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# Insulin receptor substrate 1 modulates the transcriptional activity and the stability of androgen receptor in breast cancer cells

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**Abstract** Breast cancer development and progression is regulated by growth factors and steroid hormones. Although the majority of human breast cancers expresses androgen receptor (AR), the role of androgens in breast tumorigenesis remains largely unexplored. Here we demonstrate that an AR ligand, 5- $\alpha$ -dihydrotestosterone (DHT), inhibits MCF-7 breast cancer cell growth induced by insulin like growth factor 1 (IGF-I). Our results show that DHT induces association of AR with IRS-1, the major IGF-1 receptor signaling molecule. The AR/IRS-1 complex translocates to the nucleus and is recruited to gene promoters containing androgen responsive elements causing an increase of AR transcriptional activity. Moreover, IRS-1

knockdown suggests that IRS-1/AR interaction decreases the ubiquitin/proteasome dependent degradation of AR, increasing its stability. Taken together, these data indicate that nuclear IRS-1 is a novel AR regulator required to sustain AR activity and demonstrate, for the first time in breast cancer cells, the existence of a functional interplay between the IGF system and AR. This interplay may represent the molecular basis of mechanisms through which androgens exert their inhibitory role on the proliferation of breast cancer cells.

**Keywords** Androgen receptor · Breast cancer · IRS-1 · IGF-I

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Marilena Lanzino and Cecilia Garofalo contributed equally to this work.

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## Introduction

Breast cancer development and progression depend on complex crosstalk between steroid hormones and growth factors [1–5]. Emerging evidences indicate that androgens have inhibitory effects on the growth of breast epithelial cells and play a protective role in the pathogenesis of breast cancer [6, 7].

Recent *in vivo* studies in rhesus monkeys demonstrated the ability of androgens to counterbalance positive growth stimuli in breast, with low doses of testosterone completely inhibiting estrogen-mediated mammary cell proliferation in ovariectomized animals [8]. In human, androgens antagonize the effects of estrogens in mammary gland development, so that they could be considered endogenous anti-estrogens [9]. Moreover, androgens, via androgen receptor (AR) activation, inhibit the basal and estradiol-induced growth of AR-positive breast cancer cell lines [10, 11], through a mechanism involving a decrease of estrogen receptor alpha (ER) content and transcriptional activity [12].

The majority of human breast cancers express AR [13–15], and many metastatic breast tumors, which are ER and progesterone receptor (PR) negative, still express a significant amount of AR [16]. Moreover, 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 [17]. Other events involved in breast cancer development or progression have been shown to alter AR expression or function. In BRCA1-mutated tumors, loss of AR expression, and thus loss of AR signaling, supports neoplastic transformation of mammary epithelial cells [18]. Moreover, in HER2-positive breast cancers, generally associated with a worse outcome, a trend toward a loss of AR signaling has been demonstrated [19]. Taken together, these observations suggest that androgen signaling in breast may play a protective role in cancer development and progression.

Members of the insulin-like growth factor (IGF) family exert a crucial role in the regulation of breast epithelial cell proliferation. Recently we have demonstrated that Insulin Receptor Substrate 1 (IRS-1), a major substrate for IGF-I receptor, interacts with ER and negatively influences ER function in breast cancer cells [20].

In breast cancer, IRS-1 overexpression has been associated with tumor development, hormone independence, and antiestrogen resistance [21]. These effects have been attributed to increased tyrosine phosphorylation of cytoplasmic IRS-1 and potentiation of its downstream signaling to Akt [21, 22]. 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 [20, 23–25]. Experimental data suggest that nuclear IRS-1 might function as transcriptional co-regulator for polymerase I and II [20, 26].

The observation that IRS-1 is a crucial factor involved in breast tumor development and that negatively regulates ER function, has led us to investigate the existence of a functional interplay between IRS-1 and AR in breast cancer cells. Here we demonstrate that IRS-1 modulates AR mediated transcription through two different mechanisms: by participating in AR-recruited transcriptional machinery and by increasing AR stability.

## Materials and methods

### Cell culture and treatments

Breast cancer epithelial cell line MCF-7 and 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. 5 $\alpha$ -Dihydrotestosterone (DHT, Sigma,

USA) and hydroxyflutamide (OH-FI, Sigma, USA) were used at a concentration of 10<sup>-7</sup> M and 10<sup>-6</sup> M, respectively. Before each experiment, cells were serum starved for 24 h in phenol red-free DMEM (PRF), and then shifted to PRF containing 5% charcoal-treated fetal calf serum (PRF-CT).

### Cell proliferation assay

Cell proliferation assay was performed using MCF-7 cells seeded on six well plates (10<sup>5</sup> cells/well), serum starved for 48 h and grown for 3 and 6 days in PRF-CT containing DHT (10<sup>-7</sup> M), or IGF-1 (10<sup>-9</sup> M), or OH-FI (10<sup>-6</sup> M). Media were renewed every other day. At the indicated time points, cells were harvested by trypsin, and counted using Burker's chamber.

### Immunoprecipitation and Western blotting

Total cell proteins and the cytoplasmic and nuclear fractions were obtained from 70% confluent cell cultures. Immunoprecipitation (IP) and Western blotting (WB) were performed as previously described [20]. The following monoclonal (m) and polyclonal (p) antibodies (Ab) were used: anti-IRS-1 pAb (Upstate, USA); anti p-Akt and anti p-MAPK (Cell signaling, USA); anti-AR mAb (441), anti-c-jun mAb (KM-1), anti-GAPDH pAb (FL-335), anti ubiquitin mAb (P4D1), anti-Akt pAb (H-136), anti-p21 pAb (H-164) (Santa Cruz, USA), and normal mouse immunoglobulin G (Ig) (Santa Cruz, USA).

### Confocal laser scanning microscopy

MCF-7 cells were grown on 12-mm glass coverslips, serum starved for 24 h and treated in PRF-CT containing DHT (10<sup>-7</sup> M) for additional 24 h. After incubation cells were fixed with 3% paraformaldehyde, permeabilized with 0.2% Triton X-100, and non specific sites were blocked by BSA (3% for 30 min). At the end of each step samples were washed three times with phosphate buffer saline (PBS, Gibco, USA). Blocked samples were incubated for 1 h with anti IRS-1 pAb (2 mg/ml), washed and then incubated with a mixture of fluorescein-conjugated goat anti-rabbit IgG (1:1000) (Santa Cruz Biotechnology, USA) and rhodamine-phalloidin (1:200) (Cytoskeleton, USA) diluted in PBS containing 0.3% goat serum. Secondary antibody incubation was for 1 h, followed by five washes in PBS. The cellular localization of IRS-1 was examined under a Leica TCS SP2 Confocal Laser Scanning Microscope at 400 $\times$  magnification. The optical sections were taken at the central plane. The fluorophores were imaged separately to ensure no excitation/emission wavelength overlap.

## Plasmids, transfections and luciferase reporter assays

The following plasmids were used: pcDNA3-AR (AR) encoding full-length AR [27]; pCMV-IRS-1 (IRS-1) encoding IRS-1 [20]; the reporter plasmid pARE2-tk-LUC (2X-ARE-Luc) that drives the expression of luciferase by two androgen responsive elements (a gift from Dr O. Janne); pSilencer-IRS-1 (shIRS1) encoding a specific small interference RNA for IRS-1 [28]; WWP-Luc (p21-Luc) that drives the expression of luciferase by human p21<sup>WAF1/CIP1</sup> promoter and p5.3 PSaP-Luc (PSA-Luc) that drives the expression of luciferase by PSA promoter (gifts from Dr. W. El-Deiry and Dr. Kakizuka, respectively). The *Renilla reniformis* luciferase expression vector used was pRL-Tk (Promega, USA).

MCF-7 cells were transfected using Fugene 6 (Roche, CH) according to the manufacturer's instructions. pRL-Tk was used to assess transfection efficiency. Luciferase activity was measured using dual luciferase assay System (Promega, USA), normalized to renilla luciferase activity and expressed as relative luciferase units.

For reverse transcription of total RNA, and WB assays, MCF-7 cells were plated on 60 mm dishes and transfected with an appropriate amount of various plasmids, as indicated in 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. Following treatment, ChIP assay was performed as described previously (20). The immuno-cleared chromatin was precipitated with anti-AR mAb for AR, anti-IRS-1 pAb for IRS-1 and anti-polymerase II pAb (Santa Cruz, USA) for Pol II. A 4  $\mu$ l volume of each sample was used as template for PCR with specific primers.

The following pairs of specific 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 proximal promoter containing ARE-sequence 5'-GATCTAGGCACGTGAGGCTTTGTA-3' (forward) and 5'-CATGCTGCTGGAGGCTGGAC-3' (reverse).

For reverse ChIP, the pellets obtained by IP of soluble chromatin with IRS-1 and AR were eluted with 500  $\mu$ l of Re-ChIP buffer. Next the eluate from AR IP was precipitated with anti-IRS-1 pAb and the eluate from IRS-1 IP was precipitated with anti-AR mAb. The presence of the PSA and p21 promoter sequences in the resulting Re-ChIP pellets was examined as described above for one-step ChIP [20].

## RT-PCR

MCF-7 cells were transfected with non specific shRNA (scrambled) and shIRS1 plasmid for 24 h, as previously described, then 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  $\mu$ g of total RNA were reverse transcribed using M-MLV reverse transcriptase (Promega, USA) and 2  $\mu$ l of RT products were then amplified. The following specific primers were used: IRS-1 (762 bp): 5'-TCCACTGTGACACCAGAATAAT-3' (forward), 5'-CGCCAACATTGTTTCATTCCAA-3' (reverse); PSA (754 bp): 5'-TGCGCAAGTTCACCCTCA-3' (forward), 5'-CCCTCTCCTTACTCATCC-3' (reverse); AR: (416 bp): 5'-CACAGGCACTGGTCTCTGG-3', 5'-CTGCCTTACACAACCTCTTG GC-3' (reverse); p21 (270 bp): 5'-GCTTCATGCCAGCTACTTCC-3' (forward), 5'-CTGTGCTCACTTCAGGGTCA-3' (reverse). 36B4 was amplified as internal control gene (408 bp): 5'-CTCAACATCTCCCCCTTCTC-3' (forward), 5'-CAAATCCCATATCCTCGTCC-3' (reverse).

## Statistical analysis

All data were expressed as the mean  $\pm$  SD (standard deviation) of at least three independent experiments. Statistical significances were tested using Student's *t*-test.

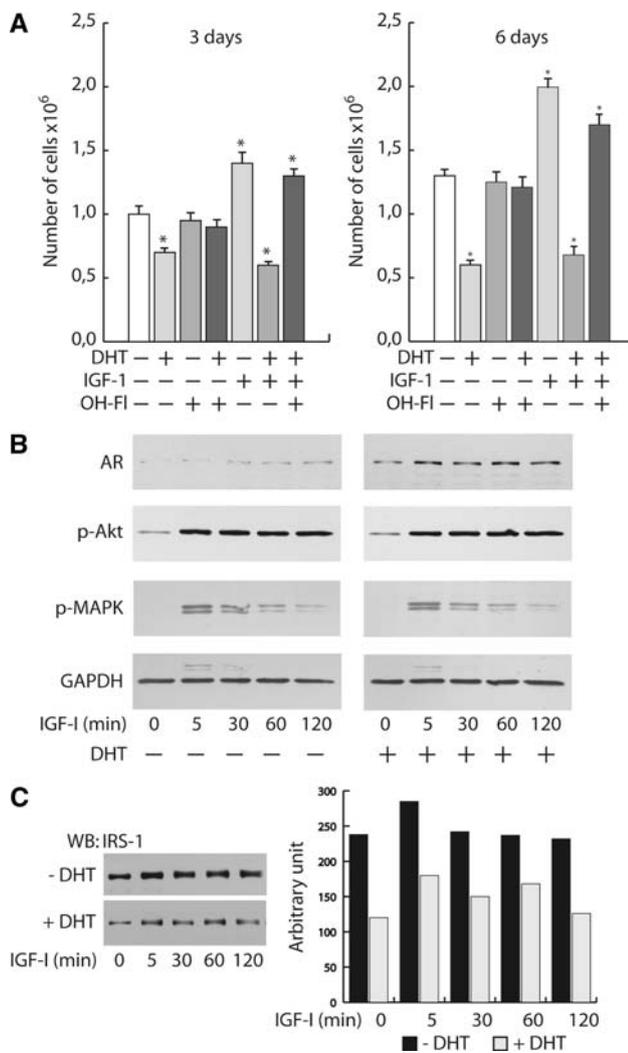
## Results

### DHT inhibits breast cancer cell growth induced by IGF-I

It is well established that IGF-I and estradiol (E2) are important positive modulators of mammary gland development as well as breast cancer progression [22]. We previously demonstrated that androgens antagonize E2 dependent cell proliferation [12], but the effect of DHT on IGF-I induced proliferation [21, 29] was not evaluated.

To investigate whether DHT influences breast cancer cell growth in response to IGF-I, MCF-7 cells were synchronized in serum free medium (PRF) for 24 h and treated with DHT and IGF-I in PRF-CT. DHT concentration was chosen based on previous studies demonstrating dose-dependent inhibitory effects of DHT on MCF-7 cells proliferation [10, 30]. As expected, the addition of  $10^{-7}$  M DHT inhibited MCF-7 cell proliferation by 30% (3 days) and 60% (6 days) with respect to controls (Fig. 1a). Moreover, DHT treatment of MCF-7 cultures impeded IGF-I induced proliferation observed over 6 days of culture.

The counteractive effect of DHT on IGF-I induced breast cancer cell proliferation was mediated by AR as the



**Fig. 1** DHT interferes with IGF-I induced MCF-7 cells proliferation. (a) MCF-7 cells synchronized in PRF were treated with  $10^{-9}$  M IGF-I,  $10^{-7}$  M DHT, and  $10^{-6}$  M OH-FI in PRF-CT for 3 and 6 days. Then cells were detached, stained with trypan blue and counted using Burker Chamber. Data, representing a mean  $\pm$  sd of three independent experiments, each in duplicate, were statistically analysed by T student test,  $*P < 0.05$  (b and c) Synchronized MCF-7 cells were pre-treated with  $10^{-7}$  M DHT for 24 h, or left untreated, and then stimulated with  $10^{-9}$  M IGF-I for the indicated times. The expression of AR, phospho-Akt, phospho-MAPK (b) and IRS-1 (c) was determined by western blotting (WB) using 30  $\mu$ g of protein lysates. The expression of GAPDH was assessed as control of protein loading of lysate fractions. The results were obtained after repetitive stripping and reprobing of the same filters. IRS-1 expression level (c) were quantified and reported as optical density (OD)

addition of the androgen antagonist hydroxyflutamide (OH-FI) reversed cell growth. Interestingly, a 24 h pre-treatment with DHT did not interfere with cell behavior, since downstream proteins of the IGF-I pathway such as Erk1/2 and Akt were normally phosphorylated in response to IGF-I stimulation (Fig. 1b). In contrast, cytosolic IRS-1 was reduced by DHT pre-treatment (Fig. 1c).

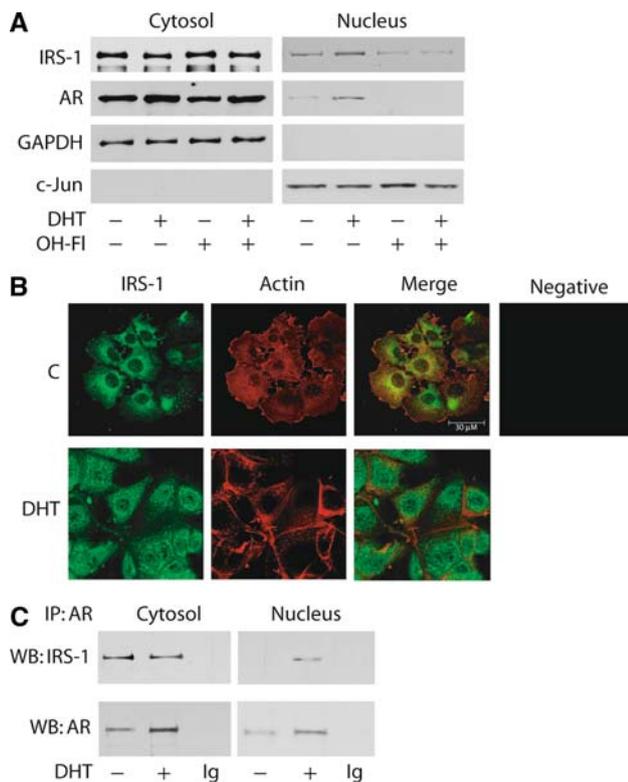
## DHT regulates cellular localization of IRS-1 in breast cancer cells

The effect of DHT on IGF-I-induced MCF-7 cell proliferation and the changes in IRS-1 cytosolic levels led us to investigate the molecular mechanisms by which DHT interfered with the IGF-I pathway. First, IRS-1 abundance was analyzed 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 as demonstrated by WB (Fig. 2a) and confocal microscopy (Fig. 2b). Moreover, the increased localization of IRS-1 in the nuclear fraction appeared to be specifically mediated by AR, since it was inhibited by the addition of the androgen antagonist OH-FI (Fig. 2a).

To assess whether the DHT-regulated intracellular localization of IRS-1 could involve a physical interaction between AR and IRS-1, a co-immunoprecipitation 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 into the nucleus with a consequent decrease of its abundance in the cytoplasm (Fig. 2c). The fraction of AR and IRS-1 precipitated was about 1/20 of the relative amount of each protein in the nuclear compartment (Fig. 2a). These observations suggest that cytosolic AR associates with IRS-1 and that DHT induces the nuclear translocation of the AR/IRS-1 complex.

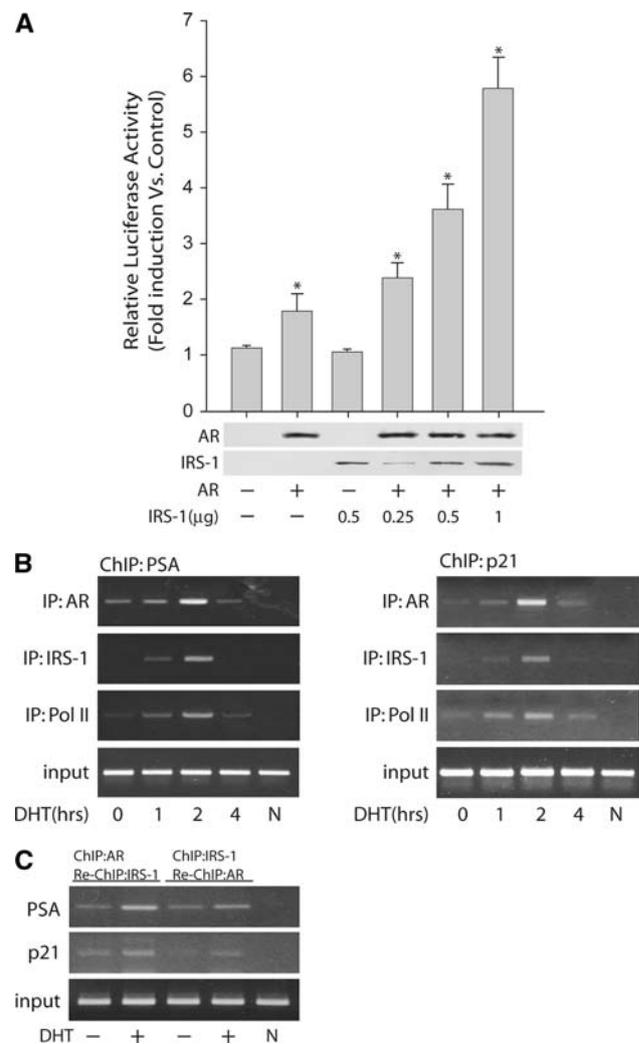
## IRS-1 is a transcriptional regulator of AR

The existence of the nuclear AR/IRS-1 complex led 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, which are negative for IRS-1 and AR, were co-transfected with an AR expression vector and increasing amounts of an IRS-1-encoding plasmid. The increasing levels of IRS-1 did not exert any effect on reporter plasmid expression in the absence of DHT stimulation. On the contrary, the ectopic expression of IRS-1 increased the transcriptional response to DHT stimulation in a dose-dependent manner, with a maximal twofold enhancement above the level observed in the absence of IRS-1 (Fig. 3a). The positive effect of IRS-1 expression on DHT induced AR transcriptional activity, was no longer detected following treatment of MCF-7 cells with the AR antagonist, OH-FI, (data not shown), suggesting a direct involvement of AR in this process.

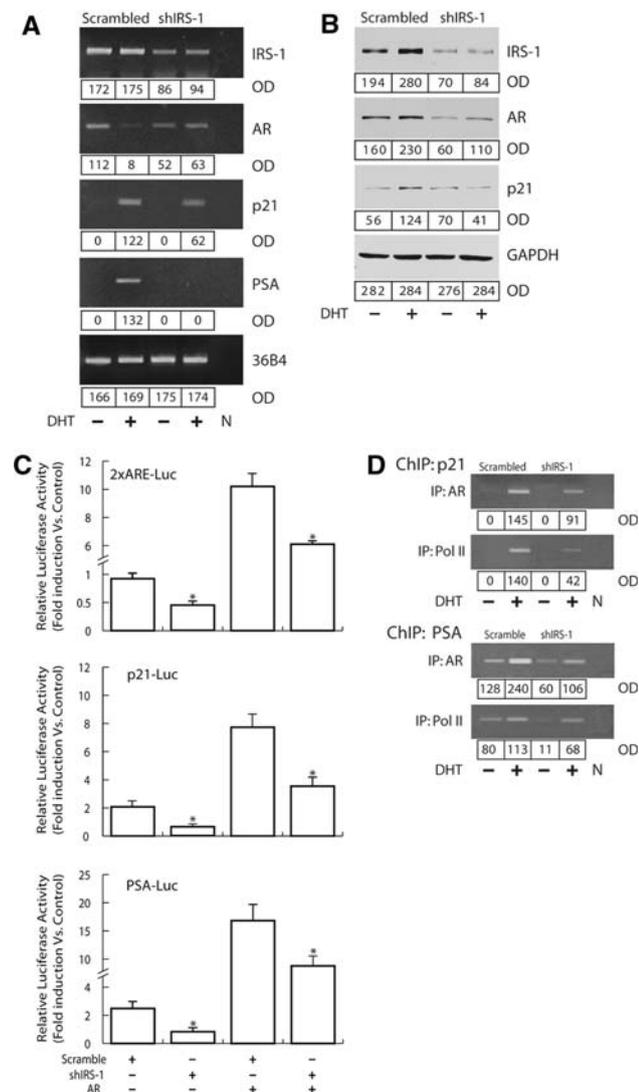


**Fig. 2** DHT induces the translocation of AR/IRS-1 complex into the nucleus. **(a)** MCF-7 cells synchronized in PRF were treated with  $10^{-7}$  M DHT and/or  $10^{-6}$  M OH-FI in PRF-CT for 24 h. The expression of IRS-1 and AR was determined by western blotting (WB) using 50  $\mu$ g of cytoplasmic and 100  $\mu$ g of nuclear protein lysates. 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 filters. **(b)** MCF-7 cells synchronized in PRF for 24 h were treated with  $10^{-7}$  M DHT for 24 h (DHT) or left untreated **(c)**. Subcellular localization of IRS-1 was evaluated by immunostaining and confocal microscopy as detailed in Materials and methods. The captured images of IRS-1, organization of actin into stress fibers (actin), merged IRS-1 and phalloidin (Merge), and normal rabbit IgG (Negative) are shown. Scale bar equals 30  $\mu$ m. **(c)** Nuclear and cytoplasmic lysates from MCF-7 cells (500  $\mu$ g) were immunoprecipitated (IP) with anti-AR mAb (2  $\mu$ g/sample) or normal mouse IgG (2  $\mu$ g/sample) and immunoblotted (WB) to detect IRS-1 and AR protein levels. Input lysates are reported in A. Results are representative of three independent experiments

To determine whether the involvement of IRS-1 in AR-mediated transcription was related to IRS-1 recruitment on ARE-containing regions of androgen target gene promoters, ChIP assay was performed. We focused on the regulatory region of two genes such as PSA (prostatic specific antigen) [31, 32] and p21<sup>WAF1/CIP1</sup> (p21) that are known to be modulated by androgens in breast cancer cells. Particularly, p21 plays a crucial role on cell cycle regulation and it is up



**Fig. 3** IRS-1 is required to sustain AR transcriptional activity. **(a)** HEK 293 cells, that are AR and IRS-1 negative, were co-transfected with 0.5  $\mu$ g pARE2-tk-LUC plus increasing quantities ( $\mu$ g) of pCMV-IRS-1 (IRS-1) together with 0.1  $\mu$ g pcDNA3-AR (AR). Upon transfection, cells were treated with  $10^{-7}$  M DHT, or left untreated in PRF-CT for 18 h. Firefly luciferase activity was detected and expressed as Relative Luciferase Activity with respect to the untreated samples. Linear relation between transfected IRS-1 plasmid and expressed IRS-1 protein quantity, as well as AR expression, was evaluated by WB. Results represent the mean  $\pm$  sd from five independent experiments. Data were statistically analysed by *T* student test, \**P* < 0.05. **(b)** ChIPs were carried out in MCF-7 cells over a 4 h time-course after stimulation with  $10^{-7}$  M DHT using AR, IRS-1 and Pol II antibodies (2  $\mu$ g/sample each). The PSA and p21 promoter regions containing ARE sequence were detected by PCR with specific primers listed in Materials and Methods. 3  $\mu$ l of initial preparations of soluble chromatin were amplified to control input DNA. In control samples (N), normal mouse or rabbit IgG (2  $\mu$ g/sample) was used instead of the primary Abs as control of Ab specificity. **(c)** AR/IRS-1 direct and reverse Re-ChIPs, were carried out as described in Materials and methods, in MCF-7 cells after 2 h of stimulation with  $10^{-7}$  M DHT. ChIP with normal mouse or rabbit IgG (2  $\mu$ g/sample) was used as a negative control (N)



**Fig. 4** IRS-1 knockdown reduces AR regulated transcription decreasing the expression of AR regulated genes. Total cellular RNA (a) and cytoplasmic proteins (b) were isolated from MCF-7 cells transfected with 0.5 μg shIRS-1 or 0.5 μg scrambled control shRNA and treated for 24 h with  $10^{-7}$  M DHT or left untreated in PRF-CT. The expression of AR, IRS-1, PSA, and p21 mRNAs was evaluated by RT-PCR as described in Materials and Methods. 36B4 was amplified as control. MCF-7 RNA sample without the addition of reverse transcriptase was amplified as negative control (N). A 50 μg of protein lysates were analyzed by WB to evaluate the expression of IRS-1, AR, p21, and GAPDH. Results, representative of three independent experiments, were quantified and reported as optical density (OD). (c) MCF-7 cells were co-transfected with 0.5 μg shIRS-1 or 0.5 μg scrambled control shRNA (Scrambled) and AR or 0.1 μg of the empty vector together with the following specific reporter constructs: 0.5 μg 2X-ARE-Luc; 0.25 μg p21-Luc; 0.5 μg PSA-Luc. Upon transfection, cells were serum starved, then stimulated with  $10^{-7}$  M DHT, or left untreated in PRF-CT for 24 h. Luciferase activities were determined and expressed as Relative Luciferase Activity with respect to the untreated samples. Results represent the mean  $\pm$  sd of five independent experiments. Data were statistically analyzed by *T* student test, \**P* < 0.05. (d) ChIP was carried out on MCF-7 cells transfected with 3 μg shIRS-1 or 3 μg scrambled control shRNA (Scrambled). Cells were treated with  $10^{-7}$  M DHT for 2 h and DNA-associated proteins were precipitated using AR, and Pol II antibodies (2 μg/sample each). The PSA and p21 promoter sequences containing ARE were detected by PCR with specific primers listed in Materials and Methods. 3 μl of initial preparations of soluble chromatin were amplified to control input DNA. In control samples (N) normal mouse or rabbit IgG (2 μg/sample) was used instead of the primary Abs

regulated by DHT in breast cancer cells [33]. As illustrated in Fig. 3b, basal occupancy of AR and RNA Pol II on PSA and p21 promoters occurs in untreated cells. The recruitment of AR and RNA Pol II to both promoters is increased in response to DHT reaching the maximum at 2 h treatment. Concomitantly, in response to DHT treatment, IRS-1 is recruited to the same promoter regions (Fig. 3b) in association with AR (Fig. 3c), reaching the maximal promoter occupancy after 2 h of hormonal stimulation.

#### IRS-1 knockdown decreases AR transcriptional activity

To determine whether modulation of the AR/IRS-1 interaction could influence AR function in response to DHT, RNA silencing was used to knockdown the expression of IRS-1 in MCF-7 cells. A 50% decrease of IRS-1 mRNA levels, achieved with anti-IRS-1 shRNA expression in MCF-7 cells (Fig. 4a), correlated with a 70% reduction of

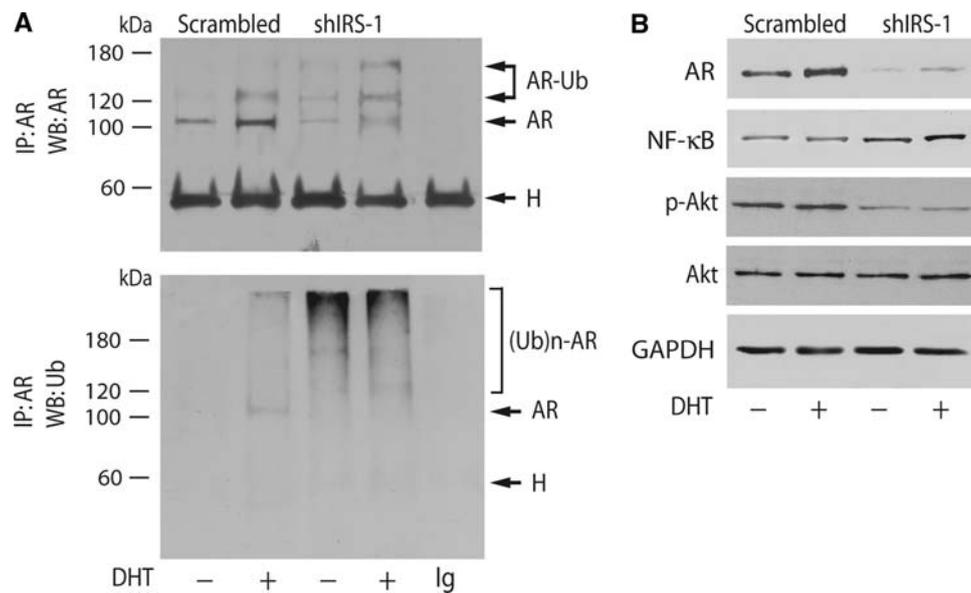
IRS-1 protein levels (Fig. 4b). Down-regulation of IRS-1 resulted in a dramatic reduction of AR protein levels leading to a decrease in PSA and p21 expression (Fig. 4a and b). Interestingly, the reduced AR protein content was not paralleled by a decrease in mRNA expression under DHT treatment, which reflected physiological regulation of active AR on its own expression [34, 35].

Luciferase assay demonstrated that IRS-1 knockdown by shRNA resulted in a 40% decrease in transcriptional activation of ARE-containing promoter in both wild-type and in AR overexpressing MCF-7 cells. Similarly, down-regulation of IRS-1 impeded activation of the PSA and p21 promoters by 50% (Fig. 4c).

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 (Fig. 4d). These observations suggest a role for IRS-1 in modulating AR expression, as well as AR functional activity.

#### IRS-1 knockdown induces ubiquitination of AR

Ubiquitin-dependent proteolysis represents an important mechanism for controlling protein turnover and it is pertinent to the regulation of numerous transcription factors, including AR [36, 37]. Given that polyubiquitination, in



**Fig. 5** IRS-1 modulates AR ubiquitination. MCF-7 cells, transfected with 3  $\mu$ g shIRS-1 or 3  $\mu$ g scrambled control shRNA (Scrambled) were treated for 6 h with  $10^{-7}$  M DHT or left untreated in PRF-CT. **(a)** Total protein lysates were immunoprecipitated (IP) with an anti-AR mAb (2  $\mu$ g/sample) and then probed (WB) for AR [upper panel]. AR mAb recognized three forms of AR protein, ranging from 110 to

170 kDa. The large band at  $\sim$ 60 kD represents the heavy chain of anti-AR mAb (H). The filter was stripped and re-probed (WB) with anti-Ub mAb [lower panel]. **(b)** The expression of AR, NF- $\kappa$ B, p-Akt, Akt and GAPDH was assessed by WB on 50  $\mu$ g of protein lysates. The results are representative of three independent experiments

general, constitutes a destructive signal that is recognized by the proteasome, and that the cellular abundance of both IRS-1 and AR is regulated through the 26S proteasome [38, 39], we investigated the ubiquitination 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. Ubiquitinated AR (Ub-AR) was detected by IP and WB. 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-ubiquitinated receptor, and the poly-ubiquitinated form ranging from 140–170 kDa, as elsewhere described [40, 41] (Fig. 5a, upper panel). In MCF-7 cells, ubiquitination of AR was ligand dependent since in the absence of DHT only marginal Ub-AR was observed (Fig. 5a, lower panel).

In IRS-1-shRNA MCF-7 cells, mono-ubiquitinated AR was still present but, it is worth noting that, in these experimental conditions a dramatic increase in the poly-ubiquitination status of AR was observed in both DHT treated and untreated samples (Fig. 5a, lower panel). Thus, in the shIRS-1 MCF-7 cells the reduction of AR protein content (Fig. 5b) might be explained by the increase of poly-ubiquitination of AR (Fig. 5a). Since AR ubiquitination and degradation has been shown to be dependent on active Akt [42], we evaluated Akt phosphorylation in response to IRS-1 knockdown. As shown in Fig. 5b, phosphorylated Akt was significantly reduced in shIRS-1 MCF-7 cells.

Interestingly, IRS-1 knockdown appears to enhance the general proteasomal activity in MCF-7 cells, as indicated by the increased levels of the DNA binding subunit of NF- $\kappa$ B, that is produced from a precursor via ubiquitin-proteasomal processing [43] (Fig. 5b). These data suggest that IRS-1 interferes with proteasomal pathways of various proteins. Particularly, the presence of IRS-1 seems to protect AR from ubiquitin-mediated degradation, probably by desegregating proteins involved in common proteolytic processes.

## Discussion

Several studies demonstrated the interplay between the IGFs and the ER or PR [5, 41] pathways in breast cancer cells. Here we investigated the existence of a functional crosstalk between IGF system and AR signaling. We report that DHT interferes with IGF-I induced proliferation in MCF-7 cells. The molecular mechanism underlying such functional interference seems to involve nuclear fraction of IRS-1 [5, 21]. Indeed, in MCF-7 cells, DHT stimulation promotes the binding of IRS-1 with AR in the cytoplasm and increases the translocation of IRS-1 into the nucleus. DHT-dependent nuclear translocation of IRS-1 seems to be strictly mediated by AR, since the addition of an androgen antagonist (OH-FI) inhibits this process. Our results are consistent with previous reports demonstrating that IRS-1 is chaperoned to the nucleus by other proteins [20, 23].

Future experiments will be required to delineate the stoichiometry, nature, and strength of the interaction between IRS-1 and AR.

Nuclear localization of IRS-1 has already been documented in breast cancer cells [20], and many other cellular systems [23, 25], suggesting that IRS-1 can function not only as a signal transducer but also as a transcriptional regulator [20, 26]. Despite the evidence that IRS-1 signaling may have a critical role in tumorigenesis [44], some studies, examining the clinical significance of IRS-1 in human breast cancer specimens, evidenced that IRS-1 marks a more differentiated phenotype and a better prognosis [24, 45]. In particular, in breast ductal cancer and benign tumors, nuclear IRS-1 is positively correlated with ER expression and both are negatively correlated with tumor grade, size, mitotic index and lymph node involvement [24]. Moreover, we have previously demonstrated that nuclear IRS-1 negatively modulates the transcriptional activity of ER [20].

Here we have extended the functional role of nuclear IRS-1, demonstrating that it is important to sustain AR transcriptional activity. Indeed in MCF-7 cells, upon DHT treatment, IRS-1 is recruited with AR on ARE-containing promoter regions increasing the transcription of classically androgen regulated gene, such as PSA that is considered a favorable prognostic marker in breast cancer [32]. Moreover, DHT stimulation induces the recruitment of AR and IRS-1 on the promoter of  $p21^{WAF1/CIP1}$ , increasing its transcription and expression. These observations support the inhibitory role of DHT on MCF-7 cells proliferation, since  $p21^{WAF1/CIP1}$  is a crucial regulator of the normal progression of cells through G1 phase of the cell cycle [46], representing the major cyclin-dependent kinase inhibitor in MCF-7 breast cancer cells [47].

Thus, it is reasonable to suggest that the nuclear translocation of IRS-1 in response to DHT stimulation participates in reducing the sensitivity of breast cancer cells to the proliferative effect of IGF-I. This effect appears to be associated to the increased expression of factors that negatively control cell growth, such as the cell cycle inhibitor p21, and not associated to the decrease in IRS-1 cytoplasmic availability since the phosphorylation of downstream proteins of the IGF-I system is unaffected by DHT treatment.

Our results are consistent with the notion that in breast cancer nuclear IRS-1 appears to exert a negative role on the development of neoplastic phenotype [20, 22, 24, 48]. Moreover, in JC Virus T-antigen positive medulloblastoma cells, IRS-1 nuclear translocation and co-localization with Rad51, sensitize cells to genotoxic agents such as cisplatin and gamma-irradiation [49].

Surprisingly, IRS-1 also appears to modulate AR expression levels, since IRS-1 silencing markedly

decreases AR protein content, altering both AR mRNA and AR protein levels. AR, like many nuclear receptors, is a target for several post-translational modifications. Of these, ubiquitination has been demonstrated to signal receptor destruction providing a mechanism for AR inactivation [42]. While AR mono-ubiquitination correlates with enhanced transactivating capacity [40, 41, 50, 51], poly-ubiquitination of AR is widely viewed as signal for the destruction by the proteasome. In keeping with this notion, in MCF-7 cells, IRS-1 knockdown causes the formation of higher molecular weight AR species that are indicative of poly-ubiquitination and coincides with a reduction of the receptor protein. This reduction results in a decrease of  $p21^{WAF1/CIP1}$  and PSA expression, suggesting that IRS-1 is required for maximal AR-dependent transcription.

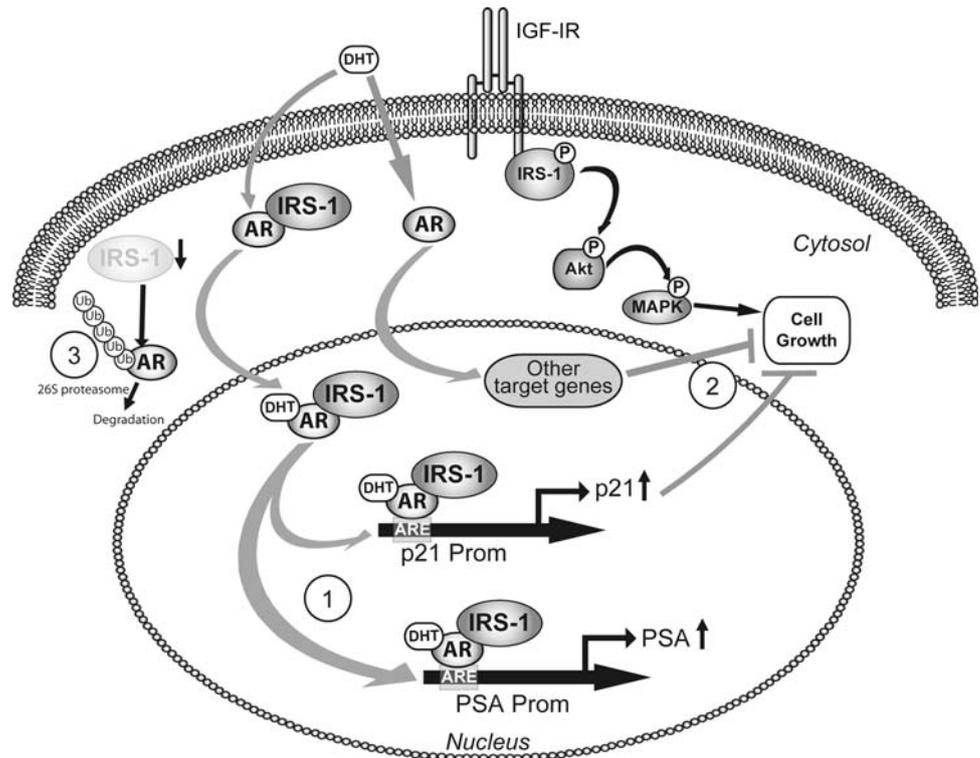
Lin et al. have previously demonstrated that activated Akt, through Mdm2, induces the ubiquitination and proteosomal degradation of AR [42]. However, this mechanism does not seem to be involved in the increased AR degradation following IRS-1 silencing. Indeed, in our experimental condition, IRS-1 knockdown reduces phosphorylated Akt without affecting Akt expression, consistent with previous study by Cesarone et al. [28]. It is likely that other mechanisms might participate in the ubiquitin-dependent proteolysis of AR. For instance, it has been proposed that recruitment of Mdm2 results in modification of various members of the AR transcriptosome, such as histone deacetylase 1, predisposing AR for ubiquitination and proteosomal degradation [41]. Therefore, further studies are required to better understand the mechanisms by which IRS-1 prevents AR proteosomal degradation thus sustaining its expression.

In summary, we provide evidence that in MCF-7 cells, the activation of AR by its ligand interferes with breast cancer cell proliferation in response to IGF-I, suggesting a negative interplay between the two systems. Specifically, our data provide novel insights into the role of IRS-1 as nuclear regulator of AR action in breast cancer cells (Fig. 6). IRS-1 directly acts to modulate AR-mediated transcription by participating in the transcriptional machinery on AR-regulated promoters. Moreover, IRS-1 modulates AR mRNA levels and stabilizes AR protein. This latter event appears to be effected by reducing the ubiquitin-dependent proteolysis of AR.

In conclusion, the results reported in this work have identified mechanisms by which androgens inhibit the proliferation of breast cancer cells and demonstrate the critical role of IRS-1 as a modulator of the functional response to different steroid hormones stimulation in breast cancer cells.

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**Fig. 6** Model for AR/IRS-1 functional interaction. DHT increases AR/IRS-1 association and nuclear translocation, leading to sustained AR transcriptional activity on promoters of genes (1) involved in the negative control of cell growth (2). This mechanism does not affect IGF-IR-mediated intracellular signaling. IRS-1 expression protects the receptor from proteasome-mediated degradation (3)



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