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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

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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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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).

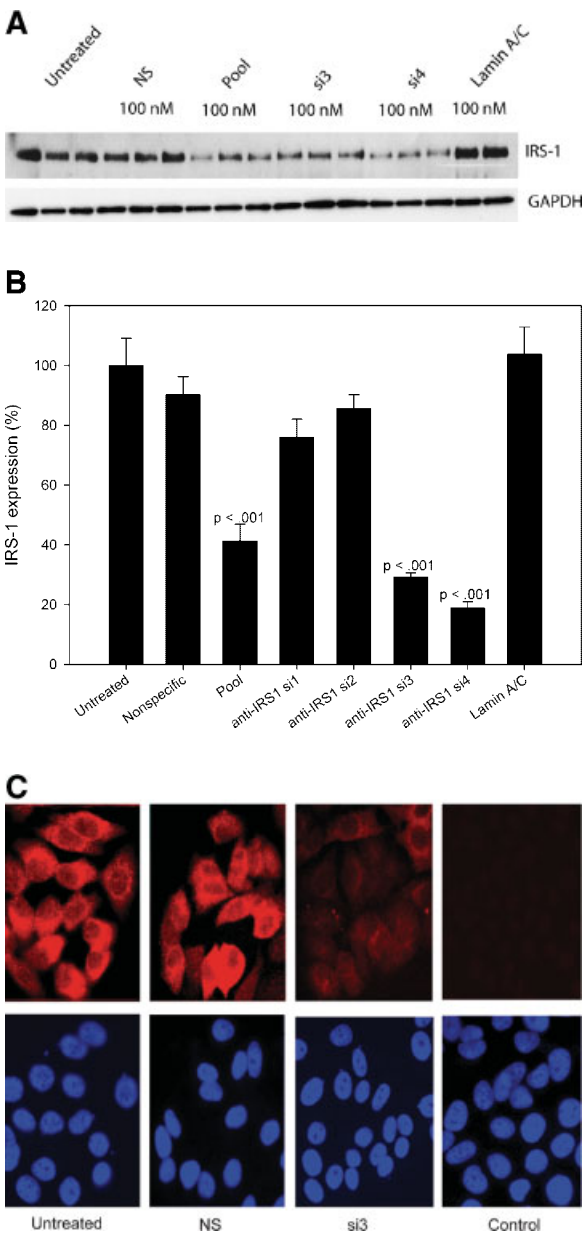

Fig. 1. Knockdown of IRS-1 protein in siRNA treated MCF-7 cells. MCF-7 cells transfected with siRNAs for 4 days, as described in Materials and Methods. **A**: Expression of IRS-1 was assessed by WB by loading 40 μg of cytoplasmic protein lysate per lane isolated from cells transfected with 100 nM of 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

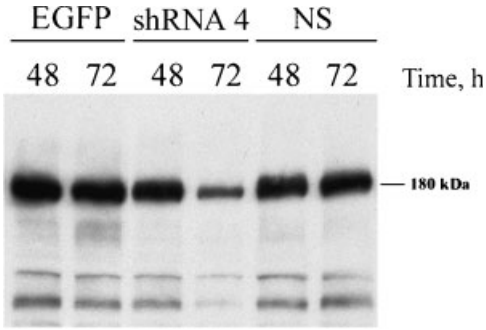

Fig. 2. Anti-*IRS1* shRNA expression leads to reduced IRS-1 protein. WB of cytoplasmic protein lysates from MCF-7 cells transfected with a shRNA directed to EGFP sequence (EGFP), a scrambled anti-*IRS1* siRNA 4 sequence (NS), and anti-*IRS1* sequence 4 (shRNA 4), respectively. Each lane was loaded with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

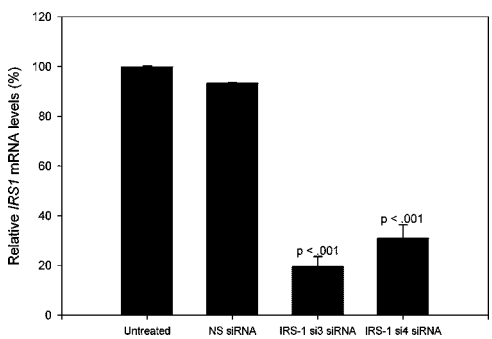

Fig. 3. *IRS1*-specific *IRS1* mRNA reduction analyzed by quantitative real time-PCR. MCF-7 cells were transfected with 100 nM of NS siRNA, siRNA 3, or siRNA 4. Total RNA was purified 24 hr post transfection and the expression of *IRS1* mRNA was assessed by QRT-PCR, as described in Materials and Methods.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

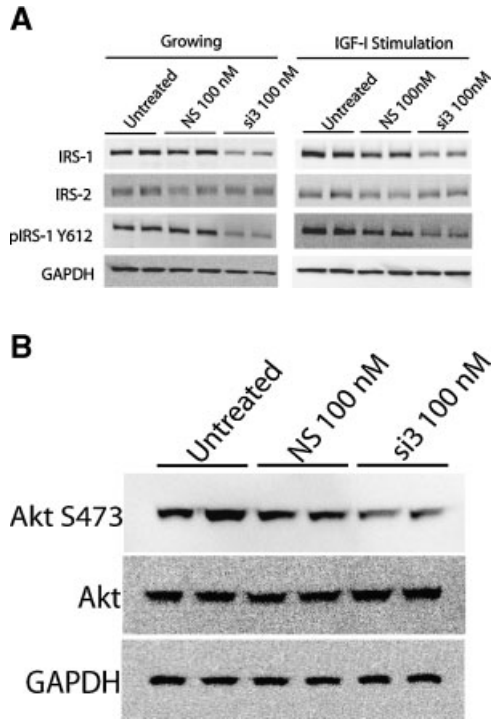

Fig. 4. Anti-*IRS1* siRNA inhibits IRS-1 Tyr phosphorylation and downstream Akt phosphorylation but does not affect IRS-2 expression. **A**: Left column (Growing): MCF-7 cells were transfected with 100 nM anti-*IRS1* siRNA 3, 100 nM nonspecific siRNA, or left untreated for 4 days. Then 40 μg of cell 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

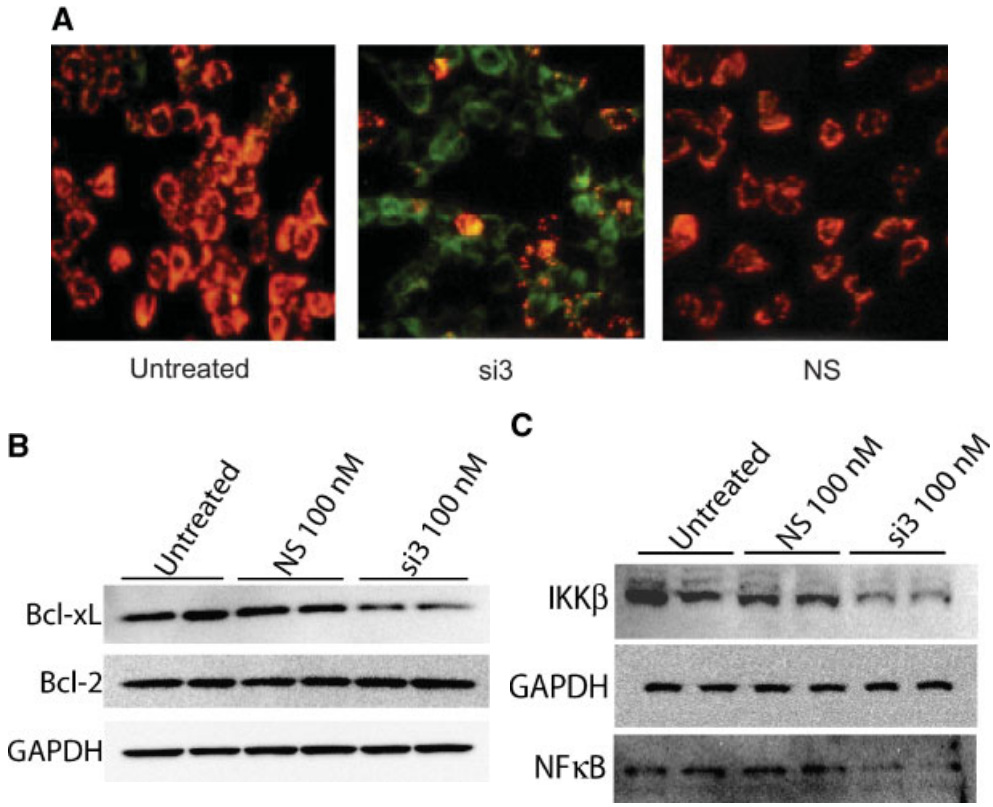

Fig. 5. Anti-*IRS1* siRNA decreases ψ and Bcl-xL expression. **A**: Decreased mitochondrial membrane potential in MCF-7 cells treated with anti-*IRS1* siRNA. **Left**, Untreated MCF-7 cells after 4 days of growth display normal ψ illustrated by the orange fluorescence present in the overlay of both the 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.

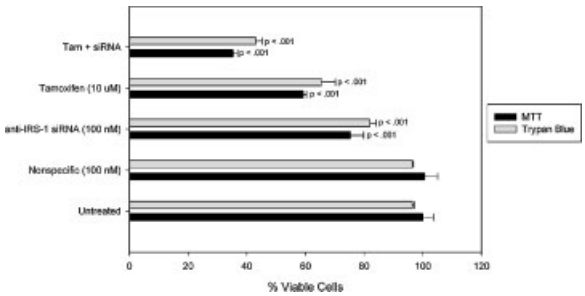

Fig. 6. An additive effect of a combined anti-*IRS1* siRNA and TAM treatment on MCF-7 cell death while in the presence of serum. Percentage of cell death in MCF-7 cells after 4 days treatment. Treatment conditions are noted in the left hand column of the graph. A minimum of 400 cells were counted for 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.

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