whether the identified activated ER-protein can bind to these putative EREs in the IGFBP-6 gene promoter, direct and competitive binding of both the wild-type and the mutant-type ERE oligonucleotides with nuclear extracts was determined using EMSA (Figure 4 and 5). The effi-cient retarded band appeared after addition of the labeled ERE-1 probe, indicating that the activated ER-protein interacts directly with this natural ERE-1 (Figure 4, Lane 1). In competitive binding studies, this retarded band was subjected to competition with a 50 and 100-fold excess of the unlabeled ERE-1 probe (Figure 4, Lane 4, 5). Addition of the nonspecific labeled probe (mutant-type ERE-1 oligonucleotides) did not result in binding (Figure 4, Lane 6). A similar retarded band did not appear when the labeled wild-type ERE-2 probe was incubated with nuclear extracts (Figure 5, Lane 2). To identify the specific ER in DNA-ER complexes, the ERα antibody was used in EMSA. As shown in Figure 4, the ERα antibody did bind specifically to labeled DNA-ER complexes and formed a supershifted band (Figure 4, Lane 2).

Positive effect of E2 on IGFBP-6 gene transcription through the functional ERE located in the IGFBP-6 gene promoter

To examine the function of these two putative EREs, SaOS-2 cells were transiently co-trans-
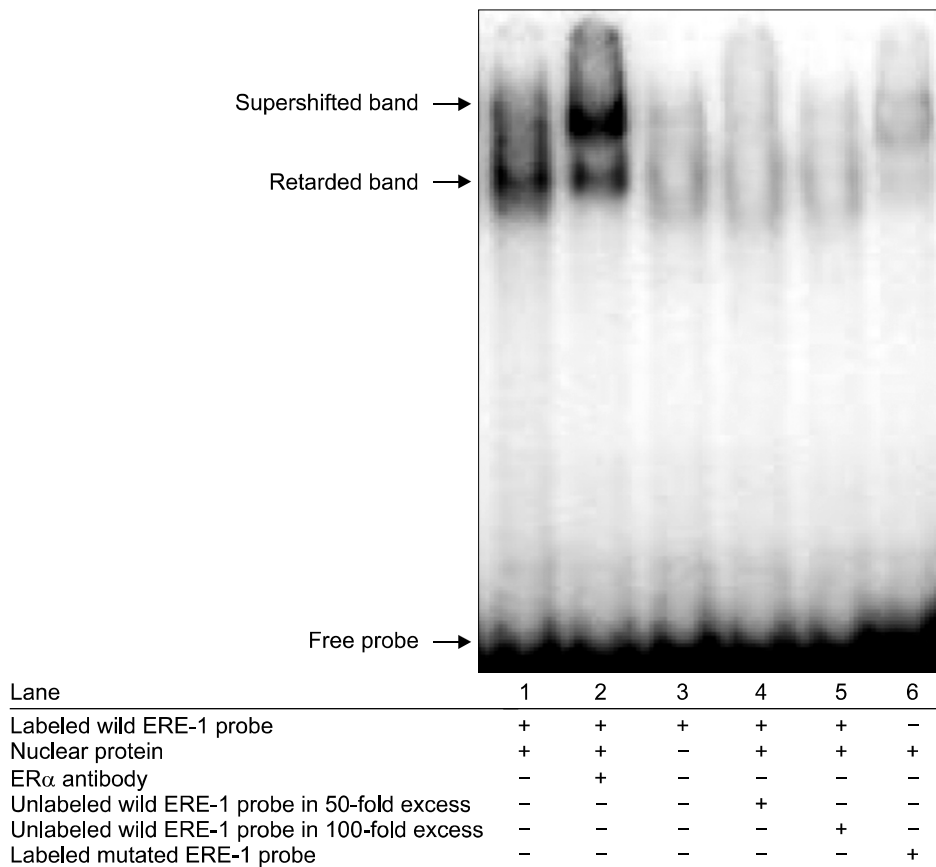
Figure 4. Binding of ER to the identified ERE-1 in the GFBP-6 gene (EMSA). Nuclear extracts from SaOS-2 cells were incubated with a ^{32}P-labeled probe harboring the ERE-1 (Lane 1-5) of the IGFBP-6 gene. The resulting complexes were resolved using non-denaturing PAGE. A labeled mutant-type ERE-1 probe was used as a negative control (Lane 6). For competition EMSA, 50-fold and 100-fold excesses of the unlabeled probe harboring the wild-type ERE-1 (Lane 4, 5) of the IGFBP-6 gene promoter were added during the pre-incubation period. Responsive banding (see arrow in Figure 4) of the wild-type ERE-1 binding complex was observed. Addition of a cold consensus ERE-1 (Lane 4, 5) decreased or eliminated the formation of specific complexes. The retarded band of the mutant-type ERE-1 binding complex was not observed when labeled mutant-type ERE-1 was incubated with nuclear extracts (Lanes 6). The ERα antibody did bind to the putative ERE-1-ER complexes. These specific complexes (Lane 3) were delayed and formed a supershifted band, compared with the ERE-1-ER complexes (Lane 2). The arrow indicates ER binding.

Lane	1	2	3	4	5	6
Labeled wild ERE-1 probe	+	+	+	+	+	−
Nuclear protein	+	+	−	+	+	+
ERα antibody	−	+	−	−	−	−
Unlabeled wild ERE-1 probe in 50-fold excess	−	−	−	+	−	−
Unlabeled wild ERE-1 probe in 100-fold excess	−	−	−	−	+	−
Labeled mutated ERE-1 probe	−	−	−	−	−	+

fected with two constructed pCAT vectors (containing these putative EREs) uisng an ERα expression vector. As shown in Figure 6, 1 μM E2 significantly stimulated CAT activity in -44 IGFBP-6-pCAT [containing ERE-1 (-9 to+1) and ERE-2 (+105 to +115)] transfected cells, but not in +29 IGFBP-6-pCAT [containing only ERE-2 (+105 to +115)] transfected cells. Similar to E2 induction of IGFBP-6 mRNA in SaOS-2 cells, 1 μM E2 induced a 3-fold increase in CAT activity in -44 IGFBP-6-pCAT transfected cells compared to cells transfected with +29 IGFBP-6-pCAT (data not shown). Neither ERE-1 nor E2 alone significantly altered CAT activity in -44 IGFBP-6-CAT transfected cells. These results indicate that the functional ERE-1 (-9 to+1) is located in the IGFBP-6 gene promoter. In the presence of ER, E2 up-regulates CAT activity through ERE-1 (-9 to+1) in the IGFBP-6 gene promoter.

Discussion

IGFBP-6, a crucial regulator in the IGF system, is involved in osteogenesis and bone function (Schmid *et al.*, 1999; Milne *et al.*, 2001). Proli-

feration and differentiation of bone marrow mesenchymal cells are associated with IGFBP-6 and other components of the IGF system (Baddoo *et al.*, 2003). IGFBP-6 is involved in biological effects in an IGF-dependent manner. However, there is growing evidence that IGFBP-6 has IGF-independent effects, including induction of apoptosis and modulation of cell migration (Fu *et al.*, 2007). IGFBP-6 mRNA and proteins are induced by estradiol in ER-positive MCF-7 cells (Martin *et al.*, 1995). Our results confirm that IGFBP-6 is an estradiol-responsive gene. Dailly (Dailly *et al.*, 2001) analyzed the promoter activity of the IGFBP-6 gene 5'-flanking region in the SaOS-2 and U-2OS cell lines. Deletion analysis showed the promoted activity of IGFBP-6 gene requires the -1,731 to +154 bp sequence of the gene promoter, and the important elements include a putative RARE, an AP-2 site, a CACCC box, and an Sp1 site (Dailly *et al.*, 2001; Song *et al.*, 2001). We found that estrogen activity in SaOS-2 cells is primarily associated with the -44 to +118 region of the IGFBP-6 gene promoter that contains the liganded-ER binding site in the -9 to +1 region (Figure 2 and 4).

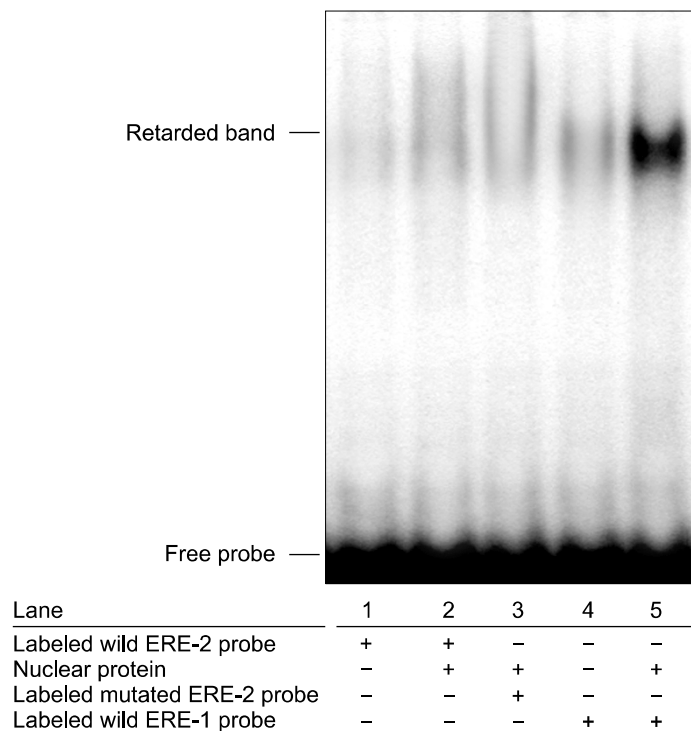Estrogen is involved in embryogenesis and

Figure 5. Identification of ERE-2 in the GFBP-6 gene promoter (EMSA). Nuclear extracts from SaOS-2 cells were incubated with a ^{32}P-labeled probe harboring the ERE-2 (Lane 1, 2) of the IGFBP-6 gene. The labeled mutant-type ERE-2 probe was used as a negative control (Lane 3). A ^{32}P-labeled probe harboring ERE-1 (Lane 1-5) was used as a positive control (Lane 5). Responsive banding (see arrow in Figure 5) of the wild-type ERE-1 binding complex was observed (Lane 5). The retarded band of the wild-type and mutant-type ERE-2 binding complex was not observed when a labeled wild-type and mutant-type ERE-2 probe was incubated with nuclear extracts (Lane 2, 3).

Lane	1	2	3	4	5
Labeled wild ERE-2 probe	+	+	−	−	−
Nuclear protein	−	+	+	−	+
Labeled mutated ERE-2 probe	−	−	+	−	−
Labeled wild ERE-1 probe	−	−	−	+	+

development of various tissues (Cummings *et al.*, 2000; Ma *et al.*, 2003; Nautiyal *et al.*, 2004). We previously reported that gene expression of IGF-II is positively regulated by estradiol in MC-3T3-E1 cells. Herein, we found that estradiol strongly stimulates gene transcription of IGFBP-6 in SaOS-2 cells and results in respective 2- and 3.5-fold increases in the gene expressions of IGFBP-6 mRNA and protein. Estradiol differentially regulates production of IGFBP-3 and IGFBP-6 in the MCF-7 cell line (Guo *et al.*, 2007; Martin *et al.*, 1995). E2 ($10^{-8} \sim 10^{-10}$ M) has similar ability to stimulate the induction of collagenase-3 mRNA in UMR 106-01 cells (Partridge *et al.*, 2000). Transfection with either ERα or ERβ causes U2OS cell sensitivity to E2 (Lima *et al.*, 2004; Kallio *et al.*, 2008). We previously reported that E2 (1 μM) does not positively regulate cell proliferation in MC-3T3-E1 cells (Guo *et al.*, 2007). However, herein, SaOS-2 cells were transfected with the wild-type human ERα expression vector and E2 up-regulated cell proliferation 2 fold in SaOS-2 cells, but in dose independent manner within the range of concentration of 0.01 to 1 μM.

Estrogen regulates some gene expression and the activities of proteins in osteoblasts. However, results concerning some of the reported effects are conflicting. Osteoblasts contain both ERα and ERβ estrogen receptors (Arts *et al.*, 1997; Spelsberg *et al.*, 1999). In addition, the level of ERα increases during osteoblastic cell differentiation (Bodine *et*

al., 1998). Stimulation of estrogen-induced gene expression is mediated by the "classical" mechanism, where by estrogen-liganded ER binds directly to the ERE and interacts with coactivator proteins and components of the RNA polymerase II transcription machinery, resulting in either enhanced or inhibited transcription. The consensus ERE contains a palindrome of PuGGTCA motifs separated by 3 bp (Nardulli *et al.*, 1996; Kuntz *et al.*, 1997). By analyzing the human IGFBP-6 gene promoter sequences, we found two putative EREs (from -9 to +1, and from +105 to +115) in the proximal region of the promoter. In the presence of ERE-1 (-9 to +1) in the IGFBP-6 gene promoter, E2 inducted a significant increase in CAT activity in IGFBP-6-pCAT transfected cells (data not shown). Neither ERE-1 nor E2 alone altered CAT activity in IGFBP-6-CAT transfected cells. The results of EMSA using either ^{32}P-labeled consensus ERE-1 (-9 to +1) or ERE-2 (+105 to +115) oligonucleotides (Figure 4 and 5) demonstrated that ERE-1 (-9 to +1) oligonucleotides formed specifically bound ERE-DNA complexes. Co-incubation with ER resulted in a supershifted ER/ERE-DNA retarded band. These results indicate that the functional ERE (-9 to +1) is located in the IGFBP-6 gene promoter. In the presence of ER, E2 up-regulates CAT activity through this ERE in the IGFBP-6 gene promoter. Therefore, estrogen-liganded ER probably binds to the specific functional ERE (-9 to +1)
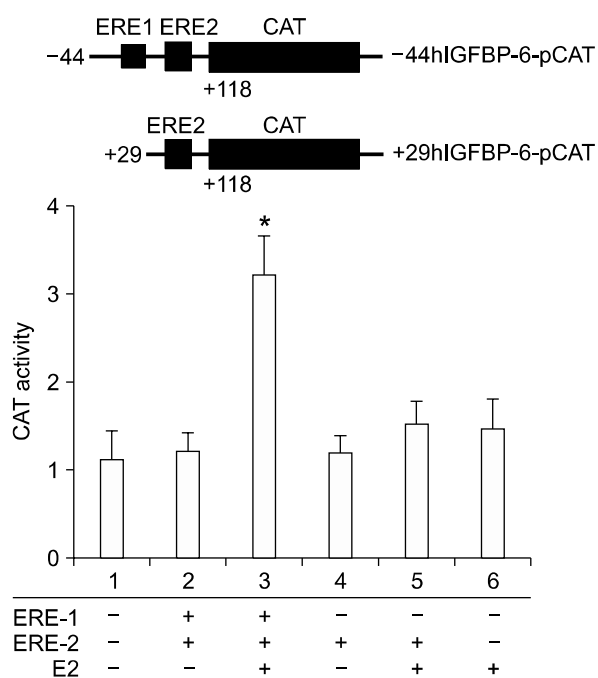
Figure 6. Plasmid constructs and CAT activity in transfected SaOS-2 cells. PCR products from the IGFBP-6 gene promoter were inserted upstream from the luciferase gene in the promoterless pCAT-Basic vector. The putative transcription activation sites are indicated as ERE-1 and ERE-2 in a schematic representation of the constructs (above panel). These constructs were cotransfected with the ERα expression vector into SaOS-2 cells. The transfected cells were then treated with E2 (1 μM) for 24 h. Cellular extracts were prepared and the β-gal and CAT activities were then determined. The CAT activity was normalized to β-gal units and is presented as fold of vehicle control. CAT activities are expressed as the mean ± SEM for three separate determinations in each treatment group. Statistical significance of differences between groups was determined. *indicates a significant difference between the ERE construct and the control group.

located in the IGFBP-6 gene promoter and up-regulates IGFBP-6 gene transcription.

We have identified ERE (5'-CCTTCACCTG-3', -9 to +1) in the IGFBP-6 gene promoter, which is important for E2 responsiveness. ER-mediated transactivation via this ERE is involved in induction of the IGFBP-6 gene by E2 in SaOS-2 cells. Acting as an ER genomic binding site, this functional ERE in the IGFBP-6 promoter region is required for estrogen regulation of IGFBP-6 gene transcription. We are currently investigating the integrating role of E2 regulation of several E2-responsive genes via other nuclear transcription factors.

Methods

Cell culture and treatment with E2

SaOS-2 cells (human osteoblastic-like cell line) were routinely cultivated as a monolayer in DMEM (Invitrogen,

Merelbeke, Belgium) supplemented with 10% FBS (Hyclone, Erebodegem-Aalst, Belgium), 100 IU/ml of penicillin, and 100 μg/ml of streptomycin (Gemini Bio-Products) in an incubator with 5% CO_2 at 37°C. For transient transfection, SaOS-2 cells were plated in 6-well plates and grown to 80% confluence, and then transfected with the 0.2 μg of pSG5HEO (the wild-type human ERα expression vector, Invitrogen) and 10 μl of lipofectamine (Invitrogen). After 16 h, the cells were grown in the presence of E2 (1 μM, 0.1 μM, 0.01 μM) for 24 h before harvesting (Cesario *et al.*, 2007, Guo *et al.*, 2007). E2 was dissolved in 1 μM DMSO. The cells were collected after incubation for 24 h.

Measurement of cell proliferation

After an initial overnight incubation, cells were washed and cell proliferation was determined using the MTT (Sigma-Aldrich) assay. The cells were washed with PBS. MTT was diluted using serum-free medium to obtain a concentration of 0.5 mg/ml, which was added to the culture prior to incubation for 3 h. Formazan extraction was performed using isopropanol, and the quantity of protein was determined using an enzyme linked-immunosorbent assay at 492 nm with four individual samples per group.

RT-PCR

Total RNA was prepared from SaOS-2 cells and used as a template for first strand cDNA synthesis using reverse transcriptase (Invitrogen). Primer sets for semi-quantitative PCR were as follows: IGFBP-6 forward primer, 5'-CTC-TCCTCCTGCCCTGTTCTA-3'; IGFBP-6 reverse primer, 5'-CATGGTCAGGGTTTGTGCCC-3' (508 bp); β-actin forward primer, 5'-AGAGCTACGAGCTGCCTGAC-3'; β-actin reverse primer, 5'- AGTACTTGCGCTCAGGAGGA -3' (299 bp). The PCR reaction mixture contained 50 μl of the single-stranded cDNA product, 4 units of Vent polymerase, 1 μM of each primer, 400 μM level of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$ in a final volume of 100 μl. The mixture was amplified for 30 cycles under the following conditions: denaturing at 94°C for 45 s; primer annealing at 55°C for 45 s; and primer extension at 72°C for 45 s. The PCR products were separated using 2% agarose gel electrophoresis with 100 ng/ml of ethidium bromide. Gels were visualized on a transilluminator and photographed using a gel-automatic formatter (GDS8000, Bio-Rad). Band intensities were analyzed using Fluorchem 2.0 software (Alpha Innotech Corporation). The relative amount of each sample was calculated by normalization to the levels of β-actin mRNA.

Western blot analysis

For immunodetection of the IGFBP-6 protein, SaOS-2 cells were treated with 1 μM E2, 0.1 μM E2, or 0.01 μM E2 for 24 h in 25-cm^2 flasks, and then harvested via trypsinization followed by resuspension in 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.5% desoxycholic acid, and 1 mM dithiothreitol containing aprotinin (10 μg/ml) at 4°C for 30 min. Cells were centrifuged at 100,000 r/min for 30 min. The amount of protein was determined at 595 nm using a protein assay kit

(BioRad, Munich, Germany). Protein extracts (10 μg/lane) were run on 12% SDS polyacrylamide gel, and separated proteins were blotted onto a nitrocellulose membrane (Amersham, Freiburg, Germany). Unbound sites were blocked overnight at 4°C in 10 mM Tris and 0.15 M NaCl (pH 7.4; TBS) containing 5% (wt/vol) skim milk powder. Blots were washed three times for 10 min each time with TBS containing 0.05% Tween-20 (TBST) and incubated for 1 h with the polyclonal anti-human IGFBP-6 antibody (1:1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For verification and quantitation of protein loading, stripped membranes were incubated with the GAPDH antibody (1:400) (Lab Vision Corporation, CA). Blots were again washed three times with TBST as described above, and immunoreactive bands were visualized using ECL plus Western blotting detection reagents (Amersham Pharmacia Biotech Inc, Piscataway, NJ). The antibody-bound protein intensities were analyzed using a Chemi-imager-5500 V 2.03 electrophoresis gel image system. The housekeeping gene product GAPDH was used as an internal control for protein integrity. The target proteins are quantified as relative values against GAPDH as an internal reference.

Electromobility shift assays (EMSA)

Nuclear extracts from SaOS-2 cells were prepared using the method of Guo *et al.* (2007). The concentrations of nuclear extracts were determined according to the Bradford method (Bradford *et al.*, 1976). The putative consensus motifs of EREs are mnCnsTGACC and mrCnnTGACC (m=A, C; n=T, C, G, A) as predicted using Transcription Element Search Software (TESS), while the sequences in a human IGFBP-6 promoter DNA fragment are 5'-CCT-TCACCTG-3' (-9 to +1) and 5'-AACCCTGACC-3' (+105 to +115). The initiation site for transcription is marked by a star and numbered as +1. The putative ERE probes (oligonucleotides) in the human IGFBP-6 promoter were synthesized, including the wild-type ERE-1 probe (DNA sequence is 5'-CCACCCCCCTTCACCTGGCTCTTA A-3' and 5'-TTA-AGAGCCAGGTGAAGGGG-3') (-15 to +9), the wild-type ERE-2 probe (DNA sequence is 5'-GACGGGGCACAA-ACCCTGACCATGA-3' and 5'-TCATGGTCAGGGTTTGT-GCCCCGTC-3') (+94 to +119), and the mutant- type ERE-1 probe (DNA sequence is 5'-CCACCCCCCTCCGAACT-GCTCTTAA-3' and 5'-TTAAGAGCAGTTCGGAGGGGGG-TGG-3'). This mutant-type ERE-1 probe was used as a negative control. It does not contain any known binding sequences. The ERE oligonucleotides were chemically synthesized, annealed, and labeled at the 5'-ends using polynucleotide kinase and [γ-^{32}P] d-ATP (3,000 Ci/mmol). The labeled wild-type EREs and the mutant ERE probe were incubated with 5 μg of nuclear extract and 1 μg of poly (dI-dC) in a buffer containing 10 mM HEPES (pH 7.5), 50 mM KCl, 5 mM MgCl$_2$, 0.5 mM EDTA, 1 mM dithiothreitol, and 12.5% glycerol on ice for 90 min. Reaction mixtures were loaded onto 8% polyacrylamide gel (acrylamide/bisacrylamide ratio, 29: 1) and run at 110 V in 0.375 × TBE (0.09 M Tris, 0.09 M borate, 2 mM EDTA, pH 8.3). For competition analysis, 50-fold and 100-fold molar excesses of the cold ERE probe were added to the reaction mixture before addition of the labeled probe. To

identify the specific ER in DNA-ER complexes, the ERα antibody (1:1000) (rabbit polyclonal IgG against the C terminus of the ERα, Santa Cruz Biotechnology, CA) was added to the reaction mixture after labeled wild-type EREs were incubated with nuclear extracts for 30 min at 4°C. The protein-nucleic acid complexes were identified using autoradiography for 48 h at -70°C.

Plasmid constructs

The chloramphenicol acetyltransferase (CAT) reporter constracts, -44IGFBP-6-pCAT and +29IGFBP-6-pCAT containing the human IGFBP-6 gene promoter sequences from -44 to +118 and from +29 to +118, respectively, were prepared using PCR-amplified DNA fragments and CAT reporter plasmids derived from pCAT-Basic (Promega).

These two DNA fragments were amplified by PCR with the forward primers 5'-AGTTTAGGGAATGCCCGT-3' and 5'-CGGCTACTTAAGACAGAG-3', and the common reverse primer 5'-CATGGTCAGGGTTTGTGCCC-3' using human genomic DNA as a template. The -44IGFBP-6-pCAT construct contains putative ERE-1 (-9 to +1) and ERE-2 (+105 to +115) located in the promoter region of the IGFBP-6 gene. In contrast, the +29 IGFBP-6-CAT construct contains only putative ERE-2. All constructs were confirmed using nucleotide sequence analysis (Figure 6).

Cell culture and transient cotransfection assays

The SaOS-2 cells were grown in DMEM supplemented with 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Cells were maintained in a 5% CO$_2$, 95% air humidified atmosphere at 37°C. For transient transfection, SaOS-2 cells were plated in 6-well plates and grown to 80% confluence, and then transfected with 5 μg of the reporter construct (-44 IGFBP-6-pCAT or +29 IGFBP-6-pCAT), 0.2 μg of pSG5HEO (the wild-type human ERα expression vector, Invitrogen) and 10 μl of Lipofectamine (Invitrogen, 11668-027). An amount of 0.2 μg of the pSV-β-Galactosidase vector (Promega) was added as an internal control. After 16 h, the cells were grown in the presence of E2 (1 μM) for 24 h before harvesting. Cell extracts were prepared by three rapid freeze /thaw cycles and the CAT activity was assayed using a CAT-ELISA kit (Roche, Germany). The transfection efficiency was normalized by measuring the β-galactosidase (β-gal) activity in the extracts. The β-gal activity was measured by adding 50 μl of cell lysate to 500 μl of buffer A [100 nM Na$_2$HPO$_4$ (pH 7.2), 10 mM KCl, 1 mM MgCl$_2$, 10 mM β-mercaptoethanol, and 15 mg/ml of β-D-galactoside sodium (Calbiochem, Germany)] and read at O.D.574. CAT activity is presented as fold of vehicle control.

Data analysis

Data are presented as the mean ± SEM. A one-way analysis of variance (ANOVA) followed by the post hoc Tukey-Kramer test was used to determine differences among multiple groups. Student's *t*-test was used to determine differences between two groups. $P < 0.05$ was defined as the level of statistical significance.

Acknowledgements

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