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Insulin Receptor Substrate 1 modulates the transcriptional activity and turnover of Androgen Receptor in breast cancer cells.

Docente Tutor
Ch.mo Prof. Sebastiano ANDO'

Dottoranda
Dott.ssa Cecilia GAROFALO

Coordinatore
Ch.mo Prof. Sebastiano ANDO'

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- ✓ RNAi-mediated silencing of insulin receptor substrate 1 (IRS-1) enhances tamoxifen-induced cell death in MCF-7 breast cancer cells. *J Cell Biochem. 2006 May 15;98(2):440-50.*

- ✓ Leptin and cancer. *J Cell Physiol. 2006 Apr;207(1):12-22. Review.*

- ✓ Functional significance of type 1 insulin-like growth factor-mediated nuclear translocation of the insulin receptor substrate-1 and beta-catenin. *J Biol Chem. 2005 Aug 19;280(33):29912-20. Epub 2005 Jun 20.*

- ✓ Leptin interferes with the effects of the antiestrogen ICI 182,780 in MCF-7 breast cancer cells. *Clin Cancer Res. 2004 Oct 1;10(19):6466-75.*

- ✓ Nuclear insulin receptor substrate 1 interacts with estrogen receptor alpha at ERE promoters. *Oncogene. 2004 Sep 30;23(45):7517-26.*

- ✓ Leptin expression in breast nipple aspirate fluid (NAF) and serum is influenced by body mass index (BMI) but not by the presence of breast cancer. *Horm Metab Res. 2004 May;36(5):336-40.*

- ✓ Low calcium intake is associated with decreased adrenal androgens and reduced bone age in premenarcheal girls in the last pubertal stages. *J Bone Miner Metab. 2004;22(1):64-70.*

Summary

Breast cancer growth is responsive to various growth factors and steroid hormones and also reflects the interplay between the respective signaling pathways. Even though the majority of human breast cancers expresses androgen receptor, the existence and/or the mechanism of a crosstalk between androgen receptor (AR) and Insulin-like Growth Factors (IGFs) in breast has not been clearly defined. To gain insight into this functional interplay we investigated whether Insulin Receptor Substrate-1 (IRS-1), the major IGF-IR signaling molecule, affects AR function. In MCF-7 breast cancer cells, upon 5-α-Dihydrotestosterone stimulation, IRS-1 associates with AR and is recruited to Androgen Responsive Elements of the androgen target genes PSA and p21 and it appear to be required for AR transcriptional activity. Indeed, the ectopic expression of IRS-1 enhances AR transcriptional activity in a dose dependent manner, while the silencing of IRS-1 significantly represses PSA and p21 mRNA and protein levels. Moreover, IRS-1 knockdown experiments suggest that IRS-1/AR interaction decreases the ubiquitin/proteasome dependent degradation of AR, reducing AR turnover. Thus, our data provide novel insights into AR/IGF crosstalk and suggest that IRS-1 might represent a novel AR regulator.

Introduction

Breast cancer development and progression depend on a complex crosstalk between steroid hormones and growth factors(Aronica and Katzenellenbogen, 1993; Ignar-Trowbridge et al., 1993; Reddy et al., 1994; Ruohola et al., 1999). Several authors have reported that the majority of human breast cancers express androgen receptor (AR) (Kuenen-Boumeester et al., 1992; Lea et al., 1989; Wilson and McPhaul, 1996), and that many metastatic breast tumors, which are estrogen receptor (ER) and progesterone receptor negative, still express a significant amount of AR (Bayer-Garner and Smoller, 2000). Androgens have been demonstrated to exert differential effects on breast cancer progression, and may either stimulate or inhibit the growth of AR-positive breast cancer cell lines in vitro (Birrell et al., 1995; Hackenberg et al., 1991; Yeap et al., 1999). Patients with AR-negative breast tumors had a significantly poorer response rate to hormone therapy and shorter overall survival than those with AR-positive ones (Bryan et al., 1984). Androgens have been found to negatively regulate the growth of mammary epithelial and breast cancer cells in vitro (Birrell et al., 1995; Lanzino et al., 2005; Szelei et al., 1997). In breast cancer cells, 5α-dihydrotestosterone (DHT) induces post-transcriptional destabilization and downregulation of AR mRNA, while AR expression is increased (Yeap et al., 1999). These observations suggest that the role of androgens and their receptor in breast cancer growth and progression needs to be clarified (Birrell et al., 1995; Kuenen-Boumeester et al., 1996; Lea et al., 1989).

Upon ligand binding, AR undergoes conformational changes that render it capable of interacting not only with androgen responsive elements (ARE) in DNA,

but also with basal transcription factors responsible for modulating the expression of androgen-regulated genes (McEwan and Gustafsson, 1997). In addition to this classical pathway, a second mechanism of AR action consists of the receptor interaction with proteins involved in various trnsductional signals (Bubulya et al., 1996; Castoria et al., 2003; Lobaccaro et al., 1999; Oettgen et al., 2000);. A substantial body of literature suggests that AR is directly or indirectly regulated by growth factor in the absence of androgens (Culig et al., 1995; Nazareth and Weigel, 1996).

A major substrate for Insulin-like Growth Factors (IGFs) is Insulin Receptor Substrate 1 (IRS-1). IRS-1 is generally a cytoplasmic/membrane localized signaling protein. In addition to its conventional role, IRS-1 has been found in the nuclear compartment in several cell types, including breast cancer cells and breast tumors, (Lassak et al., 2002; Morelli et al., 2004; Prisco et al., 2002; Sisci et al., 2006; Sun et al., 2003). Experimental data suggest that nuclear IRS-1 might function as transcriptional co-regulator for polymerase I and II (Morelli et al., 2004; Tu et al., 2002). Recently, we demonstrated that in breast cancer cells IRS-1 can bind ER and that the IRS-1/ER complex can be translocated to the nucleus upon estradiol (E2) stimulation. In the nucleus, IRS-1 is recruited to Estrogen Responsive Element-containing promoters and negatively modulates ER-dependent transcription (Morelli et al., 2004).

Here we demonstrate that androgen stimulation of breast cancer cells promotes the binding of IRS-1 with AR and increases nuclear translocation of IRS-1. We also show that nuclear IRS-1 is recruited to ARE regions of androgen responsive genes, stimulating AR-mediated transcription in response to DHT. Our results

suggest that IRS-1 modulates AR mediated transcription through two different mechanisms, by participating in AR-recruited transcriptional machinery and by decreasing AR turnover. Thus, we provide novel information on the regulation of AR activity and its crosstalk with the IGF system in breast cancer cells.

Materials and Methods

Cell culture and treatments

A breast cancer epithelial cell line MCF-7 and a human embryonic kidney cell line HEK-293 were grown in DMEM/F12 (Gibco, USA) supplemented with 5% calf serum (CS, Gibco, USA) and in DMEM plus 10% fetal calf serum, respectively. 5α-Dihydrotestosterone (DHT, Sigma, USA) and hydroxyflutamide (OHFl, Sigma, USA) were used at a concentration of 10^{-7} M. Before each experiment, cells were serum starved for 24 h in phenol red-free DMEM (PRF), and than shifted to PRF containing 5% charcoal-treated fetal calf serum (PRF-CT).

Immunoprecipitation and Western blotting

Total cell proteins and the cytoplasmic and nuclear fractions were obtained from 70% confluent cell cultures, as previously described (Garofalo et al., 2004). The following monoclonal (m) and policlonal (p) antibodies (Abs) were used: anti-AR 441 mAb (Santa Cruz, USA); anti-IRS-1 pAb (Upstate, USA); anti-c-jun mAb (Santa Cruz, USA); anti-GAP-DH mAb (Santa Cruz, USA); anti ubiquitin P4D1 mAb (Santa Cruz, USA); p21 pAb (Santa Cruz, USA).

Plasmids, transfections and luciferase reporter assays

A full-length androgen AR expression plasmid pcDNA3 AR (AR) was described by Tilley *et al.* (Tilley et al., 1990). Firefly luciferase reporter plasmid containing ARE, pARE2-tk-LUC (ARE-Luc), was a gift from Dr O. Janne. The IRS-1 knockdown cells were obtained using pSilencer-IRS-1 plasmid (shIRS1), as described by Cesarone *et al.* (Cesarone et al., 2006), with a scrabled shRNA plasmid used as a control (Scrambled). The plasmid WWP-Luc containing human

$p21^{WAF1/Cip1}$ promoter (p21-Luc) and p5.3PSAp-Luc encoding the PSA promoter (PSA-Luc) were gifts from Dr. W. El-Deiry and Dr. Kakizuka, respectively.

For transient transfection assay, MCF-7 cells were grown in 24-well plates. At 70% confluence, the cultures were transfected for 6 h using Fugene 6 (DNA:Fugene 3:1; Roche, CH). In addition, to assess transfection efficiency, each DNA mixture contained 5ng of pRL-TK-Luc, a plasmid encoding Renilla Luciferase (Promega, USA). Empty vectors were used to ensure that DNA concentrations were constant in each transfection. At 6 h after transfection cocktail addition, cells were shifted to PRF for 24 h and then treated with 10^{-7}M DHT or left untreated in PRF-CT for 24 h. Luciferase activity was measured using Dual luciferase assay System (Promega, USA). The firefly luciferase data for each sample were normalized on the basis of transfection efficiency measured by renilla luciferase activity.

To monitor the influence of IRS-1 on AR activity, ARE-Luc was co-transfected with AR expression vector alone or in combination with IRS-1 expression vector pCMV-IRS-1 (IRS-1). The experiment was done using an AR- and IRS1-negative cell line HEK-293.

To asses mRNA and protein levels, MCF-7 cells were plated on 60mm dishes and transfected with an appropriate amount of various plasmids, as indicated in the Figure legends. Upon transfection, cells were shifted to PRF for 24 h and then treated with 10^{-7}M DHT or left untreated in PRF-CT for 24 h.

Chromatin immunoprecipitation (ChIP).

MCF-7 cells were grown in 10 cm plates. Confluent cultures (90%) were shifted to PRF for 24 h and then treated with 10^{-7}M DHT or left untreated in PRF-CT for 2 h. Following treatment, ChIP methodology was performed as described previously

(Morelli et al., 2004). The precleared chromatin was precipitated with anti-AR mAb (Santa Cruz, USA) for AR, anti-IRS-1 pAb (Upstate, USA) for IRS-1 and anti-Polymerase II pAb (Santa Cruz, USA) for Pol II. A 4 μl volume of each sample was used as template for PCR with specific primers.

The following pairs of primers were used to amplify 296 bp of the ARE-containing p21 promoter 5'-CAGCGCACCAACGCAGGCG-3' (forward); 5'-CAGCTCCGGCTCCACAAGGA-3' (reverse), and 233 bp of the PSA promoter including ARE-sequence in proximal region 5'-GATCTAGGCACGTGAGGCTTTGTA-3' (forward) and 5'-CATGCTGCTGGAGGCTGGAC-3' (reverse). The PCR conditions for p21 promoter were: 1min at 94 C, 1 min at 65 C, and 2 min at 72 C; for PSA promoter: 40 s at 94 C, 40 s at 57 C, and 1 min 72 C. The amplification products obtained in 28 and 30 cycles were analyzed in a 2% agarose gel and visualized by ethidium bromide staining.

RT–PCR

MCF-7 cells were transfected with the scrambled and shIRS1 plasmid for 24 h, as described in the transactivation assays methodology than treated with 10^{-7}M DHT or left untreated in PRF-CT for 24 h. Total RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. 2 μg of total RNA were reverse transcribed using M-MLV reverse transcriptase (Promega, USA) and 2 μl of RT products were then amplified. The following primers were used: IRS-1 forward primer 5'-TCCACTGTGACACCAGAATAAT-3', IRS-1 reverse primer 5'-CGCCAACATTGTTCATTCCAA-3' (762 bp); PSA forward 5'-TGCGCAAGTTCACCCTCA-3', PSA reverse 5'-CCCTCTCCTTACTTCATCC-3'

(754 bp); AR forward 5'-CACAGGCACCTGGTCCTGG-3', AR reverse 5'-CTGCCTTACACAACTCCTTGGC-3' (416 bp); p21 forward 5'-GCTTCATGCCAGCTACTTCC-3', p21 reverse 5'-CTGTGCTCACTTCAGGGTCA-3' (270 bp). For the internal control gene 36B4, the primers were: 36B4 forward 5'-CTCAACATCTCCCCCTTCTC-3', 36B4 reverse 5'-CAAATCCCATATCCTCGTCC-3' (408 bp). PCR was performed using the following conditions: for IRS-1, 1 min at 94 C, 1 min at 50 C, 2 min at 72 C; for PSA, 30 s at 94 C, 30 s at 58 C, 30 s at 72 C; for p21, 1 min at 94 C, 1 min at 58 C, 1min 72 C; for AR, 45 s at 94 C, 45 s at 60 C, 1 min 72 C; for 36B4 1min at 94 C, 1 min at 60 C, 2 min at 72 C. The amplification products were analyzed in a 1,5% agarose gel.

Results

DHT regulates cellular localization of IRS-1 in breast cancer cells.

To determine whether androgens may regulate the expression and localization of IRS-1 in breast cancer cells, we analyzed IRS-1 abundance in cytoplasmic and nuclear protein fractions obtained from MCF-7 cells stimulated or not with 10^{-7}M DHT. In absence of DHT, IRS-1 was mainly present in the cytoplasmic compartment, while upon DHT treatment, the nuclear abundance of IRS-1 significantly increased, with a parallel decrease of cytoplasmic IRS-1 expression. Moreover, the increased localization of IRS-1 in the nuclear fraction appeared to be specifically mediated by AR, since it is inhibited by the addition of the androgen antagonist hydroxy-flutamide (OH-Fl) (Figure 1).

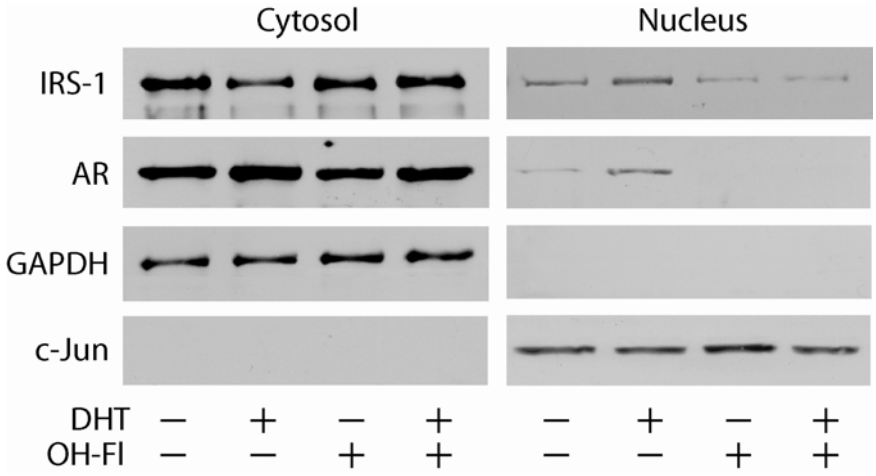

Figure 1. IRS-1 colocalizes with AR. MCF-7 cells synchronized in SFM were treated with 10^{-7} M DHT or 10^{-7} M OHFl in PRF-CT for 24 h. The expression of IRS-1 and AR was determined by Western blotting in 50 μg of cytoplasmic or in 100 μg of nuclear proteins using specifics Abs, as described in Materials and Methods. The expression of GAPDH and c-jun was assessed as control of protein loading and purity of lysate fractions. The results were obtained after repetitive stripping and reprobing of the same filter. The results are representative of three independent experiments.

To assess whether the DHT-regulated intracellular localization of IRS-1 could involve a physical interaction between AR and IRS-1, a coimmunoprecipitation assay

was carried out on nuclear and cytoplasmic protein fractions from MCF-7 cells. Under basal conditions, a constitutive association between AR and IRS-1 was observed in the cytoplasm, but not in the nuclear fraction. DHT treatment induced the translocation of AR/IRS-1 complex in the nucleus with a consequent decrease of its abundance in the cytosol (Figure 2).

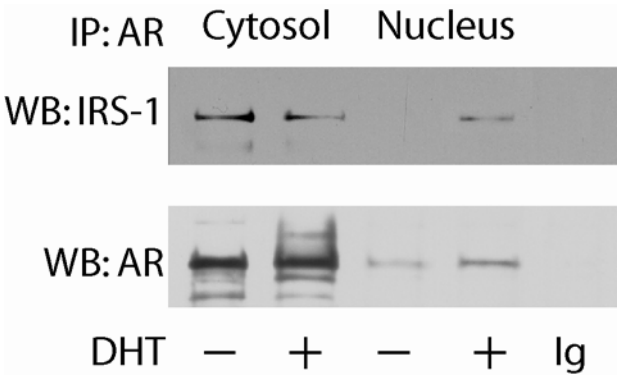

Figure 2. IRS-1 coprecipitates with AR. Cytoplasmic and nuclear fractions were immunoprecipitated (IP) with an anti-AR antibody and immunoblotted (WB) with an anti-IRS-1 pAb and an anti-AR mAb. In control samples, the primary Abs were substituted with non-immune IgGs (rabbit or mouse, depending on the source of the primary Abs). The results are representative of three independent experiments.

These observations suggest that DHT could promote direct interaction between AR and IRS-1 thereby inducing nuclear translocation of IRS-1.

IRS-1 is a transcriptional regulator of AR.

The existence of the nuclear AR/IRS-1 complex lead us to investigate the role of nuclear IRS-1 on the transcriptional activity of AR. We evaluated the effects of ectopic IRS-1 expression on the transcriptional activity of AR using the androgen-response reporter plasmid 2X-ARE-Luc. HEK-293 cells that are IRS-1- and AR-negative were cotransfected with an AR expression vector and increasing amounts of an IRS-1-encoding plasmid. The ectopic expression of IRS-1 increased the

transcriptional response to DHT stimulation in a dose-dependent manner, with a maximal two-fold enhancement above the level observed in the absence of IRS-1 (Figure 3). This stimulatory effect of IRS-1 on AR was blocked by the addition of OH-Fl (data not shown), suggesting a direct involvement of AR in this process.

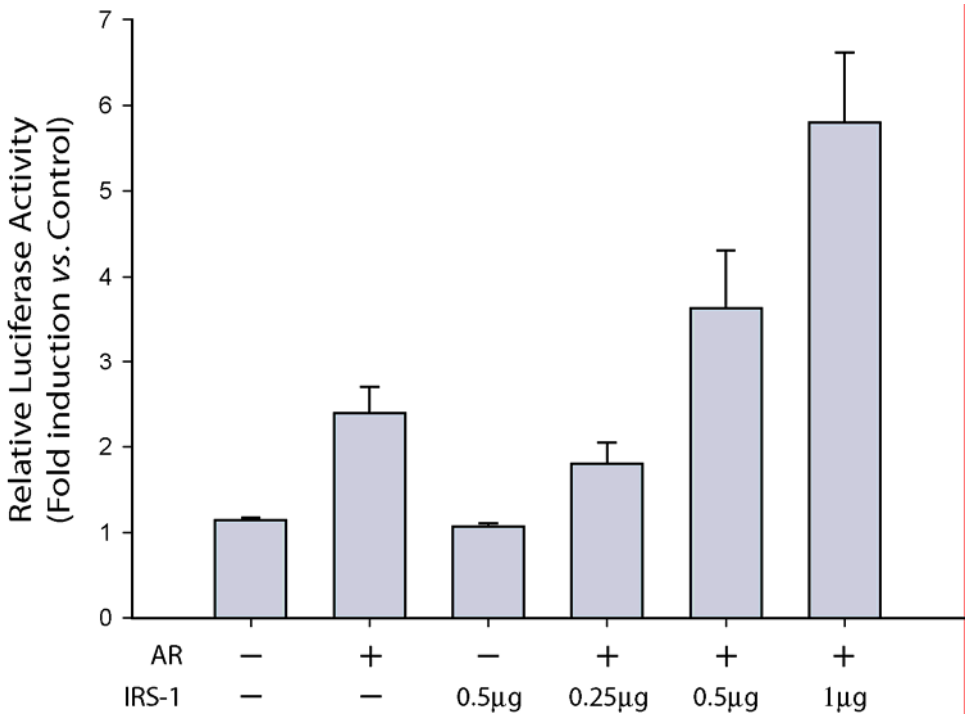

Figure 3.Transcriptional activity of AR on ARE promoter in the presence or absence of IRS-1. HEK 293 cells were transfected with 0.5 µg ARE luciferase reporter construct pARE2-tk-LUC plus increasing quantities of pCMV-IRS-1 (0, 0.25, 0.5, and 1 µg) together with 0.1 µg pcDNA3-AR. Upon transfection, the cells were shifted to SFM for 16 h and than treated with 10^{-7} M DHT, or left untreated in PRF-CT for 18 h. Firefly luciferase activity was internally normalized to Renilla luciferase and expressed as Relative Luciferase Activity (RLA) with respect to the untreated samples. Results represent the mean±s.d. from several experiments.

To determine whether the involvement of IRS-1 in AR-mediated transcription was related to IRS-1 recruitment to ARE-containing regions of androgen target gene promoters, we used chromatin immunoprecipitation assays (ChIPs). We focused our attention on the prostatic specific antigen (PSA) and $p21^{CIP/WAF}$ (p21) genes that are known to be regulated by androgens in breast cancer cells (Lu et al., 1999). As illustrated in Figure 4, basal occupancy of AR on PSA and

p21 promoters occurs in untreated cells. The recruitment of AR to both promoters is increased in response to DHT treatment, reaching the maximum at 2 h (4-fold increase vs. untreated). IRS-1 is recruited to the same promoter regions in response to DHT treatment only, reaching the maximal association after 2 h of hormonal stimulation. The association of AR and IRS-1 with the PSA and p21 promoters coincided with the loading of RNA polymerase II (Pol II) on the same regions.

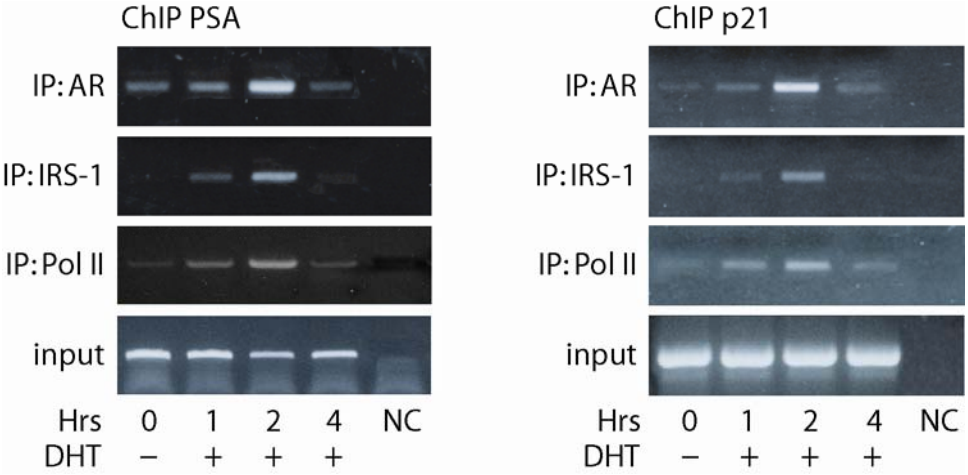

Figure 4. IRS-1 is a transcriptional co-regulator of AR. ChIPs were carried out over a 4 h time-course after stimulation with DHT using AR, IRS-1 and Pol II antibodies , as described in Materials and Methods . The PSA and p21 promoter sequences containing androgen responsive element were detected by PCR with specific primers listed in Materials and Methods (fragments of 233 and 296 bp respectively). To control input DNA, PSA and p21 promoter was amplified from 30 µl of initial preparations of soluble chromatin (before IP). In control samples (NC), non-immune IgG (rabbit for IRS-1 Ab and mouse for AR Ab) was used instead of the primary Abs.

IRS-1 knockdown decreases AR transcriptional activity.

To delineate whether modulation of the AR/IRS-1 interaction could influence AR function in response to DHT, a silencing RNA technology was used to knockdown the expression of IRS-1 in MCF-7. A 70% reduction of IRS-1 levels achieved with anti-IRS-1 shRNA expression in MCF-7 cells correlated with a 40% decrease of IRS-1 mRNA levels (Figure 4C and B).

Luciferase assays, demonstrated that IRS-1 knockdown with shRNA resulted in a 40% decrease in transcriptional activation of ARE-containing promoters in both wild-type and in AR overexpressing MCF-7 (Figure 5A). Similarly, downregulation of IRS-1 impeded activation of the PSA and p21 promoters by 50% (Figure 5B and C).

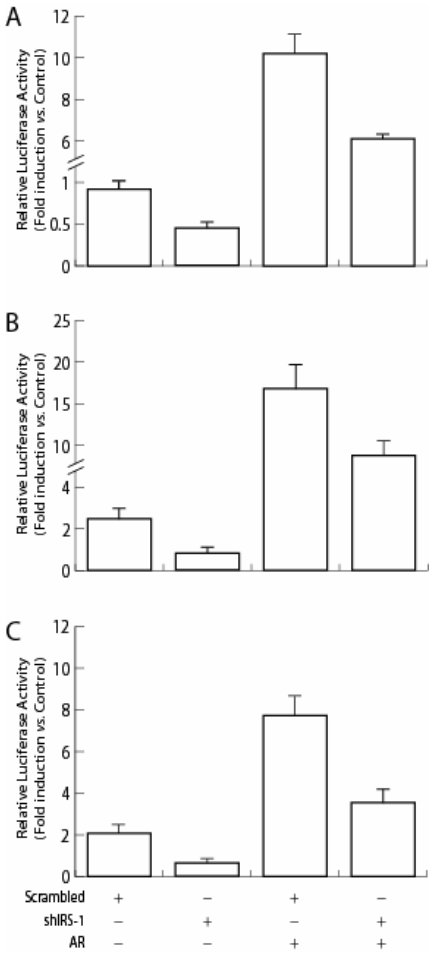

Figure 5. IRS-1 knockdown reduces AR regulated transcription. MCF-7 cells were transiently cotransfected, as described in Materials and Methods, with 0.5 μg IRS-1 shRNA (shIRS-1) or 0.5 μg scrambled control shRNA (Scrambled) either in the absence or presence of 0.1 μg pcDNA3-AR (AR) together with the following specific reporter constructs: 0.5 μg pARE2-tk-LUC **(A)**; 0.5 μg p5.3PSAp-Luc **(B)**; 0.25 μg WWP-Luc containing human $p21^{WAF1/Cip1}$ promoter **(C)**. Upon transfection, cells were serum starved overnight, then stimulated with 10^{-7} M DHT, or left untreated in PRF-CT for 24 h, and than luciferase activities were determined. Firefly luciferase activity was internally normalized to Renilla luciferase and expressed as Relative Luciferase Activity (RLA) with respect to the untreated samples. Results represent the mean±s.d. of at least five independent experiments.

These results were confirmed by ChIP analysis demonstrating that IRS-1 knockdown resulted in diminished recruitment of AR and Pol II on the p21 and PSA promoters (Figure 6).

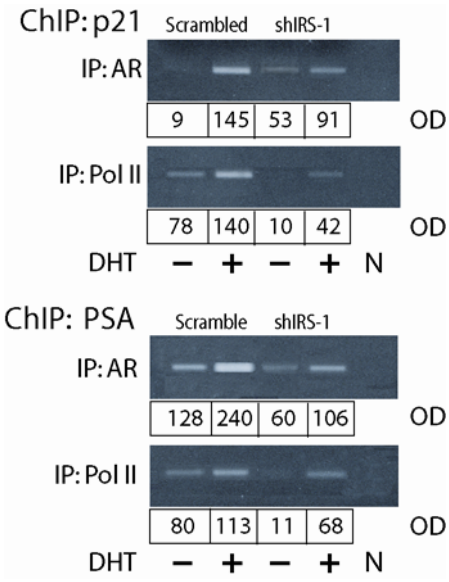

Figure 6. IRS-1 knockdown decreases AR loading on the promoters of AR-regulated genes. Chromatin immunoprecipitation was carried out on MCF-7 cells transiently transfected with 3 μg IRS-1 shRNA (shIRS-1) or 3 μg scrambled control shRNA (Scrambled), as described in Materials and Methods. Cells were treated with 10^{-7}M DHT for 2 h and DNA-associated proteins were precipitated using AR, and Pol II antibodies. The PSA and p21 promoter sequences containing ARE were detected by PCR with specific primers listed in Materials and Methods (fragments of 233 and 296 bp respectively). To control input DNA, PSA and p21 promoters were amplified from 30 μl of initial preparations of soluble chromatin (before IP). In control samples (N), non-immune IgG (rabbit for IRS-1 Ab and mouse for AR Ab) was used instead of the primary Abs.

Furthermore, down-regulation of IRS-1 resulted in reduced PSA and p21 mRNA and protein levels (Figure 4B and C). Most importantly, all the above effects were paralleled by decreased expression of AR protein (Figure 4C), which evidenced a role for IRS-1 in regulating AR expression. Interestingly, inhibition of AR protein expression in IRS-1-shRNA MCF-7 cells was not related to decreased AR mRNA levels (Figure 4B), suggesting that regulation of AR by IRS-1 occurs on the posttranscriptional level.

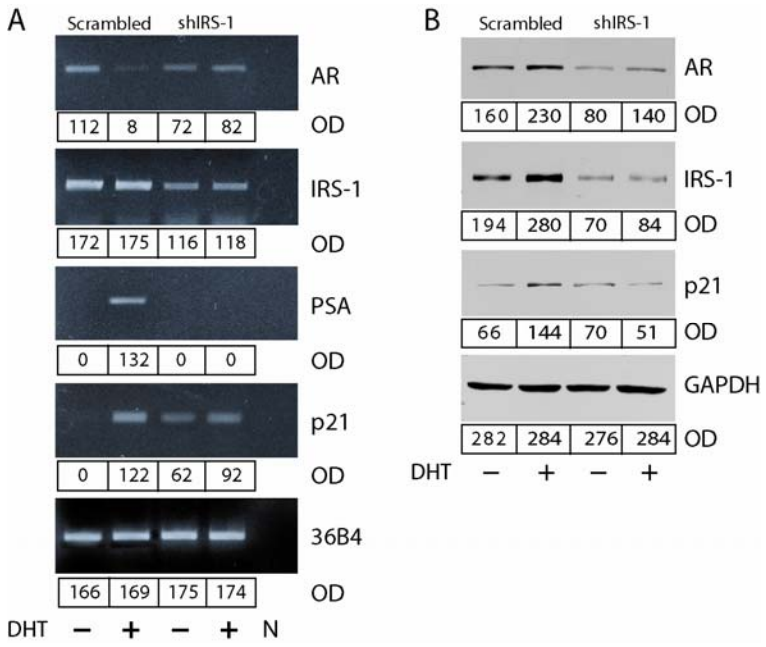

Figure 7. IRS-1 knockdown reduces the expression of AR regulated genes. Total cellular RNA **(A)** and cytoplamic proteins **(B)** were isolated from transiently transfected MCF-7 cells treated for 24 h with 10^{-7} M DHT or left untreated in PRF-CT. The expression of AR, IRS-1, PSA, p21, and 36B4 (control of RNA input) mRNA was evaluated by RT–PCR as described in Materials and Methods. The PCR products corresponding to AR, IRS-1, PSA, p21 and 36B4 cDNA fragments (416, 762, 754, 270, and 408 bp respectively) were obtained at 25 and 30 PCR cycles. To control the purity of RNA, the RT step was omitted before the samples were amplified by PCR. 50 μg of protein lysates were probed by WB for the expression of AR, p21, IRS-1, and GAP-DH, as described in Materials and methods. The results, representative of three independent experiments, were quantified and reported as optical density (OD).

IRS-1 knockdown induces ubiquitylation of AR

Ubiquitin-dependent proteolysis represents an important mechanism for controlling protein turnover and is pertinent to the regulation of numerous transcription factors, including AR (Freiman and Tjian, 2003; Nawaz and O'Malley, 2004). Given that polyubiquitylation, in general, constitutes a destructive signal recognized by the proteasome, we investigated the ubiquitylation status of AR following downregulation of IRS-1 with shRNA.

MCF-7 and IRS-1-shRNA MCF-7 cells were either left untreated or treated with 10^{-7}M DHT for 6 h. Ubiquitylated AR (Ub-AR) was detected by

immunoprecipitation and Western blotting. Three forms of AR were detected, the regular form of 110 kDa, a form of an apparent molecular weight of 120-130 kDa consistent with the mono-ubiquitylated receptor, and the poly-ubiquitylated form ranging from 140-170 kDa. In untransfected MCF-7 cells, mono-ubiquitylation of AR was ligand dependent, since in the absence of DHT the upper bands were almost absent and only marginal Ub-AR was observed (Figure 8A).

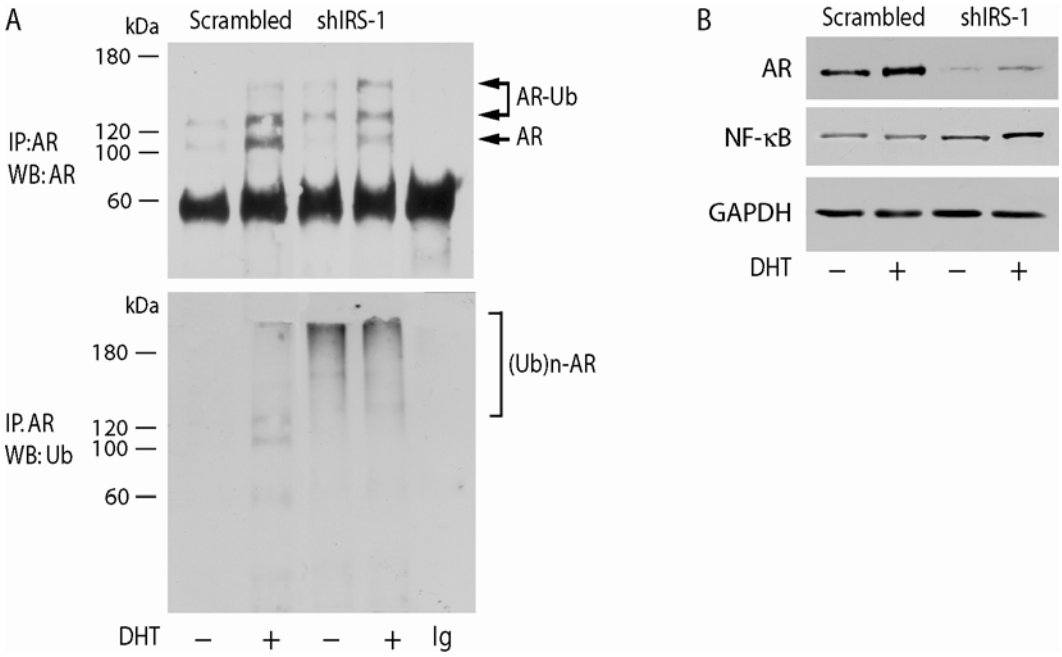

Figure 8. Effects of IRS-1 on AR ubiquitylation. MCF-7 cells, transiently transfected with 3 μg shIRS-1 or 3 μg scrambled control shRNA (Scrambled) were treated for 6 h with 10^{-7} M DHT or left untreated in PRF CT. AR was immunoprecipitated (IP) from 500 μg of total protein lysates, and probed with AR mAb **(A).** The same filter was stripped and re-probed with anti-Ub mAb. The expression of AR, NF-kB, and GAP-DH was assessed by WB on 50 μg of protein lysates **(B).** The results are representative of three independent experiments.

In IRS-1-shRNA MCF-7 cells, mono-ubiquitylated AR is still present. It is worth noting that in the same experimental conditions a dramatic increase in the poly-ubiquitylation status of AR was observed in both DHT-treated and -untreated samples (Figure 8A). Thus, poly-ubiquitylation of AR may explain its reduced expression in the shIRS-1 MCF-7 cells (Figure 8B). These results are confirmed by

treatment with the proteasomal inhibitor MG132, evidencing a dramatic increase in Ub-AR level (Figure 9).

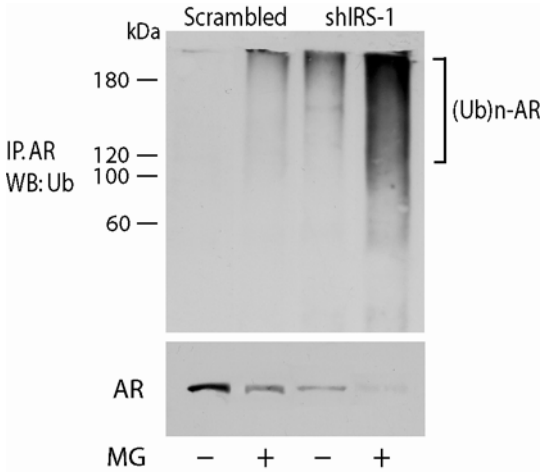

Figure 9. Proteasomal inhibitor MG132 increase Ub-AR level. MCF-7 cells, transfected as before, were treated with or without 1μM MG132 for 8 h. Total protein lysate were immunoprecipitated with an AR mAb and probed with Ub mAb. AR content is shown in the bottom panel. The results are representative of three independent experiments.

Interestingly, IRS-1 knockdown in MCF-7 cells appears to enhance basal proteosomal activity, as indicated by the increased levels of the DNA binding subunit of NF-κB, that is produced from a precursor via ubiquitin-proteosomal processing (Figure 8B). These data suggest that IRS-1 interferes with proteasomal pathways of different proteins, particularly, the presence of IRS-1 protects AR from ubiquitin-mediated degradation.

Discussion

In addition to the conventional hormone-dependent regulation of steroid receptor activity, many studies have demonstrated the existence of crosstalk between growth factor signal transduction pathways and steroid receptors. The modulation of kinase/phosphatase activity in cells leads either to the direct activation of steroid receptors in the absence of hormone (Weigel and Zhang, 1998) or to the activation and recruitment of transcription regulators.

In this study, we report on new aspects of the interaction between AR and an IGF-I signaling molecule, IRS-1. We demonstrated that IRS-1 associates with AR and can be translocated to the nucleus in response to DHT. The nuclear trafficking of IRS-1 seems to be strictly mediated by AR, since the addition of an androgen antagonist (OH-Fl) inhibits this process. Nuclear localization of IRS-1 has already been documented in breast cancer cells (Morelli et al., 2004), and many other cellular systems (Lassak et al., 2002; Prisco et al., 2002; Sun et al., 2003), suggesting that the protein can function not only as signal transducer but also as transcriptional regulator (Morelli et al., 2004; Tu et al., 2002).

The possibility that nuclear IRS-1 modulates AR-dependent transcription was addressed with transient transfection reporter assays in IRS-1 negative cells. The transcriptional response to DHT stimulation was enhanced by IRS-1 expression in a dose dependent manner (Figure 3). Moreover, nuclear IRS-1 was co-recruited with AR on ARE-containing regions of the PSA and p21 promoters (Figure 4A and B), which are regulated by androgens in breast cancer cells (Lu et al., 1999). The requirement of IRS-1 expression for AR-dependent transcription was also assessed by down-regulating IRS-1 in MCF-7 cells by RNA interference. IRS-1 knockdown

markedly reduced AR transcriptional response and inhibited the expression of AR-regulated genes in several experimental models (Figure 5, 7A and B).

Unoccupied AR is labile and its expression can be regulated by posttranscriptional events in tissues and cell culture models, independently from changes in receptor mRNA levels (Krongrad et al., 1991; Wolf et al., 1993; Yeap et al., 1999; Zhou et al., 1995). The binding of ligand to AR, alters receptor interactions with chaperone proteins, decreasing the turnover and enhancing its ability to activate androgen-responsive gene expression (Brinkmann et al., 1999; Wang et al., 1999; Wilson and McPhaul, 1996; Zhou et al., 1995). Since IRS-1 knockdown produced a reduction in basal AR protein levels (Figure 7B), we investigated whether IRS-1 could regulate AR turnover.

Previous findings demonstrated that proteasome-mediated degradation of transcriptional activators are coupled to the transcription process (Conaway et al., 2002; Ferdous et al., 2001; Salghetti et al., 2001) and that hormone binding increases ubiquitylation and degradation of nuclear receptors (Lange et al., 2000; Nawaz et al., 1999; Wallace and Cidlowski, 2001). Accumulating evidence indicates that AR may be targeted for degradation via ubiquitin-proteasome pathways and that this mechanism is required for AR transactivation in response to DHT (Gaughan et al., 2005; Lin et al., 2002; Pajonk et al., 2005; Reddy et al., 2006). AR contains an highly conserved PEST (proline, glutamate, serine and threonine-rich) sequence, located in the hinge region, which targets proteins for ubiquitylation and degradation through proteasomal pathways (Sheflin et al., 2000).

The cellular abundance of IRS-1 and AR are both regulated through the 26S proteasome (Lee et al., 2000; Pederson et al., 2001; Sun et al., 1999; Zhang et al.,

2000), Thus, we hypothesized that IRS-1 knockdown could increase the degradation of AR by desegregating proteins involved in common proteolytic processes. Indeed, our findings demonstrate that IRS-1 knockdown dramatically enhances the basal poly-ubiquitylation of AR (Figure 8A).

Consistently, poly ubiquitylation of AR targets it for rapid proteosomal degradation, while the mono ubiquitylated form represents the trascriptional active form of the receptor (Burgdorf et al., 2004; Reddy et al., 2006)

Moreover, basic enhancement in cellular proteasomal activity was observed in IRS-1 knockdown MCF-7 cells since an increase of the NF-κB DNA binding subunit, which is released from a precursor by the ubiquitin/proteosome-dependent process, was detected (Figure 8B).

This is in agreement with the increased AR gene transcription (Figure 7B), since NF-κB DNA binding subunit have been shown to stimulate the AR promoter activity (Delfino et al., 2003; Zhang et al., 2004).

In summary, our data provide novel insights into the role of IRS-1 as nuclear regulator of AR function in breast cancer cells. IRS-1 stabilizes AR protein levels reducing the ubiquitin-dependent proteolysis of AR and optimizes AR-mediated transcription by participating in the transcriptional machinery on AR regulated promoters. Further study should help to better understand the mechanisms by which IRS-1 triggers the ubiquitylation and proteosomal degradation of AR.

In conclusion, taking into account that androgens are potent growth inhibitors in breast cancer cells (Ando et al., 2002), and that IRS-1 negatively modulates estradiol-dependent transcription in MCF-7 cells (Morelli et al., 2004), the results

reported in this work underline the critical role of IRS-1 in modulating breast cancer cell proliferation in response to different steroid hormone stimulation.

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Scientific Publications
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Expression of Nuclear Insulin Receptor Substrate 1 (IRS-1) in Breast Cancer

Diego Sisci[1§] , Catia Morelli[1§], Cecilia Garofalo[1], Francesco Romeo[2], Lucio Morabito[2], Filomena Casaburi[2], Emilia Middea[1] , Sandra Cascio[3], Elvira Brunelli[4], Sebastiano Andò[5] and Eva Surmacz[3*]

[1] Department of Pharmaco-Biology, Faculty of Pharmacy, University of Calabria, Arcavacata di Rende, Italy; [2] Division of Anatomo-Pathology, Annunziata Hospital, Cosenza, Italy; [3]Sbarro Institute for Cancer Research and Molecular Medicine, Temple University, Philadelphia, PA, USA, [4]Department of Natural Science and [5]Department of Cellular Biology, Faculty of Pharmacy, University of Calabria, Arcavacata di Rende, Italy.

[§] D.S. and C.M. contributed equally to this work

***Corresponding author:** Eva Surmacz, Ph.D.
Sbarro Institute for cancer Research and Molecular Medicine
College of Science and Technology, Temple University
1900 N 12th St. Rm. 446, Philadelphia, PA 19122
Tel. 001/215-204-0306, Fax 001/215-204-0303
e-mail surmacz@temple.edu

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Abstract

Aims: Insulin receptor substrate 1 (IRS-1), a cytoplasmic protein transmitting signals from the insulin and insulin-like growth factor 1 receptors, has been implicated in breast cancer. Previously, we reported that IRS-1 can be translocated to the nucleus and modulate estrogen receptor α (ERα) activity in vitro. However, the expression of nuclear IRS-1 in breast cancer biopsies has never been examined. Consequently, we assessed whether nuclear IRS-1 is present in breast cancer and non-cancer mammary epithelium and if it correlates with other markers, especially ERα. Parallel studies were done for cytoplasmatic IRS-1.

Methods: IRS-1 and ERα expression was assessed by immunohistochemistry. Data were evaluated using Pearson correlation, linear regression, and ROC analysis.

Results: Median nuclear IRS-1 expression was low in normal mammary epithelial cells (1.6%) and higher in benign tumors (20.5%), ductal grade 2 carcinoma (11.0%), and lobular carcinoma (~30%). Median ERα expression in normal epithelium, benign tumors, ductal cancer grade 2 and 3 and lobular cancer grade 2 and 3 was 10.5, 20.5, 65.0, 0.0, 80, and 15%, respectively. Nuclear IRS-1 and ERα positively correlated in ductal cancer ($p<0.001$) and benign tumors ($p< 0.01$), but were not associated in lobular cancer and normal mammary epithelium. In ductal carcinoma, both nuclear IRS-1 and ERα negatively correlated with tumor grade, size, mitotic index, and lymph node involvement. Cytoplasmic IRS-1 was expressed in all specimens and positively correlated with ERα in ductal cancer.

Conclusions: A positive association between nuclear IRS-1 and ERα is a characteristic for ductal breast cancer and marks a more differentiated, non-metastatic phenotype.

Take-home messages

1. This is the first report examining the expression of nuclear IRS-1 in normal mammary tissue, benign breast tumors and breast cancer in relation to ERα and clinicopathological features.

2. Nuclear IRS-1 is more prevalent in cancer specimens than in normal mammary tissues.
3. Nuclear IRS-1 and ERα negatively correlated with tumor grade, size, mitotic index, and lymph node involvement.
4. A positive association between nuclear IRS-1 and ERα is a characteristic for ductal breast cancer and marks a more differentiated, non-metastatic phenotype.

Introduction

Recent experimental and clinical evidence suggests the involvement of the insulin-like growth factor I (IGF-I) receptor (IGF-IR) in breast cancer development and progression [1-6]. The tumorigenic action of IGF-IR is executed through multiple antiapoptotic, growth promoting, and/or pro-metastatic pathways [5-9]. Many of these pathways stem from IRS-1, a major IGF-I signaling molecule that becomes phosphorylated on multiple tyrosine residues upon IGF-IR activation. Tyrosine phosphorylated IRS-1 acts as a scaffolding protein sequestering downstream signaling molecules and propagating IGF-I signal through the PI-3K/Akt, Ras/Raf/ERK1/2, Jak2/Stat3 and other pathways [10-13].

Overexpression or downregulation of IRS-1 in breast cancer cell models suggested that the molecule controls several aspects of the neoplastic phenotype, especially anchorage-dependent and -independent cell growth and survival [14 15]. In breast cancer cell lines, IRS-1 appears to be expressed at higher levels in ERα-positive than in ERα-negative cells and there is evidence supporting the existence of a crosstalk between IRS-1 and ERα systems [1 4 6 16-18]. Overexpression of IRS-1 in MCF-7 ERα-positive cells has been shown to induce estrogen-independence and mediate antiestrogen-resistance [14 19 20]. High expression of IRS-1 can be in part attributed to ERα activity, as 17β-estradiol (E2) can upregulate IRS-1 expression and function [16 21 22], while antiestrogens reduce IRS-1 mRNA and protein levels and inhibit IRS-1 signaling [19 20 23]. In addition, ERα can directly interact with IRS-1, increasing its stability and potentiating its downstream signaling to Akt [24]. Notably, increased activity of IRS-1 is likely to modulate ERα, via ERK1/2- and Akt-mediated phosphorylation of ERα on Ser-118 and Ser-167, respectively [25-27].

Recent reports suggested that in addition to its cytoplasmic signaling function, IRS-1 is able to regulate nuclear processes in different cell models [28-33]. For instance, in mouse fibroblasts treated with IGF-I, a fraction of IRS-1 is translocated from the cytoplasm to the nuclear and nucleolar

compartments where it modulates the expression of genes controlling cell proliferation (i.e., Cyclin D1) and cell growth in size (i.e., rDNA) by physically interacting with transcriptional complexes of β-catenin and upstream binding factor 1 (UBF1), respectively [31 32]. Our recent work demonstrated that nuclear IRS-1 is also found in breast cancer cell lines. For instance, in MCF-7 cells treated with E2 nuclear IRS-1 physically interacted with ERα modulating its transcriptional activity at estrogen response element (ERE) DNA motifs [33]. The exact mechanism of nuclear IRS-1 transport is not clear, but most likely it involves other proteins containing nuclear localization signals (ERα, T antigen, importins).

Despite the evidence that IRS-1 signaling may play a critical role in tumorigenesis, only limited studies examined the clinical significance of IRS-1 expression in human breast cancer specimens [18 34-36]. In one study, cytoplasmatic IRS-1 has been reported to correlate with poorly differentiated breast tumor phenotype (G3) and lymph node involvement [35]. Another study correlated IRS-1 with shorter disease-free survival in patients with smaller tumors [18]. In contrast, Schnarr et al. found that IRS-1 marks a more differentiated phenotype and better prognosis [34]. Furthermore, one study examining cancer and normal specimens reported similar IRS-1 tyrosine phosphorylation in all tissues [36], while other analysis found decreased IRS-1 levels in poorly differentiated cancers relative to normal tissue and benign tumors [34].

Regarding nuclear IRS-1, its presence in breast cancer specimens has been noted by Schnarr et al. [34] and Koda et al. [35], but any association with the disease has never been formally addressed. Consequently, we examined the expression of nuclear IRS-1 in normal mammary tissue, benign breast tumors and breast cancer in relation to ERα and clinicopathological features. Parallel studies were done for cytoplasmatic IRS-1.

Materials and Methods

Patients and tissue specimens

Table 1 summarizes information on patient and specimen characteristics. The histopathological examination of sections was based on the WHO and pTN classification of breast tumors. Tumor size (pT) was scored as follows: 0, primary tumor not detectable; 1, tumor largest diameter <2cm; 2, diameter <5cm; 3, diameter >5cm; 4, inflammatory carcinoma of any size. Lymph node status (pN) was scored from 0, no node involvement; 1, proximal node involved; 2, distal node involved. The protocol of the present study was reviewed and approved by the local ethical committee.

Table 1. Patient characteristics and clinical parameters of breast tissues and cancers

Sample characteristics

	Cancers	**Controls**
Total specimens	60	34
Ductal carcinoma	38	
Lobular carcinoma	22	
Benign tumors		19
Macromasty		14

Patient Age

	Normal	**Benign**	**Ductal**	**Lobular**
Mean±SE	53.6±3.3	45.4±3.1	62.9±2.4	64.5±2.7
Median (Range)	56.5 (33-68)	43 (20-68)	61.5 (43-94)	66 (48-78)
Menopause (%)	64	39	87	82

Clinical parameters of breast cancer tissues

	Ductal (38)		**Lobular (22)**	
	G2 (19)	**G3 (19)**	**G2 (10)**	**G3 (12)**
pT	1-4	0-4	2-4	0-4
pN	0-2	0-2	0-1	0-2
Ki67	7.7 ± 0.9 (4-14)	14.2±1.3 (6-21)	7.2±1.5 (4-12)	9.0±1.9 (3-15)

The age of patients in each group is given as mean value ± SE with median age (range) for each population. The percentage of postmenopausal patients is indicated in each group. The range is

reported for tumor size (pT), and lymph node involvement (pN); median frequency of expression ±SE (range) is shown for Ki67.

Immunohistochemistry and confocal microscopy

Samples preparation

Immediately after excision, tissue samples were fixed in 10% buffered formaldehyde solution and embedded in paraffin blocks at 56°C. ERα and IRS-1 were analyzed by immunohistochemical (IHC) staining using 3µm-thick consecutive paraffin sections. The sections were dewaxed in xylene and rehydrated in graded alcohols. After antigen retrieval by boiling in 0.01M citrate buffer pH 6.

Immunohistochemistry

Endogenous peroxidase was removed with 3% H_2O_2; nonspecific binding was blocked by incubating the slides for 30 min with 1.5% BSA in PBS. Next, the sections were incubated with the primary antibodies (Abs) for 1h at room temperature. ERα was detected using ERα mouse monoclonal Ab (mAb) (DakoCytomation, Denmark) at dilution 1:35. IRS-1 was detected using the C-terminus IRS-1 rabbit polyclonal Ab (pAb) (Upstate, USA) at a concentration 4µg/ml. Ab-antigen reactions were revealed using Streptavidin-biotin-peroxidase complex (LSAB kit, DakoCytomation, Denmark). All slides were counterstained with hematoxylin. Breast specimens previously classified as positive for the expression of the studied markers were used for control and protocol standardization. In negative controls, primary Abs were omitted. The expression of ERα and IRS-1 was independently scored by two investigators (CM and CG) by light microscopy in 10 different section fields. For all nuclear markers, mean and median percentage, and the range of epithelial cells displaying positive staining was scored. In some analyses, specimens were grouped into ERα-negative (less than 5% of epithelial cells exhibiting ERα expression) and ERα-positive (5% or more of cells with ERα). The expression of cytoplasmic IRS-1 was classified using a four-point scale: 0, <10% positive cells with any staining

intensity; 1+, 10-50% positive cells with weak or moderate staining; 2+, >50% positive cells with weak or moderate staining; 3+, >50% positive cells with strong staining. No samples with less than 50% of positive cells with strong staining were recorded.

Confocal microscopy

Tissues sections were incubated for 30 min with 3% BSA in PBS to avoid nonspecific bindin, then for 1 h with a mixture of primary Abs (pAbs) recognizing IRS-1 and ERα.

The anti-IRS-1 pAb (UBI) at 4 μg/ml was used for IRS-1 staining; anti-ERa F-10 monoclonal Ab (mAb) (Santa Cruz) at 2 μg/ml was used to detect ERα. Following the incubation with primary Abs, the slides were washed three times with PBS, and incubated with a mixture of secondary Abs. A rhodamine-conjugated donkey anti-mouse IgG (Calbiochem) was used as a secondary Ab for ERα and a fluorescein-conjugated donkey anti-rabbit IgG (Calbiochem) was used for IRS-1. The cellular localization of IRS-1 and ERα was studied using the Bio-Rad MRC 1024 confocal microscope connected to a Zeiss Axiovert 135M inverted microscope with x1000 magnification. The optical sections were taken at the central plane. The fluorophores were imaged separately to ensure no excitation/emission wavelength overlap. In control samples, the staining was performed with the omission of the primary Abs.

Statistical analysis

Descriptive statistic for nuclear IRS-1 and ERα in normal, benign and tumor samples was reported as mean, standard error (±SE), median value and range. The relationship between nuclear IRS-1 and ERα was analyzed by linear regression and the statistical significance was evaluated by the Pearson correlation test. The distribution of ERα and nuclear IRS-1 in respect to tumor size, grade, and lymph node involvement are reported in scatterplots. The correlations between nuclear IRS-1, ERα,

cytoplasmatic IRS-1 and selected clinicopathologic features were examined with the Pearson correlation test.

The value of nuclear ERα or IRS-1 expression as diagnostic marker of tumor grade, pT, pN and Ki67 was evaluated calculating the areas under the receiver operating characteristic (ROC) curves [37], which assess the performance of a diagnostic test [38-40]. In the graphical representation of the ROC curve, the X-axis is the false-positive rate (1-specificity) and the Y-axis is the true positive rate (sensitivity). The diagonal line (from 0,0 to 1,1) reflects the characteristics of a test with no discriminating power. ROC curve was analyzed using MedCalc (MedCalc Software, Mariakerke, B).

Results

Nuclear IRS-1and ERα expression in normal mammary epithelium and benign breast tumors.

In general, the expression of nuclear IRS-1 in normal tissues was very low (~2% of positive cells) (Tab. 2). ERα was expressed in 11 of 14 samples; the median frequency of ERα in all samples was 10.5% (Fig. 1A, B and Tab. 2). Nuclear IRS-1 was found in 9 of 11 ERα-positive specimens at the median frequency 1.8%. Low expression (3.5%) of nuclear IRS-1 was also recorded in 2 specimens that did not express ERα (data not shown).

Compared with normal epithelium, benign tumors expressed higher median levels of nuclear IRS-1 (20.5%) and ERα (20.5.0%) (Tab. 2). Nuclear IRS-1 was found in 16 of 19 ERα-positive specimens, but was not present in any of ERα-negative cases. (Fig. 1C, D and data not shown).

Cytoplasmic IRS-1 was expressed in all epithelial cells of normal epithelium and benign tumors at the levels 1+ to 3+ (Fig. 1B, D and Tab. 3), while no evidence of cytoplasmic ERα staining was revealed in any of the specimens (Fig. 1A). The co-localization of nuclear IRS-1 and ERα was determined by confocal microscopy (Fig. 2).

Table 2. Descriptive statistics of nuclear IRS-1 and ERα in all samples.

	Normal Epithelium		Benign Tumors	
	ERα	**IRS-1**	**ERα**	**IRS-1**
Mean±SE	21.7±6.1	2.3±0.6	23.4±4.2	23±4.5
Median (Range)	10.5 (0-60)	1.6 (0-7)	20.5 (0-70)	20.5 (0-60)

Ductal Carcinoma	G2		G3	
	ERα	**IRS-1**	**ERα**	**IRS-1**
Mean±SE	51.8±10.1	23.4±7.1	6.2±3.4	4.1±1.8
Median (Range)	65 (0-92)	11 (0-72)	0 (0-40)	0 (0-20)

Lobular Carcinoma	G2		G3	
	ERα	**IRS-1**	**ERα**	**IRS-1**
Mean±SE	64.8±11.3	32±9.7	26.7±8.9	30.7±4.7
Median (Range)	80 (0-90)	35 (0-80)	15 (0-80)	33.5 (0-52)

The mean (±SE) expression with median (range) values for nuclear IRS-1 and ERα in all specimens (ERα-positive and ERα-negative) is given. Cancer samples of ductal and lobular origin were grouped into separate G2 and G3 populations.

Table 3. Descriptive statistics of cytoplasmatic IRS-1 in all samples

Cytoplasmic IRS-1 Expression (% of Cases in Class)				
Class	**Normal**	**Benign**	**Ductal**	**Lobular**
0	0	0	0	0
1+	29	21	16	0
2+	29	21	52	63
3+	42	58	32	37

Samples are grouped in 4 classes as described in Materials and Methods. The percentage of specimens with cytoplasmic IRS-1 in each staining category is given.

IRS-1 expression in ERα-positive and ERα-negative breast carcinoma.

In invasive ductal carcinoma, nuclear IRS-1 was found in 22 of 38 of specimens. The median level of expression in these samples was 13.7%. ERα was detected in 20 of 38 of specimens with a median expression of 29.2% (Fig. 1E, F). Twenty two specimens (15 of 19 in G2, and 7 of 19 in G3) expressed nuclear IRS-1 (Fig. 1F, Tab. 2). Among nuclear IRS-1-positive samples, 18 also expressed ERα, while 4 were ERα-negative. Thirteen of G2 ductal carcinomas and 5 of G3 cancers were positive for both IRS-1 and ERα. In 2 of 38 specimens, ERα was expressed in the absence of nuclear IRS-1.

In lobular cancer, nuclear IRS-1 staining was observed in 16 of 22 samples with the median frequency 31.2% (Fig. 1H). Eleven of these 16 samples were also ERα-positive. Within G2 lobular carcinomas, 6 of 10 specimens displayed nuclear IRS-1 at the median level 35.0%; all these samples expressed ERα at the median frequency 80.0% (Tab. 2). In the G3 subgroup, 10 of 12 tumors expressed nuclear IRS-1 (median 33.5%) and 5 of 10 expressed ERα (median 15.0%). In 5 of 16 lobular cancers, nuclear IRS-1 was found in the absence of ERα (Tab. 2).

Cytoplasmatic IRS-1 was identified in all ductal and lobular cancer samples displaying a weak to strong staining intensity (Tab. 3). In all specimens, the neoplasm surrounding tissue appeared normal and the pattern of ERα and IRS-staining comparable to that of the normal samples.

Correlation between nuclear IRS-1and ERα in breast cancer, benign tumors, and normal mammary epithelium.

A very strong positive correlation ($p<0.001$) between nuclear IRS-1 and ERα was found in invasive ductal breast cancer. The markers were also positively associated ($p<0.01$) in benign tumors cancer samples (Fig. 4). However, no correlations were found between nuclear IRS-1 and ERα in normal tissues ($p=0.28$) and lobular breast cancer ($p=0.24$) (Fig. 4).

Nuclear IRS-1 and ERα are correlated with some clinicopathological features in invasive ductal carcinomas.

The distribution of nuclear ERα and nuclear IRS-1 was analyzed with respect to tumor grade, tumor size, lymph node involvement, and proliferation index (Fig. 3). The frequency of both ERα and nuclear IRS-1 expression was the highest in node-negative G2 invasive ductal carcinomas of smaller size (Fig. 3). In the same group, a significant negative correlation between nuclear IRS-1 or ERα and differentiation grade, the tumor size, lymph node involvement and proliferation rate was found (Tab. 4).

In contrast, in lobular breast carcinomas, the distribution of nuclear IRS-1 or ERα appeared to be independent of and not correlated with tumor grade, size, or Ki67 expression (Fig. 3 and Tab. 4). Interestingly, both nuclear IRS-1 and ERα were more abundant in lymph node-negative samples (Fig. 3), but no significant associations were determined between these markers and lymph node status (Tab. 4).

The specificity and sensitivity of nuclear IRS-1 or ERα as a marker of tumor differentiation grade, tumor size and lymph node involvement was evaluated by the ROC curve analysis. The comparison of the areas under the ROC curves obtained for nuclear IRS-1 and ERα indicated that both nuclear IRS-1 and ERα are good markers for tumor grading in invasive ductal carcinomas, while in lobular carcinomas only ERα could be considered a marker for grading (Tab. 5 and Fig. 5).

Neither ERα nor nuclear IRS-1 was a useful marker of tumor size, node involvement, or tumor proliferation (data not shown). The distribution of nuclear IRS-1 or ERα was not related to patient's age and menopausal status in cancer, benign and normal samples (data not shown).

Table 4. Correlation between nuclear IRS-1, ERα and selected clinicopathological tumor features.

		Ductal Carcinoma		Lobular Carcinoma	
		ERα	IRS-1	ERα	IRS-1
G	r	**-0.573**	**-0.511**	-0.563	0.029
	p	**0.0015**	**0.0057**	0.065	0.94
pT	r	**-0.393**	**-0.382**	-0.326	0.153
	p	**0.039**	**0.044**	0.310	0.633
pN	r	**-0.381**	**-0.454**	-0.082	-0.122
	p	**0.044**	**0.015**	0.797	0.714
Ki67	r	**-0.591**	**-0.538**	-0.329	-0.016
	p	**0.0001**	**0.003**	0.31	0.94

The association between nuclear IRS-1 or ERa and tumor grade (G), size (pT), lymph node involvement (pN), and the expression of the proliferation marker Ki67 was statistically analyzed with Pearson correlation test; r, correlation coefficient; p, statistical significance. The statistically significant correlations are bolded.

Table 5. Association between nuclear IRS-1, ERα and tumor grade.

Diagnostic Marker	**ROC analysis for tumor grade**		
	AUC estimate (95% CI)	**Area under the ROC curve**	**Mann-Whitney test (p value)**
Ductal Carcinoma			
ERα	71.4 (41.9-91.4)	0.809	**0.001**
IRS-1	78.6 (49.2-95.1)	0.778	**0.001**
Lobular Carcinoma			
ERα	80.0 (28.8-96.7)	0.817	**0.02**
IRS-1	60.0 (15.4-93.5)	0.533	0.85

The analysis was performed with ROC curves, as described in Materials and Methods. The area under the ROC (receiver operating characteristic) curve (AUC) describes the value of nuclear IRS-1 or ERα to discriminate between G2 and G3 tumors. AUC estimate reports the confidence intervals considering an error of 5%. The statistical significance was evaluated by Mann-Whitney test for an area =0.5. Statistical significances are bolded.

Relationship between cytoplasmatic IRS-1 and clinicopathological features.

In ductal carcinomas, cytoplasmic IRS-1 (each staining intensity group) positively correlated with ERα. Moreover, in ductal cancer low and moderate IRS-1 expression was positively associated with tumor size, while high IRS-1 levels negatively correlated with tumor grade (Tab. 6).

In lobular carcinomas, high expression of cytoplasmic IRS-1 directly correlated with Ki67 (Tab. 6). In benign tumors, low expression of cytoplasmatic IRS-1 was negatively associated with ERα, while higher IRS-1 levels were not linked to ERα. No correlations between the two markers were found in normal samples (data not shown). Similarly, cytoplasmic IRS-1 expression was not related to age or menopausal status in all analyzed material (data not shown).

Table 6. Correlations between cytoplasmatic IRS-1 and selected clinicopathological features in ERα-positive tumors.

		Ductal				Lobular			
		0	**1+**	**2+**	**3+**	**0+**	**1+**	**2+**	**3+**
ERα	**r**	-	**0.978**	**0.637**	**0.987**	-	-	0.198	-0.029
	p		**0.025**	**0.019**	**0.013**			0.671	0.970
G	**r**	-	0.375	-0.082	**-0.962**	-	-	0.204	-0.376
	p		0.625	0.790	**0.037**			0.661	0.624
pT	**r**	-	**0.973**	**0.553**	-0.577	-	-	0.009	-0.225
	p		**0.026**	**0.050**	0.423			0.984	0.775
pN	**r**	-	0.00	0.301	-	-	-	-0.069	-
	p		1.00	0.318				0.883	
Ki67	**r**	-	0.724	-0.241	-0.905	-	-	-0.223	**0.978**
	p		0.276	0.428	0.095			0.631	**0.022**

The associations between cytoplasmatic IRS-1 and ERα positivity (ERα), tumor grade (G), tumor size (pT), lymph node involvement (pN), and the expression of the proliferation marker Ki67 were statistically analyzed with the Pearson correlation test; r, correlation coefficient; p, statistical significance. The statistically significant correlations are bolded. The absence of value is due to either the absence of samples in the group or to the homogeneity of samples (variance =0).

Discussion

Studies in cellular and animal models established that breast cancer cell growth is controlled by complex crosstalk between ERα and IGF-I systems [4-6 14 19 41-44]. However, while ERα is an established marker for breast cancer diagnosis and prognosis and a target for breast cancer therapy and prevention, the value of critical IGF-I system components like IGF-IR and IRS-1 as breast cancer markers needs

further examination. Until now, analysis of breast cancer samples did not establish a clear association between IGF-IR and breast cancer progression. Several studies demonstrated higher expression of IGF-IR compared with non-cancer mammary epithelium, however this feature has been associated with either favorable or unfavorable breast cancer prognosis [4 45-53]. The value of cytoplasmatic IRS-1 as a breast cancer marker is even less clear. Some studies provided evidence that IRS-1 expression is higher in cancer than in non-cancer breast epithelium, while others (including this study) reported that IRS-1 levels do not increase (but can decrease) during cancer development and progression [18 34 36]. Moreover, cytoplasmatic IRS-1 has been found either to correlate with ERα and associate with a more differentiated phenotype or be independent from ERα and associated with a more aggressive phenotype [16 34 41 52]. The significance of nuclear IRS-1 in breast cancer has never been addressed.

In view of the importance of cytoplasmatic and nuclear IRS-1 in breast cancer growth evidenced in vitro and conflicting or lacking data in vivo, we set out to investigate IRS-1 expression in normal mammary epithelium, benign tumors and breast cancer. Using IHC, we assessed cytoplasmic and nuclear IRS-1 abundance and examined its relations with some prognostic markers, especially ERα, and clinicopathological features.

Our data on cytoplasmic IRS-1 are consistent with those reported by Schnarr *et al.* who noted moderate to strong IRS-1 expression in normal and benign tissues, and in well differentiated carcinomas of both ductal and lobular origin [34]. Similarly, Finlayson *et al.* found no difference of IRS-1 phosphorylation in homogenates of normal and breast cancer tissues [36]. On the other hand, other groups reported low IRS-1 expression in normal tissue and overexpression in poorly differentiated tumors [18 35 48]. In agreement with Schnarr et al. we found a positive association between cytoplasmatic IRS-1 and ERα and a negative correlation between high expression of IRS-1 and tumor grade in ductal carcinomas. This observation is also consistent with coexpression of IRS-1 and ERα noted in less

invasive breast cancer cell lines [6]. In other studies ERα and IRS-1 were not positively correlated in primary tumors [18 35]. The causes for these different results are unclear, but could be related to different IHC protocols, including different Abs used.

We did not find any correlation between cytoplasmic IRS1 and lymph node involvement in ductal and lobular cancers. This partially confirms data of Koda *et al.*, who did not observe such a correlation in the whole group of primary tumours, but only in the subgroup of better differentiated (G2) cancers [35]. Our results also suggested a positive correlation between cytoplasmatic IRS-1 (weak to moderate) and tumor size in ERα-positive ductal cancers. This association has not been noted by others. Regarding cell proliferation, we found a positive correlation of IRS-1 and Ki-67 only in ERα-positive lobular cancers expressing high levels of IRS-1 and no associations in all other samples. Similarly, no link between cell proliferation and cytoplasmatic IRS-1 levels was reported by Rocha *et al*. In contrast, a negative correlation was reported by Schnarr *et al*., while Koda *et al*., noted a positive IRS-1/Ki-67 correlation in ERα-positive primary tumors [34 35]. Taken together, these data are still too few and inconsistent to suggest cytoplasmic IRS-1 as a marker for breast cancer prognosis and diagnosis.

Instead, our results suggest that nuclear IRS-1 is tightly linked to ERα expression and might serve as an additional clinical breast cancer marker. As expected, ERα levels were low in normal mammary epithelium, higher in benign tumors, and strongly increased in moderately differentiated (G2) cancers. ERα expression was downregulated in poorly differentiated (G3) ductal cancers but not in G3 lobular cancers, confirming the value of ERα as a marker of differentiation in ductal carcinoma [54-56]. Notably, the levels of nuclear IRS-1 were very low in normal tissue, increased in benign tumors and G2 ductal cancer, and decreased in G3 ductal cancer, displaying an expression trend similar to that of ERα.

In lobular cancer, the levels of nuclear IRS-1 were relatively high in both G2 and G3 tumors (~30%) and were not related to the abundance of ERα. Indeed, statistical analysis of data confirmed a very strong correlation between nuclear IRS-1 and ERα in ductal, but not lobular, cancers. Importantly, in ductal, but again not in lobular cancers, both nuclear IRS-1 and ERα negatively correlated with tumor grade, tumor size, lymph node involvement and proliferation rate, suggesting their association with a less aggressive phenotype. The ROC analysis confirmed that nuclear IRS-1 as for ERα, is highly reliable as diagnostic marker of differentiation grade. The observation that nuclear IRS-1 expression increases in benign as well as in highly and moderately differentiated tumors, compared to normal tissues, strongly supports this assumption.

Taken together, our data indicate that nuclear IRS-1 could serve as a novel predictive marker of good prognosis in ductal cancer. The lack of association between nuclear IRS-1 and ERα in lobular cancer and benign tumors, might suggest that, in this settings, IGF-I and ERα systems are not tightly linked.

List of Abbreviations

ERα (Estrogen Receptor alpha), IRS-1 (Insulin Receptor Substrate 1).

Authors'contributions

DS and CM participated in the design of the study, performed the statistical analysis and drafted the manuscript, CG carried out the immunostaining and participated to the statistical analysis, FR participated in the design of the study, LM participated to the statistical analysis, FC prepared the histological samples, EM carried out the immunostaining, SC participated to the statistical analysis, SA

participated in the design of the study and drafted the manuscript, ES designed the study and drafted the manuscript.

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Figure Legends

Fig. 1. ERα and IRS-1 expression in normal mammary epithelium, benign breast tumors and breast cancers.

The expression of ERα (ER) and IRS-1 (IRS) were examined by IHC, as described in Materials and Methods. Normal breast tissue (A, B); benign breast tumor (C, D); invasive ductal ERα-positive carcinoma (E, F); ERα-positive lobular breast cancer (G, H). Negative control; IHC of lobular carcinomas with primary Abs substituted with PBS. Higher magnification of specific areas is reported as inset in the original images.

Fig. 2. Subcellular localization of IRS-1 and ERα in breast tumors.

The localization of IRS-1 and ERα in ductal cancers was analyzed by immunostaining and confocal microscopy as detailed in Materials and methods. The captured images of IRS-1 (green fluorescence), ERα (red fluorescence) and merged IRS-1 and ERα (yellow fluorescence) are shown in a representative ductal cancer tissue section.

Fig. 3. Correlations between nuclear IRS-1 and ERα in normal breast tissues, benign breast tumors and breast cancers.

Associations between nuclear IRS-1 and ERα in different tissues were analyzed with Pearson correlation test. For each linear regression graph, the linear equation, the correlation coefficient (R), and the statistical significance (p) is reported.

Fig. 4. Distribution of nuclear IRS-1 and ERα in ductal and lobular breast cancers.

Distributions of nuclear IRS-1 (%) and ERα (%) relative to tumor grade (Grade), size (pT), and the lymph node involvement (pN) in ductal and lobular breast cancers are shown in scatterplots.

Fig. 5. Value of nuclear IRS-1 and ERα as diagnostic markers of tumor grading.

Graphic evaluation of ERα and nuclear IRS-1 in respect to tumor differentiation grade in invasive ductal and lobular carcinomas, showing the true-positive rate (sensitivity) and the false-positive rate (specificity) of the analysis as a function of all possible cut-points for the two markers. ERα, solid line; nuclear IRS-1, dotted line.

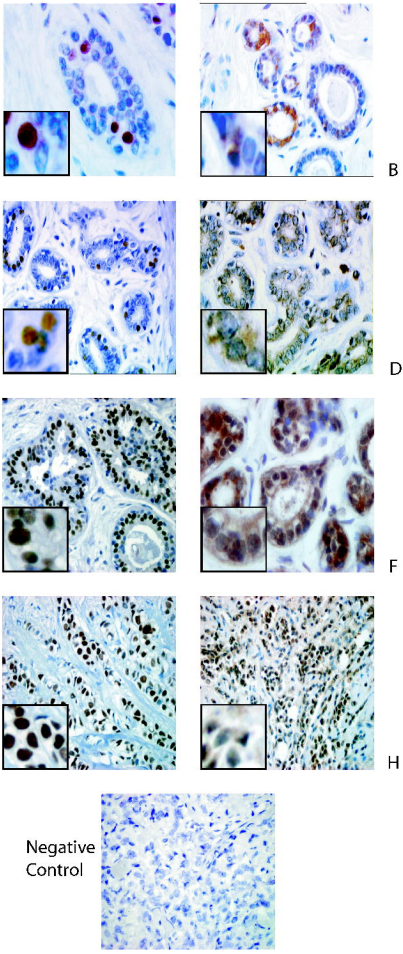


Figure 1

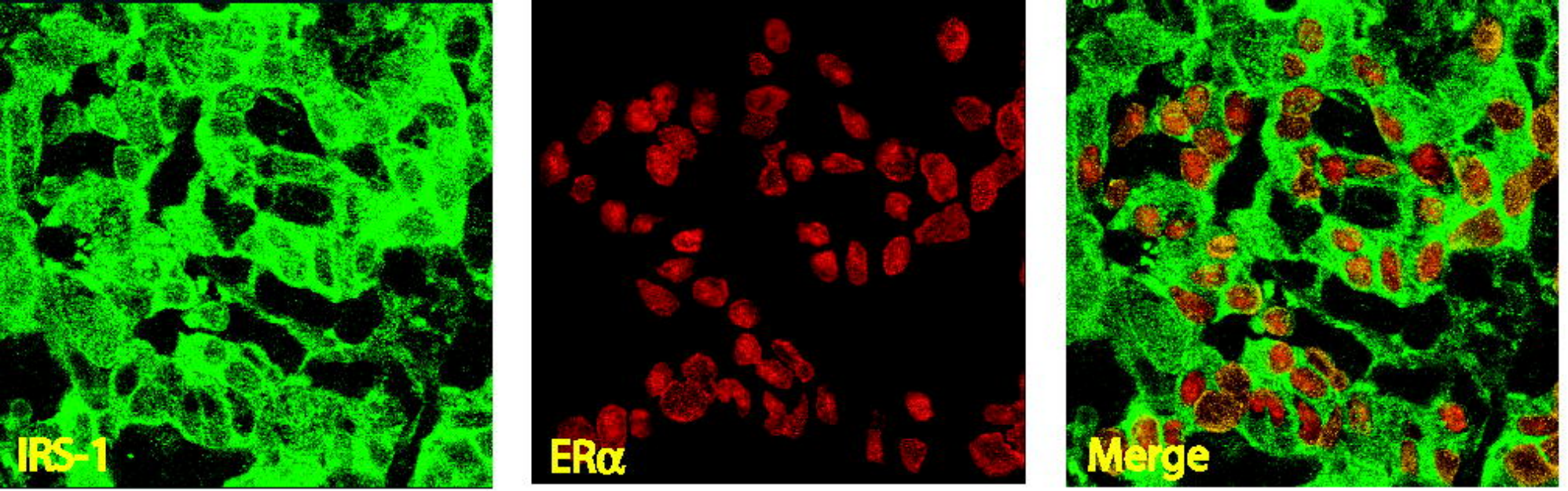


Figure 2

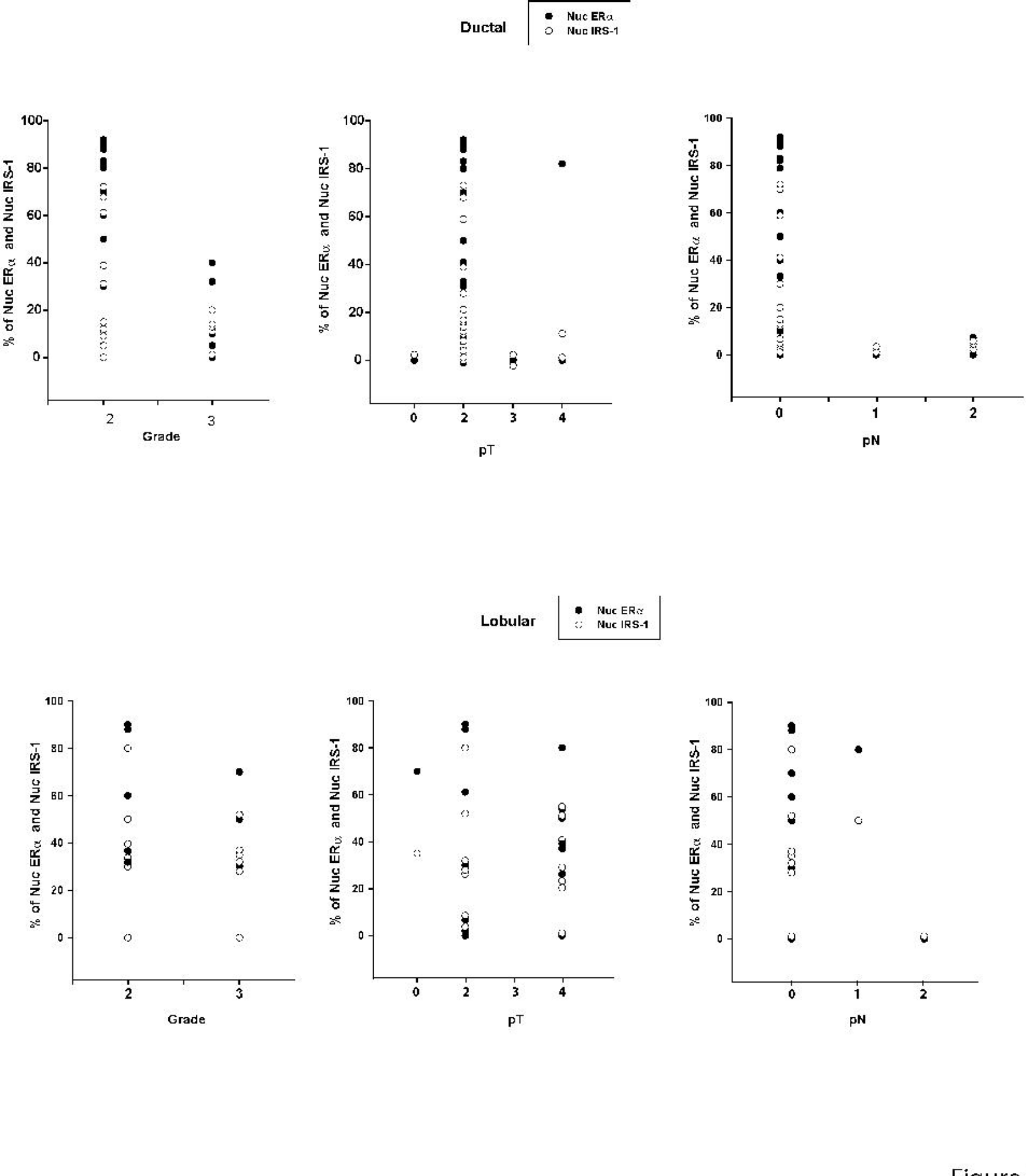
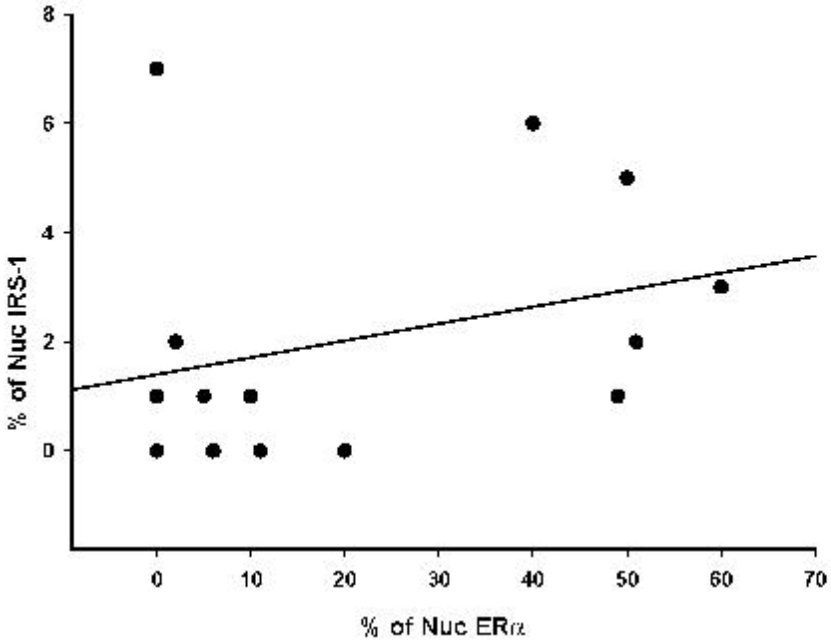

Ductal
Nuc ERα
Nuc IRS-1
% of Nuc ERα and Nuc IRS-1
Grade
pT
pN
Lobular
Nuc ERα
Nuc IRS-1
% of Nuc ERα and Nuc IRS-1
Grade
pT
pN

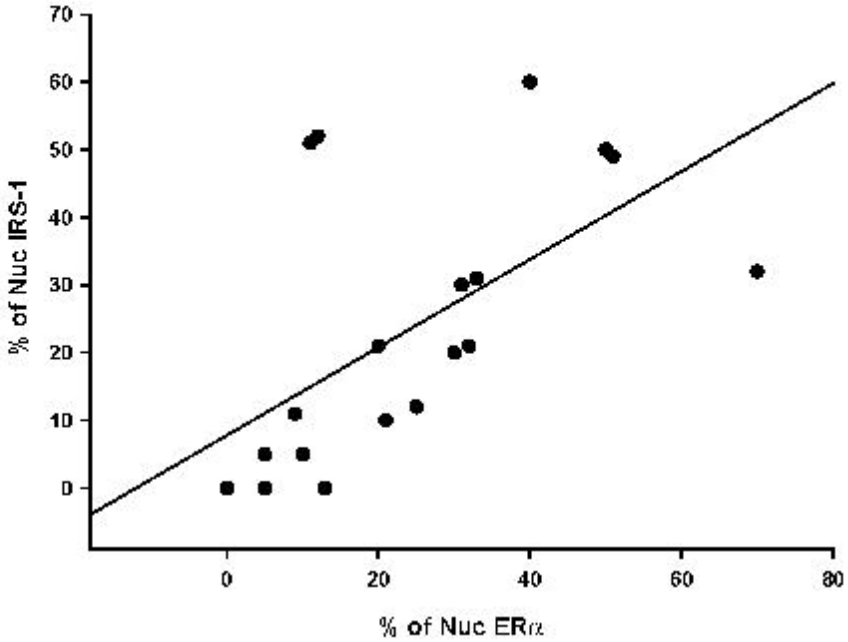
Figure 3

Normal



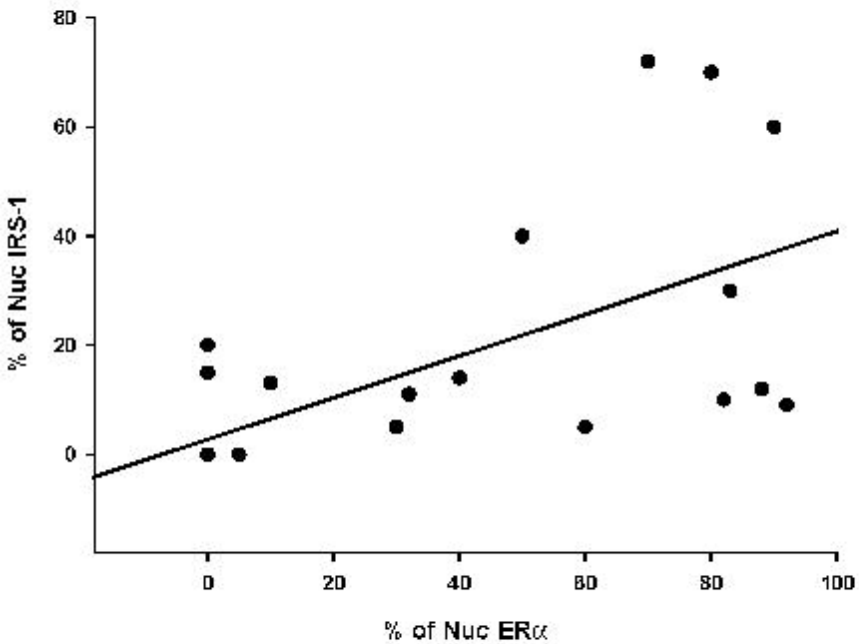
IRS Nuc=1.756+(0.03*ER) R=0.309 P=0.28

Benign



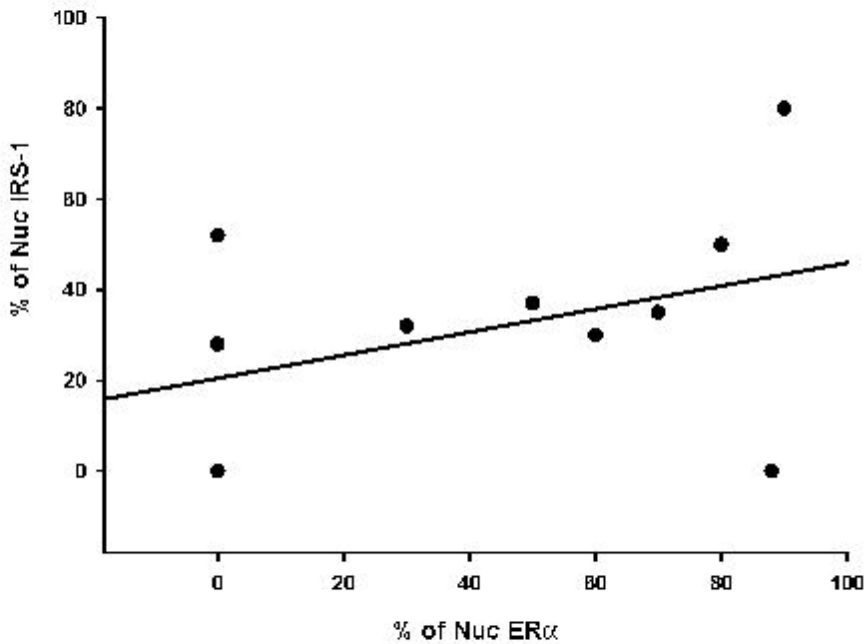
IRS Nuc=7.796+(0.650*ER) R=0.604 P<0.01

Ductal



IRS Nuc=2.707+(0.382*ER) R=0.647 P<0.001

Lobular



IRS Nuc=20.439+(0.255*ER) R=0.387 P=0.239

Figure 4

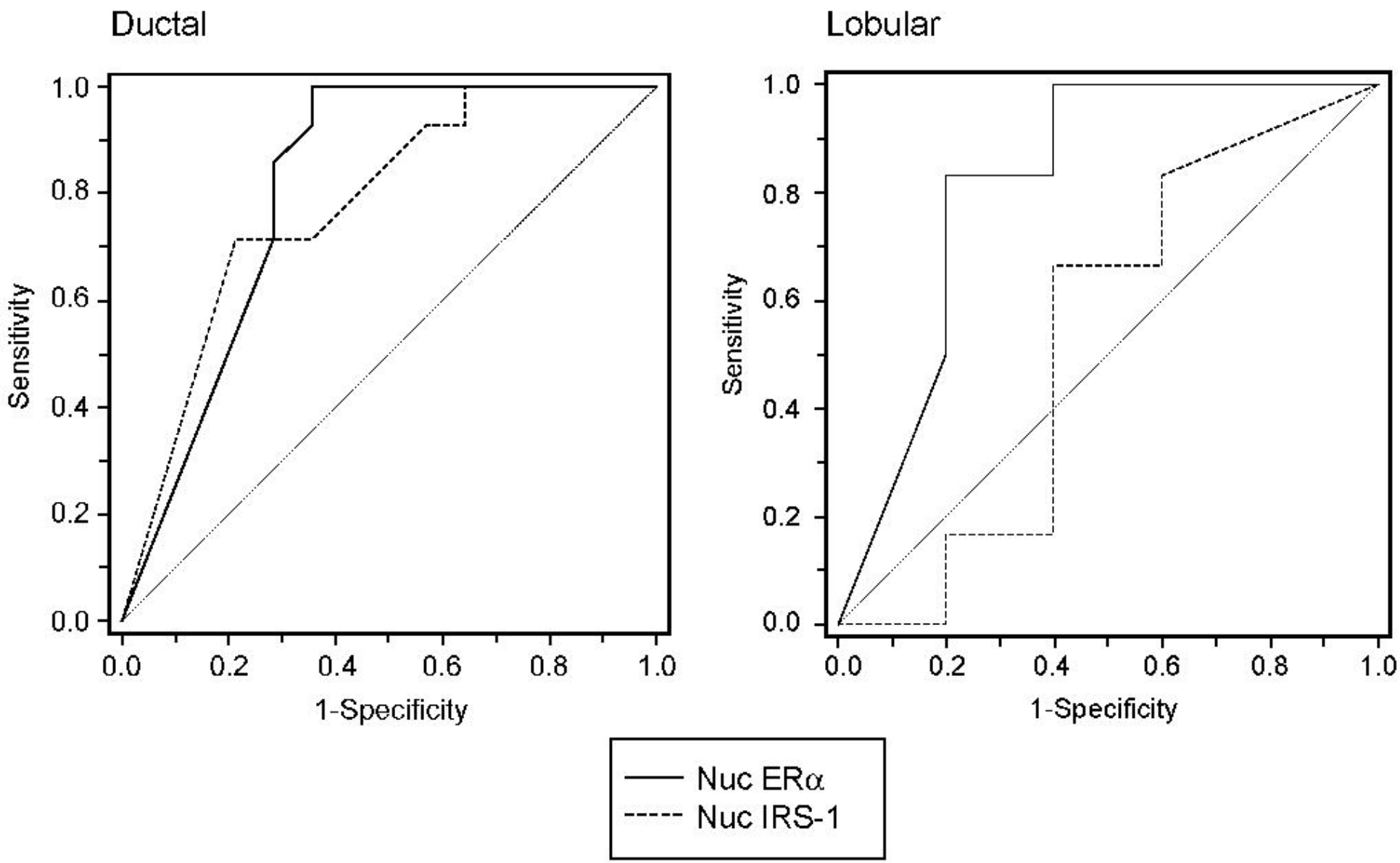

Ductal
Lobular
Sensitivity
1-Specificity
0.0
0.2
0.4
0.6
0.8
1.0
Nuc ERα
Nuc IRS-1

Figure 5

Increased Expression of Leptin and the Leptin Receptor as a Marker of Breast Cancer Progression: Possible Role of Obesity-Related Stimuli

Cecilia Garofalo,[1,2] Mariusz Koda,[3] Sandra Cascio,[1,4] Mariola Sulkowska,[3] Luiza Kanczuga-Koda,[3] Jolanta Golaszewska,[3] Antonio Russo,[4] Stanislaw Sulkowski,[3] and Eva Surmacz[1]

Abstract

Purpose: Recent *in vitro* studies suggested that the autocrine leptin loop might contribute to breast cancer development by enhancing cell growth and survival. To evaluate whether the leptin system could become a target in breast cancer therapy, we examined the expression of leptin and its receptor (ObR) in primary and metastatic breast cancer and noncancer mammary epithelium. We also studied whether the expression of leptin/ObR in breast cancer can be induced by obesity-related stimuli, such as elevated levels of insulin, insulin-like growth factor-I (IGF-I), estradiol, or hypoxic conditions.

Experimental Design: The expression of leptin and ObR was examined by immunohistochemistry in 148 primary breast cancers and 66 breast cancer metastases as well as in 90 benign mammary lesions. The effects of insulin, IGF-I, estradiol, and hypoxia on leptin and ObR mRNA expression were assessed by reverse transcription-PCR in MCF-7 and MDA-MB-231 breast cancer cell lines.

Results: Leptin and ObR were significantly overexpressed in primary and metastatic breast cancer relative to noncancer tissues. In primary tumors, leptin positively correlated with ObR, and both biomarkers were most abundant in G3 tumors. The expression of leptin mRNA was enhanced by insulin and hypoxia in MCF-7 and MDA-MB-231 cells, whereas IGF-I and estradiol stimulated leptin mRNA only in MCF-7 cells. ObR mRNA was induced by insulin, IGF-I, and estradiol in MCF-7 cells and by insulin and hypoxia in MDA-MB-231 cells.

Conclusions: Leptin and ObR are overexpressed in breast cancer, possibly due to hypoxia and/or overexposure of cells to insulin, IGF-I, and/or estradiol.

Obesity increases postmenopausal breast cancer risk by 30% to 50% (1). The exact mechanism of this phenomenon is not known, but it is assumed that different biologically active factors that are secreted by adipose tissue, such as estrogens, insulin, insulin-like growth factor-I (IGF-I), and leptin, might be implicated (1–5). Although the role of estrogens, insulin, and IGF-I in breast tumorigenesis has been extensively studied, the potential role of leptin is just being recognized (2).

The adipokine leptin (obesity protein) acts as a neurohormone-regulating energy balance and food intake in the hypothalamus. Additionally, leptin has been shown to influence various processes in peripheral organs (6, 7). In the breast, leptin is required for normal mammary gland development and lactation (8), but it might also contribute to mammary tumorigenesis (2). In support of the latter, there is evidence that different breast cancer cell lines can express various isoforms of the leptin receptor (ObR), including the long signaling form ObRl (9–13). Furthermore, in breast cancer cells, leptin has been shown to stimulate DNA synthesis and cell growth acting through multiple signaling cascades, such as the Janus-activated kinase 2/signal transducers and activators of transcription 3, extracellular signal-regulated kinase 1/2, protein kinase Cα, and Akt/GSK3 pathways (9–16). Leptin-induced cell cycle progression was accompanied by up-regulation of cyclin-dependent kinase2 and cyclin D1 levels (15) and hyperphosphorylation/inactivation of the cell cycle inhibitor pRb (13). Noteworthy, in T47D breast cancer cells, but not in normal mammary epithelial cells, leptin stimulated not only cell growth but also cellular transformation (10).

The involvement of leptin in breast carcinogenesis could be additionally supported by the fact that the hormone can potentiate estrogen signaling. Specifically, in MCF-7 cells, leptin induced aromatase gene expression, elevating aromatase activity and increasing estrogen synthesis (16). Leptin was also

Authors' Affiliations: [1]Sbarro Institute for Cancer Research and Molecular Medicine, College of Science and Technology, Temple University, Philadelphia, Pennsylvania; [2]Department of Pharmaco-Biology, University of Calabria, Arcavacata di Rende CS, Italy; [3]Department of Pathology, Medical University of Bialystok, Bialystok, Poland; and [4]Department of Oncology, Medical School, University of Palermo, Palermo, Italy

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Note: C. Garofalo and M. Koda contributed equally to this work.

Requests for reprints: Eva Surmacz, Sbarro Institute for Cancer Research and Molecular Medicine, College of Science and Technology, Temple University, Room 446, 1900 North 12th Street, Philadelphia, PA 19122. Phone: 215-204-0306; Fax: 215-204-0303; E-mail: surmacz@temple.edu.

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able to enhance estrogen receptor α (ERα) – dependent transcription by decreasing ERα ubiquitination and degradation, especially in the presence of the antiestrogen ICI 182,780 (13). Furthermore, leptin has been shown to transactivate ERα via the extracellular signal-regulated kinase 1/2 pathway (17).

Limited studies on cancer and noncancer breast biopsies indicated that both leptin and ObR are present in the human breast tissue, suggesting that mammary gland can be influenced by leptin not only through paracrine or endocrine mechanisms but also by via autocrine pathways (18 – 21). Importantly, one previous report (18) and our present study suggest that leptin and ObR are overexpressed in primary and metastatic invasive ductal breast carcinoma compared with noncancer mammary tissue.

The mechanism regulating leptin/ObR overexpression in mammary epithelium is not known. The synthesis of leptin in other cellular systems is influenced by different humoral factors, among them insulin (22, 23), IGF-I, and estrogens (24). In addition, leptin expression can be up-regulated by hypoxia via hypoxia-inducible factor 1 – mediated transcription (25, 26). The regulation of ObR is much less understood, but preliminary evidence suggested that ObR expression can be stimulated by estradiol and hypoxia in rodents (27). Because estrogens, insulin, and IGF-I are overabundant in obese subjects (5), and obesity is associated with tissue hypoxia (28), we explored whether the expression of the leptin/ObR loop in breast cancer cells can be affected by these stimuli.

Materials and Methods

Tissue samples

The expression of leptin, ObR, and other breast cancer markers was assessed in breast cancer and noncancer mammary epithelium. Tissue samples were obtained from 148 women who underwent partial or total mastectomy and lymph node dissection for primary breast cancer as well as from 48 women treated surgically for intraductal proliferative lesions. Immediately after excision, tissue samples were fixed in 10% buffered formaldehyde solution, embedded in paraffin blocks at 56°C, and stained with H&E. Histopathologic examination of sections was based on the WHO and pT_N classification of breast tumors (29). The protocol of the present study was reviewed and approved by the local ethical committee.

Breast cancer samples. Breast cancer samples included invasive ductal carcinomas in grades G_2 (57.4%) and G_3 (42.6%); in stages pT_1 (54.7%) and pT_2 (45.3%); 52.7% (78 of 148) of patients had involved lymph nodes at the time of diagnosis; 66 cases of lymph node metastases were analyzed in parallel with primary tumors. The age of patients with breast cancer ranged from 30 to 80 years (mean, 54.5 years); 55.4% of women were premenopausal, and 44.6% were postmenopausal.

Noncancer samples. Ninety cases of intraductal proliferative lesions were analyzed: 48 cases without accompanying breast cancer (37 usual ductal hyperplasias and 11 atypical ductal hyperplasias) and 42 cases of noncancer tissue adjacent to breast cancer. The latter group included 20 cases of usual ductal hyperplasia and 22 cases of atypical ductal hyperplasia. The age of patients with intraductal proliferative lesions ranged from 24 to 68 years (mean, 46.8 years); 76.2% of the subjects were premenopausal, 23.8% postmenopausal.

Immunohistochemistry

The immunohistochemical analysis of leptin, ObR, ERα, ERβ, and Ki-67 expression was carried out using 5-μm consecutive tissue sections obtained from tissue samples, as described by us previously in detail (30). The sections were dewaxed in xylene and rehydrated in graded alcohols. After antigen unmasking and endogenous peroxidase removal, nonspecific binding was blocked by incubating the slides for 1 hour with 1.5% normal serum in PBS. Next, the sections were incubated with the primary antibodies. The following antibodies were used for immunohistochemistry: for leptin, rabbit polyclonal antibody A-20 (Santa Cruz Biotechnology, Santa Cruz, CA), dilution 1:100; for ObR, rabbit polyclonal antibody H-300 (Santa Cruz Biotechnology), dilution 1:75; for ERα, mouse monoclonal antibody F-10 (Santa Cruz Biotechnology), dilution 1:200; for ERβ, rabbit polyclonal antibody H-150 recognizing primarily the cytoplasmic form of ERβ (Santa Cruz Biotechnology), dilution 1:200; and for Ki-67, mouse monoclonal antibody MIB-1 (DAKO, Glostrup, Denmark), dilution 1:100. The studies for leptin, ObR, ERα, and ERβ were done with avidin-biotin-peroxidase complex (ABC Staining System, Santa Cruz Biotechnology), and for Ki-67 with streptavidin-biotin-peroxidase complex (LSAB kit, DAKO) to reveal antibody-antigen reactions. All slides were counterstained with hematoxylin. Breast specimens previously classified as positive for the expression of the studied markers were used for control and protocol standardization. In negative controls, primary antibodies were omitted. The expression of leptin, ObR, ERα, ERβ, and Ki-67 was analyzed by light microscopy in 10 different section fields, and the mean percentage of tumor cells displaying positive staining was scored. The expression of leptin and ObR in cancer samples was classified using a four-point scale: 0, <10% positive cells; 1+, 10 to 50% positive cells with weak staining; 2+, >50% positive cells with weak staining; 3+, >50% positive cells with strong staining. The expression of leptin and ObR in noncancer tissues was classified as negative (<5% of positive cells) or positive (≥5% positive cells). ERα and ERβ were classified as follows: 0, <10% cells with positive staining; 1+, 10% to 50% cells with positive staining; 2+, 50% to 80% cells with positive staining; 3+ >80% cells with positive staining. Ki-67 expression was classified as follows: 0, <10% cells with positive staining; 1+, 10% to 40% cells with positive staining; 2+ >40% cells with positive staining.

Cell lines

MCF-7 ERα-positive and MDA-MB-231 ERα-negative breast cancer cell lines were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM:F12 plus 5% calf serum, as described by us before (31).

Cell treatments

Eighty percent confluent cell cultures were placed in phenol red – free serum-free DMEM/F12 medium for 24 hours and then treated for 4 hours with 10 nmol/L 17-β-estradiol (E2), 50 ng/mL IGF-I, 100 ng/mL insulin, or 100 nmol/L $CoCl_2$ (to induce hypoxia). The time and dose response for all treatments was tested in advance and the conditions eliciting the maximal leptin and ObR induction were applied.

Reverse transcription-PCR

RNA was isolated from untreated and treated cells using Trizol (Invitrogen, Carlsbad, CA). Total RNA (2 μg) was reverse transcribed with Superscript2 (Invitrogen). RT product (2 μL) was amplified by PCR using the following conditions: for leptin, 95°C for 5 minutes, and then 40 cycles of 95°C for 50 seconds, 60°C for 60 seconds, 72°C for 80 seconds, extension 72°C for 10 minutes. Leptin primers: forward, 5′-CTGTGCCCATCCAAAAAGTCC-3′; reverse, 5′-CCCCCAGGCTGTC-CAAGGTC-3′ (product size 336 bp). Primers for ObR (common domain ObR and ObRl): 95°C for 5 minutes, and then 30 cycles of 95°C for 40 seconds, 60°C for 50 seconds, 72°C for 50 seconds, 72°C for 10 minutes. Primers for ObR common domain: forward, 5′-CATTTTAT-CCCCATTGAGAAGTA-3′; reverse, 5′-CTGAAAATTAAGTCCTTGTGC-CCA-3′ (product size 270 bp). Primers for ObRl: forward, 5-CAGAAG-CCAGAAACGTTTCAG-3′; reverse, 5-AGCCCTTGTTCTTCACCAGT-3′ (product size 344 bp). The expression of a constitutive 36B4 mRNA was assessed as control of RNA input using primers described before (32). The PCR products were run on a 2% agarose gel, and the intensity of bands was quantified by Scion Image laser densitometry program, as described before (31).

Statistical analysis

Spearman test was used to analyze correlations among studied biomarkers in primary breast cancer and in lymph node metastases. Analyses of correlations were not corrected for multiple comparisons. The associations of leptin and ObR with clinicopathologic features were evaluated using χ^2 and Spearman tests. The significance of reverse transcription-PCR results was assessed by Student's t test. Ps < 0.05 were taken as statistically significant.

Results

Low expression of leptin in benign mammary lesions. The characteristics of leptin immunostaining in usual and atypical ductal hyperplasias were similar; therefore, all intraductal proliferative lesions were treated as one group. Within this group, positive cytoplasmic leptin immunoreactivity was found in 15 of 48 (31.3%) of intraductal proliferative lesions without accompanying breast cancer and in 24 of 42 (57.1%) of benign mammary lesions adjacent to breast cancer (Table 1; Fig. 1).

Enhanced expression of leptin in breast cancer. In primary breast cancers, leptin was detected in 128 of 148 (86.4%) cases. Most frequently (64 of 128, 50.0%), leptin immunostaining was classified as 2+, whereas lower expression (1+) was observed in 45 of 128 (35.2%) of samples, and high (3+) expression was found in 19 of 128 (14.8%) of positive tissues (Table 2; Fig. 1).

In lymph node metastases, the presence of leptin was noted in 62 of 66 (93.9%) of cases. Like in primary breast cancer, the expression of leptin in metastatic cancer was most frequent at the 2+ level (33 of 62, 53.2% of leptin-positive lymph node metastases) and less frequent at 3+ (16 of 62, 25.8%) and 1+ (13 of 62, 20.9%) levels (Table 2; Fig. 1). The expression of leptin was undetectable in primary and metastatic cancer samples when immunostaining was done with the omission of the primary antibody.

Expression of ObR is elevated in breast cancer. The expression of ObR was examined with the antibody recognizing a common domain of ObRl and ObRs, allowing for detection of all ObR isoforms. ObR immunostaining was negative in almost all studied noncancer tissues (Table 1; Fig. 1). Only in five specimens of intraductal proliferative lesions, focally positive cytoplasmic immunostaining for ObR was observed.

In contrast, ObR was often expressed in primary breast cancers, where cytoplasmic immunoreactivity for ObR was noted in 61 of 148 (41.2%) of cases. Most frequently (41 of 61, 67.2%), the expression of ObR was weak; however, ObR staining at 2+ and 3+ levels was also noted in some tissues (15 of 61 and 5 of 61 of positive samples, respectively; Table 2; Fig. 1).

In lymph node metastases, ObR was found in 34 of 66 (51.5%) of specimens. In the majority of positive samples, the expression of ObR was weak (14 of 34, 41.2%) or medium (14 of 34, 41.2%). Some metastatic cancers (6 of 34,17.6% of positive cases) expressed high levels of ObR (Table 2; Fig. 1). ObR immunoreactivity was undetectable in the control samples where the primary antibody was omitted.

Leptin and ObR are coexpressed in primary breast cancer. The expression of leptin in the group of all primary tumors as well as in the subgroups of ERα-positive and ERα-negative primary tumors positively correlated with the expression of ObR (P = 0.002, r = 0.275; P = 0.005, r = 0.393; P = 0.003, r = 0.411, respectively). In all lymph node metastases as well as in subgroups derived from ERα-positive or ERα-negative tumors, the expression of leptin was not significantly associated with ObR expression (Table 3).

Expression of leptin and ObR is maintained during metastasis to lymph nodes in ERα-positive tumors. In the group of all cancer cases, the presence of leptin in primary breast cancer positively correlated with its expression in matched cases of lymph node metastases (P = 0.046, r = 0.270; Table 3). After division of samples into ERα-positive and ERα-negative subgroups (according to the initial diagnosis of primary tumor), a strong link between leptin expression in primary tumor and its metastasis was found only in the subgroup of ERα-positive tumors (P = 0.008, r = 0.507; Table 3). Similarly, the expression of ObR in primary tumors positively correlated with its expression in lymph node metastases only in the subgroup of ERα-positive tumors (Table 3).

Relationships between the leptin/ObR system and ERα, ERβ, and Ki-67 in primary breast cancers. Because leptin is a mitogen for breast cancer cells, we assessed the relationship between the leptin/ObR system and cell proliferation (Ki-67 expression). Furthermore, because leptin is a modulator of ERα function, we explored the association between leptin/ObR and ER.

ERα, ERβ, and Ki-67 were found in 60.8%, 80.4%, and 64.2% of primary tumors, respectively. In primary tumors, leptin positively correlated with ERβ (P = 0.001, r = 0.327) but not with ERα or Ki-67 (Table 4). A positive correlation (P = 0.006, r = 0.378) between leptin and ERβ was also found in the subgroup of ERα-positive but not ERα-negative primary tumors (Table 4). The expression of ObR in primary tumors was not significantly associated with the expression of ERα, ERβ, or Ki-67 (Table 4).

Table 1. Leptin and ObR expression levels in noncancerous mammary epithelium

Tissue type	Leptin expression		ObR expression	
	Negative	Positive	Negative	Positive
Noncancerous tissue without accompanying breast cancer (n = 48)	33	15	47	1
Noncancerous tissue adjacent to breast cancer (n = 42)	18	24	38	4

NOTE: The expression of leptin and ObR was determined in noncancerous mammary tissue, as described in Materials and Methods. The number of cases (n) in each staining category is shown.

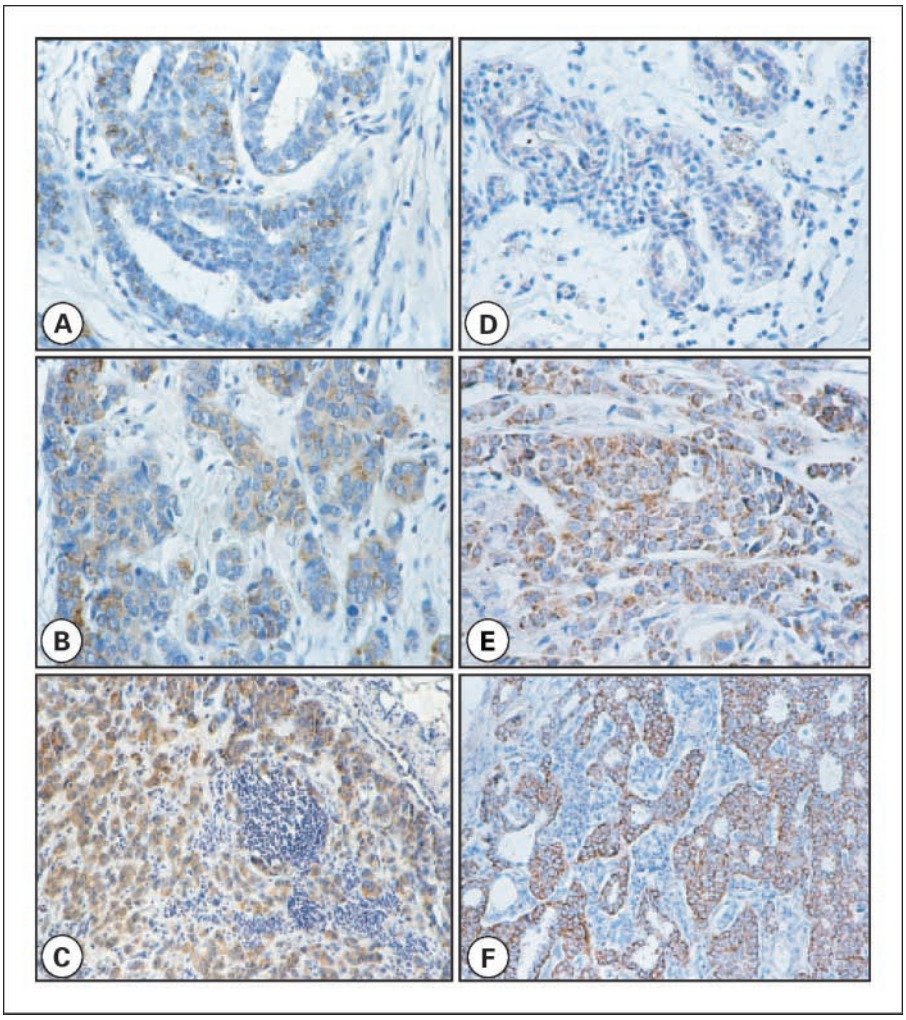

Fig. 1. Immunohistochemical detection of leptin and ObR expression. The expression of leptin (*A-C*) and ObR (*D-F*) in noncancer and breast cancer tissues was studied by immunohistochemistry, as described in Materials and Methods. In this representative image, cytoplasmic leptin immunostaining is seen in intraductal proliferative lesion (*A*). In primary breast cancer (*B*), weak leptin immunostaining is observed in >50% of cancer cells (assessed as 2+); strong staining is found in lymph node metastasis (*C*) in >50% of cancer cells (assessed as 3+). A weak ObR immunoreactivity is present in a few epithelial cells in noncancer mammary gland (*D*), whereas strong ObR immunostaining can be seen in primary tumor (*E*) and lymph node metastasis (*F*). Original magnification, ×200 (*A, B, D,* and *E*) and ×100 (*C* and *F*).

Relationships between the leptin/ObR system and ERα, ERβ, and Ki-67 in lymph node metastases. ERα, ERβ, and Ki-67 expression were detected in 60.6%, 83.3%, and 68.2% of lymph node metastases, respectively. Like in primary tumors, leptin expression in lymph node metastases was associated with ERβ ($P = 0.014$, $r = 0.338$; Table 5) but not with ERα. This relationship was also noted in lymph node metastases derived from ERα-positive ($P = 0.029$, $r = 0.400$) but not ERα-negative primary tumors (Table 5). Interestingly, a negative association between leptin expression and Ki-67 was found in the subgroup of metastases derived from ERα-positive but not ERα-negative primary tumors (Table 5).

The expression of ObR in lymph node metastases positively correlated with ERα ($P < 0.0001$, $r = 0.442$; Table 5) but not ERβ. In addition, ObR negatively correlated with Ki-67 ($P = 0.021$, $r = -0.310$; Table 5). These relationships were lost when we separately analyzed subgroups of lymph node metastases derived from ERα-positive or ERα-negative primary tumors (Table 5).

Associations of leptin/ObR with clinicopathologic features. We studied associations between the leptin/ObR system and lymph node involvement (pN), tumor size (pT), histologic differentiation (G), menopausal status, and patient age. Notably, elevated leptin expression was characteristic for less differentiated tumors, specifically high (3+) leptin content positively correlated with G3 grade ($P = 0.031$), whereas in tumors with medium (2+) leptin expression, there was a trend toward a positive correlation with G3 grade ($P = 0.069$). On the other hand, weak (1+) leptin expression was not significantly associated with tumor differentiation. Similarly, high ObR expression in primary cancers was more frequent in G3 tumors, but the association did not reach statistical significance ($P = 0.074$). No statistically significant correlations were found between leptin or ObR and lymph node involvement, tumor size, menopausal status, and age of patients.

Table 2. Leptin and ObR expression levels in primary and metastatic breast cancer

Tissue type	Leptin expression				ObR expression			
	0	1+	2+	3+	0	1+	2+	3+
PT ($n = 148$)	20	45	64	19	87	41	15	5
LNM ($n = 66$)	4	13	33	16	32	14	14	6

NOTE: The expression of leptin and ObR was determined in primary breast cancers and in lymph node metastases, as described in Materials and Methods. The number of cases (n) in each staining category is shown.
Abbreviations: PT, primary tumors; LNM, lymph node metastases.

Table 3. Associations between leptin and ObR in primary tumors and lymph node metastases

Compared biomarkers	Leptin (PT), ObR (PT), $n = 148$, P (r)	Leptin (LNM), ObR (LNM), $n = 66$, P (r)	Leptin (PT), leptin (LNM), $n = 66$, P (r)	ObR (PT), ObR (LNM), $n = 66$, P (r)
All tumors ($n = 148$)	**0.002 (0.275)**	0.154 (0.186)	**0.046 (0.270)**	0.144 (0.191)
ERα$^+$ tumors ($n = 90$)	**0.005 (0.393)**	0.120 (0.290)	**0.008 (0.507)**	**0.046 (0.355)**
ERα$^-$ tumors ($n = 58$)	**0.003 (0.411)**	0.419 (0.308)	0.449 (0.271)	0.818 (0.055)

NOTE: The associations between the expression of leptin and ObR in primary tumors and lymph node metastases in the group of all tumors and in the subgroups of ERα-positive and ERα-negative tumors (according to the initial diagnosis of primary tumors) were evaluated by Spearman correlation; *P*, statistical significance; *r*, correlation coefficient; *n*, number of cases. Statistically significant values are in bold.
Abbreviations: PT, primary tumors; LNM, lymph node metastases.

Leptin and ObR expression can be induced by different stimuli in ERα-positive and ERα-negative breast cancer cells. We studied the possible mechanism of leptin/ObR overexpression in breast cancer using ERα-positive MCF-7 and ERα-negative MDA-MB-231 breast cancer cell lines. We focused on factors and conditions that are known to induce leptin or ObR expression in other cell systems, especially insulin, IGF-I, E2, and hypoxia. Insulin, IGF-I and E2 are mitogens for breast cancer cells, and their levels are often elevated in obese women.

The induction of leptin, ObR (common domain), and ObRl mRNAs were assessed by reverse transcription-PCR in cells stimulated with E2, IGF-I, insulin, or $CoCl_2$. In MCF-7 cells, all stimuli significantly induced leptin mRNA expression, whereas ObRl and ObR mRNAs were increased by E2, IGF-I and insulin but not by hypoxia (Fig. 2).

In MDA-MB-231 cells, leptin and ObR mRNAs, but not ObRl mRNA, were induced by hypoxia. In addition, insulin stimulated the expression of leptin, ObR, and ObRl mRNAs. E2 and IGF-I did not produce significant effects on the leptin/ObR system (Fig. 2). In both cells lines, the expression of the control gene 36B4 was not affected by the treatments (Fig. 2).

Discussion

Recent reports suggested that leptin, a hormone whose expression is elevated in overweight and obese individuals, might be involved in the development and/or progression of different cancers. This concept is supported by experimental evidence that leptin can stimulate cell growth, counteract apoptosis, and induce migration and expression of matrix degrading enzymes and angiogenic factors in different cellular cancer models (2). For instance, in different breast cancer cell lines, leptin has been shown to stimulate cell proliferation, survival, and transformation, acting through ObRl, the signaling form of the leptin receptor (2, 10, 11, 13).

The involvement of leptin in mammary carcinogenesis awaits further validation in animal models and human clinical material. In this context, new data suggested that leptin is necessary for mammary tumor development in transforming growth factor-α transgenic Lep(ob)Lep(ob) mice (33). In addition, preliminary immunhistochemistry studies described the expression of ObR and/or leptin in human breast tumors and normal mammary gland (19). One recent report suggested that leptin and ObRl are overexpressed in primary breast tumors relative to normal mammary epithelium (18). No prior studies were done using clinical samples obtained from matched pairs of primary breast tumors and lymph node metastases. Similarly, the regulation of leptin/ObR expression in breast cancer cells has never been characterized.

Consequently, our goals were (*a*) to examine the relative expression of leptin and ObR in primary and metastatic breast cancer versus noncancer tissue; (*b*) to evaluate whether the expression of leptin/ObR system is maintained during metastasis to lymph nodes; (*c*) to assess the association between leptin/ObR and other clinicopathologic features, especially tumor differentiation, expression of ER, and cell proliferation; (*d*) to examine whether the expression of the leptin system can be influenced by obesity-related stimuli, such as high levels of insulin, IGF-I, estradiol, and hypoxic conditions in ERα-positive and ERα-negative cells.

We found that leptin and ObR were expressed at low levels in noncancer tissues, and both markers were overexpressed in primary breast tumors as well as in lymph node metastases. The notion that leptin is overexpressed in primary breast tumors is

Table 4. Relationships between the leptin system and ERα, ERβ, and Ki-67 in primary breast cancers

Compared biomarkers	Leptin ERα, P (r)	Leptin ERβ, P (r)	Leptin Ki-67, P (r)	ObR ERα, P (r)	ObR ERβ, P (r)	ObR Ki-67, P (r)
All PT ($n = 148$)	0.523 (−0.056)	**0.001 (0.327)**	0.611 (−0.056)	0.705 (0.032)	0.353 (0.091)	0.291 (−0.103)
ERα$^+$ PT ($n = 90$)	0.836 (−0.024)	**0.006 (0.378)**	0.289 (−0.150)	0.346 (−0.102)	0.175 (0.173)	0.263 (−0.143)
ERα$^-$ PT ($n = 58$)	—	0.683 (0.092)	0.456 (−0.166)	—	0.451 (−0.164)	0.246 (−0.252)

NOTE: The associations were evaluated in ERα-positive and ERα-negative primary breast tumors by Spearman correlation; *P*, statistical significance; *r*, correlation coefficient; (−), no cases in this category. Statistically significant values are in bold.
Abbreviations: PT, primary tumors; LNM, lymph node metastases.

Table 5. Relationships between the leptin system and ERα, ERβ, and Ki-67 in lymph node metastases

Compared markers	Leptin ERα, *P* (*r*)	Leptin ERβ, *P* (*r*)	Leptin Ki-67, *P* (*r*)	ObR ERα, *P* (*r*)	ObR ERβ, *P* (*r*)	ObR Ki-67, *P* (*r*)
All LNM ($n = 66$)	0.334 (0.124)	**0.014 (0.338)**	**0.016 (−0.331)**	**<0.0001 (0.442)**	0.092 (0.230)	**0.021 (−0.310)**
LNM derived from $ER\alpha^+$ PT ($n = 40$)	0.282 (0.172)	**0.029 (0.400)**	**0.031 (−0.394)**	**0.001 (0.507)**	0.099 (0.292)	0.388 (−0.155)
LNM derived from $ER\alpha^-$ PT ($n = 26$)	0.356 (0.207)*	0.433 (0.280)	0.512 (−0.236)	0.965 (0.010)*	0.176 (0.494)	0.296 (−0.393)

NOTE: The associations were evaluated in lymph node metastases derived from ERα-positive and ERα-negative primary tumors using Spearman correlation; *P*, statistical significance; *r*, correlation coefficient. Statistically significant values are in bold.
Abbreviations: PT, primary tumors; LNM, lymph node metastases.
*In several cases, ERα-positive metastases originated from ERα-negative primary tumors.

consistent with the results of Ishikawa et al. (18), whereas the present finding of increased expression of leptin and ObR in lymph node metastasis versus noncancer breast epithelium is original. We also report for the first time that in intraductal proliferative lesions bordering on breast cancer, leptin expression is higher relative to proliferative lesions without accompanying breast cancer, which might imply that leptin abundance is related to disease progression.

The above results further indicate that breast cancer cells can be influenced not only by endocrine and/or paracrine leptin but also via a potent autocrine leptin loop. The function of the leptin autocrine system might be especially important in primary tumors where the expression of leptin correlated with the presence of ObR in both ERα-positive and ERα-negative tumors. This observation is in agreement with the results of Ishikawa et al. who found coexpression of leptin and ObRl in primary ductal breast cancer (18). Here, we additionally identified a correlation between a less differentiated phenotype (G3 grade) and the expression of the leptin system in primary tumors. This notion is consistent with the fact that breast cancer dedifferentiation can be promoted by hypoxia (34, 35), which also can induce leptin/ObR expression (see also below).

Notably, the expression of both leptin and ObR in lymph node metastases was more frequent than their levels in primary tumors. Whether leptin is truly involved in breast cancer metastasis is still not known, but a limited analysis of Ishikawa et al. (18) suggested that the expression of leptin and ObRl is associated with cancer recurrence in distant organs and a shorter 5-year disease-free survival. Interestingly, in metastases, but not in primary tumors, both leptin and ObR negatively correlated with Ki-67, which could suggest that in metastases the leptin system is not involved in proliferation.

The mechanisms responsible for leptin/ObR overexpression in primary and metastatic breast cancer are not clear. Our results suggest that different stimuli associated with obesity can induce leptin and ObR mRNA. Most notably, high concentrations of insulin and hypoxia stimulated leptin mRNA in both ERα-positive MCF-7 and ERα-negative MDA-MB 231 cell lines. ObRl mRNA was induced by hypoxia only in MDA-MB-231 cells. On the other hand, IGF-I and E2 stimulated leptin and ObR mRNAs in MCF-7 cells. The differential response of MCF-7 and MDA-MB-231 cells to E2 and IGF-I is in agreement with our previous results (36, 37).

Previous reports suggested a link between leptin and ER. Leptin has been found to enhance ERα activity and stimulate

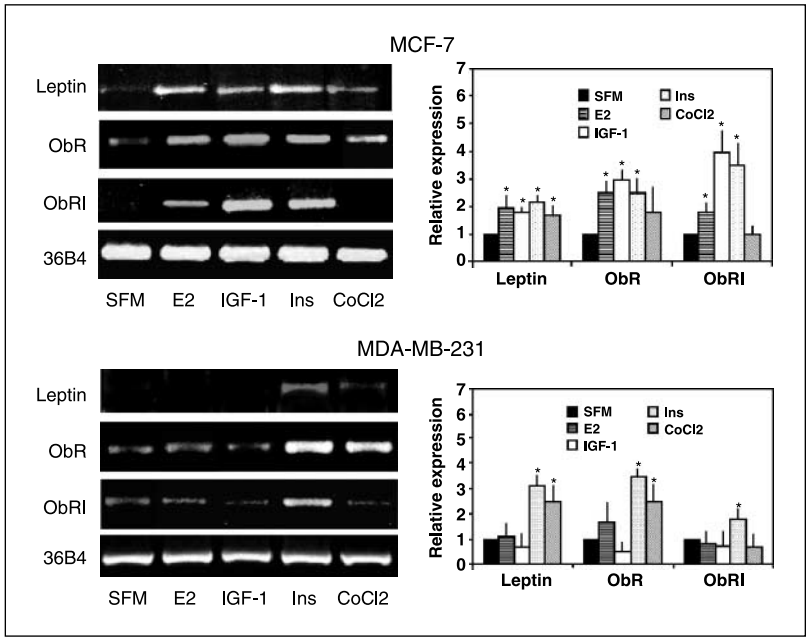

Fig. 2. Effects of E2, IGF-I, insulin, and hypoxia on leptin and ObR mRNA expression in breast cancer cells. MCF-7 and MDA-MB-231 cells were placed in serum-free medium (*SFM*) for 24 hours and then stimulated with E2, IGF-I, insulin (*Ins*), or $CoCl_2$, as described in Materials and Methods. The expression of leptin, ObR (all isoforms), and ObRl (the long form of the receptor only) mRNAs was probed by reverse transcription-PCR using conditions and primers listed in Materials and Methods. Obtained from at least three independent experiments. The abundance of leptin, ObR, and ObRl mRNAs is shown relative to the levels of 36B4 control mRNA. In all cases, the relative expression in serum-free medium is taken as 1. Bars, SD.
*, statistically significant differences between treated and untreated cells.

the synthesis of estradiol (13, 16, 17). Reciprocally, estradiol can induce leptin and ObR expression, as shown by this study and earlier reports in other models (24, 27, 38). It is possible that ERα effects on leptin/ObR is mediated in part by IGF-I and insulin systems, as E2 is known to up-regulate both pathways in breast cancer cells (39–41). Interestingly, in our study, the expression of leptin and ObR in primary tumors positively correlated with their presence in matched lymph node metastases but only in ERα-positive cases, which might suggests greater stability of the leptin system in this cell context.

Our study also suggested a relationship between leptin/ObR and ERβ (in particular the cytoplasmatic pool of ERβ recognized by our antibody). The significance of this link is not clear, especially in light of the controversial role of ERβ in breast cancer (42). However, some reports suggested the association of ERβ with poor prognostic features in breast cancer (42, 43), which would agree with our and other findings that the leptin system might be involved in metastasis (2).

In summary, we show that leptin and ObR are overexpressed in primary breast cancer and lymph node metastasis. This overexpression could be related to exposure of cells to high levels of insulin, IGF-I, and estradiol as well as due to hypoxic conditions. Thus, targeting leptin signaling could be beneficial for breast cancer therapy and prevention.

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RNAi-Mediated Silencing of Insulin Receptor Substrate 1 (IRS-1) Enhances Tamoxifen-Induced Cell Death in MCF-7 Breast Cancer Cells

Gregory Cesarone,[1] Cecilia Garofalo,[2,4] Marc T. Abrams,[1] Olga Igoucheva,[3] Vitali Alexeev,[3] Kyonggeun Yoon,[1,3] Eva Surmacz,[2,4] and Eric Wickstrom[1,2]*

[1]Department of Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107
[2]Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania 19107
[3]Department of Dermatology and Cutaneous Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107
[4]Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, Temple University, Philadelphia, Pennsylvania 19122

Abstract Insulin receptor substrate 1 (IRS-1) is a major downstream signaling protein for insulin and insulin-like growth factor I (IGF-I) receptors, conveying signals to PI-3K/Akt and ERK1/2 pathways. In breast cancer, IRS-1 overexpression has been associated with tumor development, hormone-independence and antiestrogen-resistance. In part, these effects are related to potentiation of IRS-1/PI-3K/Akt signaling. In estrogen sensitive breast cancer cell lines, tamoxifen treatment reduces IRS-1 expression and function; consequently, inhibiting IRS-1/PI-3K signaling. We tested whether anti-*IRS1* siRNA could inhibit growth and survival of estrogen-sensitive MCF-7 breast cancer cells, when used alone or in combination with TAM. Our results indicated: (a) out of four tested anti-*IRS1* siRNAs, two siRNAs reduced IRS-1 protein by approximately three-fold in both growing and IGF-I-stimulated cells without affecting a closely related protein, IRS-2; (b) these effects paralleled *IRS1* mRNA downregulation by approximately three-fold, measured by quantitative real time-polymerase chain reaction; (c) action of anti-*IRS1* siRNAs induced the apoptotic response, observed by altered mitochondrial membrane potential coupled with downregulation of NF-κB target Bcl-xL and reduced cell viability; (d) anti-*IRS1* siRNA treatment enhanced the cytotoxic effects of TAM by ~20%. In summary, anti-*IRS1* RNAi strategy could become a potent tool to induce breast cancer cell death, especially if combined with standard TAM therapy. J. Cell. Biochem. 98: 440–450, 2006.

Key words: apoptosis; IRS-1; signaling; siRNA; tamoxifen

IRS-1, a member of the IRS family of structurally related scaffolding molecules (IRS-1-4), is a ~135 kDa signaling protein that is a major substrate for the IGF-I and insulin receptors. IRS-1 contains multiple functional domains for protein–protein interactions and intracellular signal transduction. In addition, IRS-1 contains ~20 Tyr residues that, upon phosphorylation by

Abbreviations used: DAPI, 4′,6-diamidino-2-phenylindole; E_2, 17-β-estradiol; EGFP, enhanced green fluorescent protein; ERα, estrogen receptor-α; GSK3, glycogen synthase kinase 3; IGF-IR, insulin-like growth factor I receptor; IKK, I-kappaB kinase; IR, insulin receptor; IRS, insulin receptor substrate; PBS, phosphate-buffered saline; PI-3K, phosphatidyl inositol 3 kinase; QRT-PCR, quantitative real time-polymerase chain reaction; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, short interfering RNA; TAM, tamoxifen.

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Marc T. Abrams's present address is Department of Oncology, Merck Research Laboratories, West Point, PA, 19486.

*Correspondence to: Eric Wickstrom, Department of Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107.
E-mail: eric@tesla.jci.tju.edu

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activated tyrosine kinase receptors, may create binding sites for SH2-containing proteins [White, 1998].

The principal signal induced by activated IRS-1 in many cellular systems is the PI-3K/Akt pathway [Shepherd et al., 1998]. In addition, IRS-1 is known to convey signals through the MAP kinase cascade [Tanaka et al., 1996], some isoforms of protein kinase C [deVente et al., 1996], JAK/STAT pathway [Gual et al., 1998], SHP2 phosphatase [Hayashi et al., 2004], and the pathways mediated by the adapters Nck and Crk [McCarty, 1998]. The remarkable signaling potential of IRS-1 has been emphasized by findings that it can interact with multiple proteins including integrins [Vuori and Ruoslahti, 1994], cadherins [Hellawell et al., 2002], steroid hormone receptors [Mauro et al., 2003], and viral oncogenic proteins [Prisco et al., 2002].

In part, overexpression of IRS-1 leads to increased activation of survival pathways, notably the PI-3K/Akt pathway [Surmacz, 2000] where Akt can interfere with apoptosis mediated by mitochondrial and non-mitochondrial pathways [Franke et al., 2003]. Activation of the mitochondrial pathway involves depolarization of the mitochondrial membrane leading to the release of AIF and cytochrome c, with subsequent stimulation of caspases. In the non-mitochondrial extrinsic apoptotic pathway, Akt phosphorylates and blocks activity of FKHR, a member of the Forkhead family of transcription factors that induce the expression of Fas, the ligand of the death receptor [Vivanco and Sawyers, 2002]. Furthermore, Akt phosphorylates and inhibits GSK3 kinase leading to stabilization of essential cell cycle regulators cyclin D1, β-catenin and I-κB [Vivanco and Sawyers, 2002]. Finally, Akt can activate NF-κB indirectly by activating IKKs [Madrid et al., 2000] as well as stimulate protein synthesis through the mTOR/$p70^{S6}$ (RSK) pathway [Vivanco and Sawyers, 2002].

Importantly, the expression and function of IRS-1 and downstream signaling is regulated by estrogens in breast cancer cells. For instance, E_2 can stimulate *IRS1* mRNA and IRS-1 protein expression and potentiate IRS-1 signaling to Akt [Bartucci et al., 2001; Mauro et al., 2003]. IRS-1 can also bind to cytoplasmic estrogen receptors, resulting in increased IRS-1 stability and improved signaling to Akt [Mauro et al., 2003]. Conversely, both pure and non-steroidal antiestrogens reduce IRS-1 expression and function [Guvakova and Surmacz, 1997; Mauro et al., 1999].

Hence, we investigated whether IRS-1 expression can be effectively and specifically reduced with siRNA technology. We also asked whether knockdown of IRS-1 might enhance cellular response to TAM, a non-steroidal antiestrogen commonly used for breast cancer therapy and prevention [Colletti et al., 1989].

MATERIALS AND METHODS

Anti-*IRS1* siRNAs

The *IRS1* siRNA sequences (Dharmacon) were as follows: siRNA 1 5′-AAAGAGGUCUGGCAAGUGAdTdT-3′; siRNA 2 5′-GAACCUGAUUGGUAUCUACdTdT-3′; siRNA 3 5′-CCAC GGCGAUCUAGUGCUUdTdT-3′; siRNA 4 5′-GUCAGUCUGUCGUCCAGUAdTdT-3′; and a nonspecific (NS) siRNA 5′-ACAAGACCUAAGUGCACUG dTdT-3′. Lamin A/C siRNA was purchased as a control (Qiagen). All anti-*IRS1* sequences were analyzed with the BLASTn program and were found to have no significant homology to other human genes.

Construction of Plasmids Expressing shRNA Directed Against *IRS1* mRNA

Sequences for shRNA corresponding to siRNA 4 were generated by annealing two oligonucleotides, 5′-GATCCCGTCAGTCTGTCGTCCAGTATTCAAGAGATACTGGACGACAGACTGACTTTTTTGGAAA-3′ and 5′-AGCTT-TTCCAAAAAAGTCAGTCTGTCGTCCAGTATCTCTTGAAT ACTGGACGACAGACTGACGG-3′. The annealed product was cloned into *Bam*HI and *Hind*III sites of the pSilencer 2.1 Neo plasmid (Ambion), which constitutively expresses shRNA from a U6 promoter. As controls, shRNA directed against enhanced green fluorescent protein (EGFP) and scrambled siRNA 4 sequences were cloned to *Bam*HI and *Hind*III sites of the pSilencer 2.1 Neo plasmid.

Cell Growth and Transfection

MCF-7 (ATCC) human breast epithelial cells were grown in DMEM/F12 medium (Cellgro) supplemented with 5% calf serum, 50 U/ml penicillin, 5 μg/ml streptomycin, and 2 mM glutamine under 5% CO_2 in a humidified incubator at 37°C. For transfection, 70% confluent cultures were used. siRNA was complexed with the transfection agent RNAiFect (Qiagen) used according to manufacturer's instructions.

All final siRNA concentrations were 100 nM. When TAM (Sigma) was used alone or in combination with siRNA, it was added at a final concentration of 10 μM in growth medium. Cells not treated with TAM were given growth medium with0.05% vehicle (methanol). For transfection of anti-*IRS1* shRNA, MCF-7 cells were transfected with plasmid using FuGENE 6 (Roche Applied Science) according to manufacturer's instructions. Transfection experiments were performed a minimum of two independent experiments.

Western Immunoblot Analysis

At 4 days post-siRNA transfection, plates were lysed in 50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM EGTA, pH 7.5, 10% glycerin, 1% Triton X-100 with a Complete Mini protease inhibitor cocktail tablet (Roche Applied Science). Cytoplasmic lysates were electrophoresed in 4%–12% polyacrylamide tris-glycine gels (Invitrogen). Nuclei were lysed in 20 mM KOH, HEPES pH 8, 20% glycerol, 1% NP-40, 0.1 mM EDTA, 5 mM $MgCl_2$, 0.5 M NaCl with a Complete Mini. Antibodies used were IRS-1, IRS-2, and NFκB p65 (Santa Cruz), pY^{612} IRS-1 (Biosource), Bcl-xL, Akt and pS^{473} Akt (Cell Signaling), Bcl-2 (Upstate), IKKβ (Labvision) and GAPDH (Ambion). Substrate for HRP-conjugated secondary antibodies was SuperSignal® West Femto (Pierce). Detection and quantitation of protein was conducted with a Kodak Image Station 2000R. For IGF-I stimulation, MCF-7 cells were subjected to a 24 hr serum starvation at day 3 (72 hr) followed by a 15 min stimulation with IGF-I (50 ng/ml) just prior to cell lysis on day 4 (96 hr).

Immunofluorescence

MCF-7 cells were grown to 50% confluence in two-well chamber slides. Ninty-six hours after transfection of siRNA, cells were fixed in 3% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS, washed with PBS, and incubated for 1 hr with 2 μg/ml primary antibody recognizing IRS-1 (Santa Cruz Biotechnology). Next, the slides were washed with PBS, and incubated with a secondary rhodamine-conjugated donkey anti-rabbit IgG antibody, and then sealed with DAPI-containing Vectashield (Vector Labs). Cells were photographed at magnification 600× using a confocal laser scanning microscope (Bio-Rad).

Quantitative Real Time-PCR (QRT-PCR)

Total RNA was purified using Trizol (Molecular Research Center) from siRNA-treated cells after 24 hr. Reverse transcription of total RNA was performed using the TaqMan RT Kit (ABI) according to the vendor's instructions with 500 ng total RNA per reaction. Probes were designed to span the *IRS1* mRNA sequence where the siRNA would bind and induce cleavage. For siRNA 3 the set used was as follows: forward primer, 5′-CTCCACCTCGGATTGTCTCTTC-3′; reverse primer, 5′-GAAACCGCCATCGCTGG-3′; probe, 5′-6FAM-CAC GGCGATCTAGTGCTTCGGTGTC-TAMRA-3′, and for siRNA 4 forward primer, 5′-TTCGG CCACCAGCCC-3′; reverse primer, 5′-GAAGAGACAATCCGAGGTGGAG-3′; and its probe, 5′-6FAM-TCAGTCTGTCGTCCAGTAGCACCAG TGG-TAMRA-3′. The expression of the endogenous control gene β-actin was assessed using a pre-made primer probe set (ABI). An average C_T value was obtained for replicate reactions. Changes in *IRS1* mRNA content relative to β-actin mRNA were determined using the comparative C_T method (ABI User Bulletin no. 2) to calculate change in C_T and ultimately fold and percent change.

Measurements of Mitochondrial Membrane Integrity

To detect apoptosis following siRNA treatment after 4 days, cells were treated with BD MitoSensor™ Reagent according to manufacturer instructions. Cultures were photographed at magnification 100× with a blue filter (for detecting green fluorescence) or a green filter (red fluorescence) with an Olympus CK40 fluorescence microscope with a SPOT camera. For final analysis, green and red images were overlaid using SPOT v 4.01 Advanced Software (Diagnostic Instruments).

Cell Viability Assays

Once stained with 0.4% Trypan blue solution (Cellgro), the percentage of dead cells from pooled adherent and floating cells was determined by direct cell counting using a hemacytometer. At least 400 cells were counted with each experimental condition performed in triplicate. For the MTT assay (Chemicon), transfections were scaled for 96-well plates.

The assay was carried out according to manufacturer's instructions with absorbances read using a μQuant plate reader (Bio-Tek) coupled with KCjunior software (v1.22).

Statistical Analysis

Student's *t*-test was employed for statistical analysis for assessing significance of changes in experiments. The null probability *P*-value of $P < 0.05$ (2σ) was considered statistically significant. All experiments were performed with an $n = 3$ unless otherwise noted.

RESULTS

Anti-*IRS1* siRNA Inhibits *IRS1* mRNA and IRS-1 Protein Expression

We determined the effects of 4 anti-*IRS1* siRNAs on IRS-1 protein levels at 1, 2, and 4 days of treatment. Nonspecific siRNAs, a siRNA specific to Lamin A/C, and untreated cells were used as controls. The most effective inhibition was achieved by siRNA 3 ($67.6 \pm 2.2\%$ reduction vs. nonspecific) and siRNA 4 ($76.2 \pm 3.7\%$ reduction vs. nonspecific) (Fig. 1A and B) at 100 nM, respectively. The reduction by siRNA 3 and siRNA 4 was within a 5% difference when normalized for GAPDH expression, and therefore they were used interchangeably for subsequent experiments. siRNAs 1 and 2, which did not produce significant silencing of IRS-1 relative to a nonspecific siRNA treatment (Fig. 1B), were excluded from further experiments. A nonspecific siRNA and a siRNA specific to Lamin A/C, both used at 100 nM, did not modulate IRS-1 protein levels.

To test IRS-1 protein knockdown with synthetic siRNA 3 by an independent method, we treated MCF-7 cells with siRNA 3 and observed IRS-1 protein expression by immunofluorescence (Fig. 1C). DAPI was used to stain nuclei and show the presence of intact cells. Compared with untreated cells, strong silencing of cytoplasmic IRS-1 protein by siRNA 3 was apparent after 4 days of treatment. Nonspecific siRNA did not change IRS-1 protein expression.

Plasmid-encoded shRNA has been shown as an efficient means of expressing siRNA in cells in a constitutive manner [Carmell and Hannon, 2004]. Testing the reliability of our synthetic siRNA 3 results, plasmid-encoded shRNA corresponding to anti-*IRS1* siRNA 4 was transiently transfected into MCF-7 cells. This shRNA reduced IRS-1 protein levels (Fig. 2) similarly to cells treated with anti-*IRS1* siRNA 4 (Fig. 1), with the IRS-1 protein silenced 60%–70% at 72 hr post transfection. Transfection of plasmids expressing shRNA directed to *EGFP* or a nonspecific siRNA sequence showed no silencing of IRS-1.

We used QRT-PCR to determine whether siRNA 3 and siRNA 4 reduced *IRS1* mRNA levels. The results revealed consistent and statistically significant reduction in *IRS1* mRNA following a 24 hr treatment with siRNA 3 or siRNA 4 relative to untreated cells (Fig. 3). The effects of siRNA 3 and siRNA 4 on *IRS1* mRNA were statistically similar.

Anti-*IRS1* siRNA Inhibits IRS-1 Tyrosine Phosphorylation and Downstream Akt Activating Phosphorylation But Does Not Affect IRS-2 Expression and Phosphorylation

We sought to determine if signaling via IRS-1 was altered as a result of siRNA 3 treatment in growing as well as IGF-I stimulated MCF-7 cells. P^{Y612} on IRS-1 is an essential binding site for the p85 regulatory subunit of PI-3K, leading to activation [Esposito et al., 2001]. Compared with untreated cells, siRNA 3 treatment reduced P^{Y612} by $69.3 \pm 3.7\%$ in growing MCF-7 cells, and $51.6 \pm 1.5\%$ in IGF-I-stimulated MCF-7 cells, respectively (Fig. 4A). To confirm the effect on the PI-3K/Akt pathway, we tested the canonical [Vivanco and Sawyers, 2002] activating Akt P^{S473} phosphorylation site and observed downregulation of $38.3 \pm 10.4\%$ (Fig. 4B) compared to untreated cells.

We then tested whether anti-*IRS1* siRNA treatment affects expression of IRS-2, a structurally and functionally related signaling substrate. We found IRS-2 levels remained unchanged in both growing and IGF-I stimulated cells (Fig. 4A), whether the cells were untreated, treated with nonspecific siRNA, or treated with siRNA 3. These results suggest that signaling via the PI-3K/Akt pathway is reduced by siRNA knockdown of IRS-1, but that closely related IRS-2 was unaffected.

Anti-*IRS1* siRNA Induces the Apoptotic Phenotype in MCF-7 Cells

Early steps in apoptosis include cytochrome c release and altered mitochondrial membrane potential (ψ), whereas membrane blebbing and DNA fragmentation are difficult to observe, if at all, in MCF-7 cells due to the absence of functional Caspase 3 [Janicke et al., 1998]. In

order to assess the viability of MCF-7 cells transfected with siRNA 3 at 96 hr, ψ was studied using the JC-1 dye, MitoSensor™. A cationic dye, MitoSensor™ is taken up by mitochondria in healthy cells and forms aggregates that display red fluorescence. In apoptotic cells, MitoSensor™ remains as monomers in the cytoplasm producing green fluorescence. A disruption in ψ was observed in ~80% of cells treated with siRNA 3 and only in a small fraction (~3%) of cells treated with nonspecific siRNA or in untreated cultures (Fig. 5A). Therefore, disrupted ψ correlated with reduction in IRS-1 protein. Since altered ψ and cytochrome c release occur together, we tested whether Bcl-xL or Bcl-2 levels changed as a result of siRNA treatment, since they function to prevent cytochrome c release [Tsujimoto, 1998]. Figure 5B illustrates that Bcl-xL was reduced by 48 ± 2.3% relative to untreated MCF-7 cells, but not by nonspecific siRNA; Bcl-2 levels were unaffected. Hence, Bcl-xL levels correlate with altered ψ in MCF-7 cells treated with siRNA 3. Because Bcl-xL is a transcriptional target of NF-κB, we tested for levels of IKKβ, which when present and active phosphorylates IκB leading to its proteasomal degradation and the release of NF-κB [Lin and Karin, 2003]. Downstream IKKβ was indeed observed to be decreased (41.4 ± 4.0%) upon treatment with anti-*IRS1* siRNA 3 (Fig. 5C). An effect of this downregulation was confirmed in observing reduced nuclear NF-κB p65 as well (Fig. 5C).

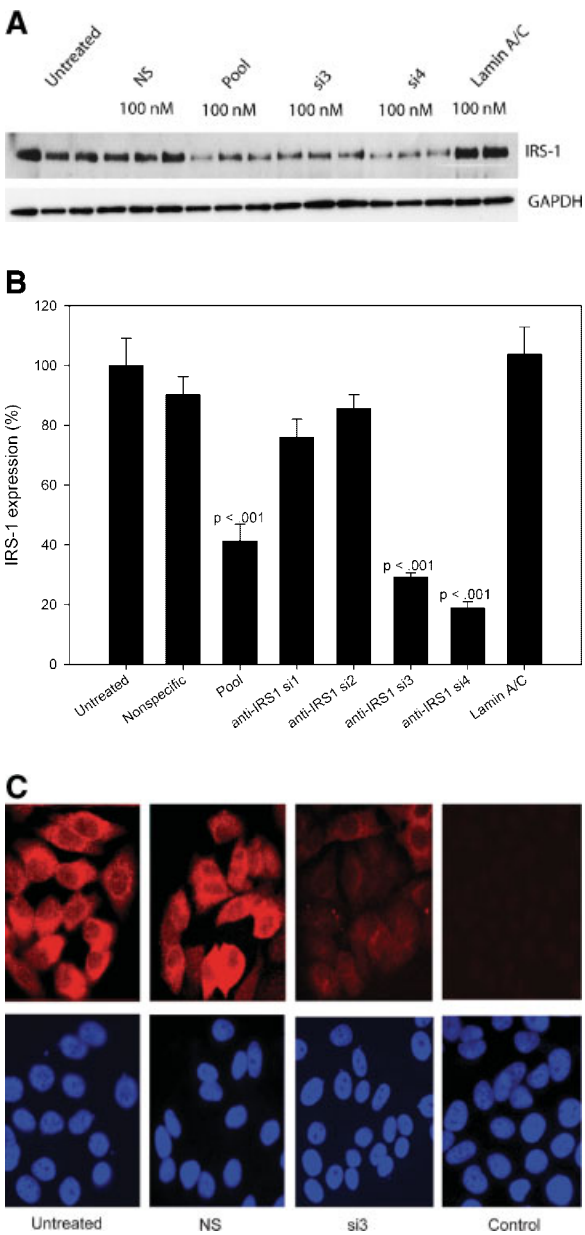

Fig. 1. Knockdown of IRS-1 protein in siRNA treated MCF-7 cells. MCF-7 cells transfected with siRNAs for 4 days, as described in Materials and Methods. **A**: Expression of IRS-1 was assessed by WB by loading 40 μg of cytoplasmic protein lysate per lane isolated from cells transfected with 100 nM of nonspecific siRNA, anti-*IRS1* pool siRNAs (25 nM each of 1, 2, 3 and 4), siRNA 1, siRNA 2, siRNA 3, siRNA 4, or anti-*Lamin-A/C* siRNA. Proteins were analyzed from three separately transfected plates, except that proteins from anti-*Lamin-A/C* siRNA treated MCF-7 cells were analyzed from two separately transfected plates. The expression of a cytoplasmic enzyme, GAPDH, was probed as a loading control. Data are not shown for siRNA 1 and siRNA 2, but a WB was performed just as in Figure 2A with the exception of siRNA 1 and siRNA 2 replacing siRNA 3 and siRNA 4. **B**: Relative IRS-1 expression in siRNA transfected cells was measured by densitometry of WB images. Each bar represents relative IRS-1 protein expression with standard deviation, with the expression in untreated cells taken as 100%. **C**: Expression of IRS-1 in MCF-7 cells treated with siRNA 3 after 4 days assessed by fluorescent microscopy. **Top panels**: Rhodamine staining from secondary rhodamine-conjugated donkey anti-rabbit IgG antibody bound to rabbit anti-IRS-1 IgG antibody. **Bottom panels**: DNA binding DAPI was used as a counter stain for visualizing nuclei. The control represents non-siRNA treated MCF-7 cells that were not incubated with primary antibody against IRS-1 prior to incubation with secondary rhodamine-conjugated donkey anti-rabbit IgG antibody.

Anti-*IRS1* siRNA Enhanced TAM-Induced Cell Death

In hormone-dependent breast cancer cells, IRS-1 is often overexpressed and hyperphosphorylated [Surmacz, 2000], eventually leading cells to become estrogen-independent and resistant to antiestrogens [Mauro et al., 1999]. In part, overexpression of IRS-1 leads to

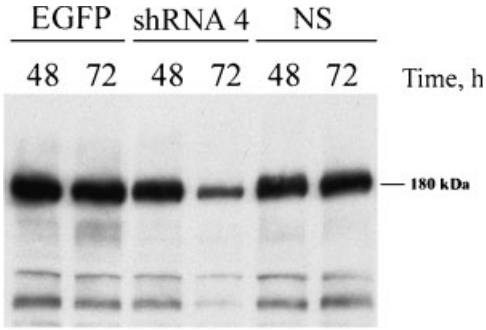

Fig. 2. Anti-*IRS1* shRNA expression leads to reduced IRS-1 protein. WB of cytoplasmic protein lysates from MCF-7 cells transfected with a shRNA directed to EGFP sequence (EGFP), a scrambled anti-*IRS1* siRNA 4 sequence (NS), and anti-*IRS1* sequence 4 (shRNA 4), respectively. Each lane was loaded with 40 μg of cytoplasmic protein lysate.

increased activation of survival pathways, most notably the PI-3K/Akt pathway [Surmacz, 2000]. Therefore, we determined the rate of MCF-7 cell death after siRNA treatment alone or in combination with TAM. Trypan blue exclusion assays (Fig. 6, gray bars) were performed at 96 hr. With TAM alone, viability dropped to $64.5 \pm 4.7\%$ of untreated, while with siRNA 3 alone, $82.0 \pm 2.2\%$ viable cells were seen. The combination of both treatments decreased viability to $42.1 \pm 2.2\%$. These results indicated additive effects of siRNA 3 and TAM. A more objective assay was also used to determine the viable cells remaining following treatment. An MTT assay (Fig. 6, black bars), where live cells cleave MTT to form blackish-purple formazan crystals, was used. The crystals can then be dissolved, and absorbance

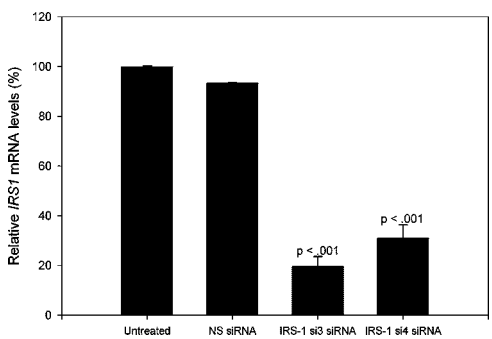

Fig. 3. *IRS1*-specific *IRS1* mRNA reduction analyzed by quantitative real time-PCR. MCF-7 cells were transfected with 100 nM of NS siRNA, siRNA 3, or siRNA 4. Total RNA was purified 24 hr post transfection and the expression of *IRS1* mRNA was assessed by QRT-PCR, as described in Materials and Methods. Each bar represents relative *IRS1* mRNA expression ± standard deviation, with the expression in untreated cells taken as 100%.

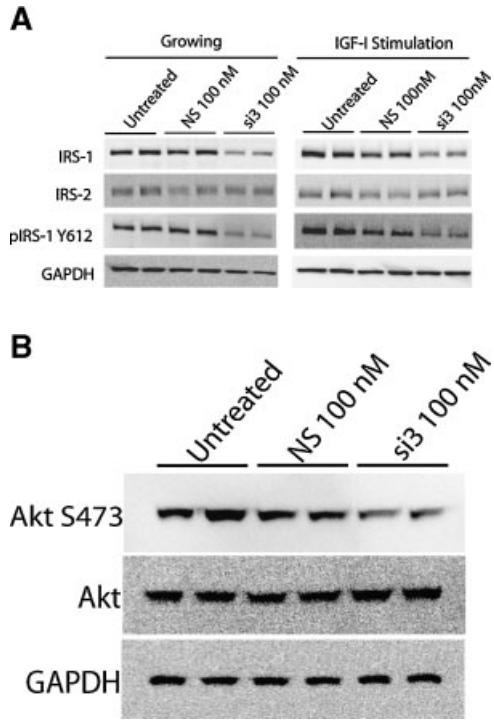

Fig. 4. Anti-*IRS1* siRNA inhibits IRS-1 Tyr phosphorylation and downstream Akt phosphorylation but does not affect IRS-2 expression. **A**: Left column (Growing): MCF-7 cells were transfected with 100 nM anti-*IRS1* siRNA 3, 100 nM nonspecific siRNA, or left untreated for 4 days. Then 40 μg of cell lysate per lane was probed by WB for the expression of IRS-1. The same WB filters were stripped and reprobed for IRS-1 P^{Y612}, and IRS-2. The expression of GAPDH was probed as a control of loading. Two samples of each treatment were analyzed in parallel. Right column (IGF-I stimulation): The treatment of cells and the evaluation of IRS-1, IRS-1 P^{Y612}, and IRS-2 were performed as described in Materials and Methods for IGF-I stimulation. **B**: MCF-7 cells were transfected with 100 nM anti-*IRS1* siRNA 3, 100 nM nonspecific siRNA, or left untreated for 4 days. Then 75 μg of cell lysate per lane was probed by WB for the expression of Akt P^{S473}, Akt, and GAPDH as a loading control. Two samples of each treatment were analyzed in parallel.

measured. Tam alone showed $59.3 \pm 1.1\%$ viable cells, while siRNA 3 dropped viability to $75.4 \pm 4.5\%$. The combination of both treatments lead to $35.4 \pm 1.6\%$ viable cells remaining; hence the additive effect was repeated with a more objective independent assay.

DISCUSSION

One of the key mechanisms controlling growth and survival of hormone-responsive breast cancer is functional crosstalk between IGF-IR and ER. IRS-1 is a major signaling substrate mediating growth and antiapoptotic signals from activated IGF-IRs through the

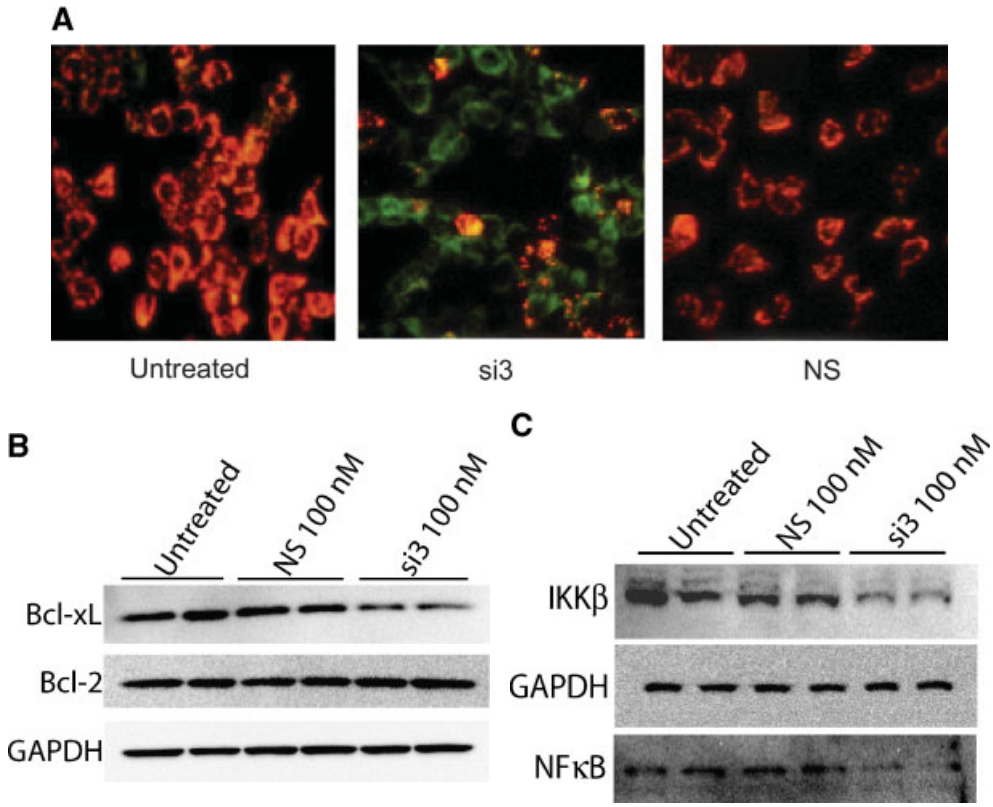

Fig. 5. Anti-*IRS1* siRNA decreases ψ and Bcl-xL expression. **A**: Decreased mitochondrial membrane potential in MCF-7 cells treated with anti-*IRS1* siRNA. **Left**, Untreated MCF-7 cells after 4 days of growth display normal ψ illustrated by the orange fluorescence present in the overlay of both the rhodamine (JC-1 aggregates in mitochondria) and FITC (JC-1 monomers in the cytoplasm) fluorescence. **Center**, MCF-7 cells treated with anti-*IRS1* siRNA 3 after 4 days of growth showed cells that have lost their normal ψ (JC-1 monomers in cytoplasm only). **Right**, MCF-7 cells treated with 100 nM NS siRNA after 4 days of growth display normal ψ akin to untreated cells. **B**: Downregulation of Bcl-xL, but not Bcl-2 was observed upon silencing of IRS-1 protein. Seventy micrograms of cytoplasmic lysate per lane were analyzed by WB from MCF-7 cells untreated or treated with NS siRNA or anti-*IRS1* siRNA 3 after 4 days of growth. **C**: Downstream IKKβ was also detected with an observed reduction in siRNA 3 treated MCF-7 cells. MCF-7 cells were transfected with 100 nM anti-*IRS1* siRNA 3, 100 nM nonspecific siRNA, or left untreated for 4 days. Then 75 μg of cell lysate per lane was probed by WB for the expression of IKKβ and GAPDH as a loading control (lysates same as those used in phospho-Akt WB with two independent samples of each treatment analyzed in parallel). Confirmation of this effect is observed with downregulation of nuclear NF-κB p65.

PI-3K pathway. In breast cancer cells, this pathway can also be induced by estrogens. Here we demonstrate that IRS-1 expression and function can be effectively inhibited with RNAi technology. Moreover, we show reduction of IRS-1 expression can improve cytotoxic activity of a commonly used antiestrogen, TAM.

We observed the greatest silencing of IRS-1 at 4 days with 100 nM anti-*IRS1* siRNA. IRS-1 appears to degrade via ubiquitination and proteasomal degradation [Zhande et al., 2002] but can be delayed by enhanced stability through binding to ERα [Mauro et al., 2003]. We hypothesize that after *IRS1* mRNA is degraded via an RNAi mechanism, that turnover of pre-existing IRS-1 protein in growing MCF-7 cells takes approximately 3 days, and that turnover reaches a maximum in MCF-7 cells treated with synthetic siRNA at 4 days (Fig. 1). Recently, synthetic shRNAs with 29-base-pair stems and 2-nucleotide 3′ overhangs were shown to produce efficiently predictable homogeneous small RNAs comprising the 22 bases at the 3′ end [Siolas et al., 2004], while shRNAs with shorter stems (22 bases) did not. The 29-mer shRNAs were reported by Siolas et al. to perform better than siRNAs, yet the 29-mer shRNA (Fig. 2) we used was not more effective than the siRNA we used. However, Siolas et al. did state that equivalent activity might occur in a few instances. With our shRNA and siRNA being comparable in their effects, we decided to continue our study with siRNA 3 only.

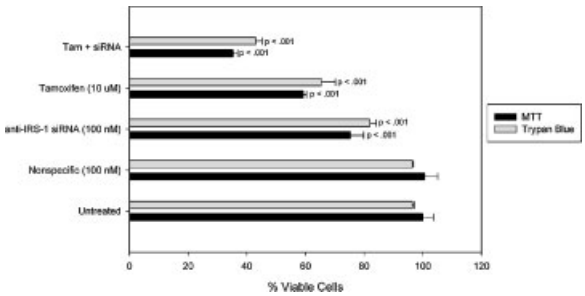

Fig. 6. An additive effect of a combined anti-*IRS1* siRNA and TAM treatment on MCF-7 cell death while in the presence of serum. Percentage of cell death in MCF-7 cells after 4 days treatment. Treatment conditions are noted in the left hand column of the graph. A minimum of 400 cells were counted for each condition in trypan blue assay. Absorbance read at 570 nm (630 nm) for MTT assay. Each bar represents the percentage of viable cells with standard deviation. Data are from at least three independent experiments.

There are eight criteria that have been established for the prediction of functional siRNAs [Reynolds et al., 2004]. It is of note that all four siRNA sequences used in this work meet no less than six of these criteria, but they displayed very different inhibition. Only siRNA 3 and 4 had significant activity silencing IRS-1, and siRNA 3 and 4 have AU base pairs at positions 18 and 19 of the sense strand whereas siRNA 1 and 2 have a GC and AU base pair. The reduced thermodynamic stability caused by 2 AU base pairs at the 5′ end of the antisense strand for siRNAs 3 and 4 would most likely facilitate their enhanced incorporation into RISC; this sequence motif has previously been associated with asymmetry of entry into RISC [Khvorova et al., 2003; Schwarz et al., 2003].

We found that phosphorylated IRS-1 and downstream Akt were reduced by treatment with anti-*IRS1* siRNA 3, as well as total IRS-1 protein levels, suggesting decreased proliferative and survival signaling downstream. Interestingly, the basal IRS-1 phosphorylation on P^{Y612} was significant even in growing cells [Esposito et al., 2001] allowing us to observe the reduction of the phosphorylation upon siRNA treatment. The reduction was less pronounced in cells stimulated with IGF-I (Fig. 4). The significant reduction ($69.3 \pm 3.7\%$) of IRS-1 P^{Y612} in growing cells treated with siRNA 3 reflected the extent of IRS-1 protein reduction ($67.2 \pm 2.2\%$), suggesting that the phosphorylation changes simply correlate with reduction of the IRS-1 protein expression. However, since we observed comparable reduction of IRS-1 and IRS-1 P^{Y612}, we hypothesize that residual IRS-1 could still take part in some survival signaling downstream of IGF-IR. Hence, in IGF-I stimulated MCF-7 cells we saw ~20% more phosphorylation than in regularly growing MCF-7 cells, even though both were treated with siRNA 3, suggesting acute phosphorylation of existing IRS-1 in response to IGF-I stimulation. In the case of growing cells, which more closely resemble an actual system with multiple signaling pathways functioning simultaneously, we observed downregulation of phosphorylated Akt at its S^{473} activating site.

The silencing of IRS-1 protein by siRNAs, but not with controls, was paralleled by the appearance of dead cells. MCF-7 cells do not have functional caspase 3 [Janicke et al., 1998] and therefore no activity of caspase 3 would be expected with altered ψ and the release of cytochrome c [Mooney et al., 2002; Ruiz-Ruiz and Lopez-Rivas, 2002]. Correlating with the altered ψ, reduced IRS-1 P^{Y612} and Akt P^{S473} observed upon IRS-1 silencing we also witnessed reduction of Bcl-xL (Fig. 5B), which has been shown to interact directly with cytochrome c [Kharbanda et al., 1997]. The reduction of IRS-1 P^{Y612} indicates downregulation of PI-3K [Esposito et al., 2001], which reduces the level of active Akt (Fig. 4B) able to phosphorylate proapoptotic Bad, giving the possible scenario of allowing nonphosphorylated Bad to bind and inhibit antiapoptotic Bcl-xL [Leverrier et al., 1999]. Previous work has shown IRS-1 to interact directly with Bcl-2 and Bcl-xL [Ueno et al., 2000]. Therefore it is possible that an unknown positive feedback loop between IRS-1 and Bcl-xL proteins was interrupted by the silencing of IRS-1.

Yet since it is known that Bcl-xL is a transcriptional target of NF-κB [Lin and Karin, 2003], we hypothesized that the key regulator to degradation of IκB and release of NF-κB, IKKβ, was effected. In the presence of IKKβ which can be effected by Akt, the NF-κB pathway can be activated to respond to cellular stress such as the ROS that would be released [Chen et al., 2003] upon altered mitochondrial membrane potential seen in Figure 5A leading to an oxidative, caspase-independent apoptotic death mechanism [Pozo-Guisado et al., 2005]. The difference in the percentage of MCF-7 cells with altered ψ after siRNA 3 treatment alone and the lesser percentage that were observed to be dead by Trypan blue staining

or the MTT assay for siRNA 3 treatment alone most probably occurred because of the aforementioned deficiency in caspase 3 in MCF-7 cells blocking a more common caspase-dependent apoptotic pathway.

Paralleling the molecular mechanisms elucidated, an additive effect was observed when MCF-7 cells were treated with anti-*IRS1* siRNA in combination with the antiestrogen TAM. TAM is commonly used for breast cancer treatment and prevention; however, resistance to TAM develops after prolonged treatment. In hormone-dependent breast cancer cells, IRS-1 is often overexpressed and hyperphosphorylated [Surmacz, 2000], eventually rendering cells to become estrogen-independent and resistant to antiestrogens [Mauro et al., 1999]. In vitro data suggested that one underlying mechanism is hyperactivation of common intracellular signaling pathways, such as PI-3K/Akt [Jordan et al., 2004].

IRS-1 is a potent alternative target to IGF-IR because of the ability of IRS-1 to relieve stoichiometric limitations from IGF-IR signaling. IRS-1 accomplishes this by allowing multiple p85 regulatory subunit molecules of PI-3K to bind a single IRS-1 molecule via multiple YMXM motifs [White, 1998]. Its downregulation could halt signaling at a point in the signaling pathway where there is great amplification potential. Additionally, $IRS1^{-/-}$ mice never develop diabetes due to an alternative pathway of insulin signaling with vigorous proliferation and survival of pancreatic β-cells [Araki et al., 1994]. The future prospects for improving the treatment of breast cancer will most probably include using current therapy in combination with new second line therapies, such as RNAi.

The additive effects that we observed with siRNA-mediated reduction of IRS-1 protein, in combination with antiestrogen treatment, poses the possibility of more effectively targeting survival signaling in tamoxifen-sensitive breast cancers and preventing de novo/acquired tamoxifen resistance [Gee et al., 2005]. A study has now looked at simultaneous assessment of IRS-1 expression in primary breast cancer and metastases suggesting a role for IRS-1 in breast cancer progression [Koda et al., 2005]. While modifications to siRNAs enhance therapeutic efficacy by increasing the half-life of the siRNAs and reducing off-target effects [Chiu and Rana, 2003; Czauderna et al., 2003; Banan and Puri, 2004; Hall et al., 2004], the last major hurdle, as with antisense oligonucleotides, will be efficient delivery to distant sites of action. Several groups are attempting tackle the delivery hurdle via a range of routes using plasmids containing shRNA delivered via pegylated immunoliposomes, viral vectors incorporating the CRE-loxP system, and siRNA complexed with atelocollagen [Devroe and Silver, 2004; Minakuchi et al., 2004; Pardridge, 2004; Tiscornia et al., 2004]. Further work encompassing in vivo studies is warranted to elucidate the effects of reducing IRS-1 expression with RNAi in combination with antiestrogens in ER-positive breast cancer, or without antiestrogens in ER-positive, antiestrogen-resistant breast cancer.

ACKNOWLEDGMENTS

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Leptin and Cancer

CECILIA GAROFALO[1] AND EVA SURMACZ[2]*
[1]Department of Pharmaco-Biology, University of Calabria, Arcavacata di Rende, Cosenza, Italy
[2]Sbarro Institute for Cancer Research and Molecular Medicine, College of Science and Technology, Temple University, Philadelphia, Pennsylvania

The prevalence of obesity has markedly increased over the past two decades, especially in the industrialized countries. While the impact of excess body weight on the development of cardiac disease and diabetes has been well documented, the link between obesity and carcinogenesis is just being recognized. This review will focus on the link between leptin, a cytokine that is elevated in obese individuals, and cancer development. First, we briefly discuss the biological functions of leptin and its signaling pathways. Then, we summarize the effects of leptin on different cancer types in experimental cellular and animal models. Next, we analyze epidemiological data on the relationship between obesity and the presence of cancer or cancer risk in patients. Finally, leptin as a target for cancer treatment and prevention will be discussed. J. Cell. Physiol. 207: 12–22, 2006. © 2005 Wiley-Liss, Inc.

The prevalence of obesity has markedly increased over the past two decades, especially in the industrialized countries. According to the Centers for Disease Control and Prevention (www.cdc.gov), in the United States alone, approximately 66% of adults are overweight as defined by body mass index (BMI, weight in kilograms divided by the square of the height in meters) in excess of 25, and 31% of adults are obese (BMI > 30) (Hedley et al., 2004). Globally, the overweight population has exceeded 1 billion (www.cdc.gov). Epidemiological data gathered over the past two decades clearly demonstrate that obesity in adults is associated with increased risk of cardiovascular disease, diabetes, some forms of cancer, and numerous other health disorders (Klein et al., 2002). Consequently, the regulation of body weight and obesity-related pathology is rapidly becoming a critical concern for public health experts and medical scientists worldwide (Kopelman, 2000).

While the impact of excess body weight on the development of cardiac disease and diabetes has been well documented, the link between obesity and carcinogenesis is just being recognized. The mechanism of adipose tissue-induced cancer is not known, but several possible scenarios can be envisioned. In the case of hormone-dependent neoplasms such as breast cancer, increased production and secretion of estrogenic compounds, growth factors, and angiogenic stimulators by excess fat tissue could contribute to tumor growth and metastasis (Sierra-Honigmann et al., 1998; Ahima and Flier, 2000; Miyazawa-Hoshimoto et al., 2004).

This review will focus on the link between leptin, a cytokine that is elevated in obese individuals, and cancer development. First, we briefly discuss the biological functions of leptin and the regulation of its production. Next, we summarize the effects of leptin on different cancer types in experimental cellular and animal models. Finally, we analyze epidemiological data on the relationship between circulating leptin levels and the presence of cancer or cancer risk in patients. The potential of targeting leptin in cancer treatment or prevention will be discussed.

LEPTIN AND ITS ACTIVITIES

Leptin, a product of the obese (*ob*) gene, is a 16 kDa cytokine that was discovered in 1994 as a regulator of body weight and energy balance acting in the hypothalamus (Zhang et al., 1994). *Ob*/*ob* mice with mutations in the gene encoding leptin do not produce leptin and become morbidly obese, infertile, hyperphagic, hypothermic, and diabetic due to unopposed appetite (Huang and Li, 2000). In humans, like in animals, leptin is an important regulator of energy balance. However, unlike in mice, human obesity is not related to leptin deficiency but to the development of leptin resistance (Bjorbaek and Kahn, 2004; Correia and Haynes, 2004; Hukshorn and Saris, 2004; Mark et al., 2004). In fact, mutations in the human *ob* gene are exceptionally rare (Chagnon et al., 2003).

In adult animals, leptin mRNA is primarily detected in white and brown adipose tissue (Masuzaki et al., 1996). In addition, a number of non-adipocyte tissues have been shown to synthesize and secrete leptin. The

Abbreviations: aa, amino acid; Ab, antibody; Akt, protein kinase B; AML, acute myeloid leukemia; Bcl2, B-cell CLL/lymphoma 2; BMI, body mass index; cdk2, cyclin dependent kinase 2; EOC, epithelial ovarian cancer; ERα, estrogen receptor alpha; ERK1/2, extracellular signal-regulated kinases 1/2; FGF-β, fibroblast growth factor beta; G-CSF, granulocyte-colony stimulating factor; GSK-3, glycogen synthase kinase-3; HIF-1, hypoxia-induced factor 1; IARC, International Agency for Research on Cancer; IGF-1, insulin-like growth factor 1; IL-3, interleukin 3; JAK2, Janus kinase 2; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NF-kappaB, nuclear factor kappa B; ob, obesity gene; ObR, leptin receptor; ObRl, leptin receptor long form; ObRs, leptin receptor short form; PI-3K, phospho inositide 3 kinase; PKC, protein kinase C; PLC gamma, phospholipase C gamma; PR, progesterone receptor; pRB, retinoblastoma protein; RT-PCR, reverse transcription-polymerase chain reaction; SCF, stem cell factor; SLR, soluble leptin receptor; SRC-1, steroid receptor coactivator 1; STAT, signal transducer and activator of transcription 3; TGF-β1, transforming growth factor beta 1; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor; WHR, waist-to-hip ratio.

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*Correspondence to: Eva Surmacz, Sbarro Institute for Cancer Research and Molecular Medicine, College of Science and Technology, Temple University, Philadelphia, PA 19122.
E-mail: surmacz@temple.edu

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other leptin sources include gastric mucosa cells (Bado et al., 1998), mammary epithelial cells (Smith-Kirwin et al., 1998), myocytes (Wang et al., 1998), and the placenta (Senaris et al., 1997). Leptin expression has also been reported in the testes, ovary, and hair follicles (Hoggard et al., 1997). Subsequent studies documented that in addition to its primary function as a regulator of food intake, leptin can affect fetal development, sex maturation, lactation, hematopoiesis, and immune responses (Wauters et al., 2000; Bonnet et al., 2002; Brann et al., 2002; Neville et al., 2002; Goumenou et al., 2003).

In humans, the major factor influencing plasma leptin concentrations is adipose tissue mass (Maffei et al., 1995). Circulating leptin levels exhibit a particularly strong positive correlation with total body fat, and to a lesser degree with BMI (Frederich et al., 1995; Ahima et al., 1996; Boden et al., 1996; Sinha et al., 1996; der Merwe et al., 1999; Thomas et al., 2000). Higher concentration of serum leptin in obese individuals is associated with both increased fat mass and increased leptin release from larger adipocytes (Hamilton et al., 1995; Kolaczynski et al., 1996a,b). Importantly, serum leptin levels are significantly higher in women than in men, even after the adjustment for total body fat mass (Havel et al., 1996; Rosenbaum et al., 1996). One explanation for this tendency is differential regulation of leptin expression by sex hormones, with estrogens reported to upregulate (Casabiell et al., 1998; Castracane et al., 1998) and testosterone observed to decrease leptin levels (Blum et al., 1997; Elbers et al., 1997; Jockenhovel et al., 1997).

The synthesis of leptin in adipocytes is influenced by different humoral factors, most notably insulin (Cusin et al., 1995; Leroy et al., 1996), tumor necrosis factor alpha (TNF-α) (Zhang et al., 2000), glucocorticoids (De Vos et al., 1995; Dagogo-Jack et al., 1997), reproductive hormones (Machinal-Quelin et al., 2002), and prostaglandins (Fain et al., 2000). Importantly, many of these factors have been shown to be associated with neoplastic processes.

In the context of cancer, it is noteworthy that leptin expression can be induced under hypoxic conditions, which often occur in solid tumors (Ambrosini et al., 2002; Grosfeld et al., 2002). Hypoxia and chemical inducers of cellular hypoxia are able to activate the leptin gene promoter through the hypoxia-induced factor-1 (HIF-1) in human adipocytes and fibroblasts (Ambrosini et al., 2002; Grosfeld et al., 2002). These data suggest that leptin may play a role in vascular remodeling (Stenmark et al., 2002). Indeed, leptin has been shown to regulate neoangiogenesis by itself and in concert with vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) 2 (Bouloumie et al., 1998; Sierra-Honigmann et al., 1998; Cao et al., 2001). In addition to the proangiogenic function, leptin can enhance endothelial cell growth (Bouloumie et al., 1998; Sierra-Honigmann et al., 1998) and suppress apoptosis through a Bcl-2-dependent mechanism (Artwohl et al., 2002). The role of leptin in neovascularization is supported additionally by the observation that the hormone can increase the levels and activity of enzymes involved in angiogenesis, for example, matrix metalloproteinases (MMPs) 2 and 9 (Park et al., 2001; Kume et al., 2002).

In addition to its involvement in endothelial cell function, leptin has been shown to act as a mitogen, transforming factor, or migration factor for many different cell types, including smooth muscle cells (Oda et al., 2001), normal and neoplastic colon cells (Hardwick et al., 2001; Liu et al., 2001), and normal and malignant mammary epithelial cells (Dieudonne et al., 2002; Laud et al., 2002).

LEPTIN SIGNALING

The activities of leptin are mediated through the transmembrane leptin receptor (ObR) (Tartaglia, 1997; White et al., 1997). In human tissues, at least four isoforms of ObR with different COOH-terminal cytoplasmic domains have been described (Barr et al., 1999). The full (long) form of ObR (ObRl) is 1,165 amino acids long (Mr ~ 150,000–190,000) and contains the extracellular, transmembrane, and intracellular domain (Fig. 1). The extracellular domain binds ligand, whereas the intracellular tail recruits and activates signaling substrates. Only ObRl has a full signaling potential, whereas short ObR isoforms (ObRs) lack major domains recruiting downstream effectors and have diminished or abolished signaling capability (Fig. 1) (Bjorbaek et al., 1997, 2001; Sweeney, 2002; Zabeau et al., 2003). ObRl is highly expressed in the hypothalamus (Bjorbaek et al., 1997). However, lower levels of ObRl have been identified in many peripheral organs (Morton et al., 1999; Frank et al., 2000; Buyse et al., 2001; Goiot et al., 2001; Akerman et al., 2002; Ebenbichler et al., 2002; Lee et al., 2002; Kim et al., 2003).

The signaling pathways activated by ObRl include the classic cytokine JAK2/STAT3 (Janus kinase 2/signal transducer and activator of transcription 3) pathway; the Ras/ERK1/2 (Ras/extracellular signal-regulated kinases 1/2) signaling cascade; and the PI-3K/Akt/GSK3 (phosphoinositide 3 kinase/protein kinase B/glycogen synthase kinase 3) growth/antiapoptotic pathway. In addition, leptin has been found to induce phospholipase C (PLC)-gamma, protein kinase C (PKC), p38 kinase, and nitric oxide (NO) (Bjorbaek et al., 1997; Sweeney, 2002; Zabeau et al., 2003) (Fig. 2). Ultimately, induction of ObRl can activate several genes involved in cell proliferation, including *c-fos*, *c-jun*, *jun*B, *egr-1*, and *socs3*, and upregulate the expression angiogenic factors,

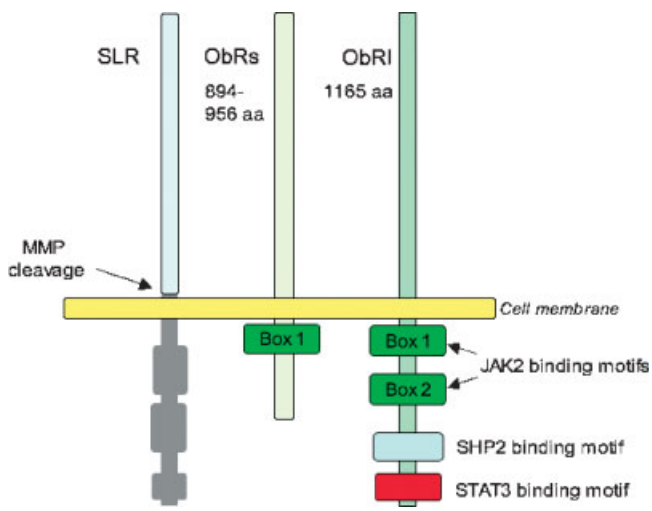

Fig. 1. Schematic representation of ObR isoforms. ObRl and ObRs share a common extracellular leptin-binding domain, but contain intracellular domains of variable lengths. The intracellular portion of ObRl (303 aa) contains two JAK2 binding motifs, Box 1 and Box 2, a binding site for SHP2 and a motif recruiting STAT3. Three human ObRs variants containing different portions of the intracellular domain (34, 44, or 96 aa) do not contain Box 2, STAT3, and SHP2 binding sites, but retain Box1 (Barr et al., 1999). SRL is a soluble leptin receptor generated by MMPs-mediated shedding of ObRl or ObRs ectodomains. SRL lacks the transmembrane domain as well as intracellular motifs.

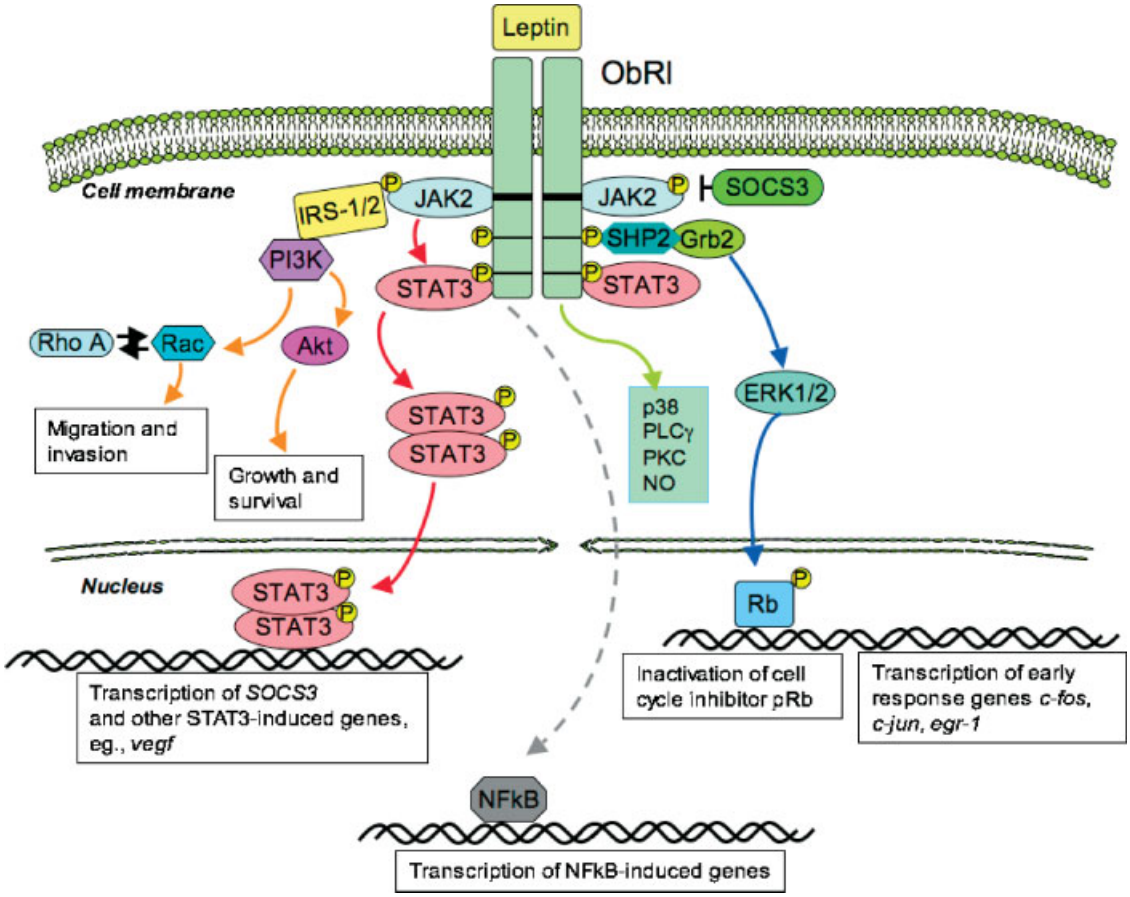

Fig. 2. Leptin receptor signaling. ObRl stimulates a broad spectrum of intracellular signaling pathways. Leptin binding to ObRl results in conformational changes and receptor oligomerization. These events stimulate tyrosine phosphorylation and activation of JAK2 that is constitutively associated with the receptor. JAK2 phosphorylates the intracellular domain of ObRl, especially tyrosines within the SHP2 and STAT3 binding sites. Recruitment of SHP2 leads to its tyrosine phosphorylation, binding to GRB2 and activation of the ERK1/2 cascade. Increased activity of ERK1/2 inactivates the cell cycle inhibitor, pRb and stimulates transcription of immediate early response genes (*c-fos, c-jun, egr-1*). In parallel, binding of STAT3 to ObRl induces STAT3 tyrosine phosphorylation, dimerization, nuclear translocation, and induction of target genes. These include *socs3* (whose product inhibits JAK-induced phosphorylation of ObRl) and *vegf* (whose product promotes angiogenesis). Induction of JAK2 can also stimulate PI-3K, possibly through the recruitment and phosphorylation of scaffolding proteins IRS-1/2. Activation of PI-3K can increase cell migration and invasion via the Rac/Rho pathways and stimulate the major growth/survival pathway Akt. ObRl activates, through yet unclear mechanisms, several other responses, for example, PLC-gamma, PKC and p38 kinases, nitric oxide (NO), and NFkappaB.

such as VEGF (Sweeney, 2002; Zabeau et al., 2003; Frankenberry et al., 2004) (Fig. 2).

The short forms of ObR are ubiquitously expressed (Fei et al., 1997). Their function is not clear, but there is evidence that ObRs can be involved in intra- and transcellular leptin transport (Hileman et al., 2000). In addition to the cellular isoforms of ObR, a soluble leptin receptor (SLR) that is generated by MMP-mediated shedding of the ObR ectodomain has been identified (Maamra et al., 2001; Ge et al., 2002) (Fig. 1). In human plasma as well as in vitro models, SLR sequesters leptin, preventing the activation of ObR signaling (Ge et al., 2002; Yang et al., 2004). The abundance of SLR is regulated by different physiological and pathological conditions, thus this receptor may serve as a potent modulator of leptin action in various tissues (Chan et al., 2002).

Recent studies suggested that leptin signaling can crosstalk with both polypeptide growth factor signaling and with steroid receptor function. For instance, insulin is known to increase leptin expression (Cusin et al., 1995; Saladin et al., 1995; Hardie et al., 1996; Leroy et al., 1996), but it can also induce leptin resistance by the inhibition of leptin signaling through JAK2 (Kellerer et al., 2001). In addition, the identification of leptin receptor on pancreatic beta-cells suggests the existence of a "adipoinsular" feedback loop whereby leptin may inhibit insulin secretion (Kieffer et al., 1996; Kulkarni et al., 1997).

Our work demonstrated that leptin modulates cellular response to estrogens. Specifically, leptin enhances the stability of the estrogen receptor alpha (ERα), leading to the maintenance of ERα-dependent transcription in breast cancer cells in the presence of antiestrogens (Garofalo et al., 2004). Leptin can also increase estrogen levels through the activation of aromatase expression and activity (Kitawaki et al., 1999; Magoffin et al., 1999; Catalano et al., 2003).

EFFECTS OF LEPTIN ON CANCER CELLS IN VITRO

The effects of leptin on cancer cells in vitro are summarized in Table 1.

Breast cancer

Leptin is necessary for normal mammary gland development in rodents and humans (Smith-Kirwin et al., 1998; Hu et al., 2002; Neville et al., 2002). How-

TABLE 1. Effects of leptin on cancer cells in vitro

Type of cancer	Effects of leptin	Cell model	References
Breast cancer	Increased cell proliferation	Human breast cancer cell lines; T47D, MCF-7, ZR75-1	Dieudonne et al. (2002); Hu et al. (2002); Laud et al. (2002); Okumura et al. (2002); Catalano et al. (2003); Somasundar et al. (2003); Garofalo et al. (2004); Yin et al. (2004)
	Increased cell transformation (anchorage-independent growth)	T47D human breast cancer cells	Hu et al. (2002)
	Activation of the ERK1/2, STAT3, Akt/GSK3, and PKC-α pathways	Human breast cancer cell lines; T-47D, MCF-7	Dieudonne et al. (2002); Hu et al. (2002); Laud et al. (2002); Okumura et al. (2002); Catalano et al. (2003); Garofalo et al. (2004); Yin et al. (2004)
	Increased AP-1 activation, upregulation of cdk2, cyclinD1, hyperphosphorylation of pRb	MCF-7, T47-D breast cancer cells	Hu et al. (2002); Okumura et al. (2002); Garofalo et al. (2004)
	Increased aromatase expression via, AP-1-dependent mechanism	MCF-7 breast cancer cells	Catalano et al. (2003)
	Induced expression of *c-myc*	MCF-7 breast cancer cells	Yin et al. (2004)
	Stabilization of ERα expression	MCF-7 cells treated with the antiestrogen ICI 182, 780	Garofalo et al. (2004)
Colorectal cancer	Increased cell invasion via an PI-3K, Rho- and Rac-dependent pathway	Premalignant familial adenomatous colonic cells PC/AA/C1 and human adenocarcinoma colonic cells LoVo and HCT-8/S11	Attoub et al. (2000)
	Increased cell growth through the ERK1/2 pathway	Human colon adenocarcinoma HT29 cell line	Hardwick et al. (2001)
	Reduced cell apoptosis Stimulation of NF-kB signaling	Human colon cancer HT29 cells treated with sodium butyrate	Rouet-Benzineb et al. (2004)
Prostate cancer	Increased cell proliferation and suppression of apoptosis	DU145, PC3 human prostate cancer cells	Somasundar et al. (2004)
Pancreatic cancer	Decreased cell proliferation	Mia-PaCa and PANC-1 human pancreatic cancer cells	Somasundar et al. (2003)
	Stimulation of STAT3 and STAT5b phosphorylation	BRIN-BD11 rat insulinoma cell line	Briscoe et al. (2001)
Ovarian cancer	Increased proliferation via the ERK1/2 pathway	BG-1 ovarian carcinoma cell line	Choi et al. (2004)
Lung cancer	Stimulation of cell proliferation the ERK1/2 pathway	SQ-5 human lung squamous cell cancer	Tsuchiya et al. (1999)

ever, new data suggest that leptin might be involved in mammary carcinogenesis (O'Brien et al., 1999; Dieudonne et al., 2002; Hu et al., 2002; Laud et al., 2002; Okumura et al., 2002; Cleary et al., 2003). Leptin and ObR have been found in normal and cancer mammary epithelium by immunohistochemistry with specific antibodies (Abs) (Ishikawa et al., 2004). Interestingly, both leptin and ObR appear to be significantly overexpressed in cancer tissue relative to non-cancer epithelium (Ishikawa et al., 2004).

The expression of ObR protein has also been demonstrated in cultured breast cancer cells with Ab against the common domain of ObR (Garofalo et al., 2004), or a specific ObRl region (Dieudonne et al., 2002; Hu et al., 2002; Laud et al., 2002; Garofalo et al., 2004; Yin et al., 2004). Interestingly, we observed that ERα-positive breast cancer cells MCF-7 and T47D express higher levels of ObRl than ERα-negative cell lines MDA-MB-231 and MDA-MB-435 (Garofalo et al., 2004). RT-PCR methods confirmed that human breast cancer cells express detectable levels of ObR mRNA, including ObRl-encoding RNA (Dieudonne et al., 2002).

In ERα-positive breast cancer cell lines, T47D, MCF-7, and ZR75-1, leptin stimulates DNA synthesis and cell growth acting through multiple signaling cascades, including the JAK/STAT, ERK1/2, and PKC-α pathways (Dieudonne et al., 2002; Hu et al., 2002; Laud et al., 2002; Okumura et al., 2002; Catalano et al., 2003; Somasundar et al., 2003; Garofalo et al., 2004; Yin et al., 2004). In addition to the classic cytokine signals, leptin is able to induce the Akt/GSK3 survival pathway in breast cancer cells (Garofalo et al., 2004). Interestingly, the stimulation of Akt/GSK3 occurs several hours after leptin addition, suggesting the involvement of intermediate signaling mechanisms (Garofalo et al., 2004).

Leptin-induced cell cycle progression is accompanied by upregulation of cdk2 and cyclin D1 levels (Okumura et al., 2002) and hyperphosphorylation/inactivation of the cell cycle inhibitor, pRb (Garofalo et al., 2004). Noteworthy, in ERα-positive T47D breast cancer cells, leptin induced not only cell growth but also cellular transformation (anchorage-independent growth). This activity of leptin was not observed in normal breast epithelial cells (Hu et al., 2002).

Of particular interest is the link between leptin activity and ERα. Recent reports demonstrated that ERα and ObR are coexpressed in malignant mammary tissue and breast cancer cell lines (Dieudonne et al., 2002; Hu et al., 2002; Laud et al., 2002). Notably, mitogenic effects of leptin and leptin-dependent activation of STAT3 require SRC-1, a member of the p160 family of steroid receptor modulators (Yin et al., 2004), which might represent crosstalk between steroid receptor- and leptin-induced transcriptional mechanisms. Furthermore, leptin has been found to modulate both estrogen synthesis and ERα activity. For instance, leptin can upregulate the aromatase gene expression and aromatase activity in MCF-7 cells, possibly leading to increased estrogen synthesis (Catalano et al., 2003). We reported that leptin is able to interfere with the action of antiestrogens via post-transcriptional modulation of ERα. Specifically, in MCF-7 cells treated with the pure antiestrogen ICI 182, 780, leptin markedly decreased ERα ubiquitination, increasing its half-life.

These effects coincided with elevated nuclear ERα expression, increased ERα recruitment to estradiol-sensitive promoters, and increased estrogen response element-dependent transcription (Garofalo et al., 2004). All these observations suggest that high levels of leptin in obese breast cancer patients might contribute to tumor growth and the development of antiestrogen resistance.

Colon cancer

There is accumulating evidence that leptin signaling might be involved in colon cancer. The presence of ObRs and ObRl mRNA has been documented using RT-PCR in colon cancer cell lines, human colon tumors, polyps, and adjacent mucosa (Attoub et al., 2000; Hardwick et al., 2001; Rouet-Benzineb et al., 2004). In support of these data, the expression of ObR in colon cancer tissues and cell lines has been confirmed by immunodetection with Abs against the intracellular domain of ObR (Hardwick et al., 2001).

Several in vitro experiments demonstrated mitogenic activity of leptin in colonic epithelial cells. In this model, leptin can induce proliferation through the activation of the NF-kappaB and ERK1/2-dependent pathways as well as upregulate *c-fos* expression (Hardwick et al., 2001; Liu et al., 2001; Rouet-Benzineb et al., 2004). Furthermore, leptin-induced NF-kappaB is able to decrease apoptotic effects of butyrate in colon cancer HT-29 cells (Rouet-Benzineb et al., 2004). Moreover, leptin can activate the invasiveness of PC/AA/C1 early stage colon cancer cells through PI-3K, Rho-, and Rac-dependent pathways (Attoub et al., 2000).

Prostate cancer

There is some evidence suggesting that leptin might play a role in the development of prostate cancer. The presence of ObR has been detected in normal prostate epithelia (Cioffi et al., 1996) and in benign and malignant prostate epithelial cells stained with an Ab recognizing a common domain of the receptor (Stattin et al., 2001). In agreement with this, both ObRs and ObRl mRNAs have been found in malignant prostate cells with RT-PCR-based methods (Stattin et al., 2001; Onuma et al., 2003; Somasundar et al., 2004). Recently, Somasundar et al. (2004) reported that leptin can increase growth and survival of prostate cancer cells through either the PI3K/Akt or ERK1/2 pathways, depending on cell type. In addition, leptin also induced a proliferative response, via JNK activation, in androgen-independent PC-3 and DU145 human prostate cancer cells, but not in androgen-dependent, ObRl expressing LNCaP-FGC cells (Onuma et al., 2003).

In addition to the mitogenic effects, leptin has been found to act as a motility factor well as an inducer of prometastatic factors VEGF, transforming growth factor TGF-β1 and FGF-β in prostate cancer cells in vitro (Frankenberry et al., 2004).

Ovarian and endometrial cancer

Epithelial ovarian cancer (EOC) is a leading cause of death from gynecological malignancies. The involvement of leptin in EOC is not clear. One study (Choi et al., 2004) found both ObRs and ObRl mRNAs in immortalized ovarian surface epithelium cell lines and in BG-1, OVCAR-3, and SKOV-3 ovarian cancer cell lines. Despite this ubiquitous expression of ObR, only BG-1 cells responded to leptin with proliferation via the ERK1/2 MAPK pathway (Choi et al., 2004).

Like in ovarian cancer, the involvement of leptin in endometrial carcinogenesis still needs further investigation. In a study on 70 cases, Yuan et al. (2004) demonstrated by RT-PCR and immunoblotting that ObRs and ObRl are expressed in both cancer and non-cancer endometrium. The abundance of ObRl was similar in cancer and normal tissues, but the levels of ObRs were significantly decreased in malignant cells. Moreover, forced expression of ObRs in the endometrial cancer cells RL95-2 suppressed cell proliferation (Yuan et al., 2004), suggesting that loss of ObRs in endometrial cancer might contribute to malignant progression.

Pancreatic cancer

Several authors proposed a link between obesity and pancreatic beta cell cancer, but the data on the role of leptin in this disease are controversial, and only a few studies have been completed using human cell models. Okuya et al. (2001) demonstrated that leptin can suppress apoptosis and stimulate the proliferation of rat insulin-secreting tumor cell lines. An induction of *c-fos* expression and proliferative response was also observed in RINm5F rat-insulinoma-derived cell line (Islam et al., 1997). Similarly, in BRIN-BD11 rat insulinoma cell line, leptin stimulated STAT3 and STAT5b phosphorylation (Briscoe et al., 2001). However, Somasundar et al. (2003) reported that leptin reduced cell growth of Mia-PaCa and PANC-1 human pancreatic cancer cells, suggesting that leptin action might be dependent on cell context.

The evaluation of ObR expression in human cell lines and tissues has not been attempted. ObRs and ObRl mRNAs were detected in rat insulinoma RIN m5F cells, mouse insulinoma cells BetaCT6 (Islam et al., 1997; Kulkarni et al., 1997), and BRIN-BD11 cells (fusion between rat beta-cells and rat insulinoma cells RINm5F cells) (Briscoe et al., 2001).

Lung cancer

The involvement of leptin in lung cancer is unknown. Preliminary reports demonstrated the existence of ObRl in human lung tissue and SQ-5 cells derived from human lung squamous cell carcinoma. In this cell line, leptin enhanced cell growth via the ERK1/2 pathway (Tsuchiya et al., 1999).

Adrenal cancer

The data on leptin and adrenal cancer are limited. Immunohistochemical analysis demonstrated a strong expression of ObRl in both benign and malignant adrenal tumors, and a weak ObRl staining in pheochromocytomas (Glasow et al., 1999). Although leptin is known to inhibit adrenal steroidogenesis (Malendowicz et al., 1997; Pralong et al., 1998), it apparently does not affect the growth of human adrenal cells and human adrenocortical carcinoma cell line, NCI-H295 (Glasow et al., 1999).

Pituitary tumors

Preliminary studies of Jin et al. (1999) suggested that leptin, ObRl, and ObRs are commonly expressed in normal and neoplastic anterior pituitary cells. Interestingly, leptin immunoreactivity in adenoma was decreased compared with its abundance in normal pituitaries. In addition, leptin was found to inhibit proliferation of the human HP75 and in the rat pituitary GH3 cell lines, but stimulate pancreastatin secretion in vitro. Thus, in this system, leptin might play a role in the growth and differentiation of anterior pituitary cells, but not in tumorigenesis (Jin et al., 1999).

Leukemia

In addition to solid tumors, ObRl and ObRs mRNAs have been detected in several myeloid and lymphoid leukemic cell lines (Bennett et al., 1996; Cioffi et al., 1996; Nakao et al., 1998). Leptin has been found to stimulate the proliferation of human myeloid leukemia cell lines OCI/AML2 and MO7E, although the induction of postreceptor signaling in these cells did not reflect the levels of ObRl or ObRs (Konopleva et al., 1999). Noteworthy, Konopleva et al. (1999) reported that freshly prepared leukemic cells from some acute myeloid leukemia patients exhibited a proliferative response to leptin alone, and that combinations of leptin with other hematopoietic cytokines (IL-3, G-CSF, and SCF) induced an additive or synergistic mitogenic response in 7 of 14 acute myeloid leukemia cases.

OBESITY, SERUM LEPTIN LEVELS, AND CANCER

The major reports on the association between circulating leptin levels and cancer are summarized in Table 2.

Breast cancer

Numerous studies have established that obesity is a risk factor for breast cancer development in postmenopausal women (Cleary and Maihle, 1997; Chlebowski et al., 2002; Rose et al., 2002; Stephenson and Rose, 2003). Notably, a large body of evidence suggests that postmenopausal obesity, assessed by BMI, is associated with the increased risk of developing hormone-dependent breast cancer (i.e., ER and progesterone receptor (PR)-positive tumors), but not with the incidence of ER/PR-negative tumors (Potter et al., 1995; Enger et al., 2000; Huang et al., 2000). In support of this notion, increased upper body obesity, defined by waist-to-hip ratio (WHR) above 0.8, has been found to correlate with poor prognosis, but only in postmenopausal women with ER-positive tumors (Borugian et al., 2003). Consistent with these observations, obesity increased tumorigenic potential of ERα-positive, but not of ERα-negative, breast cancer xenografts grown in MMTV-neu animal models (Cleary et al., 2004). The mechanism of obesity-induced hormone-dependent breast cancer is not known, but the possible contributing factors are the increased exposure of mammary epithelial cells to estrogens locally produced by adipose tissue and down-regulation of sex hormone-binding globulin occurring in women with high WHR (Soler et al., 1989).

The impact of excess body weight on breast cancer in premenopausal women is still unclear. Several studies have suggested that in the premenopausal population, obesity assessed by BMI might protect against breast cancer development, although the physiological mechanism of this protection remains speculative (London et al., 1989; Vatten and Kvinnsland, 1992; Huang et al., 1997). On the other hand, some new data indicated that excess upper body obesity increases breast cancer risk even in premenopausal women (Harvie et al., 2003). Moreover, high BMI is significantly associated with an increased risk of inflammatory breast cancer, the most lethal form of breast cancer in both premenopausal and postmenopausal populations (Chang et al., 1998).

Despite the unquestionable link between obesity and certain forms of breast cancer and the large body of in vitro data suggesting the role of leptin in breast cancer development, the association between circulating leptin and breast cancer or breast cancer risk is still unclear. Some reports found that serum leptin was associated with breast cancer regardless of the menopausal status (Tessitore et al., 2000; Han et al., 2005), while other data suggested a negative correlation between leptin and breast cancer in the premenopausal, but not postmenopausal, group (Petridou et al., 2000). Furthermore, several authors described that circulating leptin is not significantly associated with breast cancer or breast cancer risk in pre- and postmenopausal women (Mantzoros et al., 1999; Sauter et al., 2004; Stattin et al., 2004).

The inconsistent data obtained with circulating leptin as a breast cancer marker could be, at least in part,

TABLE 2. Association between serum leptin levels and cancer in vivo

Type of cancer	Association of serum leptin levels with other markers	References
Breast cancer	Positive association with elevated values of ER and PR in patients with breast cancer	Tessitore et al. (2000)
	Positive correlation with breast cancer risk but not with menopausal status	Han et al. (2005)
	Lack of association with the risk of premenopausal breast cancer in situ. Lack of association with cancer risk in pre- and postmenopausal women	Mantzoros et al. (1999); Sauter et al. (2004)
	Negative association with breast cancer risk in pre- and postmenopausal women	Petridou et al. (2000); Stattin et al. (2004)
	High levels associated with the use of tamoxifene, toremifene, and raloxifene	Marttunen et al. (2000); Ozet et al. (2001); Eng-Wong et al. (2003)
Colorectal cancer	Positive association with cancer risk marker in men, but not in women	Stattin et al. (2003b)
	No association with cancer	Tessitore et al. (2000)
	Decreased levels associated with cancer	Arpaci et al. (2002)
Prostate cancer	Positive association with the risk of cancer development	Stattin et al. (2001); Saglam et al. (2003)
	Positive association with larger tumors	Chang et al. (2001); Saglam et al. (2003)
	Increased risk of cancer in association with markers related to abdominal obesity	Hsing et al. (2001)
	No association with prostate cancer	Lagiou et al. (1998); Stattin et al. (2003a)
Pancreatic cancer	Increased levels in patients with insulinoma. Normalization of levels after surgical removal of insulinoma	Popovic et al. (1998)
	High levels not associated with cancer-induced cachexia	Brown et al. (2001)
Lung cancer	No association with the development of cancer-associated cachexia	Simons et al. (1997)
	No association with anorexia and cachexia in non-small cell lung cancer	Aleman et al. (2002)
Endometrial cancer	Positive association with cancer	Petridou et al. (2002); Yuan et al. (2004)

explained by differences in detection techniques, or by differences in sample preparation where some influencing factors, such as food intake and circadian rhythm, were not controlled. Clearly, better-controlled studies are needed to unequivocally establish whether serum leptin is associated with breast cancer etiology. Moreover, it is possible that breast carcinogenesis is induced by overabundance of local rather then systemic leptin. This concept could be addressed by direct examination of the leptin system in breast tumors. For instance, one recent study documented overexpression of leptin and ObR in breast cancer tissues versus non-cancer breast epithelium. In addition, there was an increased incidence of hematogenous metastasis or cancer recurrence in distant organs in patients with increased ObRl expression in primary breast tumors, while patients with ObR-negative and low leptin-expressing tumors displayed good outcome (Ishikawa et al., 2004).

Colorectal cancer

Obesity has been consistently associated with higher risk of colorectal cancer in men (relative risks of approximately 1.5–2.0) and women (relative risks of approximately 1.2–1.5), in both case control and cohort studies (Calle and Thun, 2004). Similar relationships have been noticed for colon adenomas, with stronger association for larger adenomas (Giovannucci et al., 1996). The link between colorectal cancer and obesity is more significant in men than in women (Terry et al., 2001, 2002), but the possible reasons for this gender difference remain speculative. One hypothesis is that central adiposity (characteristic for men) is a stronger predictor of colon cancer risk than peripheral adiposity or general overweight. In fact, waist circumference and WHR are related strongly to risk of colorectal cancer and large adenomas in men (Giovannucci et al., 1995). However, the association between WHR and colorectal cancer in women was not stronger than the association between BMI and colorectal cancer in several studies that examined both parameters, implying that body fat distribution alone cannot account for the gender difference (Caan et al., 1998; Giacosa et al., 1999). Another possible explanation is that there may be an offsetting beneficial effect of obesity on colorectal cancer risk in women. Substantial evidence supports the protective role of exogenous estrogens (in the form of postmenopausal hormone therapy) on the risk of colorectal cancer in women (Calle et al., 1995; Rossouw et al., 2002). Thus, high levels of circulating estrogens associated with postmenopausal obesity in women may diminish the obesity-associated risk of colorectal cancer.

The studies on circulating leptin levels and colorectal cancer are not conclusive. For instance, Stattin et al. (2003b) observed that elevated levels of circulating leptin were associated with about a twofold increase in risk of colorectal cancer in men, but not in women. On the other hand, Tessitore et al. (2000) found no difference between serum leptin levels in cancer patients and controls. Other authors noted that serum leptin levels in patients with colon cancer were significantly decreased (Arpaci et al., 2002). In the same study, leptin levels were lower in colon cancer patients despite lack of weight loss and BMI measurements comparable to that of control subjects.

Circulating leptin is increased by high-fat diets, which are also implicated in stimulating colon cell proliferation (Lin et al., 1998; Bahceci et al., 1999; Baile et al., 2000). It has been reported that the presence of dietary fiber can decrease serum leptin levels and reduce colon carcinogenesis through reduced colonocyte cell proliferation (Alberts et al., 1990; Agus et al., 2000). A positive relationship between dietary fat, serum leptin, and colonic epithelial cell proliferation has also been reported in animal models (Hardwick et al., 2001; Liu et al., 2001). Globally, these preliminary data suggest that high fat diet-related colon carcinogenesis is, at least, in part mediated through a mechanism involving higher systemic leptin levels.

Prostate cancer

Results of studies examining the association between BMI and prostate cancer risk are inconclusive. Although several large studies have found an increased BMI in adulthood to be associated with an increased risk of prostate cancer development, others have shown no such association (Freedland and Aronson, 2005). Recently, Giovannucci et al. (2003) published the analysis of a large cohort of men followed in the Health Professionals Follow-Up Study 1986–2000. In this study, prostate cancer risk was inversely associated with BMI in men younger than 60 years of age, but no association was found in men older than 60 (Giovannucci et al., 2003). The lack of significant impact of obesity on prostate cancer in older men was confirmed by other authors (Porter and Stanford, 2005). Interestingly, the protective effect of obesity was not observed in cases of advanced metastatic prostate cancer, regardless of patient age (Giovannucci et al., 2003).

In contrast, two large prospective studies of the American Cancer Society reported a positive association between obesity and increased risk of dying from prostate cancer (Moyad, 2002; Calle et al., 2003). A similar link has been observed for adolescent obesity and prostate cancer deaths, implicating early life events in prostate cancer development (Kaaks et al., 2000). The reasons for the discrepancies observed in the above studies are not clear and could be related to different methodology of data collection and analysis.

Like with the studies on obesity and prostate cancer, many reports on circulating leptin levels and prostate cancer risk have yielded conflicting results. Several authors noted a positive correlation between serum leptin and prostate cancer risk (Stattin et al., 2001; Saglam et al., 2003). Hsing et al. (2001) found that prostate cancer risk is associated with higher leptin levels and with WHR values above 0.87, which suggested that leptin could interact with humoral factors related to abdominal obesity, such as sex hormones, insulin and IGF-1, to increase the risk of prostate cancer. Interestingly, two studies that examined serum leptin in men with prostate cancer reported that higher leptin levels were associated with more advanced tumors, characterized by larger size and higher grade (Chang et al., 2001; Saglam et al., 2003). However, several other studies concluded that circulating leptin is not associated with prostate cancer (Lagiou et al., 1998; Hsing et al., 2001; Stattin et al., 2003a).

Endometrial cancer

There is convincing and consistent evidence from both case-control and cohort studies that obesity is strongly related to endometrial cancer (Calle and Thun, 2004). In fact, a linear increase in the risk of endometrial cancer with increasing weight or BMI has been observed in most of the relevant studies (Calle et al., 2003; Soliman et al., 2005). It is thought that the key factor involved in endometrial cancer etiology in obese women is excess available estrogen, either insufficiently balanced by

progesterone in premenopausal women or synthesized by adipose tissue in postmenopausal women. Furthermore, there is evidence that chronic hyperinsulinemia that is associated with obesity might increase the risk of endometrial cancer (Kaaks et al., 2002).

Like with other obesity-related cancers, the link between circulating leptin and endometrial cancer is unclear. Limited studies by Petridou et al. (2002) indicated that serum leptin levels were positively associated with endometrial cancer. Similarly, Yuan et al. (2004) reported that circulating leptin was significantly higher in endometrial cancer patients than in normal controls, however this association was not observed after BMI normalization.

Ovarian cancer

Numerous epidemiological studies have examined the association between BMI and ovarian cancer. The extensive review of Purdie et al. (2001) concludes that there is a weak association between increased BMI and ovarian cancer based on the results of cohort and population based case-control studies. In addition, the link between ovarian cancer risk and higher WHR has been reported by the Iowa Women's Health Study Cohort (Mink et al., 1996) and an Italian multicenter case-control study (Dal Maso et al., 2002). Furthermore, higher BMI in young adulthood has been found associated with an increased risk of premenopausal ovarian cancer (Fairfield et al., 2002), especially for serous borderline tumors (Kuper et al., 2002). No large studies addressed the correlation between circulating leptin and ovarian cancer.

Pancreatic cancer

Several recent studies have suggested that obesity may be associated with increased risk for pancreatic cancer development in man and women (Moller et al., 1994; Silverman et al., 1998; Michaud et al., 2001; Calle et al., 2003). However, other studies found no such association (Stolzenberg-Solomon et al., 2002; Lee et al., 2003), or reported an association in men but not in women (Gapstur et al., 2000).

Serum leptin concentrations were significantly elevated in patients with insulinomas producing chronically high insulin levels. On the other hand, leptin levels return to normal, after surgical treatment and normalization of insulin values (Popovic et al., 1998). In weight-losing patients with pancreatic cancer, low leptin concentration is associated with increased insulin resistance (Barber et al., 2004). However, high plasma leptin levels do not appear to contribute to cachexia in these patients (Brown et al., 2001).

Lung cancer

BMI has been reported to be inversely associated with lung cancer in several study populations that did not exclude smokers from the analysis (Calle and Thun, 2004). This negative correlation can be explained by the confounding effects of smoking, which is inversely associated with BMI (Henley et al., 2002). No association between BMI and lung cancer was observed in non-smoking populations (Calle et al., 2003).

The impact of circulating leptin on lung cancer progression has not been studied. Studies that addressed the role of circulating leptin in the development of anorexia and cachexia in lung cancer patients concluded that leptin is not a primary regulator of these processes (Simons et al., 1997).

SUMMARY

Epidemiological data suggest that obesity is associated with increased risk of certain types of cancer. In humans, high BMI is directly associated with elevated levels of the obesity hormone, leptin. Leptin, in addition to its neuroendocrine function, can act as a mitogen and an angiogenic factor. Consequently, several recent studies addressed the possible role of leptin in cancer development and progression. The possibility that the hormone might activate cell growth, transformation, or drug resistance has been assessed with different cellular and animal cancer models. The resulting data indicate that many types of cancer cells can respond to leptin as a mitogen/survival factor. To date, the best evidence that the hormone can indeed be involved in neoplastic processes has been provided by studies on breast and colorectal cancer models, while the results for other cancer types are very limited and often inconsistent or inconclusive. In any case, more research is needed to examine molecular mechanisms of leptin involvement in breast and colorectal cancers and to prove or exclude its role in other types of neoplasms.

Epidemiological studies measuring cancer risk in relation to obesity (assessed by BMI or WHR) confirmed that excess body fat can increase the risk of developing postmenopausal breast cancer and endometrial cancer. There is also supporting evidence for the association between obesity and colorectal cancer. Unfortunately, the attempts to correlate serum leptin abundance with cancer incidence or progression were not conclusive, regardless of the disease studied. This perhaps was related to differences in sample preparation and measurement techniques as well as the lack of control for other factors that influence leptin expression, such as food intake.

Taking into consideration data obtained with cultured cells and tumor specimens, one cannot exclude that local, not systemic, leptin concentrations are critical for tumor progression. Leptin abundance in tumor environment can be regulated by surrounding adipose tissue. In addition, tumor cells themselves, as shown in breast cancer specimens, can produce the hormone. This paracrine/autocrine leptin axis could become a target for leptin-inhibiting drugs, which might prove effective in cancer treatment and prevention.

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Functional Significance of Type 1 Insulin-like Growth Factor-mediated Nuclear Translocation of the Insulin Receptor Substrate-1 and β-Catenin*

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Jia Chen‡, An Wu‡, Hongzhi Sun‡, Robert Drakas‡, Cecilia Garofalo§, Sandra Cascio§, Eva Surmacz§, and Renato Baserga‡¶

From the ‡Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 and the §Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, Temple University, Philadelphia, Pennsylvania 19122

Previous work has shown that the transcriptional regulator β-catenin can translocate to the nuclei when cells are stimulated with the type 1 insulin-like growth factor (IGF-1). We show by immunocoprecipitation and by confocal microscopy that β-catenin binds to and co-localizes with the insulin receptor substrate-1 (IRS-1), a docking protein for both the insulin and the IGF-1 receptors. IRS-1 is required for IGF-1-mediated nuclear translocation of β-catenin, resulting in the activation of the β-catenin target genes. IGF-1-mediated nuclear translocation of β-catenin is facilitated by the nuclear translocation of IRS-1. Both IRS-1 and β-catenin are recruited to the cyclin D1 promoter, an established target for β-catenin, but only IRS-1 is recruited to the ribosomal DNA (rDNA) promoter. UBF proteins (known to interact with both IRS-1 and β-catenin) are also detectable in the cyclin D1 and rDNA promoters. These results indicate that IRS-1 (activated by the IGF-1 receptor) is one of several proteins that regulate the subcellular localization and activity of β-catenin. The ability of IRS-1 to localize to both RNA polymerase II (with β-catenin) and RNA polymerase I-regulated promoters suggest an explanation for the effect of IRS-1 on both cell growth in size and cell proliferation. This possibility is supported by the demonstration that enforced nuclear localization of IRS-1 causes nuclear translocation of β-catenin and transformation of normal mouse embryo fibroblasts (colony formation in soft agar).

The important roles played by β-catenin in adhesion, cancer, and development and its connections to Wnt and APC have been discussed in recent reviews (1–3). Briefly, there is usually a large pool of β-catenin in the cytoplasm, where it is targeted for destruction by phosphorylation of the N terminus (2, 4). Under certain circumstances, for instance Wnt signaling, β-catenin is stabilized and transferred to the nuclei where it binds members of the family of T-cell factor/lymphoid enhancer factors (Tcf/Lef) and activates transcription of target genes (5, 6). Among genes regulated by β-catenin are c-*myc* (7) and cyclin D1 (8, 9), which encode critical cell-cycle progression proteins (a list of target genes can be found at www.stanford.edu/~rnusse/pathways/targets.html). Recently, we found by our modified TAPtag technique that the insulin receptor substrate-1 (IRS-1)[1] interacts in the nuclei with β-catenin (10). IRS-1, a docking protein for both the IGF-1 and insulin receptors, sends a strong mitogenic, anti-apoptotic, and anti-differentiation signal (11, 12). Overexpression or ectopic expression of IRS-1 can cause cell transformation, including the ability of cells to form colonies in soft agar and tumors in mice (13). Under certain circumstances, IRS-1 translocates to the nuclei (14–16) where it interacts with nuclear proteins, including viral oncoproteins (14, 17), the upstream binding factor 1 (UBF1) (15, 16), and the estrogen receptor (18).

IGFs are known to cause translocation of β-catenin to the nuclei, where it activates the target genes (5, 19–21). We have previously reported that IGF-1 stimulates the expression of both c-*myc* and cyclin D1 (22, 23), two targets of β-catenin. The exact molecular mechanism of this effect has not been fully explored. Our recent discovery of a direct binding between IRS-1 and β-catenin has prompted us to investigate the mechanism(s) and the functional significance of the interaction, in the context of IGF-1R signaling. Because IRS-1 is known to interact with UBF1 (a regulator of RNA polymerase I activity), whereas β-catenin has been reported to interact with UBF2 in the cyclin D1 promoter (24), we also examined the possibility that IGF-1 stimulation may recruit IRS-1 and β-catenin together or separately to the cyclin D1 and the rDNA promoters.

We report here that β-catenin and IRS-1 co-immunoprecipitate in nucleus and cytosol of mouse embryo fibroblasts (MEFs). IGF-1 promotes β-catenin translocation in R+ cells, where IRS-1 is also nuclear, but not in R12 cells, where IRS-1 is confined to the cytosol. The nuclear translocation of IRS-1 and β-catenin to the nuclei activates the Tc/Lef reporter. We find also that IRS-1 and β-catenin are both recruited to the cyclin D1 promoter with the UBF proteins, as already reported for β-catenin (24). IRS-1, but not β-catenin, is recruited to the rDNA promoter, where it is known to bind UBF1 and stimulate the synthesis of rRNA (16). Using R12 and BT20 mammary cancer cells (25), we show that IRS-1 is required for IGF-1-mediated nuclear translocation of β-catenin. The role of IRS-1 in the nuclear translocation of β-catenin has been confirmed by

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¶ To whom correspondence should be addressed: Kimmel Cancer Center, Thomas Jefferson University, 233 S. 10th St., 624 Bluemle Life Sciences Bldg., Philadelphia, PA 19107. Tel.: 215-503-4507; Fax: 215-923-0249; E-mail: B_lupo@mail.jci.tju.edu.

[1] The abbreviations used are: IRS-1, insulin receptor substrate-1; UBF1, upstream binding factor 1; IGF-1, insulin-like growth factor, type 1; IGF-1R, IGF-1 receptor; rDNA, ribosomal DNA; MEF, mouse embryo fibroblast; NLS, nuclear localization signal; CMV, cytomegalovirus; IP, immunoprecipitation; GST, glutathione *S*-transferase; ChIP, chromosomal immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SFM, serum-free media; PI, propidium iodide.

using a plasmid in which IRS-1 is expressed in fusion to a Nuclear Localization Signal (NLS). Stable expression of this plasmid in growth-regulated, contact-inhibited mouse fibroblast R12 cells (where both IRS-1 and β-catenin are normally cytoplasmic) causes both proteins to co-localize to the nuclei and induces the transformation of R12 cells into cells capable of forming colonies in soft agar (the best criteria for *in vitro* transformation). Although β-catenin can be translocated to the nuclei by different stimuli and pathways (see above), independently of IGF-1R signaling, these results indicate that IRS-1 can be considered one of the proteins that regulate the subcellular localization and activity of β-catenin, especially in cells responsive to the mitogenic action of IGF-1.

MATERIALS AND METHODS

Cells and Cell Cultures—R− cells and R−-derived cells are 3T3-like cells originating from mouse embryos with a targeted disruption of the IGF-1R genes (26). They were described in previous reports (22, 27, 28) and are briefly described again under "Results." Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum plus L-glutamine plus the appropriate antibiotics. For stimulation with IGF-1 (20 ng/ml), cells were starved in serum-free medium for 24–48 h before addition of IGF-1.

Plasmids—The UBF1 plasmid with a FLAG tag has been described by Maiorana *et al.* (29). The UBF2 with a FLAG tag was constructed in the same manner. The mutant plasmids of β-catenin (∂C and ∂N), a kind gift of Dr. Kamel Khalili (Temple University, Philadelphia, PA), are described in the report by Gan and Khalili (30). The plasmid with a nuclear localization signal was the pCMV/myc/nuc plasmid (Invitrogen). IRS-1 was cloned in the XhoI/NotI site.

Immunoprecipitation and Western Blots—Western blots and immunoprecipitations (IPs) were carried out according to standard procedures, described in detail in previous reports from this laboratory (15, 16). Unless otherwise indicated, 20 μg of cytoplasmic or nuclear fractions was separated on a 4–14% gradient gel (Bio-Rad) and transferred to a nitrocellulose membrane. For immunoprecipitation, 100–200 μg of proteins was used, depending on the protein to be precipitated.

Subcellular Fractionation—Cell lysates and subcellular fractionation have been described in detail in Wu *et al.* (31). The purity of the subcellular fractions was routinely monitored with appropriate antibodies to either nuclear or cytoplasmic proteins. In this latter case, the Western blot was done directly on the nuclear or cytoplasmic lysates, without immunoprecipitation.

Confocal Microscopy—Confocal microscopy studies followed the same procedures described in detail in previous reports from our laboratory (16, 17, 29). The antibodies used are indicated in the appropriate figures.

GST Pull-down Assays—GST fusion proteins were constructed using PCR products corresponding to different regions of IRS-1 coding for amino acids 1–300, 301–700, 701–1000, and 1001–1234. These regions were generated using specific oligonucleotide primers containing the appropriate restriction sites (all of the start primers contain XhoI restriction site and end primers contain EcoRI restriction sites in the overhangs). Purified PCR products were then digested with XhoI and EcoRI and ligated into XhoI/EcoRI cloning sites of pGEX-5X-1 vector (Amersham Biosciences). All plasmids constructs were confirmed by DNA sequencing and protein expression to guarantee accuracy and amounts of GST proteins in each reaction. The detailed cloning strategies are available upon request. Binding and elution of proteins were carried out by standard procedures.

TOPFLASH Assay—The activity of β-catenin was measured using the TOPFLASH/FLOPFLASH luciferase assay (32). The plasmids used were the same as those reported by Korinek *et al.* (25). The activity is usually determined after transient expression. We followed the procedure given in detail by Playford *et al.* (20). We used for transient expression the Nucleofector (Amaxa Biosystem at www.amaxa.com), with which we have been obtaining high levels of transfection (70% with difficult cells like 32D cells, even higher in MEFs). Both R+ and other R− and R−-derived cells were used for these experiments.

ChIP Assays—Chromatin immunoprecipitation (ChIP) assays were carried out by standard methods (33). Subconfluent cultures were made quiescent and then stimulated with IGF-1 (see "Materials and Methods"). Following treatment, the cells were cross-linked with 1% formaldehyde at 37 °C for 10 min. The cells were collected and resuspended in 200 μl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) and left on ice for 10 min. They were sonicated 4× for 10 s at 30% of maximal power (Fisher Sonic Dismembrator) and collected by centrifugation at 4 °C for 10 min at 14,000 rpm. The supernatants were collected and diluted in 1.3 ml of IP buffer (0.01% SDS, 1.1% Triton, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 16.7 mM NaCl) followed by immunoclearing with 80 μl of sonicated salmon sperm DNA/protein A-agarose (Upstate Biotechnology Inc.) for 1 h at 41 °C. The pre-cleared chromatin was immunoprecipitated for 12 h with specific antibodies (see below). After IP, 60 μl of salmon sperm/protein A-agarose was added and precipitation continued for 2 h at 41 °C. After pelletting, precipitates were washed sequentially for 5 min with the following buffers: wash A (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris HCl, pH 8.1, 150 mM NaCl), wash B (same as wash A but with 500 mM NaCl), wash C (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and then twice with TE buffer (10 mM Tris, 1 mM EDTA). The immune complexes were eluted with elution buffer (1% SDS, 0.1 M $NaCO_3$). The eluates were reverse cross-linked by heating at 65 °C for 12 h and digested with proteinase K (0.5 μg/ml) for 1 h. DNA was obtained by phenol and phenol/chloroform extraction, precipitated with ethanol at 4 °C for 12 h, and then re-suspended in 20 μl of TE buffer. For PCR, 5 μl of each sample was used with specific primers. For the cyclin D1 promoter, chromatin was immunoprecipitated first with an antibody to β-catenin, which served as the positive control, because β-catenin is known to bind to the cyclin D1 promoter. Enrichment was detected with the primers for the Tcf sequence of the cyclin D1 promoter. The primers were the following: left, cggactacaggggagttttgttg; right, tccagcatccaggtggcgacgat (34). For ChIP assays with the rDNA promoter, we used the methodology of James and Zanerdijk (35) but different primers, because we were dealing with a mouse rDNA promoter. The primers we used were as follows: forward P1, 5′-CCC TGT ACG TCT GAG GCC GAG-3′ (−250); rDNA promoter reverse P2, 5′-GTT AAT AGG GAA AGG ACA GCG TG-3′ (+26). We also used two other primers of the rDNA gene, located in a transcribed spacer (see text). These primers were as follows: left, gtgggtgctgcgcggctgggagt; right, accagtctttctcggtcccgtgcc. For the mouse GAPDH promoter, the primers were forward P1 (5′-AGTGCCAGC-CTCGTCCCGTAGACAAAATG-3) and promoter reverse P2 (5′-AAGT-GGGCCCCGGCCTTCTCCAT-3′). By trial and error, we established that the best number of cycles during the PCR reaction should not be above 29 cycles. At 30–32 cycles, one could get a weak false-positive. All the data presented in this report are based on 29 cycles. The amplification products were analyzed in a 2% agarose gel and visualized by ethidium bromide staining.

Colony Formation in Soft Agar—The methodology previously described was followed (29). Briefly, to compare anchorage-independent growth of different cell lines, cells were plated at 2×10^3 in essential modified Eagle's medium containing 10% fetal bovine serum (plus or minus IGF-1) and 0.2% agarose (with 0.4% agarose underlay). The number of colonies larger than 125 μm in diameter was determined at 3 weeks following plating.

Antibodies—The antibodies used were the following: β-catenin antibody (catalog no. MAB13291, R&D Systems Inc, Minneapolis, MN); IRS-1 rabbit polyclonal IgG (catalog no. G3003, Santa Cruz Biotechnology, Santa Cruz, CA); UBF, mouse monoclonal IgG1 (catalog no. SC-13125 Santa Cruz Biotechnology); anti-flag.M2-peroxidase conjugate (catalog no. a8592, Sigma); the phospho-β-catenin (Ser-33/47/Thr-41) antibody (Cell Signaling, www.cellsignal.com); anti-Grb2 monoclonal antibody (catalog no. 610111BD, BD Transduction Laboratory); c-Jun Sc4, rabbit polyclonal IgG (catalog no. F9, Santa Cruz Biotechnology); second antibody anti-IgG, mouse (SC-45, Oncogene, San Diego, CA); Second Antibody Peroxidase-conjugated AffiniPure Rabbit-mouse IgG (Jackson ImmunoResearch); goat anti-mouse IgG2a-FITC,(SC2079, Santa Cruz Biotechnology); and donkey anti-rabbit IgG-R (SC-2095, Santa Cruz Biotechnology).

RESULTS

IRS-1 and β-Catenin Co-immunoprecipitate in the Nuclei and Cytoplasm of R−-derived Cells—Our first step was to confirm by co-immunoprecipitation the IRS-1-/β-catenin interaction we originally detected by our modified TAPtag technique (10). We used cell lines derived from R− cells, the original MEFs obtained from mouse embryos with a targeted disruption of the IGF-1R genes (26). R12 cells are derived from R− cells and express 7×10^3 IGF-R/cell (27), whereas R+ cells are R− cells stably transfected with a cDNA plasmid expressing the human IGF-R at high levels (28). R12 cells are not transformed, do not grow in serum-free medium (SFM), and do not respond to IGF-1 (22, 27). However, IGF-1 induces in R12 cells tyrosine

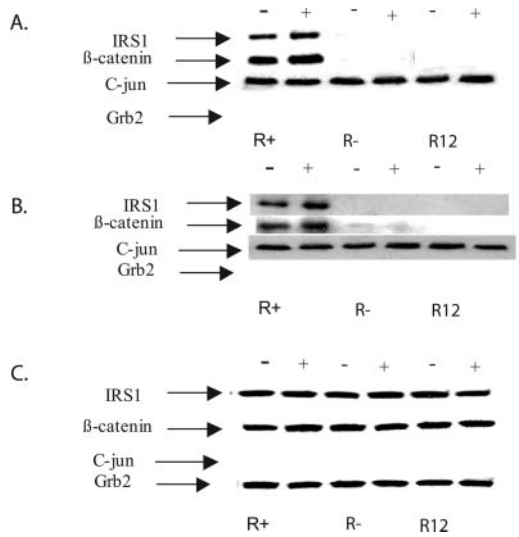

FIG. 1. **Co-immunoprecipitation of IRS-1 with β-catenin in subcellular fractions of R− and R+ cells.** Immunoprecipitations and Western blots were carried out as described under "Materials and Methods," where subcellular fractionation is also described. The Western blots with c-Jun and Grb2 antibodies were done on lysates from fractions, without previous immunoprecipitation. In *panel A*, nuclear extracts were immunoprecipitated with an antibody to β-catenin, Western blot with antibodies to IRS-1and β-catenin (c-Jun, a nuclear protein, and Grb2, a cytosol protein, were used to monitor the purity of the fractions). IRS-1 is nuclear only in R+ cells and is detectable when an antibody to β-catenin is used for immunoprecipitation. In *panel B*, nuclear extracts were immunoprecipitated with an antibody to IRS-1, and probed with antibodies to IRS-1and β-catenin (control proteins as usual). Only in R+ cells do β-catenin and IRS-1 interact in the nuclei. In *panel C*, cytoplasmic fractions were immunoprecipitated with an antibody to β-catenin, and probed with antibodies to β-catenin and IRS-1. β-Catenin and IRS-1 interact also in the cytoplasm. The reverse experiment (immunoprecipitation with an antibody to IRS-1 on cytoplasmic extracts) was also done with the same results (not shown). +, cells stimulated with IGF-1; −, cells left in serum-free medium.

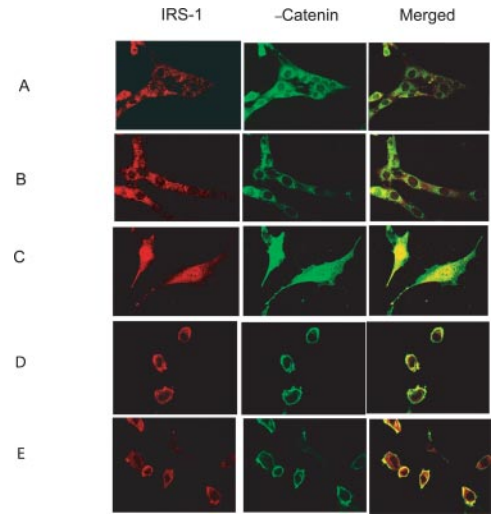

FIG. 2. **Co-localization of IRS-1 and β-catenin in mouse embryo fibroblasts by confocal microscopy.** The cells were stained with antibodies to IRS-1 (*red*) or to β-catenin (*green*), and the pictures were merged in the *third panel*. *A*, R− cells stimulated for 16 h with IGF-1; *B*, unstimulated R+ cells; *C*, R+ cells 24 h after stimulation with IGF-1. IGF-1 stimulation causes both IRS-1 and β-catenin to move significantly to the nuclei. *D*, R12 cells in serum-free medium and (*E*) 24 h after IGF-1 (20 ng/ml). Both IRS-1 and β-catenin are essentially cytoplasmic in R− and R12 cells, even after IGF-1 stimulation.

phosphorylation of IRS-1 and an increase in c-*myc* expression (22, 27). R+ cells respond to IGF-1 with cell proliferation and form colonies in soft agar (28). We first determined in whole cell lysates of these cells the levels of expression of IRS-1, β-catenin, UBF1, and Grb2. We chose the latter two proteins because UBF1 interacts with IRS-1 and is an exclusively nuclear/nucleolar protein (36), whereas Grb2 is an exclusively cytoplasmic protein that can be used to monitor protein amounts in each lane of a Western blot. All four proteins are well expressed in both cell lines, regardless of whether the cells are stimulated or not with IGF-1 (data not shown).

We then tested the interaction between IRS-1 and β-catenin in nuclear and cytoplasmic fractions. Fig. 1*A* shows a Western blot from nuclear extracts immunoprecipitated with an antibody to β-catenin, and stained with antibodies to IRS-1 and β-catenin. The purity of the fractions was monitored with antibodies to c-Jun (a nuclear marker) and Grb2 (a cytoplasmic marker), directly on the lysates, without previous immunoprecipitation. There is interaction between the two proteins only in R+ cells, where IRS-1 is nuclear (15, 16). There is some nuclear IRS-1 in unstimulated R+ cells. We reported before (16) that R+ cells secrete some IGF-1, which allows them to grow, albeit slowly, in SFM. The reverse immunoprecipitation experiment (with an antibody to IRS-1) is shown in Fig. 1*B*. Again, both proteins are detectable only in R+ cells. There is no apparent interaction between IRS-1 and β-catenin in R− and R12 cells, where IRS-1 is cytoplasmic (Ref. 16 and see below). However, Fig. 1*C* shows that β-catenin and IRS-1 interact in the cytoplasm of all cell lines. The same result was obtained in the reverse experiment, where cytoplasmic extracts were immunoprecipitated with an antibody to IRS-1 and stained with antibodies to β-catenin and IRS-1 (not shown). These results confirm an interaction between IRS-1 and β-catenin, both in the cytoplasm and in the nuclei of R+ cells. In R− and R12 cells, stimulated with IGF-1, the interaction is limited to the cytoplasm, in agreement with the finding that IRS-1 is not found in the nuclei of these cells.

Confocal Microscopy of R−-derived Cells—To confirm the IRS-1/β-catenin interaction, we studied it by confocal microscopy on R−, R12, and R+ cells. The cells were stained with antibodies to IRS-1 (*red*) and β-catenin (*green*) as shown in Fig. 2, where the merged pictures are also presented. In R− cells (here stimulated with IGF-1, to which they do not respond), both IRS-1 and β-catenin are largely cytoplasmic (*panel A*), although there is a little β-catenin in the nuclei. In R+ cells, before stimulation with IGF-1, most of the IRS-1 is cytoplasmic, and in fact so is most of β-catenin (*panel B*), although the nuclei are partially stained. After stimulation of R+ cells by IGF-1, both IRS-1 and β-catenin are much more localized to the nuclei (*panel C*). We show in *panel C* a representative field for R+ cells after stimulation with IGF-1 for 24 h (*panel C*), but the results were the same at 16 h. In R+ cells, nuclear localization of IRS-1 after IGF-1 stimulation reaches a peak at 16–24 h (16). In R12 cells, unresponsive to mitogenic stimulation by IGF-1, both IRS-1 and β-catenin are cytoplasmic before (*panel D*) or after 24 h stimulation with IGF-1 (*panel E*). The merged pictures clearly show the co-localization of the two proteins.

It could be objected that Fig. 2 does not show a nuclear staining of the cells, to validate the subcellular localization of either IRS-1 or β-catenin. This is shown in Fig. 3, where R12 cells were stained with antibodies to IRS-1 (*upper panels*) or β-catenin (*lower panels*) and counterstained with propidium iodide (PI). Whether in SFM or after IGF-1, in R12 cells, both IRS-1 and β-catenin are essentially localized to the cytoplasm. We repeated the PI experiment with R+ and R− cells (not shown, but see Tu *et al.* (16) and Sun *et al.* (15)).

IRS-1 Is Required for IGF-1-mediated Nuclear Translocation of β-Catenin—The next question is whether the IGF-1-mediated nuclear translocation of β-catenin requires IRS-1. For this purpose, we used BT20 cells, a breast cancer cell line that does

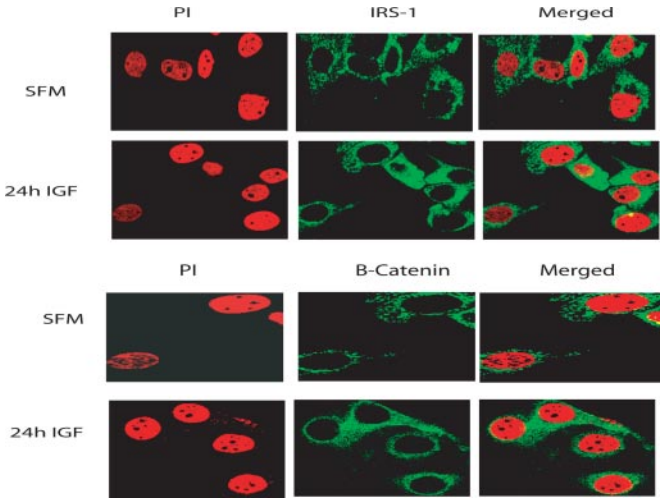

FIG. 3. **Cytoplasmic localization of IRS-1 and β-catenin in R12 cells.** Confocal microscopy of R12 cells stained with antibodies to IRS-1 (*upper panels*) or β-catenin (*lower panels*) and counterstained with PI (*red*). The cells were either in serum-free medium (*SFM*) or 24 h after IGF-1 stimulation. Both IRS-1 and β-catenin are excluded from the nuclei of R12 cells stimulated with IGF-1.

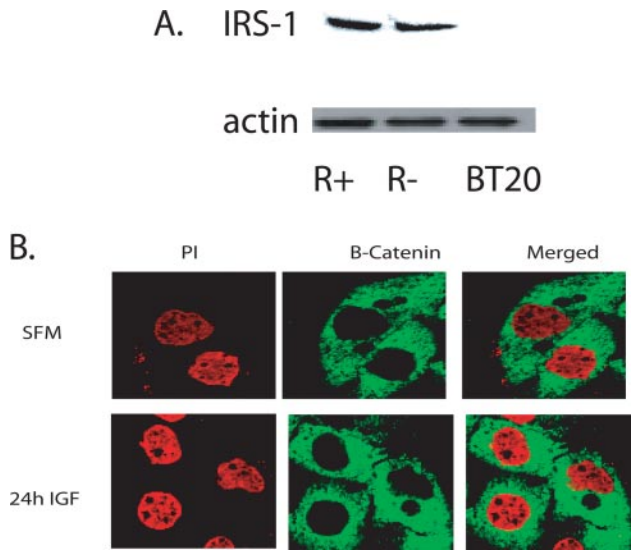

FIG. 4. **IGF-1 stimulation does not promote nuclear translocation of β-catenin in the absence of IRS-1.** BT20 cells are a breast cancer cell line that does not express IRS-1. This is confirmed in *A*, a Western blot of whole cell lysates stained with an antibody to IRS-1 (R− and R+ cells are the positive controls). An actin antibody was used to monitor the amount of protein in each lane. *B*, confocal microscopy of BT20 cells. The cells were stained with an antibody to β-catenin (*green*) and counterstained with PI (*red*). β-Catenin remains cytoplasmic even after the serum starved cells are stimulated with IGF-1 (20 ng/ml).

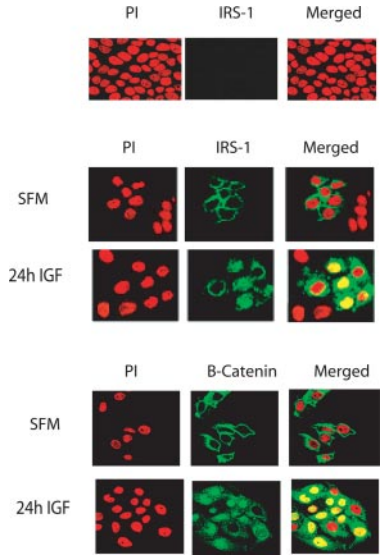

FIG. 5. **IRS-1 restores IGF-1-mediated translocation of β-catenin.** BT20 cells (see Fig. 4) were transfected in transient with a plasmid expressing wild-type IRS-1. Confocal microscopy was carried out on the transfected cells, all counter-stained with PI (*red*). In the *upper panels*, the cells were stained with an antibody to IRS-1; in the *lower panels* cells were stained with an antibody to β-catenin. IRS-1 moves to the nucleus of most, but not all, cells, and the same can be said for β-catenin. An occasional untransfected cell can be seen in the *merged images* of *both panels*.

not express IRS-1 (Castles *et al.* (25)). Fig. 4*B* shows a confocal microscopy of BT20 cells, either in SFM (*upper panels*) or after IGF-1 stimulation. The cells were again stained with an antibody to β-catenin and counterstained with PI. The images clearly show that β-catenin in these cells remains cytoplasmic after stimulation with IGF-1. BT20 cells grow slowly but significantly after IGF-1 stimulation.[2] Fig. 4*A* confirms in Western blots that BT20 cells do not express IRS-1 (R+ and R− cells are the positive controls). To confirm the importance of IRS-1 in IGF-1-mediated translocation of β-catenin, we transfected BT20 cells with a plasmid expressing wild type mouse IRS-1 (13). Cells in SFM or 24 h after stimulation with IGF-1, counterstained with PI, were stained with antibodies to either IRS-1 or β-catenin. Fig. 5 shows the results. IRS-1 is now detectable in transfected cells (in the 24-h *merged panel*, one can see a few untransfected cells that stain only with PI). In some IRS-1-positive cells, IRS-1 is cytoplasmic, which is not unexpected, given the lower than average number of IGF-1Rs in these cells. After IGF-1 stimulation, most of the cells show a nuclear β-catenin. The cells with a cytoplasmic β-catenin could be cells not responding to IGF-1 or untransfected cells.

Taken together, these results indicate that β-catenin can be translocated to the nuclei by IGF-1 stimulation, and that IRS-1 plays a crucial role in IGF-1-mediated translocation. Whether IRS-1 must also be nuclear will be taken up later.

Sequences Required for the Interaction of IRS-1 and β-Catenin—To determine the sequences of IRS-1 that interact with β-catenin, we used GST constructs of IRS-1 (described under "Materials and Methods") and cell lysates from R+ cells. The results of representative experiments are shown in Fig. 6. Fig. 6*A* shows that β-catenin binds two fragments of IRS-1, the sequence between 601 and 900, and the sequence between residue 900 and the C terminus. IRS-1 is a scaffolding protein, prone to widespread interactions (37) and has at least two binding sites for the regulatory p85 subunit of phosphatidylinositol 3-kinase (38). It is therefore not surprising that IRS-1 binds β-catenin through more than one domain. To identify the sequences of β-catenin binding to IRS-1, we used two mutants of β-catenin, with an N- and a C-terminal deletion, respectively. One mutant has a deletion of residues 1–132, whereas the second mutant has a deletion at the C terminus from residues 695 to 781 (30). These mutants were transfected into R+ cells; lysates were made and immunoprecipitated with an antibody to IRS-1. The results are shown in Fig. 6*B*. IRS-1 interacts only with the β-catenin mutant that has a deletion of the N-terminal, indicating that the C terminus is the binding site for IRS-1. The antibody used recognized both mutant forms (Fig. 6*C*). The importance of these interactions was confirmed by Western blots with an antibody to phosphorylated β-catenin. This antibody recognizes phosphorylation at serines 33 and 37

[2] J. Chen, A. Wu, H. Sun, R. Drakas, C. Garofalo, S. Cascio, E. Surmacz, and R. Baserga, unpublished data.

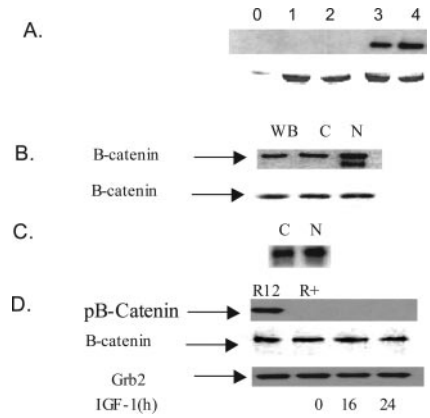

FIG. 6. **Sequences of IRS-1 and β-catenin involved in their interactions.** In *A*, we used GST constructs, expressing specific sequences of IRS-1 (see "Materials and Methods"). The sequences are from 1 to 4, respectively: amino acids 1–315, 300–600, 601–900, and 900 to C terminus. *Lane 0* is the empty GST construct. IRS-1 sequences between 601 and 900 and between 900 and the C terminus interact with β-catenin (lysates from R+ cells). The amounts of GST protein used are shown in the *lower row* of *panel A*. In *panel B*, the mutant β-catenin plasmids (see text) were transiently transfected into R+ cells. Lysates from the transfected cells were immunoprecipitated with an antibody to IRS-1 followed by Western blot with an antibody to β-catenin. IRS-1 interacts only with the mutant lacking the amino terminus. *C*, the antibody to β-catenin recognizes both mutants, Western blot on lysates from transient expression. *D*, Western blot of lysates from R12 cells and R+ cells at various times after IGF-1 stimulation, using an antibody to the phosphorylated N terminus of β-catenin (see text). In R12 cells, the N terminus is phosphorylated (marked for degradation), whereas R+ cells are negative for the same antibody at any time after IGF-1 stimulation. The protein amounts in each lane were monitored with an antibody to Grb2.

and threonine 41 of β-catenin, which are the targets of glycogen synthase kinase 3β and lead to the eventual degradation of β-catenin itself (39, 40). The antibody recognizes a phosphorylated β-catenin only in R12 cells (Fig. 6*D*); R+ cells are negative at all stages, indicating that β-catenin is targeted for destruction in R12 cells but not in R+ cells (see "Discussion").

Stimulation of β-Catenin Activity by IGF-1—Having established that the IGF/IRS-1 axis can translocate β-catenin to the nuclei, our next goal was to study the effects of these translocations. We asked first whether IGF-1 stimulation increased the activity of β-catenin in MEFs, and whether β-catenin activity may depend on the strength of IGF-1R signaling. We used the TOPFLASH/FLOPFLASH luciferase assay (32), which measures β-catenin-dependent transcription from the Tcf promoter (FLOPFLASH contains a mutant Tcf/Lef promoter and serves as the negative control). We examined the R−-derived cells described in Figs. 1 and 2 and two other R−-derived cell lines, R−/T cells (that are R− cells expressing the SV40 T antigen) and R508 cells (that have about 13×10^3 IGF-1 receptors/cell). R−, R−/T, R12, and R508 cells are not transformed, do not grow in SFM, and do not respond to IGF-1 with mitosis. However, R508 cells enter S phase, but without dividing, after IGF-1 stimulation (22, 27). R− and R−-derived cells were transiently transfected with the appropriate plasmids and the activities were determined as described under "Materials and Methods." Fig. 7 shows the results of separate experiments in which the activity was measured at 8, 16, and 24 h after stimulation with IGF-1. TOPFLASH activity is the same as FLOPFLASH activity in all cell lines, except R+ and R508 cells at 8 and 16 h. In R+ cells, TOPFLASH activity is markedly increased at 16 h, but it is also significantly increased at 8 h. The activity of TOPFLASH in R+ cells returns to base levels at 24 h after IGF-1. In R508 cells, there is a significant increase only at 16 h. These experiments have been repeated several times, with essentially similar results, and are compatible with the observation of Playford *et al.* (20) that IGF-1 stimulates β-catenin activity in colon cancer cells. The decreased response of R508 and R12 cells suggests that the levels of expression of the IGF-1 receptor may condition the response of β-catenin to IGF-1.

Recruitment of IRS-1, UBF, and β-Catenin to the Cyclin D1 Promoter—To determine whether IRS-1 and β-catenin could both be found in association with the regulatory sequences of β-catenin target genes, like cyclin D1, we studied the recruitment of β-catenin to the cyclin D1 promoter by standard ChIP procedures (33). Sonicated chromatin was also immunoprecipitated with antibodies to UBF and to IRS-1, and the procedure was repeated. The results are shown in Fig. 8, where the cells used were R+ cells in SFM (0 time) or 1, 16, and 24 h after stimulation with IGF-1. The band shown is of the correct size for the selected fragment of the cyclin D1 promoter (see "Materials and Methods"). The *main panel* shows the results with antibodies to IRS-1, UBF, and β-catenin. IRS-1 is detectable on the cyclin D1 promoter at 16 and 24 h after stimulation with IGF-1. A weak signal is already detectable with β-catenin in unstimulated cells, and it becomes quite clear after 1 h of stimulation, persisting at later hrs. UBF is detectable on the cyclin D1 promoter in cells stimulated with IGF-1 for 16 (weak) and 24 h (strong signal). The GAPDH control was negative. Other controls included immunoprecipitation with an antibody to Grb2 (a cytoplasmic protein, negative) and non-immune serum (not shown, but see Fig. 9 for an illustration). This experiment indicates that IRS-1 and UBF are recruited with β-catenin to the promoter of cyclin D1. The antibody used for UBF cannot distinguish between UBF1 and UBF2. This problem is addressed below.

Recruitment of IRS-1 and β-Catenin to the rDNA Promoter—A promoter strongly activated by IRS-1 is the rDNA promoter (15, 16, 41). The mechanism by which IRS-1 activates the rDNA promoter is, at least in part, due to its participation in a complex with UBF1 (16), one of the proteins that upregulates RNA polymerase I activity (42). We asked whether IRS-1, β-catenin, and UBF1 are recruited together to the rDNA promoter in R+ cells stimulated with IGF-1. A ChIP assay analogous to the one described above was carried out, using sequences for the mouse rDNA promoter (see "Materials and Methods" for the sequences). It is essential to use the mouse sequences, because RNA polymerase I activity is species-specific, and a human rDNA promoter is inactive in mouse cells (43). As expected (44), UBF is detectable at the rDNA promoter before and after stimulation with IGF-1 (Fig. 9*A*). IRS-1 is detectable only 16 and 24 h after IGF-1 stimulation, as with the cyclin D1 promoter. β-catenin, with this technology, is not detectable at the rDNA promoter at any time. The controls used included Grb2, non-immune mouse IgG, and non-immune rabbit Ig-G, all negative.

To validate further this result, we used the ChIP assays to assess the presence of IRS-1 in the rDNA promoter of R12 cells, where IRS-1 is cytoplasmic (see above). IRS-1 is no longer detectable on the rDNA promoter, whereas UBF still is (Fig. 9*B*). The PCR for the GAPDH promoter is negative, except when tested for input. This experiment confirms that the presence of IRS-1 on the rDNA promoter of R+ cells is not an artifact of the procedure.

Transcribed rDNA Spacer—It has been reported that the same proteins that are detectable on the proximal rDNA promoter can also be found in other segments, especially the transcribed spacers, of the rDNA genes (45, 46), suggesting that the same proteins are involved in the synthesis and processing of rRNA. To confirm the previous results on the rDNA

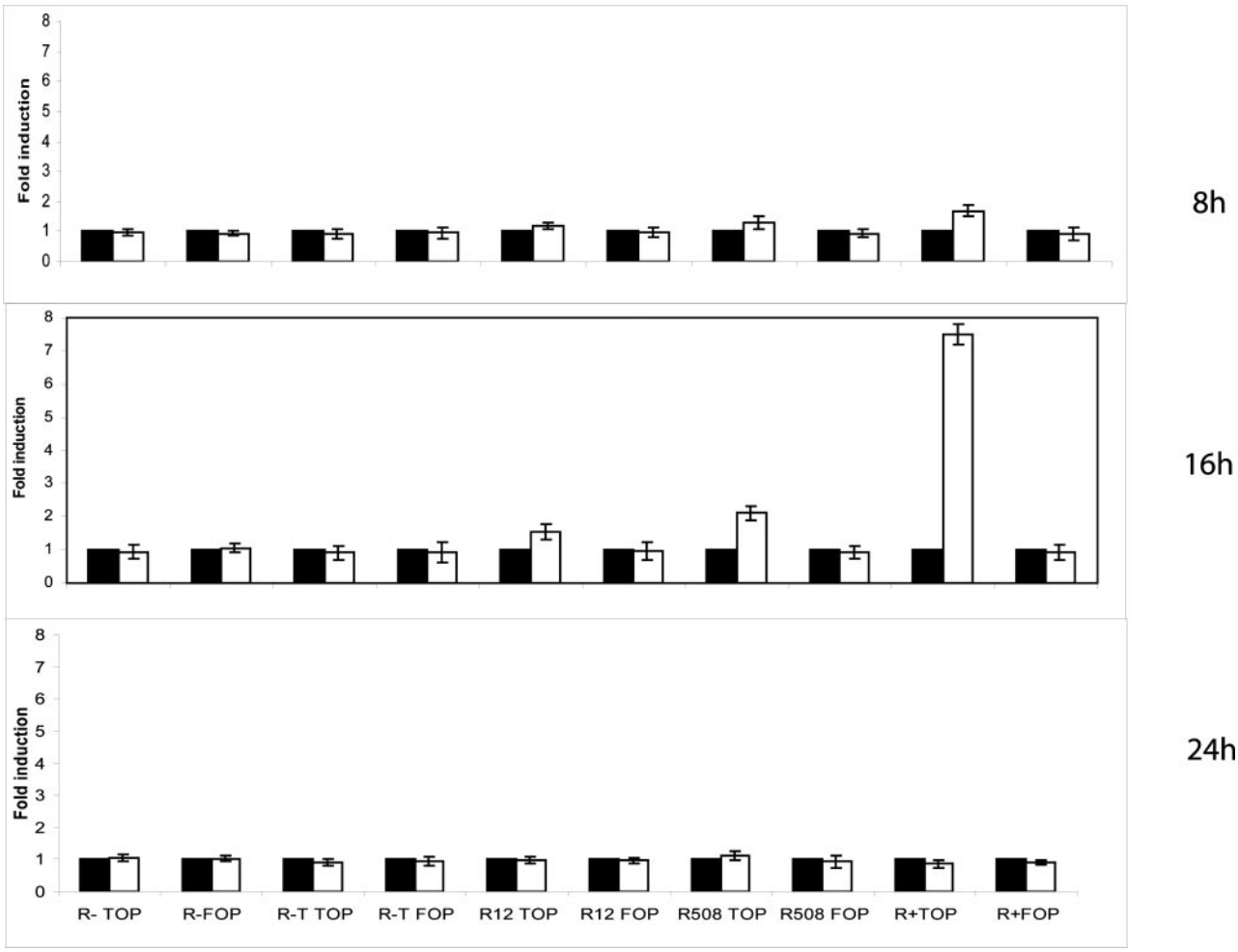

FIG. 7. **Activity of β-catenin in R−-derived cells.** The TOPFLASH/FOPFLASH plasmids were transfected (in transient) into the designated R−-derived cells, R−, R+, R12, R508, and R−/T cells, described in the text. For each cell line, one single aliquot of cells was transfected, then divided into groups that were non-stimulated (serum-free medium) or stimulated with IGF-1 (20 ng/ml) for the indicated times in hours. The data are the average (±S.D.) of three separate transfections. The experiments with R+, R−, and R508 cells were repeated several times with similar results. The experiments with the other two cell lines were repeated only twice. TOPFLASH/FLOPFLASH activity was measured at 8 (*upper row*), 16 (*middle row*), and 24 (*lower row*) h after stimulation with IGF-1. *Empty bars* are cells stimulated with IGF-1; *black bars* are cells in serum-free medium.

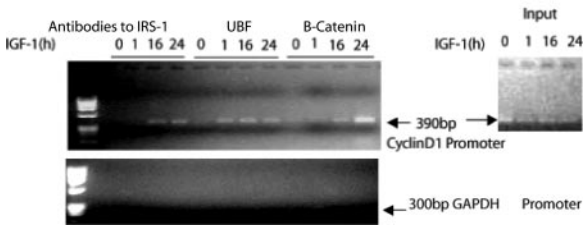

FIG. 8. **IRS-1, β-catenin, and UBF are detectable on the cyclin D1 promoter.** Chromatin immunoprecipitation (ChIP) was carried out as described under "Materials and Methods," using the published primers for amplification of the cyclin D1 promoter. The *main panel* shows the results of experiments in which the sonicated chromatin from R+ cells was immunoprecipitated with antibodies to IRS-1, β-catenin, and UBF. In all instances, the *first lane* is 0 time (no stimulation with IGF-1), and the *second*, *third*, and *fourth lanes* are from cells stimulated with IGF-1 for 1, 16 and 24 h, respectively. ChIP with GAPDH was negative. Additional controls were done and are described in the text (same as in Fig. 9).

promoter, we carried out a ChIP experiment using for PCR primers located on a transcribed spacer of rDNA (Fig. 10, see "Materials and Methods" for the primers). It shows essentially the same results as with the proximal rDNA promoter. This experiment also indirectly confirms the absence of β-catenin from the rDNA genes.

Functional Effects of Nuclear Translocation of IRS-1 and β-Catenin—For this purpose, we selected R12 cells, where IRS-1 and β-catenin are cytoplasmic even after stimulation with IGF-1 (see above). We also showed that IRS-1 is not detectable on the rDNA promoter of parental R12 cells (Fig. 10). To test the functional effect on cells, we inserted IRS-1 into an expression plasmid with a nuclear localization signal (see "Materials and Methods") and transfected it into R12 cells. The confocal microscopy image in Fig. 11*A*, shows that, in R12 cells transfected with the IRS-1/NLS plasmid, IRS-1 and β-catenin are now partially localized in the nuclei. The transfection was done in transient, and the untransfected cells serve as the internal control. We then asked whether IRS-1 would now be detectable on the cyclin D1 promoter. This experiment is shown in Fig. 11 (*B* and *C*). In R12 cells expressing an IRS-1 with a NLS, IRS-1 is now detectable on the cyclin D1 promoter (*C*), whereas it is not detectable in parental R12 cells (*B*). IRS-1/NLS also became detectable on the rDNA promoter of R12 cells, 16 and 24 h after IGF-1 (*D*).

The R12 cells expressing the NLS IRS-1 were then tested for the ability of forming colonies in soft agar, which is, at least for MEFs, the best test of transformation. The results of such an experiment are summarized in Fig. 11*E*. Parental R12 cells form few small colonies, whereas R12 cells expressing the NLS/

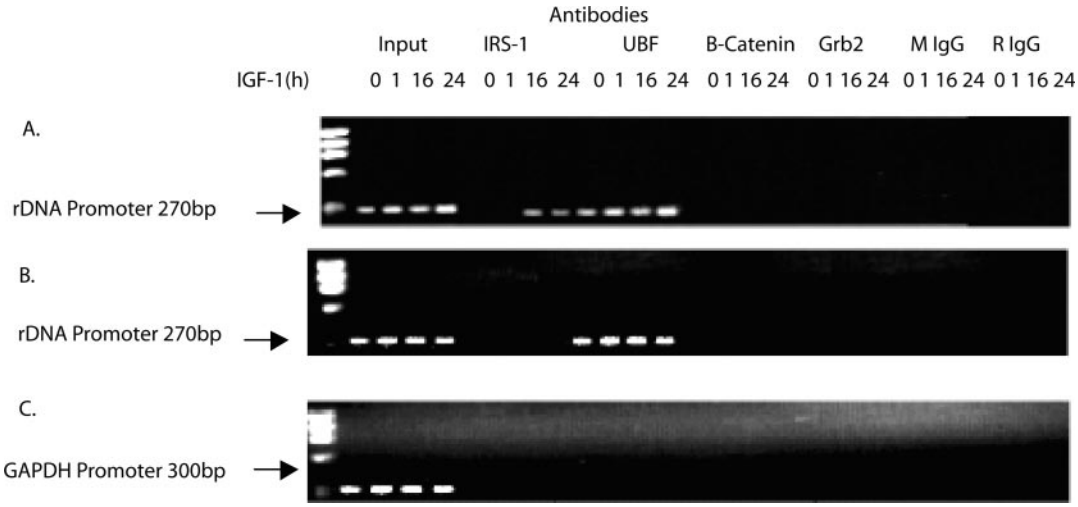

FIG. 9. **IRS-1 and UBF (but not β-catenin) are detectable on the rDNA promoter.** The sonicated chromatin was immunoprecipitated with antibodies to IRS-1, or β-catenin, or UBF. The primers used are described under "Materials and Methods," and they encompass the proximal rDNA promoter of mouse. The cells in *panel A* were R+ cells, and the *numbers above the lanes* indicate time in hours: 0, 1, 16, and 24 h after stimulation with IGF-1. UBF is present in the rDNA promoter even at 0 time, whereas IRS-1 makes its appearance only at 16 h after IGF-1. In *B*, the same experiment is shown with R12 cells, where both IRS-1 and β-catenin are cytoplasmic (see above). In these cells, only UBF can be detected in the rDNA promoter. The control input and the control GAPDH promoter (*C*) are shown as well as three other controls, in which the sonicated chromatin was immunoprecipitated with an antibody to Grb2 (a cytoplasmic protein) or with mouse or rabbit IgG.

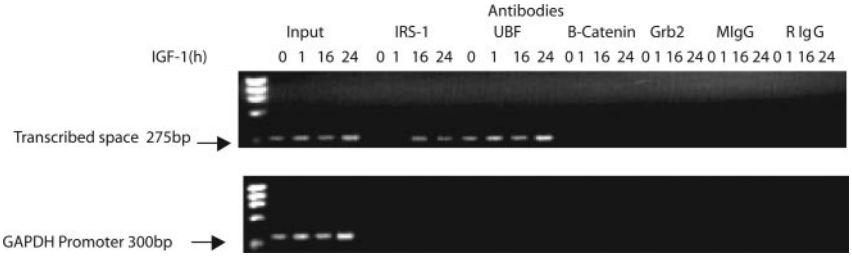

FIG. 10. **IRS-1 and UBF are detectable on the transcribed spacer of the rDNA gene.** ChIP of a transcribed spacer of the rDNA gene was performed using the primers and the technique given in the text. UBF is detectable at all times, as expected. IRS-1 is detectable only at 16 and 24 h after stimulation of R+ cells with IGF-1 (50 ng/ml). The ChIPs are negative for β-catenin, Grb2, and GAPDH or when the chromatin is immunoprecipitated with mouse or rabbit IgG.

IRS-1 form a significant number of colonies in soft agar, indicating that the enforced nuclear localization of IRS-1 causes not only the nuclear translocation of β-catenin but also the transformation of contact-inhibited MEFs. Not shown, for clarity, are R− and p6 cells. R− cells, as usual, did not form colonies in soft agar while the positive controls, p6 cells (22), formed more than 300 colonies/plate.

Interaction of β-Catenin with IRS-1, UBF1, and UBF2—The experiments described above establish the presence on the cyclin D1 promoter of IRS-1, β-catenin, and a UBF protein and of IRS-1 and a UBF protein on the rDNA promoter. The antibody against UBF commercially available cannot distinguish between UBF1 and UBF2, the latter being a slightly shorter isoform of UBF1 (47, 48). In the case of the rDNA promoter, it should be UBF1, which is known to be involved in rDNA transcription (36). UBF2 is apparently inactive in rDNA transcription (44). On the other hand, Grueneberg *et al.* (24) have reported that UBF2 interacts with RNA polymerase II as a transcription factor that enhances the β-catenin signaling pathway. The next step was to establish the connection between the IRS-1/β-catenin interaction and the two UBF proteins. For this purpose, we first used R+ cells nuclear extracts. The extracts were immunoprecipitated with an antibody to IRS-1 (Fig. 12*A*, *lanes 1* and *2*) or with an antibody to β-catenin (*lanes 3* and *4*). The Western blots were developed with an antibody to UBF that recognizes both UBFs. UBF2 is shorter than UBF1, and a shorter band can be detected in nuclear extracts immunoprecipitated with an antibody to β-catenin, but not with an antibody to IRS-1. To confirm this result, we used R+ cells stably transfected with either a UBF2 or UBF1 cDNA, tagged with FLAG at the 3′-end (see "Materials and Methods"). We prepared nuclear fractions from which lysates were immunoprecipitated with antibodies to either β-catenin or IRS-1. The blots were developed with an anti-FLAG antibody. The results are shown in Fig. 12*B*. Antibodies to β-catenin or IRS-1 now immunoprecipitate both UBF1 and UBF2. In previous work, we have shown that the antibody to FLAG is specific on Western blots, but not in immunoprecipitations. Therefore, the reverse experiment, immunoprecipitation with an anti-FLAG antibody, could not be done properly. The discrepancy between *panels A* and *B* will be taken up under "Discussion."

DISCUSSION

Our novel findings can be summarized as follows: 1) IRS-1 and β-catenin co-immunoprecipitate in both the cytosol and the nucleus of MEFs in an IGF-1-dependent manner. 2) IRS-1 plays a crucial role in IGF-1-mediated nuclear translocation of β-catenin. 3) The role of IRS-1 in IGF-1-mediated nuclear translocation of β-catenin has been confirmed by using a plasmid expressing an IRS-1 with a NLS. In R12 MEFs, in which both proteins are usually located in the cytoplasm (Fig. 3), the fusion protein translocates with β-catenin to the nuclei, and the cells now assume the transformed phenotype (colony formation in soft agar). 4) In ChIP assays, IRS-1, UBF, and β-catenin can be detected on the cyclin D1 promoter, but only UBF and

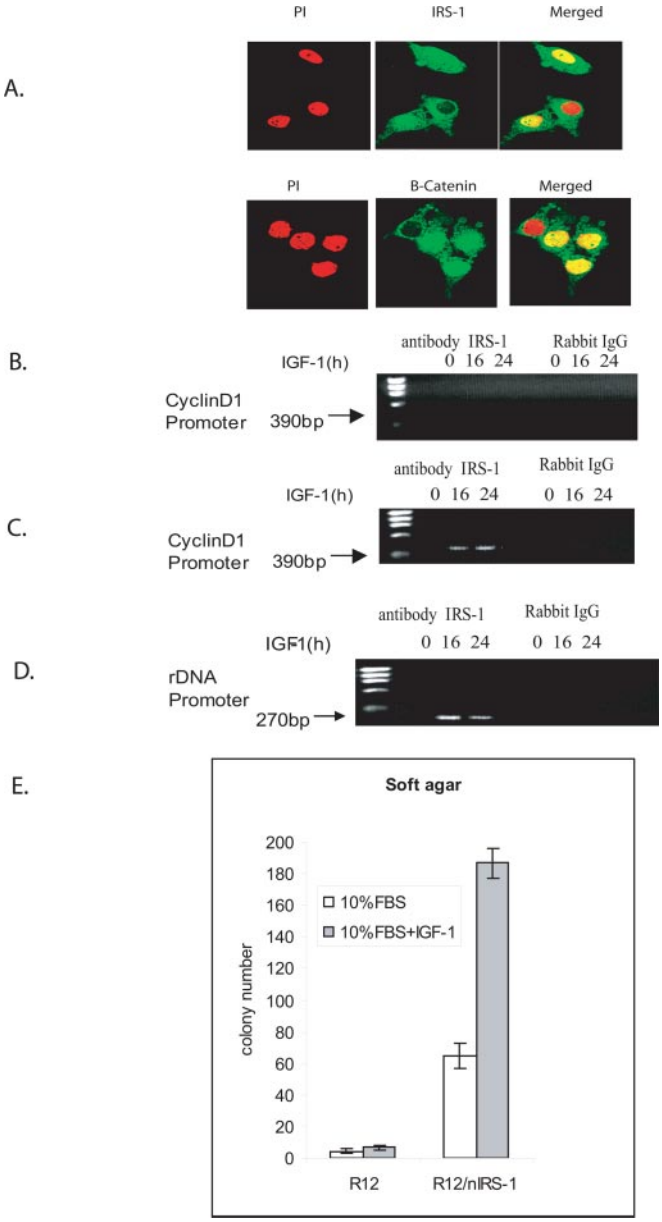

FIG. 11. **Confocal microscopy and ChIPs of the cyclin D1 promoter and rDNA promoter of R12 cells transfected with a plasmid expressing an IRS-1 with a NLS.** The subcellular localization of IRS-1 and β-catenin in parental R12 cells was shown in Fig. 2. *A* shows images of R12 cells transfected in transient with a plasmid expressing IRS-1 with an added NLS. Cells were stained with antibodies to either IRS-1 or β-catenin and counterstained with PI. Both IRS-1 and β-catenin are now detectable in the nuclei of cells, 24 h after IGF-1 stimulation. The untransfected cells serve as controls. ChIPs of the cyclin D1 and rDNA promoters are shown in the *lower panels*. *B* shows cyclin D1 promoter ChIPs of the parental R12 cells, after immunoprecipitation of the chromatin with an antibody to IRS-1. *C* and *D* show the same experiment in R12 cells transfected with the NLS/IRS-1 plasmid. IRS-1 is now detectable on the cyclin D1 and rDNA promoters of R12 cells expressing an IRS-1 with a nuclear localization signal. *E* shows the number of colonies produced in soft agar by parental R12 cells and of R12 cells stably transfected with a plasmid expressing IRS-1 with a NLS. The methodology is described under "Materials and Methods." The role of IRS-1 in transformation of these cells is confirmed by the effect of IGF-1 addition (*closed bars*).

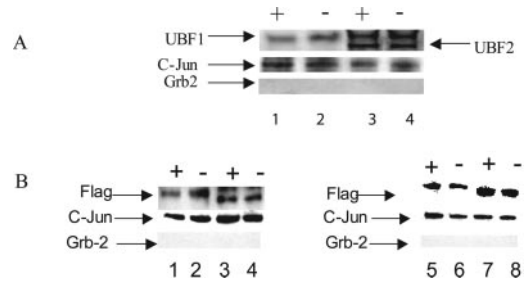

FIG. 12. **IRS-1 and β-catenin interact with both UBF1 and UBF2.** In the *upper panel*, nuclear extracts from R+ cells were immunoprecipitated with antibodies to IRS-1 (*lanes 1* and *2*) or β-catenin (*lanes 3* and *4*), and the blots were developed with an antibody to UBF that recognizes both forms of UBF proteins. The shorter form of UBF (*UBF2*) is recognizable in nuclear lysates immunoprecipitated with the antibody to β-catenin, but not in lysates immunoprecipitated with an antibody to IRS-1. Purity of nuclear lysates was monitored with antibodies to c-Jun and Grb2. In the *lower panels*, R+ cells were transiently transfected with plasmids expressing respectively UBF1 and UBF2, tagged with a FLAG epitope. Nuclear extracts were immunoprecipitated with antibodies to IRS-1 (*lanes 1–4*) or β-catenin (*lanes 5–8*), and blots were developed with an anti-FLAG antibody. *Lanes 1*, *2*, *5*, and *6* are lysates from cells transfected with the UBF1-FLAG construct; *lanes 3*, *4*, *7*, and *8* are from cells transfected with the UBF2 FLAG construct. In the absence of FLAG constructs, the Western blots are totally negative for FLAG antibodies (not shown, but shown in previous reports). +, indicates stimulation with IGF-1; −, indicates serum-free medium.

IRS-1 can be found on the rDNA promoter. In the former, IRS-1 and β-catenin associate more with UBF2 than UBF1, whereas on the rDNA promoter, IRS-1 associates mostly with UBF1, a regulatory protein for RNA polymerase I (42). 5) IRS-1 binds to β-catenin with sequences located between amino acid residue 600 and the C terminus. β-Catenin binds to IRS-1 with sequences in the C terminus. We have also confirmed that an activated IGF-1R activates the Tcf/Lef family of proteins and their target genes. These findings will be discussed separately.

Significance of the Interaction—β-Catenin plays a major role in cancer, as pointed out in the introduction. IRS-1 is known to send a strong mitogenic, anti-apoptotic, and anti-differentiation signal. Their interaction provides one mechanism for their effects on cell proliferation. We wish to emphasize that by no means we propose that IRS-1 is the exclusive, or even the main conveyor of β-catenin to the nuclei. Other growth factors and other pathways (2, 49, 50) can cause the translocation of β-catenin to the nuclei. But IRS-1 should be considered as one of the factors that regulate the subcellular localization of β-catenin. The association IRS-1/β-catenin seems to be more important for β-catenin translocation than the activation of the conventional signaling pathway. In R12 cells, IGF-1 induces IRS-1 phosphorylation and activation, all the way up to induction of c-*myc* (22), yet, with a cytoplasmic IRS-1, β-catenin remains in the cytoplasm.

Recruitment of IRS-1 and β-Catenin to the Cyclin D1 and rDNA Promoters—IRS-1 and β-catenin are found by ChIPs on the cyclin D1 promoter, a well known target of β-catenin (8, 9). UBF2 co-localizes with them on the cyclin D1 promoter, confirming and extending the findings of Grueneberg *et al.* (24) on the co-operation between β-catenin and UBF2 on the cyclin D1 promoter. Our results suggest that IRS-1 can be added to the activation complex. β-catenin, however, is absent from the rDNA promoter, at least using the methodology described in this report. Although we have used all the conventional controls to validate our ChIP experiments, the best demonstration of their validity is the experiment with R12 cells, in which IRS-1, cytoplasmic in these cells, is not found in association with the rDNA promote (this report). As to the UBF proteins, we favor the possibility that both UBFs are present in either the cyclin D1 or rDNA promoters, despite the fact that direct immunoprecipitation seems to suggest that UBF2 is not detectable in the rDNA promoter. These data with the FLAG-tagged UBFs are, in our opinion, more convincing. The recruitment of IRS-1 to both the cyclin D1 and rDNA promot-

ers may provide an explanation of why IRS-1 can accomplish both cell cycle progression and increase in cell size (51), a role that has also been suggested for c-*myc* (52).

Effects of the IGF-1R/IRS-1 Axis on β-Catenin—The previous reports that the IGF-1R could activate β-catenin (19–21, 53) have been confirmed in this report. We now show that this is mediated by the IRS-1 interaction with β-catenin that leads to nuclear translocation. This leads in turn to increased β-catenin nuclear activity. IGF-1, however, is not the only growth factor that activates β-catenin, which can also be activated by the epidermal growth factor (54).

IRS-1/β-Catenin Interaction—The interaction between IRS-1 and β-catenin, first detected using our modified TAPtag purification method (10), has been confirmed in this report by co-immunoprecipitation. The interaction occurs both in the cytoplasm and in the nucleus. It was further confirmed by the use of GST constructs for IRS-1 and mutants of β-catenin. The IRS-1 sequences (between residues 600 and the C terminus) binding β-catenin are not the IRS-1 sequences required for its nuclear translocation, which is the PTB domain (17). The sequences interacting with β-catenin contain binding sites for several proteins, such as the p85 subunit of phosphatidylinositol 3-kinase, Grb2, and 14.3.3 (38). In turn, β-catenin binds to IRS-1 with its C terminus, between residues 695 and 781. The C terminus of β-catenin also binds the JC virus T antigen (30). Binding to this sequence may prevent the phosphorylation of the N terminus of β-catenin, an event that causes β-catenin ubiquitination and degradation (2, 4, 55). Alternatively, the IRS-1/β-catenin interaction may protect β-catenin from degradation simply by favoring its nuclear translocation or by inhibiting glycogen synthase kinase 3β (56). The interaction of β-catenin with the JC virus T antigen is particularly interesting, because IRS-1 interacts with the simian homolog of JC virus, the SV40 T antigen in the nucleus (16). It provides an added indication that IRS-1 may participate in complexes with β-catenin.

In conclusion, we have shown that IRS-1 and β-catenin interact in the nuclei of IGF-1-stimulated cells. They are both recruited to the cyclinD1 promoter, a known target of activated β-catenin. IRS-1 by itself (see the experiment with the NLS/IRS-1) can translocate β-catenin to the nuclei of R12 cells, although we are aware that β-catenin translocation to the nuclei can be induced by other conditions, independently of IRS-1 or any signaling from the IGF-1R. In the cyclin D1 promoter, IRS-1 and β-catenin co-operate with the UBF proteins (24). We could not detect β-catenin on the rDNA promoter or a transcribed spacer of the rDNA gene. We propose that IRS-1 (through signaling from the IGF-1R) should be considered among the factors that cause nuclear translocation and activation of β-catenin. The presence of IRS-1 on both the cyclin D1 and rDNA promoters suggests an explanation for the powerful effects of IRS-1 on both mitogenesis (12) and cell size (54).

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Featured Article

Leptin Interferes with the Effects of the Antiestrogen ICI 182,780 in MCF-7 Breast Cancer Cells

Cecilia Garofalo,[1] Diego Sisci,[2] and Eva Surmacz[1]

[1]Kimmel Cancer Center, Thomas Jefferson University, Philadelphia Pennsylvania; and [2]Faculty of Pharmacy, University of Calabria, Cosenza, Italy

ABSTRACT

Purpose: **Obesity is a risk factor for breast cancer development in postmenopausal women and correlates with shorter disease-free and overall survival in breast cancer patients, regardless of menopausal status. Adipose tissue is a major source of leptin, a cytokine regulating energy balance and controlling different processes in peripheral tissues, including breast cancer cell growth. Here, we investigated whether leptin can counteract antitumorigenic activities of the antiestrogen ICI 182,780 in breast cancer cells.**

Experimental Design: **Mitogenic response to leptin and the effects of leptin on ICI 182,780-dependent growth inhibition were studied in MCF-7 estrogen receptor α-positive breast cancer cells. The expression of leptin receptor and the activation of signaling pathways were studied by Western immunoblotting. The interference of leptin with ICI 182,780-induced estrogen receptor α degradation was probed by Western immunoblotting, fluorescence microscopy, and pulse-chase experiments. Leptin effects on estrogen receptor α–dependent transcription in the presence and absence of ICI 182,780 were studied by luciferase reporter assays and chromatin immunoprecipitation.**

Results: **MCF-7 cells were found to express the leptin receptor and respond to leptin with cell growth and activation the signal transducers and activators of transcription 3, extracellular signal-regulated kinase-1/2, and Akt/GSK3/pRb pathways. The exposure of cells to 10 nmol/L ICI 182,780 blocked cell proliferation, induced rapid estrogen receptor α degradation, inhibited nuclear estrogen receptor α expression, and reduced estrogen receptor α–dependent transcription from estrogen response element–containing promoters. All of these effects of ICI 182,780 were significantly attenuated by simultaneous treatment of cells with 100 ng/mL leptin.**

Conclusions: **Leptin interferes with the effects of ICI 182,780 on estrogen receptor α in breast cancer cells. Thus, high leptin levels in obese breast cancer patients might contribute to the development of antiestrogen resistance.**

INTRODUCTION

Numerous epidemiologic studies documented that obesity is a risk factor for postmenopausal breast cancer (1–4). Furthermore, increased body weight and body mass index have been associated with shorter disease-free and overall survival in breast cancer patients, regardless of age and menopausal status (4). Some studies also suggested that obesity can reduce the efficacy of anti-breast cancer chemotherapy (5). In animal models, high adiposity has been linked with increased incidence of spontaneous and chemically induced mammary tumors (6–9).

Human obesity is associated with increased levels of leptin, a M_r 16,000 circulating hormone controlling food intake and energy balance by providing signals to the hypothalamus (10). In addition to its central nervous system activities, leptin regulates multiple processes in peripheral tissues, including hematopoiesis, immune responses, puberty, pregnancy, and lactation (10–14). In cellular models, leptin has been shown to activate proliferation, angiogenesis, motility, and invasion (10, 15–22). The major source of leptin is adipose tissue; however, leptin can be produced by other organs, including the mammary gland (10–12).

The activities of leptin are mediated through the transmembrane leptin receptor (ObR; ref. 23). In human tissues, at least four isoforms of ObR with different COOH-terminal cytoplasmic domains have been described previously (24). The full (long) form of ObR (ObR_l) is 1165 amino acids long (M_r ~150,000–190,000) and contains extracellular, transmembrane, and intracellular domains. The extracellular domain binds ligand, whereas intracellular tail recruits and activates signaling substrates. Only ObR_l has a full signaling potential, whereas the shorter ObR isoforms have diminished or abolished signaling capability (25–28). The signaling pathways known to be activated by ObR_l include the classic cytokine JAK2/signal transducers and activators of transcription 3 (STAT3) pathway; the Ras/extracellular signal-regulated kinase (ERK) signaling cascade; the kinases phosphatidylinositol 3′-kinase, Akt, p38, and protein kinase C; nitric oxide; and phospholipase Cγ (25–27). Ultimately, induction of ObR_l can activate the expression of several genes involved in cell proliferation, including c-*fos*, c-*jun*, *jun*B, *egr-1*, and *socs3* (25, 26).

Although leptin is necessary for normal mammary gland development in rodents and humans (11, 12, 18, 29), recent studies suggested that the hormone might be involved in mam-

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Requests for reprints: Eva Surmacz, Kimmel Cancer Center, Thomas Jefferson University, 233 South 10th Street, Bluemle Life Sciences Building 631, Philadelphia, PA 19107. Phone: 215-503-4512; Fax: 215-923-0249; E-mail: eva. surmacz@jefferson.edu.

mary carcinogenesis (18–22, 30, 31). Notably, leptin (31) and ObR (20) have been detected in human breast cancer specimens. In breast cancer cell lines T47D and MCF-7, leptin has been shown to stimulate DNA synthesis and cell growth acting through the STAT3 and ERK1/2 signaling pathways (18–20, 22). Leptin has also been shown to induce transformation (anchorage-independent growth) of cancer but not normal breast epithelial cells (18). Finally, leptin-deficient mice have decreased incidence of spontaneous and oncogene-induced mammary tumors (30).

The possible impact of leptin produced in mammary tissue on breast cancer development is yet unknown. The role of circulating leptin remains unclear, with clinical studies reporting positive (32), negative (33), or no association (34) of serum leptin levels with breast cancer.

In addition to leptin, adipose tissue is a source of estrogens produced, via aromatase conversion, from androstenedione in postmenopausal women (35). Recent studies suggest that leptin and estrogen systems are involved in functional cross-talk. For instance, leptin has been shown to modulate, either positively (36–38) or negatively (39, 40), aromatase activity. Reciprocally, 17-β-estradiol (E_2) has been found to up-regulate leptin mRNA and protein synthesis in adipocytes (41). E_2 can also modulate ObR expression (42), possibly through the putative estrogen-responsive element in the *ObR* gene promoter (43).

In this study, we explored a new aspect of leptin/estrogen cross-talk. Specifically, we asked whether leptin can interfere with antitumorigenic effects of the antiestrogen ICI 182,780. ICI 182,780 [Faslodex (fulvestrant); AstraZeneca, Macclestield, United Kingdom], which induces estrogen receptor α degradation through ubiquitin-mediated mechanism (44–47), is currently used for treatment of hormone receptor–positive metastatic breast cancer in post-menopausal women with disease progression following other hormonal therapy (44).

MATERIALS AND METHODS

Cell Lines and Cell Culture. MCF-7, T47D, MDA-MB-231, and MDA-MB-435 cells were obtained from American Type Culture Collection (Manassas, VA). The cells were grown in Dulbecco's modified Eagle's medium:Ham's F-12 containing 5% calf serum. In the experiments requiring E_2- and serum-free conditions, the cells were cultured in phenol red-free serum-free medium (48, 49).

Cell Growth. MCF-7 cells were plated in 35-mm plates at a concentration of 1.5 to 2.0 × 105 cells/plate in Dulbecco's modified Eagle's medium:Ham's F-12 (1:1) containing 5% calf serum. The following day (day 0), the cells at approximately 70% confluence were shifted to serum-free medium and treated with 10 nmol/L E_2 (Sigma. St. Louis, MO), 10 nmol/L ICI 182,780 (Tocris Cookson, Ellisville, MI), 100 ng/mL leptin (R&D Systems, Minneapolis, MN), or 10 nmol/L ICI 182,780 + 100 ng/mL leptin, singly or in combination. Cell number was determined by direct cell counting at days 0, 1, and 3. The number of cells at day 0 was taken as 100%, and the relative values at days 1 and 3 were calculated for each treatment.

Fluorescence Microscopy. Fifty percent confluent MCF-7 cells grown on coverslips were fixed in 3% paraformaldehyde, permeabilized with 0.2% Triton X-100, washed three times with PBS, and incubated for 1 hour with 2 μg/mL estrogen receptor α Ab H-184 (Santa Cruz Biotechnology, Santa Cruz, CA). Next, the cells were washed three times with PBS, and incubated with the rhodamine-conjugated goat antirabbit immunoglobulin G (Calbiochem, San Diego, CA) used as a secondary Ab. After this step, the slides were covered with Vectashield containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) to allow visualization of cellular nuclei. Nuclear abundance of estrogen receptor α under different conditions was assessed using Zeiss Axiovert zoom microscope with magnification ×100.

Immunoprecipitation and Western Blotting. The expression of ObR, activation of leptin signaling pathways, and the abundance of estrogen receptor α were assessed by Western blotting or immunoprecipitation followed by Western blotting using total protein lysates or fractionated proteins, where appropriate. Total cell proteins were obtained using RIPA buffer containing 1% Nonidet P40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS. Cytoplasmic proteins were obtained using the lysis buffer containing 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 1.5 mmol/L $MgCl_2$, EGTA, 10 mmol/L (pH 7.5), glycerol 10%, and inhibitors (0.1 mmol/L Na_3VO_4, 1% phenylmethylsulfonyl fluoride, and 20 mg/mL aprotinin). After the collection of cytoplasmic proteins, the nuclei were lysed with the nuclear buffer containing 20 mmol/L HEPES (pH 8), 0.1 mmol/L EDTA, 5 mmol/L $MgCl_2$, 0.5 mol/L NaCl, 20% glycerol, 1% Nonidet P40, and inhibitors (as above). For Western blotting, 50 mg of protein lysates were separated on a 4 to 15% polyacrylamide denaturing gel (PAGE), and proteins of interest were detected with specific antibodies (Abs) and visualized by ECL chemiluminescence (Amersham Biosciences, Piscataway, NJ). The intensity of bands representing relevant proteins was measured by Scion Image laser densitometry scanning program.

For immunoprecipitations, 500 μg of protein lysates were incubated with primary Abs at 4°C or 18 hours in HNTG buffer [20 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 0.1% Triton X-100, 10% glycerol, and 0.1 mmol/L Na_3VO_4], and then the antigen/Ab complexes were precipitated with Protein A agarose (Calbiochem) for pAbs or Protein G for mAbs (Calbiochem) at for 2 hours in HNTG buffer. In control samples, primary immunoprecipitating Abs were replaced with normal rabbit immunoglobulin G (Santa Cruz Biotechnology). The immunoprecipitated proteins were washed three times with HNTG buffer, separated on PAGE, and processed by Western blotting.

Antibodies for Western Blotting and Immunoprecipitation. ObR expression was studied by Western blotting with the anti-ObR H300 pAb (Santa Cruz Biotechnology). Estrogen receptor α was assessed by Western blotting with the anti-estrogen receptor α F-10 mAb (Santa Cruz Biotechnology). Ubiquitination of estrogen receptor α was assessed by immunoprecipitation/Western blotting in 500 μg of total proteins. In this assay, estrogen receptor α was immunoprecipitated with the anti-estrogen receptor α F10 mAb, and ubiquitination was detected by Western blotting with the anti-ubiquitin P4D1 mAb (Santa Cruz Biotechnology). The expression of STAT3 was probed in 500 μg of total proteins by immunoprecipitation and Western blotting with the anti-STAT 3 pAb (Santa Cruz Biotechnology). The activation of STAT 3 was measured in STAT3

immunoprecipitations with the anti-STAT3 Ser^{727} pAb (UBI, Lake Placid, NY) and the anti-STAT Tyr^{705} pAb (Cell Signaling, Beverly, MA). The following Abs were used to study other elements of leptin signaling by Western blotting: anti-phospho-ERK1/2 Thr^{202}/Tyr^{204} mAb (Cell Signaling); anti-p44/42 MAP kinase pAb (Cell Signaling); anti-phospho-Akt Ser^{473} pAb (Cell Signaling); anti-Akt pAb (Cell Signaling); anti-phospho-GSK3β pAb (Cell Signaling); and anti-phospho-pRB pAb (Cell Signaling). The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and nucleolin was assessed by Western blotting as controls of loading and purity of lysates with the anti-GAPDH mAb (Research Diagnostics Inc., Flanders, NJ) and the anti-nucleolin C23 mAb (Santa Cruz Biotechnology), respectively. The expression of β-catenin was probed with the anti-β-catenin mAb (BD Transduction Laboratories, San Jose, CA). All Abs were used at concentrations recommended by the manufacturers.

Estrogen Response Element Reporter Assays. MCF-7 cells were grown in 24-well plates. At 70% confluence, the cultures were transfected for 6 hours with 0.5 μg DNA/well using Fugene 6 (DNA:Fugene 3:1; Roche, Gipf-Oberfrick, Switzerland). All transfection mixtures contained 0.5 μg of the reporter plasmid, estrogen response element-Luc, encoding the firefly luciferase (Luc) cDNA under the control of the TK promoter and three estrogen response element sequences. In addition, to test transfection efficiency, each DNA mixture contained 50 ng of pRL-TKLuc, a plasmid encoding renilla luciferase (RI Luc; Promega, Madison, WI). Upon transfection, the cells were shifted to serum-free medium for 16 hours and then treated with 10 nmol/L E_2, 10 nmol/L ICI 182,780, 100 ng/mL leptin, and ICI 182,780 + leptin for 24 hours. Untreated cells in serum-free medium served as controls. Luciferase activity (Luc and RI Luc) in cell lysates was measured using Dual Luciferase Assay System (Promega) following the manufacturer's instructions. The values obtained for Luc were normalized to that of RI Luc to generate relative Luc units.

Chromatin Immunoprecipitation. We followed the chromatin immunoprecipitation methodology described by Shang *et al.* (50) with minor modifications. MCF-7 were grown in 100-mm plates. Ninety percent confluent cultures were shifted to serum-free medium for 24 hours and then treated for 4 hours with 10 nmol/L E_2, 10 nmol/L ICI 182,780, 100 ng/mL leptin, 10 nmol/L E_2 + 10 nmol/L ICI 182,780, or 100 ng/mL leptin + 10 nmol/L ICI 182,780, or left untreated in serum-free medium. After treatment, the cells were washed twice with PBS and cross-linked with 1% formaldehyde at 37°C for 10 minutes. Next, the cells were washed twice with PBS at 4°C, collected, and resuspended in 200 mL of lysis buffer [1% SDS, 10 mmol/L EDTA, and 50 mmol/L Tris-Cl (pH 8.1)] and left on ice for 10 minutes. Then, the cells were sonicated four times for 10 seconds at 40% maximal power (Fisher Sonic Dismembrator, Pittsburgh, PA), and insoluble material was collected by centrifugation at 4°C for 10 minutes at 14,000 rpm. Supernatants were diluted in 1.3 mL of immunoprecipitation buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris-Cl (pH 8.1), and 16.7 mmol/L NaCl] and precleared with 80 mL of sonicated salmon sperm DNA/protein A agarose (UBI) for 1 hour at 4°C. The precleared chromatin was immunoprecipitated with either the anti-estrogen receptor α mAb F-10 (Santa Cruz Biotechnology) or the anti-polymerase II CTD4H8 mAb (UBI) for 12 hours. After that, 60 mL of salmon sperm DNA/protein A agarose were added, and precipitation continued for 4 hours at 4°C. After pelleting, the precipitates were washed sequentially for 5 minutes with the following buffers: wash A [0.1% SDS, 1% Trition X-100, 2 mmol/L EDTA, 20 mmol/L Tris-Cl (pH 8.1), and 150 mmol/L NaCl], wash B [0.1% SDS, 1% Trition X-100, 2 mmol/L EDTA, 20 mmol/L Tris-Cl (pH 8.1), and 500 mmol/L NaCl], and wash C [0.25 mol/L LiCl, 1% Nonidet P40, 1% sodium deoxycholate, 1 mmol/L EDTA, and 10 mmol/L Tris-Cl (pH 8.1)]. The precipitates were then washed twice with 10 mmol/L Tris and 1 mmol/L EDTA. The immune complexes were eluted with the buffer containing 1% SDS and 0.1 mol/L $NaHCO_3$. The eluates were reverse cross-linked by heating at 65°C for 12 hours and digested with 0.5 mg/mL proteinase K at 45°C for 1 hour. DNA was obtained by phenol and phenol/chloroform extractions. Two mL of 10 mg/mL yeast tRNA were added to each sample, and DNA was precipitated with ethanol for 12 hours at 4°C and resuspended in 20 mL of 10 mmol/L Tris and 1 mmol/L EDTA. Four mL of each sample were used for PCR with pS2 promoter sequences containing estrogen response element: upstream, 5′-TGG CCA GGC TAG TCT CAA AC-3′; and downstream, 5′-CTT AAT CCA GGT CCT ACT CAT A-3′. The PCR conditions were: 30 seconds at 94°C, 50 seconds at 60°C, and 2 minutes at 72°C. The amplification products obtained in 35 cycles were analyzed in a 2% agarose gel and visualized by ethidium bromide staining. The intensity of bands was measured by laser scanning.

Pulse-Chase Labeling. Seventy percent of cultures were shifted to methionine- and cysteine-free Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD) for 16 hours and then metabolically labeled with 100 mCi/mL ^{35}S (Express protein labeling mix; Perkin-Elmer, Fremont, CA) for 1 hour. After that, the labeling medium was replaced with serum-free medium containing 10 nmol/L ICI 182,780 or 10 nmol/L ICI 182,780 + 100 ng/mL leptin, and the cultures were grown for 1, 2, 4, 6, and 8 hours. Untreated cells in serum-free medium served as controls. At specific time points, the cells were lysed in RIPA buffer, and 500 mg of proteins were precipitated with the anti-estrogen receptor α F10 mAb. The estrogen receptor α immunoprecipitations were separated by SDS-PAGE, and labeled estrogen receptor α was identified by autoradiography.

Statistical Analysis. Data were analyzed with Student's *t* test, where appropriate. Means ± SE are shown.

RESULTS

ObR_l Is Expressed in Estrogen Receptor α–Positive Breast Cancer Cell Lines. To study possible effects of leptin on ICI 182,780 action, we first assessed the expression of ObR_l, a signaling form of ObR, in different breast cancer cell lines. Several ObR isoforms (M_r ~190,000–150,000) were detected in estrogen receptor α–positive and estrogen receptor α–negative breast cancer cells by Western blotting (Fig. 1). Notably, the greatest expression of ObR_l M_r 190,000 was found in estrogen receptor α–positive cell lines, MCF-7 and T47D. The shorter isoforms of ObR were abundant in estrogen receptor α–negative cells (Fig. 1). For additional experiments, we selected MCF-7

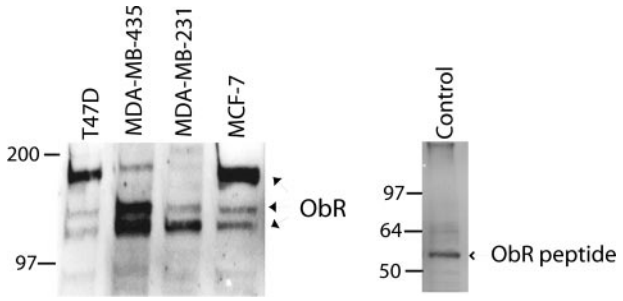

Fig. 1 ObR is expressed in breast cancer cell lines. *Left panel*. The expression of ObR was determined by Western blotting in 50 μg of cytoplasmic protein lysates obtained from proliferating estrogen receptor α–positive (*MCF-7* and *T47D*) and estrogen receptor α–negative (*MDA-MB-231* and *MDA-MB-435*) cells. The ObR Ab used for Western blotting recognizes a common domain in ObR, revealing several isoforms of ObR (M_r 150,000–190,000) indicated by *arrows*. The M_r 190,000 isoform represents ObR_l, which is highly expressed in MCF-7 and T47D cells. *Right panel*. The specificity of the ObR Ab was tested using 250 ng of a M_r 60,000 ObR-tagged fusion protein (amino acids 541–840 of human ObR) provided as a positive control by the manufacturer of ObR Abs (Santa Cruz Biotechnology). The molecular weight markers are indicated on the *left* of both panels.

cells because they are E_2- and ICI 182,780-responsive and express high levels of ObR_l

Leptin Induces Multiple Signaling Pathways in MCF-7 Cells. We examined leptin effects on the activation of different ObR_l signaling pathways in MCF-7 cells. In addition to ObR_l pathways known to be induced in breast cancer cells, i.e., STAT3 and ERK1/2 (18–20), we studied whether leptin can activate Akt/GSK3 antiapoptotic signaling and whether it can phosphorylate (and thereby block) a key cell cycle inhibitor, pRb.

The stimulation of ObR_l by leptin was assessed at different time points, from 5 minutes to 24 hours. We used leptin at a concentration of 100 ng/mL, which in our preliminary dose-response experiments proved to exert maximal mitogenic effects (data not shown). The stimulation of MCF-7 cells with leptin induced multiple signaling elements, including STAT3, ERK1/2, Akt, and GSK3β (Fig. 2). The phosphorylation of STAT3 on Tyr^{705} and on Ser^{727}, reflecting STAT3 activation, was maximal at 5 minutes of leptin treatment and then declined to basal levels (Fig. 2*A*). The stimulation of ERK1/2 become detectable at 15 minutes, was maximal at 1 hour, and persisted up to 24 hours. The activation of Akt appeared at 15 minutes, was maximal at 1 hour, and was reduced to basal levels at 4 hours. GSK3β, a downstream effector of Akt and other kinases was induced at 5 minutes, reached the maximal activation at 1 hour, and then declined to basal levels at 24 hours (Fig. 2*B*). These leptin effects coincided with the phosphorylation of pRb on Ser^{795} (maximum at 1–4 hours; Fig. 2*B*).

Leptin Stimulates the Proliferation of MCF-7 Cells and Interferes with ICI 182,780-Dependent Growth Inhibition. The mitogenic effects of leptin at doses 1 to 1000 ng/mL were studied in MCF-7 cells at 1 and 3 days of treatment. Confirming the results of other investigators (18, 20), we found that the highest proliferation rates were induced with 100 ng/mL leptin, whereas lower leptin concentrations (1 and 10 ng/mL) were less mitogenic (data not shown). Increasing the dose over 100 ng/mL did not improve growth response (data not shown).

At days 1 and 3, 100 ng/mL leptin increased cell growth over that seen in serum-free medium by 20 and 38%, respectively (Fig. 3). Leptin did not affect cell proliferation in the presence of E_2 at any time point. However, leptin consistently counteracted cytostatic effects of ICI 182,780. Specifically, at day 1 and 3, the addition of leptin to ICI 182,780-treated cells increased proliferation by ~30 and ~45%, respectively (Fig. 3). In these studies, ICI 182,780 was used at a concentration of 10 nmol/L, which is cytostatic but not cytotoxic for MCF-7 cells, as demonstrated by us previously (48).

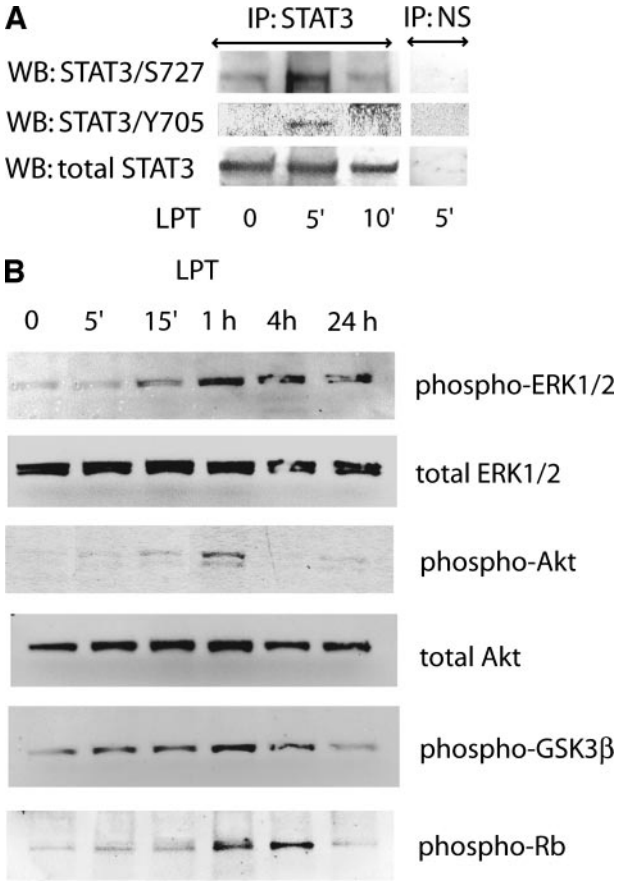

Fig. 2 Leptin activates multiple signaling pathways in MCF-7 cells. *A*, activation of STAT3. MCF-7 cells were synchronized in serum-free medium for 16 hours and then stimulated with 100 ng/mL leptin (*LPT*) for 5 and 10 minutes or left untreated in serum-free medium. STAT3 was immunoprecipitated (*IP*) with the anti-STAT3 pAb (Santa Cruz Biotechnology) from 500 μg of total protein lysates, and the activation of STAT3 was visualized with the STAT3 Ser^{727} pAb (*STAT3/S727*; UBI) and then after stripping of the membrane with the anti-$STAT3^{Tyr705}$ pAb (*STAT3/Y705*; Cell Signaling). In control experiments, the proteins were precipitated with control rabbit immunoglobulin G and processed for Western blotting (*WB*), as described above. *B*, activation of ERK1/2, Akt, GSK3, and Rb. MCF-7 cells were synchronized in serum-free medium for 16 hours and then stimulated with 100 ng/mL leptin (*LPT*) for 5 minutes to 24 hours or left untreated in serum-free medium. The activation (*phospho*) and levels of ERK1/2, Akt, GSK3β, and pRb were assessed by Western blotting in 50 μg of proteins using specific Abs.

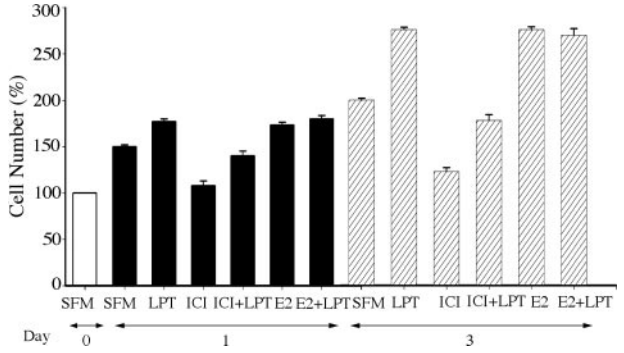

Fig. 3 Leptin stimulates the growth of MCF-7 cells and counteracts the effects of ICI 182,780. Seventy percent confluent MCF-7 cells were synchronized in serum-free medium for 16 hours and treated with 100 ng/mL leptin (*LPT*), 10 nmol/L ICI 182,780 (*ICI*), leptin + ICI 182,780 (*LPT+ICI*), 10 nmol/L E_2, or E_2 + ICI 182,780 (*E2+ICI*) for 1 and 3 days or were left untreated (*SFM*). Cell number was determined by direct cell counting. Please note that MCF-7 cells grow in serum-free medium due to activation of autocrine pathways, as described by us previously (57, 58). Cell number at day 0 in serum-free medium was taken as 100%. The experiments were performed at least four times. The *bars* demonstrate relative cell number (±*SEM*) at different time points. The differences between serum-free medium and leptin values and between leptin and ICI 182,780 + leptin values were statistically significant at days 1 and 3 ($P < 0.05$).

Effects of Leptin on the Nuclear Abundance of Estrogen Receptor α in ICI 182,780-Treated MCF-7 Cells. To study the mechanism of leptin interference with ICI 182,780, we assessed the abundance of cytoplasmic and nuclear estrogen receptor α in MCF-7 cells treated with E_2, E_2 + ICI 182,780, ICI 182,780, ICI 182,780 + leptin, and leptin alone (Fig. 4). As expected, E_2 significantly (by ~50%) decreased the cytoplasmic expression of estrogen receptor α and increased (by ~150%) its nuclear levels, relative to estrogen receptor α under serum-free medium conditions. Also predictably, ICI 182,780 treatment induced the degradation of estrogen receptor α, resulting in reduced estrogen receptor α abundance in the cytoplasm and nucleus (~85 and 70%, respectively). These effects of ICI 182,780 were partially reversed in the presence of E_2 (Fig. 4). The addition of leptin to ICI 182,780-treated cells significantly improved nuclear estrogen receptor α expression but had only minimal effects on the cytoplasmic estrogen receptor α levels. Leptin alone had no significant effects on estrogen receptor α expression in the cytoplasmic and nuclear compartments (Fig. 4).

The above observations were confirmed by fluorescence microscopy of estrogen receptor α in MCF-7 cells treated with ICI 182,780 in the presence or absence of leptin. estrogen receptor α accumulated in the nucleus upon E_2 stimulation, whereas a 24-hour treatment with ICI 182,780 dramatically reduced nuclear estrogen receptor α expression. The effect of ICI 182,780 was prevented by the addition of leptin (Fig. 4*B*).

Leptin Increases Estrogen Receptor α Recruitment to the pS2 Promoter in ICI 182,780-Treated MCF-7 Cells. The function of nuclear estrogen receptor α under different conditions was addressed with chromatin immunoprecipitation assay (Fig. 5). We found that the stimulation of MCF-7 cells with E_2 increased (~5-fold) the recruitment of estrogen receptor α to the classical E_2-responsive estrogen response element–containing pS2 gene promoter. This effect coincided with the greater association of polymerase II to the pS2 regulatory sequences (Fig. 5). In the presence of ICI 182,780, the recruitment of estrogen receptor α to the pS2 promoter was similar to that seen in untreated cells, and the recruitment of polymerase II was completely blocked. The addition of leptin counteracted the inhibitory action of ICI 182,780, resulting in a greater association of polymerase II (increased by ~2-fold) and estrogen receptor α (~3-fold) to the pS2 promoter. Leptin alone did not stimulate the recruitment of either estrogen receptor α or polymerase II to the pS2 promoter (Fig. 5).

Effects of Leptin on Estrogen Receptor α Transcriptional Activity in ICI 182,780-Treated MCF-7 Cells. We validated the information obtained with chromatin immunoprecipitation assays using estrogen response element–luciferase reporter system (Fig. 6). In control experiments, E_2 significantly (by ~250%) stimulated estrogen response element-dependent transcription above the basal level, whereas the addition of ICI 182,780 to E_2 abolished this effect (Fig. 6). Leptin alone did not activate estrogen response element transcription above that seen under serum-free medium conditions. Similarly, leptin did not improve E_2-dependent estrogen response element activation. In the presence of ICI 182,780, estrogen response element activity decreased ~60% below basal levels. In contrast, in ICI 182,780 + leptin cotreated cells, estrogen response element activity was increased ~50% above the level recorded in untreated cells (Fig. 6).

Leptin Increases Estrogen Receptor α Half-Life and Reduces Estrogen Receptor α Ubiquitination in ICI 182,780-Treated MCF-7 Cells. ICI 182,780 is known to induce rapid degradation of estrogen receptor α in MCF-7 (46, 51). We probed the possibility that leptin treatment competes with ICI 182,780 action and increases estrogen receptor α stability. Using pulse-chase assay, we confirmed previous observations that estrogen receptor α half-life in untreated cells is ~4 hours, and in ICI 182,780-treated cells, ~1.5 hours (refs. 47 and 51; Fig. 7*A*). The addition of leptin increased estrogen receptor α half-life to ~2.5 hours (Fig. 7*A*).

Next, we addressed the mechanism by which leptin might decrease estrogen receptor α turnover. Because ICI 182,780- and E_2-dependent degradation of estrogen receptor α occurs through the ubiquitin-proteasome pathway (46, 47), we studied the effects of leptin on estrogen receptor α ubiquitination (Fig. 7*B*). The ubiquitination of estrogen receptor α was undetectable in untreated cells, whereas it was increased when the cells were treated for 1 hour with ICI 182,780 or E_2. The addition of leptin greatly reduced estrogen receptor α ubiquitination in ICI 182,780-treated cells (Fig. 7*B*). Leptin alone did not induce estrogen receptor α ubiquitination. However, estrogen receptor α ubiquitination was still observed when ICI 182,780 was challenged with E_2 (data not shown).

The above treatments had no effects on the expression and ubiquitination of β-catenin, a known target of proteasome (ref. 52; Fig. 7*B*; data not shown).

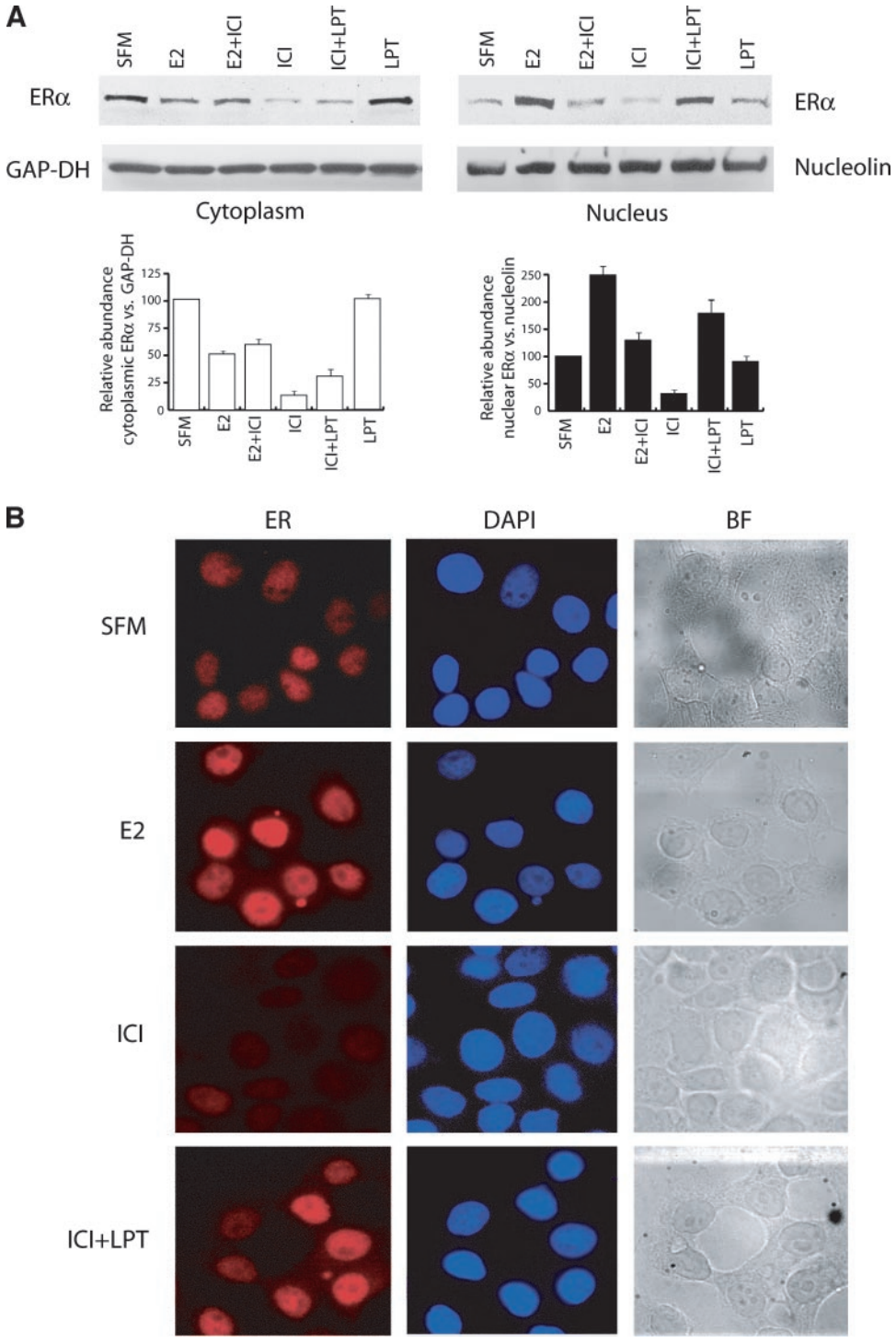

Fig. 4 Leptin increases nuclear abundance of estrogen receptor α (*ERα*) in ICI 182,780-treated MCF-7 cells. *A*, effects of leptin on subcellular estrogen receptor α expression. MCF-7 cells were treated with 10 nmol/L E_2, 10 nmol/L ICI 182,780 (*ICI*), E_2 + ICI 182,780 (*E2+ICI*), 100 ng/mL leptin (*LPT*), or ICI 182,780 + leptin (*ICI+LPT*) for 24 hours or were left untreated (*SFM*). The expression of estrogen receptor α was determined by Western blotting in 50 μg of cytoplasmic or nuclear proteins. The expression of GAPDH (cytoplasmic enzyme) and nucleolin (nuclear protein) was assessed as a control of protein loading. The experiments were performed three times. The *bars* represent mean levels of estrogen receptor α expression (±*SEM*). The differences between ICI 182,780 and ICI 182,780 + leptin values were statistically significant ($P < 0.05$). *B*, fluorescence microscopy. The expression of estrogen receptor α (*ER*) was assessed by immunostaining and fluorescence microscopy in MCF-7 cells treated for 24 hours with 10 nmol/L E_2, 10 nmol/L ICI 182,780 (*ICI*), or 10 nmol/L ICI 182,780 + 100 ng/mL leptin (*ICI+LPT*) or left untreated in SFM. 4′,6-Diamidino-2-phenylindole (*DAPI*) staining and bright field (*BF*) of the same fields is shown to visualize cell nuclei and general morphology. Magnification, ×100.

DISCUSSION

Obesity is a risk factor for the development of breast cancer in postmenopausal women (1–4) and for tumor recurrence in all breast cancer patients, regardless of age and menopausal status (4). However, molecular mechanisms by which excessive fat accumulation could promote mammary carcinogenesis remain unknown. One possibility is that the process is mediated by elevated estrogen levels produced by adipose tissue in postmenopausal women (35, 53). In addition, it has been suggested that the development and progression of breast cancer could be stimulated by mitogenic and transforming activity of leptin (18), the levels of which rise proportionally to body mass index and

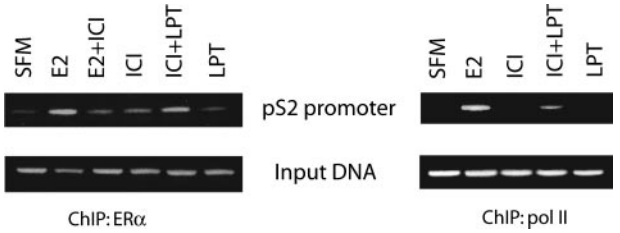

Fig. 5 Leptin increases estrogen receptor α recruitment to pS2 promoter in ICI 182,780-treated MCF-7 cells. The cells were treated for 4 hours with 10 nmol/L E_2, 10 nmol/L ICI 182,780 (*ICI*), 100 ng/mL leptin (*LPT*), ICI 182,780 + leptin (*ICI+LPT*), E_2 + ICI 182,780 (*E2+ICI*) or left untreated (*SFM*). The cells were then cross-linked with formaldehyde and lysed, and soluble, precleared chromatin was obtained. The soluble chromatin was immunoprecipitated with either the anti-estrogen receptor α F-10 mAb (Santa Cruz Biotechnology; *ChIP: ERα*) or the anti-polymerase II CTD4H8 mAb (UBI; *ChIP:pol II*). The estrogen receptor α and polymerase II immunocomplexes were reverse cross-linked, and DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The pS2 promoter sequences containing estrogen response element were detected by PCR with specific primers, as detailed in Materials and Methods. To control input DNA, pS2 promoter was amplified from 30 μL of initial preparations of soluble chromatin (before immunoprecipitations).

are generally higher in women than in men (10). Furthermore, because estrogen receptor α and ObR have been found coexpressed in malignant mammary tissue and breast cancer cell lines (18–20), it is also possible, that both signaling systems are involved in a functional cross-talk contributing to carcinogenesis. However, leptin/E_2 interactions and their possible role in breast cancer have not been extensively studied. In this work, we investigated whether the presence of leptin could compete with antiestrogenic effects produced by ICI 182,780.

First, we provided evidence that estrogen receptor α–positive breast cancer cells MCF-7 and T47D express higher levels of ObRl than estrogen receptor α–negative cell lines MDA-MB-231 and MDA-MB-435. These results confirmed the data of other investigators who demonstrated ObR_l expression in T47D and MCF-7 cells (18–20) but lack of ObR_l mRNA in MDA-MB-231 cells (19). As a cellular model of this study, we selected estrogen receptor α–positive and ICI 182,780-sensitive MCF-7 breast cancer cells. We demonstrated that MCF-7 cells respond to leptin stimulation with the activation of several signaling intermediates, including the STAT3, ERK1/2, and Akt pathways (Figs. 1 and 2). In MCF-7 cells, leptin was also able to inactivate the cell cycle inhibitor pRb and stimulate cell growth (Fig. 2). These results extend the observations of Xu *et al.* (18), Dieudonne *et al.* (19), Laud *et al.* (20), and Okamura *et al.* (22), who described leptin-dependent proliferation and leptin-induced STAT3 and ERK1/2 signaling in different estrogen receptor α–positive breast cancer cell lines. The maximal mitogenic concentrations of leptin used in our and other studies (100 ng/mL) are in the range of serum leptin levels found in obese and morbidly obese (body mass index > 40) individuals (54, 55).

The growth of estrogen receptor α–positive breast cancer cells can be effectively inhibited by ICI 182,780, which induces rapid proteasome-mediated degradation of estrogen receptor α (44–46). We report here, for the first time, that antiestrogenic action of ICI 182,780 can be significantly reduced in the presence of leptin. Specifically, in ICI 182,780-treated MCF-7 cells, leptin increased estrogen receptor α half-life and decreased estrogen receptor α ubiquitination. These effects coincided with elevated nuclear estrogen receptor α expression, increased estrogen receptor α recruitment to the E_2-sensitive gene promoter, and increased estrogen response element–dependent transcription. Leptin also counteracted cytostatic effects of ICI 182,780, resulting in increased cell proliferation (Figs. 3–7).

Interestingly, the mechanism by which leptin competes with ICI 182,780 appears to be different from that exerted by E_2. For instance, estrogen receptor α is still ubiquitinated in ICI 182,780 + E_2-treated cells, whereas it is not ubiquitinated in ICI 182,780 + leptin-treated cells (Fig. 7*B*; data not shown). Similarly, the abundance of nuclear estrogen receptor α is higher under ICI 182,780 + leptin conditions than that seen in ICI 182,780 + E_2-treated cells (Fig. 4*A*). In part, this phenomenon could be explained by the recent discovery that estrogen receptor α turnover is differentially regulated depending on whether the receptor is unliganded, agonist bound, or antagonist bound and whether other cellular pathways (*e.g.*, MAP kinases) are induced by cell surface receptors (46). Thus, it is possible that leptin can exert its action only on ICI 182,780-dependent estrogen receptor α processing. Indeed, in different assays, we did not observe any effects of leptin on basal or E_2-induced activity

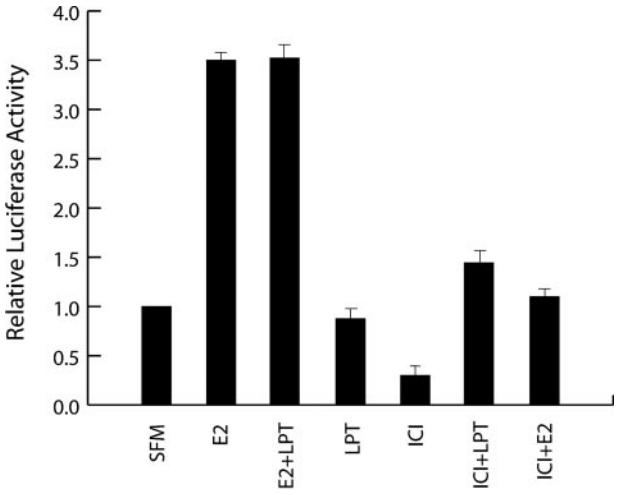

Fig. 6 Leptin increases estrogen receptor α transcription at estrogen response element promoters in ICI 182,780-treated cells. MCF-7 cells grown in 24-well plates were transfected for 6 hours with 0.5 mg of DNA per well using Fugene 6. All transfection mixtures contained 0.5 mg of estrogen response element reporter plasmid estrogen response element-TK-Luc. In addition, each of the DNA mixtures contained 50 ng of pRL-TK-Luc plasmid encoding renilla luciferase to assess transfection efficiency. Upon transfection, the cells were shifted to SFM for 16 hours and then treated for 24 hours with 10 nmol/L E_2, 10 nmol/L ICI 182,780 (*ICI*), 100 ng/mL leptin (*LPT*), ICI 182,780 + leptin (*ICI+LPT*), E_2 + ICI 182,780 (*E2+ICI*) or left untreated (*SFM*). Luciferase activity (Luc and RI Luc) was measured in cell lysates with a luminometer. Relative Luc activity in each sample was obtained upon normalization of Luc to RI-Luc values. The mean relative Luc activity (±*SEM*) obtained in five experiments is shown. The differences between leptin and ICI 182,780 values and between ICI 182,780 and leptin + ICI 182,780 values were statistically significant ($P < 0.05$).

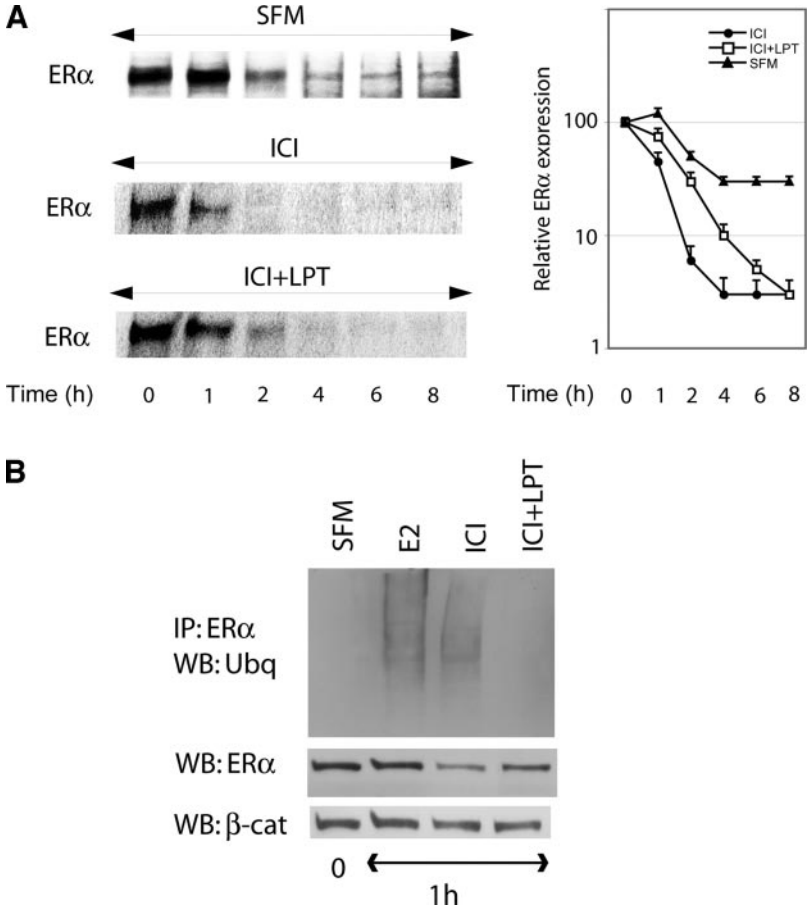

Fig. 7 Leptin effects of estrogen receptor α half-life and ubiquitination in ICI 182,780-treated MCF-7 cells. *A*, effects of leptin on estrogen receptor α half-life. The half-life of estrogen receptor α was determined by ^{35}S pulse-chase labeling, as described in Materials and Methods. The abundance of estrogen receptor α was analyzed at different time points (0, 2, 4, 6, and 8 hours) in untreated cells (*SFM*) and in cells treated with 10 nmol/L ICI 182,780 and 10 nmol/L ICI 182,780 +100 ng/mL leptin (*ICI+LPT*). The expression of estrogen receptor α at time 0 was assigned a value of 100. The relative estrogen receptor α expression (±*SEM*) under different experimental conditions is presented in the graph. This experiment was repeated three times. The differences between ICI 182,780 and ICI 182,780 + leptin values were statistically significant ($P < 0.05$) at 1, 2, and 4 hours. *B*, effects of leptin on estrogen receptor α ubiquitination. MCF-7 cells were treated for 1 hour with 10 nmol/L E_2, 10 nmol/L ICI 182,780 (*ICI*), or 10 nmol/L ICI 182,780 + 100 ng/mL leptin (*ICI+LPT*) or left untreated in SFM. Estrogen receptor α was immunoprecipitated (*IP*) from 500 μg of total protein lysates, and its levels and ubiquitination were evaluated by Western blotting (*WB*) with specific Abs, as described in Materials and Methods. The expression of β-catenin (β-*cat*) in 50 μg of total protein lysates is shown as a control of protein loading.

of estrogen receptor α. These data suggest that in our cell model, leptin did not modulate the synthesis of endogenous E_2. This latter point is worth discussion because leptin has been suggested as a potential modulator of E_2 production (36–40). In some cell models (36, 37), including breast cancer cells (38), leptin has been shown to stimulate the aromatase gene promoter and aromatase activity. Furthermore, pharmacologic doses of leptin (1000 ng/mL) apparently activate estrogen response element promoters, presumably through the stimulation of E_2 synthesis (56), however, increased E_2 expression has not been formally shown in this setting. Our data included in Figs. 4*A*, 5, and 6 suggest that the exogenous E_2 levels were similar in untreated and leptin-induced MCF-7 cell cultures.

In summary, we demonstrated that leptin interferes with the action of ICI 182,780 in MCF-7 cells. Our results suggest that the mechanism of this phenomenon involves increased nuclear expression and activity of estrogen receptor α but is independent of E_2. Future studies should explore whether obesity might impede the benefits of ICI 182,780 therapy in breast cancer patients.

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Nuclear insulin receptor substrate 1 interacts with estrogen receptor α at ERE promoters

Catia Morelli[1,2,6], Cecilia Garofalo[1,2,6], Diego Sisci[1,2], Sonia del Rincon[3], Sandra Cascio[1,4], Xiao Tu[1], Andrea Vecchione[1], Edward R Sauter[5], Wilson H Miller Jr[3] and Eva Surmacz*[,1]

[1]Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107, USA; [2]Postgraduate School in Clinical Pathology, University of Calabria, Cosenza, Italy; [3]McGill University, Lady Davis Institute for Medical Research, Montreal, Canada; [4]Section of Molecular Oncology, Department of Oncology, University of Palermo, Palermo, Italy; [5]Ellis Fischel Cancer Center, University of Missouri-Columbia, Columbia, MO 65212, USA

Insulin receptor substrate 1 (IRS-1) is a major signaling molecule activated by the insulin and insulin-like growth factor I receptors. Recent data obtained in different cell models suggested that in addition to its conventional role as a cytoplasmic signal transducer, IRS-1 has a function in the nuclear compartment. However, the role of nuclear IRS-1 in breast cancer has never been addressed. Here we report that in estrogen receptor α (ERα)-positive MCF-7 cells, (1) a fraction of IRS-1 was translocated to the nucleus upon 17-β-estradiol (E2) treatment; (2) E2-dependent nuclear translocation of IRS-1 was blocked with the antiestrogen ICI 182,780; (3) nuclear IRS-1 colocalized and co-precipitated with ERα; (4) the IRS-1:ERα complex was recruited to the E2-sensitive pS2 gene promoter. Notably, IRS-1 interaction with the pS2 promoter did not occur in ERα-negative MDA-MB-231 cells, but was observed in MDA-MB-231 cells retransfected with ERα. Transcription reporter assays with E2-sensitive promoters suggested that the presence of IRS-1 inhibits ERα activity at estrogen-responsive element-containing DNA. In summary, our data suggested that nuclear IRS-1 interacts with ERα and that this interaction might influence ERα transcriptional activity.
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Introduction

The insulin receptor substrate 1 (IRS-1) is a major signaling substrate of the insulin receptor (IR) and the insulin-like growth factor I (IGF-I) receptor (IGF-IR) (Yenush and White, 1997; White, 1998; Burks and White, 2001). Aberrant expression of IRS-1 has been associated with pathogenesis of many diseases, including diabetes and cancer (Yenush and White, 1997; White, 1998; Surmacz, 2000; Burks and White, 2001). Activated IRS-1 transmits signals from IGF-IR and IR by sequestering multiple effector molecules and stimulating different signaling pathways, including the PI-3K/Akt and ERK1/2 pathways (Yenush and White, 1997; White, 1998; Burks and White, 2001). In addition to its conventional role as a cytoplasmic signaling molecule, IRS-1 appears to function in the nuclear compartment. Several rigorously controlled studies demonstrated that nuclear IRS-1 can be found in cells transformed by oncogenic proteins, for example, T antigens of the JCV (Lassak *et al*., 2002) and SV40 (Prisco *et al*., 2002) viruses, and v-src (Tu *et al*., 2002). Nuclear translocation of IRS-1 has also been described in mouse embryo fibroblasts stimulated with IGF-I (Tu *et al*., 2002; Sun *et al*., 2003), 32D murine cells (Sciacca *et al*., 2003), osteoblasts (Seol and Kim, 2003), and hepatocytes (Boylan and Gruppuso, 2002). The mechanism by which IRS-1 is targeted to the nucleus is unknown. The observations from different cell models suggested that although IRS-1 contains putative nuclear localization signals (NLSs), it is most likely chaperoned to the nucleus by other proteins, such as viral antigens (Prisco *et al*, 2002; Tu *et al*., 2002). The nuclear localization of IRS-1 requires specific IRS-1 domains, but these requirements appear to be different depending on the experimental system. For instance, in JCV T-antigen-expressing cells, nuclear localization of IRS-1 depends on its pleckstrin homology domain (Lassak *et al*., 2002), while in IGF-I-stimulated cells, the phosphotyrosine binding domain is required (Prisco *et al*., 2002).

The biological relevance of nuclear IRS-1 in various cell backgrounds has yet to be determined. One recent study demonstrated that in mouse embryo fibroblasts stimulated with IGF-I, IRS-1 accumulated in the nucleoli and interacted with the upstream binding factor 1 (UBF1), a regulator of RNA polymerase I (Tu *et al*., 2002). In this cell model, the presence of nucleolar IRS-1 coincided with increased rRNA synthesis (Tu *et al*., 2002).

*Correspondence: E Surmacz, Kimmel Cancer Center, Thomas Jefferson University, 233 S 10th St, BLSB 631, Philadelphia, PA 19107, USA; E-mail: eva.surmacz@jefferson.edu
[6]These two authors contributed equally to this work
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In breast cancer, IRS-1 overexpression has been associated with tumor development, hormone independence, and antiestrogen resistance (Surmacz, 2000). These effects have been attributed to increased tyrosine phosphorylation of IRS-1 and potentiation of its downstream signaling to Akt (Surmacz, 2000; Sachdev and Yee, 2001). In hormone-dependent breast cancer cell lines and breast tumors, the expression of IRS-1 is strongly correlated with estrogen receptor α (ERα), and numerous studies demonstrated that IRS-1 is one of the central elements of ERα/IGF-I crosstalk (Surmacz, 2000; Chan *et al*., 2001; Sachdev and Yee, 2001). It has been well established that ERα can activate IRS-1 transcription acting on IRS-1 promoter (Lee *et al*., 1999; Molloy *et al*., 2000; Mauro *et al*., 2001). In addition, our recent data suggested that unliganded ERα can upregulate IGF-I signaling by decreasing IRS-1 degradation through proteasomal pathways (Morelli *et al*., 2003). The existence of nuclear IRS-1 in breast cancer cells and its possible role in the regulation of gene expression have never been addressed. Here we studied whether 17-β-estradiol (E2) can induce nuclear translocation of IRS-1 and if nuclear IRS-1 can associate with and modulate the action of ERα.

Results

E2 stimulates nuclear translocation of IRS-1 in MCF-7 cells, and nuclear IRS-1 interacts with ERα

The subcellular localization of IRS-1 and ERα was studied in MCF-7 cells stimulated with E2 for different times, from 15 min to 72 h. The images obtained by immunostaining and confocal microscopy are shown in Figure 1. Under serum-free medium (SFM) conditions, IRS-1 was present mainly in the cytoplasm, especially in the perinuclear area, while ERα localized in the nucleus and was weakly detectable in the cytoplasm (Figure 1). In 95% of untreated cells (SFM), colocalization of IRS-1 and ERα was not observed. At 15 min of E2 treatment, the staining of both proteins resembled that at time 0. At 1 and 4 h, 80% of cells displayed weak nuclear IRS-1 staining and strong nuclear expression of ERα. At these time points, nuclear colocalization of ERα and IRS-1 was detectable in ~25% of cells (data not shown). At 8, 24, and 48 h of E2 treatment, ERα was expressed almost exclusively in the nucleus, while IRS-1 was abundant in both cellular compartments (Figure 1). Furthermore, at these time points, evident nuclear colocalization of ERα and IRS-1 was observed in 60–70% of the cells (Figure 1). At 72 h, nuclear presence of IRS-1 became greatly reduced compared with that of earlier time points, while ERα remained nuclear. At this time, colocalization of ERα and IRS-1 was nearly undetectable (data not shown).

The above experiments were repeated several times with reproducible results. The specificity of IRS-1 staining was confirmed with other anti-IRS-1 polyclonal antibodies (pAbs), specifically anti-IRS-1 CT and anti-IRS-1 pre-CT (both from UBI), and pAb C20 (Santa Cruz). The staining was negative when the primary Abs were omitted or blocking peptide was used, as shown by us before in other cell models (Tu *et al*., 2002). In addition, we evaluated the specificity of staining procedures using BT-20 breast cancer cells, which are ERα and IRS-1 negative but express IRS-2 (Figure 1, inset). BT-20 cells were treated for 24 and 48 h with E2 and subjected to the same staining protocol as described for MCF-7 cells. Both IRS-1 and ERα were undetectable in BT-20 cells (Figure 1).

The localization of ERα and IRS-1 was further pursued in subcellular protein fractions. Cytoplasmic and nuclear proteins were obtained from MCF-7 cells

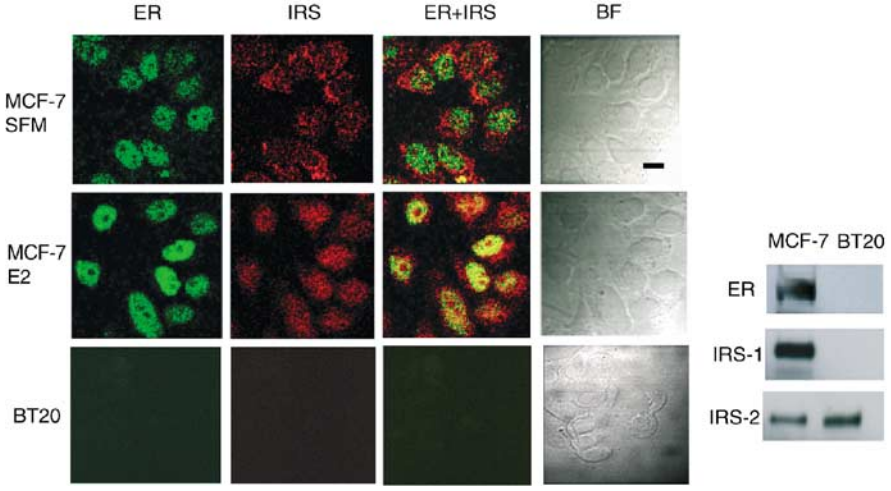


Figure 1 Subcellular localization of IRS-1 and ERα by confocal microscopy. MCF-7 cells synchronized in SFM for 24 h were treated with 10 nM E2 for 24 h (E2) or were left untreated (SFM). BT-20 cells were stimulated with E2 for 24 h. The localization of IRS-1 and ERα was studied by immunostaining and confocal microscopy as detailed in Materials and methods. The captured images of IRS-1 (IRS, red fluorescence), ERα (ER, green fluorescence), merged IRS-1 and ERα (IRS + ER, yellow fluorescence), and bright field (BF) are shown. Scale bar equals 20 μm. Inset: The expression of ERα (ER), IRS-1, and IRS-2 was detected by WB in 50 μg of total protein lysates obtained from growing MCF-7 and BT-20 cells

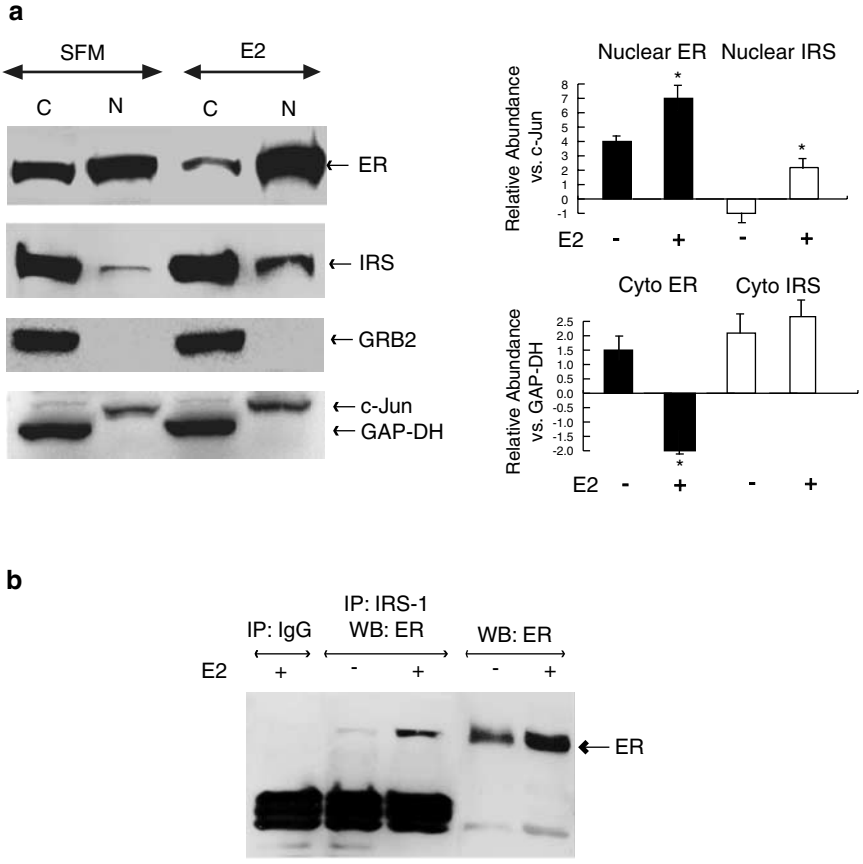


Figure 2 Subcellular localization of ERα and IRS-1 in E2-treated MCF-7 cells. (**a**) MCF-7 cells synchronized in SFM were treated with 10 nM E2 for 24 h (E2) or were left untreated (SFM). The expression of ERα (ER, ~67 kDa), IRS-1 (IRS, ~180 kDa), GRB-2 (~25 kDa), c-Jun (~39 kDa), and GAP-DH (~36 kDa) was assessed by WB in 100 μg of cytoplasmic (C) and nuclear (N) proteins using specific Abs, as described in Materials and methods. The graphs represent relative abundance of nuclear and cytoplasmic (Cyto) ERα and IRS-1 in unstimulated and E2-stimulated cells. Nuclear and cytoplasmic levels of ERα and IRS-1 were normalized to c-Jun and GAP-DH, respectively (relative values = 1). The asterisks indicate statistically significant ($P<0.05$) differences between the amounts in stimulated *vs* unstimulated cells. The results were obtained after repetitive stripping and reprobing of the same filter. (**b**) Nuclear lysates from MCF-7 cells (300 μg) were immunoprecipitated with anti-IRS-1 Ab (CT-IRS-1, UBI) or nonimmune rabbit IgG, and the amounts of ERα in the IPs were probed by WB. A 50 μg portion of nuclear proteins was run in parallel

treated with E2 for 24 h or left untreated. Under SFM conditions, ERα was present in the cytoplasmic and nuclear compartments. As expected, upon E2 treatment the nuclear abundance of ERα significantly increased, while the abundance of the cytoplasmic ERα significantly decreased (Figure 2a). In parallel, E2 stimulation significantly (~ 3.0-fold) upregulated nuclear amounts of IRS-1 (Figure 2). Despite nuclear translocation of IRS-1, its abundance in the cytoplasm remained similar in treated and untreated cells (Figure 2a), which is consistent with the fact that E2 can induce IRS-1 expression (Lee *et al*., 1999; Molloy *et al*., 2000). The expression of two cytoplasmic proteins GRB-2 and GAP-DH, and a nuclear protein c-Jun, was assessed as control of lysate purity (Figure 2a).

Confocal microscopy results suggested nuclear colocalization of IRS-1 and ERα. To confirm this observation, we studied IRS-1 and ERα interactions by immunoprecipitation (IP) and Western blotting (WB) using nuclear protein fractions obtained form MCF-7 cells grown in SFM or treated with E2 for 24 h (Figure 2b). ERα was found in IRS-1 immunoprecipitates in treated and untreated cells, with greater abundance of ERα/IRS-1 complexes in E2-stimulated cells (Figure 2b). Similarly, IRS-1 co-precipitated with nuclear ERα under E2 treatment (data not shown). Comparison of ERα content in total nuclear proteins *vs* IRS-1-associated proteins suggested that only a fraction (~10%) of ERα co-precipitates with IRS-1.

IRS-1 is recruited to the ERE-containing pS2 promoter in MCF-7 cells

Nuclear colocalization and co-precipitation of ERα and IRS-1 suggested that both molecules could be recruited to the same regulatory sequences in DNA. The binding of ERα and IRS-1 to the estrogen-responsive element (ERE)-containing domain of the pS2 gene promoter was assessed with chromatin immunoprecipitation (ChIP) and reverse ChIP (Re-ChIP) assays (Figure 3). First, we tested the dynamics of ERα association with the pS2 promoter sequences. Soluble chromatin obtained from

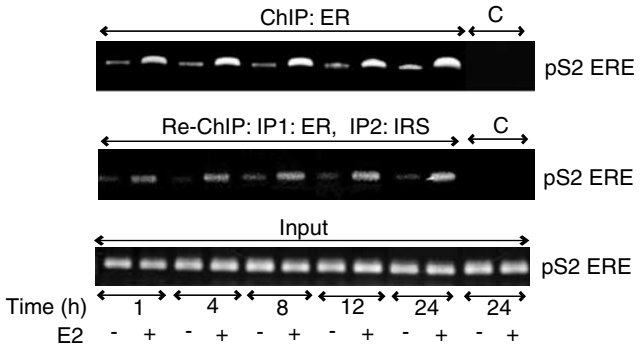


Figure 3 Time course of ERα and IRS-1 association with the pS2 promoter. Soluble chromatin was isolated from MCF-7 cells stimulated with E2 for 1, 4, 8, 12, and 24 h, and from untreated cells at the same time points. ERα ChIP (ChIP: ER) experiments were performed as described in Materials and methods. IRS-1 Re-ChIPs were obtained from ERα ChIP samples after reprecipitation with IRS-1 Abs (Re-ChIP: IP1: ER, IP2: IRS). DNA recovered from the immune complexes was tested for the presence of the ERE-containing pS2 promoter sequences (pS2 ERE) by PCR with specific primers listed in Materials and methods. PCR products obtained after 35 cycles are shown. ChIP pellets obtained using nonimmune IgG were analysed as controls of Ab specificity (C). The abundance of the pS2 promoter sequences in all samples before IP is shown as control of input DNA (Input)

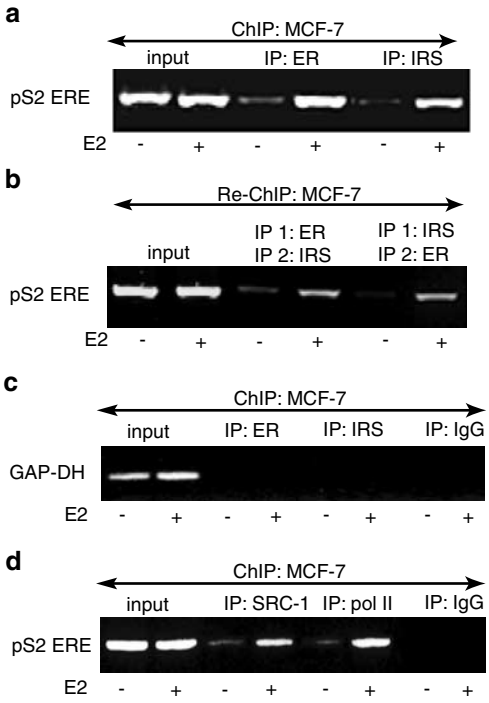


Figure 4 Association of the ERα:IRS-1 complex, SRC-1, and pol II with the pS2 promoter. MCF-7 cells were stimulated with E2 for 24 h, and the presence of the pS2 promoter sequences (pS2 ERE) in (**a**) ERα and IRS-1 ChIPs, (**b**) ERα:IRS-1 direct and reverse Re-ChIPs, and (**d**) SRC-1 and pol II ChIPs was detected by PCR as described in Materials and methods. The occupancy of ERα and IRS-1 on the GAP-DH promoter (not regulated by E2) was tested with specific GAP-DH primers in ERα and IRS-1 ChIP preparations (**c**). ChIP with nonimmune IgG was used as a control (IP: IgG)

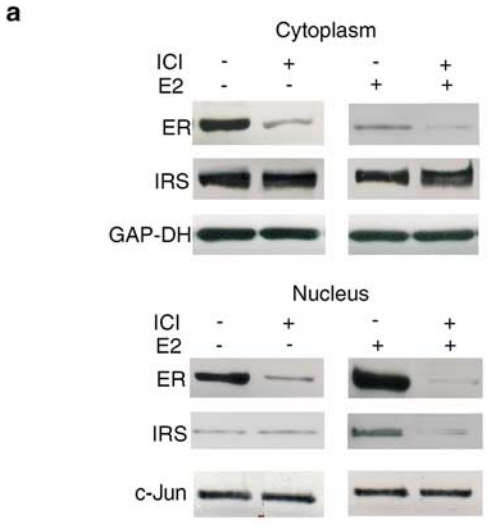
MCF-7 cells untreated or treated with E2 for 1, 4, 8, 12, and 24 h was immunoprecipitated with anti-ERα Abs and the presence of pS2 promoter DNA in ERα precipitates was detected by PCR. As illustrated in Figure 3, E2 treatment increased ERα occupancy on the pS2 promoter at all time points. The association of ERα with pS2 DNA was maximal at 24 h after E2 addition (~3.5-fold increase *vs* untreated) (Figure 3).

To test whether IRS-1 belongs to the ERα multi-complex recruited to the pS2 promoter, we performed Re-ChIP experiments, following the protocol described for ERα interacting proteins (Reid *et al*., 2003). In our Re-ChIP experiments, the original ERα ChIP pellets were eluted and precipitated with IRS-1 Abs and the pS2 promoter sequences were detected in IRS-1 Re-ChIPs by PCR. We found pS2 DNA in IRS-1 Re-ChIPs at all time points, which indicated that IRS-1 and ERα belong to the same protein complex, and that the complex is associated with the ERE-pS2 promoter in E2-stimulated MCF-7 cells. Notably, the greatest amounts of pS2 DNA in IRS-1 Re-ChIPs were present in cells stimulated with E2 for 24 h (Figure 3).

To extend the above observations, we examined the presence of other regulatory proteins in ERα transcriptional complexes in MCF-7 cells stimulated with E2 for 24 h. Figure 4 illustrates pS2 promoter occupancy by two proteins known to regulate ERα-dependent transcription, ERα coactivator SRC-1 and polymerase II (pol II). In parallel, the association of ERα and IRS-1 under the same conditions was assessed by ChIP and two-way Re-ChIP assays. The results confirmed that E2 stimulates the recruitment of the ERα:IRS-1 complex to the pS2 promoter in MCF-7 cells (Figure 4a and b). In the same experiment, neither ERα nor IRS-1 was recruited to the GAP-DH promoter that is not regulated by ERα (Metiver *et al*., 2002) (Figure 4c). Notably, the association of ERα and IRS-1 with pS2 DNA coincided with the recruitment of SRC-1 and pol II to the same promoter (Figure 4d).

Absence of ERα blocks nuclear IRS-1 translocation

The role of ERα in the nuclear translocation of IRS-1 was probed in MCF-7 cells pretreated with the antiestrogen ICI 182,780 (ICI) for 6 h (Figure 5). This treatment has been chosen based on preliminary tests establishing the dynamics of ICI-dependent downregulation of ERα and IRS-1. IRS-1 is a stable protein with a half-life of ~10 h (Morelli *et al*., 2003) and only a long-term ICI treatment (48–74 h) can substantially decrease its levels (Salerno *et al*., 1999), while short-term ICI exposure is sufficient to degrade ERα (Reid *et al*., 2003). Indeed, a 6 h ICI treatment dramatically reduced cytoplasmatic and nuclear ERα expression without affecting IRS-1 levels (Figure 5a and b). In ICI-pretreated cells, E2 did not stimulate nuclear translocation of IRS-1, as demonstrated by WB (Figure 5a) and confocal microscopy (Figure 5b). However, E2 induced nuclear translocation of IRS-1 in untreated cells expressing normal ERα levels (Figures 1, 2 and 5a). Low amounts of nuclear IRS-1 were found under SFM



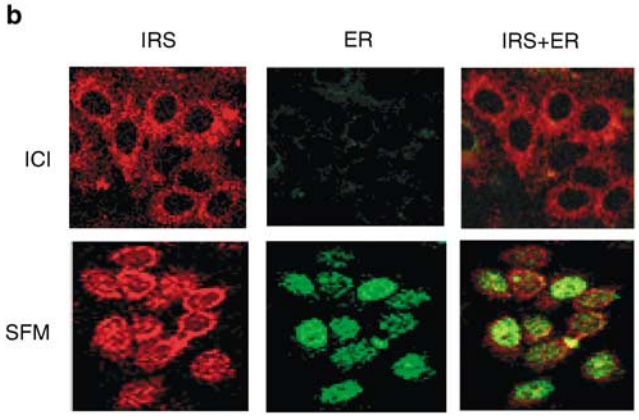


Figure 5 Effects of ERα downregulation on nuclear translocation of IRS-1. MCF-7 cells synchronized in SFM were pretreated with 10 nM ICI 182,780 for 6 h, and then stimulated with 10 nM E2 for 24 h or left untreated in SFM (**a**). The levels of IRS-1 (IRS) and ERα (ER) were detected in 50 μg of cytoplasmatic and nuclear proteins with specific Abs, as described in Materials and methods. The results were obtained after repetitive stripping and reprobing of the same filter. (**b**) MCF-7 cells were pretreated with ICI and then stimulated with E2 or left in SFM as described in (**a**). The localization of IRS-1 (IRS) and ERα (ER) was studied by confocal microscopy, as described in Figure 1. Scale bar equals 50 μm

conditions, possibly reflecting IRS-1 translocation induced by basal ERα activity (Figures 1, 2, 5a and b).

IRS-1 does not associate with the pS2 promoter in ERα-negative MDA-MB-231 cells

The requirement of ERα for E2-dependent nuclear translocation of IRS-1 was further investigated with MDA-MB-231 and MDA-MB-231/ER breast cancer cells (Figure 6). MDA-MB-231 cells are ERα negative but express IRS-1 on a level similar to that found in MCF-7 cells (Bartucci *et al*., 2001; Morelli *et al*., 2003). MDA-MB-231/ER cells have been developed in our laboratory by stable transfection of MDA-MB-231 cells with an ERα expression vector (Morelli *et al*., 2003). The association of ERα and IRS-1 with the pS2 promoter

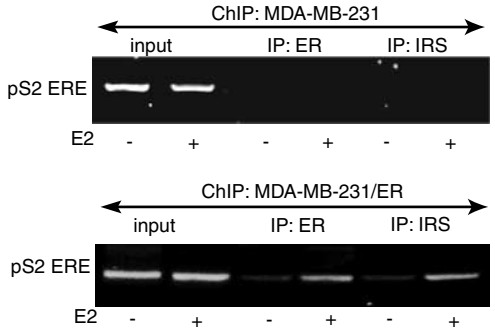


Figure 6 Recruitment of ERα and IRS-1 to the pS2 gene promoter in MDA-MB-231 and MDA-MB-231/ER cells. MDA-MB-231 and MDA-MB-231/ER cells were stimulated with E2 for 24 h or were left untreated in SFM. The presence of ERα and IRS-1 on the pS2 promoter (pS2 ERE) was detected by ChIP assays, as described in Materials and methods

was studied in both cell lines by ChIP assays. In MDA-MB-231 cells, neither ERα nor IRS-1 was found on the pS2 promoter. However, reintroduction of ERα allowed the association of both molecules with pS2 ERE sequences in response to E2 treatment (Figure 6).

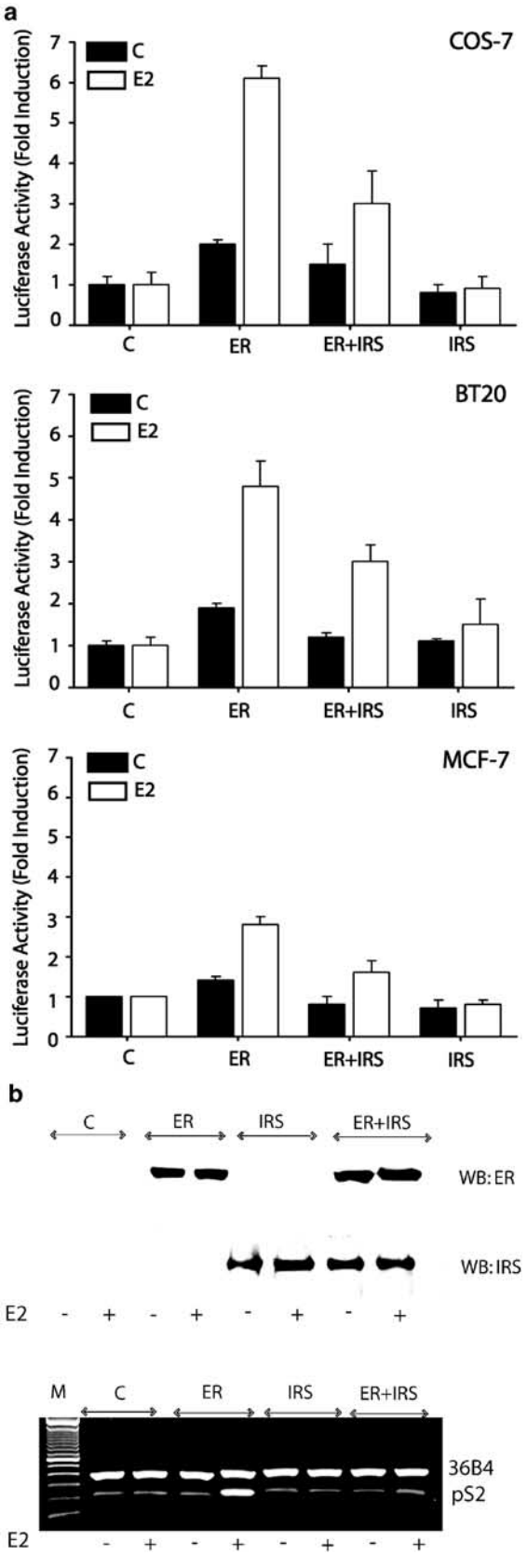
IRS-1 modulates ERα transcriptional activity

Because IRS-1 and ERα are recruited to E2-sensitive promoters, we tested whether the presence of IRS-1 may affect ERα transcriptional activity at ERE sites. This possibility was examined with transactivation assays employing an ERE reporter plasmid. The plasmid, ERE-Luc, was transiently transfected into cells either together with ERα expression vector only or with a mixture of ERα and IRS-1 expression plasmids. The transfected cells were left untreated or were treated with E2 for 24 h (Figure 7).

To assess E2-dependent transcription in a controlled environment, we used ERα- and IRS-1-negative COS-7 and BT-20 cells (Figures 1 and 7b), which allowed measurements of transcriptional activity in the presence or absence of studied molecules. In addition, transactivation experiments were performed in MCF-7 cells expressing endogenous IRS-1 and ERα.

The transactivation assays indicated that the presence of IRS-1 significantly decreased ERα activation of ERE promoters in all cell lines stimulated with E2 (Figure 7a). Specifically, in COS-7, BT-20, and MCF-7 cells, cotransfection of IRS-1 reduced ERα activation of ERE by ~50, ~39, and ~44%, respectively. The transfection of IRS-1 alone did not stimulate ERE transcription (Figure 7a).

In addition to ERE reporter assays with reporter plasmids, we assessed transcriptional activation of the pS2 gene in COS-7 cells transfected with either ERα, IRS-1, or a combination of ERα plus IRS-1. COS-7 cells were selected for this assay as they proved to be the most E2-responsive and the best transfectable cell model (Figure 7a). The levels of pS2 mRNA in COS-7 cells transfected with different plasmids were studied using



RT–PCR (Figure 7b). The amounts of a constitutively expressed 36B4 mRNA were assessed in the same samples. The results suggested that E2 stimulated pS2 mRNA expression (~3-fold) in ERα-transfected cells, compared with vector-only-transfected cells. This effect of E2 was significantly reduced in cells cotransfected with ERα and IRS-1, confirming the trend observed in ERE luciferase reporter assays. Notably, ERα expression was similar in the 'ER' and 'ER + IRS' populations, ruling out the possibility that differences in pS2 transcription were related to unequal ERα expression (Figure 7b).

Discussion

The interactions between IGF-IR and ER signaling systems have been implicated in the development of the neoplastic phenotype in mammary epithelial cells (Surmacz, 2000; Yee and Lee, 2000; Sachdev and Yee, 2001). In this context, IRS-1, a molecule that activates multiple growth and survival pathways, has been found to be one of the central elements of IGF-IR/ER crosstalk. Several reports documented that E2 can increase IRS-1 transcription, while ICI inhibits IRS-1 mRNA levels (Surmacz, 2000; Yee and Lee, 2000; Sachdev and Yee, 2001). Furthermore, the expression of ERα seems to stabilize IRS-1 protein and potentiate IRS-1 signaling through the PI-3K/Akt pathway (Morelli *et al*., 2003). In turn, the activation of IRS-1/PI-3K/Akt by growth factors can stimulate ERα by increasing its phosphorylation (Lannigan, 2003). In MCF-7 cells, overexpression of IRS-1 has been shown to induce estrogen independence (Surmacz and Burgaud, 1995), while downregulation of IRS-1 resulted in increased sensitivity to E2 (Ando *et al*., 1998).

Here we report on a novel aspect of ERα/IGF-I crosstalk involving nuclear ERα/IRS-1 interactions. Specifically, we demonstrated that (1) in MCF-7 cells, IRS-1 can be translocated from the cytoplasm to the nucleus following E2 treatment; (2) nuclear transloca-

Figure 7 Effects of IRS-1 on ERα transcriptional activity at ERE promoters. (**a**) Transactivation assays. The transcriptional activity of ERα on ERE promoters in the presence or absence of IRS-1 was evaluated using luciferase reporter system, as described in Materials and methods. COS-7, BT-20, and MCF-7 cells were transfected with DNA mixtures containing ERE reporter plasmid alone (C), ERE + plasmid pHEGO encoding ERα (ER), ERE + ER + plasmid encoding IRS-1 (ER + IRS), or ERE + IRS-1 (IRS). The activity of the ERE promoter in each experimental setting is represented by relative Luc units. The results are means ± s.e. from several experiments. In all experimental systems, the difference between E2-stimulated Luc activities in ER *vs* ER + IRS transfectants was statistically significant ($P < 0.05$). (**b**) Effect of IRS-1 expression on pS2 mRNA levels. Upper panel: COS-7 cells were transfected with different plasmids and stimulated with E2 or left untreated, as described above. ERα and IRS-1 expression in transfected cells was detected by WB in 50 μg of total protein lysates. Lower panel: The abundance of pS2 and 36B4 mRNAs in COS-7 cells transfected with different plasmids was detected by RT–PCR, as described in Materials and methods

tion of IRS-1 is blocked with ICI and does not occur in ERα-negative cells; (3) nuclear IRS-1 interacts with ERα; (4) nuclear IRS-1 is corecruited with ERα to the ERE-containing pS2 promoter; and (5) the presence of IRS-1 decreases ERα transcription at ERE promoters.

Nuclear localization of IRS-1 has recently been described in different cellular systems (Lassak *et al*., 2002; Prisco *et al*., 2002; Sun *et al*., 2003; Tu *et al*., 2002; Sciacca *et al*., 2003). The mechanism by which IRS-1 enters cell nucleus is still not clear. Although IRS-1 contains putative NLS, it is thought that IRS-1 is chaperoned to the nucleus by other proteins, for instance, by T antigens of the SV40 and JC viruses (Lassak *et al*., 2002). The transporting molecules involved in IGF-IR-dependent IRS-1 nuclear translocation are yet unknown.

In our experimental system, E2-dependent nuclear translocation of IRS-1 and its interaction with the pS2 promoter were totally blocked when ERα was down-regulated by ICI (Figure 5) and did not occur in MDA-MB-231 cells that are ERα negative but express ERα (Vladusic *et al*., 2000) (Figure 6). However, re-expression of ERα allowed association of IRS-1 with the pS2 sequences. These observations suggest that nuclear function of IRS-1 in response to E2 requires ERα. Notably, a small amount of nuclear IRS-1 was found in unstimulated MCF-7 cells, which could result from basal ERα activity.

The prerequisite for nuclear translocation of IRS-1 in response to E2 is most likely the formation of the ERα:IRS-1 complex in the cytoplasm. ERα association with cytoplasmic signaling molecules is not unusual. Recently, ERα has been shown to bind the PI-3K/Akt complex (Simoncini *et al*., 2000; Sun *et al*., 2001), and to interact with growth factor receptor docking protein Shc (Song *et al*., 2002) as well as with IGF-IR (Kahlert *et al*., 2000). Similarly, we reported that unliganded ERα can associate with cytoplasmic IRS-1 in MDA-MB-231/ER cells (Morelli *et al*., 2003). Our preliminary data suggest that ERα/IRS-1 binding involves at least two different IRS-1 domains, and does not depend on IRS-1 tyrosine phosphorylation (Surmacz *et al*., unpublished data).

Our previous findings (Morelli *et al*., 2003) and this report suggest that only a fraction of ERα binds to IRS-1 (~10% of nuclear ERα) (Figure 2b), according to rough estimations based on coprecipitation procedures. However, if the linkage between ERα and IRS-1 is labile, coprecipitation assays might underestimate the actual extent of their association. In fact, the results obtained with confocal microscopy in intact cells suggested that in some cells, ~30% of ERα colocalized with IRS-1. Because only a fraction of ERα associates with IRS-1, it is understandable that nuclear accumulation of IRS-1 upon E2 stimulation might occur slower than that of ERα. The nuclear presence of IRS-1 in E2-treated MCF-7 cells was limited to ~72 h, while ERα remained nuclear for longer times. It needs to be discovered whether IRS-1 disappearance from the nucleus is caused by proteolysis or by translocation to the cytoplasmic compartment.

In this work, we report for the first time that nuclear IRS-1 can interact with ERα on ERE-containing chromatin regions. In our experimental system, IRS-1 was recruited together with ERα and other proteins involved in ERα transcription (SRC-1 and pol II) to the pS2 promoter (Figures 3, 4, and 6). The possibility that IRS-1 modulates ERα-dependent transcription was addressed with transient transfection reporter assays. With this methodology, we noted inhibition of ERα activity by IRS-1 in several cell lines (Figure 7a). We also found that overexpression of IRS-1 inhibits E2/ERα-dependent transcription of the endogenous pS2 gene in COS-7 cells (Figure 7b). A hypothetical model explaining the inhibitory effect of IRS-1 could be proposed on the basis of the recent discovery that ERα-dependent transcription from ERE sites requires cyclic proteasomal degradation of ERα (Reid *et al*., 2003). Because IRS-1 and ERα compete for the same degradation machinery (Morelli *et al*., 2003), it is possible that the presence of nuclear IRS-1 interferes with ERα proteolysis, and thus with ERα transcription.

IRS-1 modification of ERα activity is probably restricted to certain transcriptional complexes, as we did not observe significant effects of IRS-1 on ERα-dependent transcription at AP-1 sites. In addition, our new data suggest that the association of IRS-1 with ERE promoters can be transiently inhibited by its recruitment to activated IGF-I receptors (Surmacz *et al*., unpublished data).

The presence of nuclear IRS-1 in cellular systems needs to be further evaluated in human clinical material. The expression of nuclear IRS-1 in primary breast tumors has been reported by Schnarr *et al*. (2000), but the authors did not speculate on the biological relevance of this phenomenon. Our preliminary data confirmed that nuclear IRS-1 can be detected in mammary tissue sections and that its expression correlates with ERα (data not shown). Larger studies evaluating the correlations of cytoplasmatic and nuclear IRS-1 with other tumor markers are underway in our laboratory.

In summary, our data suggest that IRS-1 can interact with ERα in the nucleus of breast cancer cells and modulate ERα transcriptional activity. We postulate that nuclear ERα/IRS-1 interactions represent a new paradigm in IGF-IR/ER crosstalk.

Materials and methods

Cell lines

MCF-7, MDA-MB-231, BT-20, and COS-7 cells were obtained from ATCC. MDA-MB-231 cells stably expressing ERα have been developed in our laboratory (Morelli *et al*., 2003).

Cell culture

MCF-7 and MDA-MB-231 cells were grown in DMEM:F12 containing 5% calf serum (CS). MDA-MB-231/ER cells were grown in DMEM:F12 plus 5% CS plus 0.05 mg/ml

G418. BT-20 cells were grown in DMEM:F12 with 10% fetal bovine serum (FBS). COS-7 and HeLa cells were grown in DMEM supplemented with 10% FBS. In the experiments requiring E2- and serum-free conditions, the cells were cultured in phenol red-free SFM (Guvakova and Surmacz, 1997).

Cell treatments

E2 (Sigma) and the antiestrogen ICI 182,780 (Tocris Cookson) were used at a concentration of 10 nM.

Detection of IRS-1 and ERα by confocal microscopy

Confluent cultures (50%) grown on coverslips were fixed in 3% paraformaldehyde, permeabilized with 0.2% Triton X-100, washed three times with PBS, and incubated for 1 h with a mixture of primary Abs (pAbs) recognizing IRS-1 and ERα. The anti-IRS-1 CT pAb (UBI) or anti-IRS-1 pre-CT pAb (UBI) at 2 μg/ml was used for IRS-1 staining; F-10 monoclonal Ab (mAb) (Santa Cruz) at 2 μg/ml was used to detect ERα. Following the incubation with primary Abs, the slides were washed three times with PBS, and incubated with a mixture of secondary Abs. A fluorescein-conjugated donkey anti-mouse IgG (Calbiochem) was used as a secondary Ab for ERα and a rhodamine-conjugated donkey anti-rabbit IgG (Calbiochem) was used for IRS-1. The cellular localization of IRS-1 and ERα was studied with Bio-Rad MRC 1024 confocal microscope connected to a Zeiss Axiovert 135M inverted microscope with ×600 magnification. The optical sections were taken at the central plane. The fluorophores were imaged separately to ensure no excitation/emission wavelength overlap. In control samples, the staining was performed with the omission of the primary Abs.

Immunoprecipitation and Western blotting

The cytoplasmic and nuclear proteins were obtained from 70% cultures. The cytoplasmic lysis buffer contained the following: 50 mM HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100, 1.5 mM $MgCl_2$, EGTA 10 mM pH 7.5, glycerol 10%, inhibitors (0.1 mM Na_3VO_4, 1% PMSF, 20 μg/ml aprotinin). Following the collection of cytoplasmic proteins, the nuclei were lysed with the buffer containing 20 mM HEPES pH 8, 0.1 mM EDTA, 5 mM $MgCl_2$, 0.5 M NaCl, 20% glycerol, 1% NP-40, inhibitors (as above). A 25–50 μg portion of protein lysates was used for WB, while 500 μg was used for IP. The following mAbs and pAbs were employed: anti-IRS-1 CT pAb (UBI) for WB and IP; anti-ERα F-10 mAb (Santa Cruz) for WB and IP; anti-GAP-DH mAb (Research Diagnostics Inc.) for WB, anti-c-Jun mAb (Santa Cruz) for WB, anti-GRB2 mAb (Transduction Laboratories) for WB.

In all IPs, protein lysates were first incubated with primary Abs at 4°C for 4 h in HNTG buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.1 mM Na_3VO_4), and then immune complexes were precipitated for 1 h with appropriate beads, specifically with protein A agarose (Calbiochem) for IPs with polyclonal Abs and anti-mouse IgG agarose (Sigma) for IPs with mouse mAbs. In control samples, the primary Abs were substituted with nonimmune IgGs (rabbit or mouse, depending on the source of the primary Abs). The immunoprecipitated proteins were washed three times with HNTG buffer, separated on a 4–15% polyacrylamide denaturing gel, analysed by WB, and visualized by ECL chemiluminescence (Amersham). The intensity of bands representing relevant proteins was measured by Scion Image laser densitometry scanning program.

Luciferase reporter assays

The experiments were performed using COS-7, BT-20, and MCF-7 cell lines. The cells were grown in 24-well plates. At 70% confluence, the cultures were transfected for 6 h with 0.5 μg DNA/well using Fugene 6 (Roche) (DNA : Fugene 3 : 1). All transfection mixtures contained 0.3 μg of the reporter plasmid, ERE-Luc, encoding the firefly luciferase (Luc) cDNA under the control of the TK promoter and three ERE sequences. ERE-Luc was cotransfected with the ERα expression vector pSG5-HEGO, alone or in combination with the IRS-1 expression vector pCMV-IRS-1. To maintain the same DNA input in all transfection mixtures, the samples were adjusted with an empty vector (pcDNA3 or pSG5). In addition, to assess transfection efficiency, each of the DNA mixtures contained 50 ng of pRL-TK-Luc, a plasmid encoding *Renilla* luciferase (RI Luc) (Promega). Upon transfection, the cells were shifted to SFM for 12 h and then treated with 10 nM E2 for 24 h, or left untreated in SFM. Luciferase activity (Luc and RI Luc) in cell lysates was measured using Dual Luciferase Assay System (Promega) following the manufacturer's instructions. The values obtained for Luc were normalized to that of RI Luc to generate relative Luc units representing ERE-dependent transcription.

Chromatin immunoprecipitation

We followed ChIP methodology described by Shang *et al*. (2000) with minor modifications. MCF-7, MDA-MB-231, and MDA-MB-231/ER cells were grown in 100 mm plates. Confluent cultures (90%) were shifted to SFM for 24 h and then treated with 10 nM E2 for 1–24 h, or left untreated in SFM. Following treatment, the cells were washed twice with PBS and crosslinked with 1% formaldehyde at 37°C for 10 min. Next, the cells were washed twice with PBS at 4°C, collected and resuspended in 200 μl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1) and left on ice for 10 min. Then, the cells were sonicated four times for 10 s at 30% of maximal power (Fisher Sonic Dismembrator) and collected by centrifugation at 4°C for 10 min at 14 000 rpm. The supernatants were collected and diluted in 1.3 ml of IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 16.7 mM NaCl) followed by immunoclearing with 80 μl of sonicated salmon sperm DNA/protein A agarose (UBI) for 1 h at 4°C. The precleared chromatin was immunoprecipitated for 12 h with specific Abs, specifically anti-ERα C-terminus mAb F-10 (Santa Cruz) for ERα, and anti-IRS-1 C-terminus pAb (UBI) for IRS-1, anti-pol II CTD4H8 mAb for pol II (UBI), and anti-SRC1 1135 mAb for SRC1 (UBI). After this, 60 μl of salmon sperm DNA/protein A agarose was added and precipitation was continued for 2 h at 4°C. After pelleting, precipitates were washed sequentially for 5 min with the following buffers: Wash A (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), Wash B (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), and Wash C (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1), and then twice with TE buffer (10 mM Tris, 1 mM EDTA). The immune complexes were eluted with elution buffer (1% SDS, 0.1 M $NaHCO_3$). The eluates were reverse crosslinked by heating at 65°C for 12 h and digested with proteinase K (0.5 mg/ml) at 45°C for 1 h. DNA was obtained by phenol and phenol/chloroform extractions. A 2 μl portion of 10 mg/ml of yeast tRNA was added to each sample and DNA was precipitated with EtOH for 12 h at 4°C and then resuspended in 20 μl of TE buffer. A 5 μl volume of each sample was used for PCR with pS2 promoter primers

flanking ERE-containing pS2 promoter fragment: upstream 5′-GATTACAGCGTGAGCCACTG-3′, and downstream 5′-TGGTCAAGCTACATGGAAGG-3′. The primers for GAPDH promoter were 5′-GCTACTAGCGGTTTTACGGG-3′ (forward) and 5′-AAGATGCGGCTGACTGTCGAA-3′ (reverse). The PCR conditions were 45 s at 94°C, 40 s at 58°C, and 90 s at 72°C. The amplification products obtained in 25 and 35 cycles were analysed in a 2% agarose gel and visualized by ethidium bromide staining. The intensity of bands representing relevant proteins was measured by Scion Image laser densitometry scanning program. In control samples, non-immune IgG (rabbit for IRS-1 Abs and mouse for all other Abs, Santa Cruz) was used instead of the primary Abs.

Reverse ChIP

We followed the methodology described by Reid *et al*. (2003). The pellets obtained by IP of soluble chromatin with IRS-1 and ERα Abs were eluted with 500 μl of Re-ChIP buffer (0.5 mM DTT, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8.1). Next, the eluate from ERα IP was precipitated with IRS-1 pAb (UBI) and the eluate from IRS-1 IP was precipitated with ERα mAb (Santa Cruz). The presence of the pS2 promoter sequences in the resulting Re-ChIP pellets was examined as described above for one-step ChIP.

RT–PCR

COS-7 cells were transfected with different plasmids for 24 h, as described in the transactivation assays methodology. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. A 5 μg portion of total RNA was reverse transcribed (RT) at 37°C for 30 min in 20 μl of buffer containing 200 U of M-MLV reverse transcriptase (Promega), 0.4 μg oligo-dT, 0.5 μM deoxynucleotide triphosphate (dNTP), and 24 U RNAsin. The reaction was terminated by heat denaturation for 5 min at 95°C.

A 2 μl portion of RT products was used to simultaneously amplify pS2 and 36B4 (control) DNA sequences. The pS2 cDNA fragment (210 bp) was amplified using the following primers: 5′-TTCTATCCTAATACCATCGACG-3′ (forward) and 5′-TTTGAGTAGTCAAAGTCAGAGC-3′ (reverse). The 408 bp fragment of the 36B4 ribosomal phosphoprotein DNA was amplified with the following primers: 5′-CTCAACATCTCCCCCTTCTC-3′ (forward) and 5′-CAAATCCCATATCCTCGTCC-3′ (reverse) (Maggiolini *et al*., 2001). The PCR amplification was performed using 1.25 U GoTaq DNA polymerase (Promega), 1 × PCR buffer (10 mM Tris-HCl, 50 mM KCl), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 1.5 pM of primers for pS2 or 0.5 pM primers for 36B4. PCR conditions were 30 s at 94°C, 40 s at 59°C, and 60 s at 72°C for 30 cycles. PCR products (10 μl) were separated on a 1.2% agarose gel.

Statistical analysis

Student's *t*-test was used to analyze WB and transactivation data. Statistical significance was assumed at $P<0.05$.

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E. R. Sauter
C. Garofalo
J. Hewett
J. E. Hewett
C. Morelli
E. Surmacz

Leptin Expression In Breast Nipple Aspirate Fluid (NAF) and Serum Is Influenced By Body Mass Index (BMI) But Not by the Presence of Breast Cancer

Abstract

While obesity is a known risk factor for postmenopausal breast cancer, the molecular mechanisms involved are unclear. Systemic levels of leptin, the product of the *ob* (obesity) gene, are increased in obese individuals (body mass index, BMI, over 25) and are higher in women than men. Leptin has been found to stimulate the growth of breast cancer cells *in vitro*. Our goal was to determine whether leptin was 1) present in nipple aspirate fluid (NAF), and 2) whether NAF leptin levels were associated with a) levels in serum, b) obesity, and c) breast cancer. We collected and evaluated NAF specimens from 83 subjects and serum specimens from 49 subjects. NAF leptin was detectable in 16/41 (39%) of premenopausal and 21/42 (50%) postmenopausal subjects. NAF leptin was significantly lower ($p = 0.042$) in premenopausal than postmenopausal women with a BMI < 25, but not in those with a higher BMI. NAF leptin was significantly associated with BMI in premenopausal ($p = 0.011$) but not in postmenopausal women. Serum leptin was associated with BMI in both premenopausal and postmenopausal women ($p = 0.0001$ for both). NAF and serum leptin were associated in premenopausal ($p = 0.02$) but not postmenopausal women. Neither NAF nor serum leptin was associated with premenopausal or postmenopausal breast cancer. Our findings include that 1) leptin is present in the breast and detectable in a subset of NAF samples, 2) NAF leptin in premenopausal but not postmenopausal women parallels serum leptin levels, and 3) neither NAF nor serum levels of leptin were associated with premenopausal or postmenopausal breast cancer.

Key words
Obesity · Premenopause · Postmenopause · Protein concentration · Leptin release

Abbreviations: BCA, bicinchoninic acid; BMI, body mass index; DCIS, ductal carcinoma *in situ*; ELISA, enzyme linked immunosorbent assay; IBC, invasive breast cancer; IRB, institutional review board; NAF, nipple aspirate fluid; ob, obesity; OB-R, obesity receptor; OB-Rs, short form of the obesity receptor

Introduction

Leptin, the product of the *ob* gene, is a 16 kDa cytokine acting as a circulating satiety factor [1] concentrated in adipose tissue. The expression of leptin is 2 – 3 times as high in women as in men [2]. This is likely both because women have a higher percentage of subcutaneous fat [3] and because leptin expression may be affected by female sex hormones [2]. Leptin mRNA is detectable in human mammary epithelial cells, and immunoreactive leptin has

Affiliation
Ellis Fischel Cancer Center, University of Missouri-Columbia, Columbia, MO [E.R.S., J.H., J.E.H.] and Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA [C.G., C.M., E.S.]

Correspondence
E. R. Sauter · University of Missouri – Columbia · One Hospital Drive · Rm 588 · Columbia · MO 65212 · Phone: +1(573)8824471 · Fax: +1(573)8844585 · E-Mail: sautere@health.missouri.edu

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been found in breast milk [4,5]. Leptin is considered a mammary hormone that may induce differentiation of breast cells [6].

The association between leptin and breast cancer has been addressed by recent studies. *In vitro* experiments demonstrate that estrogen receptor-positive MCF-7 [7] and T47-D [8] cells express leptin receptors and are stimulated to proliferate by leptin. In clinical studies, leptin has been directly associated with breast cancer in one report [9], and inversely associated with premenopausal breast cancer in another [10]; no association was found in the third report [11].

Obesity seems to protect premenopausal women and increase a postmenopausal woman's breast cancer risk [12]. The ideal method of evaluating the role of leptin in the breast should be non-invasive and organ-specific. Nipple aspiration, which provides NAF, is organ-specific (unlike plasma or serum), causes minimal or no discomfort, is non-invasive and provides both cells and extracellular fluid from the breast ductal epithelium, the cells which give rise to cancer. NAF can be reliably collected from both premenopausal and postmenopausal subjects [13] and biological markers measured in the fluid using as little as 1 µl of sample [14].

Material And Methods

Subjects

Informed consent was obtained from 91 subjects using a protocol approved by the Institutional Review Board. Eighty-two enrolled subjects had NAF samples collected in the breast evaluation clinics at our institutions. Forty-nine of the subjects provided blood for serum leptin analysis, including nine who did not have nipple aspiration attempted. The median age was 47 (range 23–81) years. Forty (49%) subjects who provided NAF were premenopausal. Women who had nursed or been pregnant within two years before enrollment were excluded. Subjects provided their height and weight as part of a comprehensive breast health history questionnaire. Women belonging to any breast cancer risk category were enrolled, including women with breast cancer, those with an abnormal mammogram or a palpable mass, or women undergoing nipple aspiration without breast cancer concerns. Women who required diagnostic breast surgery had NAF and serum samples obtained just prior to (within three weeks) or on the same day as definitive surgery to treat their cancer. Women that had received prior radiation to the breast from which NAF was collected were also excluded from the study.

Specimen collection and preparation

Nipple fluid was aspirated from the breast by a trained physician or nurse clinician using a modified breast pump [15]. Samples were collected into capillary tubes and stored at – 80 °C until use; 8 ml of blood was also collected if the subject agreed, and stored after serum separation at – 80 °C until use.

Leptin analysis

NAF and serum leptin were measured using a human leptin ELISA kit (Linco Research, St. Charles, MO) following the manufacturer's instructions. Total protein concentration in NAF was assessed by the BCA method (Pierce Chemicals, Rockford, IL). Pure NAF samples were adjusted to 100 µl with PBS. Leptin was measured in 50 µl of adjusted samples in duplicate. Leptin in serum was analyzed in pure form. Several NAF and serum samples were measured repeatedly to determine the interassay variation. The interassay variation was < 9% for NAF and < 12% for serum.

Statistical analysis

Since the data were not normally distributed, ranking procedures were used for all analyses with continuous variables. Data were analyzed using all NAF leptin results, including those below the level of detection, which were considered as zero. To compare leptin expression between the premenopausal and postmenopausal groups, the Wilcoxon Rank Sum Test or Fisher's Exact Test was used. Spearman Rank Correlations were calculated to measure the association between leptin expression and BMI and between NAF leptin and serum leptin.
Where multiple samples contained detectable leptin, the median value of these samples was used for our analyses. In all BMI analyses, controlling for total NAF protein identified a stronger association than when total protein was not controlled for. For this reason, all Spearman's calculations are reported as NAF leptin/mg total protein.

Results

Leptin is measurable in NAF and serum

Leptin was measurable in the NAF of 44.6% (37/82) of subjects (Table **1**), 39% of premenopausal and 50% of postmenopausal women. When comparing premenopausal and postmenopausal women based on BMI (0 – 24.99, 25 – 29.99, and > = 30), NAF leptin levels were lower (p = 0.042) in premenopausal than postmenopausal women for the lowest but not for the intermediate (p = 0.33) or highest (p = 0.60) BMI groups. The ability to detect leptin increased with increasing BMI among premenopausal (p = 0.011) but not postmenopausal women (Table **1**, Fig. **1**). When considering only women without breast cancer (Table **2**), the ability to detect leptin in NAF remained associated with increased BMI in premenopausal women.

Leptin was detectable in the serum of 49/49 subjects (Table **3**). Median serum leptin was 11.0 ng/ml in premenopausal subjects

Table **1** Detection of NAF Leptin Expression (ng/g total protein) Based on Body Mass Index (BMI)

	BMI (kg/m²)			
	0 – 24.99	*25 – 29.99*	*≥ 30*	*P value*
Overall				
Yes[1]	12	9	16	
No	23	8	15	0.15
Premenopausal				
Yes	4	5	7	
No	18	2	5	**0.011**
Postmenopausal				
Yes	8	4	9	
No	5	6	10	0.56

Yes[1]: NAF leptin level was measurable; No: NAF leptin was not measurable.

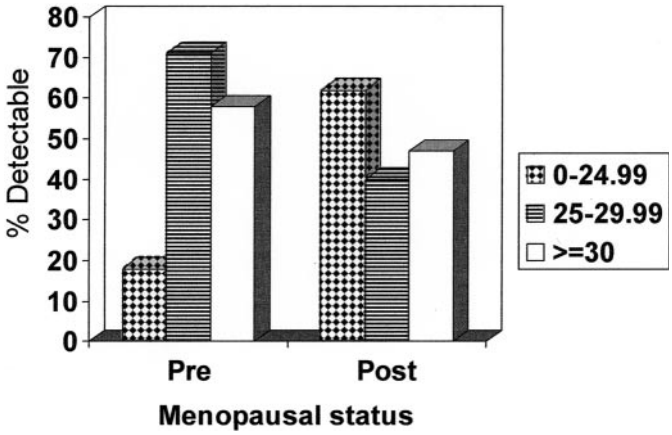

Fig. **1** Percentage of NAF samples in which leptin was detectable based on body mass index and menopausal status.

and 21.4 ng/ml in postmenopausal subjects. The difference was not statistically significant. Serum leptin was associated with BMI in both premenopausal ($r = 0.79$, $p = 0.0001$, $n = 23$) and postmenopausal ($r = 0.73$, $p = 0.0001$, $n = 26$) women.

NAF leptin levels are associated with BMI and serum leptin levels in premenopausal women

We next evaluated NAF leptin expression based on the actual measurement (Table **4**), rather than whether classified as detectable or not. Using this approach, NAF leptin expression was associated with BMI in premenopausal ($r = 0.38$, $p = 0.01$) but not in postmenopausal women ($r = -0.003$, $p = 0.98$). There were 40 subjects in whom we had matched NAF and serum samples. NAF and serum leptin levels were associated in premenopausal ($r = 0.51$, $p = 0.02$, $n = 20$) but not in postmenopausal women ($r = -0.12$, $p = 0.60$, $n = 20$).

Table **2** Detection of NAF Leptin Expression in Women with/without Breast Cancer

	BMI 0–24.99		*25–29.99*		*≥30*		*P value*	
Overall subjects (%)	CA	No CA	CA	No CA	CA	No CA	CA	No CA
NAF								
Yes	4	7	5	4	6	10		
No	4	19	1	7	8	7	0.24	0.11
Premenopausal								
Yes	0	4	2	3	2	5		
No	2	16	0	2	2	3	0.14	**0.053**
Postmenopausal								
NAF								
Yes	4	3	3	1	4	5		
No	2	3	1	5	6	4	0.39	0.32

CA: cancer; Yes, No: Indicates whether NAF leptin was detectable

Table **3** Serum Leptin Expression (ng/ml) Based on Body Mass Index (BMI)

	BMI (kg/m²) 0–24.99	*25–29.99*	*≥30*
Overall			
n	21	8	20
Median	7.80	16.39	27.10
Range	0.71–29.41	10.06–22.56	20.47–88.41
Premenopausal			
n	14	1	8
Median	5.96	10.06	30.63
Range	0.71–29.41	10.06–10.06	22.71–71.06
Postmenopausal			
n	7	7	12
Median	9.67	17.32	25.11
Range	3.32–26.14	10.28–22.56	20.47–88.41

Table **4** NAF Leptin Expression (ng/g total protein) Based on Body Mass Index (BMI)

	BMI (kg/m²) 0–24.99	*25–29.99*	*≥30*
Overall			
n	35	17	31
Median	0	0.047	0.037
Range	0–0.47	0–1.52	0–0.73
Premenopausal			
n	22	7	12
Median	0	0.068	0.067
Range	0–0.47	0–1.52	0–0.42
Postmenopausal			
n	13	10	19
Median	0.013	0	0
Range	0–0.41	0–0.74	0–0.73

Overall[1]: Eight women who had blood serum analyzed for leptin did not provide NAF samples. n: Number of subjects.

Table **5** Detection of NAF Leptin in Ductal Carcinoma In Situ (DCIS) and Invasive Breast Cancer (IBC)

Diagnosis		*BMI*					
		19.3 – 24.9		*25 – 29.9*		*≥ 30*	
	n	*Yes*	*No*	*Yes*	*No*	*Yes*	*No*
NAF							
DCIS	*6*	1	2	1	0	2	0
IBC	*22*	3	2	4	1	4	8

Association of NAF and serum leptin with breast cancer

We used two approaches for evaluating the association of NAF leptin with breast cancer. In the first approach, we considered whether there was an association between our ability to detect leptin and breast cancer. The two were not associated in pre ($p = 0.14$) nor in postmenopausal ($p = 0.39$) subjects (Table **2**). In the second approach, we considered the actual leptin measurement. Using this approach and Spearman's rank correlation, NAF leptin was not associated with breast cancer in either premenopausal ($p = 0.59$) or postmenopausal women ($p = 0.60$). Similarly, there was no association between serum leptin levels and the presence of breast cancer in either premenopausal ($p = 0.28$) or postmenopausal women ($p = 0.68$).

Finally, we evaluated whether there was an association between our ability to detect NAF leptin and breast cancer progression (Table **5**). Specifically, we compared non-invasive (ductal carcinoma *in situ*, DCIS) and invasive breast cancer (IBC). The limited number of samples from subjects with DCIS did not allow statistical analysis comparing NAF leptin detection in DCIS *vs.* IBC, nor leptin detection in DCIS based on BMI. There was no association between leptin detection and IBC.

Discussion and Conclusions

The link between obesity and breast cancer has been established fairly well [16]. Since circulating leptin levels rise with increasing BMI, we hypothesized that leptin expression might be associated with breast cancer risk. As a first step, we assessed the expression of this hormone in NAF, an organ specific specimen, and compared it to systemic levels in serum. Because leptin has been found in breast milk [4], we anticipated that it would be measurable in NAF. Indeed, leptin was detectable in 34% of samples. Leptin was probably also expressed in some samples below the threshold level of sensitivity of our ELISA detection kit (0.5 ng/ml).

Leptin was detectable in all serum samples (mean 21.2, median 20.3 ng/ml). Consistent with published findings, the expression of leptin in serum was significantly correlated with BMI [17]. According to previous reports, there is no consensus on the correlation between serum leptin and menopausal status [18]. In our study, serum leptin levels did not differ by menopausal status. Interestingly, the expression of leptin in NAF only correlated with BMI in premenopausal women. We speculate that leptin in NAF is transported from neighboring adipose tissue by the means of transcellular and intracellular passage. Although the mechanism of transcellular leptin transport is still unclear, some data suggest that this process could be controlled by premenopausal female sex hormones. Experiments demonstrated that transcellular transport of leptin is mediated by short-form leptin receptors (Ob-Rs) [19] whose expression appears to be regulated by estrogens [20, 21].

We observed that leptin levels in NAF and serum are significantly associated ($p = 0.02$). Generally, leptin concentration in NAF was lower than in serum. NAF leptin was detectable in 44.6% of samples overall, and was significantly lower ($p = 0.042$) in premenopausal than in postmenopausal women with a BMI less than 25, but not in those with a higher BMI. There are at least two explanations for the lower leptin levels in NAF than in serum. First, NAF leptin may originate in and be secreted from mammary epithelial cells, which produce only small amounts of the hormone. Indeed, normal mammary cells and breast cancer cells have been reported to express leptin mRNA in vitro, even though leptin protein is not consistently detected [22]. Second, it is possible that in addition to (or instead of) local leptin secretion, the hormone is transported from neighboring breast adipose tissue or serum, which results in its dilution. The mechanism of such passage could involve transepithelial transport similar to that suggested in the transport of leptin through the blood-brain barrier [23]. The association between NAF leptin levels and BMI suggests that the second possibility should be considered.

Prior reports link obesity with both premenopausal and postmenopausal breast cancer [12] and two clinical reports analyzing leptin levels in serum have linked the protein with breast cancer risk, although the conclusions were conflicting, one report indicating a direct [9] and the other [10] an inverse associated with premenopausal breast cancer. Our findings are consistent with and extend a third report [11], which evaluated serum leptin levels in premenopausal women with DCIS, while our study evaluated women with both DCIS and invasive breast cancer (combined into one group as cancer) and both premenopausal and postmenopausal women, concluding that there was no significant association between serum leptin and breast cancer.

In conclusion, our study indicates that leptin can be detected in a subset of NAF samples, that NAF leptin is associated with BMI and serum leptin in premenopausal women, and that serum leptin is associated with BMI in both premenopausal and postmenopausal women. Contrary to some other reports suggesting that serum leptin was associated with breast cancer, we did not observe an association between leptin and cancer in either NAF or serum. It would be important to test our hypothesis on transcellular transport of leptin by measuring the level of the Ob-R in mammary epithelium in premenopausal and postmenopausal women and correlating the levels with NAF leptin.

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Low calcium intake is associated with decreased adrenal androgens and reduced bone age in premenarcheal girls in the last pubertal stages

Daniela Bonofiglio[1], Cecilia Garofalo[2], Stefania Catalano[2], Stefania Marsico[2], Saveria Aquila[2], and Sebastiano Andò[3]

[1] Department of Pharmaco-Biology, University of Calabria, Cosenza, Italy
[2] Health Center, University of Calabria, Cosenza, Italy
[3] Department of Cellular Biology, University of Calabria, 87036 Rende, Cosenza, Italy

Abstract In 50 premenarcheal girls selected from the lowest and highest end of the calcium-intake distribution of a large population sample, we evaluated bone mineral density (BMD), together with the following hormonal-metabolic parameters: androstenedione (ASD), dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), testosterone (T), estradiol (E2), the apparent free fractions of T (AFTC) and E2 (AFEC), osteocalcin (OC), parathyroid hormone (PTH), and 25-hydroxyvitamin D. Dietary calcium was assessed by 3-day food records, and BMD was measured at ultradistal (ud) and proximal (pr) radial sites, using dual-energy X-ray absorptiometry. Calcium intake, which was below the recommended levels set for the Italian population and below the recommended daily allowance (RDA) in both subgroups of girls, did not show any apparent relationship with ud- and pr- BMD. However, despite the similar chronological age of the two premenarcheal groups, in the low-calcium consumers, we found lower bone age, delayed pubertal development, and lower circulating adrenal androgens. Of interest, in girls who had a low calcium intake, PTH levels were significantly higher. In all premenarcheals, we observed that DHEA, T, and AFTC were positively correlated with bone age and with bone density at both radial sites. Even though bone density at the two radial sites did not show any apparent relationship to calcium consumption, the increased mean PTH in the girls with low calcium intake seems to underscore the hormonal attempt in maintaining calcium homeostasis. In conclusion, low calcium intake and reduced levels of adrenal androgens, leading to decreased bone age and delayed pubertal development, indicate a link between calcium intake, the hormonal milieu, and skeletal maturation.

Key words calcium intake · adrenal and gonadal androgens · bone mineral density · bone age

Offprint requests to: S. Andò (e-mail:sebando@tin.it)
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Introduction

During adolescence, calcium intake is generally known to affect the genetically determined attainment of peak bone mass [1,2]. The acquisition of bone mass in girls parallels body growth, showing the greatest increase throughout pubertal development, when the sharp rise of estrogens with the onset of menarche strongly contributes to skeletal maturation, acting in siynergy with other hormones and growth factors [3–7].

The pivotal role of estrogens in increasing and maintaining bone mass is now generally accepted [8], although some studies report a lack of correlation of estrogen with the risk of bone fracture in elderly women [9]. However, the determination of total estrogen circulating levels without measuring the estrogen free fraction (bioavailable) can be misleading [10]. In the presence of relatively low total serum estradiol levels, estrogen deficiency could be amplified by a concomitant enhancement of sex hormone-binding globulin levels, resulting in reduced hormonal bioavailability to the target tissues [11].

Our understanding of the role of androgens in bone tissue was recently reviewed [12]. The suggestion is that androgens are mainly involved in stimulating periosteal bone growth and enhancing cortical bone remodelling [12,13].

In the context of a European multicenter investigation of bone density and dietary calcium, anthropometric characteristics, puberty, and lifestyle factors [14], we studied the role of adrenal and gonadal steroids, together with osteocalcin and parathyroid hormone (PTH), in modulating bone turnover in 50 premenarcheal girls. In particular, androgens, a potential source of estradiol via the aromatization process, were also evaluated, in relation to skeletal accrual, during the crucial period of bone maturation.

Subjects and methods

All girls (n = 1079; age range, 11–14 years) attending secondary schools in the town of Rende (Cosenza, southern Italy) were invited to participate in the present study.

Subjects were excluded for the following reasons: non-Caucasian origin, chronic systemic disease, use of corticosteroids, vigorous sports activity (more than 7 h per week), and vegetarianism or adherence to any prescribed diet (except for an energy-restricted diet). Subsequently, 722 girls filled out a 20-item food frequency questionnaire (FFQ), adapted for Italian adolescents, in order to select our population sample from the lowest (100 adolescents) and highest (100 adolescents) end of the calcium-intake distribution. All premenarcheals (n = 50) at both the low (n = 22) and high (n = 28) end of the calcium-intake distribution were enrolled in this study.

The definitive values of calcium consumption were re-evaluated in the selected population (n = 50) using a 3-day food-record method. Therefore, subjects were invited to record everything they consumed during a consecutive Wednesday, Thursday, and Friday, the week before their visit to the Institute. Food and quantity, and recipes for composite dishes were recorded, and, if necessary, the parent responsible for meal preparation was invited to assist in checking and compiling the food diaries. Mean daily consumption of food products was converted into calcium intake (mg/day), using the National Food Composition Tables [15].

The local Medics-Ethics Committee approved our study protocol, and parents of all participants gave their written consent.

Morning serum samples were drawn from each subject (after an overnight fast) by arm venipuncture to measure serum levels of hormones. After centrifugation, the serum samples were frozen at −70°C in individual aliquots for later assay in duplicate.

Radioimmunoassays (RIA) were used to determine total testosterone (T; ICN Pharmaceuticals, Costa Mesa, CA, USA; intra- and interassay coefficients of variation [CVs], 10.5% and 8.2%, respectively), total estradiol (E2; Immunotech, Marseille, France; intra- and interassay CVs, 5.6% and 6.7%, respectively), total androstenedione (ASD; Diagnostic Systems Laboratories, Webster, TX, USA; intra- and interassay CVs, 2.8% and 7.0%, respectively), total dehydroepiandrosterone (DHEA; ICN Pharmaceuticals; intra- and interassay CVs, 7.3% and 7.0%, respectively), and total dehydroepiandrosterone sulfate (DHEAS; ICN Biomedicals; intra- and interassay CVs, 8.5% and 7.6%, respectively). Apparent free fractions of T (AFTC) and E2 (AFEC) were measured using the technique of dialysis described by Vermeulen et al. [16]. Briefly, dialysis tubes, 1 cm in diameter and 15 cm long (Viskase, Chicago, IL, USA) were washed and left overnight in distilled water. The tubes were filled with 1 ml of plasma and were sealed at each end with a double knot, bent into a U-shape, and placed in 20-ml scintillation vials with 10 ml of phosphate buffer (pH 7.4) containing about 10 000 cpm ^{3}H-T (specific activity 79 Ci/mmol, obtained from Amersham Life Science, Milan, Italy). Estimation of free steroid fractions was derived after shaking tubes in a water bath at 37°C for 24 h when the equilibrium of dialysis was determined in the closed system, as previously described [16].

Immunoradiometric assays (IRMA) were used to quantify serum osteocalcin (OC; (Diagnostic Systems Laboratories; intra- and interassay CVs, 2.9% and 4.7%, respectively), serum PTH (ICN Pharmaceuticals; intra- and interassay CVs, 3% and 5.2%, respectively), and serum sex hormone binding globulin (SHBG) levels (Diagnostic Systems Laboratories) after 100-fold sample dilution prior to assay (intra- and interassay CVs, 2.8% and 8.8%, respectively). Serum levels of 25-hydroxyvitamin D (25-OH-D) were determined, after extraction with acetonitrile, with an RIA kit (Incstar, Stillwater, MN, USA (intra- and interassay CVs, 7.6% and 8.2%, respectively).

One investigator measured height and weight and assigned pubertal stages to the girls according to Tanner [17].

Bone mineral content (BMC) and bone area were evaluated, by dual-energy X-ray absorptiometry (DXA; Osteoscan; Nederburg, Bunschoten, Netherlands), in the nondominant arm at the ultradistal (ud) and proximal (pr) radius (one-third distal point between the styloid process and the tip of the olecranon of the elbow) representing the trabecular and cortical bone components, respectively [18]. DXA, a rapid, accurate, and reproducible method of assessment of different bone components, with a very low radiation exposure (0.02–0.03 mSV per scan) was used to calculate bone mineral density (BMD; g/cm^2), by dividing the BMC value (expressed in grams) by the projected area of bone. Two technicians collected all densitometric data, calibrating the Osteoscan every day against a reference phantom. The CVs for ten measurements of the same subject (with repositioning) were 2.15% for ud-BMD and 1.75% for pr-BMD.

Left-hand and wrist X-rays were performed in girls to estabilish bone age, by the Tanner-Whitehouse (TW2) method [19].

Statistics

Data values for all variables were presented as means with standard error. Comparisons of means were made using unpaired Student's t-test. Pearson's correlation,

and linear and multiple regression analyses were performed using SIMSTAT 3.5 (Provalis Research, Montreal, Canada). Because variations of BMD could be due to differences in bone size between individuals, we included height, weight, and bone area as covariables on multiple regressions, with BMD as a dependent variable. *P* values of less than 0.05 were considered significant.

Results

Table 1 shows anthropometric characteristics, radial BMD, and calcium intake of girls selected according to the criteria indicated in the "Methods" section. It is worth noting that the dietary calcium consumption was below the recommended levels set for the Italian population, and below the recommended daily allowance (RDA) [20], even in the girls with high calcium intake (Table 1).

No relationship was observed between calcium intake and radial BMD adjusted for height, weight, and bone area, while calcium intake was related to bone age in all girls ($r = 0.34$; $P = 0.018$).

Despite the similar chronological age of the two subgroups, low-calcium consumers exhibited markedly delayed pubertal stage, well fitting with the decreased bone age (Table 1).

It is important to note that, in low-calcium consumers, a decrease in all aromatizable adrenal androgens (DHEA, DHEAS, and ASD) predicted a lower peripheral action of estrogens (Table 2). Furthermore, in the same subgroup, the enhanced SHBG reduced the amount of biologically active estradiol, resulting in a significant lowering of AFEC concentrations in these subjects (Table 2).

It is worth remarking that, in the subjects who had a low calcium intake, PTH levels appearred to be significantly enhanced, while serum levels of OC and 25-OH-D were similar in the two subgroups (Table 3).

Table 1. Anthropometric characteristics, dietary calcium, and ultradistal/proximal-bone mineral density (ud/pr-BMD; X ± SE and *P* values) in premenarcheal girls with low and high calcium intake (Ca I)

	Premenarcheals ($n = 50$)		
	Low Ca I	High Ca I	*P*
Subjects (*n*)	22	28	
Age (years)	12.01 ± 0.10	12.33 ± 0.18	NS
Bone age (years)	11.96 ± 0.30	12.89 ± 0.14	<0.01
Tanner stage (*n*)	3.50 ± 0.21	4.10 ± 0.11	<0.01
Height (cm)	150.68 ± 1.76	152.59 ± 1.18	NS
Weight (kg)	46.06 ± 2.46	45.16 ± 1.49	NS
Body mass index (kg/m^2)	20.22 ± 0.85	19.87 ± 0.52	NS
Dietary calcium (mg/day)	420 ± 12.38	839 ± 20.01	<0.001
ud-BMD (g/cm^2)	0.29 ± 0.01	0.30 ± 0.01	NS
pr-BMD (g/cm^2)	0.61 ± 0.01	0.60 ± 0.01	NS

NS, not significant

Table 2. Levels of testosterone (T), estradiol (E2), androstenedione (ASD), dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), sex hormone binding globulin (SHBG), and apparent free fractions of T (AFTC) and E2 (AFEC) (X ± SE and *P* values) in premenarcheal girls with low and high calcium intake (Ca I)

	Premenarcheals		
	Low Ca I ($n = 22$)	High Ca I ($n = 28$)	*P*
T (nM)	0.92 ± 0.07	0.88 ± 0.06	NS
E2 (pM)	136.67 ± 15.94	171.72 ± 12.75	NS
ASD (nM)	3.56 ± 0.25	5.38 ± 0.44	<0.01
DHEA (nM)	21.87 ± 1.97	27.78 ± 1.74	<0.05
DHEAS (μM)	1.67 ± 0.20	2.32 ± 0.19	<0.05
SHBG (nM)	175.72 ± 15.38	119.60 ± 8.45	<0.01
AFTC (pM)	14.90 ± 2.01	14.60 ± 1.01	NS
AFEC (pM)	2.05 ± 0.08	2.41 ± 0.10	<0.01

Of interest, in all premenarcheals, DHEA, T, and its free fraction (AFTC) were positively related to bone age (Fig. 1); moreover DHEA, T, and AFTC were positively related to both radial BMD values (Figs. 2, 3, and 4).

Table 3. Serum osteocalcin (OC), parathyroid hormone (PTH), and 25-hydroxyvitamin D (25-OH-D) levels (X ± SE and *P* values) in premenarcheal girls with low and high calcium intake (Ca I)

	Premenarcheals		
	Low Ca I (n = 22)	High Ca I (n = 28)	*P*
OC (nM)	1.36 ± 0.08	1.44 ± 0.09	NS
PTH (pM)	3.48 ± 0.44	2.39 ± 0.22	<0.05
25-OH-D (nM)	50.82 ± 6.01	49.67 ± 4.47	NS

Discussion

In our investigation, the average dietary calcium intake was below the RDA [20] in premenarcheal girls in the presence of normal body mass index (BMI) and body weight. Even though bone density at both radial sites measured did not show any substantial difference between the two groups of girls, lower calcium intake was associated with lower mean bone age and delayed pubertal development. However, in this regard, we would point out how the bone density detected at the two radial sites may be not adequately representative of the systemic influence of the low calcium intake on bone maturation. Such effects, indeed, appear to be evaluated in a more reliable way by bone age determination, which was positively related to calcium intake in all girls. In low-calcium consumers, a reduction in circulating adrenal androgens suggests that, along with other nutritional factors [21 and references therein], calcium

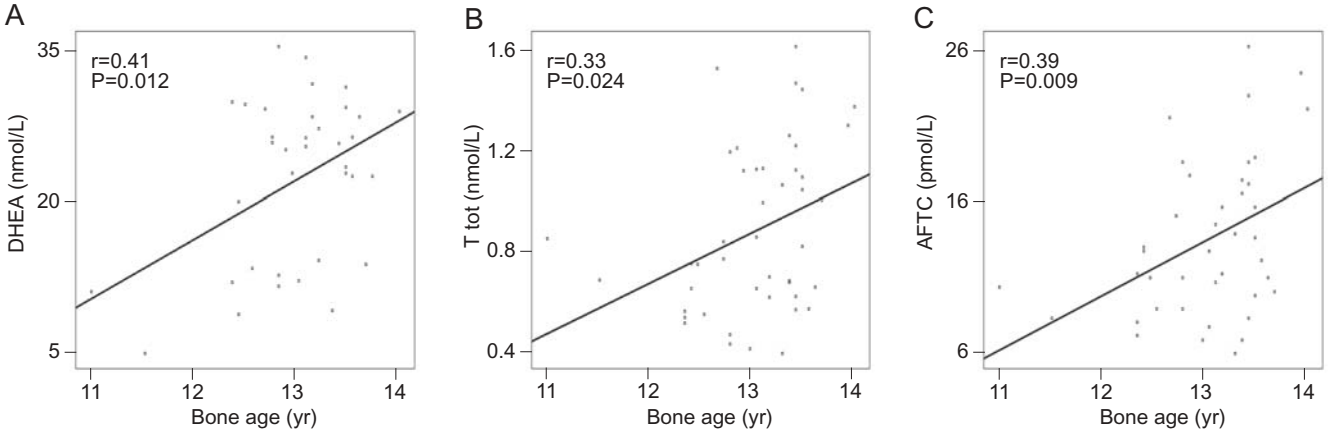

Fig. 1. Correlations between **A** dehydroepiandrosterone (*DHEA*), total and apparent free fraction of testosterone (*T tot* and *AFTC* **B** and **C**, respectively) and bone age in premenarcheal girls. *yr*, years

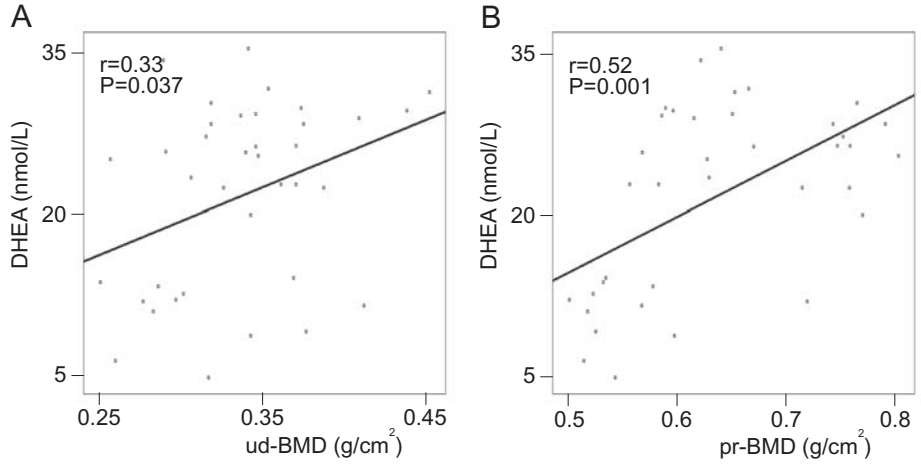

Fig. 2. Correlations between dehydroepiandrosterone (*DHEA*) and ultradistal/proximal bone mineral density (*ud/pr-BMD* **A**/**B**) in premenarcheal girls

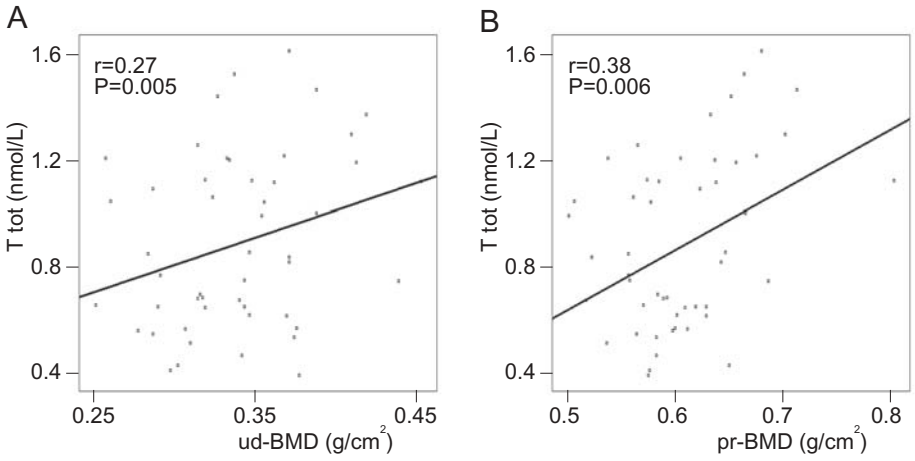

Fig. 3. Correlations between total testosterone (*T tot*) and ultradistal/proximal bone mineral density (*ud/pr-BMD* **A**/**B**) in premenarcheal girls

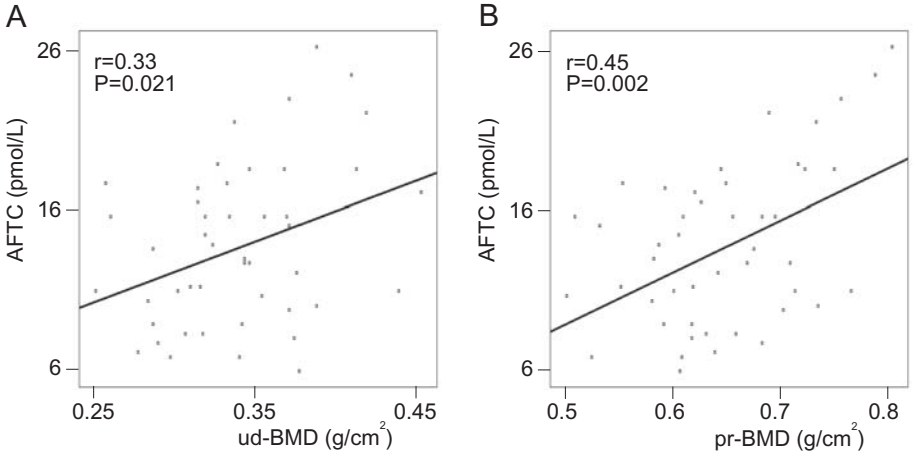

Fig. 4. Correlations between apparent free fraction of testosterone (*AFTC*) and ultradistal/proximal bone mineral density (*ud/pr-BMD* **A**/**B**) in premenarcheal girls

may influence enzymatic adrenal steroidogenesis. On the other hand, there is increasing evidence that anabolic changes are involved in the enhanced bioactivity of insulin and/or insulin-like growth factor-I (IGF-I) [22,23]. As the adrenal cortex expresses insulin and IGF-I receptors [24], and both mitogenic peptides enhance adrenal secretion in human adrenocortical cells [24–26], it becomes plausible to regard nutritional status as an important regulator of adrenarche.

In our premenarcheal girls, both adrenal and gonadal androgens were positively related to bone density. These data fit with the results of previous studies showing that AFTC and DHEA were the androgens more consistently correlated with bone density [27–29]. In the same vein, we observed in the present study that DHEA, T, and AFTC were positively related to bone age, even though the relationship between AFTC and bone age was much more significant than DHEA or T and bone age. For instance, testosterone, the major gonadal androgen, circulates largely bound to albumin and SHBG, while AFTC represents the hormone that is bioavailable to the target tissue. DHEA and ASD are the major circulating adrenal androgens in both women and men [30]. DHEA is metabolized to DHEAS, which has little androgenic activity, and thus, a direct stimulatory effect of DHEAS on bone, although possible, is unlikely. In this regard, we would point out that DHEAS may serve, rather, as a surrogate marker for the effect of DHEA, which can directly stimulate both osteoblast proliferation and differentiation (similar to testosterone and dihydrotestosterone [DHT]) [31] or for the effect of ASD [6].The detection of functional androgen receptors (ARs) in various bone cells [32–35] has implicated bone as a target tissue for androgen action and has fuelled an increase in further investigations of the direct and indirect effects of androgens on bone cells in vitro, as well as on bone metabolism in vivo. In addition, the presence of the enzymes aromatase, 17-β-hydroxysteroid-dehydrogenase, 3-β-hydroxysteroid-dehydrogenase, and 5-α-reductase, detected in osteoblastic cells [36–41], indicates the ability of the bone microenvironment to locally form biologically

potent estrogens and androgens from weak circulating sex steroid hormones such as DHEA, DHEAS, and ASD. Thus, the biological effects of these compounds may result from activation of the ER or AR, as demonstrated in several in vitro and in vivo studies [12 and references therein]. For instance, ERs have been generally demonstrated in cultured bone cells of the osteoblast lineage [42,43]. Besides, there is a large mass of data demonstrating that estrogens decrease bone resorption, promoting the apoptosis of osteoclasts, and that they also increase skeletal mass, reducing apoptosis in osteoblasts and osteocytes [44,45]. Indeed, during skeletal growth and maturation, estrogens, in combination with other hormones, induce major beneficial effects on skeletal development consistent with a combination of increases in bone height, bone diameter, cortical bone width, and cancellous bone mass [6,8]. Moreover, estrogens have indirect actions that affect bone metabolism, which include promoting the intestinal absorption of calcium and decreasing the renal excretion of calcium. In the present study, the increase of SHBG observed in low-calcium consumers produced a significant lowering of AFEC, which may negatively influence bone maturation, as previously evidenced [8,46].

Moreover, in low-calcium consumers, we found an enhancement of PTH, a major osteotropic factor which plays a critical role in calcium homeostasis and in regulating the rate of bone turnover.

In conclusion, our observations support the notion that low dietary calcium consumption in premenarcheal girls is associated with a lower adrenal androgen secretion. Both a low calcium intake and reduced levels of adrenal androgens appear to cooperate in decreasing bone age and in delaying pubertal development in girls with low calcium intake. Testosterone and DHEA may also act through an intracrine conversion into estrogens, to contribute to bone mass acquisition during this important period of skeletal maturation.

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