Biomarkers, Genomics, Proteomics, and Gene Regulation

A Reduction in Pten Tumor Suppressor Activity Promotes ErbB-2-Induced Mouse Prostate Adenocarcinoma Formation through the Activation of Signaling Cascades Downstream of PDK1

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Loss of function at the *Pten* tumor-suppressor locus is a common genetic modification found in human prostate cancer. While recent *in vivo* and *in vitro* data support an important role of aberrant ErbB-2 signaling to clinically relevant prostate target genes, such as cyclin D1, the role of Pten in ErbB-2-induced prostate epithelial proliferation is not well understood. In the Pten-deficient prostate cancer cell line, LNCaP, restoration of Pten was able to inhibit ErbB-2- and heregulin-induced cell cycle progression, as well as cyclin D1 protein levels and promoter activity. Previously, we established that probasin-driven ErbB-2 transgenic mice presented with high-grade prostate intraepithelial neoplasia and increased nuclear cyclin D1 levels. We show that mono-allelic loss of *pten* in the probasin-driven-ErbB-2 model resulted in increased nuclear cyclin D1 and proliferating cell nuclear antigen levels and decreased disease latency compared to either individual genetic model and, unlike the probasin-driven-ErbB-2 mice, progression to adenocarcinoma. Activated 3-phosphoinositide-dependent protein kinase-1 was observed during cancer initiation combined with the activation of p70S6K

(phospho-T389) and inactivation of the 4E-binding protein-1 (phosphorylated on T37/46) and was primarily restricted to those cases of prostate cancer that had progressed to adenocarcinoma. Activation of mTOR was not seen. Our data demonstrates that Pten functions downstream of ErbB-2 to restrict prostate epithelial transformation by blocking full activation of the PDK1 signaling cascade. (Am J Pathol 2009, 174:2051–2060; DOI: 10.2353/ajpath.2009.080859)

The proto-oncogene ErbB-2 (Neu or HER2) is an 185-kDa receptor tyrosine kinase with no known ligand and is the preferential dimerization partner for all members of the epidermal growth factor receptor family. Enhanced ErbB-2 signaling has been demonstrated in a number of cancers, including those of the breast, head and neck, pancreas, and colon, and recently, increased ErbB-2 levels in the absence of gene amplification was shown to correlate with poor prognosis in prostate cancer (PCa) patients with progressive disease.^{1,2} In previous studies, we established that increased ErbB-2 membrane expression correlated with increased nuclear cyclin D1 staining in clinical PCa specimens.³ We also established in human PCa cell lines that ErbB-2 induced both cell cycle proliferation and cyclin D1, and that small interfering RNA

Supported by grants from the National Institutes of Health (NIH) R01CA129003, U54CA100970-02, and the American Institute for Cancer Research (AICR05B131), (to C.A.); NIH R01CA111482 (R.I.G.); and in part by grants from the Intramural Research Program of the NIH and the National Institute for Child Health and Human Development (to E.W.L. and K.P.).

Accepted for publication February 17, 2009.

A guest editor acted as editor-in-chief for this manuscript. No person at Thomas Jefferson University or Albert Einstein College of Medicine was involved in the peer review process or final disposition for this article.

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targeting cyclin D1 blocked a significant proportion of the ErbB-2 or heregulin-induced cell cycle progression. Furthermore, we established that probasin-driven ErbB-2 transgene mice (PB-ErbB-2) presented with high-grade prostate intraepithelial neoplasia, (PIN), a localized adenoma, and induced epithelial cyclin D1 expression; trans-formation to adenocarcinoma was not observed.³

The phosphatidylinositol-3-kinase (PI3K) signaling pathway is a major mediator of receptor tyrosine kinase signaling and plays an important role in controlling cell proliferation and cell survival. Pten is a lipid phosphatase that catalyzes the dephosphorylation of phosphatidylinositol-3,4,5-tri-phosphate and phosphatidylinositol-3,4-bis-phosphate, thereby inhibiting PI3K signaling. Modifications at the *pten* locus are frequently found in human diseases, and *pten* is one of the most frequently mutated genes identified in human PCa.⁴ Pten levels were reduced in as many as 50% of the tumors examined,⁵ and haploinsufficiency of *pten* was associated with early stage PCa.⁶ Additionally, loss of heterozygosity at the *pten* locus (and thereby loss of Pten expression) has been associated with increased Gleason score and poor clinical outcome.⁷ Mutations in *pten* may also serve as a molecular marker for metastatic PCa progression in humans,⁸ further supporting the hypothesis that *pten* is a clinically important PCa tumor suppressor gene.

In preclinical models of prostate disease, prostate intraepithelial neoplasia has been observed in mice deleted of candidate tumor suppressor genes, and combinatorial genetic manipulations allow for the accurate modeling of known human genetic lesions *in vivo* (reviewed in⁹). Mice harboring heterozygous deficiency at the *pten* locus (*pten*^{+/-}) displayed intermittent PIN with long latency.¹⁰ In some models where genetic modification induced PIN, but not adenocarcinoma, the extent of glandular involvement and PCa progression could be induced through the combination of *pten* haploinsufficiency and alterations in the function of key cell regulatory genes, such as *p27Kip1*¹⁰ or *nkx3.1*.¹¹ Pten haploinsufficiency has recently been shown to interact cooperatively with the overexpression of the mTOR regulatory protein Rheb, to induce PCa.¹² Additionally, the targeted homozygous ablation of *pten* induced latent PCa, which was dependent on the p110 β catalytic subunit of PI3K,^{13,14} Pten ablation in a *p53* knockout background resulted in the induction of early invasive PCa and the loss of cellular senescence,¹⁵ while modeling studies have further established that in FGF8b transgenic $\times$ *pten*^{+/-} mice where prostate cancer is seen, the expression from both *pten* alleles was lost.¹⁶

To better understand the role of Pten in regulating ErbB-2-induced tumorigenesis in the prostate epithelium, we analyzed the effect of alterations in Pten levels on ErbB-2 signaling both *in vitro* and *in vivo*. Herein, we demonstrate that the heterozygous loss of *pten* when integrated into the PB-ErbB-2 mouse model (PB-ErbB-2 $\times$ Pten^{+/-}) resulted in increased cyclin D1 and proliferating cell nuclear antigen (PCNA) nuclear positivity and decreased disease latency compared with either singly modified genetic model. Notably, the PB-ErbB-2 $\times$ Pten^{+/-} mice also developed prostate adenocarcinomas

while retaining Pten expression. Pten re-expression in the Pten-deficient prostate cancer cell line LNCaP inhibited ErbB-2-induced *cyclin D1* promoter activity. Mechanistically, modest activation of phosphoinositide-dependent kinase (PDK)1 (phosphorylated at S241) was observed in PIN lesions, and which was further increased in adenocarcinomas. In contrast, the combined activation of 70S6K (phosphorylated at T389) and inactivation of the eIF4E-binding protein-1 (4E-BP1, phosphorylated at pT37/46) was primarily restricted to those glands that had progressed to adenocarcinoma, interestingly however, activation of mTOR was not observed.

Collectively, these data indicate a role for Pten in the suppression of ErbB-2-induced prostate epithelial transformation through an inhibition of proteins that function downstream of PDK-1 that are involved in the regulation of cell proliferation and protein biosynthesis.

Materials and Methods

Generation of Compound Animals Used

The PB-ErbB-2 transgenic mice have been previously described.^{3,17} Briefly the minimal rat probasin promoter was used to drive prostate-specific expression of an activated ErbB-2 growth factor receptor.^{3,17} The *pten*^{+/-} mice, which harbor hemizygous inactivation of one *pten* allele and PB-CRE $\times$ Pten^{PC1} mice, which delete both *pten* alleles in the prostate epithelium as previously reported^{13,18} were kindly provided by Dr. Pier Paolo Pandolfi, Memorial Sloan-Kettering Cancer Center/Beth Israel Deaconess Medical Center. The compound-engineered PB-ErbB-2 $\times$ *pten*^{+/-} mice in an FVBN background resulted from the repeated cross-breeding of the *Pten*^{+/-} mice into the PB-ErbB-2 line for six or more generations. The genotypes were established as previously described.^{3,17,18} Male wild-type FVBN mice used in these studies were littermates to the genetically engineered animals.

Cell Culture

The human prostate cancer cell line, LNCaP, was maintained in RPMI with 10% fetal calf serum, 0.1 mmol/L non-essential amino acids, 100u/ml penicillin-streptomycin, and 1 mmol/L sodium pyruvate at 37°C in 5% CO₂ as previously described.^{3,19} For heregulin 1 β (HRG) stimulation studies, subconfluent (50%) LNCaP cells were placed in RPMI with 2.0% fetal calf serum, and HRG (R&D Systems, Minneapolis, MN) was added to a concentration of 1 ng/ml³. The chemical inhibitors PD98059 (30 μ mol/L), the PI3K inhibitor LY24002 (20 μ mol/L), the mTOR/raptor complex inhibitor, rapamycin (1 ng/ml) or vehicle (dimethyl sulfoxide) were added and the cells were cultured for an additional 30 minutes or 12 hours.

Plasmids

The cyclin D1 promoter construct and transfection methodologies have been previously described by our laborat-

ory.^{3,20–22} The pcDNA3 and pcDNA3-ErbB-2 expression vectors have been previously described.^{3,23} CMV5-Pten expresses the wild-type human Pten cDNA and was a generous gift from Dr. Todd Waldman.

Luciferase Assays

The co-transfection of reporter constructs and expression vector DNA was accomplished using Lipofectamine Plus or Lipofectamine 2000 (Invitrogen, Carlsbad, CA), following the manufacturers conditions. Luciferase activity was measured in a Bertold Autolumat 963 luminometer as previously described^{3,22} and was measured in arbitrary light units by calculating the light emitted during the initial 10 seconds of the reaction. Background activity from cell extracts was typically <100 arbitrary light units/10s. Co-transfection of Renilla luciferase (TK-renilla) was used to control for transfection efficiency.^{3,22} Plasmid concentrations of the ErbB-2 and Pten expression vectors used in the dose response curves were 0.22, 0.45 and 0.9 μ g per well. Statistical analyses were performed using the Student's *t*-test with significant differences established as at least $P \leq 0.05$ on $N \geq 3$ independent transfections. Data were plotted as average fold-induction $\pm$ SEM versus empty vector controls.^{3,22}

Flow Cytometry

LNCaP cells were collected by trypsinization, fixed in 10% citrate buffer and resuspended in PBS containing 20 mg/ml propidium iodide and 5U RNase A. DNA content measured using a FACStar Plus dual laser FACS sort system as previously described.^{3,21,22}

Western Blotting

Protein extracts were separated on 4% to 20% Tris-glycine gels and electro-blotted onto nitrocellulose.³ ErbB-2 expression levels were assessed using the antibody OP15 (Calbiochem). Induction of signal transduction cascades was assessed using antibodies against total- and phospho-AKT (Cell Signaling, S473, 9271; total, 9272), PDK1 (Abcam, S241, ab32800; total, ab31406), S6Kinase (Cell Signaling T389, 9205), and 4E-BP1 (Cell Signaling T37/46, 9644). Cyclin D1 protein levels were assessed using an anti-cyclin D1 polyclonal antibody, AB3 (NeoMarker).^{3,22} β -actin (Cell Signaling, 4967) was used as loading control.

Immunohistochemical Staining

Immunohistochemical staining was performed on prostate tissue using the following antibodies: PCNA (BD, 610664), Her2 (Calbiochem OP15),³ cyclin D1 (Neomarkers, AB3),³ Pten (Cell Signaling, 138G6),²⁴ phospho-70S6K T389 (Abcam, ab32359), phospho-PDK1 S241 (Abcam, ab32800), phospho-4E-BP1 (Cell Signaling, 2855), and phospho-mTOR (Cell Signaling, 2976). The slides were blocked for 20 minutes, and incubated over-

night at 4° with the primary antibody. Detection was performed using DakoCytomation kits (Dako, Carpinteria, CA). Statistical analyses were performed using the Student's *t*-test with significant differences established as at least $P \leq 0.05$.

Semiquantitative Image Analysis

Semiquantitative immunohistochemical analyses of anti-ErbB-2 or anti-Pten with diaminobenzidine (DAB)- and hematoxylin-stained sections were performed by multispectral analysis using a Nikon E600 upright microscope system fitted with a Nuance 2 spectral imaging system (CRI Inc, MA) running Nuance 2.4 software. To perform semiquantitative image analysis, individual spectral databases or "spectral libraries" for DAB and hematoxylin were generated using a $\times 60$ lens and transmitted light at wavelengths from 440 to 680 nm in 10-nm steps. Background staining data for DAB was established using prostate tissue slides incubated with the secondary antibody (in the absence of the primary antibody) followed by treatment with DAB. The Nuance software and spectral libraries were used to separate, or "unmix" the individual signals that represent DAB (antigen staining) and hematoxylin (nuclei). Quantification of the DAB staining per exposure (in milliseconds) was performed and the average $\pm$ SD for numerous regions of interest in multiple mice was calculated. Staining associated with ductal secretions or areas of the slide devoid of tissue were not used in the analyses.

Results

Heterozygous Loss of pten in PB-ErbB2 Mice Induces Adenocarcinoma in Vivo

Previously, we reported that PB-ErbB-2 transgenic mice develop widespread PIN within the dorsal prostate, dorsolateral prostate (DLP), and ventral prostate (VP), and that approximately 50% of the mice exhibited moderate to high-grade PIN, but no progression to adenocarcinoma by 18 months of age.³ In addition, we demonstrated that heterozygous deletion of the tumor suppressor gene, *pten* in the context of PB-ErbB-2 resulted in an increase in total prostate volume and an alteration in the prostatic choline to citrate ratio, as measured by magnetic resonance imaging and magnetic resonance spectroscopy, respectively, commensurate with induction of prostate disease.⁹ To more fully investigate the pathobiology of prostate disease in these models, comprehensive pathological and immunohistochemical analyses were performed on over 25 mice from the compound engineered (PB-ErbB-2 $\times$ *pten*) line. The latency of initiation of prostate disease in both the DLP and VP was found to be greatly reduced in the PB-ErbB-2 $\times$ *pten*^{+/-} model versus the singly modified PB-ErbB-2 model,³ with 100% of the animals presenting with prostate disease by 16 months of age. Importantly, adenocarcinomas of the DLP and VP were found in 15% of the PB-ErbB-2 $\times$ *pten*^{+/-} mice, some occurring as early as 8 months of

Figure 1. Pathology of dorsolateral prostate (DLP) and ventral prostate (VP) sections from (A, DLP) non-transgenic and (B, VP; C, VP) genetically modified mouse models.

age (Figure 1C). The *pten*^{+/-} mice in an FVB background, but lacking the PB-ErbB-2 transgene, primarily presented with sporadic, low-grade PIN (Figure 1, A–B).

Cyclin D1 and PCNA Levels Are Induced in the PB-ErbB-2 × *pten*^{+/-} Adenocarcinomas

We had previously shown that approximately 25% of the cells in PB-ErbB-2 PIN IV lesions stained for nuclear cyclin D1.³ Immunostaining for cyclin D1 performed on DLP and VP sections from the various mouse models (Figure 2A) revealed that cyclin D1 nuclear positivity was less than 5% in the nontransgenic (Figure 2A and Casimiro et al³) and less than 20% in the *pten*^{+/-} PIN lesions (not shown). In addition, there was a statistically significant increase in cyclin D1 nuclear positivity (30% ± 5%, *P* < 0.05) in the PB-ErbB-2 × *pten*^{+/-} adenocarcinoma samples versus *pten*^{+/-} or the previously reported PB-ErbB-2 PIN IV lesions. Immunostaining for the prolifera-

tion marker, PCNA revealed that 54% (± 12%) of the cells were moderately to strongly positive for nuclear PCNA in the PB-ErbB-2 × *pten*^{+/-} adenocarcinomas (Figure 2A) versus 11% (± 6) of cell in the PB-ErbB-2 × *pten*^{+/-} low-grade PIN lesions and 30% (± 5%) in PIN IV lesions (not shown). These data confirm that ErbB-2 signaling in the prostate epithelium is sensitive to *pten* genocopy number.

Our previous data in human prostate cancer cell lines established that the *cyclin D1* gene and promoter was induced by the p110α catalytic subunit of PI3Kinase²² and by ErbB-2.³ To evaluate the functional effect of Pten in regulating ErbB-2 induced cyclin D1 expression in LNCaP cells, the -1745 cyclin D1 luciferase reporter plasmid^{3,20} was cotransfected with activated CMV-ErbB-2, both with and without CMV-Pten, and luciferase activity was measured. Expression of ErbB-2 induced the -1745 cyclin D1 luciferase promoter approximately two-fold, and increasing amounts of Pten significantly inhib-

Figure 2. Pten regulates ErbB-2-induced proliferation markers *in vivo* and *in vitro*. **A:** Immunohistochemical staining for either cyclin D1 (left) or PCNA (right), performed on normal non-transgenic or PB-ErbB-2 × *pten*^{+/-} PCa ventral prostate tissue. **B:** Analysis of the effect of Pten rescue on activated ErbB-2 signaling to the cyclin D1 promoter in the Pten deficient cell line, LNCaP. The average fold change (±SD) in promoter activity versus CMV control transfections for *N* ≥ 3 separate experiments is shown **P* ≤ 0.05, ***P* ≤ 0.01.

Figure 3. Pten levels are reduced but not eliminated in adenocarcinomas. **A:** Bright field image (top) and unmixed pseudo-colored (bottom) images of Pten staining performed on normal and cancerous ventral prostate tissue. Blue staining in the unmixed images represent nuclei, red is Pten staining. **B:** Multispectral imaging data showing the individual spectral profiles of DAB and hematoxylin. **C:** Average ($\pm$ SD) Pten signal obtained using Nuance spectroscopy. PB-Cre $\times$ *pten*^{PC1} PCa tissue was used as a negative control for Pten staining.

ited its activity (Figure 2B). In reciprocal experiments, increasing amounts of ErbB-2 partially reversed the inhibition of -1745 cyclin D1 luciferase brought about by Pten overexpression (Figure 2B). Data are the mean $\pm$ SD of ≥ 3 separate experiments. Pten, therefore, is capable of abrogating ErbB2-mediated cyclin D1 promoter activity.

*Pten Expression Is Retained in PB-ErbB-2 $\times$ *pten*^{+/-} Induced Prostate Adenocarcinomas*

Our *in vivo* and *in vitro* data established that ErbB-2 signaling in the prostate is sensitive to changes in Pten. However since loss of *pten* expression was frequently required to induce adenocarcinomas in the mouse prostate,^{13,16} immunohistochemistry was performed on prostate sections to establish whether Pten expression was retained in the cancerous tissue. Low but detectable levels of Pten were seen both in the normal, non-transgenic prostate and PCa samples from PB-ErbB-2 $\times$ *pten*^{+/-} mice (Figure 3A). To better assess glandular Pten expression, absorbance levels were determined by spectral imaging analyses performed on the stained samples. The bright field images were subjected to multispectral analysis using a Nuance imaging module affixed to a Nikon E600 upright microscope. Individual spectra for hematoxylin and DAB were first acquired and used to "unmix" the DAB and hematoxylin staining (Figure 3, A and B). The unmixed spectra from multiple regions of interest in each sample (wild-type, *pten*^{+/-}, PB-ErbB-2 $\times$ *pten*^{+/-}, and PB-Cre $\times$ *pten*^{PC1}) were then converted to gray-scale and used for analysis. An example of the individual spectral tracings for DAB (red and brown), hematoxylin (blue) and intraductal non-specific staining and non-tissue areas (black) are shown (Figure 3B). The unmixed Pten/DAB spectroscopy data are shown (Figure 3C). Loss of one *pten* allele resulted in reduction in Pten staining, however Pten expression was

retained both in normal tissue as well as in PIN and cancerous lesions from the PB-ErbB-2 $\times$ *pten*^{+/-} mice (Figure 3C, lanes 1–5). PB-Cre $\times$ *pten*^{PC1} mice, which conditionally ablate *pten* in the prostate epithelium, were used as negative controls to adjust for non-specific staining of the primary antibody. The level of Pten staining in the PB-Cre $\times$ *pten*^{PC1} region of interest was at or near the lowest level of detection (Figure 3C, lane 6). In addition, immunohistochemistry for ErbB-2 was performed on prostate sections as previously described³ to assess whether ErbB-2 expression differed between the cancerous versus neoplastic tissue (not shown). Semi quantitative analysis using Nuance multispectral imaging demonstrated that no significant difference in ErbB-2 staining was seen ($P = 0.83$). These data demonstrate that transgene expression per se was not altered and a complete loss of Pten expression was not required for transformation of prostate epithelium in the PB-ErbB-2 $\times$ *pten*^{+/-} model.

PDK1 Signaling

Since PDK1 may serve as both a prognostic proliferation indicator and a potential therapeutic target in cancer, we investigated the levels of both total and activated (phospho-S244) PDK1 in our mouse models. As seen in Figure 4, PDK1 levels were detected in both the normal and transgenic epithelium (Figure 4A), however phospho-PDK1 not detected in the normal epithelium of both the non-transgenic and PB-ErbB-2 $\times$ *pten*^{+/-} mice (Figure 4B, left hand panels). Weak phospho-PDK1 staining was observed in 18% (± 4.45) of the epithelial cells within the PB-ErbB-2 $\times$ *pten*^{+/-} PIN lesions while strong immunopositivity was seen in 70% (± 3.95) of those in the PCa lesions, a statistically significant increase over PIN ($P < 0.001$) (Figure 4B, right panels).

Figure 4. PDK1 levels are increased during PCA initiation and progression. **A:** Total PDK1 and **(B)** phospho-PDK1 immunostaining of non-transgenic and PB-ErbB-2 × *Pten*^{+/-} DLP (**B top** and **bottom right**) and VP (**B bottom left**) sections.

Signal Transduction Activity Downstream of PDK1 in PCA

Both p70^{S6K} and 4E-BP1 are known regulators of cell growth and protein translation. Mechanistically, phosphorylation of these proteins regulates their functional affect on cell cycle progression and protein synthesis. Phosphorylation of p70^{S6K} by upstream kinases, including PDK1, activates p70^{S6K} while the inhibitory activity of the eIF4E regulatory protein 4E-BP1 is repressed by phosphorylation.²⁵ In breast cancer cells, ErbB-2 signaling has been found to affect the phosphorylation status of p70^{S6K} and 4E-BP1,²⁶ however little is known of ErbB-2's role in regulating p70^{S6K} and 4E-BP1 activity in the prostate epithelium.

Immunohistochemistry for phosphorylated-p70^{S6K} (p-p70^{S6K}) or phosphorylated-4E-BP1 (p-4E-BP1) was therefore performed on normal, PIN and PCA samples. Staining for p-p70^{S6K} was negligible in normal tissue (Figure 5, A and B) and was undetectable in low-grade PIN lesions from all genotypes (not shown). Levels of p-p70^{S6K} were marginally increased in the PB-ErbB-2 × *pten*^{+/-} in high-grade PIN IV lesions with 12% (±6.2%) of the cells staining positive. Conversely, p-p70^{S6K} was induced in the ErbB-2 × *pten*^{+/-} adenocarcinomas with 70% (±7%) of the cells in the lesions staining strongly positive (Figure 5, C and D). Similarly, p-4E-BP1 levels were increased in the PCA lesions of the PB-ErbB-2 × *pten*^{+/-} prostate, with 71% (±12%) of the cells staining strongly positive within the PCA, but not the adjacent normal epithelium, a statistically significant increase (*P* < 0.01) versus high-

grade PIN lesions, where diffuse, low level staining was observed in 8.2% (±6.3%) of the cells (Figure 5, E–H). These results strongly indicate that the reduction in *Pten* tumor suppressor function and the subsequent modulation of p70^{S6K} and 4E-BP1 activities were critical for the progression from an adenoma to PCA. Surprisingly, the levels of the phosphorylated mTOR (mammalian target of rapamycin), a known regulator of p70^{S6K} phosphorylation status and activity, were undetectable in the PB-ErbB-2 × *pten*^{+/-} adenocarcinomas (Figure 6 A–B), suggesting that mTOR per se may not be a key ErbB-2 target protein in the prostate epithelium. Activation of mTOR was observed throughout pheochromocytoma samples and PCA from the PB-Cre × *pten*^{PC1} mouse model (Figure 6, C–D).

To explore the possible mechanisms by which ErbB-2-induced signal transduction resulted in enhanced phosphorylation of p70^{S6K} and 4E-BP1, *in vitro* analyses were performed in LNCaP cells treated with HRG and either the ERK inhibitor, PD98059 (30 μmol/L), the PI3K inhibitor LY294002 (20 μmol/L), or the mTOR/raptor complex (mTORC1) inhibitor, rapamycin (1 ng/ml). The addition of LY294002 inhibited HRG-induced AKT, p70^{S6K}, and 4E-BP1 phosphorylation while PD98059 and rapamycin reduced phosphorylation levels to a lesser extent (Figure 7A). Cell cycle analyses established that both LY294002 and rapamycin were potent inhibitors of HRG-induced proliferation, while PD98059 was less effective in inhibiting HRG-induced cell cycle progression (Figure 7B).

Figure 5. p70^{S6K} and 4E-BP1 immunostaining. **Left** panels, phospho-p70^{S6K} staining in (A) Normal tissue from non-transgenic prostate, (B) Normal or (C) PB-ErbB-2 PIN. **D:** PCA lesions from PB-ErbB-2 $\times$ *pten*^{+/-} mice. **Right** panels, phospho-4E-BP1 staining in (E) normal tissue from non-transgenic mice, (F) normal, or (G) PB-ErbB-2PIN. **H:** PCA lesions from PB-ErbB-2 $\times$ *pten*^{+/-} prostate. Both DLP (D, E and H) and VP (A, B, C, F, and G) sections are shown.

Discussion

In this study, we found that Pten inhibited ErbB-2-induced prostate cancer cell proliferation *in vitro* while *in vivo*, the monoallelic loss of *pten* increased ErbB-2-induced cyclin D1 and PCNA nuclear positivity in the PB-ErbB-2 $\times$ *pten*^{+/-} model. Importantly, the PB-ErbB-2 $\times$ *pten*^{+/-} epithelium progressed to prostate adenocarcinoma. In addition, while PDK1 activity was moderately increased in PIN lesions and was further induced in prostate adenocarcinomas, the induction of p70^{S6K} and inhibition of

4E-BP1 occurred primarily in adenocarcinomas. Loss of Pten was not observed. We conclude therefore that a modest reduction in normal Pten function in the prostate epithelium creates a permissive environment for ErbB-2 to overcome intrinsic tumor-suppressive mechanisms downstream of PDK1. Surprisingly, mTOR was not induced in this model. We speculate that these Pten-sensitive mechanisms are involved in the suppression of both cell proliferation and translation initiation. Therefore, we conclude that within the context of enhanced ErbB-2 signaling, even modest reductions in Pten activity are sufficient to allow ErbB-2 to overcome the normal tumor-suppressive environment and promote prostate epithelial transformation (Figure 8).

De-repression of the PI3K signaling pathway plays a significant role in tumorigenesis. Investigations into the

Figure 6. mTOR activity in PCA. **A, B:** Immunostaining adenocarcinomas of the DLP (A, B, C, and D) and VP showing lack of mTOR activity. Immunostaining for phospho-mTOR in (C) mouse pheochromocytoma and (D) PB-Cre $\times$ *pten*^{PC1} DLP PCA tissue are shown as a positive controls.

Figure 7. Pathways of ErbB-2-induced signaling in human PCA cells. **A:** Short-term (30 minutes) effect of chemical inhibitors on protein phosphorylation by Western blotting. **B:** Effect of prolonged exposure (16 hours) to inhibitors on cell cycle progression in randomly cycling prostate cancer cells. Data are average $\pm$ SD $\geq$ 3 separate experiments **P* < 0.05, ***P* < 0.01.

Figure 8. Mechanisms of prevention of growth factor-induced prostate cancer by Pten, and loss thereof, *in vivo*. In the normal prostate epithelium, expression of ErbB-2 induces proliferation and hypertrophy, which over time results in PIN. Transformation is blocked in part through an inhibition of signaling downstream of PDK1. Since Pten functions in part to inhibit signaling downstream of ErbB-2, a reduction in Pten anti-tumor surveillance function allows for engaged PI3Kinase signaling by ErbB-2, inducing PDK1 and p70S6K signaling and inhibiting 4E-BP1, thereby driving transformation. We propose therefore that p70S6K and 4E-BP1 are key prostate-disease-inducing proteins whose activities are highly sensitive to modest changes in Pten. Phosphorylated proteins associated with either (*) PIN induction or (**) PCa transformation.

expression profiles of key proteins within this pathway in human prostate disease found that Pten levels were more frequently reduced in human PCa samples than in PIN. In clinical PCa samples, p-4E-BP1 levels have been found to be significantly elevated, and altered levels of PTEN, mTOR, and 4E-BP1 were reported as potential biomarkers of PCa progression,²⁷ while in the genetic knockout of *pten*, mTOR was induced (Figure 6 and as previously reported¹³). While increased p-4E-BP1 levels identified patients at high risk for progression from PIN to PCa,²⁸ a possible role for ErbB-2 in 4E-BP1 regulation was not established. In breast cancer, ErbB-2 signaling induces the synthesis of vascular endothelial growth factor and promotes metastases via mTOR and p70^{S6K},²⁹ and linkage of ErbB-2 to activation of the Akt/mTOR/4E-BP1 pathway may be a predictor of breast cancer progression.³⁰ In ovarian cancer cell lines, inhibition of ErbB-2 signaling by the interferon- γ -induced retinoid-inducible gene 1 resulted in a reduction in AKT phosphorylation and repression of mTOR.³¹ Our *in vivo* data are consistent with these previous studies and indicate that the progression from PIN to PCa in the PB-ErbB-2 $\times$ *pten*^{+/-} model correlates with increased PCNA and cyclin D1 nuclear positivity, increased levels of phosphorylated PDK1, and importantly, increased levels of p-p70^{S6K} and p-4E-BP1. Interestingly however, levels of p-mTOR remained low. While prostate specific expression of Rheb as been shown to facilitate PCa in *pten*^{+/-} through induction of mTOR,¹² the enhanced ErbB-2 signaling present in epithelium of our model may supersede the requirement for mTOR activation in the regulation of p70^{S6K} and p-4E-BP1 activity and prostate tumorigenesis. These findings may have important implications in the design of clinical trials of mTOR inhibitors for the treatment of advanced prostate cancer.

Cyclin D1 regulation can occur at the level of transcription, translation, and/or protein stability. We have previously shown that PI3K induced cyclin D1 promoter activity through an IKK α -dependent mechanism.²² While the percentage of the cells staining strongly positive for cy-

clin D1 was significantly increased in the prostate adenocarcinomas, versus the PIN lesions, the total number of D1 positive cells was less than that of the S-phase marker, PCNA. These data suggest that additional regulatory proteins may also be altered, such as the known prostate tumor suppressor protein p27KIP1, or downstream cyclins, such as cyclin E and cyclin A. Further investigations are underway to assess the levels these cell cycle regulatory proteins in the PIN versus PCa lesions, and how they correlate, if at all, with Pten levels and disease progression.

In MCF7 cells, cyclin D1 protein levels were modulated in part through the regulation of translation via p70^{S6K} and 4E-BP1.^{32,33} Conversely, in LAPC cells low levels of AKT induced, while high levels of AKT repressed, cyclin D1 (and *c-myc*) translation, despite the fact that p-p70^{S6K} and p-4E-BP1 levels were similar within the different AKT environments.³⁴ While the mechanisms responsible for the differential regulation of cyclin D1 translation were not defined, speculation emerged that the extensive GC polynucleotide tracts present in the 5' region of the cyclin D1 promoter may function as an AKT-sensitive internal ribosome entry sequences.³⁴ In the current studies, Pten rescue experiments performed in LNCaP cells demonstrated that Pten inhibited ErbB-2-induced cyclin D1 promoter-luciferase activity, raising the possibility that a component of the cyclin D1 induction may occur, in part, through alterations in translation. Since the 1745 cyclin D1 promoter-luciferase reporter construct we have developed²⁰ contains the entire 5' untranslated region of *cyclin D1* (from nucleotide +1 to nucleotide +133) putative IRES/poly-GC sequences, if they exist, should be contained within the promoter construct. This reporter plasmid is therefore an excellent molecular platform to investigate the possible effects of ErbB-2 and Pten activity on cyclin D1 translation.

Chemical inhibition of key components of the PI3-kinase and MAP-kinase signaling pathways *in vitro* indicated that general inhibition of PI3K signaling by LY294002 was most effective in reducing HRG-in-

duced AKT, p70^{S6K}, and 4E-BP1 phosphorylation, while both rapamycin and PD90859 were less effective in reducing levels of target-protein hyperphosphorylation. Conversely, the prolonged exposure of LNCaP cells to LY294002, rapamycin and PD90859 resulted in a significant inhibition of HRG-induced cell cycle progression. The results are consistent with our previous data^{3,22} and indicate that both the Erk pathway and PI3K pathways have distinct but perhaps overlapping roles in ErbB-2-regulated signal transduction. Genetic modeling in the mouse prostate has also established that potentially different roles for the PI3K catalytic subunits, p110 α vs p110 β , exist in Pten-induced PCa. Using Cre-induced ablation of *pten* and either p110 α or p110 β , AKT signaling and PCa were both repressed by the loss of p110 β but not p110 α , perhaps through differential integration of receptor signaling.¹⁴ Furthermore, since targeting both the MAPK and PI3K pathways blocks PCa in *nkx3.1*^{-/-} $\times$ *pten*^{+/-} mice following castration,³⁵ *in vivo* experiments are certainly warranted in our ErbB-2-based models to more clearly define the cross talk between the PI3K and MAPK signaling cascades as they relate to gene regulation, mRNA translation, PIN induction, and/or PCa progression. Overexpression of either the wild-type or a kinase-inactivated PDK1, both alone and in the context of the genetic models described herein, will further help define the contribution of PDK1 -dependent and -independent signaling intermediary proteins in PCa progression. Additional experiments will also be required to assess the mechanism by which the PB-ErbB-2 $\times$ *pten*^{+/-} mice evade the requirement for mTOR activation during tumorigenesis, and the effect that enhanced ErbB-2 signaling may have on other components of the eIF4 translation complex as they relate to PCa progression.

Since both ErbB-2 and cyclin D1 can be regulated at the level of translation initiation,³⁶ our studies provide additional support for the development of novel therapeutics that target tumor-restricted signaling that is associated with PCa progression. The PB-ErbB-2 $\times$ *pten*^{+/-} engineered mice described herein represent an important preclinical *in vivo* platform for detailed investigations into the role of these (and other) pathways in prostate cancer initiation and progression.

Acknowledgments

We thank Dr. Pier Paolo Pandolfi for the genetically modified *pten* mice. Fluorescence-activated cell sorting analyses were performed in the Lombardi Comprehensive Cancer Center's Flow Cytometry and Cell Sorting Shared Resource; microscopy was performed in the Lombardi Comprehensive Cancer Center's Microscopy and Imaging Shared Resource, while mouse tissue embedding, tissue sectioning and prostate pathology were performed in the Lombardi Comprehensive Cancer Center's Histology and Tissue Shared Resource.

References

- Shi Y, Chatterjee SJ, Brands FH, Shi SR, Pootrakul L, Taylor CR, Datar R, Cote RJ: Role of coordinated molecular alterations in the development of androgen-independent prostate cancer: an *in vitro* model that corroborates clinical observations. *BJU Int* 2006, 97:170–178
- Dai B, Kong YY, Ye DW, Ma CG, Zhou XY, Yao XD: Human epidermal growth factor receptor type 2 protein expression in Chinese metastatic prostate cancer patients correlates with cancer specific survival and increases after exposure to hormonal therapy. *Asian J Androl* 2008, 10:701–709
- Casimiro MC, Rodriguez O, Pootrakul L, Fricke ST, Ostrowski M, Rabbani S, Datar R, Cote RJ, Pestell R, Albanese C: ErbB-2 induces the cyclin D1 gene in prostate epithelial cells *in vitro* and *in vivo*. *Cancer Res* 2007, 67:4364–4372.
- Cantley LC, Neel BG: New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci USA* 1999, 96:4240–4245
- Whang YE, Wu X, Suzuki H, Reiter RE, Tran C, Vessella RL, Said JW, Isaacs WB, Sawyers CL: Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression. *Proc Natl Acad Sci USA* 1998, 95:5246–5250
- Di Cristofano A, Pandolfi PP: The multiple roles of PTEN in tumor suppression. *Cell* 2000, 100:387–390
- McMenamin ME, Soung P, Perera S, Kaplan I, Loda M, Sellers WR: Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage. *Cancer Res* 1999, 59:4291–4296
- Rubin MA, Gerstein A, Reid K, Bostwick DG, Cheng L, Parsons R, Papadopoulos N: 10q23.3 loss of heterozygosity is higher in lymph node-positive (pT2–3,N+) versus lymph node-negative (pT2–3,N0) prostate cancer. *Hum Pathol* 2000, 31:504–508
- Albanese C, Rodriguez O, Johnson MD, Fricke S: Models of prostate cancer. *Drug Discov Today: Disease Models* 2005, 2:7–13
- Di Cristofano A, De Acetis M, Koff A, Cordon-Cardo C, Pandolfi PP: Pten and p27KIP1 cooperate in prostate cancer tumor suppression in the mouse. *Nat Genet* 2001, 27:222–224
- Abate-Shen C, Banach-Petrosky WA, Sun X, Economides KD, Desai N, Gregg JP, Borowsky AD, Cardiff RD, Shen MM: Nkx3.1; Pten mutant mice develop invasive prostate adenocarcinoma and lymph node metastases. *Cancer Res* 2003, 63:3886–3890
- Nardella C, Chen Z, Salmena L, Carracedo A, Alimonti A, Egia A, Carver B, Gerald W, Cordon-Cardo C, Pandolfi PP: Aberrant Rheb-mediated mTORC1 activation and Pten haploinsufficiency are cooperative oncogenic events. *Genes Dev* 2008, 22:2172–2177
- Trotman LC, Niki M, Dotan ZA, Koutcher JA, Cristofano AD, Xiao A, Khoo AS, Roy-Burman P, Greenberg NM, Dyke TV, Cordon-Cardo C, Pandolfi P: Pten dose dictates cancer progression in the prostate. *PLoS Biol* 2003, 1:E59
- Jia S, Liu Z, Zhang S, Liu P, Zhang L, Lee SH, Zhang J, Signoretti S, Loda M, Roberts TM, Zhao JJ: Essential roles of PI(3)K-p110beta in cell growth, metabolism and tumorigenesis. *Nature* 2008, 454:776–779
- Chen Z, Trotman LC, Shaffer D, Lin HK, Dotan ZA, Niki M, Koutcher JA, Scher HI, Ludwig T, Gerald W, Cordon-Cardo C, Pandolfi PP: Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* 2005, 436:725–730
- Zhong C, Saribekyan G, Liao CP, Cohen MB, Roy-Burman P: Cooperation between FGF8b overexpression and PTEN deficiency in prostate tumorigenesis. *Cancer Res* 2006, 66:2188–2194
- Fricke S, Rodriguez O, Vanmeter J, Dettin L, Casimiro M, Chien C, Newell T, Johnson K, Ileva L, Johnson MD, Albanese C: *In vivo* magnetic resonance volumetric and spectroscopic analysis of mouse prostate cancer models. *Prostate* 2006, 66:708–717
- Di Cristofano A, Pesce B, Cordon-Cardo C, Pandolfi PP: PTEN is essential for embryonic development and tumour suppression. *Nat Genet* 1998, 19:348–355
- Rodriguez O, Fricke S, Chien C, Dettin L, Vanmeter J, Shapiro E, Dai HN, Casimiro M, Ileva L, Dagata J, Johnson MD, Lisanti MP, Koretsky A, Albanese C: Contrast-enhanced *in vivo* imaging of breast and prostate cancer cells by MRI. *Cell Cycle* 2006, 5:113–119
- Albanese C, Johnson J, Watanabe G, Eklund N, Vu D, Arnold A, Pestell RG: Transforming p21^{ras} mutants and c-Ets-2 activate the

- cyclin D1 promoter through distinguishable regions. *J Biol Chem* 1995, 270:23589–23597
21. Albanese C, D'Amico M, Reutens AT, Fu M, Watanabe G, Lee RJ, Kitsis RN, Henglein B, Avantaggiati M, Somasundaram K, Thimmappaya B, Pestell RG: Activation of the cyclin D1 gene by the E1A-associated protein p300 through AP-1 inhibits cellular apoptosis. *J Biol Chem* 1999, 274:34186–34195
22. Albanese C, Wu K, D'Amico M, Jarrett C, Joyce D, Hughes J, Hulit J, Sakamaki T, Fu M, Ben-Ze'ev A, Bromberg JF, Lamberti C, Verma U, Gaynor RB, Byers SW, Pestell RG: IKK α regulates mitogenic signaling through transcriptional induction of cyclin D1 via Tcf. *Mol Biol Cell* 2003, 14:585–599
23. Kwong KY, Hung MC: A novel splice variant of HER2 with increased transformation activity. *Mol Carcinog* 1998, 23:62–68
24. Lai EW, Rodriguez OC, Aventian M, Cromelin C, Fricke ST, Martiniova L, Lubensky IA, Lisanti MP, Picard KL, Powers JF, Tischler AS, Pacak K, Albanese C: ErbB-2 induces bilateral adrenal pheochromocytoma formation in mice. *Cell Cycle* 2007, 6:1946–1950
- 25.ingar DC, Richardson CJ, Tee AR, Cheatham L, Tsou C, Blenis J: mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factor 4E. *Mol Cell Biol* 2004, 24:200–216
26. Kim KK, Lee JJ, Yang Y, You KH, Lee JH: Macrophage inhibitory cytokine-1 activates AKT and ERK-1/2 via the transactivation of ErbB2 in human breast and gastric cancer cells. *Carcinogenesis* 2008, 29:704–712
27. Kremer CL, Klein RR, Mendelson J, Browne W, Samadzadeh LK, Vanpatten K, Highstrom L, Pestano GA, Nagle RB: Expression of mTOR signaling pathway markers in prostate cancer progression. *Prostate* 2006, 66:1203–1212
28. Armengol G, Rojo F, Castellvi J, Iglesias C, Cuatrecasas M, Pons B, Baselga J, Ramon y Cajal S: 4E-binding protein 1: a key molecular “funnel factor” in human cancer with clinical implications. *Cancer Res* 2007, 67:7551–7555
29. Klos KS, Wyszomierski SL, Sun M, Tan M, Zhou X, Li P, Yang W, Yin G, Hittelman WN, Yu D: ErbB2 increases vascular endothelial growth factor protein synthesis via activation of mammalian target of rapamycin/p70S6K leading to increased angiogenesis and spontaneous metastasis of human breast cancer cells. *Cancer Res* 2006, 66:2028–2037
30. Zhou X, Tan M, Stone Hawthorne V, Klos KS, Lan KH, Yang Y, Yang W, Smith TL, Shi D, Yu D: Activation of the Akt/mammalian target of rapamycin/4E-BP1 pathway by ErbB2 overexpression predicts tumor progression in breast cancers. *Clin Cancer Res* 2004, 10:6779–6788
31. Ou CC, Hsu SC, Hsieh YH, Tsou WL, Chuang TC, Liu JY, Kao MC: Downregulation of HER2 by RIG1 involves the PI3K/Akt pathway in ovarian cancer cells. *Carcinogenesis* 2008, 29:299–306
32. Averous J, Fonseca BD, Proud CG: Regulation of cyclin D1 expression by mTORC1 signaling requires eukaryotic initiation factor 4E-binding protein 1. *Oncogene* 2008, 27:1106–1113
33. MacManus CF, Pettigrew J, Seaton A, Wilson C, Maxwell PJ, Berlingeri S, Purcell C, McGurk M, Johnston PG, Waugh DJ: Interleukin-8 signaling promotes translational regulation of cyclin D in androgen-independent prostate cancer cells. *Mol Cancer Res* 2007, 5:737–748
34. Gera JF, Mellinghoff IK, Shi Y, Rettig MB, Tran C, Hsu JH, Sawyers CL, Lichtenstein AK: AKT activity determines sensitivity to mammalian target of rapamycin (mTOR) inhibitors by regulating cyclin D1 and c-myc expression. *J Biol Chem* 2004, 279:2737–2746
35. Kinkade CW, Castillo-Martin M, Puzio-Kuter A, Yan J, Foster TH, Gao H, Sun Y, Ouyang X, Gerald WL, Cordon-Cardo C, Abate-Shen C: Targeting AKT/mTOR and ERK MAPK signaling inhibits hormone-refractory prostate cancer in a preclinical mouse model. *J Clin Invest* 2008, 118:3051–3064
36. De Benedetti A, Graff JR: eIF-4E expression and its role in malignancies and metastases. *Oncogene* 2004, 23:3189–3199