ErbB-2 Induces the Cyclin D1 Gene in Prostate Epithelial Cells In vitro and In vivo

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Abstract
The receptor tyrosine kinase ErbB-2 plays an important role in the regulation of growth factor–induced signal transduction cascades in the epithelium, and ErbB-2 is frequently overexpressed in epithelial tumors. Our previous studies on clinical prostate cancer specimens indicated that ErbB-2 expression was increased in patients undergoing hormone ablation therapy. We had also shown that the critical cell cycle regulatory gene cyclin D1 and its promoter were targets of proliferative signaling in prostate cancer cell lines, and that cyclin D1 was required for ErbB-2–induced mammary tumorigenesis. In the current studies, we found that increased ErbB-2 membrane expression correlated with increased nuclear cyclin D1 staining in clinical prostate cancer specimens, and that expression of ErbB-2 was capable of inducing cell cycle progression in human prostate cancer cell lines. We further showed that ErbB-2 induced the cyclin D1 promoter in DU145 cells, and that small interfering RNA knockdown of cyclin D1 blocked a significant proportion of the DU145 cell cycle progression. Probasin promoter–targeted expression of an activated ErbB-2 isoform induced cyclin D1 expression in the mouse prostate, commensurate with prostate intraepithelial neoplasia. Together, these in vitro and in vivo studies identify cyclin D1 as a critical downstream target of ErbB-2 in the prostate epithelium, both of which are possible therapeutic targets for cancer intervention. Furthermore, our novel mouse model provides a useful platform for ongoing in vivo investigations of ErbB-2 signaling in the prostate epithelium. [Cancer Res 2007;67(9):4364–72]

Introduction
The induction of ErbB-2 (Neu or HER2) signaling, a member of the epidermal growth factor receptor (EGFR) family (which also includes the EGFR, ErbB-3, and ErbB-4), has been associated with a wide variety of cancers. Although ErbB2 has no known ligand, it is the preferential dimerization partner for all members of the EGFR family and may therefore represent a key growth factor receptor regulatory protein. There is clinical evidence implicating ErbB-2 in several common cancers, including prostate cancer. In prostate cancer patients, increased ErbB-2 levels correlated with poor histochemical features; however, results varied in different studies involving low-grade cancer due to degree of disease, study criteria and lesion, and the lack of an effective standardized protocol (1). Importantly, the correlation between increased ErbB-2 expression and tumorigenesis is more clearly defined as the disease advances. For example, in patients undergoing radiotherapy for localized prostate cancer, increased expression of ErbB-2 is inversely proportional to disease-free survival and clinical outcome in both univariate and multivariate analyses (2). A significant proportion of patients undergoing androgen ablation therapy whose disease had progressed to the androgen-independent phase were shown to overexpress ErbB2 in the prostate epithelium (3–6), and elevated levels of ErbB-2 in patient serum correlated with metastatic prostate cancer (7). Although the incidence of ErbB-2 positivity increased following total androgen ablation, being highest in androgen-independent prostate cancer (AIPC), amplification of the ErbB-2 locus was infrequent (5).

Despite the absence of a known ligand, ErbB-2 can affect signaling through a variety of means. For example, ErbB-2 can form an active heterodimer with the EGFR, and the high-affinity ligands for the EGFR, including EGF, heparin-binding epidermal growth factor–like factor, and transforming growth factor–α (TGF-α), are found in the prostate epithelium and stroma. Additionally, ErbB-3 expression is consistently observed in prostate epithelial cells. The ErbB-3 ligand, heregulin (also referred to as Neu Differentiation Factor), is expressed in the stroma and basal epithelium of the normal prostate gland (8), and heregulin acts as a potent mitogen in prostate cancer cells. The signaling pathways induced through ErbB-2 in prostate cancer cell lines include phosphoinositide-3-kinase (PI3K)/AKT (9–11), c-Src, and mitogen-activated protein kinase (MAPK; ref. 12). ErbB-2 also induced nuclear factor-κB nuclear activity through a Pten-sensitive, PI3K-dependent signaling pathway (13), which may represent a prognostic indicator of tumor grade (14). ErbB-2 is also the preferential ErbB-3 binding partner in signaling to the androgen receptor (AR) in androgen-independent prostate cancer cells (15). Activation of ErbB-2 signaling also occurs through receptor overexpression, mutations, or deletions and by alternate splicing. An alternatively splice receptor isolated from mammary epithelial tumors, ErbB-2Δ, enhanced tyrosine phosphorylation, increased MAPK activation, and supported cellular transformation in the absence of ligand in vitro (16).
Cyclin D1 functions as the rate-limiting regulatory subunit of a multiprotein cyclin/Cdk complex that is critical for controlling progression through the G1 to S phase of the cell cycle (17). Deregelation of cyclin D1 gene expression has been described in numerous types of human tumors, and the tissue-specific dependency of certain oncogenes on cyclin D1, including Ras, ErbB-2, and β-catenin (18–20), is well documented.

Cyclin D1 mRNA levels were induced by EGF in human prostate cancer cell lines (21) and were found to be increased in both primary prostate cancer samples (22) and androgen-independent bone metastases (23). Increased expression of cyclin D1 in LNCaP human prostate cancer cells enhanced cell growth and tumorigenicity (21, 24), whereas inhibition of ErbB-2/ErbB-3 signaling by the flavonoid, quercetin, reduced cyclin D1 protein levels, resulting in an inhibition of cell cycle progression both in prostate cancer cell lines (25) and in vivo (26). These data are all consistent with a role for cyclin D1 as a mediator of prostate epithelial cell proliferation.

Despite an apparent link between ErbB-2 signaling and cyclin D1 expression in prostate epithelial proliferation and tumorigenesis, some studies to date have investigated the effect of enhanced ErbB-2 signaling on alterations in cyclin D1 gene expression in the prostate. Our previous data indicated that tissue-specific expression of a constitutively active ErbB-2 splice variant in the mouse prostate epithelium resulted in prostate enlargement. To better understand role of the ErbB-2 oncoprotein in prostate cancer and to investigate its association with cyclin D1 abundance in clinical prostate cancer, we assessed the effect of ErbB-2 on cyclin D1 expression in both human prostate cancer cell lines in vitro and in the human and mouse prostate epithelium in vivo. We present evidence that cyclin D1 is a target of ErbB-2 and is modulated in both androgen-independent and androgen-sensitive prostate cancer cells. We further showed that heregulin-induced cell cycle progression is cyclin D1 dependent, and that the transgene expression of activated ErbB-2 in the mouse prostate epithelium resulted in prostate intraepithelial neoplasia and a commensurate induction of both cyclin D1 mRNA and protein levels. We further showed that there is strong, statistically significant correlation between ErbB-2 positivity and cyclin D1 expression in human prostate cancer specimens. Our data describe a causal link between ErbB-2 expression and the regulation of the cyclin D1 gene in prostate epithelial cells and in prostate cancer.

Materials and Methods

Cell lines and cell culture. Three human prostate cancer cell lines, DU145, PC3, and LnCaP, were maintained as previously described (27). Cells were cultured in RPMI with 10% FCS, 0.1 mmol/L non–essential amino acids, 100 U/mL penicillin-streptomycin, and 1 mmol/L sodium pyruvate at 37°C in 5% CO2 (27). LNCaP cells treated with heregulin 1β (R&D Systems) were seeded to a density of 1 × 105 cells per 10-cm dish in normal growth media followed by prolonged serum starvation (0.1% FCS in RPMI for 72 h); heregulin was added at a concentration of 5 mmol/L for 12 to 24 h.

Plasmids. The cyclin D1 promoter construct and transfection methodologies have been previously described by our laboratory (28–31). The mouse urokinase-type plasminogen activator (uPA) promoter reporter construct (32), the human uPA (33) and uPAR promoter reporter constructs (34), and the pcDNAs, pcDNAs-ErbB-2WT, and pcDNAs-ErbB-Δ2 expression vectors have been previously described (16). ErbB-Δ2 encodes a constitutively activated ErbB-2 receptor splice variant that was isolated from breast cancer epithelial cells as previously described (35).

Flow cytometry. The prostate cancer cell lines (1 × 106 cells per 10-cm dish) were transfected in serum-free media with either ErbB-2 expression vectors or the control vector (pcDNA3) and spectrin–green fluorescent protein (GFP) at a 5:1 molar ratio, respectively using either LipofectAMINE 2000 or LipofectAMINE Plus (Invitrogen) following the manufacturer’s recommendations. After 4 h, the transfection media was replaced with RPMI containing 2% fetal bovine serum (FBS) and resuspended in PBS containing 20 mg/ml propidium iodide and 5 U RNase A. DNA content was analyzed as previously described (29, 30) using a FACStar Plus dual-laser FACSort system. Statistical analyses were done on LNCaP cell data using the Mann-Whitney U test, with significant differences established as P < 0.05.

Western blotting. A total of 35 μg of protein per lane were separated on 12% acrylamide gels and electroblotted onto polyvinylidene difluoride membranes. ErbB-2 expression levels were assessed using the antibody OP15 (Calbiochem). Induction of signal transduction cascades were assessed using antibodies for total- and phospho-Erk (p21/p44 MAPK; Cell Signaling, Danvers, MA). Cyclin D1 protein levels were assessed by using an anti-cyclin D1 polyclonal antibody, AB3 (NeoMarkers; ref. 30), and p27Kip1 levels were assessed using an anti-p27 antibody from Santa Cruz Biotechnology (sc-528), either β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. Statistical analyses were done using the Mann-Whitney U test with significant differences established as P < 0.05 for cyclin D1 protein levels in the ErbB-2 versus control samples.

Immunohistochemical staining. Immunohistochemical staining for ErbB-2 levels in ≥16-month-old mouse prostate tissue was done using the antibody OP15 (Calbiochem) following the manufacturer’s prescribed conditions. Immunohistochemical staining on embedded mouse prostate sections was done using the anti-cyclin D1 antibody, AB3 (Novoceastra) as previously described (20, 36). Detection was done using a DAKOcystomation mouse kit (DAKO). Immunohistochemical staining for cyclin D1 was also done on human prostate cancer specimens, which we had previously assessed immunohistochemically for Her-2/neu (37). A 3% hydrogen peroxide solution in absolute methanol was used to quench endogenous peroxidase, and antigen retrieval was done using citrate buffer (pH, 6) and microwaving for 30 min (36). The slides were blocked with normal horse serum for 20 min and incubated for 1 h with anti-cyclin D1 monoclonal antibody (Novoceastra) at a 1:20 dilution in PBS. Subsequent incubation with biotinylated horse anti-mouse secondary antibody at a 1:200 dilution was followed by avidin-biotin-conjugate (Vector Laboratories, Inc.), Chromogen (3,3′ diaminobenzidine) was then applied with diaminobenzidine staining. Negative controls consisting of diluent with no antibody and positive tonsilar tissue controls with heterogeneous immunoreactivity were used in all experiments.

Immunoreactivity assessment of clinical samples. All slides examined for cyclin D1 immunoreactivity were interpreted by a pathologist who was blinded to all outcome data. Tumor scores for cyclin D1 were categorized based on two criteria: (a) percent of tumor cells demonstrating nuclear immunoreactivity and (b) intensity of nuclear immunostaining. For assessment according to percent of cells with nuclear reactivity, tumors were classified as showing weak cyclin D1 expression (<5%), moderate cyclin D1 expression (5–10%) or strong cyclin D1 expression (>50%). For intensity of cytoplasmic immunoreactivity, tumors were classified as having weak or no cyclin D1 expression (1+), moderate cyclin D1 expression (2+), or strong cyclin D1 expression (3+). Cyclin D1 expression was then categorized as a combined measure (i.e., status) of percent of immunoreactive tumor cells and intensity of nuclear immunoreactivity. Cyclin D1 status was assigned as negative to cases with <5% cyclin D1 immunoreactivity or weak (1+) staining. All other cases were assigned positive cyclin D1 status. Scoring was done via an overall evaluation of the entire tissue section and by counting the average percentage of immunoreactive tumor cells per total number of tumor cells in five high power fields per slide. ErbB-2 immunoreactivity was evaluated as previously described (37). χ2 analysis was used to compare cyclin D1 and ErbB-2 expression in the clinical cohort. Results were considered significant at P < 0.05 for two-sided analyses.
Luciferase assays. The cotransfection of reporter constructs and expression vector DNA was accomplished using LipofectAMINE Plus or LipofectAMINE 2000 (Invitrogen) following the manufacturer’s conditions, and luciferase activity was measured in a Bertold Autolumat 963 luminometer as previously described (30). Luciferase content was measured in arbitrary light units (ALU) by calculating the light emitted during the initial 10 s of the reaction. Background activity from cell extracts was typically <100 ALU/10 s. Internal controls (either Renilla luciferase or cytomegalovirus β-Gal) were used as necessary as previously reported (30). Statistical analyses were done using the Mann-Whitney U test, with significant differences established as P < 0.05 on at least four independent pcDNA3 ErbB-2 WT transfections and eight pcDNA3 ErbB-Δ2 transfections. Data were plotted as average fold induction ±SE versus pcDNA3 empty vector control (30).

Transgene construction. The construction of the transgene, PB-ErbB-Δ, has been previously described (33).

Transgene expression. Functional analysis of the expression of the transgene was determined using Q-PCR. The prostate was dissected away from other urogenital tissues (e.g., seminal vesicles, bladder, and urethra). Total RNA was isolated and purified from the ventral and dorsolateral prostate gland lobes with TRIzol reagent (Life Technologies BRL) and an RNaseasy Kit (Qiagen) according to the manufacturers’ protocols. Traces of genomic DNA were removed by treating the RNA samples with DNA-free DNase (Ambion). About 1 μg of total RNA was reverse transcribed with SuperScript II RNase H− reverse transcriptase (Invitrogen).

Semiquantitative real-time PCR (Q-PCR). Q-PCR for the ErbB-Δ2 transgene, endogenous ErbB-2, cyclin D1, and UPAR was done on total RNA from >16-month-old normal and PB-ErbB-Δ2 prostate tissue (dorsolateral or ventral lobes). Approximately 1 μg of total RNA was reverse transcribed with SuperScript II RNase H− reverse transcriptase (Invitrogen) as described above. PCR was done using SYBR Green PCR Core Reagents (Applied Biosystems). Reverse transcription-PCR reaction mixes contained cDNA generated by reverse transcription using 900 nmol/L of primers. Primer sequences are as follows: for GAPDH, GCAAGTGGGATTTGTCG-CAT (forward) and CCTTGACTGTCGTTGAATTT (reverse); for the ErbB-2 transgene, TGTTGTATCAGTTGGAACG (forward) and GTCTCCTGAAATGGTGCACG (reverse); for endogenous (mouse) ErbB-2, TCTGGAACATGTCGGAAACA (forward) and TGAAACAGC-GAATCTTCTC (reverse); and for UPAR, AAACTGTCGCTGGACGACTT (forward) and ATCTCAGATGTCGCTGAA (reverse). Q-PCR for cyclin D1 was done with the Assay by Design mix, Mm00432359-m1 (forward) or ventral lobes) (39). Approximately 1 μg of total RNA was reverse transcribed and luciferase activity was measured in a Bertold Autolumat 963 luciferase reporter and either pcDNA3, ErbB-2 WT, or ErbB-2 Δ transfections.

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Results

ErbB-2 expression induces prostate epithelial cell cycle progression in vitro. To explore the possibility that ErbB-2 regulated cell cycle progression in prostate cancer cells, randomly cycling DU145, PC3, or LNCaP cells were cotransfected with either the control vector (pcDNA3), the wild-type ErbB-2, or the activated ErbB-2 Δ isoform (ErbB-2 Δ) and GFP at a 5:1 molar ratio of ErbB-2 to GFP. The cells were collected by fluorescence-activated cell sorting (FACS) and assayed for DNA content at 16 h. The expression of ErbB-2 WT or ErbB-2 Δ in random cycling LNCaP cells resulted in a 4.5 ± 1.4% (n = 4; P < 0.05) and 4.1 ± 0.9% (n = 4; P < 0.05) decrease in G1 phase versus pcDNA3, respectively. The S phase fraction was increased by 3.8 ± 1.1% (n = 4; P < 0.05) and 4.2 ± 1.2% (n = 4; P < 0.05), respectively. The ErbB-2/ErB-3 ligand heregulin increased the S phase fraction by ~10% at 24 h (data not shown).

Experiments done in DU145 and PC3 cells produced similar results, with the G1 phase fraction of cells reduced by 9% to 12% (n = 2) by both receptor isoforms and a concomitant increase in S phase fraction (data not shown). In separate sets of experiments, the time course of cell cycle progression by ErbB-2 Δ was assessed in DU145 cells. The greatest effect of ErbB-2 Δ on the G1 and S phases was seen at 16 to 20 h and was reduced by 24 to 36 h, consistent with the short doubling time of DU145 cells (data not shown).

ErbB-2 induces cyclin D1 expression. Because our previous data showed that cyclin D1 was involved in proliferative signaling in prostate cancer cell lines (30, 38), immunoblot against cyclin D1 were done on cell extracts from LNCaP cells transfected with the ErbB-2 Δ expression vector (Fig. 1B). ErbB-2 Δ increased cyclin D1 protein levels an average of 2.5 ± 0.91-fold (P < 0.05, n = 4) and Erk-phosphorylation 1.7 ± 0.43-fold (n = 2). MCF7 cell extracts and LNCaP cells stimulated with heregulin were used as control samples. A representative experiment is shown. We had previously shown that levels of p27kip1 were reduced following androgen depletion (6) or by overexpression of ErbB-2 in prostate cancer (6) and breast cancer cell lines (39). Furthermore, because agents that inhibit prostate cancer cell proliferation, such as vitamin D, do so in part through p27kip1 stabilization (40), we assessed the effect of transfection with ErbB-2 Δ on p27kip1 levels in LNCaP cells. ErbB-2 Δ overexpression reduced p27kip1 protein levels in LNCaP cells by 39 ± 17% (n = 3; Fig. 1B).

These data support our earlier studies that showed that ErbB-2 inhibited p27kip1 and further establishes that another key regulator of the cell cycle, the cyclin D1 gene, is induced by ErbB-2 signaling in prostate cancer cell lines.

ErbB-2 induces the cyclin D1 promoter. We had previously established that the cyclin D1 promoter was induced by ErbB-2 in breast cancer cell lines (18) as well as by mitogenic signaling cascades in DU145 cells (30). To establish whether ErbB-2 regulated cyclin D1 promoter activity in prostate cancer cells, DU145 cells were cotransfected with the full-length −1745 cyclin D1 promoter luciferase reporter and either pcDNA3, ErbB-2 WT, or ErbB-2 Δ. The activity of the −1745 cyclin D1 promoter was induced 2.2-fold by ErbB-2 WT (data not shown) and 3.1-fold by ErbB-2 Δ (Fig. 1C). Because the uPA system has been implicated in cancer progression...
and metastasis and because we have previously shown that the uPA and uPAR promoters are regulated by heregulin in breast cancer cells (33), we tested whether the promoters of these genes were activated by ErbB2 in DU145 cells. The mouse uPA, the human uPA, and the human uPAR promoters were induced from 1.5- to approximately 8-fold by ErbB-2WT (data not shown) and by ErbB-2Δ (Fig. 1C), all of which were statistically significant at P < 0.05. These data confirm that cyclin D1 was a transcriptional target of ErbB-2 in prostate cancer cell lines. They do not, however, indicate whether cyclin D1 is a target of ErbB-2 in vivo.

Figure 1. ErbB-2 induces cell cycle progression and cyclin D1 in human prostate cancer cell lines. A, effect of ErbB-2WT or ErbB-2Δ on cell cycle progression in randomly cycling LNCaP cells. Values are percent change with ErbB-2 expression vector versus empty control vector (average % change ± SE; n = 4). *, P < 0.05. B, Western blotting of LNCaP cells transfected with pcDNA3 or ErbB-2Δ. Average fold change for cyclin D1 (n = 4), p27KIP1 (n = 3), and p-Erk (n = 2) is shown. *, P < 0.05. MCF-7 cell extracts or LNCaP cells treated with heregulin served as controls. C, the human cyclin D1, mouse uPA (muPA), human uPA (huPA), and human uPAR (huPAR) promoter luciferase reporter constructs (fold change ± SE; n = 8) were induced by ErbB-2Δ. **, P < 0.01.

Figure 2. Cyclin D1 expression in prostate cancer specimens. Immunohistochemical staining for cyclin D1 was done on prostate cancer specimens previously screened for ErbB-2 expression. A, tumor from a T3N0M0 patient negative for cyclin D1 nuclear immunoreactivity. B, T3N0M0 tumor showing weak (1+) focal cyclin D1 nuclear immunoreactivity in >10% of tumor cells. C, T3N0M0 tumor showing moderate (2+; arrow) focal immunoreactivity in 5% to 10% of tumor cells. D, T3N0M0 tumor showing moderate (arrowhead) and strong (arrow) cyclin D1 nuclear immunoreactivity. E, correlation between immunostaining for ErbB-2 and cyclin D1 in clinical prostate cancer specimens. Specimens staining for both increased ErbB-2 and increased cyclin D1 are in italics.
Cyclin D1 expression correlates with ErbB-2 expression in clinical prostate cancer. Expression levels of cyclin D1 (Fig. 2) and ErbB-2 (37) were assessed in 36-stage T2N0M0 and T3N0M0 prostate tumors by immunohistochemistry. Of the 36 prostate tumors examined, 33% (n = 12) of tumors were found to be positive for cyclin D1 (combined intensity and percent tumor cells reactive; Fig. 2E). ErbB-2 expression was considered positive in 9 (25%) of these 36 cases (Fig. 2E). Cyclin D1 expression was strongly associated with ErbB-2 expression (P < 0.0001). Furthermore, when ErbB-2 status was analyzed for the expression of cyclin D1, either by intensity of immunoreactivity or for percent immunoreactive tumor cells, the expression levels of cyclin D1 and ErbB-2 were also significantly correlated (P = 0.0081 and P = 0.0018, respectively). These data show that increased ErbB-2 expression was correlated with increased cyclin D1 protein levels in clinical prostate cancer.

ErbB2-Δ induces prostate intraepithelial neoplasia in vivo. Our previous data indicated that the prostate gland volume was increased in PB-ErbB-2Δ mice versus nontransgenic control mice, and that the prostate of the transgenic mice displayed widespread prostate intraepithelial neoplasia (PIN; ref. 35). Further histologic evaluations were done, and prostate pathology was evaluated on the basis of Park et al. (41). Analyses of prostate from 5- to 11-month-old PB-ErbB-2Δ mice revealed that three of eight mice presented with PIN. All but one of the 12- to 17-month-old PB-ErbB-2Δ mice had PIN, three of which were PIN III or PIN IV (Table 1). By 18 months of age and older, all prostates showed significant pathology, and 10 of the 15 transgenic mice presented with PIN, 6 of which were PIN III or PIN IV (Table 1). Advanced PIN lesions (Fig. 3) were typified by extensive thickening of the fibromuscular stroma, along with a loss of the normal glandular architecture. These disorganized ductal structures were enlarged, lacked an organized basal epithelium, and displayed extensive epithelial expansion, which, in many cases, filled the lumen of the gland. The atypically shaped epithelial cells contained dense, irregular nuclei, and many seemed necrotic, especially those in the center of the duct. Fully involved PIN IV lesions invaded adjacent ducts (Fig. 3, right). Infiltrating immune cells were frequently observed. Atypical hyper trophy and especially PIN were rarely seen in normal mice, regardless of age (Table 1).

ErbB2-Δ induces cyclin D1 in vivo. To assess transgene expression and its effect on transcriptional activity, Q-PCR was done on RNA isolated from the dorsolateral and ventral lobes from either normal or PB-ErbB-2Δ prostates. Oligonucleotide primers directed against the ErbB-2Δ transgene, endogenous mouse ErbB-2 (mHER2), cyclin D1, or uPAR were used. Primers for GAPDH or β-actin were used as internal controls. ErbB-2Δ transcripts were abundant in the transgenic prostate but not in the control (P = 3; Fig. 4A). RNA from PB-ErbB-2Δ epithelium not reverse transcribed into cDNA (no RT) showed no product (Fig. 4A, inset). Q-PCR revealed that endogenous HER2 mRNA levels were similar between the normal and transgenic mice (1.3 ± 0.96-fold; n = 4), which was not significant. In contrast, mouse cyclin D1 mRNA levels were

Table 1. Prevalence of prostate disease in normal versus PB-ErbB-2Δ mice

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Abbreviation: AH, atypical hyperplasia.
induced 3.7 ± 1.5-fold (n = 4; P < 0.05) in the PB-ErbB-2Δ epithelium (Fig. 4B). Primers directed against mouse uPAR revealed a 3.5-fold induction in uPAR mRNA abundance (data not shown), consistent with the promoter induction.

Immunohistochemical staining for ErbB-2 expression was done on prostate tissue from littermate nontransgenic or PB-ErbB-2Δ mice (Fig. 4C). Strong ErbB-2 immunoreactivity was found throughout the PB-ErbB-2Δ PIN lesions. Quantification of cyclin D1 nuclear positivity was carried out on >300 cells. In the nontransgenic tissue, 7% of the normal prostate epithelial cells stained weakly positive for nuclear cyclin D1. In contrast, 24% of the prostate epithelial cells in the PB-ErbB-2Δ PIN lesions were strongly positive for nuclear cyclin D1 (Fig. 4C). Analyses of normal adjacent glands in the PB-ErbB-2Δ prostate revealed that 20% of the cells were cyclin D1 positive versus 13% in the rare PIN lesion in the nontransgenic prostate (data not shown).

**Cyclin D1 is required for ErbB-2–induced cell cycle progression.** To determine whether cyclin D1 was required for ErbB-2–induced cell cycle progression in prostate epithelial cells, LNCaP cells were serum starved for 72 h, with a majority of cells...
arresting in G1. The cells were transfected with either control- or cyclin D1–siRNA and stimulated with heregulin. Subsets of cells were collected for both Western blotting and flow cytometry, and analyses were carried out on data from each single experiment, done on five separate occasions. Cyclin D1–siRNA reduced cyclin D1 protein abundance by $64 \pm 20\%$ ($n = 5, P < 0.01$) versus control-siRNA when normalized to β-actin (a representative immunoblot is shown in Fig. 5). Cell cycle analysis done on a subset of cells used for protein analysis revealed that control-siRNA LNCaP cells stimulated with heregulin exhibited a $2.5 \pm 0.2\%$ ($n = 5, P < 0.01$) increase in S phase fraction versus unstimulated cells, with a concomitant decrease in the G1 phase (a representative experiment, corresponding to the expression data in Fig. 5A), is shown in Fig. 5B). In cyclin D1–siRNA–transfected cells, heregulin treatment resulted in only a $0.9 \pm 0.35\%$ ($n = 5$) increase in S phase versus unstimulated cells, which failed to reach significance at $P < 0.05$ (the cell cycle data, corresponding to the expression data in Fig. 5A, is shown in Fig. 5B). Conversely, the abrogation of heregulin-induced cell cycle progression by cyclin D1–siRNA knockdown was highly significant at $P < 0.01$ compared with heregulin-treated control-siRNA cells.

Discussion

Accumulating clinical evidence from our group and others has clearly shown that increased expression of ErbB-2 contributes to human prostate disease (see refs. 1, 3–6). These findings are further supported by both in vitro (6, 15, 42) and in vivo (15, 43) studies. Because our previous data had also established that the activity of the cell cycle regulatory protein, cyclin D1, is important for both cellular proliferation and regulation of AR function (44, 45), we assessed the possibility that cyclin D1 may be a bona fide intermediary of androgen-independent proliferative signaling in prostate cancer cell lines and establishes that activation of ErbB-2 in the mouse prostate epithelium results in localized prostate cancer, with increased cyclin D1 expression.

Activation of the MAPK/ERK signaling pathway is an important intermediary of androgen-independent proliferative signaling in prostate cancer cells through the modulation of AR and AR coactivator function (12). The AR has been shown to transduce a component of an androgen-independent proliferative signaling via the PI3K pathway/Akt pathway (10). Because our data showed that ErbB-2 induction of both cyclin D1 and cell cycle progression occurred in AR-positive and AR-negative prostate cancer cell lines, the AR may not represent an absolute requirement for ErbB-2–regulated proliferation. Additional or alternate regulatory proteins and signaling pathways may therefore be involved.

Accumulating evidence suggests that the mechanisms by which ErbB-2 signaling is increased, and the effects it has on epithelial transformation, may differ between the mammary gland and the prostate. Unlike breast cancer, ErbB-2 gene amplification is rare in clinical prostate tumors (1), suggesting that sustained or enhanced ErbB-2 expression in vivo may occur at a transcriptional or translational level and is not a product of increased gene copy number. In addition, whereas the rat Neu oncogene was able to induce metastatic adenocarcinomas in the mammary glands of mouse mammary tumor virus-Neu mice, and whereas activated ErbB-2Δ induced both localized prostate cancer induction (advanced PIN III and PIN IV) and prostate enlargement in our PB-ErbB-2Δ mouse model (35), progression to invasive prostate adenocarcinoma has not yet been observed. One possible explanation for the differential tissue response to ErbB-2 may be related to differences in the physiologic growth characteristics between the two tissues. The mammary gland is capable of undergoing a rapid expansion of both size and function during pregnancy and lactation that is fully reversed following the cessation of lactation. Conversely, both the mouse and human prostates exhibit very low proliferative indices throughout development. Because glandular tissue is only slowly accumulated,

<table>
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<tr>
<th>Treatment</th>
<th>HRG- siRNA</th>
<th>HRG+ siRNA</th>
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<tr>
<td>Western Cyclin D1</td>
<td>Control</td>
<td>CD1</td>
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<td>β-actin</td>
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Figure 5. Suppression of cyclin D1 blocks heregulin-induced cell cycle progression in prostate cancer cells. A, a representative Western blot of LNCaP cells treated either with control- or cyclin D1–siRNA in the absence or presence of heregulin. Cyclin D1–siRNA significantly reduced cyclin D1 protein abundance ($P < 0.01$). B, cell cycle analysis by flow cytometry done on a subset of cells from the same experiment shown in (A). Cyclin D1–siRNA significantly reduced heregulin-induced cell cycle progression ($P < 0.01$).
the broadest scope of signaling protein recruitment among the protein microarray that ErbB-2 and EGFR are unique among the of multigenic tumor progression in an otherwise normal prostate. Furthermore, additional engineered lesions can be incorporated into this model (35) to study the biophysical basis of high efficiency and fidelity in the prostate epithelium that may genomewide tumorsuppressorgenes(suchasthe ref. 47), our mouse model provides an important platform to more fully investigate the molecular and functional consequences of enhanced growth factor receptor tyrosine kinase signaling in the prostate. Finally, recent pioneering work by Jones et al. (48) has shown by protein microarray that ErbB-2 and EGFR are unique among the ErbB receptors with respect to the dosage sensitivity of their signaling capacity, and that the ErbB-2/ErbB-3 complex displayed the broadest scope of signaling protein recruitment among the possible ErbB-family combinations. The use of the PB-ErbB2Δ mouse described herein may provide a useful complementary model to perform comprehensive proteomic-based analyses of ErbB-2 signaling to cyclin D1 and other targets in an otherwise normal prostate. Additional investigations into the molecular signature of ErbB-2 signaling in vivo will help to elucidate both the mechanistic progression to PIN and the apparent protection from full ErbB-2– induced transformation of the prostate epithelium.

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