

Article

Identification of Insulin-Like Growth Factor-I Receptor (IGF-IR) Gene Promoter-Binding Proteins in Estrogen Receptor (ER)-Positive and ER-Depleted Breast Cancer Cells

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Abstract: The insulin-like growth factor I receptor (IGF-IR) has been implicated in the etiology of breast cancer. Overexpression of the IGF-IR gene is a typical feature of most primary breast cancers, whereas low IGF-IR levels are seen at advanced stages. Hence, evaluation of IGF-IR levels might be important for assessing prognosis. In the present study, we employed a proteomic approach based on DNA affinity chromatography followed either by mass spectroscopy (MS) or Western blot analysis to identify transcription factors that may associate with the IGF-IR promoter in estrogen receptor (ER)-positive and ER-depleted breast cancer cells. A biotinylated IGF-IR promoter fragment was bound to streptavidin magnetic beads and incubated with nuclear extracts of breast cancer cells. IGF-IR promoter-binding proteins were eluted with high salt and analyzed by MS and Western blots. Among the proteins that were found to bind to the IGF-IR promoter we identified zinc finger transcription factors Sp1 and KLF6, ER- α , p53, c-jun, and poly (ADP-ribosylation) polymerase. Furthermore, chromatin immunoprecipitation (ChIP) analysis confirmed the direct *in vivo* binding of some of these transcription factors to IGF-IR promoter DNA. The functional relevance of binding data was assessed by cotransfection experiments with specific expression vectors along with an IGF-IR promoter reporter. In summary, we identified nuclear proteins that are potentially

responsible for the differential expression of the IGF-IR gene in ER-positive and ER-depleted breast cancer cells.

Keywords: transcription factors; estrogen receptor; insulin-like growth factor I receptor promoter; biomarkers; breast cancer

Abbreviations: IGF-IR: Insulin-like growth factor I receptor; ER: Estrogen receptor

1. Introduction

The insulin-like growth factors, IGF-I and IGF-II, are mitogenic polypeptides with important roles in cell growth and differentiation [1]. The biological activities of IGF-I and IGF-II are mediated by the IGF-I receptor (IGF-IR) and are modulated by a family of IGF-binding proteins (IGFBPs) that control ligand bioavailability [2–4]. The IGF-IR is essential for normal cell cycle progression and has a crucial role in tumor growth and metastasis development. Ligand-mediated activation of the IGF-IR tyrosine kinase domain leads to mitogenic and antiapoptotic effects in multiple cells and cancer-derived cell lines [5]. In the specific context of breast tumorigenesis, the IGF-IR is overexpressed in most primary tumors [4]. In addition, and consistent with its potent antiapoptotic role, IGF-IR-null cells do not undergo transformation when exposed to different oncogenes [6,7]. On the other hand, inhibition of IGF-IR signaling by IGF-IR antibodies, antisense oligonucleotides, IGF-BPs, or specific IGF-IR kinase inhibitors results in reduced cell proliferation and cancer progression *in vivo* and *in vitro* [8–14]. Furthermore, epidemiological studies revealed that high levels of circulating IGF-I are linked to an increased risk of developing breast cancer in premenopausal women [15,16].

Regulation of IGF-IR gene expression is mainly attained at the transcription level. The IGF-IR promoter is a TATA-less, CCAAT-less, highly GC-rich, ‘initiator’-type of promoter. IGF-IR gene transcription is dependent on a number of stimulatory zinc-finger nuclear proteins, including Sp1 [17] and KLF6 [18]. In addition, IGF-IR gene transcription is negatively regulated by several tumor suppressors, including BRCA1, p53/p63/p73, the von Hippel-Lindau protein (VHL), and the Wilms’ protein-1 (WT1) [19–25]. Interactions between stimulatory and inhibitory transcription factors play an important role in IGF-IR gene regulation and, therefore, were postulated to have a major impact on the proliferative status of the cell. The molecular mechanisms and specific transcription factors responsible for regulating IGF-IR gene expression in breast cancer cells, however, have not yet been identified.

The IGF-I and estrogen signaling systems were shown to act in a synergistic fashion in breast epithelial cells [25]. Estrogens regulate IGF-I signaling and the expression of several members of the IGF system [25–31]. Moreover, activation of estrogen receptor- α (ER α) by estrogens induces a physical interaction between ER α and IGF-IR [32] that results in activation and phosphorylation of IGF-IR and downstream signaling molecules [33–36]. The aim of this study was to identify the collections of IGF-IR promoter-binding transcription factors in ER-positive and ER-depleted breast cancer cells. Using DNA affinity chromatography, mass spectroscopy (MS), and Western blot analyses, we identified a series of known and previously unidentified transcription factors that

specifically bind to the IGF-IR promoter in either cell type. The ability of selected proteins to bind and transactivate the IGF-IR promoter was confirmed by chromatin immunoprecipitation (ChIP) and transient transfection assays. In addition, we identified a number of non-DNA sequence-specific nuclear proteins that are probably involved in IGF-IR gene regulation. Identification of differentially expressed IGF-IR promoter-binding and non-binding transcription factors may help elucidate the mechanisms responsible for the differential expression of the IGF-IR gene in ER-positive and ER-depleted breast cancer cells.

2. Material and Methods

2.1. Cell Cultures

Human breast cancer-derived MCF7 cells [(ER-positive), American Type Culture Collection, Manassas, VA, USA] were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 µg/mL gentamicin sulfate, and 5.6 mg/L fungizone (Sigma-Aldrich Co., St. Louis, MO, USA). The C4.12.5 cell line was derived by clonal selection of MCF7 cells that were grown in the absence of estrogen for nine months [37]. C4.12.5 cells were maintained in phenol red-free DMEM with 10% charcoal/dextran-treated FBS, 2 mM glutamine, and antibiotics. The C4.12.5 cell line was provided by Dr. Wade V. Welshons (University of Missouri, Columbia, MO, USA). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

2.2. PCR and DNA Affinity Chromatography of the IGF-IR Promoter

For DNA affinity chromatography, a 511-bp human proximal IGF-IR promoter fragment extending from nucleotides -458 to +53 was labeled using a 5'-biotinylated antisense primer. This fragment includes most of the proximal 5'-flanking region and comprises the 'initiator' motif from which transcription starts *in vivo* (Nucleotide 1) (Figure 1).

Primer sequences were derived from genomic IGF-IR clone #R73P2 [38] as follows: sense, 5'-CTTCCAGCCGCGCTGTTGTTG-3'; antisense, 5'-(Biotin)-GGTAAACAAGAGCCCCAGCCTC-3'. PCR was performed using the TernalAceTM DNA Polymerase reagent (Invitrogen, Carlsbad, CA, USA). The biotinylated PCR product was bound to streptavidin magnetic beads (Dynabeads[®] M-270 Streptavidin; Dynak Biotech ASA, Oslo, Norway), and incubated with nuclear extracts of MCF7 or C4.12.5 cells in the presence of double stranded poly dI-dC (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Following high-salt elution, specifically-bound proteins were resolved on 10% SDS-PAGE gels and visualized by silver staining (Figure 2). Gel slices were then submitted for MS analysis at the Smoler Proteomics Center (Technion-Israel Institute of Technology, Haifa, Israel) or analyzed by Western blotting as described below.

Figure 1. IGF-IR promoter. (A) Schematic representation of the IGF-IR promoter region [5]. The initiator (INR) element is denoted by an arrow. The coding region, starting with the AUG codon, is shown in gray. The translation start site is denoted by a dashed arrow. The 5'-flanking region is denoted by a black bar, and the 5'-untranslated region (UTR) is represented by a dotted bar. The location of primers (P#1 and P#2), employed to amplify the proximal promoter is indicated. (B) PCR amplification of the human IGF-IR promoter. The PCR reaction was performed using a biotinylated antisense primer, as described under *Materials and Methods* section, and 4 μ L of the biotinylated PCR product was loaded on a 1% agarose gel. A PCR reaction without DNA served as negative control. A 100-bp DNA ladder was used as a M.W. marker.

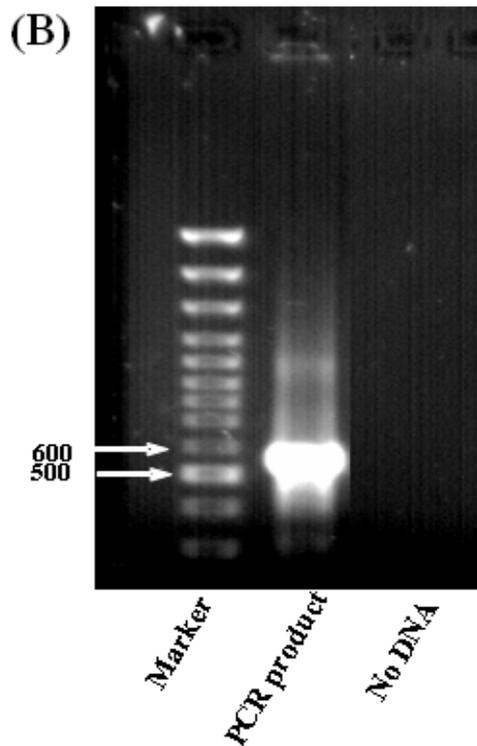
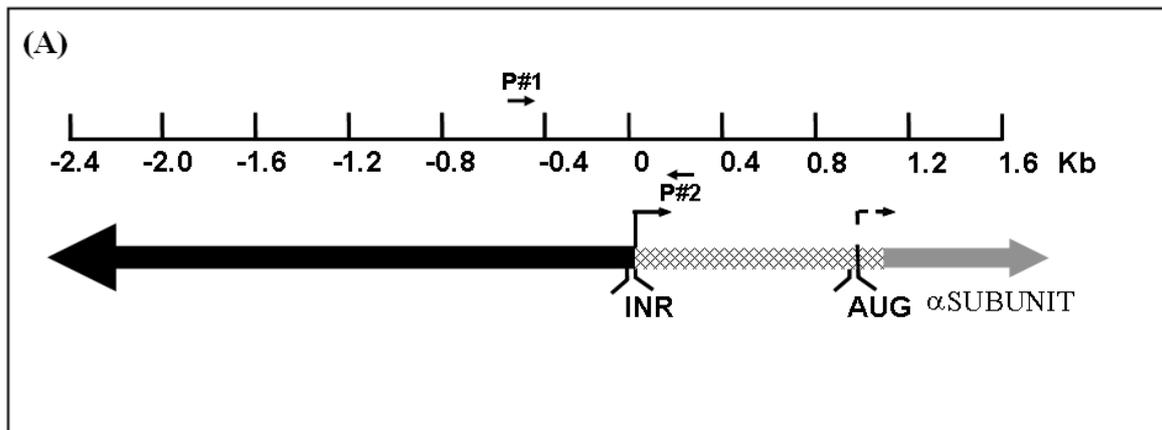
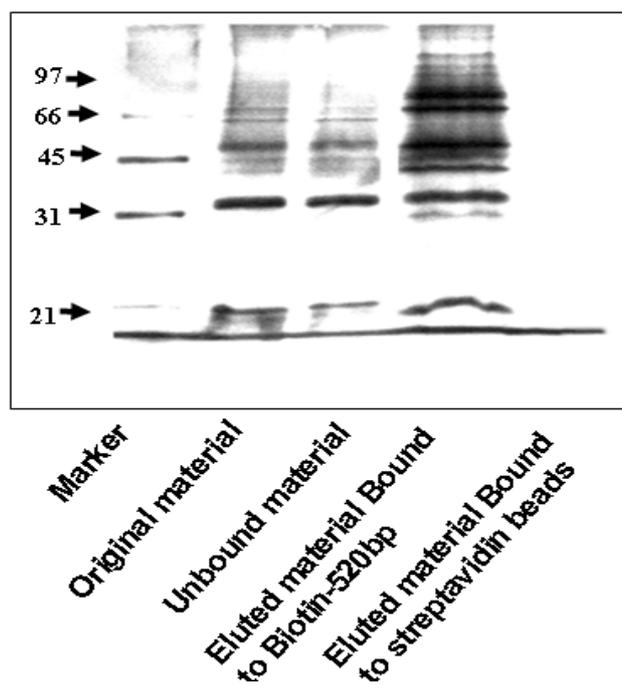


Figure 2. Silver staining of IGF-IR promoter-bound proteins identified by DNA affinity chromatography. Nuclear extracts of MCF7 cells were prepared as described under *Materials and Methods* Section. Proteins bound to IGF-IR promoter DNA (10 μ g) were electrophoresed through 10% SDS-PAGE, fixed, and stained with silver (Bio-Rad, Hercules, CA, USA). Lane 1, M.W. marker; lane 2, starting material (MCF7 nuclear extract, 4.2 μ g); lane 3, unbound material (MCF7 nuclear extract that did not bind to DNA, 4.2 μ g); lane 4, eluted material bound to Biotin-520 bp (MCF7 nuclear proteins that bound to IGF-IR promoter, 10 μ g); and lane 5, negative control (MCF7 nuclear proteins bound to streptavidin magnetic beads, 10 μ g).



2.3. Gel Slices Proteolysis and Mass Spectroscopy Analysis

The gel slices containing the specific DNA-bound proteins were reduced with 10 mM DTT, incubated at 60 °C for 30 min, carboxyamidomethylated with 40 mM iodoacetamide at room temperature for 30 min, and trypsinized overnight at 37 °C [modified trypsin (Promega)] at a 1:100 enzyme-to-substrate ratio. The tryptic peptides were resolved by reverse-phase chromatography on 0.075 \times 200-mm fused silica capillaries (J&W, Wilmington, DE, USA) packed with Repronil reversed phase material (Dr Maisch GmbH, Germany). The peptides were eluted with a linear 90 min gradient from 5 to 45% acetonitrile containing 0.1% formic acid, followed by a wash of 95% acetonitrile for 15 min. The flow rate was 0.25 μ l/min and the peptides were electrosprayed into an ion-trap mass spectrometer (Orbitrap, Thermo Fisher Scientific Inc., USA). MS was performed in a positive mode using repetitively full MS scans followed by collision induced dissociation (CID) of the 7 most dominant ions selected from the first MS scan. The MS data was clustered and analyzed using the Sequest software (Finnigan, San Jose, CA, USA) and Pep-Miner [39] searching against International Protein Index (IPI) protein IDs (part of the NR-NCBI database).

2.4. Cell Fractionation

Whole cell extracts were prepared by lysing cells in a buffer containing protease inhibitors, as described previously [20]. To prepare cytosolic and nuclear extracts, cells were washed with ice-cold phosphate-buffered saline (PBS), centrifuged, and the pellet was resuspended in buffer A [10 mM Hepes (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 2 µg/mL aprotinin, 1 mM ortovanadate, 2 µg/mL leupeptin, 4 mM pyrophosphate, 2 µg/mL pepstatin, and 1 mM PMSF] and incubated for 30 min on ice. IGEPAL (CA-630, Sigma-Aldrich Co.) was then added to a final concentration of 3%, vortexed, and centrifuged (14,000 rpm, 30 sec), after which the supernatant containing the cytosolic fraction was saved at -70°C . Buffer C [20 mM Hepes, 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, and protease inhibitors] was added to the nuclei-containing pellet, incubated for 30 min at room temperature, and spun at 14,000 rpm for 5 min. The supernatant containing the nuclear extract was frozen at -70°C . Protein concentrations were determined with the Bradford reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin (BSA) as a standard.

2.5. Western Immunoblots

Samples were electrophoresed through 12%, 10%, or 7.5% SDS-PAGE gels, followed by blotting of the proteins onto nitrocellulose membranes. After blocking with either skim milk or 3% BSA, the blots were incubated overnight with the antibodies listed below, washed and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. Antibodies against BRCA1 (C-20), TOPOII (C-15), MET (C-28), p120 (H-90), androgen receptor [(AR), N-20], IGF-IR β -subunit (C-20), Sp1 (PEP2), TCF3A (M-20), c-fos (4), ER α (MC-20), WT1 (C-19), KLF6 (R-173), lamin A/C (636), Smad2/3 (FL-425), c-Myc (9E10), p63 (4A4), E2F1 (KH95), LEF1 (C-19), TCF1A (C-21), p53 (mixture of DO-1 and Pab 1801), GSK3 β (11B7), phospho c-jun (KM-1), cyclin D (H295), cyclin G (F-5), histone1 (FL-219), β -catenin (E-5), p300 (C-20), BRCA2 (H-300), TLE-1 (M-101), p21 (C-19), normal mouse IgG [NMS (SC-2025)], and normal rabbit IgG [NRS (SC-2027)] were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An antibody against tubulin (Clone: B-5-1-2) was purchased from Sigma-Aldrich Co. Anti IRS-1 was kindly provided by Dr. Hagit Eldar-Finkelman (Tel Aviv University). Antibodies against poly ADP ribose polymerase [(PARP); 9542 and 9532], phospho-IGF-IR (3024), Foxo1 (9462), and phospho-Smad2 (3101) were obtained from Cell Signaling Technology (Beverly, MA, USA). In addition, the following antibodies were used: p73 (Ab-1; Oncogene Research Products, San Diego, CA, USA), c-jun (610326; BD Transduction Laboratories, Franklin Lakes, NJ, USA), APC (OP47L; Calbiochem, San Diego, CA, USA) and hemagglutinin (HA-11; Berkeley Antibody Company, Richmond, CA, USA). The secondary antibodies were HRP-conjugated bovine (1:500) or donkey (1:500) anti-goat IgG (both from Santa Cruz Biotechnology), goat anti-rabbit IgG (1:50,000) and donkey anti-mouse IgG (1:25,000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Proteins were detected using the SuperSignal West PicoChemiluminescent Substrate (Pierce, Rockford, IL, USA).

2.6. Chromatin Immunoprecipitation Assays

The ChIP assays were performed as described previously [40]. Briefly, MCF7 cells were cross-linked with 1% formaldehyde at 37 °C for 10 min. Cells were rinsed with ice-cold PBS and centrifuged. The pellet was then resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1 and protease inhibitor mixture: 1 mM PMSF, 1 µg/mL aprotinin and 1 µg/mL pepstatin A) and sonicated for 3 min. The supernatant fraction was immunoprecipitated with anti-PARP, anti-c-jun, anti-Sp1, anti-KLF6, anti-HA, and NRS or NMS for 18 h at 4 °C. The next day, Protein A/G plus agarose beads (SC-20003, Santa Cruz Biotechnology) was added for one hour at 4 °C. Immunoprecipitates were electrophoresed and immunoblotted as described under *Western immunoblots*. For PCR analysis of antibody-immunoprecipitated chromatin, a set of primers encompassing the IGF-IR proximal promoter region, sense, 3'-CCAGCCGCGCTGTTGTTG-5', anti-sense: 3-CCAGCCGCGCTGTTGTTG-5, and Thermolace™ DNA polymerase kit were used.

2.7. DNA Transfections

For transient cotransfection experiments an IGF-IR promoter luciferase reporter construct including 476 bp of 5'-flanking and 640 bp of 5'-untranslated regions of the IGF-IR gene [p(-476/+640) luciferase (LUC)] was employed [41,42]. An expression vector encoding c-jun (RSV-c-jun) was provided by Dr. Lili Vardimon (Tel Aviv University). An HA-HMGA1 expression vector (in pcDNA3) was provided by Dr. G. Manfioletti (University of Trieste, Italy) [43]. A KLF6 expression vector (pCI-neo-KLF6) was provided by Dr. Scott L. Friedman (Mount Sinai School of Medicine, New York, USA). The E2F1 expression vector, pcDNA-I-E2F-1, was previously described [44]. Cells were seeded in six-well plates the day before transfection and cotransfected with 1 µg of the IGF-IR promoter reporter along with 1 µg of the E2F1 expression plasmid (or empty pCDNA3), or 2 µg of the HA-HMGA1 (or empty pCDNA3), or 2 µg of the pCI-neo-KLF6 (or empty pCI-neo) using the Jet-PEI (Polyplus, Illkirch, France) transfection reagent. Cells were harvested 48 h after transfection, and luciferase activity was measured as previously described [41]. Promoter activities were expressed as luciferase values normalized to protein concentrations. Protein content was determined using the Bradford reagent (Bio-Rad, Hercules, CA, USA) using BSA as a standard.

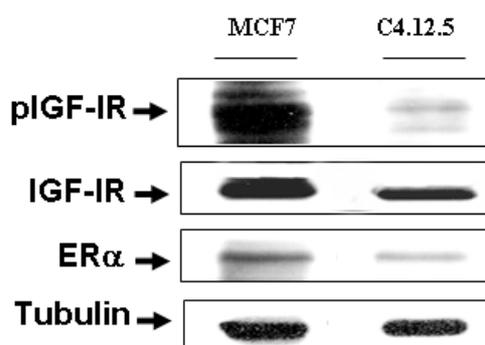
3. Results

3.1. ER and IGF-IR Expression in MCF7 and C4.12.5 Cells

The involvement of the IGF-IR in breast cancer development has been well established. To address the potential regulation of IGF-IR expression during breast cancer progression, we measured in initial experiments the levels of total and phospho-IGF-IR (pIGF-IR) in syngeneic cell lines representing different stages of the disease. For this purpose, we employed the human breast cancer-derived MCF7 (ER-positive) and MCF7-derived C4.12.5 (ER-depleted) cell lines. C4.12.5 cells were generated by clonal selection of MCF7 cells that were maintained in estrogen-free conditions for nine months [37]. Although C4.12.5 cells exhibited some variability in ER expression, the passages used in the present

study displayed consistently low levels of ER α (~59% of the ER α levels in MCF7 cells). Western blots using anti-IGF-IR β -subunit and anti-pIGF-IR antibodies showed that both total IGF-IR and pIGF-IR levels were significantly reduced in C4.12.5, in comparison to, MCF7 cells (76% for IGF-IR and 30% for pIGF-IR) (Figure 3). These results corroborate previous observations [40,45].

Figure 3. IGF-IR and ER α expression in MCF7 and C4.12.5 breast cancer cell lines. MCF7 and C4.12.5 cells were lysed as described under *Materials and Methods*. Cellular extracts (80 μ g) were electrophoresed through 10% SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-total IGF-IR- β , anti-pIGF-IR, and anti-ER α . Blots were then blotted with a tubulin antibody as a loading control.



3.2. DNA Affinity Chromatography

The MCF7 and C4.12.5 cell lines were employed to identify the sets of IGF-IR promoter-binding proteins that may be involved in IGF-IR transcription and that are responsible for the differential expression of the gene at different stages of the disease. The rationale for these studies was the fact that regulation at the transcription level has been identified as a major control step in IGF-IR regulation [46]. To this end, we used a proteomic approach based on DNA affinity chromatography followed either by MS or Western blot analyses using antibodies against previously identified or novel IGF-IR promoter-binding proteins. The basis for selecting this specific fragment was the fact that previous studies have shown that this region is responsible for most of the promoter activity. Nuclear extracts of MCF7 and C4.12.5 cells were incubated with the PCR-amplified, biotin-labeled IGF-IR proximal promoter DNA probe, after which DNA-protein complexes were adsorbed to streptavidin beads. Bound proteins were eluted with high-salt buffer and concentrated by centrifugation at reduced pressure. The eluted material was electrophoresed through 10% SDS-PAGE and proteins were visualized by silver staining (Figure 2). A number of bands were seen in the eluted material (lane 4) whereas no proteins were detected in eluates of lysates incubated with streptavidin magnetic beads alone (lane 5). Silver stained gels were cut into slices and the specific DNA-bound proteins were analyzed by MS.

3.3. MS Analysis

MS analysis of DNA affinity chromatography-purified proteins identified a number of previously reported and several novel IGF-IR promoter binding proteins. The proteins identified were categorized using the Gi (gene index) identification tool, coverage (percentage of amino acids covered by the

identified peptide), and source (Table 1). The specific proteins bound to the IGF-IR promoter were classified based on cellular localization and biological function (Table 2). Most of the proteins were localized in the nucleus, but some of them were found also in the cytoplasm as well as in the plasma membrane. Furthermore, Table 2 illustrates the variety of biological processes associated with the identified IGF-IR promoter-binding transcription factors in ER-positive and ER-depleted breast cancer cells. Out of 91 proteins identified by MS, 6 correspond to cytoskeleton-associated proteins, 6 are involved in transcription, regulation of nucleobase, nucleoside and nucleic acid metabolism, 2 in nuclear stability, chromatin structure, cycle control, and gene expression, 20 in DNA repair, breaking, replication, and cell death, 24 in RNA splicing and processing, and translation, and 33 in other functions, including proliferation, apoptosis, and proteosomal degradation.

Table 1. IGF-IR promoter-binding proteins identified by MS in MCF7 and C4.12.5 cells.

	Protein Name	Coverage		Source
		MCF7	C4.12.5	
4760598	Adenylate kinase isozyme 2	4	-	Mus musculus
61743954	AHNAK nucleoprotein isoform 1	1	3	Homo sapiens
1061128	Anpg	25	21	Homo sapiens
5453541	Anterior gradient 2 homolog	26	-	Homo sapiens
6753086	Apurinic/aprimidinic endonuclease 1	8	-	Mus musculus
10947139	Arginase, type I	-	3	Homo sapiens
6680748	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, alpha subunit, isoform 1	3	7	Mus musculus
10863945	ATP-dependent DNA helicase II	20	28	Homo sapiens
4503841	ATP-dependent DNA helicase II, 70 kDa subunit	22	28	Homo sapiens
1040689	ATP-dependent DNA helicase Q1 (DNA-dependent ATPase Q1)	-	4	Homo sapiens
42740907	Clusterin isoform 2	-	8	Homo sapiens
22208854	Cytidine monophospho-N-acetylneuraminic acid synthetase	3	3	Mus musculus
4557515	Damage-specific DNA binding protein 2 (48kD)	6	18	Homo sapiens
45476309	Damage-specific DNA binding protein 2 splicing variant D4	7	13	Homo sapiens
4758138	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	15	13	Homo sapiens
506756	Desmoyokin	-	6	
1706481	DNA ligase III	23	37	Homo sapiens
11225260	DNA topoisomerase I	10	11	Homo sapiens
347019	DnaK-type molecular chaperone hsp72-ps1	8	10	Rattus norvegicus
1722884	DNA-repair protein complementing XP-C cells	-	6	Drosophila melanogaster
38201714	ELAV-like 1	10	-	Homo sapiens
4503471	Eukaryotic translation elongation factor 1 α 1	6	2	Homo sapiens
4503477	Eukaryotic translation elongation factor 1 β 2	7	-	Homo sapiens
25453472	Eukaryotic translation elongation factor 1 δ isoform 2	22	-	Homo sapiens

Table 1. Cont.

110625979	Eukaryotic translation elongation factor 1 γ	6	–	Mus musculus
37078458	Far upstream element-binding protein 1 (FUSE-binding protein 1)	11	13	Mus musculus
27806887	Fusion (involved in t(12;16) in malignant liposarcoma)	5	–	Bos taurus
7669492	Glyceraldehyde-3-phosphate dehydrogenase	9	–	Mus musculus
74736618	Grainyhead-like protein 2 homolog	–	16	Homo sapiens
51316135	GTP-binding nuclear protein Ran, testis-specific isoform	10	–	Rattus norvegicus
4504517	Heat shock 27kDa protein 1	33	–	Homo sapiens
2495339	Heat shock 70 kDa protein 1B	8	10	Bos taurus
8393547	Heterogeneous nuclear ribonucleoprotein A1	13	11	Rattus norvegicus
14043072	Heterogeneous nuclear ribonucleoprotein A2/B1 isoform B1	19	10	Homo sapiens
31559916	Heterogeneous nuclear ribonucleoprotein A3 isoform a	11	15	Mus musculus
55956919	Heterogeneous nuclear ribonucleoprotein AB isoform a	9	3	Homo sapiens
14249959	Heterogeneous nuclear ribonucleoprotein C (C1/C2)	10	2	Homo sapiens
870747	Heterogeneous nuclear ribonucleoprotein D (hnRNP D)	11	22	Homo sapiens
10946928	Heterogeneous nuclear ribonucleoprotein H1	13	8	Mus musculus
14141157	Heterogeneous nuclear ribonucleoprotein H3 isoform a	11	–	Homo sapiens
13384620	Heterogeneous nuclear ribonucleoprotein K	23	10	Mus musculus
133274	Heterogeneous nuclear ribonucleoprotein L (hnRNP L)	4	4	Homo sapiens
14141152	Heterogeneous nuclear ribonucleoprotein M isoform a	15	18	Homo sapiens
5031755	Heterogeneous nuclear ribonucleoprotein R isoform 2	6	6	Homo sapiens
8393536	High mobility group box 2	14	–	Rattus norvegicus
1568557	Histone H2B	19	12	Homo sapiens
968888	HMG-1	13	–	Homo sapiens
32358	HnRNP U protein	11	9	Homo sapiens
37183160	HRPE773	–	29	Homo sapiens
1040689	Human Diff6, H5, CDC10 homologue, KIAA0097	6	–	Homo sapiens
13385872	Interleukin enhancer binding factor 2	8	13	Mus musculus
4504865	KH-type splicing regulatory protein (FUSE binding protein 2)	6	8	Homo sapiens
6453818	Kinesin family member 22	–	20	Homo sapiens
2055427	KSRP	5	26	Homo sapiens
27436946	Lamin A/C isoform 1 precursor	20	15	Homo sapiens
5031877	Lamin B1	8	21	Homo sapiens
14149645	Methyl CpG binding protein 2	4	4	Mus musculus
1770458	M-phase phosphoprotein 4	12	6	Homo sapiens
4038587	Mucin	–	2	Homo sapiens
55956788	Nucleolin	5	5	Homo sapiens
58037163	Nucleoporin 35	6	–	Mus musculus
35119	NuMA protein	–	2	Homo sapiens
1483131	p80 protein	3	3	Homo sapiens
115497186	Paraspeckle component 1	2	7	Bos taurus
156523968	Poly (ADP-ribose)polymerase	32	36	Homo sapiens
14141166	Poly(rC)-binding protein 2 isoform b	20	9	Homo sapiens

Table 1. *Cont.*

31543419	Polynucleotide kinase 3 phosphatase	13	11	Homo sapiens
4506243	Polypyrimidine tract-binding protein 1 isoform a	8	10	Homo sapiens
4502801	Regulator of chromosome condensation (RCC1)	12	10	Homo sapiens
4506583	Replication protein A1, 70kDa	18	26	Homo sapiens
4506585	Replication protein A2, 32kDa	7	–	Homo sapiens
4506439	Retinoblastoma binding protein 7	3	–	Homo sapiens
15718687	Ribosomal protein S3	23	–	Homo sapiens
7159943	RNA and export factor binding protein 1-II	24	9	Mus musculus
4757926	RNA binding motif protein 39 isoform b	3	–	Homo sapiens
66346679	SERPINE1 mRNA binding protein 1 isoform 1	9	9	Homo sapiens
2440078	SPIN protein	–	4	Homo sapiens
4826998	Splicing factor proline/glutamine rich	9	11	Homo sapiens
5902076	Splicing factor, arginine/serine-rich 1 isoform 1	4	–	Homo sapiens
39930517	Sterile alpha motif domain containing 1	11	17	Homo sapiens
30088600	Telomerase subunit EST1A	1	1	Homo sapiens
4507555	Thymopoietin isoform alpha	8	17	Homo sapiens
4507401	Transcription factor A, mitochondrial	4	-	Homo sapiens
14389309	Tubulin alpha 6	6	12	Homo sapiens
12963615	Tubulin, beta 3	9	8	Mus musculus
21361322	Tubulin, beta 4	9	9	Homo sapiens
1066004	Typr II DNA topoisomerase beta isoform	–	1	Mus musculus
28077011	Tyrosyl-DNA phosphodiesterase 1	4	4	Mus musculus
4506387	UV excision repair protein RAD23 homolog B	4	–	Homo sapiens
2632123	Xeroderma Pigmentosum Group E Complementing protein	7	17	Homo sapiens
5454172	X-ray repair cross complementing protein 1	9	12	Homo sapiens

Gi: identification tool; Coverage: percentage of amino acids covered by the identified peptide (n = 3 independent analyses).

Table 2. Summary of functional categories of IGF-IR promoter-bound proteins identified by MS.

Gene	Subcellular fraction	Function
<i>Cytoskeleton-associated proteins</i>		
KIAA0097 ; Human Diff6	Cytoplasm Nucleus	Microtubule associated protein.
Kinesin family member 22	Nucleus	Microtubule-dependent molecular motor.
NuMA protein	Nucleus	Component of the mitotic spindle matrix.
-Tubulin α 6 -Tubulin, β 3 -Tubulin, β 4	Cytoplasm Nucleus	Related to microtubules.

Table 2. Cont.

<i>Factors involved in transcription, regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism</i>		
-Elongation factor-1 (EF1) -Eukaryotic translation elongation factor 1 β 2 -Eukaryotic translation elongation factor 1 δ isoform 2 - Eukaryotic translation elongation factor 1 γ	Cytoplasm Nucleus	Involved in enzymatic delivery of aminoacyl tRNAs to the ribosome.
Poly(rC)-binding protein 2 isoform b	Cytoplasm Nucleus	RNA binding protein involved in regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism.
Telomerase subunit EST1A Alternative name: Chromosome 17 open reading frame 31 Est1p like protein A Ever shorter telomeres 1	Nucleus Nucleolus	Telomere regulation.
<i>Nuclear stability, chromatin structure, cycle control, gene expression</i>		
-Lamin B1 -Lamin A/C	Nucleus	Nuclear stability, chromatin structure and gene expression.
<i>Proteins with a known function in DNA repair, breaking, replication, transcription and cell death</i>		
Apurinic/apyrimidinic endonuclease 1	Nucleus	DNA repair and redox regulatory activities.
ATP-dependent DNA helicase II, 70 kDa subunit ATP-dependent (80 kda)	Nucleus	Repair of nonhomologous DNA ends.
ATP-dependent DNA helicase Q1 (DNA-dependent ATPase)	Nucleus	Repair of UV-irradiated DNA damage or other mutagens.
- Damage-specific DNA binding protein (127kDa) –DDB1 -Damage-specific DNA binding protein 2 (48kD) – DDB2 -Damage-specific DNA binding protein 2 splicing variant D4	Cytoplasm Nucleus	Repair of UV-irradiated DNA damage.
-DNA Topoisomerase I -DNA Topoisomerase II - Tyrosyl-DNA phosphodiesterase 1	Cytoplasm Nucleus Nucleoplasm	Type I topoisomerase breaks single DNA strands, whereas Type II topoisomerase breaks double stranded DNA.
-High mobility group box 2 -High Mobility Group Protein 17 (HMG17) – chromosomal protein -HMG1	Nucleus	DNA repair, recombination, replication, and transcription.
Histone 1,2 and 3	Nucleus	Play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability.

Table 2. Cont.

Poly (ADP-ribosylation) polymerase I-PARP-1	Nucleus	DNA repair, replication, transcription and cell death.
Polynucleotide kinase 3 phosphatase	Nucleus	DNA repair following ionizing radiation or oxidative damage.
Replication protein A1, 70kDa Replication Protein A2, 32-KD; RPA2	Nucleus	Replication, recombination and DNA repair.
Ribosomal Protein S3; RPS3	Cytoplasm Nucleus	DNA repair and apoptosis.
Xeroderma Pigmentosum Group E Complementing protein X-ray repair cross complementing protein 1	Cytoplasm Nucleus Nucleus	Involved in DNA repair. Repair of single-strand DNA breaks.
<i>RNA splicing, RNA processing, Transcription, Translation and other functions</i>		
-Dead/H-box-5 -RNA Helicase p68	Nucleus	Implicated in cellular processes involving alteration of RNA secondary structure.
ELAV-like 1	Cytoplasm Nucleus	RNA-binding proteins.
Fusion (involved in t(12;16) in malignant liposarcoma)	Nucleus	Plays a role in maintenance of genomic integrity.
hnRNP U protein	Cell surface Nucleus	Binds to pre-mRNA.
Interleukin enhancer binding factor 2 variant	Nucleus Nucleolus	Regulates transcription of the IL2 gene during T-cell activation.
Nucleolin	Nucleus Nucleolus	The major nucleolar protein of growing eukaryotic cells.
RNA and export factor binding protein 1	Cytoplasm Nucleus	Nuclear mRNA export pathway.
RNA binding motif protein 39 isoform b	Nucleus	Transcriptional coactivator for steroid nuclear receptors ESR1/ER α and ESR2/ER- β , and JUN/AP-1.
-Splicing factor, arginine/serine-rich 1 isoform 1 - Splicing factor proline/glutamine rich	Cytoplasm Nucleus	Prevents exon skipping.
<i>Others</i>		
Adenylate kinase isozyme 2	Membrane Microsomal Mitochondria Nucleus	Small ubiquitous enzyme.
Alkyladenine DNA glycosylase (AAG), also known as 3-methyladenine DNA glycosylase,	Nucleus	Catalyzes the first step in base excision repair.
Arginase, type I	Mitochondria	Plays a role in the regulation of extra-urea cycle arginine metabolism.

Table 2. Cont.

ATP synthase, H ⁺ transporting, mitochondrial F1 complex, α subunit, isoform 1	Mitochondria Cytoplasm Nucleus	Produces ATP from ADP in the presence of a proton gradient across the membrane.
BBF2H7/FUS protein	Nucleus	Binds both single-stranded and double-stranded DNA and promotes ATP-independent annealing of complementary single-stranded DNAs and d-loop formation in superhelical double-stranded.
Clusterin	Cytoplasm Nucleus	Undefined function, being described as being both pro-apoptotic and anti- apoptotic.
Cytidine monophospho-N-acetylneuraminic acid synthetase	Cytoplasm Nucleus	Involved in the biosynthesis of sialo-glycoproteins and gangliosides.
Desmoyokin/AHNAK	Plasma Membrane Cytoplasm Nucleus	Involved in the formation of cytoskeletal structure, calcium homeostasis, and muscle regeneration.
DNA ligase III α and β	Nucleus	Interacts with DNA-repair protein XRCC1 and corrects defective DNA strand-break repair and sister chromatid exchange.
-DnaK-type molecular chaperone hsp72-ps1 -Heat shock 27 kD protein 1 -Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8).	Cytosol Perinuclear Mitochondria Endoplasmatic Reticulum Nucleus	Chaperone activity, thermotolerance, inhibition of apoptosis, regulation of cell development and cell differentiation.
DNA-repair protein complementing	Nucleus	Nucleotide excision repair (NER) of DNA.
-Far upstream element-binding protein 1 (FUSE-binding protein 1) -KH-type splicing regulatory protein (FUSE binding protein 2) -KSRP	Nucleus (Probable).	Regulates MYC expression by binding to a far-upstream element (FUSE) upstream of the MYC promoter.
Grainyhead-like protein 2 homolog (Brother of mammalian grainyhead) (Transcription factor CP2-like 3)	Nucleus	Transcription factor involved in epidermal differentiation.
GTP-binding nuclear protein Ran, testis-specific isoform	Cytoplasm Nucleus	Nuclear transport pathways, required for the import of protein into the nucleus and also for RNA export.
Gyceraldehyde-3-phosphate dehydrogenase	Membrane Cytoplasm Nucleus	Carbohydrate metabolism and membrane trafficking in the early secretory pathway.
Methyl CpG binding protein 2	Nucleus	Nuclear proteins containing a methyl-binding domain (MBD)
M-phase phosphoprotein 4	Nucleolus	Regulation of cell cycle, cell communication and signal transduction.

Table 2. *Cont.*

Mucin	Cytoplasm Mitochondria Nucleus	Activation of Wnt target genes.
Nucleoporin 35 [Nuclear pore glycoprotein-210 (gp210)]	Membrane Cytoplasm Nucleus	Trafficking regulator in the eukaryotic nuclear pore complex.
p80 protein	Cytoplasm Nucleus	Part of the nuclear coiled bodies (CBS) involved in assembly/disassembly of nucleoplasmic snRNPs.
Paraspeckle component 1	Cytoplasm Nucleus Nucleolus	Regulates cooperatively with NONO and SFPQ, androgen receptor-mediated gene transcription activity.
PREDICTED: similar to RAN protein	Cytoplasm Nucleus	Inhibits GTP exchange on Ran.
Regulator of chromosome condensation (RCC1)	Cytoplasm Nucleus	Promotes the exchange of Ran-bound GDP by GTP.
Retinoblastoma binding protein 7	Nucleus	Modulates the functions ascribed to BRCA1 in transcriptional regulation, DNA repair, and/or cell cycle checkpoint control.
SERPINE1 mRNA binding protein 1 isoform 1	Cytoplasm Nucleus	Regulates mRNA stability.
SPIN	Nucleus	Regulation of cell cycle
Sterile alpha motif domain containing 1	Nucleus	Widespread domain in signalling and nuclear proteins.
Thymopoietin γ and β (TMPOs)	Nucleus	Directs the assembly of the nuclear lamina.
Transcription factor A, mitochondrial	Mitochondria	Involved in mitochondrial transcription regulation.
UV excision repair protein RAD23 homolog B	Cytoplasm Nucleus	Plays a role both in proteosomal degradation of misfolded proteins and DNA repair.

3.4. Identification of IGF-IR Promoter-Binding Transcription Factors by Western Blots

Western blot analysis of DNA affinity chromatography-purified proteins identified a number of previously reported as well as novel IGF-IR promoter binding proteins. The data are summarized in Figure 4 and Tables 3 and 4. Consistent with their role as positive regulators of the IGF-IR gene, the binding of zinc finger proteins Sp1 and KLF6 to the IGF-IR promoter was stronger in MCF7 than in C4.12.5 cells. Similarly, ER- α binding to the IGF-IR promoter was stronger in MCF7 than in C4.12.5 cells. On the other hand, a number of transcription factors, including AR, Smad2/3, E2F1, c-jun, TCF1A, TCF3A, GSK3 β , and p21 appear to bind to the promoter region only in the C4.12.5 cell line. p53, c-fos, and PARP bind to IGF-IR promoter elements in both cell lines with similar affinity. No DNA binding was detected in either cell line using antibodies against BRCA1, TOPOII, IRS-1, MET, p120, Foxo1, p73, lamin A/C, c-Myc, p63, cyclin D, cyclin G, histone1, and BRCA2.

Figure 4. IGF-IR promoter-binding transcription factors identified by Western blots in the MCF7 and C4.12.5 cell lines. DNA chromatography eluates were electrophoresed through 10% SDS-PAGE and blotted with the antibodies listed under *Materials and Methods* Section. The left lanes correspond to nuclear extracts (NE; 4.2 μg) and the right lanes correspond to DNA affinity chromatography eluates (E; 10 μg).

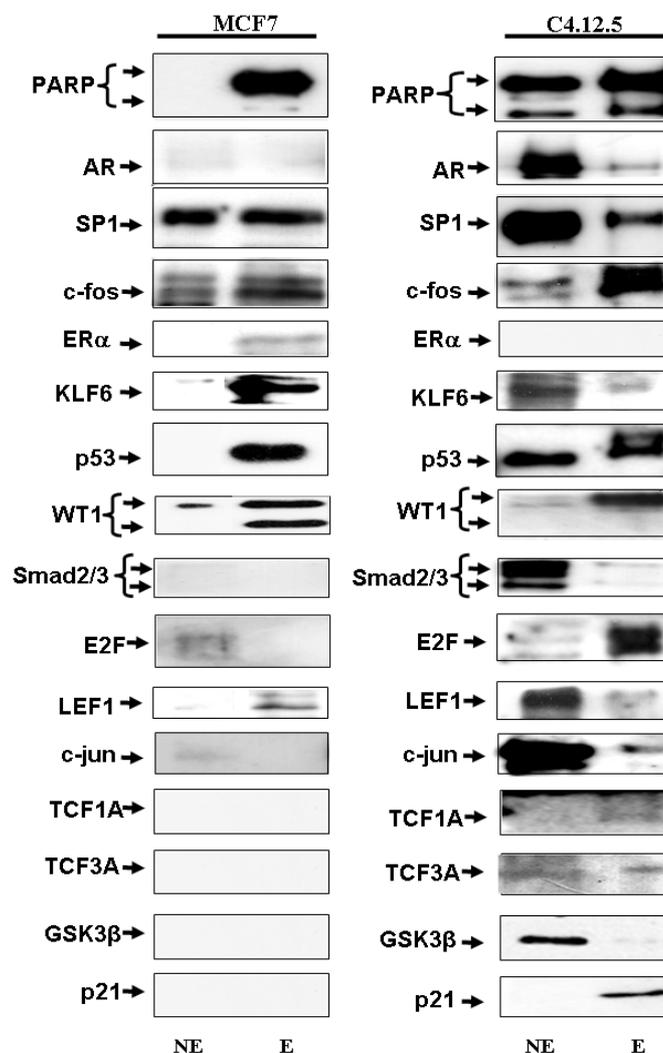


Table 3. Differentially expressed nuclear proteins identified by Western blots in MCF7 and C4.12.5 cells.

Gene	MCF7	C4.12.5	Nomenclature
BRCA1	1	0.3	Breast and ovarian cancer type I
TOPOII	1	0	Type II topoisomerase
IRS-I	0	0	Insulin receptor substrate-1
PARP	1	2	Poly ADP ribose polymerase
MET	1	0	Met tyrosine kinase
p120	1	1.39	Zinc finger protein 120
AR	1	0.64	Androgen receptor
Sp1	1	0.79	Trans-acting transcription factor 1
Foxo1	1	0.5	Forkhead Box A1

Table 3. *Cont.*

ER	1	0.59	Estrogen receptor
WT1	1	0.8	Wilms' tumor suppressor gene-1
KLF6	1	1.2	Kruppel-like factor 6
LaminA/C	1	2.4	Nuclear protein
Smad 2	1	1.12	
Smad 3	0	2.6	Smad 2/3 family
pSmad2/3	1	1.13	
c-Myc	1	1.48	Myelocytomatosis oncogene
E2F1	1	0.88	E2F transcription factor 1
TCF1	1	0.717	T-cell factor 1
TCF3A	1	0.002	High Mobility Group box transcription factor 3
LEF1	1	1.15	Lymphoid enhancer binding factor 1
p53	1	0.96	
p63	1	1.12	p53-related tumor suppressors
p73	1	0.03	
p21	1	0.91	Waf1/Cip1
GSK3 α	1	1.05	Glycogen Synthase Kinase-3
c-fos	1	0.92	
c-jun	1	10	c-fos/c-jun family of transcription factors
pc-jun	1	0.5	
Cyclin G	1	0.13	
Cyclin D	1	0	Regulatory subunits of Cdc2 p34
Histone1	1	0.74	Nuclear protein

A value of 1 was assigned to the level of expression in whole MCF7 cell lysates, normalized to tubulin.

Table 4. Function of gene products identified in MCF7 and C4.12.5 cells by Western analyses.

Gene	Function
BRCA1	Tumor suppressor involved in DNA damage repair, cell growth and apoptosis, and gene transcription.
TOPOII	Type II topoisomerase, breaks double strands of duplex DNA.
IRIS-I	Mediates insulin related activities.
PARP	DNA repair, replication, transcription and cell death.
MET	Receptor for HGF/SF.
p120	Prototypical member of a subfamily of armadillo (Arm)-domain proteins involved in intercellular adhesion and in nuclei.
AR	Androgen receptor (AR), a zinc finger transcription factor of the nuclear receptor superfamily.
SP1	Transcription factor of the zinc finger family.
Foxo1	Transcription factor of the forkhead family.
ER	Estrogen receptor (ER), a DNA binding transcription factor of the nuclear receptor superfamily.

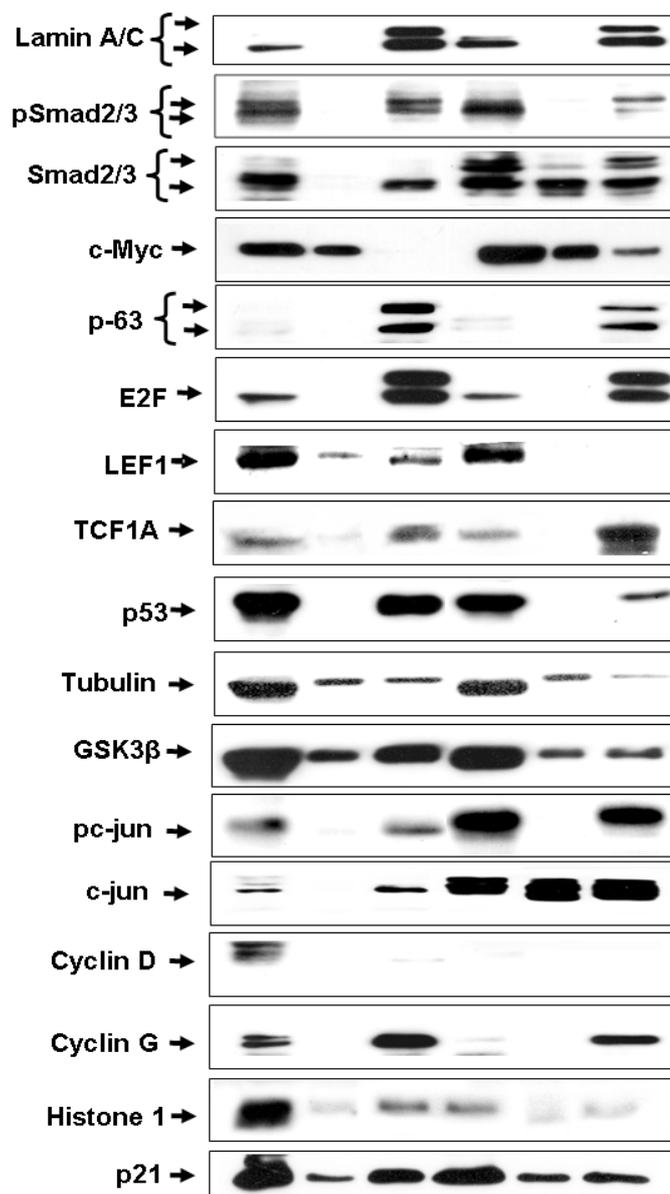
Table 4. *Cont.*

WT1	Wilm's tumor suppressor protein-1.
KLF6	Kruppel-like zinc finger transcription factor-6.
LaminA/C	Involved in nuclear stability, chromatin structure and gene expression.
pSmad 2/3	Smad transcription factors mediate the effect of TGF- β .
Smad 2/3	
c-Myc	Nuclear proteins involved in cell proliferation, differentiation and neoplastic disease.
E2F1	Involved in cell cycle progression, apoptosis and oncogenesis.
LEF1	Members of the high mobility group (HMG) DNA binding protein family of transcription factors.
TCFA1	
TCF3A	
P53	Tumor suppressor p53, p63 and p73.
p63	
p73	
GSK3 α	Involved in protein synthesis, cell adhesion, proliferation, and differentiation.
c-fos	Fos and Jun protooncogenes.
c-jun	
pc-jun	
Cyclin G	Regulatory subunits of Cdc2 p34 and related cyclin-dependent kinases (Cdk).
Cyclin D	
Histone1	Basic and water soluble nuclear proteins.
p21	Tumor suppressor protein p21/Waf1/Cip1 acts as an inhibitor of cell cycle progression.

3.5. Cellular Distribution of IGF-IR Promoter-Binding Proteins

Given that differences in binding could be due to differential expression of the transcription factors in both cell lines, we next investigated the subcellular distribution and expression of IGF-IR promoter-binding transcription factors. For this purpose, MCF7 and C4.12.5 lysates were fractionated into cytoplasmic and nuclear fractions and the subcellular distribution of the different proteins was examined by Western blots (Figure 5 and Tables 3 and 4). The cytosolic marker MET [47] was used to check for cytoplasmic contamination of the nuclear fraction, and it was found to be expressed at very low levels in both cell lines. A number of proteins, including PARP (200% of levels in MCF7 cells), lamin (240%), Smad 2/3 (260%), and c-jun (1000%) had higher expression levels in C4.12.5 than in MCF7 cells. Other proteins, such as BRCA1 (30% of levels in MCF7), AR (64%), Sp1 (79%), Foxo I (50%), p73 (3%), TCF3A (0.2%), ER (59%), TCF1 (71.7%), pc-jun (50%), cyclin G (13%), and histone (74%) were expressed at lower levels in C4.12.5 compared to MCF7 cells. No differences in expression between the cell lines were detected in p120, c-fos, WT1, KLF6, pSmad2/3, c-Myc, p63, E2F, LEF1, p53, GSK3 α , and p21. We were unable to detect expression of β -catenin, p300, TLE1, APC, and BRCA2 in neither cell line. Transcription factor WT1 was present in both cell lines, though its low molecular weight form appears to be specific to the MCF7 cell line.

Figure 5. Cellular distribution of transcription factors in the MCF7 and C4.12.5 cell lines. Cell lines were fractionated as described under *Materials and Methods* and total lysates (T; 80 μ g), cytosolic fractions (C; 20 μ g), and nuclear extracts (N; 20 μ g) were resolved on 10% SDS-PAGE and blotted with the indicated antibodies.

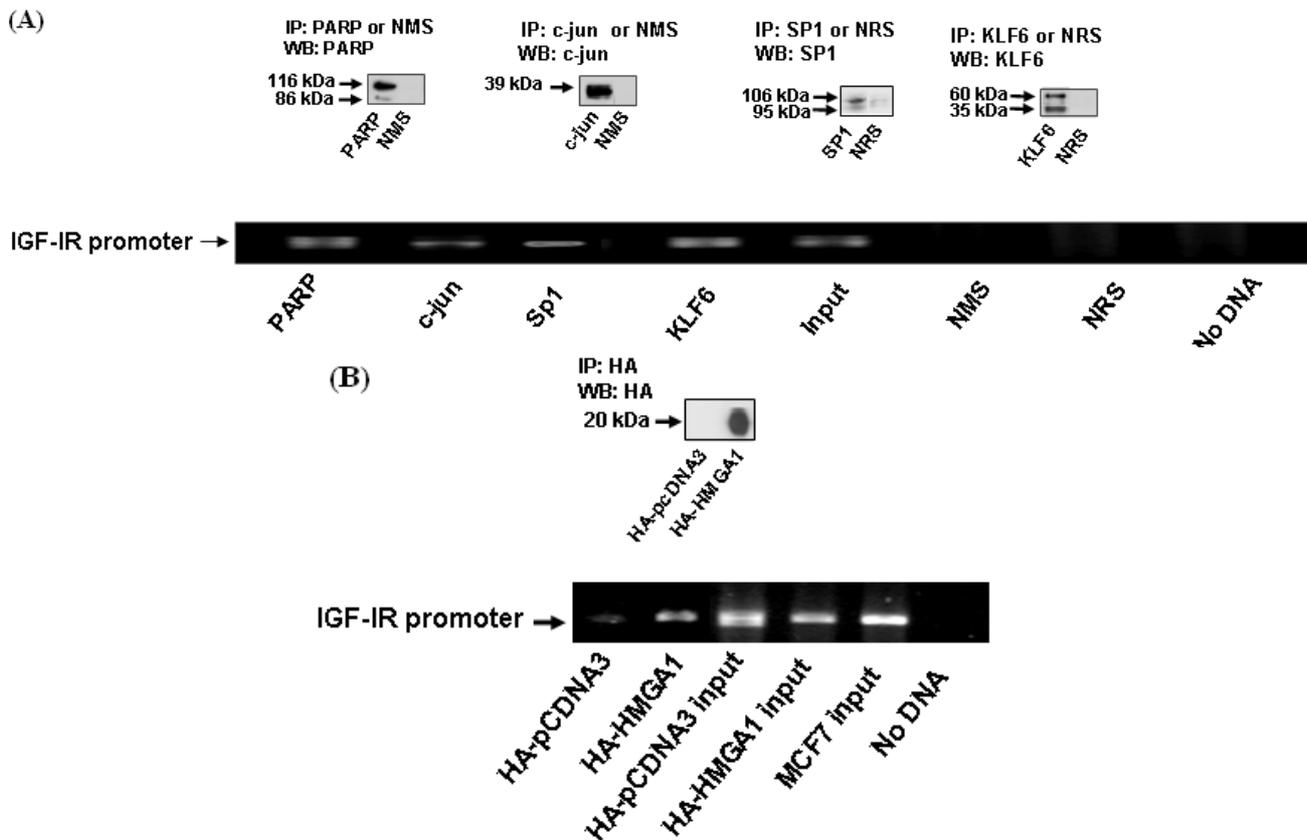


3.6. ChIP Assays

To corroborate results of DNA chromatography we measured the binding of selected proteins identified by MS or by Western blots using ChIP assays. The following proteins were chosen for ChIP assays: PARP (detected by MS and Western blots in both cell lines), Sp1 (positive control, detected in both cell lines), c-jun, (detected only in C4.12.5) and HMGA1 (detected by MS only in MCF7). Briefly, cells were lysed, sonicated for 3 min, and immunoprecipitated with the indicated antibodies (or normal mouse or rabbit sera) for 18 h (Figure 6A). In addition, MCF7 cells transfected with HA-HMGA1 (or empty pCDNA3 vector) were also immunoprecipitated with an HA monoclonal antibody (Figure 6B). For PCR analysis of immunoprecipitated chromatin, a set of primers encompassing the

human IGF-IR promoter was employed as described under *Materials and Methods* section. The input bands represent the amplified PCR product in the absence of antibodies (Figures 6A and 6B). The results of ChIP analyses confirmed that PARP, c-jun, Sp1, KLF6 and HMGA1 bound *in vivo* directly to the IGF-IR promoter and, thus, corroborate the results of MS and Western blot analyses.

Figure 6. ChIP assays of transcription factor binding to IGF-IR promoter DNA. **(A).** MCF7 cells were cross-linked with formaldehyde, lysed, sonicated, and immunoprecipitated with PARP, c-jun, Sp1, or KLF6 antibodies, or normal mouse or rabbit sera, followed by PCR amplification of precipitated chromatin using primers encompassing the IGF-IR promoter. The position of the 773 bp-amplified fragments is indicated. The input bands represent the amplified PCR product in the absence of antibodies. The insets indicate the endogenous levels of expression of the various proteins as measured by Western blots (WB) with specific antibodies or NRS or NMS as negative controls. IP, immunoprecipitation. **(B).** MCF7 cells were transfected with an expression vector encoding HA-HMGA1 (or empty pcDNA3 vector), after which the cells were cross linked with formaldehyde, lysed, sonicated and immunoprecipitated with an HA antibody, followed by PCR amplification of IGF-IR promoter DNA. HA-HMGA1 immunoprecipitated protein was detected by Western blots using an anti-HA antibody (inset).



3.7. Regulation of IGF-IR Promoter Activity by c-jun, HMGA1, KLF6 and E2F1

To evaluate the potential functional implications of our findings we next assessed the ability of selected IGF-IR promoter-binding proteins to activate the promoter. For this purpose cotransfection

experiments were performed in MCF7 and C4.12.5 cells using expression vectors encoding c-jun, HMGA1, KLF6 and E2F1, along with an IGF-IR promoter-luciferase reporter plasmid, p(-476/+640)LUC, which contains most of the proximal region of the IGF-IR promoter. The results of cotransfection experiments are presented in Figures 7A-D. Results obtained indicate that transcription factors c-jun, KLF6, and E2F1 induced a significant increase in IGF-IR promoter activity in both cells lines. Specifically, c-jun enhanced IGF-IR promoter activity by $445 \pm 12.4\%$ in MCF7 and by $209 \pm 3.1\%$ in C4.12.5 cells (*versus* empty vector), KLF6 enhanced promoter activity by $237 \pm 9.7\%$ in MCF7 and by $159 \pm 7.1\%$ in C4.12.5 cells, and E2F1 stimulated promoter activity by $631.5 \pm 44.5\%$ in MCF7 and by $1127 \pm 22.2\%$ in C4.12.5 cells. On the other hand, HMGA1 had a minimal effect ($131 \pm 4.3\%$ increase in MCF7 and $80.3 \pm 1.7\%$ reduction in C4.12.5 cells).

Figure 7. Regulation of IGF-IR promoter activity by c-jun, HMGA1, KLF6 or E2F1. MCF7 and C4.12.5 cells were cotransfected with the p(-476/+640)LUC reporter construct along with c-jun (A), HMGA1 (B), KLF6 (C) or E2F1(D) expression plasmids (or empty expression vectors). Forty eight hours after transfection the cells were harvested and luciferase activity was measured. Promoter activities are expressed as luciferase values normalized to protein concentration. A value of 100% was given to the promoter activity generated by the reporter plasmid in empty vector-transfected MCF-7 or C4.12.5 cells. The results represent the mean \pm SEM (N = 3 independent experiments in triplicate wells); *p < 0.01 versus empty vector-transfected cells and ** p < 0.01 versus specific vector-transfected MCF7 cells.

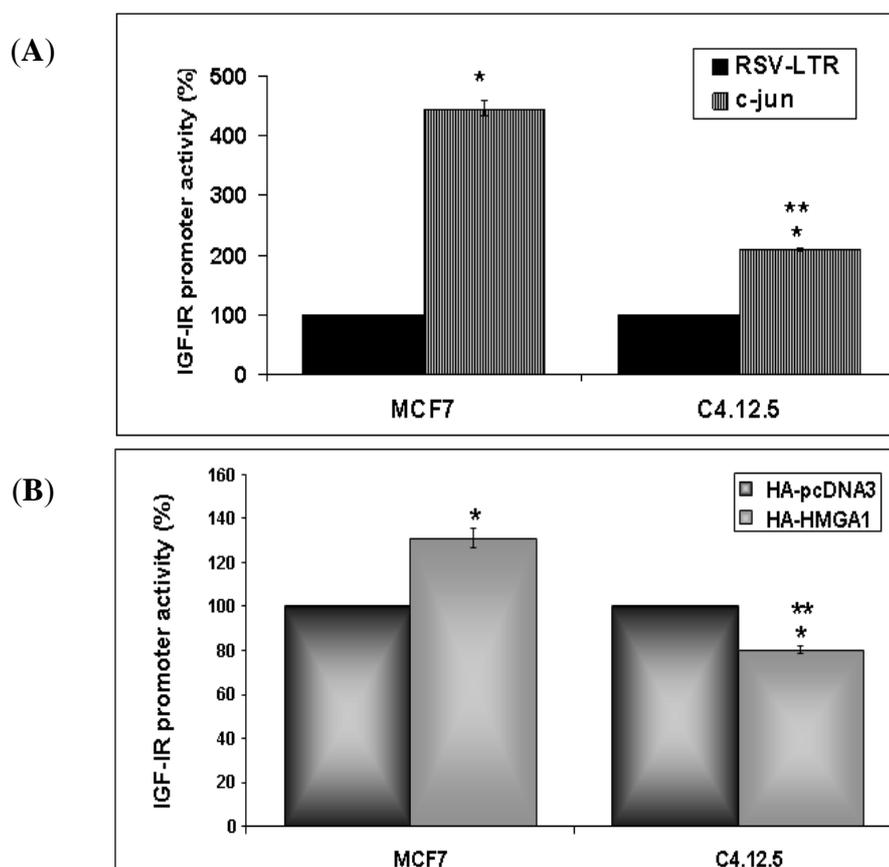
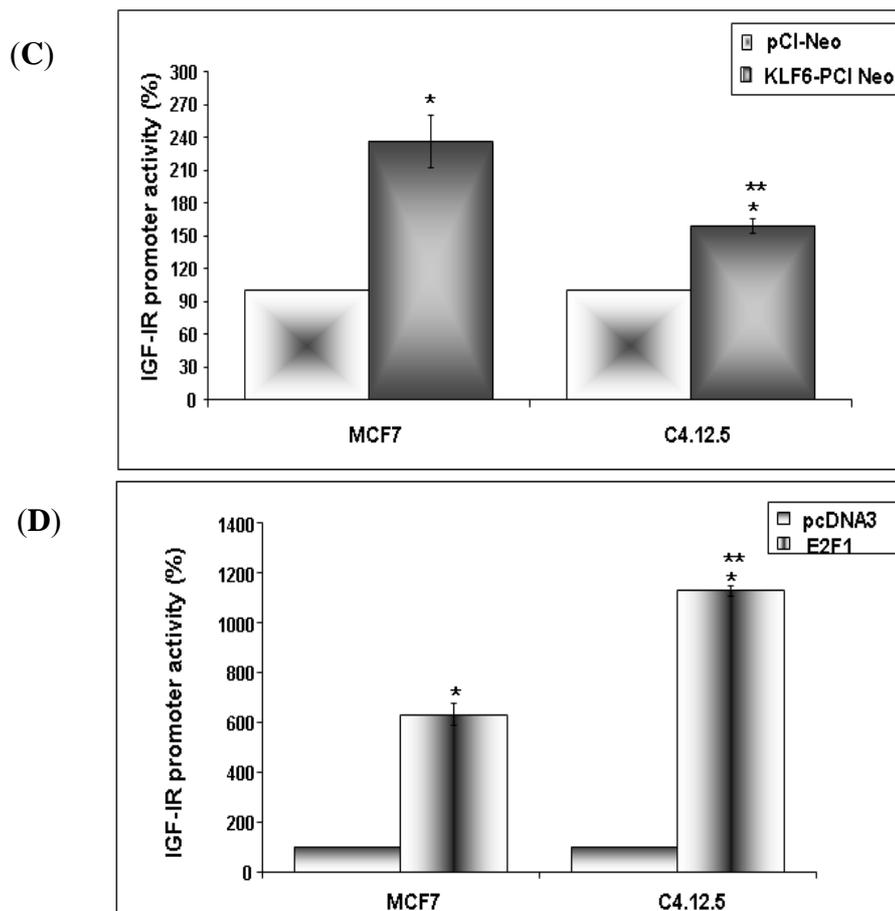


Figure 7. Cont.



4. Discussion

Breast cancer is one of the main causes of cancer-related mortality among women in economically developed regions of the world. Care of patients with breast cancer is complicated by the wide differences in the natural history of the disease, as manifested by a remarkable variability in response to treatment and survival. Over 100 potential biomarkers were reported in various studies to be differentially expressed between normal and cancerous cells and tissues. Cancer biomarkers include, among others, ER, progesterone receptor, HER2, cathepsin D, cyclin E, CA 15-3, and CA 27.29. Currently, only a few of those biomarkers are used in clinical practice. High-throughput genomic and proteomic techniques developed over the last decade offer the potential to better define the biological nature of the disease process, both for risk assessment and therapy purposes [48].

There is conflicting evidence regarding the importance of IGF-IR expression for diagnosis and prognosis of breast cancer. In different studies, IGF-IR expression was detected in 39% to 93% of the cases [49]. Whereas some studies reported that IGF-IR levels are indicative of early recurrence of the disease (*i.e.*, poor prognosis), others considered high IGF-IR values as an indication of good prognosis [4,50–54]. These disparities may be due to a number of factors, including selection of patients or the diverse approaches used to detect IGF-IR, including immunoblots of membrane fractions and/or whole tissues, ligand binding assays, *etc.* Immunohistochemical studies revealed high IGF-IR and IRS-1 levels in control breast tissues and in well-differentiated breast carcinoma, whereas

very low levels were seen in poorly differentiated cancers [3]. These results, suggesting that progression to advanced-stage disease is associated with a reduction in IGF-IR expression, indicate that IGF-IR status can be used as a marker for tumor evaluation [3]. Notably, no correlation was seen between insulin receptor levels and the differentiation stage of the tumors.

In the present study we employed a novel and reliable DNA affinity chromatography protocol in order to identify IGF-IR promoter-binding proteins in breast cancer cells. This method allowed us to detect known and new specific potential biomarkers in ER-positive and ER-depleted cells, which may reflect early and advanced stages of the disease. Our analyses identified twenty four proteins that bind to the IGF-IR promoter only in MCF7 cells whereas, on the other hand, C4.12.5 cells contain nineteen proteins that did not exhibit IGF-IR promoter binding activity in MCF7 cells. The results obtained suggest that the specific binding of different DNA-binding transcription factors and non-DNA sequence specific-binding nuclear proteins (including Smad2/3, LEF1, c-jun, TCF1A, TCF3A, GSK3 β and others) in the MCF7 and C4.12.5 cell lines could reflect differences in transcriptional regulation of the IGF-IR gene at different stages of the disease. Interestingly, sixty two proteins were shown to bind (either directly or indirectly) to IGF-IR promoter elements in both cell lines. As shown in Tables 2 and 4, the list of proteins identified in this study includes proteins involved in apoptosis, protein synthesis, cell adhesion, proliferation, differentiation, nuclear stability, chromatin structure, DNA repair, recombination, replication, tumor suppression, and oncogenesis. In this context, it has been previously shown that the regulatory region of the IGF-IR gene constitutes a molecular target for a number of transcription factors with tumor suppressor or stimulatory activity [46]. Under normal physiological conditions, IGF-IR expression is tightly regulated by complex interactions involving positively acting (Sp1, KLF-6, ER α) and negatively acting (p53, p73, BRCA1, ATM, WT1, VHL) transcription factors [17,18,22-24,40,45,55-57]. Elevated IGF-IR levels were postulated to be the result of loss-of-function mutations of tumor suppressor genes in cancer cells [58]. The approach employed in this study allowed us to identify several previously reported IGF-IR promoter-binding proteins, including Sp1, KLF6, and WT1. Moreover, results of MS and Western blots confirmed bioinformatic predictions on transcription factor binding sites (e.g., WT1, ER, E2F, Sp1, *etc.*), obtained using the TRANSFAC and AliBaba2 softwares (<http://www.biobase-international.com> and <http://www.gene-regulation.com>) (data not shown). Furthermore, the fact that we were able to detect differences in promoter binding between the two cell lines, despite the lack of differences in protein expression, indicates that the DNA affinity chromatography approach used was highly specific and sensitive. Given that differences in binding could be due to differences in the availability of these proteins, we confirmed that the proteins detected in the eluates were truly present in the nuclear fractions. Several proteins, including KLF6, Smad2/3, GSK3 β , c-jun, and p21, were present in both cytosolic and nuclear fractions. A number of transcription factors, including BRCA1, p120, Foxo1, p73, c-myc, p63, cyclin D and G, were undetectable in eluates of both cell lines. A potential explanation for these results is the fact that the expression of these transcription factors was very low in the nuclear fractions [59] or, alternatively, that they are engaged in multi-protein complexes and, therefore, cannot bind to IGF-IR promoter sequences.

The capacity of a number of proteins identified by MS and/or Western blots (PARP, Sp1, c-jun, HMGA1) to directly bind to IGF-IR promoter DNA *in vivo* was confirmed by ChIP assays. Furthermore, the functional relevance of binding data was addressed by coexpression experiments

which revealed that c-jun, KLF6, and E2F1 significantly enhanced IGF-IR promoter activity. Differences in the capacity of these factors to stimulate IGF-IR promoter activity was seen between both cell lines. The luciferase assays corroborate the results obtained by Western blots and ChIP assays. We have previously shown using EMSA and ChIP that both Sp1 and ER α bind to the IGF-IR promoter and stimulate gene transcription, whereas no ER α binding was seen in C4 cells [60]. In addition, we have reported that Sp1 increased IGF-IR promoter activity with a 5.8-fold higher potency in MC7 than in C4 cells [60]. Taken together, there is a remarkable consistency between the various technological approaches used to identify IGF-IR promoter-binding transcription factors.

5. Conclusions

In summary, using DNA affinity chromatography we identified a collection of known and novel IGF-IR promoter-binding transcription factors in breast cancer cell lines. These proteins may constitute potential biomarkers characteristic of ER-positive or ER-negative tumors. The potential clinical relevance of our results need to be confirmed by future studies on biopsies from patients at early and advanced stages, and need to be correlated with conventional prognostic factors such as tumor size, lymph nodes status, histological grade, and ER status.

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