Inhibition of cyclin D1 expression by androgen receptor in breast cancer cells—identification of a novel androgen response element

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ABSTRACT

Cyclin D1 gene (CCND1) is a critical mitogen-regulated cell-cycle control element whose transcriptional modulation plays a crucial role in breast cancer growth and progression. Here we demonstrate that the non-aromatizable androgen 5α-dihydrotestosterone (DHT) inhibits endogenous cyclin D1 expression, as evidenced by reduction of cyclin D1 mRNA and protein levels, and decrease of CCND1-promoter activity, in MCF-7 cells. The DHT-dependent inhibition of CCND1 gene activity requires the involvement and the integrity of the androgen receptor (AR) DNA-binding domain. Site directed mutagenesis, DNA affinity precipitation assay, electrophoretic mobility shift assay and chromatin immunoprecipitation analyses indicate that this inhibitory effect is ligand dependent and it is mediated by direct binding of AR to an androgen response element (CCND1-ARE) located at −570 to −556-bp upstream of the transcription start site, in the cyclin D1 proximal promoter. Moreover, AR-mediated repression of the CCND1 involves the recruitment of the atypical orphan nuclear receptor DAX1 as a component of a multiprotein repressor complex also embracing the participation of Histone Deacetylase 1. In conclusion, identification of the CCND1-ARE allows defining cyclin D1 as a specific androgen target gene in breast and might contribute to explain the molecular basis of the inhibitory role of androgens on breast cancer cells proliferation.

INTRODUCTION

Over-expression of cyclin D1 has been linked to breast cancer growth and progression (1–4), as well as development of resistance to hormone therapy (5–8). The biologically relevant role of cyclin D1 in breast tumourigenesis has been evidenced by several findings: mammary gland-targeted cyclin D1 over-expression resulted in mammary hyperplasia and adenocarcinoma in transgenic mice (9); cyclin D1 antisense blocked ErbB2-induced mammary tumour growth in vivo (10), and cyclin D1-deficient mice were resistant to ErbB2- or Ras-induced mammary tumourigenesis (11). In addition, the correlation between cyclin D1 expression levels and cellular proliferation in breast cancer cells has been further confirmed by silencing experiments (1,12).

Several hormones are involved in breast cancer cells proliferation, so that cyclin D1 represents an important target of their intracellular-signalling pathways (13–17). Emerging evidences indicate that the androgen-signalling pathway mainly exerts inhibitory effects on the growth of normal mammary epithelial cells and plays a protective role in the pathogenesis of breast cancer (18–21). Nonetheless, there are also some epidemiologic reports supporting the concept that androgens, in certain settings, can contribute to breast cancer growth (22–23). Androgens excess (e.g. in congenital adrenal hyperplasia) suppresses breast development (20), while mice lacking a functional androgen receptor (AR) display defective mammary gland development and morphogenesis (21). Furthermore, in vivo studies evidenced that blocking the action of endogenous androgens results in a significant increase in mammary epithelial cell proliferation (24–25).

In vitro, androgen signalling may counteract the proliferative effect of estrogens in AR-positive breast cancer
cells, (26), while over-expression of the AR in MCF-7 cells markedly decreases oestrogen receptor α (ERα) transcriptional activity (27–28). Furthermore, the non-aromatizable androgen 5-α-dihydrotestosterone (DHT) is able to inhibit serum as well as estradiol-induced proliferation in ERα positive breast cancer cell lines (18,28–31), through a mechanism involving an increase in AR protein cell content (28) concomitantly with the down-regulation of the G1/S transition of the cell cycle (28,30).

The AR is present in both primary breast tumours (70–90%) and metastases (75%) (32–34) and shows significant association with important clinical and pathologic prognostic factors (35), since AR expression and functional activity correlate with a low tumour grade, smaller tumour size, improved response to hormone therapy and longer patient survival (33,35–41). Conversely, reduced or impaired AR signalling has been demonstrated in hereditary male breast cancer (42) as well as in HER2-positive breast cancers, generally associated with a worse outcome (43). Androgens have been previously used in the adjuvant therapy of breast cancer in both pre-menopausal and post-menopausal women, with an efficacy comparable to that of current hormonal treatment (44–45), and combined hormonal therapy using tamoxifen plus the androgen fluoxymesterone offered some therapeutic advantage over tamoxifen alone in metastatic breast carcinoma (45). Furthermore, it has been suggested that in AR-positive breast cancer cells and breast carcinomas, the anti-proliferative effect of aromatase inhibitors seems to be due not only to the reduction in estrogens biosynthesis but also to the unmasking of the inhibitory effect of androgens acting via the AR (46–47).

Thus, AR is not only frequently expressed in breast tumours and related to prognosis (48), but it also may serve as a predictive marker for adjuvant hormonal therapy (49). However, events following AR activation and leading to inhibition of cell growth are not clearly identified in breast cancer cells.

Here we investigated whether DHT-dependent inhibition of breast cancer cell proliferation might be due to the modulation of cyclin D1, whose induction represents a key rate-limiting event in mitogenic signalling leading to S-phase entry. We report the identification of a novel androgen-mediated mechanism that controls the expression of cyclin D1 in MCF-7 breast cancer cells by negatively regulating cyclin D1 transcript and protein levels. Indeed, we identified, in the human cyclin D1 promoter a functional androgen responsive element (ARE), which binds the AR in response to DHT stimulation. Transcriptional repression of CCND1 by AR appears to be consequent to the recruitment of a multiprotein repressor complex involving the participation of the AR corepressor DAX1 and containing histone deacetylase activity.

**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) and in DMEM plus 10% foetal calf serum, respectively. 5α-DHT (Sigma, USA) and hydroxylutamide (OH-Fl; Sigma) were used at a concentration of 10⁻⁷ M and 10⁻⁶ M, respectively. Before each experiment, cells were grown in phenol red-free (PRF) DMEM, containing 5% charcoal-treated foetal calf serum (PRF–CT) for 3 days and then serum starved in PRF for 24 h to synchronize the cells. All the experiments were performed in 2.5% PRF–CT.

**Cell proliferation assays**

MCF-7 cells were seeded on six-well plates (10⁵ cells/well) in 2.5% PRF–CT. After 24 h, cells were exposed for 3 days to 10⁻⁷ M DHT and/or 10⁻⁶ M OHFl or left untreated. Media were renewed daily. The effects of the various drugs on cell proliferation were measured 0, 24, 48 and 72 h following initial exposure to treatments by counting MCF-7 cells using a Burker’s chamber, with cell viability determined by trypan blue dye exclusion.

In the same experimental conditions, cell viability was also examined using the method of transcriptional and translational (MTT) colorimetric assay (50). At the above indicated time points, 100 µl of MTT (5 mg/ml) were added to each well, and the plates were incubated for 4 h at 37°C. Then, 1 ml 0.04N HCl in isopropanol was added to solubilise the cells. The absorbance was measured with the Ultraspec 2100 Prospecrophotometer (Amersham-Biosciences, Italy) at a test wavelength of 570 nm.

**Cell-cycle analysis**

MCF-7 cells were seeded on six-well plates (10⁵ cells/well) in 2.5% PRF–CT. After 24 h, cells were exposed to 10⁻⁷ M DHT or left untreated. Cell-cycle analysis was performed 72 h following initial exposure to treatment as previously described (28).

**Plasmids, transfections and luciferase reporter assays**

The following plasmids were used: pcDNA3-AR (AR) encoding full-length AR [27]; CMV-P881 (AR(Cys574→Arg)) encoding the full-length AR carrying a mutation in the DNA-binding domain (DBD; Cys574→Arg) (51); D1Δ−2960, D1Δ−944, D1Δ−848, D1Δ−254, D1Δ−136 and D1Δ−96, carrying fragments from the human CCND1 promoter and inserted into the luciferase vector pXP2 (a gift from Dr A. Weitz, University of Naples, Italy); the vector-based pSiAR promoter and inserted into the luciferase vector pXP2 (a gift from Dr A. Weitz, University of Naples, Italy); the vector-based pSiAR plasmid, coding for small interfering RNA targeting the 5’-untranslated region of AR mRNA, and the scrambled control construct pSiCon (52); The Renilla reniformis luciferase expression vector used was pRL-Tk (Promega, USA). MCF-7 cells were transfected using Fugene 6 (Roche, CH, USA) according to the manufacturer’s instructions, pRL-Tk was used to assess transfection efficiency. Luciferase activity was measured using dual luciferase assay System (Promega), normalized to renilla luciferase activity and expressed as relative luciferase units.

For western blotting (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.

**Immunoprecipitation and WB**

Total cell proteins and the cytoplasmic and nuclear fractions were obtained from 70% confluent cell cultures. Immunoprecipitation (IP) and WB were performed as previously described (53). The following monoclonal (m) and polyclonal (p) antibodies (Ab) were used: anti-AR mAb (441), anti-DAX1 pAb (K-17), anti-Lamin B pAb (C-20), anti-GAPDH pAb (FL-335) and normal mouse immunoglobulin G (Ig) (Santa Cruz Biotechnology, USA).

**Real-time reverse transcription-PCR**

Total RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer’s instructions and treated with DNase I (Ambion, Austin, TX, USA). Two micrograms of total RNA were reverse transcribed with the ImProm-II Reverse transcription system kit (Promega); cDNA was diluted 1:3 in nuclease-free water and 5 µl were analysed in triplicates by real-time PCR in an iCycler iQ Detection System (Bio-Rad, USA) using SYBR Green Universal PCR Master Mix (Bio-Rad) with 0.1 µmol/l of each primer in a total volume of 30 µl reaction mixture. Primers used for the amplification were 5′-CGTGGCCTCTAAGATGAGGA-3′ (forward) and 5′-GGGTGTAGATGCACAGCTTCTC-3′ (reverse). Negative controls contained water instead of first strand cDNA. Each sample was normalized on its 18S rRNA content. The 18S quantification was done using a TaqMan rRNA Reagent kit (Applied Biosystems, USA) following the manufacturer instructions. The relative gene expression levels were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as n-fold differences in gene expression relative to 18S rRNA and calibrator, calculated using the ΔΔCt method as follows: 

\[ n\text{-Fold} = 2^{-\Delta\Delta C_t} \]

where ΔCt values of the sample and calibrator were determined by subtracting the average Ct value of the 18S rRNA reference gene from the average Ct value of the different genes analysed.

**Site-directed mutagenesis**

The cyclin D1 promoter plasmids bearing the AR responsive (CCND1-ARE) mutated sites (D1Δ-944mARE) were created by site-directed mutagenesis using Quick Change kit (Stratagene, La Jolla, CA, USA), as previously described (54), using the following mutagenic primers (mutations are shown as lowercase letters): CCND1-ARE (forward) 5′-TGTGGTGCCCCGCTCTCCCGGT CTTTGCAATAAAACTTTAGGAATGCAACAGGTGACACAA-3′ and CCND1-ARE (reverse) 5′-TTTTCATATACACGTTAATAATAGC AaccCTAATTtTtATGCAGGAGCGGAGGACCGGACACACA-3′; Sp1 (forward) 5′-GCCTTCCTTCCTCCCTGCaCCaaCC CCaacaCCCTCCTGCTCCAT-3′ and Sp1 (reverse) 5′-ATGGGAGGGAGGGGCGTTTGGGTTGGTTCGAG CCGGGAGGGGC-3′. The mutated expression vectors were DNA sequencing.

**DNA affinity precipitation assay**

DNA affinity precipitation assay was performed as previously described (55). The DNA motif probes were prepared by annealing a biotinylated sense oligonucleotide for CCND1-ARE, 5′-[Bio]-GCTAAATTAGTTTCTGC AATTAC-3′; for CCND1-mutatedARE, 5′-[Bio]-CAT AAAA-AATTAGGTTTGCAAT-3′; for Sp1, 5′-[Bio]- TGCACCGGCCCCCTCCCCCTGCCCGGCCCCG-GCCCCC-3′) with the respective unbiotinylated complementary oligonucleotide (for CCND1-ARE, 5′-GTAAATT TGCAAGAAGTAAATTGCACG-3′; for CCND1- mutatedARE, 5′-ATTTCGAACCCTTAATTtTAT-3′; for Sp1, 5′-AGGGGGGCGGCGGGGCGCCAG GAGGGGGGCGCCG-3′). The mutated expression vectors were end labelled with [γ-32P]ATP and T4 polynucleotide kinase and purified using Sephadex G50 spin columns. The oligonucleotides used as probes and as cold competitors (Sigma Genosys, UK) were: (nuclideotide motifs of interest are underlined): Probe: 5′-TGCAATGTG TCAATATAGTTTGCACG-3′; Mutated probe; 5′-TGCATaaaAATATAGGTGAGG-CAAC-3′. Nuclear extracts (20 µg) were incubated with 50,000 c.p.m. of labelled probe, under conditions previously reported (54). The mixture was incubated at room temperature for 20 min in the presence or absence of the unlabelled competitor oligonucleotide. Mouse anti-AR monoclonal antibody (441), or rabbit anti-AR polyclonal antibody (C-19) or normal rabbit IgG (Santa Cruz Biotechnology), were included in some of the reaction mixtures with an additional 12-h incubation at 4°C before addition of labelled probe. The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel for 3 h at 150 V.

**Electrophoretic mobility shift assay**

Nuclear protein extracts were prepared as previously described (54). The double-stranded oligonucleotides used as probes were end labelled with [γ-32P]ATP and T4 polynucleotide kinase and purified using Sephadex G50 spin columns. The oligonucleotides used as probes and as cold competitors (Sigma Genosys, UK) were: (nuclideotide motifs of interest are underlined): Probe: 5′-TGCAATGTG TCAATATAGTTTGCACG-3′; Mutated probe; 5′-TGCATaaaAATATAGGTGAGG-CAAC-3′. Nuclear extracts (20 µg) were incubated with 50,000 c.p.m. of labelled probe, under conditions previously reported (54). The mixture was incubated at room temperature for 20 min in the presence or absence of the unlabelled competitor oligonucleotide. Mouse anti-AR monoclonal antibody (441), or rabbit anti-AR polyclonal antibody (C-19) or normal rabbit IgG (Santa Cruz Biotechnology), were included in some of the reaction mixtures with an additional 12-h incubation at 4°C before addition of labelled probe. The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel for 3 h at 150 V.

**Chromatin IP**

Chromatin IP (ChIP) assay was performed as previously described (56). The immuno-cleared chromatin was precipitated with anti-AR mAb, anti-DAX1 pAb, anti-Polymerase II pAb, anti-HDAC1 mAb, anti-HDAC3 pAb and anti-AIB1 mAb (Santa Cruz, USA). A 4-µl volume of each sample was used as template for PCR with specific primers. The following specific primer pairs were used to amplify 168 bp of the ARE-containing cyclin D1 promoter fragment: 5′-TACCCCTTGGGCAATTTCG AACCAGA-3′ (forward); 5′-ACAGACGGCAAAAGAATC TCA-3′ (reverse), and 228 bp of the Sp1-sites containing cyclin D1 promoter fragment 5′-GGCGATTTTGACATTCT TATGA-3′ (forward) and 5′-CAAACACTCCCTGTAGT CGG-3′ (reverse). Immunoprecipitated DNA was also analysed in triplicates by real-time PCR by using 5 µl of the diluted (1:3) template DNA as described earlier. The following primer pairs were used: 5′-GGCCCGGAAATGAACTTTG-3′ (forward); 5′-CTGCATCTTTTCTTTCTTTTCAAC AC-3′ (reverse) to amplify the ARE-containing cyclin D1.
promoter fragment. Real-time PCR data were normalized with respect to unprocessed lysates (input DNA). Inputs DNA quantification was performed by using 5 μl of the diluted (1/50) template DNA. The relative antibody-bound fractions were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as fold differences with respect to the relative inputs.

RNA silencing

For AR gene silencing experiments, MCF-7 cells were transfected using the vector-based pSiAR plasmid or the scrambled control construct pSiCon (52), as described in the ‘Plasmids, transfections and luciferase reporter assays’ paragraph.

For DAX1 gene silencing experiments, custom synthesized siRNA (Ambion) annealed duplexes were used for effective depletion of DAX1 mRNA. A scrambled siRNA that does not match with any human mRNA was used as a control for non-sequence-specific effects (Ambion). Growing cells were switched to PRF for 24 h and then switched to PRF–CT medium for 48 h. After that, cells were trypsized and transfected in suspension with 5 nM siRNA (siDAX1 or scrambled siRNA) in 35-mm dishes, using Lipofectamine 2000 (Invitrogen), following the manufacturer’s instructions. Cells were incubated with the siRNA-Lipofectamine 2000 complex at 37°C for 4 h and then switched to fresh PRF and treated or not with DHT (10−7 M) for 72 h before analysis. For ChIP assay 100 nM siDAX1 was used to silence 60% confluent cells plated in 150-mm dishes.

Statistical analysis

All data were expressed as the mean ± SD of at least three independent experiments. Statistical significances were tested using Student’s t-test.

RESULTS

DHT administration inhibits serum-induced MCF-7 cells proliferation

We previously demonstrated that MCF-7 cells are androgen responsive and that DHT treatment induces a transient increase in AR protein levels (27), similar to those seen in other cell types (57).

To investigate the role of activated AR on breast cancer cell proliferation, the response of MCF-7 cells to the non-aromatizable androgen DHT and/or the AR antagonist OHFl was measured after 24, 48 and 72 h of treatment in PRF DMEM implemented with 2.5% of steroid-depleted serum (PRF–CT). DHT concentration was chosen based on previous studies demonstrating dose-dependent inhibitory effects of DHT on MCF-7 cells proliferation (28,30).

As expected, cells grown in presence of PRF–CT proliferated; DHT treatment instead inhibited serum-induced MCF-7 cells proliferation and, by the end of the treatment, the mean number of DHT-treated cells was ~30% of respective controls. Addition of the AR antagonist OHFl (or bicalutamide, data not shown) effectively reversed the inhibitory effect of DHT on MCF-7 cells proliferation, suggesting that it was mediated by the AR (Figure 1A). A similar pattern of DHT-dependent effect on MCF-7 cells proliferation was obtained by simultaneously performed MTT colorimetric assay (Figure 1B). These data well correlated with cell-cycle analysis showing an increase of the percentage of cells in G0/G1 phase and a concurrent decrease in the S phase, following 72 h of DHT treatment. At this time point the presence of sub-G1 apoptotic cells was undetectable (Figure 1C).

Activated AR decreases cyclin D1 expression and promoter activity

Since DHT administration reduces the G1/S phase transition in MCF-7 cells (28,30), we inquired whether DHT-induced decrease of MCF-7 cells proliferation might be consequent to the modulation of cyclin D1 expression, whose induction represents a key rate-limiting event in mitogenic signalling leading to S-phase entry. To this aim, serum starved MCF-7 cells were left untreated or treated with 10−7 M DHT for 24 h, 48 h or 72 h and cyclin D1 expression was assessed by real-time RT–PCR and WB analysis. As shown in Figure 2A, MCF-7 cells exhibited a decrease in the serum-dependent levels of cyclin D1 mRNA (Figure 2A) and protein (Figure 2B) following 48 and 72 h of DHT treatment. The involvement of the AR in the negative regulation of cyclin D1 expression was ascertained by silencing AR expression in MCF-7 cells (Figure 2B).

To test whether activated AR might negatively modulate cyclin D1 promoter activity, MCF-7 cells were transiently transfected with a cyclin D1 promoter reporter plasmid (D1Δ-2996) and left untreated or treated with 10−7 M DHT.

The cyclin D1 promoter was induced by serum, while DHT treatment inhibited basal cyclin D1 promoter activity, decreasing the serum-induced signal by ~50%. OHFl addition reversed this effect, suggesting that it was due to AR activation (Figure 2C).

To substantiate the AR involvement in the modulation of CCND1 expression, we evaluated the effects of ectopic AR expression on the transcriptional activity of the cyclin D1 promoter. HEK-293 cells, which do not express AR, were co-transfected with the D1Δ-2996 plasmid and increasing amounts of a full length AR-encoding plasmid. In the absence of exogenous AR expression, DHT treatment cannot influence cyclin D1 promoter activity. On the contrary, in the presence of ectopic AR, a dose-dependent decrease in the serum-induced cyclin D1 promoter activity was observed upon DHT administration (Figure 2D).

To investigate whether the effect of AR on cyclin D1 promoter activity is dependent on its transactivation properties, luciferase assay was performed in AR-negative HEK-293 cells transfected with an expression plasmid encoding an AR carrying a mutation (AR_Cys574→Arg) in the DBD, which disrupts its ability to bind target DNA sequences (27,51). In these
circumstances, no decrease in cyclin D1 promoter activity could be detected (Figure 2D), suggesting the existence, in the cyclin D1 promoter, of putative androgen responsive region(s).

Characterization of functional androgen responsive region(s) in the cyclin D1 promoter

To define the AR responsive region(s) of the cyclin D1 promoter, a series of 5'-promoter-deleted mutants were used and tested for both androgen sensitivity and promoter activity, in MCF-7 cells. The constructs D1Δ-2960, D1Δ-944 and D1Δ-848, which include 2.960 kb, 0.944 kb and 0.848 kb of the cyclin D1 promoter fragment, respectively, showed a decreased transcriptional activity upon DHT stimulation with respect to untreated controls. A weaker inhibition of the cyclin D1 promoter signal was evidenced using the D1Δ-254 and D1Δ-136 constructs, while the D1Δ-96 plasmid, failed to respond to DHT (Figure 3).

Sequence analysis of the −848 bp to −254 bp cyclin D1 promoter DNA fragment revealed a likely ARE sequence (TGCTAAattAGTTCT) located at the −570 bp position of the promoter. This putative ARE (CCND1-ARE) is homologous to the AREs found in the promoters of the sc (sc-ARE1.2) and slp (slp-HRE2) genes (58) (Figure 4A). To assess the relative importance of this sequence we used site-directed mutagenesis to alter it. Nucleotide substitutions were introduced into the −570-bp to −556-bp fragment of D1Δ-944 and the mutant promoter D1Δ-944-mARE was assayed in parallel with the unmutated D1Δ-944 promoter. Disruption of the putative ARE consensus site resulted in a significant loss of the inhibitory effect of DHT on cyclin D1 promoter activity (Figure 4B). These results address the CCND1-ARE as a crucial sequence in mediating cyclin D1 promoter inhibition upon DHT exposure.

Nevertheless, mutations at the putative ARE did not completely abolish cyclin D1 promoter responsiveness to DHT, as indicated by the persistence of a weak DHT-induced decrease in the luciferase activity of the D1Δ-136 construct, containing two Sp1 sites. To investigate whether this additional cis-element might be involved in the androgen response, as previously demonstrated for other AR target genes (57,58), site-directed mutagenesis was used to introduce nucleotide substitutions into the −117-bp to −101-bp fragment of D1Δ-944 and the mutant promoters D1Δ-944-mARE were assayed in functional analysis. As depicted in Figure 4B, DHT treatment is able to induce only an about 30% inhibition of D1Δ-944-mSp1 promoter activity, indicating that Sp1 sites are important for an AR full inhibitory effect on the cyclin D1 promoter. Consistent with these observations, mutation of both ARE and Sp1 motifs completely abolishes DHT responsiveness of the cyclin D1 promoter further suggesting that its regulation occurs through a functional interaction of the AR with the CCND1-ARE and Sp1 responsive sequences.

AR interacts with the ARE containing region of the cyclin D1 promoter

To support the functional importance of the identified ARE sequence, a double-stranded oligonucleotide containing the putative ARE sequence was used in a DNA affinity precipitation assay (DAPA) to determine whether AR can bind to the CCND1-ARE consensus sequence. Endogenous AR was found associated with the putative consensus oligonucleotide following DHT administration only, while AR binding was undetectable in nuclear cell lysates from untreated cells (Figure 5A). Moreover, AR
from DHT treated MCF-7 lysates was unable to bind to a cyclin D1 promoter oligo in which the putative ARE consensus site was mutated.

To further determine the specificity of the putative ARE site we performed electrophoretic mobility shift assay (EMSA). As shown in Figure 5B, factors present in MCF-7 cells nuclear extracts retarded the mobility of the putative ARE domain in a specific manner, since the formation of a protein–DNA complex was found (Figure 5B, lane 1). Of note, DHT treatment induced a strong increase in the protein–DNA association (Figure 5B, lane 2) whose appearance was competed effectively by a 100-fold molar excess of unlabelled probe (Figure 5B, lane 3), demonstrating the specificity of the DNA-binding complex. This inhibition was no longer observed using a mutated putative ARE oligonucleotide as competitor (Figure 5B, lane 4). Contemporary administration of the anti-androgen bicalutamide caused a dramatic decrease in the DNA-binding complex induced by DHT (Figure 5B, lane 6) suggesting that the AR is involved in the binding to the CCND1-ARE. Finally the specificity of these bands was proved by the drastic attenuation of the complex in the presence of PRF–CT is arbitrarily set at 100%. Linear relation between transfected AR plasmid and expressed AR protein quantity was evaluated by WB. Results represent the mean ± SD from five independent experiments. Data were statistically analysed by Student’s t-test, *P < 0.05 and **P < 0.01 versus untreated.
AR on cyclin D1 promoter, is further evidenced by the dynamic of RNA Pol II recruitment onto the cyclin D1 promoter, that appears to be drastically reduced upon DHT treatment (Figure 5C).

AR associates with the SP1 sites present in the cyclin D1 proximal promoter

To further investigate the functional importance of the Sp1 sites in the AR-mediated modulation of the cyclin D1 promoter, the ability of AR to associate to the Sp1 sites containing sequence of the cyclin D1 promoter (59) was first examined by DAPA. The association between AR and the cyclin D1-Sp1 sequence was observed only upon DHT treatment, while AR binding could not be detected in nuclear cell lysates from untreated cells (Figure 6A).

AR recruitment on the Sp1-containing region of the endogenous cyclin D1 promoter was also investigated by ChIP experiments using anti-AR or anti-RNA Pol II antibodies. AR was recruited to the Sp1-containing region in a ligand-dependent manner. Conversely, upon DHT administration, RNA Pol II was released from the investigated promoter region (Figure 6B) supporting the hypothesis that this Sp1-containing DNA region might cooperate with the CCND1-ARE site in the AR-dependent regulation of CCND1.

The AR corepressor DAX1 is recruited at the CCND1-ARE containing sequence of the cyclin D1 promoter

To assess whether the decrease of cyclin D1 promoter transcriptional activity might be caused by the cooperative interaction between AR and negative transcriptional regulators, we investigated the involvement of the orphan nuclear receptor DAX1, which has been shown to interact with and function as a negative coregulator of AR (60–62).

DAX1 abundance was analysed in cytoplasmic and nuclear protein fractions obtained from MCF-7 cells stimulated or not with 10^{-7} M DHT. DAX1 was mainly present in the nuclear compartment and its abundance increased upon DHT treatment (Figure 7A). To investigate whether the DHT-induced enhancement of DAX1 expression is paralleled by an AR/DAX1 physical interaction, co-IP assay was performed on nuclear and cytoplasmic protein fractions from MCF-7 cells. The formation of a AR/DAX1 complex was clearly evidenced into the nucleus upon DHT administration (Figure 7B), arguing for a DAX1 regulatory function on AR transcriptional activity.

To test whether DAX1 could assemble on the androgen responsive region of the cyclin D1 promoter, DAPA assay was performed. The binding affinity of DAX1 to the DNA probe containing the putative CCND1-ARE site (Figure 8A) or the Sp1 site (Figure 8B) was significantly changed in response to DHT. Furthermore, no binding of another AR corepressor, such as SMRT, to the CCND1-ARE containing DNA probe could be detected (data not shown).

To verify that DAX1 is recruited within the androgen responsive region of human cyclin D1 promoter in vivo, ChIP assay was performed using anti-DAX1 antibody. DAX1 occupancy of the CCND1-ARE containing region of the cyclin D1 promoter significantly increased following DHT treatment. No recruitment of the steroid receptor coactivator AIB1, was detected under the same conditions.
experimental condition (Figure 8C and D), showing the selectivity of corepressor recruitment. A similar pattern of DAX1 recruitment to the Sp1-containing region of the cyclin D1 promoter was observed (Figure 8E). Since histone deacetylation represents at least one of the mechanisms by which repressor proteins mediate transcriptional repression (63–64), we investigated HDAC1 and/or HDAC3 association on the AR responsive sequences within the cyclin D1 promoter by DAPA and ChIP assays. In both assays, DHT stimulation induces the recruitment of HDAC1 but not HDAC3 to either the CCND1-ARE consensus sequence (Figure 8A, C and D), or the Sp1 site (Figure 8B and E).

To confirm the role of DAX1 in the AR-dependent modulation of the CCND1, RNA silencing was used to knockdown the expression of endogenous DAX1 in MCF-7 cells. A 50% decrease of DAX1 protein levels was achieved using anti-DAX1 siRNA in MCF-7 cells after 48 h (Figure 9A). ChIP analysis was then performed on MCF-7 cells transfected with DAX1 siRNA or scrambled control siRNA. Upon DHT stimulation, DAX1 knockdown caused the recovery of RNA Pol II recruitment within the CCND1-ARE (Figure 9B and C) and Sp1 (Figure 9D) containing region of the cyclin D1 promoter, whereas the scrambled siRNA showed no effect on RNA Pol II dismissal. AR recruitment on the responsive regions of the cyclin D1 promoter was not affected by DAX1 silencing (Figure 9B, C and D). Moreover, DAX1 knockdown resulted in the loss of the DHT inhibitory effect on cyclin D1 levels (Figure 9A), strengthening the role of DAX1 as a component of the repressor complex participating in the AR-dependent inhibition of the CCND1.

DISCUSSION

The AR represents a common characteristic in breast tumors, since it is expressed in a significant subset (60–90%) of both ERα-positive and -negative breast cancers (33–35). AR expression in breast cancer tissue samples has been shown to be associated with a better prognosis (35,38–41,65–66). Conversely, lack of AR expression correlates with transformation from in situ to invasive basal subtype of high-grade ductal breast carcinoma (48) and with a poor prognosis in lymph node-positive ER/PR/Her2-negative breast cancers (41); even though a recent report suggests that the AR may participate to the development of invasive ductal carcinoma by repressing E-cadherin expression (67).

Despite the growing body of evidence indicating a protective role of AR in breast tumourigenesis, the mechanisms underlying the inhibition of the in vivo and/or in vitro
breast cancer cells growth by activated AR, remain poorly defined.

In this report we provide evidence that DHT-activated AR is a transcriptional repressor of the \(CCND1\) in MCF-7 breast cancer cells. Analysis of the molecular events associated with this hormone-induced negative regulation of cyclin D1 expression allows, for the first time, the identification of a specific ARE within the \(CCND1\) proximal promoter, and recognizes the recruitment of DAX1 and HDAC1 as components for AR-mediated transcriptional repression.

Earlier studies have shown that DHT inhibits MCF-7 cells proliferation by targeting the G1/S transition of the cell cycle (28,30). Here, we demonstrated that, in MCF-7 cells, cyclin D1 expression is negatively modulated by DHT administration as evidenced by reduction of
The study provides evidence that AR-mediated inhibition of the CCND1-ARE is consequent to direct binding of the AR to a specific androgen responsive site, located at position −570bp as indicated by deletion and sequence analyses of the cyclin D1 proximal promoter. The identified CCND1-ARE motif 5′-TGCTAattAGTTCT-3′ resembles the so-called selective AREs that exhibit characteristics of partial direct repeats, instead of the conventional inverted repeats of the 5′-TGTTTCT-3′ monomer-binding element, and are recognized exclusively by the AR (58,71,72).

This CCND1-ARE is functional, as demonstrated by transactivation studies, and capable to interact with the AR, in a ligand-dependent manner as evidenced by DAPA or EMSA. The physiological relevance of CCND1-ARE within the cyclin D1 promoter in vivo is pointed out by ChIP analysis showing that AR occupancy of the ARE containing promoter region is concomitant with a reduced in RNA Pol II recruitment, consistent with the reduced cyclin D1 transcriptional activity.

Our study also indicates that AR binding to the Sp1 sites within the proximal cyclin D1 promoter is required for a full DHT-dependent inhibition of cyclin D1 promoter activity. This observation is consistent with previous studies on the organization of transcription factor-binding sites within the regulatory regions of a selection of androgen-responsive genes, showing that a key feature of these genes is the presence of one or more AR-binding site (ARE, HRE) together with binding sequences for housekeeping (Sp1, NF-1), inducible (NF-kB, AP1) and tissue specific transcription factors (73). Therefore, androgen-regulated gene expression appears to be dependent on the coordinated interactions of the receptor protein and other transcription factors.

Intriguingly, our data indicate that on CCND1 the promoter-bound DHT–AR complex functions as an inverse agonist, causing active repression of basal gene activity, eventually creating a repressive chromatin conformation associated with reduced RNA Polymerase II recruitment, in MCF-7 breast cancer cells.
Our proposed model for AR-mediated repression of the *CCND1* involves the recruitment of the atypical orphan nuclear receptor DAX1 that is expressed in tissues directly involved in steroid hormone production and reproductive function (74–75). DAX1 inhibits ligand-dependent trans-activation by agonist-bound nuclear receptors like AR, ER and progesterone receptor (60–61,76). Moreover, DAX1 has been proposed to be involved in the development of cancers of a variety of tissues and has been found to positively correlate with AR and ER expression in breast cancer specimens (60–61,74,77). Our results suggest that, in MCF-7 cells, upon DHT stimulation, DAX1 is recruited on the androgen responsive region of the cyclin D1 promoter concomitantly to AR. The repressor complex formation also embraces the participation of HDAC1, further contributing to explain the AR-mediated inhibition of *CCND1* transcription (Figure 10).

Given the importance of cyclin D1 in breast cancer cell proliferation, androgen-mediated regulation of cyclin D1 levels appears to play a crucial role in cell-cycle control.
Figure 9. Effect of DAX1 knockdown on the modulation of CCND1 expression by AR. (A) Total cellular proteins were isolated from MCF-7 cells transfected with 5 nM DAX1 siRNA or 5 nM scrambled control siRNA (scrambled) and treated for 72 h with 10^{-7} M DHT or left untreated in PRF–CT. Fifty micrograms of protein lysates were analysed by WB to evaluate the expression of DAX1 and cyclin D1. The expression of GAPDH was assessed as control of protein loading. Results were obtained after repetitive stripping and reprobing of the same filters. (B) ChIP assays were carried out on serum starved MCF-7 cells transfected with 100 nM DAX1 siRNA or 100 nM scrambled control siRNA (scrambled). Cells were then treated with 10^{-7} M DHT for 2 h and DNA-associated proteins were precipitated using either anti-DAX1, AR or Pol II antibodies (2 μg/sample each). In control samples (Ig), normal mouse or rabbit IgG were used instead of the primary Abs, as control of Ab specificity. The region of the cyclin D1 promoter containing the CCND1-ARE site, was detected by PCR using specific primers listed in the ‘Materials and Methods’ section. Inputs DNA were amplified as loading controls. (C) Five-microliter volume of each sample and input from ChIP were used for real-time PCR as reported in the ‘Materials and Methods’ section. Data were statistically analysed by Student’s t-test, *P < 0.05 and **P < 0.05 versus scrambled DHT treated. (D) ChIP assays were carried out as above described. The 5'-flanking sequence of the CCND1 containing the Sp1 sites was detected by PCR using specific primers listed in the ‘Materials and Methods’ section. Input DNAs were amplified as loading controls. Results are representative of three independent experiments.

Figure 10. Proposed model for AR-mediated repression of the CCND1. In the absence of DHT, CCND1 basal activity is regulated by several serum factors acting through multiple regulatory region of the cyclin D1 promoter and enabling a permissive chromatin conformation and gene transcription (13,15,17). Upon DHT treatment, AR is recruited on the ARE- and Sp1-containing region of the proximal cyclin D1 promoter, causing displacement of RNA Polymerase II and recruitment of a corepressor complex containing DAX1 and HDAC1, with consequent repression of cyclin D1 expression.
The importance of our results is highlighted by recent studies demonstrating that biologically active DHT is locally produced in breast carcinoma tissues. Interestingly, intratumoural DHT levels are positively associated with AR and 5α-reductase 1 expression but inversely correlated with tumour size, Ki-67 and aromatase expression (78), suggesting that in AR-positive breast carcinomas the use of aromatase inhibitors may be more effective by accumulation of the local DHT concentration. The biological significance of DHT-induced inhibition of cyclin D1 expression is also pointed out by clinical studies using tamoxifen as an adjuvant therapy in ER-positive breast cancers, showing a higher response and better survival rate in cancers with cyclin D1 low/moderate expression (7–8). These observations, supported by the widespread expression of AR in primary and metastatic breast tumours, suggest the possibility that targeting the AR-signalling pathway could be helpful in improving new molecular and pharmacological approaches for breast cancer treatment and to potentiate the effectiveness of anti-oestrogen adjuvant therapies.

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