

# Estrogen receptor regulates insulin-like growth factor-I receptor gene expression in breast tumor cells: involvement of transcription factor Sp1

Sharon Maor, Doris Mayer<sup>1</sup>, Ronit I Yarden<sup>2</sup>, Adrian V Lee<sup>3</sup>, Rive Sarfstein, Haim Werner and Moshe Z Papa<sup>4</sup>

Department of Human Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel

<sup>1</sup>Group on Hormones and Signal Transduction, German Cancer Research Center, Heidelberg D-69120, Germany

<sup>2</sup>Laboratory of Genomic Applications, Chaim Sheba Medical Center, Tel Hashomer 52621, Israel

<sup>3</sup>Department of Medicine, Baylor College of Medicine, Houston, Texas 77030, USA

<sup>4</sup>Department of Oncological Surgery, Chaim Sheba Medical Center, Tel Hashomer 52621, Israel

(Requests for offprints should be addressed to H Werner; Email: hwerner@post.tau.ac.il)

## Abstract

The insulin-like growth factors, IGF-I and IGF-II are a family of mitogenic polypeptides with important roles in growth and differentiation. The biological actions of the IGFs are mediated by the IGF-I receptor (IGF-IR), a cell-surface tyrosine kinase, whose activation by serum IGF-I seems to be a key step in breast cancer initiation. Evidence accumulated indicates that estrogens stimulate the expression and activity of IGF axis components. The aim of our study was to examine the transcriptional mechanisms involved in regulation of *IGF-IR* gene expression by the estrogen receptor (ER). For this purpose, transient transfections using an IGF-IR promoter-luciferase reporter plasmid were performed in breast cancer-c derived ER-positive MCF-7 cells and isogenic ER-negative C4 cells. To examine the potential involvement of zinc-finger nuclear proteins in the transactivating effect of estrogens, chromatin immunoprecipitation (ChIP) experiments were performed using an Sp1 antibody,

along with the Sp1-family-binding inhibitor Mithramycin A. The results obtained indicate that basal IGF-IR promoter activity was 5–8-fold higher in MCF-7 than in C4 cells. Estradiol treatment significantly activated the IGF-IR promoter in MCF-7, but not in C4 cells. Furthermore, the estrogen responsive region in the IGF-IR promoter was mapped to a GC-rich sequence located between nucleotides –40 and –188 in the 5' flanking region. ChIP experiments revealed that at least part of the estrogen effect on IGF-IR expression was mediated through activation of the Sp1 transcription factor. In summary, our studies demonstrate that *IGF-IR* gene transcription in breast cancer cells is controlled by interactions between ER $\alpha$  and Sp1. Dysregulated expression of the *IGF-IR* gene may have pathologic consequences with relevance in breast cancer etiology.

*Journal of Endocrinology* (2006) **191**, 605–612

## Introduction

The insulin-like growth factors, IGF-I and IGF-II, are a family of mitogenic polypeptides with important roles in growth and differentiation. The biological actions of the IGFs are mediated by the IGF-I receptor (IGF-IR), a transmembrane tyrosine kinase structurally and evolutionarily related to the insulin receptor (LeRoith *et al.* 1995, Baserga *et al.* 1997, Werner & LeRoith 2000). The IGF-IR axis has a central role in cell cycle progression, as demonstrated by the fact that receptor overexpression in fibroblasts abrogates all requirements for additional growth factors in order to promote cellular proliferation (Pietrkowski *et al.* 1992). In addition, evidence is mounting for a pivotal role for the IGF-IR in tumorigenesis (Werner & LeRoith 1996, Baserga 1999). Essentially, all components of the IGF system are

expressed in breast tumors, including ligands, receptors, and binding proteins (IGFBPs) (Yee *et al.* 1989, Schnarr *et al.* 2000, Surmacz 2000). The central role of the IGF-IR as a mediator of both IGF-I and IGF-II action in breast cancer is illustrated by the results of several groups showing that blockage of the IGF-IR by a variety of methods, including anti-IGF-IR antibodies, antisense oligonucleotides against IGF-IR mRNA, and IGFBPs significantly inhibited cellular proliferation (Arteaga *et al.* 1989, Van der Burg *et al.* 1990, Surmacz *et al.* 1998). Further evidence in support of an important role for the IGF system in breast carcinogenesis is also provided by epidemiological studies, showing a positive correlation between circulating IGF-I concentrations and relative risk of breast cancer (Hankinson *et al.* 1998).

Solid evidence accumulated in recent years indicating that the biological activity of the IGF system is strongly associated

with estrogen status (Lee *et al.* 1999, Yee & Lee 2000). Estrogens were shown to increase IGF-I binding and IGF-IR mRNA levels in MCF-7 cells by sevenfold, suggesting that the potential mechanism by which estrogens stimulate breast cancer cell proliferation involves sensitization to the mitogenic effects of IGFs by enhancing IGF-IR concentration (Stewart *et al.* 1990). In addition, estrogens can modulate IGF signaling by regulating the expression of other members of the IGF family, including ligands, IGFBPs, and insulin receptor substrate (IRS)-1 (Osborne *et al.* 1989, McGuire *et al.* 1992, Salerno *et al.* 1999). Using MCF-7-derived sublines that have been selected for loss of estrogen receptor  $\alpha$  (ER $\alpha$ ) by long-term estrogen withdrawal, it was previously demonstrated that the loss of ER $\alpha$  caused reduced expression of IGF-signaling molecules and failure to proliferate in response to IGF-I or estrogen. Re-expression of ER $\alpha$  restored the IGF-responsive phenotype, suggesting that ER $\alpha$  is a crucial regulator of the IGF mitogenic loop (Oesterreich *et al.* 2001).

The IGF-IR promoter is a TATA-less, CAAT-less, and initiator type of promoter. The region flanking the transcription start site is extremely GC-rich, and contains numerous potential binding sites for transcription factor Sp1, a zinc-finger-containing nuclear protein that has been shown to strongly transactivate the IGF-IR promoter (Beitner-Johnson *et al.* 1995). Given the involvement of ER $\alpha$  in control of *IGF-IR* gene expression and IGF-I action, and in view of the fact that the transcriptional mechanisms involved in regulation of the *IGF-IR* gene by ER $\alpha$  have not yet been characterized, the present study was designed to analyze the molecular mechanisms responsible for regulation of the *IGF-IR* gene by ER $\alpha$ . Specifically, we hypothesized that ER $\alpha$  regulates *IGF-IR* gene expression through interaction with transcription factor Sp1. The results obtained prove that ER $\alpha$  is a potent transactivator of the *IGF-IR* gene and that GC-rich sequences in the proximal IGF-IR promoter region are required for this effect. Impaired interactions between ER $\alpha$  and zinc-finger proteins may lead to aberrant IGF-IR expression in breast cancer cells.

## Materials and Methods

### Cell cultures

The MCF-7 human breast cancer cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 50  $\mu$ g/ml gentamicin sulfate. C4 cells were generated by clonal selection of MCF-7 cells, cultured in estrogen-free medium for 9 months (Oesterreich *et al.* 2001). C4 cells were kindly provided by Dr Wade V Welshons (University of Missouri, Columbia, MO, USA). The C4 cell line was maintained in DMEM without phenol red supplemented with 10% charcoal-stripped FBS, 2 mM glutamine, and 50  $\mu$ g/ml gentamicin sulfate.

### Western immunoblots

Cells were serum-starved overnight and then incubated with increasing concentrations of  $\beta$ -estradiol (Sigma-Aldrich Corp.) for 24 h. Following incubation, cells were harvested and lysates were prepared as described previously (Idelman *et al.* 2002). Samples (50  $\mu$ g) were subjected to 8% SDS-PAGE, followed by electrophoretic transfer of the proteins to nitrocellulose membranes. After blocking with 3% bovine serum albumin or milk in T-TBS (20 mM Tris-HCl, pH 7.5, 135 mM NaCl, and 0.1% Tween-20), blots were incubated with polyclonal antibodies against IGF-IR  $\beta$  subunit, Sp1 and ER $\alpha$  (C-20, PEP-2, and MC-20 respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and against actin or tubulin, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Proteins were detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). In selected experiments, the Sp1-family inhibitor Mithramycin A (Sigma-Aldrich Corp.) was added at a concentration of 500 nM 1 h before the estradiol treatment.

### IGF-IR promoter activity measurements

For transient transfection experiments, luciferase reporter constructs p(-2350/+640)LUC, p(-476/+640)LUC, p(-188/+640)LUC, and p(-40/+640)LUC (nucleotide 1 corresponds to the transcription start site) were employed. These plasmids include 2350, 476, 188, or 40 nucleotides of the 5'-flanking region, linked to 640 bp of the 5'-untranslated region of the rat *IGF-IR* gene. The basal promoter activities of these constructs were previously reported (Maor *et al.* 2000). The ER $\alpha$  expression vector was generated by subcloning the human ER $\alpha$  cDNA into the pSG5 vector using EcoRI sites. The ER $\alpha$  vector was provided by Dr Yitzhak Koch, Weizmann Institute of Science, Rehovot, Israel. An Sp1 expression vector (pPacSp1), under the control of an actin promoter in the pPac0 vector, was provided by Dr Robert Tjian (University of California, Berkeley, CA, USA). Cells were seeded in six-well plates 48 h before transfection, and transfected with 0.5  $\mu$ g of the indicated IGF-IR promoter reporter plasmids, along with 1.3  $\mu$ g ER $\alpha$  expression vector (or empty pSG5 vector), 1  $\mu$ g pPacSp1 (or empty pPac0 vector), and 0.2  $\mu$ g  $\beta$ -galactosidase expression plasmid (pCMV $\beta$ , Clontech, Palo Alto, CA, USA), using the jetPEI reagent (Polyplus Transfection, Illkirch, France). Twenty-four hours after transfection, cells were treated with estradiol (1, 10 and 100 nM) or left untreated. Cells were harvested after an additional 24 h, and luciferase and  $\beta$ -galactosidase activities were measured as previously described (Maor *et al.* 2000). In addition, some experiments included Mithramycin A. Mithramycin A was added 1 h before estradiol treatment at a concentration of 500 nM.

### Chromatin immunoprecipitation (ChIP) studies

Estradiol-treated and untreated cultures were incubated with formaldehyde (1% final concentration) for 10 min at room

temperature. At the end of the incubation period, cells were washed twice and harvested using ice-cold phosphate-buffered saline. Pelleted cells were resuspended in 1% SDS-containing buffer, incubated on ice for 10 min, and sonicated for 3 min. Cell extracts were then immunoprecipitated with anti-Sp1 or anti-ER $\alpha$  for 18 h at 4°C. For PCR analysis of Sp1/ER $\alpha$ -immunoprecipitated chromatin, a set of primers encompassing the IGF-IR proximal promoter region (nt -458 to +53) was employed, using the following primers: sense, 5'-CTTTCCAGCCGCGCTGTTGTTG-3'; anti-sense, 5'-GGTAAACAAGAGCCCCAGCCTC-3'. Sequencing analyzes revealed the presence of *cis*-acting elements for transcription factor Sp1 in this particular region (Cooke *et al.* 1991). PCR was performed using the TernalAce DNA polymerase (InVitrogen).

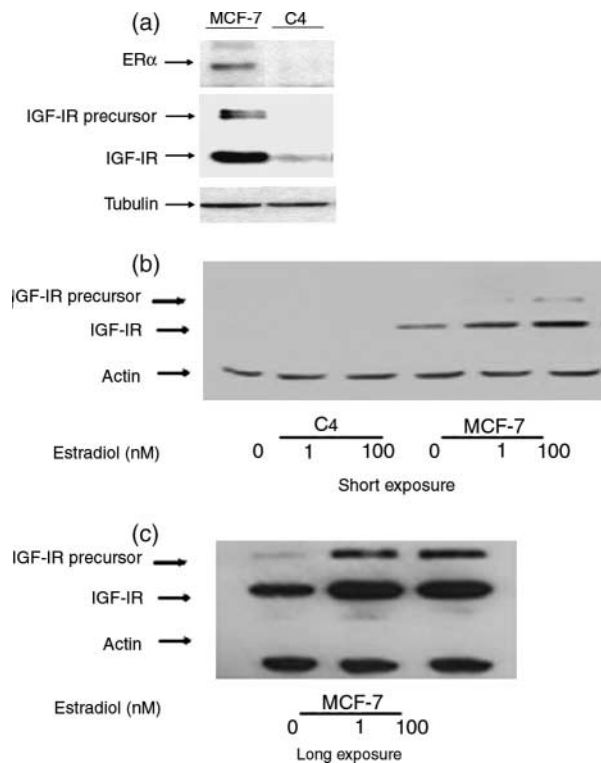
## Results

### Effect of ER status on IGF-IR expression

To evaluate the potential role of ER on IGF-IR levels, we employed the human breast cancer-derived MCF-7 (ER-positive) and MCF-7-derived C4 (ER-negative) cell lines. C4 cells were generated by clonal selection of MCF-7 cells that were maintained for 9 months in estrogen-free culture medium (Oesterreich *et al.* 2001). Although C4 cells exhibited certain variability in ER expression, the passages used in the present study displayed consistently low levels of ER $\alpha$  (Fig. 1a). Results of western immunoblots using an anti-IGF-IR  $\beta$ -subunit antibody showed that IGF-IR levels in ER $\alpha$ -depleted C4 cells were 13.6% of those in control MCF-7 cells (Fig. 1a). These results replicate previously reported results (Reizner *et al.* 2005). To address the responsiveness of MCF-7 and C4 cells to estradiol treatment, cells were serum-starved overnight and then stimulated with increasing concentrations of estradiol for 24 h, after which cell lysates were prepared. Western blot analysis revealed that estradiol stimulated precursor and mature IGF-IR levels in MCF-7 cells in a dose-dependent manner (Fig. 1b and c), whereas no changes were seen in C4 cells (Fig. 1b). Maximal stimulation was achieved at a dose of 100 nM estradiol (3.3- and 7-fold increase in mature and pre-IGF-IR levels respectively).

### Effect of estradiol on IGF-IR promoter activity

To establish whether the differences in IGF-IR gene expression between ER-positive and ER-negative cells are mediated at the level of transcription, MCF-7 and C4 cell lines were transiently transfected with a luciferase reporter construct under the control of the proximal IGF-IR promoter region (p(-476/+640)LUC). Transfected cells were incubated in the absence or presence of estradiol (100 nM) during the last 24 h of the incubation period. Forty-eight hours after transfection, cells were harvested and luciferase and  $\beta$ -galactosidase activities were measured.

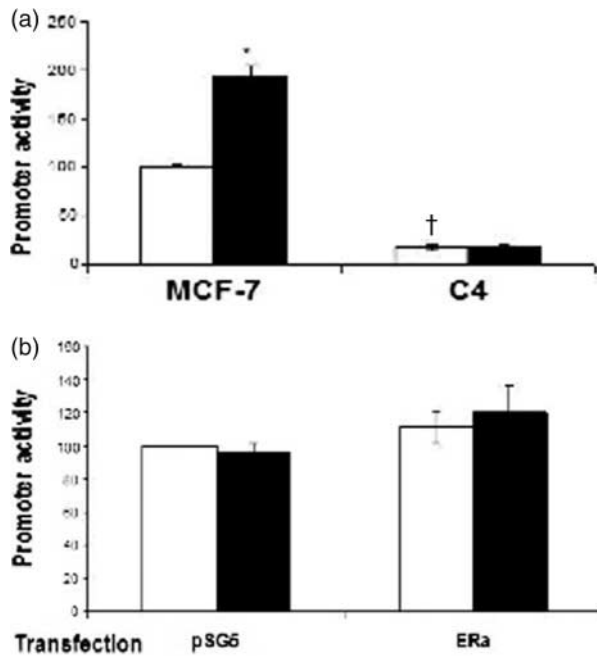


**Figure 1** Effect of ER status on IGF-IR expression. (a) Untransfected MCF-7 and C4 cells were harvested, cellular pellets were lysed as indicated in Materials and Methods, and equal amounts of protein (50  $\mu$ g) were separated by 8% SDS-PAGE. After electrophoresis, proteins were transferred onto nitrocellulose membranes, and blotted with anti-IGF-IR  $\beta$ -subunit and anti-ER $\alpha$  antibodies, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Membranes were re-probed with a tubulin antibody. The figure shows the results of a typical experiment, repeated three times. (b and c) Serum-starved MCF-7 cells and ER-negative C4 cells were incubated with 0, 1, or 100 nM estradiol for 24 h. After incubation, cells were lysed, and processed as described above. The migration positions of the ~97 kDa IGF-IR  $\beta$ -subunit and ~250 kDa IGF-IR precursor proteins are indicated. Membranes were re-probed with an actin antibody. Short and long exposures are presented to show the dose-dependent effect of estradiol on the low-abundance IGF-IR precursor. The figure shows the result of a typical experiment, repeated at least three times.

The results obtained indicated that basal IGF-IR promoter activity was 5.8-fold higher in MCF-7 than in C4 cells. In addition, estradiol activated IGF-IR promoter in MCF-7 cells (1.9-fold), whereas it had no effect in C4 cells (Fig. 2a). Furthermore, coexpression of C4 cells with an ER $\alpha$  expression vector did not confer upon the cells the ability to respond to estradiol (Fig. 2b).

### Mapping the promoter region responsible for induction of IGF-IR gene expression by estradiol

To define the IGF-IR promoter regions responsible for mediating the observed transcriptional activity of ER $\alpha$  in



**Figure 2** Regulation of IGF-IR promoter activity by estradiol. (a) MCF-7 and C4 cells were transiently transfected with a luciferase reporter gene under the control of the proximal IGF-IR promoter, p(-476/+640)LUC. Twenty-four hours after transfection, cells were incubated in the absence (open bars) or presence (solid bars) of 100 nM estradiol for another 24 h. At the end of the incubation period cells were harvested and luciferase and  $\beta$ -galactosidase activities were measured. Promoter activities are expressed as luciferase values normalized to the corresponding  $\beta$ -galactosidase values. A value of 100% was given to the basal promoter activity in MCF-7 cells. The results represent the mean  $\pm$  S.E.M. of 7–10 independent experiments, performed in duplicate dishes. \* $P < 0.01$  versus untreated cells, † $P < 0.01$  versus MCF-7 cells. (b) C4 cells were cotransfected with the p(-476/+640)LUC reporter construct along with an ER $\alpha$  expression plasmid (or empty expression vector). Twenty-four hours after transfection, cells were incubated in the absence (open bars) or presence (solid bars) of 100 nM estradiol for another 24 h. The promoter activity generated by the reporter plasmid in empty vector-transfected, untreated C4 cells was given a value of 100%. Data are means  $\pm$  S.E.M. of 6–10 experiments, performed each time in duplicate.

MCF-7 cells, cotransfections were performed using a series of IGF-IR promoter deletion constructs containing different portions of 5'-flanking and 5'-untranslated regions (Fig. 3a), along with the ER $\alpha$  expression vector. Twenty-four hours after transfection cells were treated with estradiol for an additional 24 h (or left untreated) and, after an additional 24 h cells were harvested and luciferase and  $\beta$ -galactosidase values were measured. Results of deletion analysis showed that estrogen-stimulated promoter activity of the p(-476/+640)LUC and p(-188/+640)LUC constructs by 2.8- and 2.4-fold respectively. The basal promoter activity of the full-length p(-2350/+640)LUC construct was twofold lower than that of the proximal p(-476/+640)LUC plasmid, but was still responsive to estradiol stimulation. Removal of the

promoter fragment located between -188 and -40 completely abrogated the stimulatory effect of estradiol, suggesting that the estrogen responsive region in the IGF-IR promoter was mapped to a fragment located between nucleotides -188 and -40 in the 5' flanking region (Fig. 3b).

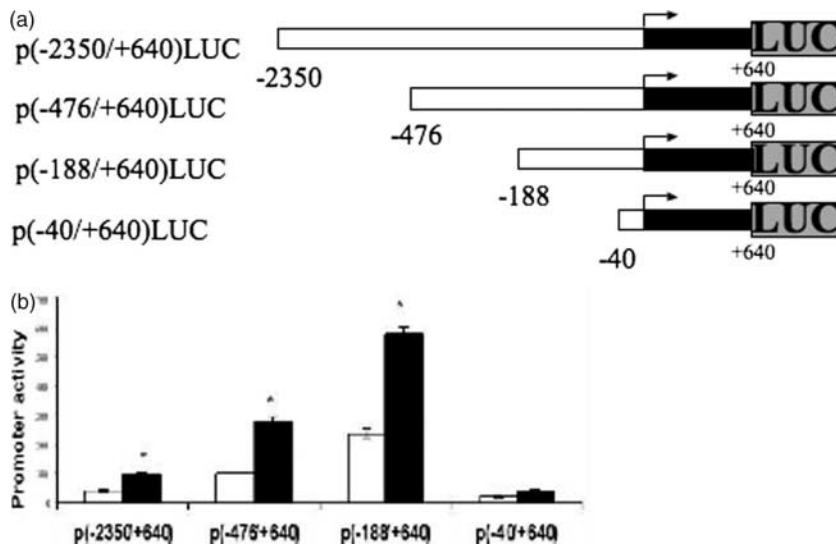
#### Estimation of Sp1 levels in MCF-7 and C4 cells

Previous studies have identified a number of Sp1-binding sites in the IGF-IR promoter region (Beitner-Johnson *et al.* 1995). To begin to examine the potential involvement of transcription factor Sp1 in the ER-induced IGF-IR expression, we measured Sp1 levels in MCF-7 and C4 cells. To this end, cells were serum-starved overnight and then stimulated with increasing concentrations of estradiol for 24 h, after which cell lysates were prepared. Western blot analysis revealed that basal Sp1 levels in MCF-7 cells were twofold higher than in C4 cells. No starvation-induced decrease in Sp1 levels was seen at 24 h (data not shown). As shown in Fig. 4a, no change in Sp1 levels was seen after estradiol treatment. To more directly evaluate whether Sp1 is involved in ER action, we cotransfected C4 cells with an Sp1 expression vector (or empty pPac0), in the presence of the ER $\alpha$  expression vector (or empty pSG5) and the p(-476/+640)LUC reporter construct. Luciferase measurements revealed that, in the presence of Sp1, ER $\alpha$ -stimulated IGF-IR promoter activity by 2.2-fold compared with cells transfected with Sp1 or ER $\alpha$  alone (Fig. 4b).

To further corroborate the role of Sp1 on the stimulatory effect of estradiol, we employed the Mithramycin A inhibitor. Mithramycin A has been shown to block the activity of Sp1-family members by binding GC-rich sequences in target promoters. Addition of Mithramycin A abrogated the estradiol-stimulated increase in IGF-IR promoter activity in cultures transfected with both the p(-476/+640)LUC and p(-188/+640) constructs (Fig. 5a).

#### ChIP analysis of the physical interactions between Sp1, ER $\alpha$ , and the IGF-IR promoter

Next, the potential physical interactions between Sp1, ER $\alpha$ , and the IGF-IR promoter region were assessed using ChIP assays. Untransfected MCF-7 and C4 cells were incubated with estradiol (100 nM) for 24 h (or left untreated, for control purposes), after which cells were processed as described under Materials and Methods. Cell lysates were immunoprecipitated with anti-Sp1 or anti-ER $\alpha$  and the precipitated chromatin was amplified by PCR using primers encompassing nt -458 to +53 of the IGF-IR promoter. Results of ChIP assays showed that estradiol treatment enhanced Sp1 binding to the IGF-IR promoter region in MCF-7 cells by 194% and ER $\alpha$  binding by 187% (Fig. 5b and c). No ER $\alpha$  binding to the IGF-IR promoter was detected in C4 cells (data not shown).



**Figure 3** Deletion analysis of the estradiol-mediated induction of IGF-IR promoter activity. (a) Schematic representation of IGF-IR promoter-luciferase reporter constructs used in transient transfection experiments. Plasmids contained 2350, 476, 188, or 40 bp of the *IGF-IR* gene 5' flanking region and 640 bp of the 5'-untranslated region, fused to a luciferase cDNA. The transcription start site is denoted by the arrow. The luciferase cDNA is not shown to scale. (b) MCF-7 cells were cotransfected with the indicated reporter constructs, together with an ER $\alpha$  expression vector. The activity generated by the p(-476/+640)LUC construct in the absence of estradiol was given a value of 100%. The open bars represent the basal activities of each of the promoter constructs, and the solid bars represent their activities after stimulation with estradiol for 24 h. Data are expressed as means  $\pm$  S.E.M. of 5–14 independent experiments, performed each time in duplicate dishes. \* $P < 0.01$  versus untreated cells.

## Discussion

The IGF system performs multiple functions in mammary gland biology. IGF-I and IGF-II, working through the IGF-IR, were shown to promote proliferation, inhibit cell death, and stimulate transformation of breast cancer cells (Sachdev & Yee 2001). Immunohistochemical and *in situ* hybridization assays revealed that the IGF-IR and ER are coexpressed in breast cancer cells, while biochemical and genetic analyzes identified a complex crosstalk between both pathways (Yee & Lee 2000). This crosstalk is of particular relevance since ER status is an important prognostic factor in breast cancer, whereas anti-estrogens such as tamoxifen are widely used in the treatment of the disease (Sachdev & Yee 2001). Several reports have shown that estrogens can affect IGF action in breast cancer cells by altering expression of various members of the IGF family. Specifically, estrogens can modulate the expression of IGF ligands, receptors, IGFBPs, and downstream signaling mediators such as IRS-1 and others (Osborne *et al.* 1989, Stewart *et al.* 1990, McGuire *et al.* 1992, Lee *et al.* 1999, Salerno *et al.* 1999). In the present study we investigated the transcriptional mechanisms involved in estrogen regulation of *IGF-IR* gene expression.

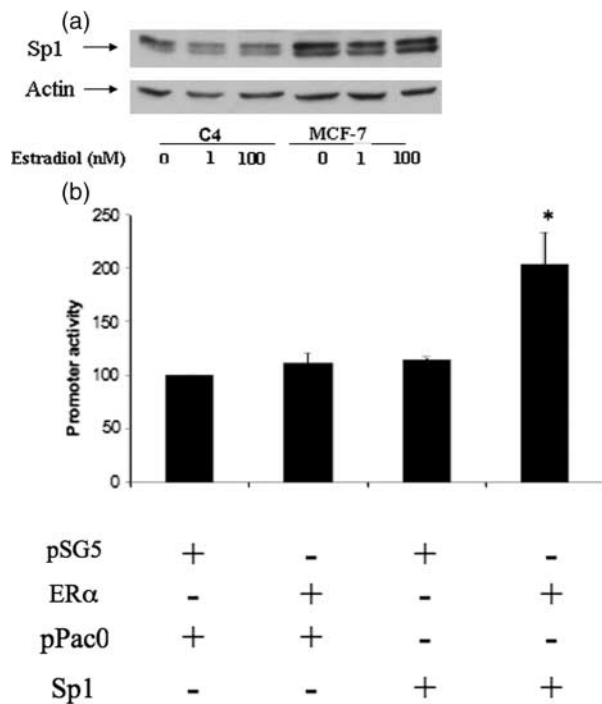
The results obtained show that ER status is strongly correlated with IGF-IR levels and that estradiol is a potent inducer of IGF-IR expression. Furthermore, results of transient transfection experiments show that the effect of

estradiol on IGF-IR levels is mediated at the transcriptional level. In addition, deletion analysis using a series of IGF-IR promoter constructs containing different portions of 5'-flanking and 5'-untranslated regions shows that removal of the promoter fragment located between -188 and -40 completely abrogated the stimulatory effect of estradiol, suggesting that the estrogen responsive region in the IGF-IR promoter was mapped to a proximal promoter fragment located between nucleotides -40 and -188. Taken together, our results are consistent with those of Bartucci *et al.* (2001) showing that IGF-IR growth-related functions in breast cancer cells depend on ER expression. Interestingly, the basal activity of the full-length IGF-IR promoter (p(-2350/+640)LUC) is significantly lower than that of proximal promoter fragments (p(-476/+640)LUC and p(-188/+640)LUC). These results are consistent with the existence of still unidentified silencer element/s in the distal promoter region. However, the biological role of these elements as well as their potential interactions with ER $\alpha$  and Sp1 are yet to be elucidated.

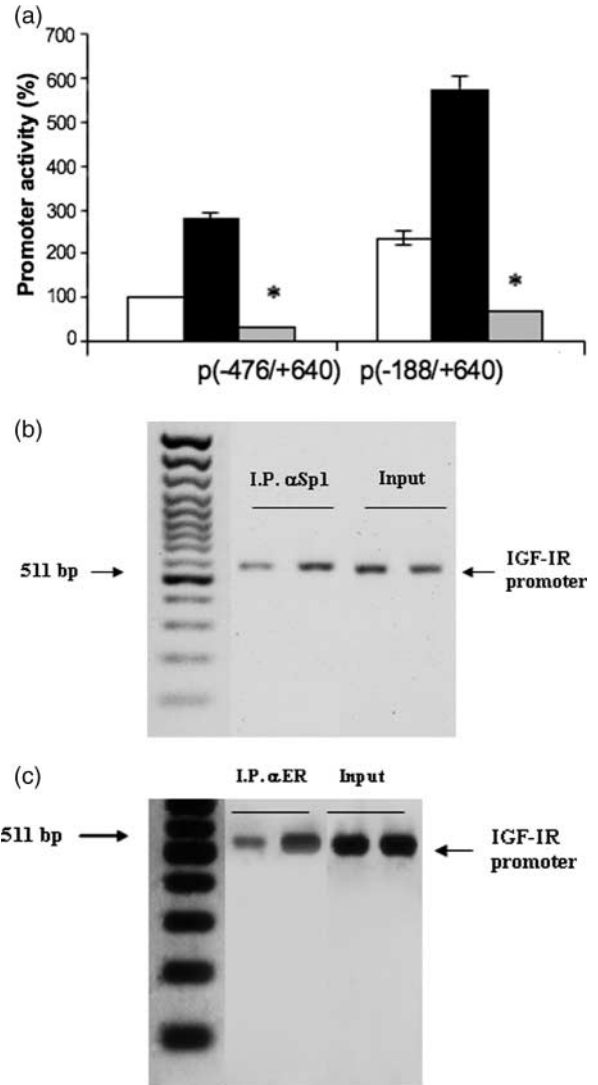
The classical estrogen response element (ERE) consensus sequence was originally defined as GGTCANNNTGACC and shown to be directly bound by ER $\alpha$ . However, several estrogen-responsive genes have been identified that utilize divergent EREs, or even other transcription factor DNA-binding sites as the target sequence for ER $\alpha$  action. This family of genes includes those in which the effect of ER $\alpha$

is mediated through activating protein-1, zinc-finger protein Sp1, or nuclear factor- $\kappa$ B (NF- $\kappa$ B), and others in, which ER $\alpha$  is thought to act through as yet unidentified protein/s (Zou *et al.* 1999).

Previous studies have identified transcription factor Sp1 as a critical transactivator of the IGF-IR gene (Beitner-Johnson *et al.* 1995). The IGF-IR promoter region includes multiple Sp1-binding sites, whose presence is crucial for Sp1 transactivation. Interestingly, Sp1 levels in ER-positive MCF-7 cells were significantly higher than in ER-negative C4 cells. No change in Sp1 levels, however, was seen following estradiol treatment. Expression of Sp1 in conjunction with ER $\alpha$ , but not ER $\alpha$  alone, in C4 cells, induced a strong stimulation of IGF-IR promoter activity. These results are consistent with a crucial role for Sp1 in the ER-induced



**Figure 4** Evaluation of Sp1 levels in MCF-7 and C4 cells. (a) Serum-starved MCF-7 and C4 cells were incubated with 0, 1, or 100 nM estradiol for 24 h. After incubation, cells were lysed as indicated in Materials and Methods, and equal amounts of protein (50  $\mu$ g) were separated by 8% SDS-PAGE and transferred onto nitrocellulose membranes. Levels of Sp1 were determined using a polyclonal antibody against Sp1, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. The positions of the 95 and 106 kDa Sp1 proteins are indicated. Membranes were re-probed with an actin antibody. The figure shows the result of a typical experiment, repeated at least three times. (b) C4 cells were cotransfected with the IGF-IR promoter p(-476/+640)LUC construct, along with ER $\alpha$  and Sp1 expression plasmids (or empty pSG5 and pPac0 vectors respectively). Promoter activities were determined 48 h after transfection. The activity generated in the absence of Sp1 and ER $\alpha$  expression plasmids was given a value of 100%. Data are means  $\pm$  S.E.M. of 5–10 experiments, performed each time in duplicate. \* $P < 0.03$  versus cells transfected with ER $\alpha$  alone.



**Figure 5** Involvement of transcription factor Sp1 in the estradiol-induced IGF-IR gene expression. (a) MCF-7 cells were cotransfected with the p(-476/+640)LUC or p(-188/+640)LUC promoter construct, along with an ER $\alpha$  expression vector, as described in Materials and Methods. After 24 h, Mithramycin A (500 nM) was added for 1 h (gray bars), after which estradiol (100 nM) was added to the cultures (solid and gray bars). Control cells were left untreated (open bars). Luciferase values were measured after an additional 24 h. A value of 100% was given to the promoter activity measured in untreated cells in the absence of Mithramycin A. Results are means  $\pm$  S.E.M. of 2–14 independent experiments. \* $P < 0.01$  versus estradiol only treated cells. (b and c) MCF-7 cells were incubated with estradiol (100 nM) for 24 h (or left untreated). After incubation, cells were lysed and immunoprecipitated with an Sp1 antibody (b) or an ER $\alpha$  antibody (c), followed by PCR amplification of precipitated chromatin using primers encompassing the IGF-IR promoter (nt -458 to +53). The position of the 511 bp-amplified fragment is indicated. The input bands represent the amplified PCR product in the absence of antibodies.

IGF-IR gene transactivation. The important role of Sp1 is further corroborated by results of experiments showing that Mithramycin A treatment abrogated the estradiol-stimulated increase in IGF-IR promoter activity. In addition, estradiol enhanced both Sp1 and ER $\alpha$  binding to the IGF-IR promoter region in MCF-7 cells, whereas no ER $\alpha$  binding was detected in C4 cells. Additional genes shown to be regulated by ER $\alpha$ /Sp1 include IGFBP-4, cyclin D1, adenosine deaminase, retinoic acid receptor  $\alpha$ 1, c-fos, cathepsin D, E2F1, transforming growth factor  $\alpha$ , progesterone receptor, epidermal growth factor receptor, vascular endothelial growth factor, and others (Duan *et al.* 1998, Sun *et al.* 1998, Qin *et al.* 1999, Wang *et al.* 1999, 2001, Xie *et al.* 1999, Petz & Nardulli 2000, Salvatori *et al.* 2000, Vyhlidal *et al.* 2000, Castro-Rivera *et al.* 2001, Safe 2001, Khan *et al.* 2003, Kim *et al.* 2005, Koos *et al.* 2005). Interestingly, results of fluorescence resonance energy transfer assays are consistent with *in vitro* studies on ER $\alpha$ /Sp1 interactions and transactivation, and confirmed that ER $\alpha$  and Sp1 interact in living breast cancer cells (Kim *et al.* 2005). Of notice, Oesterreich *et al.* (2001) reported that expression of ER $\alpha$  in C4 cells restored estrogen inducibility of the IGF-IR, whereas in the present study we were able to restore estrogen responsiveness in C4 cells only by coexpression of an Sp1 vector, in addition to ER $\alpha$ . The discrepancy between the results can be most probably explained by the fact that in the present study we employed transient transfection assays, whereas Oesterreich *et al.* (2001) used stable transfected clones.

Our bioinformatic analyzes, as well as a report by Scheidegger *et al.* (2000), indicate that there is no consensus EREs in the IGF-IR promoter. Consistently, using EMSA assays Scheidegger *et al.* (2000) were unable to demonstrate direct binding of ER to sequences in the IGF-IR promoter in rat aortic smooth muscle cells. It is conceivable, however, that half-ERE, which are present in this region, might be involved in this interaction (Vyhlidal *et al.* 2000, Koos *et al.* 2005). The results of ChIP experiments using an ER $\alpha$  antibody in the present study suggest that ER binds to the IGF-IR promoter via Sp1, and that both proteins can form a DNA-binding high molecular weight complex. We have previously demonstrated that transcription of the IGF-IR gene in breast cancer cells is negatively regulated by breast cancer susceptibility gene, BRCA1 (Maor *et al.* 2000). The mechanism of action of BRCA1 was shown to involve specific binding to Sp1, thus preventing this zinc-finger protein from binding to, and transactivating, the IGF-IR promoter (Abramovitch & Werner 2003). We speculate that Sp1 is a central player in transcriptional regulation of the IGF-IR gene that mediates the actions of both proliferative agents (e.g. estrogens) as well as tumor suppressors (e.g. BRCA1). Furthermore, the estrogen-induced downregulation of IGF-IR expression in vascular smooth muscle cells was also shown to involve binding of ER to Sp1, thus preventing Sp1 binding to the IGF-IR promoter (Scheidegger *et al.* 2000).

In summary, our results demonstrate that estradiol activates the IGF-IR promoter in an ER-dependent manner. The estrogen responsive region in the IGF-IR promoter was mapped to a GC-rich sequence located between nucleotides -40 and -188 in the 5' flanking region. In addition, activation of the Sp1 transcription factor is a critical step in estrogen-induced IGF-IR gene expression. Dysregulation of the IGF-IR gene may constitute a key step in breast cancer initiation and progression.

## Acknowledgements

This work was performed in partial fulfilment of the requirements for a PhD degree by S M in the Sackler Faculty of Medicine, Tel Aviv University. We thank Drs R Tjian, W V Welshons, and Y Koch for providing cell lines and reagents. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

## Funding

This work was supported by the Cooperation Program in Cancer Research of the Deutsches Krebsforschungszentrum (DKFZ) and Israel's Ministry of Science and Technology (MOST) (to H W, D M and M Z P).

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Received in final form 27 August 2006

Accepted 19 September 2006

Made available online as an Accepted Preprint  
3 October 2006