3-Phosphoinositide–Dependent Kinase 1 Potentiates Upstream Lesions on the Phosphatidylinositol 3-Kinase Pathway in Breast Carcinoma

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Abstract

Lesions of ERBB2, PTEN, and PIK3CA activate the phosphatidylinositol 3-kinase (PI3K) pathway during cancer development by increasing levels of phosphatidylinositol-3,4,5-triphosphate (PIP_3). 3-Phosphoinositide-dependent kinase 1 (PDK1) is the first node of the PI3K signal output and is required for activation of AKT. PIP_3 recruits PDK1 and AKT to the cell membrane through interactions with their pleckstrin homology domains, allowing PDK1 to activate AKT by phosphorylating it at residue threonine-308. We show that total PDK1 protein and mRNA were overexpressed in a majority of human breast cancers and that 21% of tumors had five or more copies of the gene encoding PDK1, *PDPK1*. We found that increased *PDPK1* copy number was associated with upstream pathway lesions (*ERBB2* amplification, PTEN loss, or *PIK3CA* mutation), as well as patient survival. Examination of an independent set of breast cancers and tumor cell lines derived from multiple forms of human cancers also found increased PDK1 protein levels associated with such upstream pathway lesions. In human mammary cells, PDK1 enhanced the ability of upstream lesions to signal to AKT, stimulate cell growth and migration, and rendered cells more resistant to PDK1 and PI3K inhibition. After orthotopic transplantation, PDK1 overexpression was not oncogenic but dramatically enhanced the ability of ERBB2 to form tumors. Our studies argue that PDK1 overexpression and increased *PDPK1* copy number are common occurrences in cancer that potentiate the oncogenic effect of upstream lesions on the PI3K pathway. Therefore, we conclude that alteration of PDK1 is a critical component of oncogenic PI3K signaling in breast cancer. [Cancer Res 2009;69(15):6299–306]

Introduction

ERBB2/HER2/Neu (human epidermal growth factor receptor 2), *PTEN* (phosphatase and tensin homologue deleted on chromosome ten), and *PIK3CA* (encodes the p110α subunit of phosphatidylinositol 3-kinase [PI3K]) frequently contribute to breast carcinoma (BC) progression through their ability to regulate the intracellular level of phosphatidylinositol-3,4,5-triphosphate (PIP_3; refs. 1–5). 3-Phosphoinositide-dependent kinase 1 (PDK1), a serine threonine kinase known as the master AGC [cyclic AMP dependent, cyclic guanosine 3′,5′-monophosphate dependent, and protein kinase C] kinase, activates the catalytic domain of over 20 other kinases by phosphorylating their T-loops (6). PDK1 is the first node of the PI3K signal output and is required for activation of AKT (protein kinase B, PKB), S6K (p70 ribosomal S6 kinase), and RSK (p90 ribosomal S6 kinase) *in vivo* (7). PDK1 kinase activity is constitutive with regulation typically occurring through phosphorylation of the substrate hydrophobic pocket by other kinases (8–10). In the case of AKT, the interaction of the pleckstrin homology (PH) domain of AKT with membrane bound PIP_3 confers a conformational change in AKT, which allows PDK1 to phosphorylate AKT at residue threonine-308 (T-308; refs. 11–13).

Although the roles of many individual PDK1 substrates remain to be defined, the oncogenic activity of aberrant PI3K pathway signaling through PDK1 to AKT has been extensively validated. Murine Akt was originally isolated as an oncogene (14), and human AKT isoforms are altered in tumors (genomic amplification of *AKT2* in pancreatic and ovarian cancer and activating PH domain mutations of *AKT1* in BC and *AKT3* in melanoma; refs. 15–17). AKT has many substrates that define its diverse oncogenic outputs from cell growth and survival to angiogenesis, migration, and invasion (18). Targeting AKT1 and AKT2 in tumor cell lines with a small molecule inhibitor has a profound antitumor effect when *PIK3CA* is mutated or *ERBB2* is amplified (19).

PDK1 is oncogenic in the Comma-1D immortal murine mammary cell model, but its role in human cancers is yet to be fully elucidated (20, 21). Its oncogenic effect in mice seems to function via the PI3K pathway, because $Pten^{+/-}$ tumor formation was severely attenuated when bred with *Pdk1* hypomorphic mice

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

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with 10% of normal Pdk1 enzyme (22). Two previous reports suggested increased phosphorylated PDK1 protein levels in the majority of human BCs, both by immunohistochemistry (IHC) analysis with a phosphorylated-specific antibody (21, 23), yet the significance of this overexpression is unclear.

We have found that total PDK1 is overexpressed in a large proportion of human BCs and have found that many harbor an increased copy number (ICN) of the gene encoding PDK1, *PDPK1*. Hypothesizing that PDK1 could amplify the PI3K signal output, we discovered that increased PDK1 was associated with PI3K pathway lesions (ERBB2/PTEN/PI3K) in a highly annotated set of human sporadic BCs (24). This notion was further validated in human mammary cell lines wherein increased PDK1 in multiple settings of upstream activation [due to ERBB2 or *PIK3CA* mutation or PTEN RNA interference (RNAi)] enhanced AKT activation and rendered some cell lines less sensitive to both PDK1 and PI3K inhibition. PDK1 overexpression was insufficient to promote tumor growth of orthotopically transplanted human mammary epithelial MCF10A cells, but dramatically enhanced the tumor growth and invasion of cells overexpressing ERBB2. We thus propose a model in which coincident lesions with PDK1 overexpression on the same signaling pathway enhance PI3K signaling to promote cellular transformation and postulate that PDK1 expression levels may alter the efficacy of PI3K pathway–targeted cancer therapy.

Materials and Methods

Patient samples. BC samples were obtained from the Columbia University Tumor Bank (Columbia cohort) in accordance with institutional review board approval. Tissue microarrays were created from 172 unique BCs and 78 corresponding normal breast tissues with three cores embedded per sample.

Plasmids. *PDPK1* sequence was PCR amplified from p-FAST-BAC-myc-PDK1 (from Dr. Dario Alessi, University of Dundee) with primers 5′-CGCGTCGACGCCAGGACCACCAGCCAGCT and 5′-GCGGCCGCCTGCA-CAGCGGCGTCCGGG and cloned into XhoI-NotI sites of pOZ-FH-N (25). pBABE-NeuT was obtained from Dr. Nancy Hynes at the Friedrich Miescher Institute.

IHC. PDK1 staining was on paraffin sections [PKB kinase (E-3) Santa Cruz, 1:300] microwave antigen retrieval in citrate, detected by EnVision+ (Dako). The PDK1 IHC score was determined by fraction of cells showing cytoplasmic staining (0–1) multiplied by staining intensity rated from 0 to 6 to give a score from 0 to 6. Both BC and nonneoplastic breast epithelium was separately evaluated. PTEN IHC was performed as described (24) with the following modifications: PTEN antibody (Cell Signaling, 138G6) 1:200, microwave retrieval in Target Retrieval Solution pH 9 (Dako), and signal detection using EnVision+ (Dako).

Fluorescence *in situ* hybridization and chromogenic *in situ* hybridization. A BAC clone (RP11-67B18) spanning *PDPK1* gene was obtained from BACPAC Resources.[13] A green-labeled CEP 16 probe (Abbott Molecular) was used for chromosome 16. A case was considered to have ICN for *PDPK1* if at least 25% of cells contained greater or equal to five copies. *ERBB2* chromogenic *in situ* hybridization (CISH) was performed as described (26).

Tissue culture. Phoenix-ampho cells for retrovirus production were provided by Dr. Gary Nolan, Stanford University. After transfection, the virus was stabilized with fetal bovine serum (FBS; 6:1 v/v viral supernatant/FBS) and passed through a 0.45-μm filter. Morphogenesis assay performed as described for MCF10A (27). Cells were fed on days 3, 5, and 7. Pictures were taken, and cells were harvested on day 16.

[13] http://bacpac.chori.org

Immunoblotting. Whole-cell lysates were used in immunoblots. Antibodies were from Cell Signaling, except for PDK1 (Upstate 06-906), PDK1 (BD Biosciences 611070) or PKB kinase (E-3; Santa Cruz sc-17765), β-tubulin (clone Tu27, Covance), PTEN (6H2.1, Cascade Bioscience), and c-Neu (Ab-3, Calbiochem).

Migration assay. Cells (8×10^4) in assay media (DMEM/F12, 0.5% horse serum, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 μg/mL insulin, penicillin/streptomycin) were placed in the upper chambers of 8-μm 24-well Transwell cell culture plates (Costar), and the assay was performed as described (27).

AKT RNAi migration. MCF10A cells with and without stable overexpression of PDK1 were infected with short hairpin RNA (shRNA) lentiviral vectors targeting AKT1 and/or AKT2 or empty pLKO.1 control vector (AKT1 hairpin sequences: sense, 5′-CCGGgagtttgagtacctgaagctgCTCGAGcagctt-caggtactcaaactcTTTTTG-3′ and antisense, 5′-AATTCAAAAAgagtttgagtacct-gaagctgCTCGAGcagcttcaggtactcaaactc-3′; AKT2 hairpin sequences: sense, 5′-CCGGgcgtggtgaatacatcaagacCTCGAGgtcttgatgtattcaccacgcTTTTTG-3′; antisense, 5′-AATTCAAAAAgcgtggtgaatacatcaagacCTCGAGgtcttgatgtatt-caccacgc-3′). At 48 h after infection, Transwell migration assays were performed. Relative migration of MCF10A cells is expressed as the ratio of the number of cells that migrated to the lower surface of the membrane over that of control (cells infected with pLKO.1 vector).

Mouse studies. Animal procedures were performed in compliance with Columbia University Institutional Animal Care and Use Committee within Institute of Comparative Medicine. Seven-week-old SCID/NCr (BALB/c background) mice (NCI catalogue number 01S11) were injected s.c. with 1.5×10^6 into inferior mammary fat pad. Mice were monitored daily for general health and tumor growth. Mice were sacrificed 6 mo after injection or when tumors reached a surface area of 1 cm^2, as measured by caliper (surface area of tumor = length × width).

Protein lysate array. As described previously (28, 29), interrogating total PDK1 (Cell Signaling 3062) and PDK1 phosphorylated on residue serine 241 (PDK1pS241; Cell Signaling 3061).

PDK1 shRNA. The shRNA lentiviral particles targeting PDK1 (SHVRS-NM_002613) and nontarget shRNA control transduction particles (SHC002V) were purchased from Sigma-Aldrich. The shRNA transductions were performed as per manufacturer's instructions. Two separate clones were used:

TRCN0000039779 (PDK1-1): ccggcgaagatgagaagaggttgttctcgagaa-caacctcttctcatcttcgtttttg.
TRCN0000039782 (PDK1-2): ccggcaaagttctgaaaggtgaaatctcgagatttcaccttt-cagaactttgtttttg.
Nontarget control (CTRL): ccggcaacaagatgaagagcaccaactcgagttggtgctctt-catcttgttgttttt.

Proliferation and IC_{50} assays. Cells ($3–5 \times 10^3$) were plated on 48-well dishes and left at room temperature for 1 h before 37°C incubation. At 12 to 16 h later, cells were washed and media was changed. At the day of harvest, cells were stained with 0.05% crystal violet in 10% formalin, washed, and incubated with 10% acetic acid before a 590-nm absorbance was measured (BIO-TEK). Curve fit (using XLfit4) with model 205 with parameters *A* and *B* locked at 0 and 100, respectively.

Statistics. We compared clinical and pathologic tumor characteristics and their association with increased PDPK1 copy number (≥5 copies and ≤4 copies) using χ^2 test (and Fisher's exact test when sample counts were small). To test the distribution differences displayed via box plot, the Mann-Whitney test was used.

Results

PDK1 is overexpressed with increased genetic copy number in human BC. Because PDK1 is overexpressed in many human BC cell lines (20), we evaluated total PDK1 expression levels by IHC in a set of human BC samples (Fig. 1*A*). Although there was variation among cases in the level of PDK1 staining in nonneoplastic breast epithelium, we found that membranous and cytoplasmic PDK1 staining was significantly higher in BC cells than in adjacent

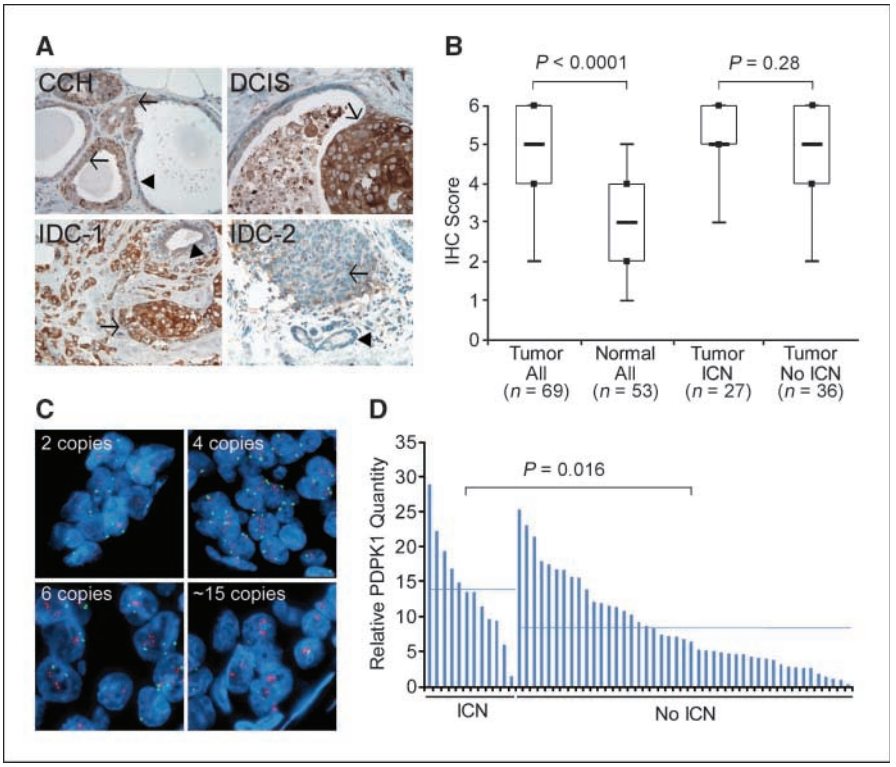

Figure 1. PDK1 is overexpressed with increased genetic copy number in human BC. *A,* IHC staining for PDK1: columnar cell hyperplasia (CCH, 20×) with moderate PDK1 expression (*arrow*) adjacent to normal epithelium (*arrowhead*) reflects variable expression of PDK1 within nonneoplastic duct epithelium, DCIS (40×) with increased PDK1 expression (*arrow*), invasive ductal carcinoma (*IDC-1*, 40×) with irregular cords of tumor cells overexpressing PDK1 (*arrow*) and adjacent normal duct (*arrowhead*). IDC-2 (40×) with low level of PDK1 expression (*arrow*) and adjacent normal duct (*arrowhead*). *B,* box plot showing PDK1 IHC score distribution between BCs versus adjacent normal ducts, as well as in tumors with *PDPK1* ICN (≥5 copies) and tumors without *PDPK1* ICN (≤4 copies). *C,* interphase FISH for *PDPK1* (*red*) with centromere chromosome 16 control (*green*) and indicated number of *PDPK1* copies. *D,* quantitative reverse transcription-PCR for *PDPK1* mRNA correlated with ICN (by FISH), comparing ICN cases (relative message quantity average, 14.0; *horizontal line*) compared with cases without ICN (average, 8.6; *horizontal line*) among BCs with at least 50% tumor density as determined by H&E staining (n = 57, Spearman coefficient of rank correlation = 0.321, P = 0.016, 95% CI = 0.066–0.537).

normal duct cells (P < 0.0001; Fig. 1*B*). Overall, increased PDK1 protein levels (defined as an IHC score of ≥5) were observed in 72% (50 of 69) of the cases. The specificity of the antibody was tested both by immunoblot and IHC of paraffin-embedded cells with RNAi knockdown of PDK1 (Supplementary Fig. S1*A* and *B*).

To test the hypothesis that the increase in PDK1 expression was due to increased gene copy number, we performed interphase fluorescence *in situ* hybridization (FISH; Fig. 1*C*). We found that 21% (27 of 129) of BCs had at least five copies of *PDPK1*, which we define as ICN. On average the ICN cases had seven copies of *PDPK1*, over a 3-fold increase above normal tissue (which always had two copies), and a 2-fold increase over the average number of chromosome 16 centromere copies. Although *PDPK1* ICN cases had increased PDK1 expression above that of normal ducts, they had only a slightly higher IHC score distribution than low copy number tumor cases (≤4 copies), indicating that ICN is only one mechanism of PDK1 overexpression (Fig. 1*B*). *PDPK1* ICN was confirmed by Southern blot (Supplementary Fig. S1*D*), in which 10 of 49 cases (20%) showed an increased signal, consistent with the frequency of ICN by FISH. Of the 24 cases in which we also had FISH data, three of four ICN cases gave an increased Southern signal, whereas only 2 of 20 cases without ICN did (P = 0.02). We also sequenced the *PDPK1* gene in 124 human BCs and found one somatic mutation (P340A). This low mutation rate is similar to that found in human colon cancers, and its significance is unclear (30).

Previous CGH studies found gains of 16p in ~40% of BCs (31, 32), with 16p13.3 (containing *PDPK1*) being the third most (57%) amplified region in invasive BCs (33). Using whole genome single-nucleotide polymorphism mapping, we found that the distribution of tumors with *PDPK1* ICN generally clustered within two separate groups, those with $16p^{+}/16q^{-}$ and those with many scattered amplicons throughout all of chromosome 16 (Supplementary Fig. S2*A*). We identified one tumor (case no. 432) with a relatively narrow amplicon containing ~85 genes (Supplementary Figs. S2*A, B* and S3). Expression mapping of this region showed 11 genes (including *PDPK1*) with at least a 3-fold increase in expression compared with control (dual channel) and at least a 10-fold increase in expression compared with the median of all genes in the sample (single channel; Supplementary Fig. S2*B*). A comprehensive genome-wide analysis of both copy number and message identified six genes (*NME3, GFER, E4F1, PDPK1, TCEB2*, and *HCFC1R1*) within this same region that had a strong correlation between copy number and message (34). Of these six genes, *PDPK1* had the strongest correlation (0.58) and lowest P value (<0.00012) and only *PDPK1* and *TCEB2* are found within the single-nucleotide polymorphism array amplicon peak of case 432 (Supplementary Fig. S2*B*). Given the more common broad amplicon in 16p (Supplementary Figs. S2*A* and S3), *PDPK1* is at least one of possibly several genes whose ICN drives increased expression.

Although there were a large number of tumors with increased PDK1 protein levels in the absence of *PDPK1* ICN, there was a significant correlation with *PDPK1* ICN and PDK1 mRNA (P = 0.0161; Fig. 1*D*). Employing protein lysates from fresh frozen tissue, we found that PDK1 levels are varied in human BC with a high level of overexpression in the two *PDPK1* ICN cases tested (Supplementary Fig. S1*E*). In addition, increased *PDPK1* copy number was associated with decreased patient survival [hazard ratio (HR) = 3.14, 95% confidence interval (95% CI) = 1.3–7.6, P = 0.04] independent of age at diagnosis and stage of disease (Supplementary Fig. S4). This association did not appreciably change when further adjusted for hormone receptor status, tumor ploidy, and race (HR = 3.30, 95% CI = 1.3–8.1). *PDPK1* ICN itself was not associated with hormone status (Supplementary Table S1) or basal cytokeratin expression (data not shown).

Table 1. Association of *PDPK1* copy number with upstream PI3K pathway lesions in breast tumors (Columbia cohort)

Pathway lesion	Total with lesion (%)	No ICN ≤ 4 copies (%)	ICN ≥ 5 copies (%)	*P*
ERBB2 amplification (CISH)	19 (16)	14 (14)	5 (22)	0.36*
PTEN loss (IHC)	22 (24)	16 (21)	6 (38)	0.20*
PIK3CA mutation	21 (22)	17 (22)	4 (22)	1.00*
ERBB2 or PTEN	39 (41)	29 (37)	10 (59)	0.10
ERRB2 or *PIK3CA*	35 (36)	26 (33)	9 (50)	0.16
PTEN or *PIK3CA*	42 (45)	32 (42)	10 (63)	0.13
ERBB2 or PTEN or *PIK3CA*	54 (57)	40 (51)	14 (82)	0.02 †

*P value was from Fisher's exact test; all other P values were from χ^2 test.
†$P < 0.05$ denoting significant enrichment in the number of breast cancers with *PDPK1* ICN (≥5 copies) among those with upstream PI3K pathway activating lesions (as noted) and the remainder of cases.

Increased PDK1 is associated with upstream PI3K pathway activation. To test the relationship of *PDPK1* ICN to known oncogenes and tumor suppressors that regulate AKT activation, we compared the pattern of *PDPK1* ICN with *PIK3CA* mutations (24), PTEN loss (scored by IHC; ref. 24), and *ERBB2* amplification (measured by CISH; ref. 26). At least one of these three lesions was found in 57% of BCs (Table 1). Importantly, there was an enrichment of *PDPK1* ICN cases among those with at least one of these upstream activators (82% with an upstream activator versus 51% without, $P = 0.02$). This concept that *PDPK1* gain correlated with a second hit on the pathway was validated using protein lysate arrays on a diverse set of 223 cancer cell lines and an independent set of 478 BCs (Table 2) in which both total and phosphorylated S241–specific PDK1 protein levels were measured. Increased PDK1 protein expression (ΔPDK1) was found in BCs with either *ERBB2* amplification or *PIK3CA* mutation compared with tumors without either of these lesions. In cancer cell lines, the relationship was again upheld with increased PDK1 levels found coincident with *ERBB2* amplification, *PIK3CA* mutation, or *PTEN* mutation, suggesting that this relationship may be present in other tumor types. Even better correlations with upstream events were observed for phosphorylated S241 PDK1. A strong association was found between the measurements of total PDK1 and phosphorylated S241–specific PDK1 protein levels in both the tumors ($r = 0.57$) and cell lines ($r = 0.95$) consistent with previous reports of efficient S241 autophosphorylation of PDK1 expressed in bacteria (10) and of increased phosphorylated S241–specific PDK1 protein levels in BCs (21, 23).

Increased PDK1 potentiates AKT signaling in the setting of upstream PI3K pathway activation. Human breast epithelial cell line MCF10A, immortalized in part through loss of the INK4/ARF locus (35), has been extensively used to validate BC oncogenes (27). To determine whether PDK1 overexpression could alter ERBB2-induced signaling, a set of four MCF10A cell lines were created from pools of cells infected with retrovirus containing the open reading frame for *PDPK1* (+PDK1), the gene of the activated mutant rat homologue of *ERBB2* (+NeuT; ref. 36), both

Table 2. Association of PDK1 protein expression with upstream PI3K pathway lesions

Pathway lesion	Total with lesion (%)	ΔPDK1* total (*P*)	ΔPDK1* p-S241 (*P*)
Cancer cell lines			
ERBB2 amplification	6 (3)	0.00 (0.973)	0.10 (0.222)
PTEN mutation	54 (24)	0.08 (0.069)	0.06 (0.221)
PIK3CA mutation	27 (12)	0.10 (0.260)	0.21 (0.061)
ERBB2 or *PTEN*	60 (27)	0.07 (0.077)	0.07 (0.147)
ERRB2 or *PIK3CA*	33 (15)	0.09 (0.252)	0.20 (0.034) †
PTEN or *PIK3CA*	81 (36)	0.11 (0.017) †	0.15 (0.008) †
ERBB2 or *PTEN* or *PIK3CA*	87 (39)	0.11 (0.018) †	0.16 (0.005) †
Breast tumors (MD Anderson cohort)			
ERBB2 amplification	20 (12)	0.216 (0.106)	0.375 (0.026) †
PIK3CA mutation	89 (24)	0.021 (0.859)	0.305 (0.002) †
ERRB2 or *PIK3CA*	45 (28)	0.239 (0.048) †	0.364 (0.008) †

*Reverse-phase protein lysate arrays probed with total PDK1 or p-S241 PDK1 antibodies. ΔPDK1 = magnitude difference in the mean signal from each antibody in the set of cases defined by a particular PI3K pathway alteration (denoted by the column heading "pathway lesion") minus the mean signal from the remainder of cases.
†$P < 0.05$ (two-tailed t test).

(+PDK1+NeuT), or empty vector controls. Consistent with the function of PDK1 as a selective T-308 AKT kinase, overexpression of PDK1 alone increased AKT phosphorylation on residue T-308 but had no effect on serine-473 (S-473), whereas NeuT overexpression alone increased both (Fig. 2*A*). When PDK1 and NeuT were both overexpressed, there were significant increases in both phosphorylation of T-308 and, surprisingly, S-473 over that of either PDK1 or NeuT overexpression alone, with a more pronounced relative activation in the setting of serum starvation. Consistent with this narrower and less pronounced effect on AKT signaling, increasing PDK1 levels alone was not sufficient to induce serum-starved MCF10A proliferation but did enhance growth when added to NeuT (Supplementary Fig. S5*A*).

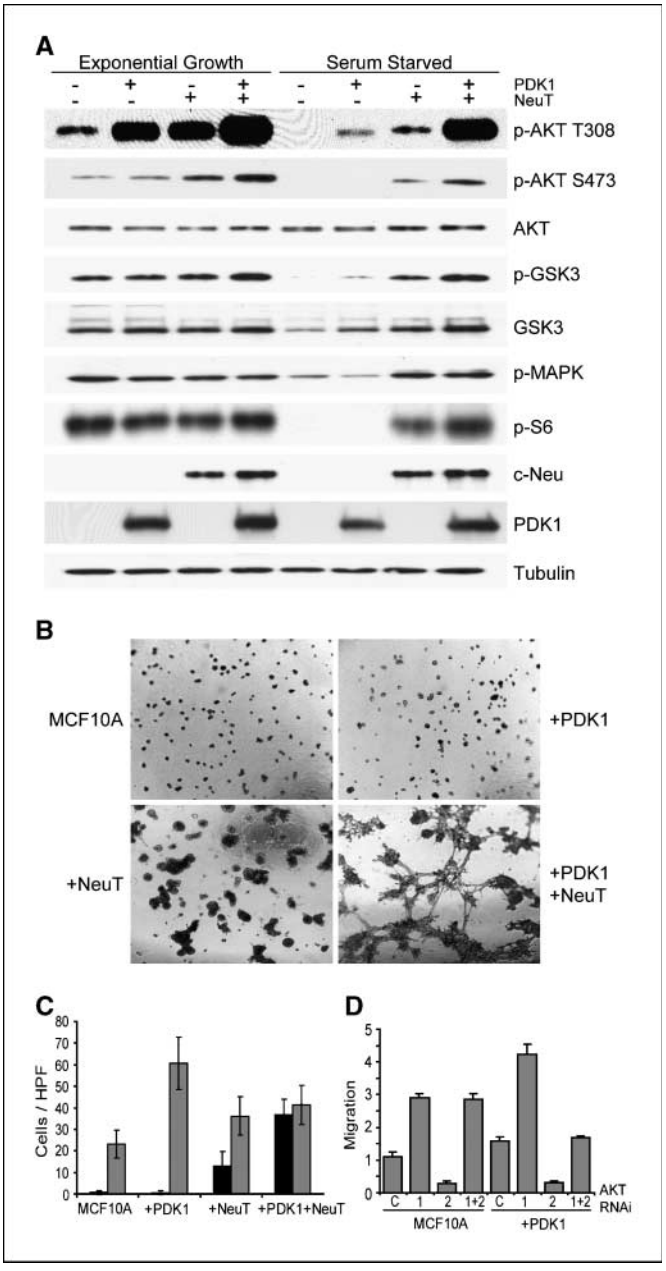

Figure 2. Overexpression of PDK1 enhances oncogenic phenotype in setting of upstream PI3K activation. *A,* immunoblots showing signaling effects of overexpressed PDK1 and NeuT on MCF10A cells under normal exponential growth or growth factor withdrawal conditions. *B,* Matrigel morphogenesis assay of stably transfected pools of MCF10A cells as indicated. *C,* transwell migration assay with same set of cells with and without chemoattractant (epidermal growth factor and 5% horse serum; SD, $n = 3$). *D,* migration assay of control MCF10A cells or those with PDK1 overexpression with small interfering RNA knockdown of AKT1, AKT2, or both. Migration (y axis) = ratio of the number of migratory cells in test versus control (SD, $n = 3$).

To determine whether increased PDK1 levels enhanced PI3K signaling induced by other genetic aberrations found in BCs, we knocked down PTEN expression in MCF10A cells and overexpressed PDK1 in *PIK3CA* mutant MCF7 cells. As with +PDK1+NeuT, increasing PDK1 levels in the context of reduced PTEN or mutant *PIK3CA* enhanced activation of AKT as indicated by increased phosphorylation of T-308 and S-473 (Supplementary Fig. S5*B* and *C*).

Increased PDK1 potentiates ERBB2-induced transformation and migration. To assess the biological affect of the enhancement of signaling of PDK1, we chose to assess elevated PDK1 levels in combination with ERBB2 because unlike PTEN or PI3K, ERBB2 activates multiple signaling pathways, such as the RAS/mitogen-activated protein kinase pathway, which can lead to evidence of oncogene cooperation. ERBB2 alone partially transforms MCF10A cells in three-dimensional culture, forming large multiacinar structures (37). In a three-dimensional culture, addition of PDK1 did not alter the control MCF10A phenotype (Fig. 2*B*). However, overexpression of PDK1 had a profound effect on the morphology of +NeuT cells in which multiacinar structures were distorted and cell foci were linked by interconnecting branching tracts. IHC analysis revealed a more complete epithelial-to-mesenchymal transition and decreased central acinar apoptosis within the +PDK1+NeuT structures compared with those of +NeuT (Supplementary Fig. S6*A*).

Given the extensive branching seen in the +PDK1+NeuT three-dimensional foci, we tested the capacity of the cells to migrate. Consistent with published data showing that PDK1 kinase activity is required for PI3K-dependent cell migration (38), we found that PDK1 overexpression alone increased migration toward a chemoattractant, but had no effect when the chemoattractant was withheld (Fig. 2*C*). Overexpression of NeuT alone allowed cells to migrate without a chemoattractant signal, yet they migrated 3-fold more toward the chemoattractant. +PDK1+NeuT cells showed increased migration to the same extent as +NeuT regardless of the presence of a chemoattractant, suggesting that the cells had completely uncoupled their migratory machinery from extracellular growth factor sensing. This effect was confirmed with a scratch test performed under serum-starved conditions (Supplementary Fig. S6*B*). Strikingly, knockdown of AKT2 inhibited PDK1-induced migration, whereas knockdown of AKT1 promoted migration (Fig. 2*D*), consistent with previous reports implicating AKT2 in motility and metastasis (39).

Increased PDK1 potentiates tumor growth *in vivo*. To test whether these effects could confer tumor growth *in vivo*, +NeuT cells or +PDK1+NeuT cells were injected into the inferior mammary fat pads of developing *scid* mice ($n = 10$ in each cohort). +PDK1+NeuT cells rapidly produced large muscle invasive tumors in all mice requiring sacrifice at a median of 30 days, whereas +Ne2uT cells formed only one tumor (sacrificed on day 50) after 140 days of observation ($P < 0.0001$; Fig. 3*A* and *B*). Control MCF10A cells and those overexpressing PDK1 alone did not form tumors ($n = 5$ in each cohort). The same combination of PDK1 and ERBB2 expressed in HMEC-hTERT cells failed to form tumors ($n = 10$).

In cells with PI3K activation, PDK1 levels are a determinant of signaling, proliferation, transformation, and pathway inhibition. Given potential off-target effects from either RNAi or drug inhibition of PDK1 (40–42), both methods were used to show the effects of altered PDK1 levels on cell proliferation and signaling. Stable RNAi knockdown of PDK1 in cells harboring *PIK3CA* mutation decreased both AKT and downstream GSK3 activation in MCF7 cells (Fig. 4*A*) with corresponding decreased proliferation of MCF7 (Fig. 4*B*) and T47D (Supplementary Fig. S7*A*) cells, all in a dose-dependent manner. The relatively selective PDK1 inhibitor BX-795 (ref. 43; also shown to inhibit Aurora A, Aurora B, Aurora C, Cdk1, and Cdk2; refs. 40, 42) inhibited growth factor–stimulated AKT T-308 phosphorylation in MCF10A cells with 50% signal inhibition corresponding to its measured IC_{50} of 1 μmol/L (Supplementary Fig. S7*B*). Increasing PDK1 levels in MCF7 cells made them more resistant to BX-795 (Fig. 4*C*) and decreasing PDK1 levels made them more sensitive (67% inhibition in control cells compared with 83% in the potent knockdown line; Fig. 4*B*), arguing that the level of PDK1 is a significant determinant of BX-795 activity.

We also found that transformation of cells via a *PIK3CA* kinase domain mutation (H1047R) was dependent on PDK1. Decreasing PDK1 levels inhibited colony formation in soft agar and growth of immortalized human mammary epithelial cells (HMEC/hTERT/p53DD) stably expressing mutant p110α (H1047R; Fig. 4*D*; Supplementary Fig. S7*C*). In the same cell background (HMEC/hTERT/p53DD/H1047R), overexpression of PDK1 conferred resistance to the selective PI3K inhibitor wortmannin (Supplementary Fig. S7*D–F*). Consistent with $PDK1^{K465E/K465E}$ knock-in mouse data showing that PDK1 membrane localization is necessary for optimal AKT activation (9), cells expressing myristolated PDK1 were more resistance than wild-type PDK1 expressing cells to PI3K inhibition (Supplementary Fig. S7*D*). This suggests that the amount of PDK1 at the membrane is a determinant of resistance to pathway inhibition and highlights another potential mechanism to therapeutically target PDK1 other than through its kinase domain.

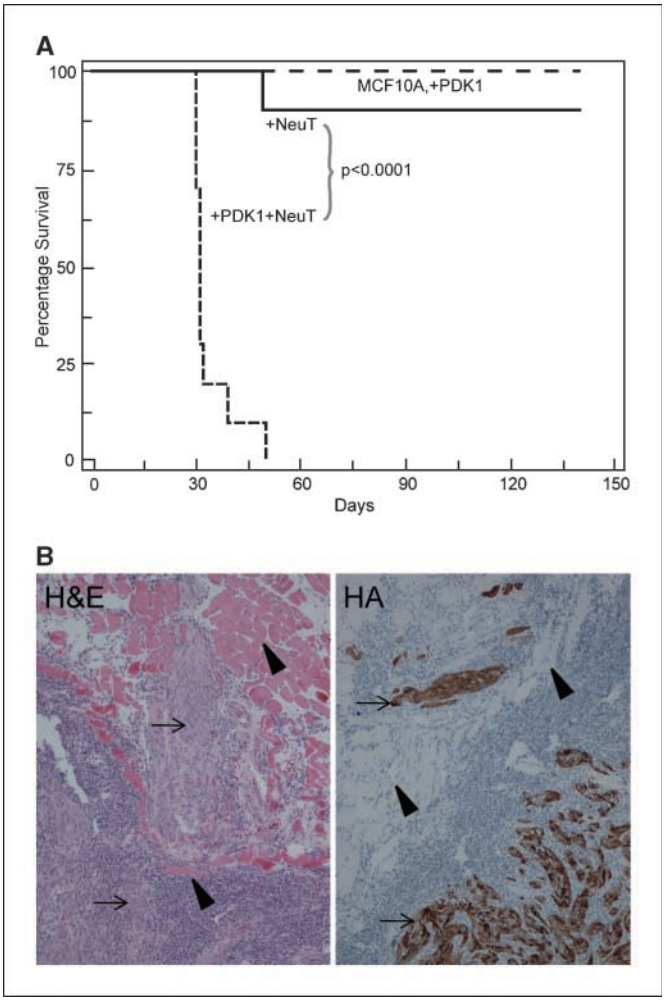

Figure 3. Increased PDK1 potentiates tumor growth *in vivo. A,* Kaplan-Meier survival curves of MCF10A cells injected into the mammary fatpads of developing *scid* mice, comparing cells overexpressing NeuT with (+PDK1+NeuT, *narrow dashed line*) or without (+NeuT, *solid line*) overexpressed PDK1 (death is defined as tumor growth to size = 1 cm^2). Injected control MCF10A cells and
cells overexpressing PDK1 alone did not form tumors (wide dashed line). *B,* IHC staining (*left,* H&E; *right,* HA antibody to HA-tagged PDK1, 40×) of xenografted tumor cells (*arrows*) invading host muscle (*arrowheads*).

Discussion

We have shown that total PDK1 protein and message up-regulation is present in almost three quarters of BCs tested, making it a common lesion of the PI3K pathway in BC. We have found that total PDK1 levels correlate strongly with S241 phosphorylated PDK1 levels, which suggests that it also is, at least in part, a measure of total PDK1 expression. We have found that one mechanism for PDK1 up-regulation occurs through an increase in gene copy number within 16p13.3 amplicons (Fig. 1), the third most frequently amplified region in BCs (33). However, *PDPK1* ICN can only explain a portion of cases with PDK1 overexpression, which suggests that additional mechanisms of overexpression remain to be elucidated.

Our data strongly argues that PDK1 overexpression coordinately occurs with upstream PI3K activation to contribute to BC progression, because we see that both PDK1 ICN and protein expression are associated in tumors to upstream PI3K pathway lesions of *PIK3CA, ERBB2*, or *PTEN* (Tables 1 and 2). The link between PDK1 and PI3K signaling is further substantiated by the observation that *PDPK1* ICN is associated with poor prognosis (Supplementary Fig. S4), which has also been established for activation of the PI3K pathway (44) and by findings by others that 16p13.3 gains correlate with gains of 17q12, the *ERBB2* locus (33). In addition to BC, we identified a coordinated increase of PDK1 with upstream PI3K pathway lesions in tumor cell lines representing a large variety of cancer. These findings suggest that PDK1 overexpression may cooperate with upstream PI3K pathway lesions in a wide variety of solid tumors to promote tumor progression by further activating the PI3K pathway.

Our data from human BCs, tissue culture, and xenografted tumors provide evidence for a model of tumor development in which BCs are selected to increase PDK1 to potentiate upstream lesions of the PI3K pathway for increased signaling and as a consequence tumor progression. Given that both *PDPK1* ICN and increased PDK1 protein levels in human BCs correlate with either one of three activators of PI3K signaling (*ERBB2* amplification, *PIK3CA* mutation, or PTEN down-regulation), we hypothesized that the effect of PDK1 up-regulation is likely to be an increased signal output. Our data from experiments with cultured mammary cells support this conclusion, because PDK1 overexpression, in the setting of upstream activation by ERBB2 or mutant *PIK3CA* or PTEN loss, increased phosphorylation of its substrate AKT T-308 as

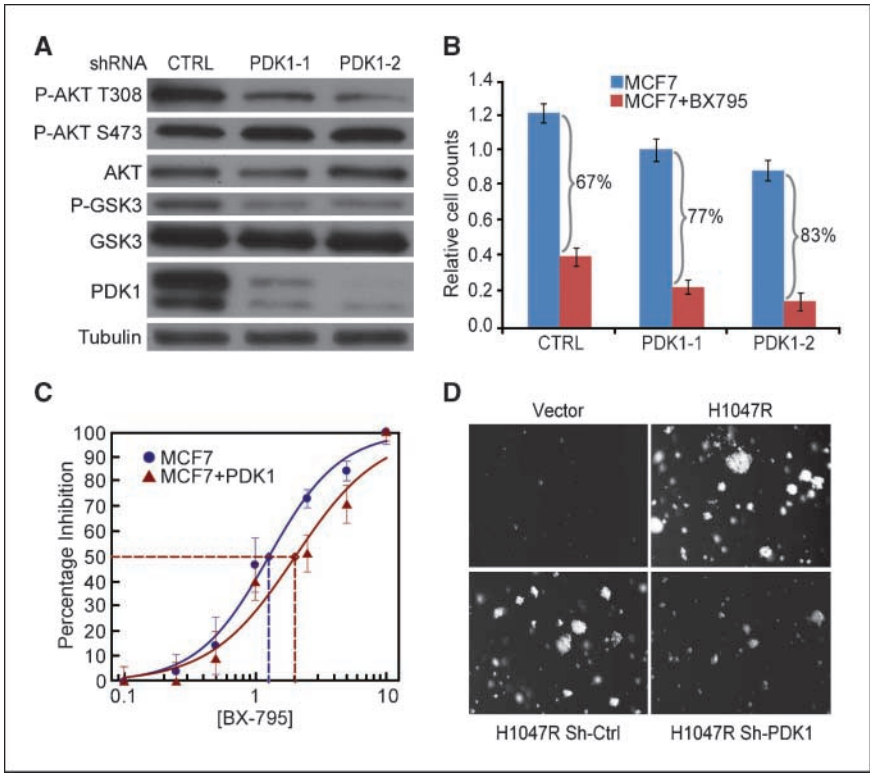

Figure 4. In cells with PI3K activation, PDK1 levels are a determinant of signaling, proliferation, transformation, and pathway inhibition. *A,* immunoblots of MCF7 cells grown with stable control shRNA (*CTRL*) or two separate PDK1 shRNA contructs (*PDK1-1* and *PDK1-2*). *B,* MCF7 cells with stable control shRNA or separate PDK1 shRNA contructs (as indicated) grown for 4 d with 1.25 μmol/L BX-795 (*orange columns*) or control (DMSO, *blue columns*; percentage of inhibition is indicated, SD, $n = 3$). *C,* BX-795 dose-response curves of control MCF7 cells or MCF7 cells overexpressing PDK1 with the IC_{50} indicated (*dotted lines*; SD, $n = 3$). *D,* colony formation assay of HMEC/hTERT/p53DD cells overexpressing mutant p110α (*H1047R*) or control, or p110α (*H1047R*) in the setting of stable PDK1 shRNA compared with control shRNA.

well as AKT S-473 (Fig. 2). The model asserts that, in cells with increased levels of PIP_3, coordinate gain of PDK1 potentiates the PI3K pathway signal to a level that maintains downstream pathway activation. The most likely mechanism of such intrapathway enhancement involving overexpression of PDK1 is the direct boosting of the signal from a defined static amount of PIP_3 due to an upstream lesion in *PIK3CA, ERBB2*, or *PTEN*.

PDK1 levels had their most prominent potentiating effect on the PI3K signal due to an upstream pathway lesion when growth factor input was low (serum starvation). Therefore, PDK1 is limiting under these conditions, perhaps recreating the selective pressure for increasing PDK1 levels found in tissues during the stress associated with tumor development. In support of this idea, a 90% reduction of PDK1 protein expression did not significantly affect ligand-activated insulin signaling in normal mice (45), whereas the same PDK1 hypomorph significantly attenuated tumor formation in *Pten* heterozygous mice (22).

We have documented that the potentiating effect of PDK1 on the PI3K signal is sufficient to have phenotypic effects on mammary cells (Fig. 2). PDK1 increased proliferation, migration, and epithelial-to-mesenchymal transition and reduced apoptosis in ERBB2 MCF10A cells. The combination of ERBB2 and PDK1 in this immortal cell line was even sufficient to cause tumor formation in the mammary fat pad of *scid* mice in all mice tested when either gene alone had little or no effect (Fig. 3). It will be interesting to determine whether PDK1 overexpression in combination with *PIK3CA* mutation or reduced PTEN expression in MCF10A cells phenocopies PDK1/ERBB2; however, we anticipate that they will be less oncogenic given their weaker ability to activate other signaling pathways.

We suspect that many of the consequences of PDK1 overexpression occur via the activation of different AKT isoforms and have shown that increased migration flows through AKT2 (Fig. 2). These data are consistent with a transgenic mouse model of concurrent ERBB2 and AKT1 overexpression showing acceleration of mammary tumor progression but lower levels of invasion (46) and argues that PDK1 overexpression may be a more efficient and potent PI3K pathway potentiator than any one of its substrates. PDK1 phosphorylates other AGC kinase substrates, including p70S6 kinase and SGK1 in a PI3K pathway–dependent manner (47), and these outputs are likely to be enhanced by PDK1 overexpression as well. In addition, PDK1 regulation of other AGC kinases remains an active area of investigation that may expose the functional role of additional PI3K-regulated substrates.

Evidence for different PI3K pathway lesions co-occurring in the same tumor has been shown in endometrial cancers, wherein PTEN disruption through gene mutation and loss of protein expression are frequently coincident with *PIK3CA* mutation or amplification and together provide increased PI3K signal output (48). It is possible that in endometrial cancers, the level of PIP_3 may be limiting and thus the determinants of the PI3K signal could be tissue specific, although it is not known whether PDK1 makes a contribution in these tumors. Alternatively, if PDK1 levels are found to be coincidentally increased in this setting, it would argue that tumors using an active PI3K pathway undergo continual selection for increased PDK1 to maintain a high signal output. Because we observe increased PDK1 levels in the ductal carcinoma *in situ* (DCIS) component of invasive tumors expressing high levels of PDK1, one could imagine a scenario in which *ERBB2* amplification is followed by PDK1 overexpression and subsequent *PIK3CA* mutation, as well as possibly other events, all to ratchet up the level of PI3K signaling.

The ability of endogenous PDK1 to contribute to PI3K signaling and tumor cell proliferation was also documented in tumor cells harboring *PIK3CA* mutations, which suggests that PDK1 amplification of PI3K signaling outputs stimulates tumor growth (Fig. 4). Our data also show that increasing PDK1 levels, at

least in some settings, could contribute to resistance to inhibitors of the PI3K pathway at the level of PDK1 and PI3K (Fig. 4).

Thus, we conclude that PDK1 overexpression in tumors increases the level of oncogenic PI3K signal due to pathogenetic activation of PI3K or inactivation of PTEN. Our findings suggest that PDK1 levels should be taken into account in any attempt to assess derangements of the PI3K pathway in cancer and that targeting PDK1 along with other components of the PI3K pathway simultaneously may be a useful approach in cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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