Androgen Receptor Controls *EGFR* and *ERBB2* Gene Expression at Different Levels in Prostate Cancer Cell Lines

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Abstract

EGFR or ERBB2 contributes to prostate cancer (PCa) progression by activating the androgen receptor (AR) in hormone-poor conditions. Here, we investigated the mechanisms by which androgens regulate *EGFR* and *ERBB2* expression in PCa cells. In steroid-depleted medium (SDM), EGFR protein was less abundant in androgen-sensitive LNCaP than in androgen ablation–resistant 22Rv1 cells, whereas transcript levels were similar. Dihydrotestosterone (DHT) treatment increased both EGFR mRNA and protein levels and stimulated RNA polymerase II recruitment to the *EGFR* gene promoter, whereas it decreased ERBB2 transcript and protein levels in LNCaP cells. DHT altered neither EGFR or ERBB2 levels nor the abundance of prostate-specific antigen (PSA), TMEPA1, or TMPRSS2 mRNAs in 22Rv1 cells, which express the full-length and a shorter AR isoform deleted from the COOH-terminal domain (ARΔCTD). The contribution of both AR isoforms to the expression of these genes was assessed by small interfering RNAs targeting only the full-length or both AR isoforms. Silencing of both isoforms strongly reduced PSA, TMEPA1, and TMPRSS2 transcript levels. Inhibition of both AR isoforms did not affect EGFR and ERBB2 transcript levels but decreased EGFR and increased ERBB2 protein levels. Proliferation of 22Rv1 cells in SDM was inhibited in the absence of AR and ARΔCTD. A further decrease was obtained with PKI166, an EGFR/ERBB2 kinase inhibitor. Overall, we showed that ARΔCTD is responsible for constitutive EGFR expression and ERBB2 repression in 22Rv1 cells and that ARΔCTD and tyrosine kinase receptors are necessary for sustained 22Rv1 cell growth. [Cancer Res 2009;69(7):2941–9]

Introduction

Prostate cancer (PCa) is the most commonly diagnosed noncutaneous malignancy in men in developed countries. At diagnosis, most PCas are androgen dependent, meaning that their growth, survival, and progression are driven by the androgen-activated androgen receptor (AR). Hence, the most efficient first-line therapy for locally advanced and metastatic PCa is surgical or chemical castration. Nevertheless, the cancer eventually evolves toward androgen ablation resistance (AAR). At this stage, cancerous cells continue to express a transcriptionally active AR, as evidenced by the expression of androgen-dependent genes, such as the prostate-specific antigen (*PSA*) gene.

The AR is a ligand-dependent transcription factor. Hormone binding induces AR translocation from the cytoplasm to the nucleus and the recruitment of different transcriptional cofactors on androgen-responsive elements (ARE) in the neighborhood of androgen-responsive genes (1–3).

Several mechanisms have been proposed to explain the maintenance of AR signaling in a hormone-poor environment. Briefly, the AR could be activated by intratumoral androgens (4). Moreover, AR overexpression, mutations, or modulation of the repertoire of cofactors, increasing the sensitivity of the receptor to low ligand levels or rendering it responsive to other hormones and even to androgen antagonists, could lead to AAR tumor progression (1, 2). In addition, the AR can be activated indirectly by growth factor receptors (1, 2), mainly the transmembrane tyrosine kinases EGFR and ERBB2, members of the epidermal growth factor receptor (EGFR) family, which are activated inappropriately in many human cancer types (5).

Several lines of evidence indicate that these receptors contribute to PCa progression. The paracrine regulation of the EGFR observed in the healthy gland seems to be replaced by an autocrine stimulation of the signaling in the PCa (6). Overexpression of ERBB2 protein constitutively activated by mutation induces PCa in transgenic mice (7). Moreover, EGFR activation (8, 9) or forced overexpression of ERBB2 (10–12) induces androgen-independent activation of AR transcriptional activity. Finally, EGFR (13, 14) and ERBB2 (15, 16) overexpression has been reported during the progression from hormone-dependent to AAR PCa.

Increased EGFR protein levels in androgen-treated LNCaP cells have been reported (17, 18). There are also reports of ERBB2 down-regulation by the hormone (19, 20). However, the mechanisms responsible for these effects were not investigated in detail. Moreover, the mechanisms by which these genes are dysregulated in hormone-depleted medium have not been investigated.

Given the role of EGFR and ERBB2 in the evolution of PCa to AAR, we investigated *EGFR* and *ERBB2* expression in PCa cell lines presenting different androgen sensitivities. LNCaP cells express the AR and their proliferation is androgen dependent (21). 22Rv1 cells also express the AR but androgens weakly increase their proliferation rate (22). PC3 and Du145 cells do not express the AR (23). We found that EGFR expression was highest in PC3 and Du145 cells. In hormone-depleted culture conditions, EGFR was more abundant in 22Rv1 than in LNCaP cells. However, dihydrotestosterone (DHT) increased EGFR levels in LNCaP but not in 22Rv1 cells. In LNCaP cells, DHT stimulated *EGFR* gene transcription, whereas in 22Rv1 cells an AR isoform lacking the COOH-terminal hormone binding domain (ARΔCTD) controlled

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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the expression of the protein but not the transcript. In contrast to *EGFR*, *ERBB2* was repressed by androgens in LNCaP cells, and ARΔCTD posttranscriptionally mediated ERBB2 repression in 22Rv1 cells. When AR and ARΔCTD were inhibited, both the expression of androgen-responsive genes and the proliferation of 22Rv1 cells cultured in steroid-depleted medium (SDM) were inhibited. PKI166, a tyrosine kinase inhibitor, further decreased proliferation. Taken together, these data suggest that ARΔCTD controls the AAR phenotype of 22Rv1 notably by posttranscriptionally regulating *EGFR* and *ERBB2* expression.

Materials and Methods

Reagents and plasmids. DHT, charcoal, cycloheximide and bicalutamide were obtained from Sigma. PKI166 was obtained from Novartis. Anti-EGFR antibody and normal rabbit IgGs were obtained from Santa Cruz Biotechnology. Anti-ERBB2 and anti-AR (pg-21) antibodies were purchased from Upstate Biotechnology. Anti-PSA antibody was from Biogenex, and anti-RNA polymerase II (4H8) and anti-β-actin antibodies were from Covance. The $pPSA_{EEP}GL3$ and the pMMTV reporter vectors were generous gifts, respectively, from Dr. N.D. James (University of Birmingham, Birmingham, United Kingdom) and Dr. M. Muller (University of Liège, Liège, Belgium) and these have been described previously (24, 25). pCH110 vector encoding the *LacZ* gene under the SV40 early promoter was obtained from Promega.

Cell lines and cell culture conditions. LNCaP and 22Rv1 cell lines were maintained in a humid atmosphere containing 5% CO_2 and were grown as monolayers in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 10 mmol/L HEPES, 10 mmol/L sodium pyruvate, and 100 units/mL penicillin/streptomycin. Steroid deprivation consisted in maintaining the cells for 5 d in SDM before DHT treatment. The SDM consisted of phenol red–free RPMI 1640 supplemented with 8% FBS treated overnight with 2% charcoal at 4°C. DHT was diluted in ethanol and appropriate ethanol controls were included. All tissue culture media were obtained from Lonza.

Reporter assay. Cells were transfected with Fugene HD transfection reagent (Roche). Cells (10^5) were seeded in six-well plates and treated with a Fugene to DNA ratio of 3 μL:1 μg for 48 h. Reporter vector (0.5 μg) and pCH110 (0.5 μg) were used. Cells were harvested and lysed, and luciferase activity was measured using the luciferase gene reporter assay kit (Roche) according to the manufacturer's instructions. β-Galactosidase activity was measured in a reaction mixture containing 400 μL β-galactosidase buffer (60 mmol/L Na_2HPO_4, 10 mmol/L NaH_2PO_4, 10 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.27% β-mercaptoethanol), 100 μL lysate, and 100 μL of 4 mg/mL orthonitrophenyl-β-D-galactopyranoside. The reaction was performed at 37°C until the development of a yellow color. The reaction was stopped with 250 μL of 1 mol/L Na_2CO_3. Absorbance was measured at 420 nm.

Testosterone dosage. Testosterone concentration was determined by electrochemiluminescence immunoassay with an Elecsys testosterone reagent kit according to the manufacturer's instructions (Roche).

Cell proliferation assay. Cell proliferation assays were performed with WST-1 reagent according to the manufacturer's instructions (Roche).

Protein extraction and Western blot. Protein extractions were performed as described by Myers and colleagues (20), except that 1× Mini Complete (Roche) protease inhibitor cocktail was added to the lysis buffer. Western blots were performed with 30 μg of protein extract, as described previously (26).

RNA isolation and semiquantitative reverse transcription-PCR. Total RNA was isolated using the NucleoSpin RNAII kit (Macherey-Nagel) according to the protocol provided by the manufacturer. Total RNA (1 μg) was reverse transcribed using 0.5 μmol/L oligo(dT) primers and 10 units of avian myeloblastosis virus reverse transcriptase (Promega) in a total volume of 50 μL. PCRs were performed with AmpliTaq DNA polymerase (Applied Biosystems), as described by the manufacturer with 1:20 reverse transcription reaction mixture in a final volume of 25 μL in the presence of 1 mmol/L $MgCl_2$. PCR cycles were 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C. Primer pairs were chosen in different exons. The primer sequences are given in Supplementary Data.

Small interfering RNA transfection. Small interfering RNAs (siRNA) targeting the AR were designed and chemically synthesized by Eurogentec. The siRNA sequences are given in Supplementary Data. Negative control siRNA (siNeg) from Eurogentec served as the negative control. Cells were transfected with 30 nmol/L siRNA with phosphate calcium or for cell proliferation assay with X-tremeGENE transfection reagent (Roche). For phosphate calcium transfection, the medium was replaced with transfection medium (DMEM without phenol red supplemented with 8% charcoal-treated FBS) and cells were transfected with siRNA using the calcium phosphate transfection protocol. After 16 h of transfection, cells were rinsed twice with PBS and incubated in SDM. For X-tremeGENE transfection, cells were directly treated in SDM with an X-tremeGENE/siRNA/medium ratio of 5 μL:30 pmol:1 mL according to the manufacturer's instructions.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were performed as described previously (27), except that chromatin was sheared with Biorupter (Diagenode). The specific primers used for PCR are given in Supplementary Data.

Results

Hormone-insensitive PCa cell lines are enriched in EGFR protein. We compared EGFR and ERBB2 protein and mRNA levels in four PCa cell lines with different androgen sensitivities. EGFR protein and transcript levels were highest in PC3 and Du145 cells. EGFR was more abundant in LNCaP than in 22Rv1 cells grown in complete medium (Fig. 1*A, top*). In contrast, higher EGFR protein levels were measured in 22Rv1 than in LNCaP cells grown in SDM, whereas mRNA levels were comparable (Fig. 1*A, bottom*). The higher EGFR protein abundance contrasting with similar transcript levels in the two cell lines could be attributed to an increased stability of the protein in 22Rv1 cells. Indeed, we estimated that EGFR protein half-life was almost twice as long in 22Rv1 as in LNCaP cells grown in SDM (data not shown).

In complete medium, ERBB2 protein was most abundant in LNCaP and Du145 cells and least abundant in 22Rv1 cells. However, under these culture conditions, comparable mRNA expression was detected in LNCaP and 22Rv1 cells. In SDM, LNCaP cells expressed higher levels of both ERBB2 protein and transcript than 22Rv1 cells (Fig. 1*A*).

To find out if the changes in expression result from the lack of androgens, we tested the effect of DHT on the expression of EGFR and ERBB2 in LNCaP and 22Rv1 cells cultured in SDM.

Different mechanisms control EGFR and ERBB2 protein levels in the different PCa cell lines. DHT treatment increased EGFR mRNA and protein levels in LNCaP cells (Fig. 1*B, left*) but not in 22Rv1 cells (Fig. 1*B, right*). The treatment repressed ERBB2 expression only in LNCaP cells. Of note was the increase in AR protein levels in LNCaP cells 24 hours after the addition of the hormone to the culture medium. Interestingly, in 22Rv1 cells, the proportion of the full-length AR increased after 24 hours of DHT treatment. High PSA levels measured in DHT-treated LNCaP cells showed the efficiency of the androgenic stimulation (Fig. 1*B*). As previously described, PSA levels were very low in 22Rv1 cells and we were unable to detect the protein by Western blot (data not shown; ref. 28).

Ligand-activated AR is known mainly as a transcription factor, but it also modulates the stability of mRNA and protein. Both mechanisms can explain the differences in EGFR and ERBB2 levels following stimulation of LNCaP cells by DHT. DHT did not

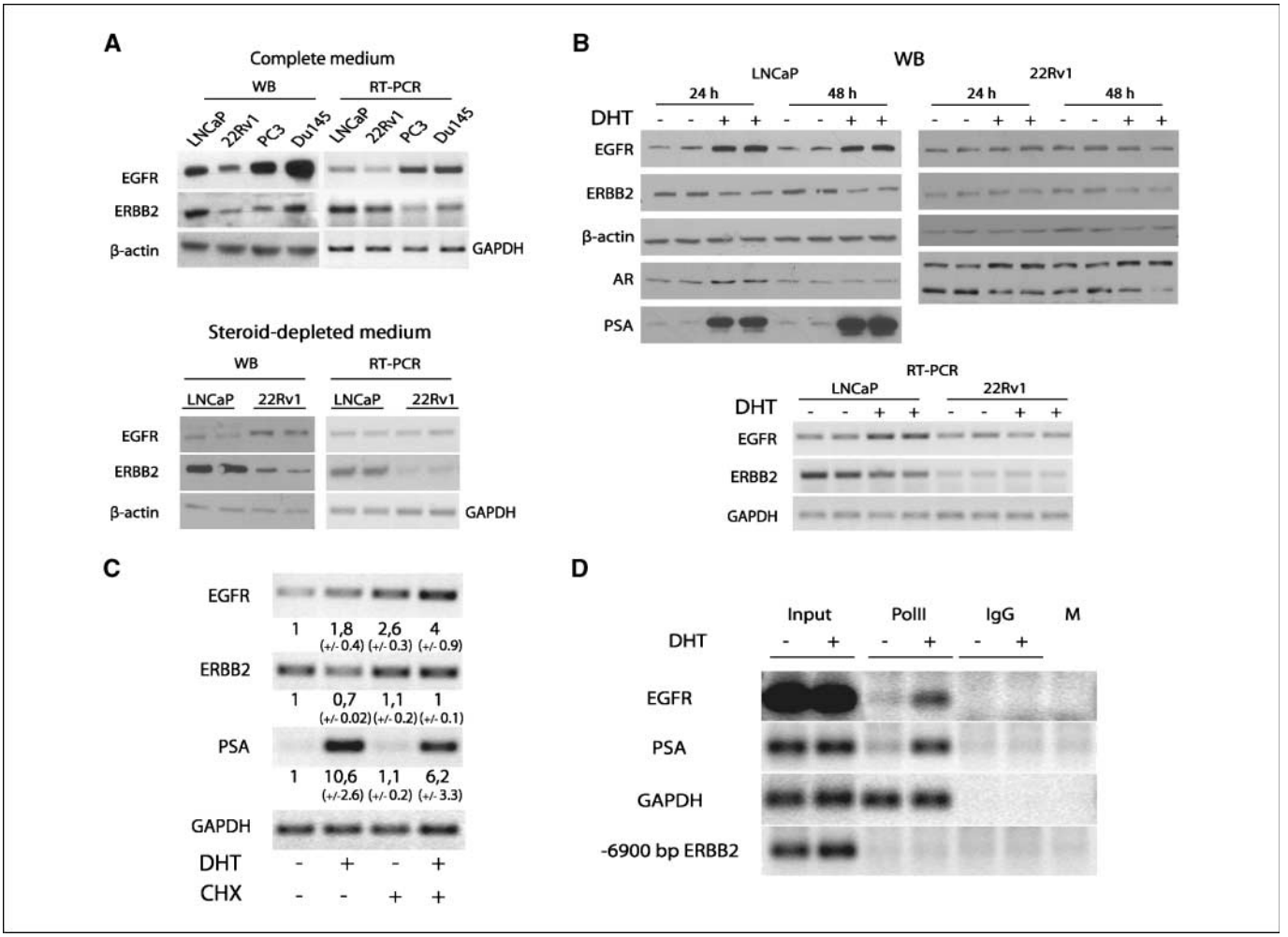

Figure 1. *A,* cells seeded at a density of 200,000 in 6-cm-diameter dishes were grown in complete medium or in SDM for 5 d. EGFR and ERBB2 mRNA and protein levels were analyzed by RT-PCR and by Western blotting (*WB*). Results are representative of two independent experiments. *B,* steroid-deprived LNCaP and 22Rv1 cells were treated with 10 nmol/L DHT or ethanol. Medium with fresh DHT was renewed 24 h after the initial treatment. RT-PCRs for EGFR, ERBB2, and GAPDH transcripts were performed 12 h after DHT treatment. EGFR, ERBB2, PSA, AR, and β-actin protein levels were estimated by Western blotting 24 and 48 h after the first DHT treatment. Results are representative of two independent experiments performed in duplicate. *C,* steroid-starved LNCaP cells were treated or not with 25 μg/mL cycloheximide (*CHX*). Thirty minutes later, 10 nmol/L DHT or ethanol was added. EGFR, ERBB2, and PSA mRNA levels were estimated by semiquantitative RT-PCR 12 h after DHT stimulation. Amplification products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide and quantified by densitometric analysis (Multianalyst software, Bio-Rad). The results are presented as relative expression levels of treated versus untreated cells, the latter being considered as equal to 1. Means and the range were calculated from three independent experiments. *D,* ChIP assays were performed on chromatin extracted from steroid-starved LNCaP cells treated with 10 nmol/L DHT or with ethanol for 4 h. Sonicated chromatin fragments were immunoprecipitated with an anti-RNA polymerase II (*PolII*)–specific antibody or with a nonrelevant antibody (*IgG*) and were PCR amplified. Input represents 2% of total cross-linked, reversed chromatin before immunoprecipitation. PCRs were also carried out on immunoprecipitates without chromatin (*M*). Results are representative of three independent experiments.

modulate the protein and transcript half-lives (data not shown), so the changes in EGFR and ERBB2 mRNA and protein levels in hormone-treated LNCaP cells are most probably the result of transcriptional regulation.

Ligand-activated AR regulates expression either by binding to regulatory sequences in the neighborhood of target genes or by modulating the expression of other transcription factors, which would control the expression of the gene of interest. To find out which of these two mechanisms occurs in the androgen modulation of *EGFR* and *ERBB2* gene expression, protein neosynthesis was blocked by cycloheximide addition to LNCaP cells before DHT treatment (Fig. 1*C*). DHT or cycloheximide alone was found to increase EGFR transcript abundance. In cycloheximide plus hormone-treated cells, a cumulative increase in EGFR transcript levels was observed, indicating that the androgen-induced increase in EGFR mRNA levels did not depend on the synthesis of novel proteins. In conclusion, androgens directly stimulated *EGFR* gene expression. In contrast, cycloheximide pretreatment inhibited the androgen-induced reduction in ERBB2 transcript levels, indicating that the repression does require *de novo* protein synthesis. *PSA* gene expression was not inhibited by cycloheximide, in agreement with the well-known regulation of this gene by the ligand-activated AR.

To substantiate the androgen-induced stimulation of *EGFR* gene transcription, we measured by ChIP RNA polymerase II binding to the *EGFR* proximal promoter in LNCaP cells treated or not with DHT. As shown in Fig. 1*D*, RNA polymerase II attached to the proximal promoter region of the *EGFR* and *PSA* genes was increased after 4 hours of DHT stimulation. In contrast, there was no difference between RNA polymerase II recruited to the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) promoter in

DHT-treated or DHT-untreated cells. No enrichment of RNA polymerase II was detected in −6,900 bp among the *ERBB2* gene, as previously described (27). This observation confirmed the stimulation of *EGFR* gene transcription by androgen-activated AR in LNCaP cells, although it did not prove that the receptor itself was bound to the promoter.

The ARΔCTD isoform is responsible for constitutive EGFR expression and for ERBB2 repression in 22Rv1 cells. The next question we asked was whether the short AR isoform could be responsible for the different response of 22Rv1 cells to the androgenic stimulation compared with LNCaP cells. An important fraction of ARΔCTD is nuclear and is able to bind DNA in the absence of ligand (28). Moreover, ARΔCTD expression leads to constitutive activation of artificial androgen-responsive promoters in SDM (29–32). This AR isoform could thus be responsible for constitutive *EGFR* gene expression and *ERBB2* gene repression and for the 22Rv1 cell AAR phenotype.

To test this, we needed to inhibit specifically one or both isoforms. 22Rv1 cells contain a single copy of the *AR* gene located on chromosome X (22). Because 22Rv1 cells also express the full-length AR, therefore, ARΔCTD could not result from a point mutation leading to a premature stop codon in the *AR* gene. So, we hypothesized that ARΔCTD might result from the alternative splicing of a single AR pre-mRNA.

To test this assumption, three siRNAs recognizing different regions of the AR mRNA were designed (Fig. 2*A*). If our hypothesis were correct, siAR1 and siAR2, targeting the 3′ end of the transcript, would be expected to silence only the full-length AR. siAR3, which targets the exon coding for the NH_2-terminal transactivation domain (NTD), should silence both transcripts. The AR transcripts from the cells transfected with the three siRNAs were identified by reverse transcription-PCR (RT-PCR) with 5′ and 3′ specific primer pairs (Fig. 2*A*). No transcript was detected by amplification with the 3′ primers in cells treated with the three siRNAs (Fig. 2*B, top*), in good agreement with your hypothesis. Amplification with 5′ primers allowed the detection of the AR transcripts in the cells transfected with siAR1 and siAR2 (Fig. 2*B, top*). Western blot analysis of proteins extracted from the same cells with an antibody recognizing the NTD showed that siAR1 and siAR2 down-regulated only the full-length receptor, whereas siAR3 inhibited both isoforms (Fig. 2*B, bottom*). These results thus confirmed our hypothesis that ARΔCTD is encoded by a transcript that is different from the one encoding the full-length AR.

We then measured the effects of the siRNAs on *EGFR* and *ERBB2* gene expression in 22Rv1 cells. Inhibition of the full-length or both AR isoforms did not modify the levels of EGFR and ERBB2 mRNAs (Fig. 2*C*). siAR1 and siAR2 did not modify EGFR and ERBB2 protein contents (Fig. 2*D, left*). In contrast, siAR3 strongly reduced EGFR

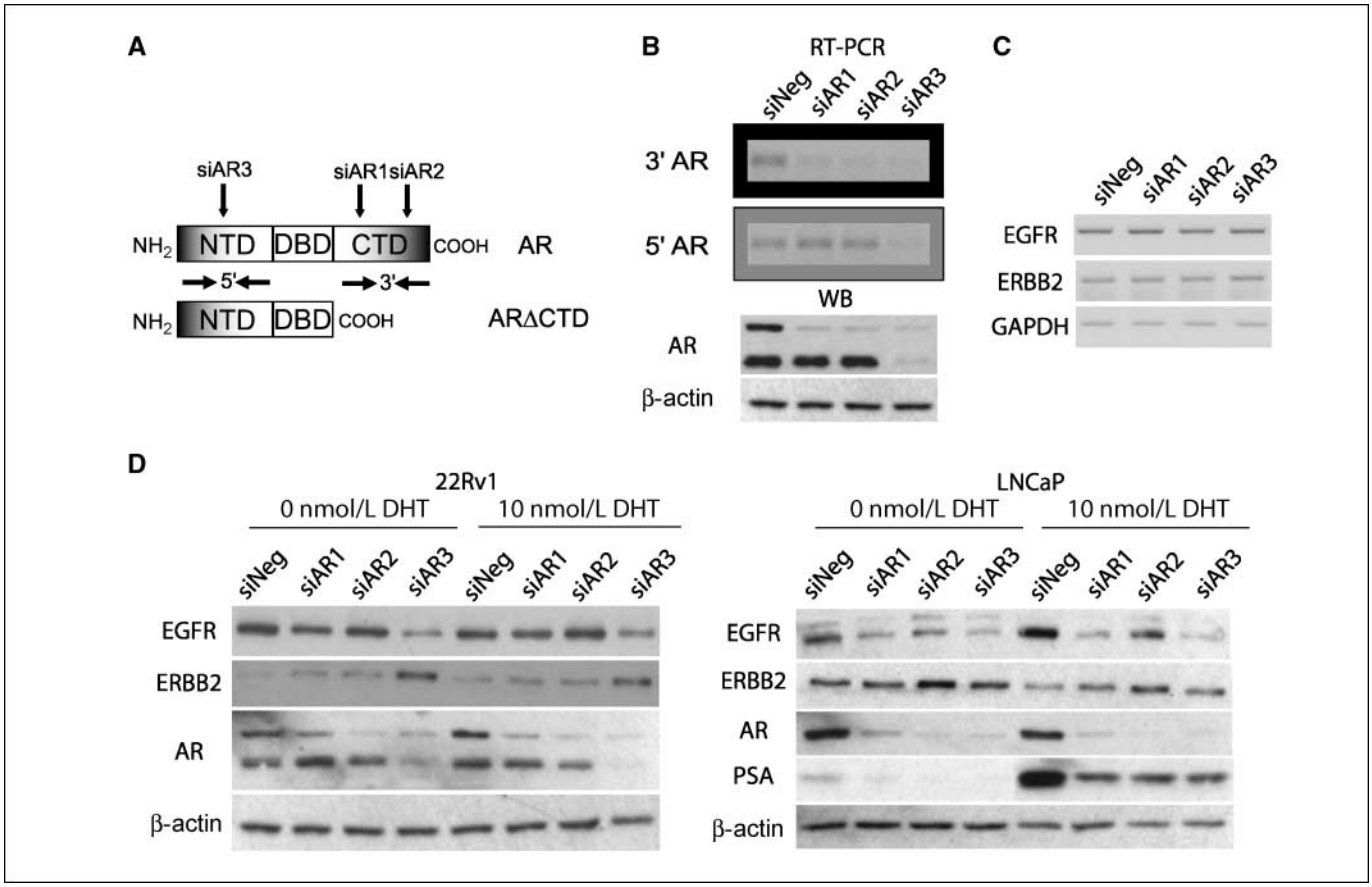

Figure 2. *A,* schematic representation of the AR and ARΔCTD domains and the positions of the regions targeted by siAR1, siAR2, and siAR3. The two pairs of arrows surrounding the numbers 5′ and 3′ indicate the region of the transcripts amplified by RT-PCR. *NTD*, NH_2-terminal transactivation domain; *DBD,* DNA-binding domain; *CTD,* COOH-terminal hormone-binding domain. *B,* 22Rv1 cells seeded at a density of 400,000 in 6-cm dishes were grown in SDM for 2 d and transfected for 2 d with siRNAs. AR transcripts were amplified by RT-PCR with the 5′ and 3′ primers shown in *A*. AR proteins were visualized by Western blotting. *C,* detection of EGFR, ERBB2, and GAPDH mRNA in 22Rv1 cells treated as described in *B. D,* LNCaP and 22Rv1 cells were seeded at a density of 400,000 in 6-cm dishes in SDM supplemented with 10 nmol/L DHT or ethanol. Medium with fresh DHT was renewed everyday. After 48 h, cells were transfected with siRNAs. Seventy-two hours later, proteins were extracted and analyzed by Western blotting for AR, EGFR, ERBB2, PSA, and β-actin levels. Results are representative of three independent experiments.

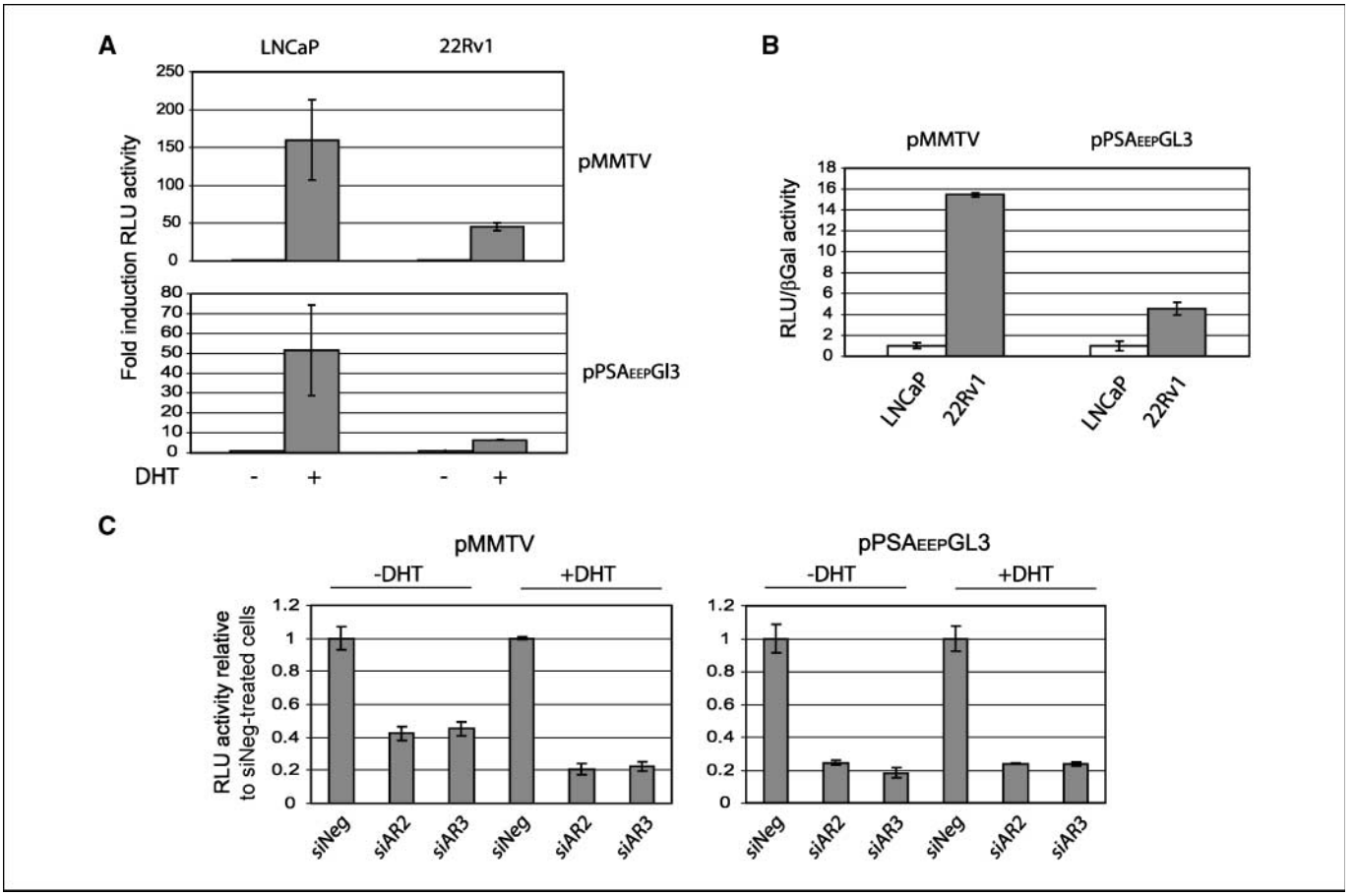

Figure 3. *A,* LNCaP and 22Rv1 cells seeded in SDM for 1 d were transfected with $pPSA_{EEP}GL3$ and pMMTV reporters and with pCH110. Twenty-four hours after transfection, cells were stimulated with 10 nmol/L DHT or with ethanol. Luciferase activity was measured after 24 h of hormonal stimulation. Results are presented as luciferase activity normalized to β-galactosidase activity in hormone-stimulated relative to vehicle-treated cells or (*B*) as luciferase activity normalized to β-galactosidase activity in vehicle-treated 22Rv1 cells relative to vehicle-treated LNCaP cells. Results are representative of three independent experiments performed in triplicate. *C,* 22Rv1 cells were seeded and grown for 1 d in SDM containing or not 10 nmol/L DHT. Cells were first transfected with the siRNAs and transfected 24 h later with the reporter vectors. Medium with fresh DHT was renewed everyday. Luciferase activity was measured 24 h after the last transfection. Results are presented as luciferase activity in siAR-transfected cells relative to siNeg-transfected cells and are representative of three experiments performed in triplicate.

protein abundance and increased ERBB2 protein levels. Contrary to the results in 22Rv1 cells, all three siRNAs reduced EGFR and increased ERBB2 protein levels in LNCaP cells treated or not with DHT (Fig. 2*D, right*).

In summary, these results indicated that AR controls EGFR and ERBB2 protein levels in LNCaP and 22Rv1 cells by different mechanisms. In LNCaP cells, androgens act at the level of transcription, whereas in 22Rv1 cells, ARΔCTD controls EGFR and ERBB2 protein levels by a posttranscriptional mechanism.

The AR is active on androgen-responsive reporter vectors but not on endogenous androgen-responsive genes in 22Rv1 cells. Because androgens did not stimulate *EGFR* gene transcription in 22Rv1 cells, we wanted to know whether the AR is transcriptionally active in these cells. We therefore tested the capacity of the AR to modulate transcription from two reporter vectors under the control of MMTV and PSA androgen-responsive promoters.

DHT induced a strong increase in luciferase activity from both reporters in LNCaP cells (Fig. 3*A*). Interestingly, luciferase activity was also increased in hormone-treated 22Rv1 cells, albeit to a lesser degree than in LNCaP cells (Fig. 3*A*). This small increase in luciferase activity in 22Rv1 cells was due to high constitutive activity of the receptors, as shown by the luciferase activity in unstimulated LNCaP and 22Rv1 cells (Fig. 3*B*).

To determine which of the two AR isoforms was responsible for the ligand-independent transcriptional activity, we cotransfected 22Rv1 cells with the reporter vectors and the siRNAs that down-regulated either the full-length (siAR2) or both AR isoforms (siAR3). As shown in Fig. 3*C*, both siRNAs induced a similar decrease in luciferase activity from the two reporters. The inhibition was more pronounced in DHT-treated than in untreated cells, transfected with the pMMTV-LUC vector (Fig. 3*C*), in agreement with the results from Fig. 3*A*. This difference was not observed with the $pPSA_{EEP}GL3$-LUC reporter, which was less sensitive to DHT than pMMTV-LUC. In summary, although DHT did not modulate *EGFR* and *ERBB2* gene expression in 22Rv1 cells, it stimulated the activity of two androgen-responsive reporter vectors.

Next, we asked whether the hormone could modulate the expression of endogenous AR target genes. To answer this question, we compared the response to DHT of *PSA, TMEPA1*, and *TMPRSS2* genes in 22Rv1 and LNCaP cells. TMPRSS2 and TMEPA1 transcripts were easily detected in 22Rv1 cells but were almost undetectable in LNCaP cells cultured in SDM. In contrast, the PSA transcript level was very low in 22Rv1. DHT increased the

expression of the three genes in LNCaP cells but not in 22Rv1 cells (Fig. 4*A*).

We then investigated the contribution of the full-length AR and ARΔCTD isoforms to the expression of the androgen-responsive genes in 22Rv1 cells grown in SDM. siAR1 and siAR2 reduced PSA and TMEPA1 mRNA levels and to a lesser degree the TMPRSS2 level. siAR3 transfection was followed by a drastic reduction in the abundance of the three androgen-responsive genes (Fig. 4*B*).

These results indicated that AR and ARΔCTD are active in 22Rv1 cells. Because the activity of the full-length receptor is ligand dependent, we asked whether the basal full-length AR activity could actually be attributed to hormones present in the culture medium. We effectively detected some residual testosterone in the SDM. Interestingly, testosterone levels were increased in the conditioned SDM (Fig. 4*C*). To test if residual testosterone could account for the full-length AR activity, 22Rv1 cells transfected with the $pPSA_{EEP}GL3$-LUC reporter were treated with the AR antagonist bicalutamide. Simultaneously, cells were stimulated with DHT to verify that bicalutamide was able to antagonize ligand-dependent activity of the full-length AR. As shown in Fig. 4*D*, bicalutamide effectively antagonized DHT-induced $pPSA_{EEP}GL3$-LUC reporter activity but was unable to decrease the constitutive activity of the reporter in the absence of exogenously added ligand. Similar results were obtained with the pMMTV-LUC vector (data not shown). These results showed that the basal activity of the full-length AR was not dependent on residual testosterone.

In summary, in LNCaP cells, ligand-activated AR stimulates the expression of androgen-responsive endogenous genes as well as of artificial promoters. In contrast, in 22Rv1 cells, androgens stimulate the activity of reporter vectors but not the expression of the androgen target genes we tested. Down-regulation of both the full-length and the truncated receptor reduced the expression of these genes. The activity of the full-length receptor is probably ligand independent and not stimulated by androgens present in the medium conditioned by 22Rv1 cells.

AR and tyrosine kinase receptors are necessary to sustain 22Rv1 cell growth. The last question we asked was whether EGFR and AR contribute to 22Rv1 cell proliferation. To answer this question, 22Rv1 cells were transfected with siRNAs targeting the full-length or both AR isoforms. Proliferation was assessed using the WST-1 assay 3 and 6 days after transfection. As shown in Fig. 5, siAR1 and siAR2 did not inhibit proliferation. However, when compared with siNeg, siAR3 reduced proliferation by 30% at day 3 and by 50% at day 6. To evaluate EGFR and ERBB2 contribution, the cells were treated with PKI166, a bispecific tyrosine kinase inhibitor. PKI166 induced a decrease of ~20% of proliferation rates at day 3 and of ~40% after 6 days of treatment. Treatment with the tyrosine kinase inhibitor concomitantly with siAR1 or siAR2 transfection induced the same degree of inhibition. However, proliferation of the cells treated with PKI166 and transfected with siAR3 was reduced by 40% at day 3 and by 70% at day 6 compared with cells transfected with siNeg.

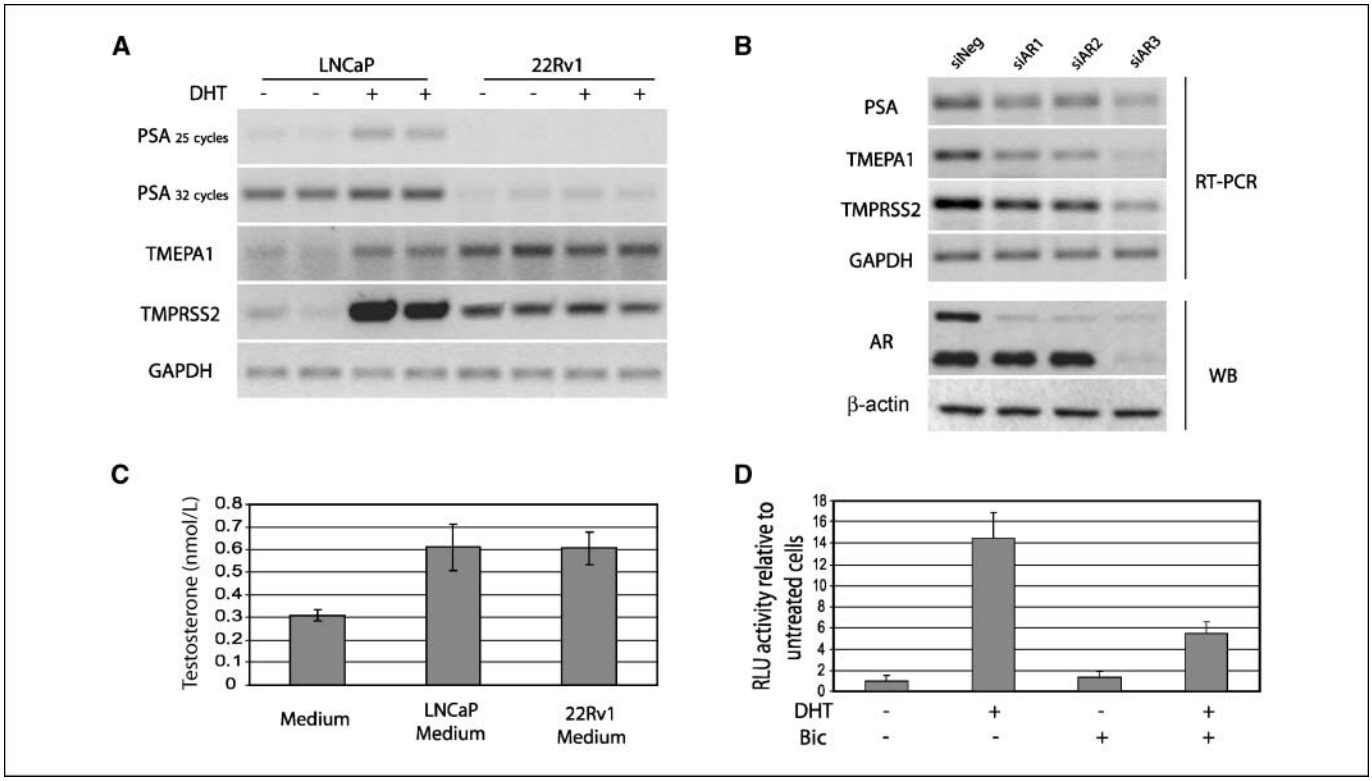

Figure 4. *A,* cells were seeded at a density of 200,000 in 6-cm dishes. After steroid deprivation, they were treated with 10 nmol/L DHT or with ethanol for 24 h. mRNA was extracted and PSA, TMEPA1, TMPRSS2, and GAPDH transcript levels were estimated by RT-PCR. Results are representative of two independent experiments performed in duplicate. *B,* 22Rv1 cells seeded at a density of 400,000 in 6-cm dishes cultured for 2 d in SDM were transfected with AR siRNAs. Forty-eight hours later, RNA and proteins were extracted. PSA, TMEPA1, TMPRSS2, and GAPDH mRNAs were detected by RT-PCR. AR and β-actin proteins were visualized by Western blotting. Results are representative of three independent experiments. *C,* cells were seeded in SDM at a density of 400,000 in 6-cm dishes and culture media were harvested after 5 d. Testosterone concentration was determined in conditioned medium as well as in the control SDM. *D,* 22Rv1 cells seeded in SDM for 24 h were transfected with pMMTV-LUC reporter vector. Eight hours thereafter, cells were treated with 10 μmol/L bicalutamide (*Bic*) or with DMSO vehicle and then with 2.5 nmol/L DHT or ethanol. Luciferase activity was measured after 14 h of hormonal stimulation. Results are presented as luciferase activity normalized to protein content of stimulated cells relative to vehicle-treated cells.

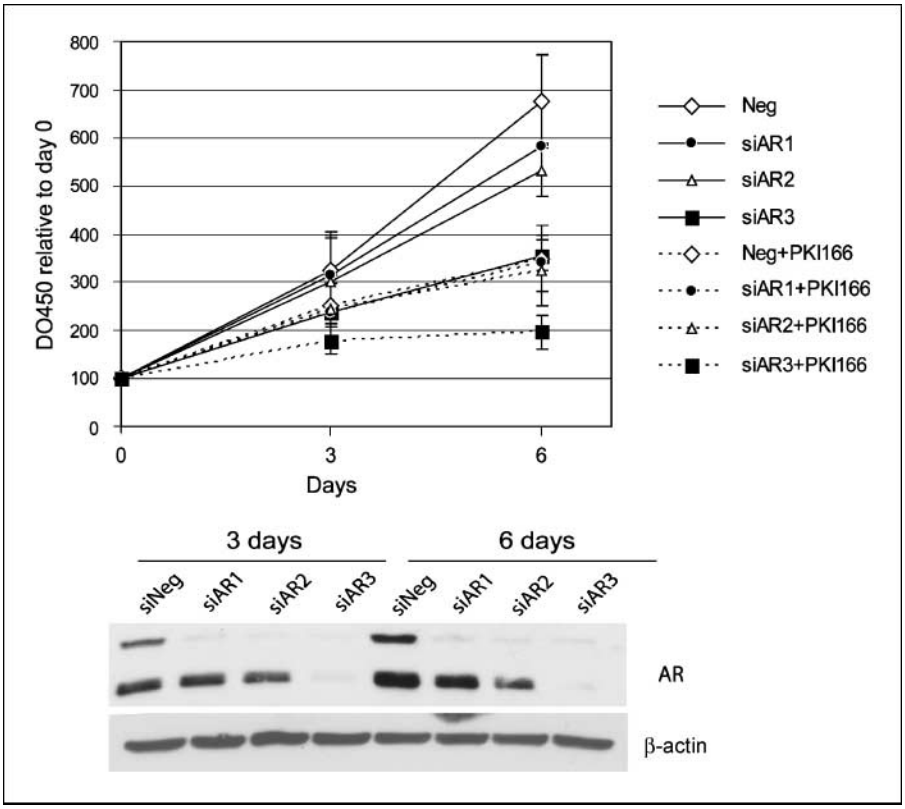

Figure 5. 22Rv1 cells were seeded in SDM at a density of 5,000 per well of a 96-well plate. After 2 d, considered as day 0 of the experiment, siRNAs were transfected. One day later, cells were treated with 1 μmol/L PKI166. At day 3, the medium was renewed and cells were transfected again and treated with PKI166. Proliferation was determined at days 0, 3, and 6. AR expression was determined by Western blotting performed on proteins extracted from siRNA-transfected 22Rv1 cells at days 3 and 6.

Discussion

Understanding the cross-talk between the AR and ERBB signaling pathways is important for the comprehension of PCa progression. Our study sought to examine this relationship by comparing the androgen responsiveness of *EGFR* and *ERBB2* genes in androgen-sensitive LNCaP cells and androgen ablation–resistant 22Rv1 cells.

Our results revealed different regulatory mechanisms of EGFR and ERBB2 protein levels in LNCaP and 22Rv1 cells. We confirmed that androgens stimulate *EGFR* gene expression in LNCaP cells (17, 18). In addition, we showed that androgenic stimulation of *EGFR* gene expression in LNCaP cells is associated with RNA polymerase II recruitment to the promoter. Although androgenic stimulation of *EGFR* gene expression did not require *de novo* protein synthesis, we do not know whether this results from the AR binding to the *EGFR* gene regulatory regions. A computer analysis identified several putative AREs in a 12-kb *EGFR* promoter region (33). However, we could not show their functionality in reporter vectors (data not shown). Recent results have pointed to the complexity of androgenic regulation of gene expression. The AR binds only to some chromatin-embedded AREs (34). Genome-wide ChIP (3, 35, 36) or ChIP display (37) revealed that the AR was binding to noncanonic AREs and even to different sequences, some located far from the promoters. Moreover, the AR can also activate transcription in complex with other transcription factors, such as Sp1 (38). Androgens are known to repress *ERBB2* gene expression in LNCaP cells (19, 20). Here, we showed that cycloheximide pretreatment abrogates the androgen-induced decrease in ERBB2 transcripts, indicating that the hormone acts through an androgen-dependent intermediary.

Interestingly, AR transcriptional activity was not required to maintain high EGFR protein levels in 22Rv1 cells. Indeed, down-regulation of both AR isoforms did not modulate EGFR and ERBB2 mRNA levels but decreased EGFR protein levels and increased ERBB2 protein levels. Posttranscriptional control of EGFR and ERBB2 receptors might result from indirect androgenic regulation of the protein stability. This mechanism has been previously observed for the androgen-induced up-regulation of p27 protein levels, which results from the transcriptional repression of the E3 ubiquitin ligase Skp2 involved in p27 degradation (39, 40). Another possibility lies in an androgenic regulation of translation. Indeed, miRNA-7 was shown recently to control EGFR mRNA translation in glioblastoma (41). Further work is needed to identify the mode of *EGFR* gene expression regulation in PCa.

We observed that AR activity is different in 22Rv1 and LNCaP cells. 22Rv1 cells were first shown by Tepper and colleagues (28) to contain two AR isoforms: a full-length and a short isoform bearing a deleted COOH-terminal domain. The origin of the deleted receptor was debated. The deletion was first reported to originate from the proteolytic cleavage of the full-length receptor by calpain (42). We were unable to reproduce the data of Libertini and colleagues by using calpeptin, a calpain inhibitor (data not shown). We therefore tested if the deletion might result from the translation of an alternatively spliced AR transcript. Down-regulation of the full-length or both isoforms with different siRNAs that recognize different domains of the AR mRNA confirmed our hypothesis. While we were completing this article, the precise identity of ARΔCTD was described by Dehm and colleagues (43). They identified the alternative transcript encoding the ARΔCTD. This transcript contains the alternative exon2b present in intron 2 of the *AR* and encoding 11 amino acids followed by a premature stop codon. Our results largely agree with their conclusions on ARΔCTD origin and on the fact that

ARΔCTD controls androgen ablation–insensitive, androgen-responsive gene expression and proliferation of 22Rv1 cells. These authors identified low levels of expression of ARΔCTD in AAR PCa xenografts. Interestingly, different point mutations introducing premature stop codons were identified in mRNA extracted from 16% of AAR PCa metastasis (29, 44–46).

By silencing specifically the full-length AR or both isoforms with specific siRNAs, we showed that both AR isoforms are responsible for the high level of activity of the reporter vectors and for the expression of the three androgen-responsive genes we have tested in 22Rv1 cells grown in SDM. Indeed, the activity of the full-length AR is probably ligand independent because it was not inhibited by bicalutamide treatment. Similar ligand-independent activity, resistant to bicalutamide treatment, was described in androgen ablation–resistant LNCaP C4-2 cells (47). These results differ from those of Dehm and colleagues (43), who did not report any activity of the full-length AR. About short isoform, the absence of the CTD could lead to the constitutive activity of AF1, the NH_2-terminal, ligand-independent transcription activation domain (29–32).

We also showed that the androgen-independent proliferation of 22Rv1 cells depends on tyrosine kinase receptors and on ARΔCTD. Our results are in good agreement with those of Jones and colleagues (48) and of Ponguta and colleagues (9). Increased EGFR in the presence of even low levels of ARΔCTD might thus contribute to PCa growth in hormone-poor medium.

Whereas in most AAR PCa cells PSA is expressed despite androgen ablation, the expression of this gene was very low in 22Rv1 cells, as previously reported (28). The reason for this lack of expression is not known. Nevertheless, the elevated activity of androgen-responsive promoters, the expression levels of *TMPRSS2* and *TMEPA1* genes, as well as the ARΔCTD-dependent proliferation clearly show that AR signaling continues to play an important role in the AAR phenotype of 22Rv1 cells.

Increased expression of ERBB family receptors in PCa cells is suspected to contribute to the progression of a proportion of the tumors toward ablation insensitivity. Unveiling the mutual regulation between androgens and the ERBB network may thus aid the understanding of the evolution of PCa toward ablation insensitivity. Indeed, androgen ablation, which induces the regression of many androgen-dependent PCas, could promote the growth or survival of a couple of cells by stimulating ERBB2 expression that is negatively regulated by androgens. We showed this here with the use of LNCaP cells. Moreover, our results indicate that maintenance of AR signaling despite androgen depletion in AAR PCa cells, as was observed in 22Rv1 cells, could contribute to EGFR overexpression observed at this stage of the disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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