Heregulin-Induced Activation of HER2 and HER3 Increases Androgen Receptor Transactivation and CWR-R1 Human Recurrent Prostate Cancer Cell Growth

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ABSTRACT

Purpose: **The androgen receptor (AR) is a ligand-dependent transcription factor that mediates gene expression and growth of normal and malignant prostate cells. In prostate tumors that recur after androgen withdrawal, the AR is highly expressed and transcriptionally active in the absence of testicular androgens. In these "androgen-independent" tumors, alternative means of AR activation have been invoked, including regulation by growth factors and their receptors in prostate cancer recurrence.**

Experimental Design and Results: **In this report, we show that HER receptor tyrosine kinases 1 through 4 are expressed in the CWR-R1 recurrent prostate cancer cell line; their stimulation by epidermal growth factor (EGF) and heregulin activates downstream signaling, including mitogen-activated protein kinase and phosphatidylinositol-3 kinase and Akt pathways. We show that heregulin activates HER2 and HER3 and increases androgen-dependent AR transactivation of reporter genes in CWR-R1 cells. Tyrosine phosphorylation of HER2 and HER3, AR transactivation, and cell proliferation induced by heregulin were more potently inhibited by the EGFR/HER2 dual tyrosine kinase inhibitor GW572016 (lapatinib) than the EGFR-specific inhibitor ZD1839 (gefitinib). Basal proliferation in the absence of growth factors was also inhibited by GW572016 to a greater extent than ZD1839, suggesting that low level HER2/HER3 activation, perhaps by an autocrine pathway contributes to the proliferation signal.**

Conclusions: **These data indicate that heregulin signaling through HER2 and HER3 increases AR transactivation and alters growth in a recurrent prostate cancer cell line. Therefore, inhibition of low-level HER2 signaling may be a potential novel therapeutic strategy in prostate cancer.**

INTRODUCTION

Prostate cancer is a common disease in men that initially develops in an environment rich in circulating androgens derived from the testis. Androgen-regulated gene expression and prostate cancer cell growth is mediated by the androgen receptor (AR), a ligand-dependent transcription factor that is activated by binding dihydrotestosterone (DHT; refs. 1–6). Typically, prostate tumors regress following androgen withdrawal by surgical or medical castration, reflecting their initial dependence on androgens for growth. Inactivation of AR by an AR-specific ribozyme or by antibody neutralization inhibited proliferation of LNCaP cells in culture (7). AR antisense oligonucleotides also inhibit LNCaP prostate tumor growth and prostate-specific antigen (PSA) production *in vivo* (8), further demonstrating the critical role of AR in prostate cancer growth. Following a remission of variable duration, prostate cancers usually recur despite reduced circulating androgen levels. Several hypotheses of mechanisms underlying recurrence of prostate cancer, including cross-talk with growth factor signaling pathways and AR overexpression have been postulated (9–12).

Growth factor regulation of AR transactivation has been postulated to play a role in prostate cancer progression after androgen withdrawal. For example, insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor (EGF) were reported to increase AR transactivation in the absence of androgen (13). Our laboratories set out to determine if additional members of the EGF/HER family of receptor tyrosine kinases, consisting of EGFR, HER2 , HER3, and HER4 might also be involved. Although the HER receptor family members are often overexpressed or activated in human cancers, this overexpression is not typically observed in prostate cancer. The EGF family of ligands such as EGF, transforming growth factor α, and heregulin stimulate the formation of receptor homodimers and heterodimers and tyrosine kinase activity. EGF preferentially binds to EGF receptor (EGFR) and induces predominantly EGFR homodimers or EGFR/HER2 heterodimers. Heregulin binding to HER3 or HER4 induces the formation of HER2/HER3 or HER2/HER4 heterodimers (14). HER2 does not bind to any ligands with high affinity, but preferentially forms heterodimers with other HER family members for activation. Vast overexpression of HER2 results in ligand-independent HER2 activation and has been reported to induce androgen-independent AR transactivation (10). A few studies have reported elevated HER2 levels in human

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recurrent prostate cancer (15–17), whereas other reports did not find increased HER2 protein or HER2 gene copy number in human recurrent prostate cancer (18, 19). Other routes of HER2 activation are possible, including autocrine or paracrine ligand–induced HER2 and HER3 activation. In fact, HER3 and heregulin-α overexpression in prostate cancer has been noted and was associated with a poor prognosis (20). Anti-HER2 recombinant humanized monoclonal antibody (rhuMAb 2C4) inhibited heregulin-induced phosphorylation of HER2 and cell proliferation of the 22Rv1 prostate cancer cell line (21). Another study showed decreased growth of the CWR22 human prostate cancer xenograft in the presence of rhuMab 2C4 (22).

To address the role for the HER receptors in AR transactivation in recurrent prostate cancer, we investigated heregulin induction of HER2 and HER3 in the CWR-R1 human prostate cancer cell line, derived from the recurrent CWR22 tumor. CWR-R1 cells express an endogenous AR that is relatively stable and show increased sensitivity to low androgen levels (23). Our results indicate that CWR-R1 cells express EGFR, HER2, HER3, and HER4. Heregulin treatment of CWR-R1 cells increases HER2, HER3, and HER4 receptor tyrosine phosphorylation and cell proliferation in the absence of androgen. Moreover, heregulin increased DHT-induced growth and AR transactivation of a reporter plasmid. The EGFR/HER2 dual tyrosine kinase inhibitor GW572016 (lapatanib) prevented HER2 and HER3 phosphorylation in response to heregulin and inhibited AR transactivation and cell growth in a dose-dependent manner. The results suggest potential approaches for therapeutic intervention.

MATERIALS AND METHODS

Plasmids. Expression vectors were described previously for full-length human AR (pCMVhAR; ref. 24) and AR NH_2-terminal and DNA binding and hinge region fragment AR1-660 (pCMVhAR1-660; refs. 25, 26). The following vectors were generously provided: prostate specific antigen-luciferase reporter PSA-Luc (previously referred to as PSE-Luc) containing the −5220 PSA promoter with an internal deletion of −2876 to −540 (10) from Charles Sawyers and Lily Wu (University of California Los Angeles) and mouse mammary tumor virus long terminal repeat-luciferase reporter vector (MMTV-Luc) from Stanley M. Hollenberg and Ronald M. Evans (Salk Institute).

Transient Expression Assays. Cotransfection assays were done using the CWR-R1 cell line derived from the CWR22 recurrent human prostate cancer xenograft (23). To study transcriptional activity of the H874Y mutant AR endogenous to CWR-R1, cells were transfected with the MMTV-Luc reporter vector. To study transcriptional activity of wild-type AR and AR1-660 that lacks the ligand binding domain, 0.01 μg pCMVhAR or pCMVhAR1-660 was transfected with 0.5 μg MMTV-Luc or 1 μg PSA-Luc reporter vector. DNA was transfected into ∼75% confluent CWR-R1 cells plated the day before at 10^6 cells per 6-cm dish using prostate growth medium without serum (23) and Effectene transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. To 3 mL fresh medium containing 10% FCS per plate was added 1 mL of a reaction mix containing 150 μL EC buffer (Qiagen), 4 μL enhancer, 4 μL Effectene reagent (Qiagen), and 1 mL 2% serum containing medium. The following day, the prostate growth medium was replaced with serum-free medium lacking phenol red with or without the indicated concentrations of DHT, growth factors, or tyrosine kinase inhibitors. Incubations were continued for 24 hours. Cells were harvested in 0.5 mL lysis buffer containing 25 mmol/L Tris phosphate (pH 7.8), 2 mmol/L EDTA, and 1% Triton X-100 (27). Luciferase activity was measured using an automated LumiStar Galaxy (BMG Labtechnologies, Offenburg, Germany) multiwell plate reader luminometer.

Immunoprecipitation and Immunoblot Analysis. Cells were plated in 10-cm tissue culture plates with appropriate growth medium. When cells reached ∼70% confluence, cells were treated with either vehicle (0.1% DMSO) or the appropriate doses of GW572016 (*N*-{3-Chloro-4-[(3-fluorobenzyl)oxy]-phenyl}-6-[5-({[2-(methylsulfonyl)ethyl]amino}methyl)-2-furyl]-4-quinazolinamine; Lapatinib ditosylate; ref. 28) or ZD1839 (4-(3-chloro-4-fluorophenylamino)-7-methoxy-6 (3-(4-morphlinyl) propoxy)-quinazoline; gefitinib, IRESSA; ref. 29), kindly provided by Dr. Tona M. Gilmer and Dr. David W. Rusnak (GlaxoSmithKline, Research Triangle Park, NC). Treated cells were returned to the incubator for 45 minutes. Cells were then treated with either EGF (100 ng/mL, BD Biosciences, San Jose, CA) or heregulin (10 ng/mL, Genentech, South San Francisco, CA) and returned to the incubator for 15 minutes. Cells were washed with cold PBS and lysed in lysis buffer containing 20 mmol/L HEPES (pH 7.3), 50 mmol/L sodium fluoride, 10% glycerol, 1% Triton X-100, 5 mmol/L EDTA, and 0.5 mol/L NaCl supplemented with the protease inhibitors aprotinin (6 μg/mL) and leupeptin (10 μg/mL) and the tyrosine phosphatase inhibitor sodium orthovanadate (1 mmol/L). Nuclei and insoluble material were removed by centrifugation at 13,000 × *g* for 10 minutes at 4°C. HER receptor proteins were precipitated with one of the following antibodies: EGFR 22, polyclonal rabbit antisera raised against recombinant glutathione *S*-transferase fusion protein containing the COOH-terminal domain of EGFR; HER2, clone 9G6.10, mouse monoclonal antibody (Neomarkers, Inc., Fremont, CA); HER3 1511, polyclonal rabbit antisera raised against recombinant glutathione *S*-transferase fusion protein containing the COOH-terminal 100 amino acids of HER3; HER4 132, polyclonal rabbit antisera raised against recombinant glutathione *S*-transferase fusion protein containing the COOH-terminal 80 amino acids of HER4 and protein A/G or protein A agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 3 hours at 4°C. Immune complexes were washed thrice with lysis buffer and denatured in SDS sample buffer. Protein samples were separated on an 8% SDS-PAGE gel and were electrophoretically transferred to a Sequi-blot polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). After blocking with 3% cold fish gelatin (Sigma Chemical Co., St. Louis, MO), the membrane was incubated for 1 hour at room temperature with anti-phosphotyrosine antibody (PY20-H; Santa Cruz Biotechnology; 1:3,000 dilution), washed thrice with TBS-Tween 20, and detected by enhanced chemiluminescence (Amersham Life Sciences, Piscataway, NJ). For membranes probed for phospho-p44/42 and phospho-Akt, the same Western blotting procedure was used, except protein samples were separated on 12% SDS-PAGE gels and the polyvinylidene difluoride membrane was blocked with 5% dry milk. The membrane was probed overnight at 4°C with either

the phospho-p44/42 antibody or phospho-Akt antibodies (Cell Signaling Technology, Beverly, MA) at 1:1,000 dilution, followed by anti-rabbit IgG conjugated with horseradish peroxidase (Cell Signaling) at 1:2,500 dilution for 1 hour at room temperature. Receptor levels were detected by enhanced chemiluminescence (Amersham Life Sciences).

For AR Western blots, CWR-R1 cells were incubated with 100 ng/mL EGF, 10 ng/mL heregulin in the absence or presence of 0.1 nmol/L DHT for 24 hours in growth medium with 0.2% lipid-rich bovine serum albumin (AlbuMax I; Invitrogen, Carlsbad, CA). Lysates were prepared as described (30). Eighty micrograms of protein per lane was separated on 10% acrylamide gels and electroblotted to nitrocellulose membranes (NitroBind, 0.22 μmol/L; Osmonics, Inc., Minnentonka, MN). Rabbit polyclonal AR antibody AR52 targeted to amino acids 544 to 558 NH_2-terminal to the DNA binding domain (31) was used at 1 μg/mL. Incubation with primary antibody was for 1 hour at room temperature. Anti-rabbit horseradish peroxidase–conjugated secondary IgG antibodies (Amersham Life Sciences) were used at 1:10,000 dilution for 1 hour at room temperature. Specific signals were detected using chemiluminescence (SuperSignal West Dura Extended Duration Substrate; Pierce, Rockford, IL).

Cell Growth Assays. For data shown in Fig. 5*A* and *B*, CWR-R1 cells (1×10^4) were plated in 96-well plates in prostate growth medium without serum or phenol red with 0.2% bovine serum albumin (Albumax, Life Technologies, Grand Island, NY) and allowed to grow for 24 hours. Culture medium with treatments was added and triplicate wells were assayed for cell growth on days 1, 2, and 3. One-tenth volume WST-8 reagent [(2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt, Dojindo Molecular Technologies, Gaithersburg, MD] was added to the wells, incubated for 2.5 hours and the absorbance determined at 450 nm using a plate reader. For data shown in Fig. 5*C*, CWR-R1 cells (1×10^4 per well) were plated in triplicate in 96-well plates in prostate growth medium containing 2% FCS and allowed to grow for 24 hours. Various doses of GW572016 and ZD1839 were added to the wells and the cells were incubated for an additional 72 hours. Relative cell growth was determined by incubating with the colorimetric dye 3[4,5 -2-yl]-2, 5-diphenyltetrazolium bromide (Sigma Chemical). The absorbance at 595 nm was determined using a plate reader after solubilization of the precipitated formazan crystals. The concentration required for growth inhibition by 50% (IC_{50}) was calculated using a curve-fitting program in Excel (Microsoft Co., Redmond, WA) as described previously (28).

RESULTS

HER Receptor Expression in CWR-R1 Cells. We initiated studies on a new CWR-R1 recurrent prostate cancer cell line derived from the recurrent CWR22 human prostate cancer xenograft (23) by determining the expression profile of the EGFR, HER2, HER3, and HER4 receptors. Following stimulation and immunoprecipitation of each of the four receptors, Western blots were done using an anti-phosphotyrosine antibody. We observed ligand-dependent phosphorylation of EGFR, HER2, HER3, and HER4 (Fig. 1*A*). Due to conflicting reports in the literature regarding HER4 expression in prostate cancer

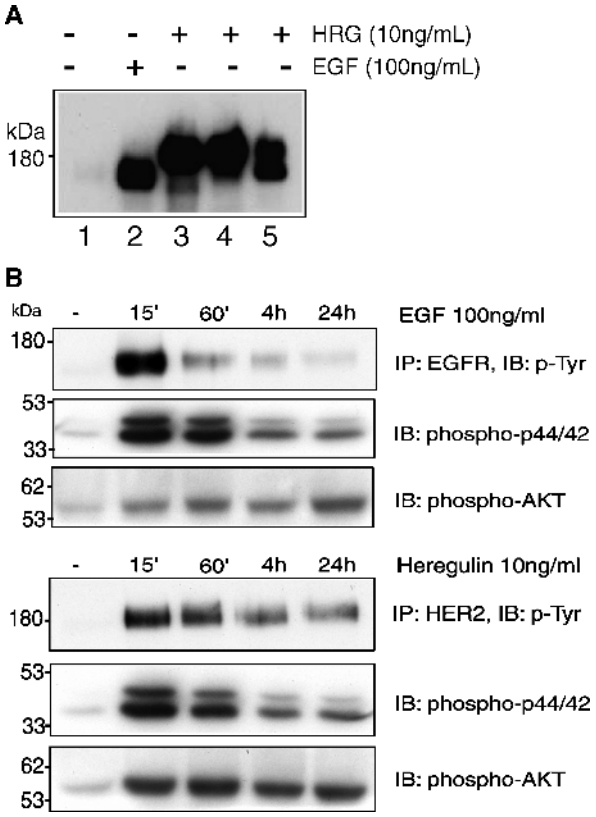

Fig. 1 Ligand induced HER, MAPK, and Akt activation in CWR-R1 cells. *A,* CWR-R1 cells were incubated without or with EGF (100 ng/mL) or heregulin (*HRG*; 10 ng/mL) for 15 minutes before immunoprecipitation with HER1 through HER4 antibodies, followed by immunoblotting with phospho-Tyr antibody to detect active receptors. Lanes 1 and 2, EGFR; lane 3, HER2; lane 4, HER3; lane 5, HER4. *B,* CWR-R1 cells were incubated with EGF (100 ng/mL) or HRG (10 ng/mL) for 15 minutes, 1, 4, and 24 hours before immunoprecipitation with EGFR or HER2 antibody followed by immunoblotting with phospho-Tyr antibody to detect active proteins. Cell extracts were immunoblotted with antibodies specific for phospho-p42/44 or phospho-Akt. Representative of at least three independent experiments.

(32–34), we used quantitative reverse transcription-PCR (19) to determine copy number in CWR-R1 cells and found 855 copies per 50 ng RNA, thus confirming the immunoprecipitation data. Activation of EGFR and HER2 in CWR-R1 cells was rapid, reaching maximum levels 15 minutes after treatment with EGF (100 ng/mL) or heregulin (10 ng/mL), respectively (Fig. 1*B*). Activation of both receptors decreased over 24 hours, although the HER2 signal was more prolonged. Mitogen-activated protein kinase (MAPK) and Akt were also activated by treatment of CWR-R1 cells with EGF or heregulin, as determined by Western blotting with phospho-specific antibodies. Phospho-p42/44 MAPK and phospho-Akt activity increased within 15 minutes of treatment. Phospho-p42/44 was reduced after 4 hours of treatment, whereas phospho-Akt levels persisted or increased by 24 hours after ligand addition. Heregulin activated phospho-Akt more potently than EGF, consistent with the previous finding that HER3 couples to the phosphatidylinositol-3 kinase pathway better than EGFR (35). Pretreatment with LY294002 before ligand stimulation completely abrogated phospho-Akt activation,

suggesting that Akt activation in CWR-R1 cells is dependent on phosphatidylinositol-3 kinase activation (data not shown). CWR-R1 cells express wild-type PTEN protein, as determined by direct sequencing and Western blot analysis (data not shown). DHT treatment of CWR-R1 cells for 15 minutes did not result in increased phospho-p42/44 or phospho-Akt activity (data not shown).

Effects of Epidermal Growth Factor and Heregulin on Androgen Receptor Transactivation and Expression in CWR-R1 Cells. We determined the effect of EGF and heregulin on AR transactivation in the CWR-R1 cell line. In the absence of DHT, EGF (100 ng/mL) or heregulin alone (10 ng/mL) induced a 1- to 2-fold increase in AR-mediated transactivation of PSA-Luc by the endogenous AR (pCMV5 empty vector control) or the transiently expressed full-length wild-type AR (AR1-919; Fig. 2*A*). In the presence of 0.1 nmol/L DHT, EGF and heregulin each increased transactivation by the endogenous and transiently expressed AR by 2-fold relative to the response with DHT alone. Transcriptional activity of a constitutively active AR NH_2-terminal fragment lacking the ligand binding domain (AR1-660) increased 2-fold with the addition of EGF or heregulin. To determine the mechanism through which EGF and heregulin increase AR transcriptional activity in the presence of androgen, we explored the effect of EGF and heregulin on AR protein levels.

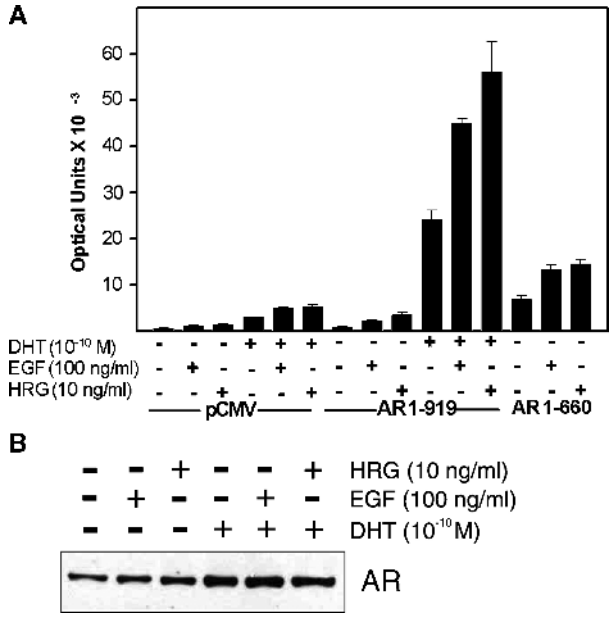

Fig. 2 EGF and heregulin (*HRG*) effects on AR transactivation and expression. *A,* CWR-R1 cells were transiently transfected with 10 ng each of pCMV5 empty vector control, pCMVAR1-919 coding for full-length wild-type AR, or pCMVAR1-660 that lacks the ligand binding domain in the presence of the PSA-Luc reported as described in Materials and Methods. Cells were incubated in the presence of 100 ng/mL EGF or 10 ng/mL HRG with or without 0.1 nmol/L DHT for 24 hours. Columns, mean luciferase activity in optical units representative of at least five independent experiments; bars, ±SE. *B,* CWR-R1 cells were incubated with and without 100 ng/mL EGF or 10 ng/mL HRG in the absence and presence of 0.1 nmol/L DHT for 24 hours. Extracts were analyzed using 80 μg of protein per lane for CWR-R1 cells. Blots were incubated with AR52, an NH_2-terminal specific antibody for AR. Representative of three independent experiments.

In CWR-R1 cells, endogenous AR protein levels increased 3-fold in the presence of DHT, but a further increase in the presence of EGF or heregulin was not detectable and there was no increase in AR in the presence of EGF or heregulin alone (Fig. 2*B*). Similar results were seen for exogenous AR in CWR-R1 (data not shown) and COS cells (30).

Inhibition of Androgen Receptor Transactivation and Phospho-HER2 and HER3 by Tyrosine Kinase Inhibitors ZD1839 and GW572016. To characterize the HER signaling pathways involved in modulating AR transactivation, CWR-R1 cells were incubated with the dual tyrosine kinase inhibitor GW572016 at increasing concentrations for 45 minutes before treatment with heregulin (10 ng/mL) for 15 minutes. HER2 and HER3 were immunoprecipitated from cell lysates and analyzed by phospho-tyrosine Western blot. GW572016 inhibited ligand-dependent activation of HER2 at concentrations ≥0.5 μmol/L and HER3 at concentrations >0.1 μmol/L (Fig. 3*A*). Phospho-MAPK and phospho-Akt activation were inhibited by GW572016 at the same concentrations. Although phospho-HER3 was not detectable in the absence of heregulin treatment, phospho-HER2 was present in untreated CWR-R1 cells.

We determined the effects of the EGFR tyrosine kinase inhibitor ZD1839 (Fig. 3*B*), and the EGFR and HER2 tyrosine kinase inhibitor GW572016 (Fig. 3*C*), on endogenous AR transactivation of the MMTV-Luc reporter in CWR-R1 cells. Viral promoters such as MMTV are typically stronger than the naturally occurring promoter and enhancer regions of androgen regulated genes, thus accounting for differences in luciferase activity compared with PSA-Luc. Nonspecific toxicity effects were not detected with concentrations up to 5 μmol/L ZD1839 and GW572016 using the constitutively active pSG5-Luc and pGL3-Luc reporter vectors in CWR-R1 cells, therefore 2.5 μmol/L concentrations were used (data not shown). ZD1839 at concentrations ≤2.5 μmol/L did not inhibit DHT-mediated endogenous AR transactivation of MMTV-Luc (Fig. 3*B*) in the absence of added EGF. The EGF-induced increase in AR transactivation determined in the presence of DHT was inhibited 40% by 0.1 μmol/L ZD1839 and completely by 1 and 2.5 μmol/L ZD1839. DHT-induced AR transactivation was inhibited 10% by 1 μmol/L GW572016 and 30% by 2.5 μmol/L GW572016 (Fig. 3*C*). In the presence of DHT, heregulin induced AR activity was inhibited 40% at 0.1 μmol/L and was completely inhibited at 1 μmol/L GW572016. To determine the signaling components downstream of the receptor tyrosine kinases, CWR-R1 cells were treated with the ME kinase kinase inhibitor U0126. As published previously (30), U0126 inhibited DHT + EGF–mediated AR transactivation of MMTV-Luc. U0126 (10 μm) inhibited DHT + heregulin–modulated AR transactivation of PSA and MMTV-Luc reporters by 50% (data not shown). This is an important observation as the MEK inhibitor U0126 abrogates the EGF effect but only inhibited the heregulin effect by 50% at 10 μmol/L, indicating that the HER2/HER3 signaling is more complex in these cells than the EGFR signaling, which is almost exclusively dependent on the MAPK signaling. These results indicate that MAPK signaling downstream of EGF and heregulin is responsible, at least in part, for AR transactivation.

We compared the inhibitory effects of ZD1839 and GW572016 on ligand-dependent EGFR and HER2 tyrosine

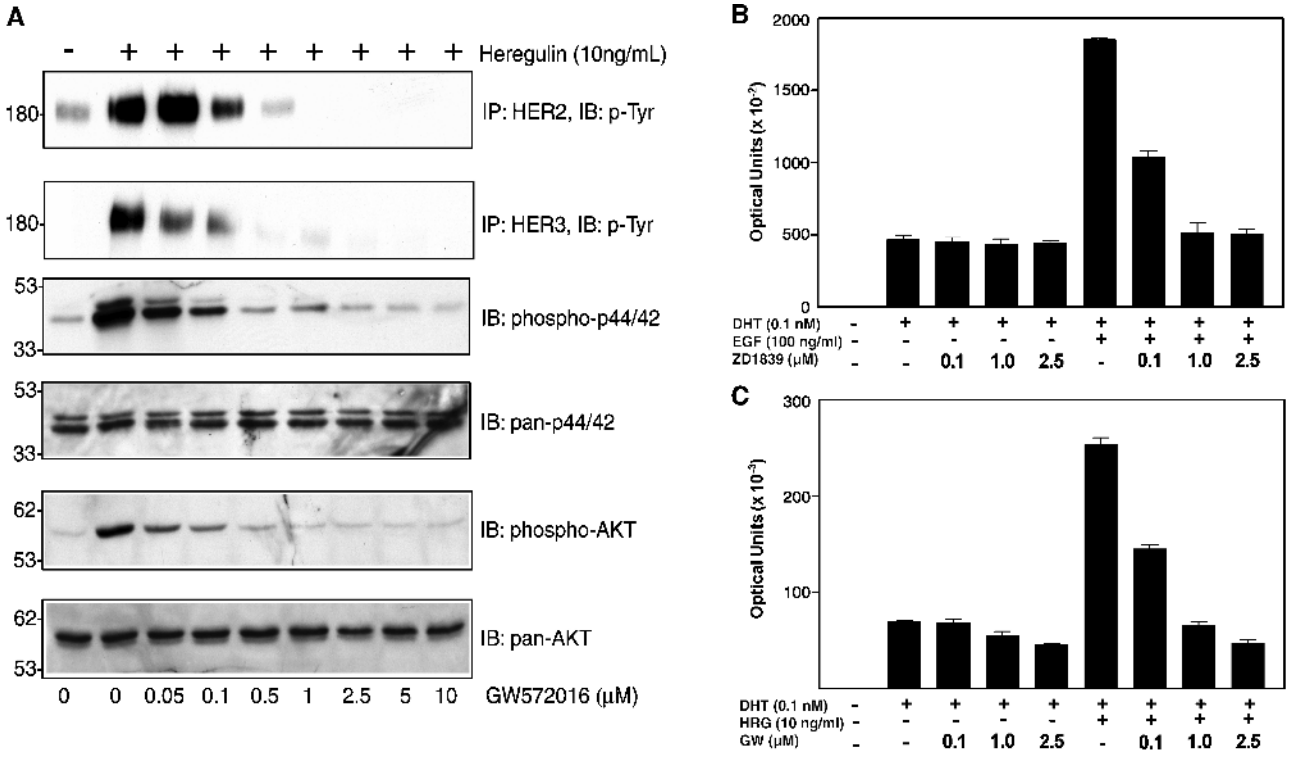

Fig. 3 Inhibition of HER2 and HER3 receptor phosphorylation and AR transactivation by GW572016 and ZD1839. *A,* CWR-R1 cells were incubated in the absence and presence of increasing concentrations of GW572016 as indicated before a 15-minute incubation with 10 ng/mL heregulin (*HRG*). Immunoblots of immunoprecipitated HER2 and HER3 were incubated with phospho-Tyr antibody or phospho-specific MAPK and Akt antibodies, respectively. Blots were stripped and reprobed with pan-MAP kinase and Akt antibodies. *B,* CWR-R1 cells were transiently transfected with MMTV-Luc as described in Materials and Methods. Cells were incubated in the presence of 100 ng/mL EGF with or without 0.1 nmol/L DHT in the absence and presence of 0.1, 1.0, or 2.5 μmol/L ZD1839. Columns, mean luciferase activity in optical units representative of at least three independent experiments; bars, ±SE. *C,* transiently transfected CWR-R1 cells were incubated in the presence of 10 ng/mL HRG with or without 0.1 nmol/L DHT in the absence and presence of 0.1, 1.0, or 2.5 μmol/L GW572016. Columns, mean luciferase activity in optical units representative of at least three independent experiments; bars, ±SE.

phosphorylation. As shown in Fig. 4*A* (*top*), 0.1 μmol/L ZD1839 and 0.1 μmol/L GW572016 each blocked EGF-induced EGFR activation. In contrast, GW572016 blocked heregulin-induced HER2 activation, but ZD1839 at doses up to 5 μmol/L did not prevent HER2 activation by heregulin (Fig. 4*B*).

Inhibition of CWR-R1 Cell Growth by ZD1839 and GW572016. To determine the effects of inhibiting EGFR- and HER2-mediated kinase signaling and AR transactivation on cell proliferation, we tested the effects of ZD1839 and GW572016 on CWR-R1 cell growth in the absence of serum (Fig. 5*A* and *B*) or in the presence of serum (Fig. 5*C*). In the absence of DHT, heregulin- and EGF-induced cell proliferation was inhibited 40% by 2.5 μmol/L GW572016 compared with no inhibition in the presence of 2.5 μmol/L ZD1839 (Fig. 5*A*), similar to its lack of effect on heregulin-induced AR transactivation and heregulin-mediated HER2 activation. GW572016 inhibited heregulin-induced growth of CWR-R1 cells by 46% on day 3. DHT or heregulin alone induced CWR-R1 growth to similar levels and together were synergistic. In the presence of both DHT and heregulin, GW572016 (2.5 μmol/L) inhibited CWR-R1 cell growth by 56% on day 3 whereas ZD1839 (2.5 μmol/L) inhibited cell growth 30% (Fig. 5*B*). We then did a dose titration of the growth inhibitory activity of GW572016 and ZD1839 on CWR-R1 cells growing in medium with 2% FCS (Fig. 5*C*). GW572016 was more potent than ZD1839 on an equimolar basis. The IC_{50} for GW572016 was 1.53 μmol/L, but the IC_{50} for ZD1839 was >10 μmol/L. Taken together, the data show that the dual tyrosine kinase inhibitor GW572016 is a strong inhibitor of DHT- or growth factor/DHT–mediated CWR-R1 cell growth, emphasizing the important role of basal HER2 expression in this cell line.

DISCUSSION

Cross-talk between growth factor signaling pathways and AR function has been implicated in the recurrence of prostate cancer in the androgen-deprived patient (9, 11, 36). Here we show that the EGF receptors HER1 through HER4 are expressed in the CWR-R1 recurrent prostate cancer cell line. We show that heregulin induces receptor activation and signaling through MAPK and phosphatidylinositol-3 kinase and Akt. Kinase signaling induced by heregulin increased DHT-dependent AR transactivation of a reporter gene. AR transactivation and CWR-R1 cell growth induced by heregulin was inhibited by the dual tyrosine kinase inhibitor GW572016, presumably by preventing phosphorylation of HER2, HER3 and downstream signaling (e.g., MAPK and Akt).

AR activation by growth factors including EGF, insulin-like growth factor-I, keratinocyte growth factor, and IL-6 has been reported in the absence and presence of DHT (37, 38), although

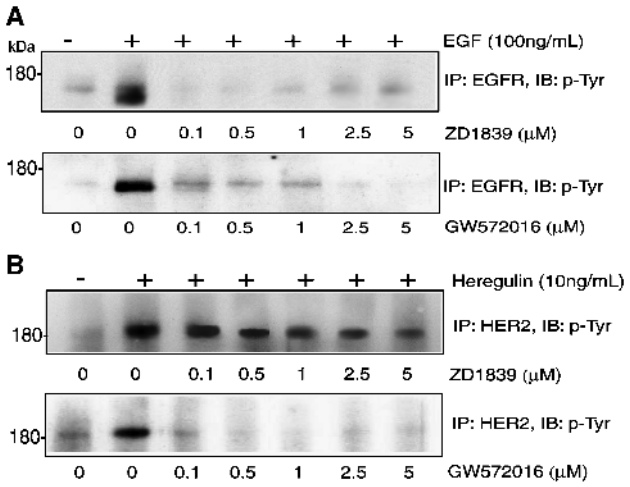

Fig. 4 ZD1839 and GW572016 inhibition of EGFR and HER2 activation. CWR-R1 cells were incubated in the absence and presence of ZD1839 or GW572016 at the indicated amounts before incubation with 100 ng/mL EGF or 10 ng/mL heregulin. Immunoblots of immunoprecipitated EGFR and HER2 were incubated with phospho-Tyr antibodies. EGF stimulation of EGFR (*A*) and heregulin stimulation of HER2 (*B*) in the absence and presence of increasing concentrations of inhibitors. Representative of at least three independent experiments.

IL-6 can also inhibit AR signaling in prostate cancer cells (39). Contrary to the findings of Culig et al. (37), AR activation by growth factors in CWR-R1 cells requires a stable (ligand-bound full-length or constitutively active truncated) AR. This study and our earlier work (30) do not support the possibility that autocrine signaling through the HER receptor family negates the requirement for androgen in recurrent prostate cancer. EGFR, HER2, and HER3 are expressed in normal prostate and prostate cancer as shown by immunohistochemistry, reverse transcription-PCR, and Western blot analysis (34, 40–42). Craft et al. (10) showed that xenograft-derived recurrent prostate cancer cells express higher levels of HER2 compared with androgen-dependent cells. Overexpression of HER2 in LNCaP cells mediates cell growth in the absence of androgen and increased the androgen-independent expression of *PSA*, an androgen-regulated gene. Yeh et al. (43) showed that overexpression of HER2 increases DHT-dependent AR activation by way of the MAPK signaling pathway and increased interaction of AR with the coactivator ARA70. IL-6 activation of HER2 and MAPK in LNCaP cells was reported to result from heterodimerization of the IL-6 receptor and HER2 (44). Taken together, these studies suggest that EGFR and HER2 activation are associated with elevated kinase signaling and AR activation in recurrent prostate cancer. We reported recently that HER2 is not amplified or overexpressed in recurrent prostate cancer tumors (19). However, the data presented here suggest that HER2 has a low level of basal activity in CWR-R1 cells and could be further activated by ligands such as heregulin. This suggests a possibility of constitutive HER2 activation by a ligand-mediated mechanism, potentially through an autocrine loop in prostate cancer cells.

Heregulin is the cognate ligand for HER3 and HER4 and heregulin binding induces heterodimerization with other HER family members such as HER2, leading to downstream kinase signaling. HER3 has no intrinsic kinase activity (45) but relies on heterodimerization with HER2 for induction of mitogenic or transforming effects (46). HER4 activation in response to heregulin can lead to proliferation (neuronal cells; ref. 47) or differentiation (breast cancer cells in culture; ref. 48). Heregulin is expressed in normal prostate (49) and overexpression of HER3 in one report of prostate cancer was associated with poor disease outcome (20). In LNCaP cells, heregulin treatment inhibited cell growth by 50% (34). In contrast, we show here that heregulin activated HER2 and HER3 and induced growth of the recurrent prostate cancer cell line CWR-R1, suggesting that HER profiles or kinase signaling pathways may differ in androgen-dependent compared with recurrent prostate cancers. Recent work described a possible mechanism by which HER3 inhibits prostate cancer cell growth. Ebp1, an HER3 binding protein, was shown to interact with AR, inhibit DHT-induced *PSA* gene transcription and slow the growth of LNCaP cells (50). CWR-R1 recurrent prostate cancer cells are stimulated by heregulin suggesting they lack this putative pathway. Further studies will be necessary to understand the factors underlying the different biological responses to HER family ligands in different cell types.

Based on the fact that AR transactivation of gene expression is associated with CWR-R1 cell growth (23, 51), we tested the ability of heregulin to modulate AR transactivation of reporter genes in the absence and presence of DHT. We found a small effect of EGF or heregulin on AR transactivation of PSA-Luc in the absence of DHT. However, in the presence of 10^{-10} mol/L DHT, EGF and heregulin induced AR transactivation was 10- to 12-fold higher compared with cells incubated in the absence of DHT. Androgen activation of AR seemed to be a requirement for the heregulin-mediated increase in transactivation of PSA-Luc. The AR1-660 ligand binding domain deletion mutant is constitutively active (26). Transactivation by this AR mutant was induced by EGF and heregulin in the absence of DHT, whereas the endogenous AR in CWR-R1 cells or an exogenously expressed wild-type AR required DHT-mediated activation before heregulin induction. The data show that heregulin is as effective as EGF at inducing AR transactivation in the presence of low levels of DHT.

Taken together, the data suggest the presence of an heregulin autocrine signaling loop in CWR-R1 cells that increases AR transactivation and cellular proliferation through HER2 and HER3 signaling. Although HER2 is not typically overexpressed in recurrent prostate cancer (19), the low level of activated HER2 present in CWR-R1 cells may heterodimerize with HER3 and activate downstream kinase signaling pathways. Heregulin mRNA is expressed in CWR-R1 cells, as shown by quantitative reverse transcriptase PCR.[1] This finding suggests that HER2 overexpression is not required for AR-mediated recurrence of prostate cancer but that heregulin signaling through HER2 expressed at low basal levels is sufficient to increase DHT-dependent AR transcriptional regulation of gene expression and growth of malignant cells. This is supported by earlier findings showing that disruption of an association between HER2 and other HER receptors using the mouse and humanized versions of

[1] Gregory, unpublished observations.

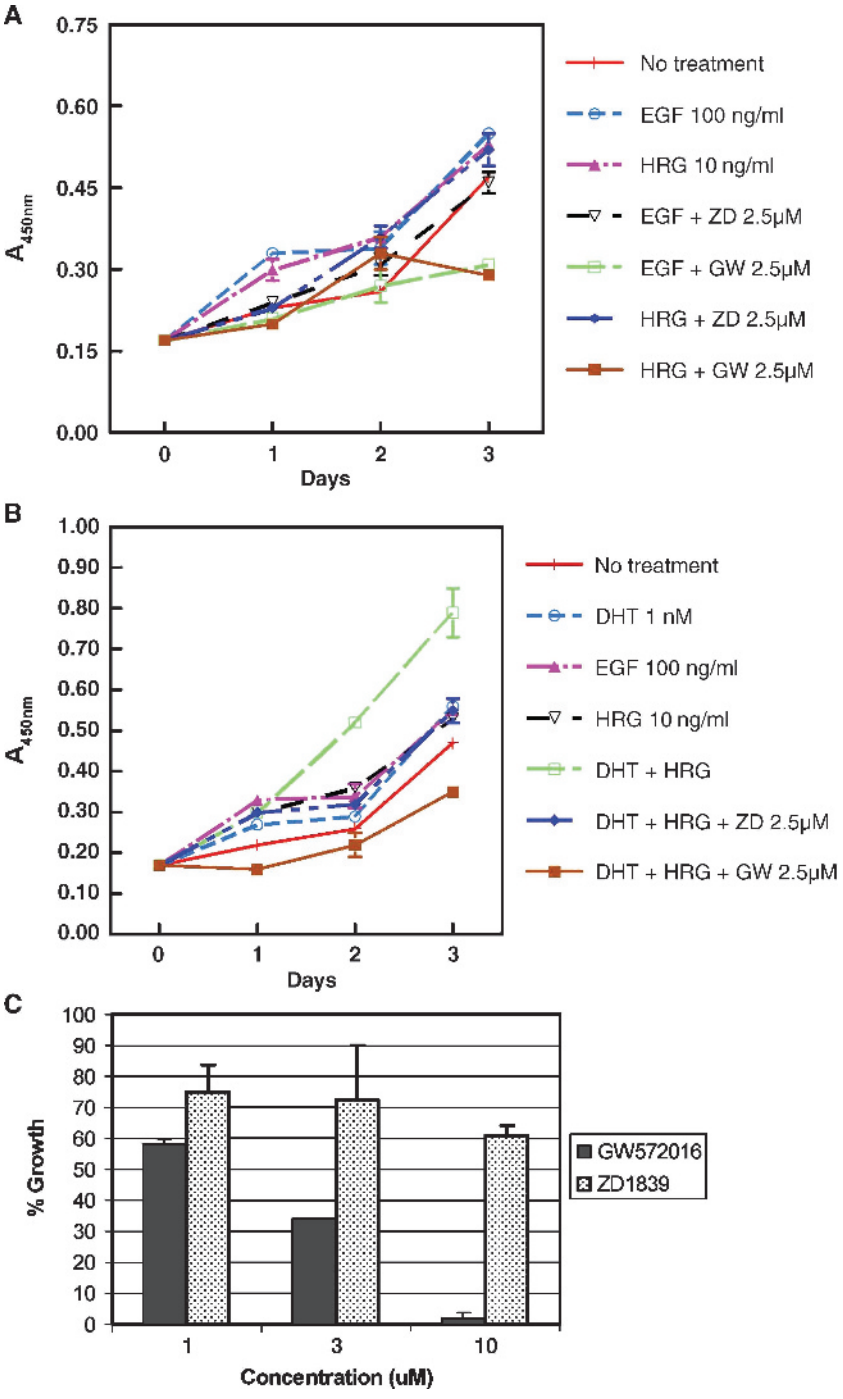

Fig. 5 GW572016 inhibition of CWR-R1 cell growth. *A* and *B,* CWR-R1 cell growth in the presence of various treatments was determined as described in Materials and Methods. Triplicate wells of CWR-R1 cells were incubated in basal prostate growth medium containing no serum and 100 ng/mL EGF, 10 ng/mL heregulin (*HRG*, *A*), or 1 nmol/L DHT *(B)* in the presence and absence of 2.5 μmol/L ZD1839 (*ZD*) or GW572016 (*GW*), as indicated. The number of viable cells was measured on days 1, 2, and 3 of treatment. Representative of at least three independent experiments. *C,* triplicate wells of CWR-R1 cells were incubated in prostate growth medium containing 2% FCS and increasing doses of GW572016 or ZD1839 were added to the wells. After 72 hours of further incubation, cell growth as determined by the colorimetric dye was normalized to the untreated control wells. Points, mean of three independent experiments; bars, SD.

the 2C4 monoclonal antibody inhibits growth of androgen-dependent and recurrent CWR22 tumors in nude mice (22). CWR-R1 cells express EGFR thereby enabling these cells to receive and process EGF, heregulin, and related growth factor signals. Phosphorylation of MAPK and Akt may lead to subsequent phosphorylation of AR and steroid receptor coactivators (10, 52–56). Our recent study showed that EGF-induced DHT-mediated AR transactivation in CWR-R1 cells is associated with phosphorylation of TIF2 (30), a p160 coactivator that is overexpressed in recurrent prostate cancer (57). The components of growth factor signaling cascades in recurrent prostate cancer may be important therapeutic targets for the treatment of this disease.

GW572016 is a reversible inhibitor of EGFR and HER2 with growth inhibitory effects shown previously on several cancer cell lines and xenograft tumors overexpressing EGFR or HER2 (28). We used GW572016 to inhibit heregulin-mediated phosphorylation of HER3 and HER2, thereby inactivating the phospho-MAPK and phosphatidylinositol-3 kinase and Akt

signaling cascades. HER2/3, MAPK, and Akt phosphorylation events were inhibited at GW572016 concentrations as low as 0.1 μmol/L. We found that the EGFR inhibitor ZD1839 only slightly inhibited DHT-induced AR transactivation in the absence of EGF (Fig. 3*B*), whereas GW572016 more effectively inhibited DHT-induced AR transactivation in the absence of exogenous heregulin, consistent with the possibility of an autocrine loop involving HER2 (Fig. 3*C*). However, ZD1839 blocked additional AR transactivation of MMTV-Luc induced by EGF in the presence of DHT. GW572016 also inhibited heregulin-induced AR activity, in agreement with the sensitivity of HER2 and HER3 phosphorylation to GW572016 treatment. The ability of these tyrosine kinase inhibitors to reduce cell growth is consistent with the idea that cell proliferation in prostate cancer cells requires AR function and that HER receptors, through an autocrine signaling loop, lead to activation of AR. Consistent with its ability to inhibit EGFR but not HER2, ZD1839 slightly inhibited EGF-induced growth but had no effect on heregulin-induced proliferation, further implicating HER2 and HER3 rather than EGFR in heregulin-induced growth. On the other hand, GW572016 (2.5 μmol/L) inhibited EGF-, heregulin-, and DHT + heregulin–induced growth of CWR-R1 cells. When compared directly, GW572016 was at least 6- to 7-fold more potent than ZD1839 on a molar basis (IC50 1.53 μmol/L for GW572016 versus >10 μmol/L for ZD1839) in inhibiting proliferation of CWR-R1 cells growing in serum-supplemented medium. LNCaP prostate cancer cells were also more potently inhibited by GW572016 than ZD1839.[2] These data underscore the importance of HER2 and HER3 signaling in driving cell proliferation and AR activation. We reported recently the presence of testosterone levels in recurrent prostate cancer tissue specimens from patients undergoing androgen withdrawal therapy that were similar to levels in benign prostate hyperplasia from untreated patients. DHT levels, although reduced to 10% of the levels in benign prostate, were nonetheless sufficient to activate AR (58). Taken together, combination therapies to inhibit HER2/HER3 signaling and androgen synthesis or AR binding may show efficacy in delaying or preventing the growth of recurrent prostate cancer.

In summary, our work shows that activation of HER2 and HER3 by heregulin enhances AR transactivation in the presence of DHT and growth of CWR-R1 recurrent prostate cancer cells. We also show inhibition of HER2 and HER3 by a novel kinase inhibitor GW572016 inhibits AR transactivation and cellular proliferation more potently than ZD1839 inhibiting EGFR selectively. Therefore, inhibition of HER2 signaling may be a potential novel therapeutic strategy in prostate cancer.

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REFERENCES

1. Lubahn DB, Joseph DR, Sullivan PM, et al. Cloning of human androgen receptor complementary DNA and localization to the X chromosome. Science 1988;240:327–30.

2. Chang CS, Kokontis J, Liao ST. Molecular cloning of human and rat complementary DNA encoding androgen receptors. Science 1988;240:324–6.

3. Roy AK, Lavrovsky Y, Song CS, et al. Regulation of androgen action. Vitam Horm 1999;55:309–52.

4. Gelmann EP. Molecular biology of the androgen receptor. J Clin Oncol 2002;20:3001–15.

5. Nelson PS, Clegg N, Arnold H, et al. The program of androgen-responsive genes in neoplastic prostate epithelium. Proc Natl Acad Sci U S A 2002;99:11890–5.

6. Gregory CW, Hamil KG, Kim D, et al. Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes. Cancer Res 1998;58:5718–24.

7. Zegarra-Moro OL, Schmidt LJ, Huang H, Tindall DJ. Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. Cancer Res 2002;62:1008–13.

8. Eder IE, Hoffmann J, Rogatsh H, et al. Inhibition of LNCaP prostate tumor growth *in vivo* by an antisense oligonucleotide directed against the human androgen receptor. Cancer Gene Ther 2002;9:117–25.

9. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. Nat Rev Cancer 2001;1:34–45.

10. Craft N, Shostak Y, Carey M, Sawyers CL. A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/*neu* tyrosine kinase. Nat Med 1999;5:280–5.

11. Grossmann ME, Huang H, Tindall DJ. Androgen receptor signaling in androgen-refractory prostate cancer. J Natl Cancer Inst 2001;93:1687–97.

12. Chen CD, Welsbie DS, Tran C, et al. Molecular determinants of resistance to antiandrogen therapy. Nat Med 2004;10:33–9.

13. Culig Z, Hobisch A, Cronauer MV, et al. Regulation of prostatic growth and function by peptide growth factors. Prostate 1996;28:392–405.

14. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2001;2:127–37.

15. Osman I, Scher HI, Drobnjak M, et al. HER-2/*neu* (p185neu) protein expression in the natural or treated history of prostate cancer. Clin Cancer Res 2001;7:2643–7.

16. Shi Y, Brands FH, Chatterjee S, et al. Her-2/*neu* expression in prostate cancer: high level of expression associated with exposure to hormone therapy and androgen independent disease. J Urol 2001;166:1514–9.

17. Signoretti S, Montironi R, Manola J, et al. Her-2-*neu* expression and progression toward androgen independence in human prostate cancer. J Natl Cancer Inst 2000;92:1918–25.

18. Savinainen KJ, Saramaki OR, Linja MJ, et al. Expression and gene copy number analysis of ERBB2 oncogene in prostate cancer. Am J Pathol 2002;160:339–45.

19. Calvo BF, Levine AM, Marcos M, et al. Human epidermal receptor-2 expression in prostate cancer. Clin Cancer Res 2003;9:1087–97.

20. Leung HY, Weston J, Gullick WJ, Williams G. A potential autocrine loop between heregulin-α and erbB-3 receptor in human prostatic adenocarcinoma. Br J Urol 1997;79:212–6.

21. Mendoza N, Phillips GL, Silva J, Schwall R, Wickramasinghe D. Inhibition of ligand-mediated HER2 activation in androgen-independent prostate cancer. Cancer Res 2002;62:5485–8.

22. Agus DB, Akita RW, Fox WD, et al. Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. Cancer Cell 2002;2:127–37.

23. Gregory CW, Johnson RT, Mohler JL, French FS, Wilson EM. Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen. Cancer Res 2001;61:2892–8.

24. Lubahn DB, Joseph DR, Sar M, et al. The human androgen receptor: complementary deoxyribonucleic acid cloning, sequence analysis and gene expression in prostate. Mol Endocrinol 1988;2:1265–75.

25. Simental JA, Sar M, Lane MV, French FS, Wilson EM. Transcriptional activation and nuclear targeting signals of the human androgen receptor. J Biol Chem 1991;266:510–8.

[2] Unpublished data.

26. Zhou ZX, Sar M, Simental JA, Lane MV, Wilson EM. A ligand dependent bipartite nuclear targeting signal in the human androgen receptor: requirement for the DNA binding domain and modulation by the NH2-terminal and carboxyl-terminal sequences. J Biol Chem 1994;269:13115–23.

27. He B, Bowen NT, Minges JT, Wilson EM. Androgen-induced NH_2- and COOH-terminal interaction inhibits p160 coactivator recruitment by activation function 2. J Biol Chem 2001;276:42293–301.

28. Rusnak DW, Lackey K, Affleck K, et al. The effects of the novel, reversible epidermal growth factor receptor/Erb-2 tyrosine kinase inhibitor, GW2016, on the growth of human normal and tumor-derived cell lines *in vitro* and *in vivo*. Mol Cancer Ther 2001;1:85–94.

29. Wakeling A, Guy S, Woodburn J, et al. ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. Cancer Res 2002;62:5749–54.

30. Gregory CW, Fei X, Ponguta LA, et al. Epidermal growth factor increases coactivation of the androgen receptor in recurrent prostate cancer. J Biol Chem 2004;279:7119–30.

31. Tan J, Joseph DR, Quarmby VE, et al. The rat androgen receptor: primary structure, autoregulation of its messenger RNA and immunocytochemical localization of the receptor protein. Mol Endocrinol 1988;2:1276–85.

32. Wainstein MA, He F, Robinson D, et al. CWR22: androgen-dependent xenograft model derived from a primary human prostatic carcinoma. Cancer Res 1994;54:6049–52.

33. Robinson MJ, Cobb MH. Mitogen-activated protein kinase pathways. Curr Opin Cell Biol 1997;9:180–6.

34. Grasso AW, Wen D, Miller CM, et al. ErbB kinases and NDF signaling in human prostate cancer cells. Oncogene 1997;15:2705–16.

35. Fedi P, Pierce JH, di Fiore PP, Kraus MH. Efficient coupling with phosphatidylinositol 3-kinase, but not phospholipase Cγ or GTPase-activating protein, distinguishes ErbB-3 signaling from that of other ErbB/EGFR family members. Mol Cell Biol 1994;14:492–500.

36. Salama El Sheikh S, Domin J, Abel P, Stamp G, Lalani EN. Androgen-independent prostate cancer: potential role of androgen and ErbB receptor signal transduction crosstalk. Neoplasia 2003;5:99–109.

37. Culig Z, Hobisch A, Cronauer MV, et al. Androgen receptor activation in prostate tumor cell lines by insulin-like growth factor-1, keratinocyte growth factor, and epidermal growth factor. Cancer Res 1994;54:5474–8.

38. Ueda T, Bruchovsky N, Sadar MD. Activation of the androgen receptor N-terminal domain by interleukin-6 via MAPK and STAT3 signal transduction pathways. J Biol Chem 2002;277:7076–85.

39. Jia L, Choong CS, Ricciardelli C, et al. Androgen receptor signaling: mechanism of interleukin-6 inhibition. Cancer Res 2004;64:2619–26.

40. Myers RB, Oelschlager D, Manne U, et al. Androgenic regulation of growth factor and growth factor receptor expression in the CWR22 model of prostatic adenocarcinoma. Int J Cancer 1999;82:424–9.

41. Robinson D, He F, Pretlow TG, Kung H-J. A tyrosine kinase profile of prostate carcinoma. Proc Natl Acad Sci U S A 1996;93:5958–62.

42. DeMiguel P, Royuela, Bethencourt R, et al. Immunohistochemical comparative analysis of transforming growth factorα, epidermal growth factor, and epidermal growth factor receptor in normal, hyperplastic and neoplastic human prostates. Cytokine 1999;11:722–7.

43. Yeh SY, Lin HK, Kang HY, et al. From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. Proc Natl Acad Sci U S A 1999;96:5458–63.

44. Qiu Y, Ravi L, Kung HJ. Requirement of ErbB2 for signaling by interleukin-6 in prostate carcinoma cells. Nature 1998;393:83–5.

45. Carraway III KL, Sliwkowski MX, Akita RW, et al. The erbB3 gene product is a receptor for heregulin. J Biol Chem 1994;269:14303–6.

46. Riese DJn, van Raaij TM, Plowman GD, Andrews GC, Stern DF. The cellular response to neuregulins is governed by complex interactions of the erbB receptor family. Mol Cell Biol 1995;15:5770–6.

47. Vaskovsky A, Lupowitz Z, Erlich S, Pinkas-Kramarski R. ErbB-4 activation promotes neurite outgrowth in PC12 cells. J Neurochem 2000;74:979–87.

48. Sartor CI, Zhou H, Kozlowska E, et al. Her4 mediates ligand-independent antiproliferative and differentiation responses in human breast cancer cells. Mol Cell Biol 2001;21:4265–75.

49. Lyne JC, Melhem MF, Finley GG, et al. Tissue expression of *neu* differentiation factor/heregulin and its receptor complex in prostate cancer and its biologic effects on prostate cancer cells *in vitro*. Cancer J Sci Am 1997;3:21–30.

50. Zhang Y, Fondell JD, Wang Q, et al. Repression of androgen receptor mediated transcription by the ErbB-3 binding protein, Ebp1. Oncogene 2002;21:5609–18.

51. Gregory CW, Johnson RT, Presnell SC, Mohler JL, French FS. Androgen receptor regulation of G_1 cyclin and cyclin-dependent kinase function in the CWR22 human prostate cancer xenograft. J Androl 2001;22:537–48.

52. Ikonen T, Palvimo JJ, Kallio PJ, Reinikainen P, Janne OA. Stimulation of androgen-regulated transactivation by modulators of protein phosphorylation. Endocrinol 1994;135:1359–99.

53. Sadar MD. Androgen-independent induction of prostate specific antigen gene expression via cross-talk between the androgen receptor and PKA signal transduction pathways. J Biol Chem 1999;274:7777–83.

54. Rowan BG, Weigel NL, O'Malley BW. Phosphorylation of steroid receptor coactivator-1. J Biol Chem 2000;275:4475–83.

55. Lamb DJ, Weigel NL, Marcelli M. Androgen receptors and their biology. Vitam Horm 2001;62:199–230.

56. Lopez GN, Turck CW, Schaufele F, Stallcup MR, Kushner PJ. Growth factors signal to steroid receptors through mitogen-activated protein kinase regulation of p160 coactivator activity. J Biol Chem 2001;276:22177–82.

57. Gregory CW, He B, Johnson RT, et al. A mechanism for androgen receptor mediated prostate cancer recurrence after androgen deprivation therapy. Cancer Res 2001;61:4315–9.

58. Mohler JL, Gregory CW, Ford OH, et al. The androgen axis in recurrent prostate cancer. Clin Cancer Res 2004;10:440–8.