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Research Article

Loss of *Phosphatase and Tensin Homologue Deleted on Chromosome 10* Engages ErbB3 and Insulin-Like Growth Factor-I Receptor Signaling to Promote Antiestrogen Resistance in Breast Cancer

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Abstract

Knockdown of the tumor suppressor phosphatase Phosphatase and tensin homologue deleted on chromosome 10 (*PTEN*) with shRNA in three estrogen receptor (ER)-positive breast cancer cell lines resulted in increased phosphatidylinositol-3 kinase (PI3K) and AKT activities, resistance to tamoxifen and fulvestrant, and hormone-independent growth. *PTEN* knockdown induced the up-regulation of ER transcriptional activity in MCF-7 cells but decreased ER protein levels and transcriptional activity in T47D and MDA-361 cells. Tamoxifen and fulvestrant treatment inhibited estradiol-induced ER transcriptional activity in all shPTEN cell lines but did not abrogate the increased cell proliferation induced by *PTEN* knockdown. *PTEN* knockdown increased basal and ligand-induced activation of the insulin-like growth factor-I (IGF-I) and ErbB3 receptor tyrosine kinases, and prolonged the association of the p85 PI3K subunit with the IGF-I receptor (IGF-IR) effector insulin receptor substrate-1 and with ErbB3, implicating PTEN in the modulation of signaling upstream of PI3K. Consistent with these data, PTEN levels inversely correlated with levels of tyrosine-phosphorylated IGF-IR in tissue lysate arrays of primary breast cancers. Inhibition of IGF-IR and/or ErbB2-mediated activation of ErbB3 with tyrosine kinase inhibitors restored hormone dependence and the growth inhibitory effect of tamoxifen and fulvestrant on shPTEN cells, suggesting that cotargeting both ER and receptor tyrosine kinase pathways holds promise for the treatment of patients with ER+, PTEN-deficient breast cancers. [Cancer Res 2009;69(10):4192–201]

Introduction

Loss-of-function mutations of the *Phosphatase and tensin homologue deleted on chromosome 10* (*PTEN*) tumor suppressor gene occur in 5% to 45% of human cancers (1), with reduced PTEN protein found in 31% to 48% of breast cancers (2–4). The main tumor suppressive action of PTEN is its lipid phosphatase activity to antagonize phosphatidylinositol-3 kinase (PI3K) by dephosphorylating its product, phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), resulting in inhibition of the serine/threonine kinase AKT and other pleckstrin homology domain-containing proteins that modulate cell growth, survival, and angiogenesis. PTEN can also act as a protein phosphatase with targets including focal adhesion kinase (5), platelet-derived growth factor receptor, epidermal growth factor receptor (EGFR; ref. 6), and itself (7), and as a binding partner to increase p53 activity (8).

Two-thirds of breast cancers express estrogen receptor (ER)-α, which drives breast cancer cell growth. Although endocrine therapies designed to block estrogen action (e.g., tamoxifen, aromatase inhibitors) have changed the natural history of hormone-dependent breast cancer, many tumors exhibit *de novo* or acquired therapeutic resistance. Crosstalk between receptor tyrosine kinase (RTK) and ER signaling promotes resistance to endocrine therapy (9). Tumor overexpression of RTKs and RTK ligands, and increased RTK pathway activation, have been linked to antiestrogen resistance (10–12). For example, the RTK effectors AKT and mitogen-activated protein kinase (MAPK) can phosphorylate ER (13, 14), and MAPK can phosphorylate the ER coactivator AIB1, to promote ER transcriptional activity (15). In turn, ER drives transcription of *insulin-like growth factor-I* (*IGF-I*), *IGF-II, IGF-I receptor* (*IGF-IR*), and its effector *insulin receptor substrate-1* (*IRS-1*). Activated IRS-1 induces PI3K activation (16) and is stabilized by ER (17). Furthermore, estrogen induces the expression of genes encoding the EGFR ligands transforming growth factor α and amphiregulin (18, 19) and can activate EGFR, HER2/ErbB2, and downstream signal transducers by transcription-independent mechanisms (20). Although the traditional role of ER as a transcription factor is central to ER+ breast cancer, ER has also been implicated in nongenomic, plasma membrane-initiated signaling with IGF-IR, EGFR, and PI3K (21, 22).

Mutational activation of the PI3K pathway, by PTEN loss and/or gain-of-function mutations in *PIK3CA* (which encodes the PI3K p110α catalytic subunit), occurs in 56% to 62% of ER+ breast cancers (23, 24). Patients with cancers exhibiting a gene expression signature of PTEN loss show poor disease outcome (24). Although PI3K mutations and PTEN loss are both thought to confer increased PI3K activity, the cellular effects of these mutations may be different, as suggested by the coexistence of these alterations in 5% to 14% of primary breast tumors (2, 23, 24). We therefore investigated the effects of PTEN loss in three ER+ human

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

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breast cancer cell lines on PI3K activation, hormone-independent growth, and response to antiestrogens.

Materials and Methods

Cell lines. MCF-7, T47D, and MDA-361 cells (American Type Culture Collection) were stably transduced with retrovirus-encoding shRNA targeting *PTEN* or mismatch control (shMM; as in Supplementary Materials and Methods). Experiments were performed using phenol red–free IMEM + dextran-charcoal–treated-fetal bovine serum (DCC-FBS; Hyclone) unless otherwise indicated.

Phospholipid analysis. MCF-7 lines were labeled ×16 h with 100 μCi/mL [^{32}P]-P_i (Perkin-Elmer) in phosphate-free DMEM + 10% dialyzed FBS (Hyclone). Radiolabeled lipids were extracted, concentrated, and separated by TLC as described (25). ^{32}P incorporation into phosphatidylinositol species was detected by autoradiography.

Cell proliferations assays. Cells were seeded in triplicate in 12-well plates (2.5×10^4 per well). The next day, medium was changed to IMEM + DCC−FBS ± 17-β-estradiol (E2), 4-hydroxy-tamoxifen (4-OH-T), fulvestrant (faslodex; ICI182780; gift from AstraZeneca), testosterone, letrozole (gift from Dean Evans, Novartis, Basel, Switzerland), the allosteric AKT1/2 inhibitor 0360263-1 (AKTi; ref. 26), BEZ235 (27), AEW541 (both provided by Carlos Garcia-Echeverria, Novartis, Basel, Switzerland; ref. 28), or lapatinib ditosylate (GW-572016; LC Laboratories). For siRNA of ErbB3/HER3, cells were transfected as in Supplementary Materials and Methods. Media were refreshed every 2 to 3 d, and after 5 to 8 d, cells were trypsinized and counted using a Coulter counter.

ER transcriptional reporter assays. Cells were plated as above and transfected with pGLB-MERE (provided by Dorraya El-Ashry, University of Miami, Miami, FL) and pCMV-Renilla (Promega) luciferase plasmids. Cells were then treated as above, and luciferase activity was measured 16 to 20 h later as described (29).

Immunoprecipitation and immunoblotting. Cells were treated as indicated (IGF-I and heregulin-β1; R&D Systems). Cells were lysed in NP40 buffer plus protease and phosphatase inhibitors, sonicated for 10 s, centrifuged at 14*k* rpm for 10 min., and protein was quantitated using BCA assay (Pierce). Immunoprecipitations were performed using Dynal protein-G beads (Invitrogen) and p85 antibody (Upstate) as described (30). Immunoprecipitates and cell lysates were subjected to SDS-PAGE and transferred to nitrocellulose. Primary antibodies for immunoblotting included PTEN, IGF-IRβ, HER3, ERα (Santa Cruz Biotechnology), AKT, P-AKT$_{S473}$, P-HER2$_{Y1248}$, P-HER3$_{Y1289}$, P-IGF-IRβ$_{Y1131}$ (Cell Signaling), PR (Dako), P-tyrosine (4G10, Vanderbilt Monoclonal Antibody Core), IRS-1 (Upstate), and actin (Sigma). Phospho-RTK arrays were performed as per manufacturer's protocol (R&D Systems).

Reverse phase protein lysate microarray analysis. Three hundred and eighty-three hormone receptor–positive, primary breast tumor samples were obtained from the Breast Tissue Frozen Tumor Bank at M. D. Anderson Cancer Center. Specimens were collected under Institutional Review Board–approved protocols. Tumor lysates were analyzed as in Supplementary Materials and Methods (31) using antibodies against PTEN, P-IGF-IRβ$_{Y1135/1136}$ (may cross-react with P-InsRβ$_{Y1150/1151}$), and IGF-IRβ. Relative protein levels were quantified, and PTEN scores were plotted against P-IGF-IRβ/IGF-IRβ ratio.

Statistical analysis. In cell proliferation assays and ER transcriptional reporter assays, significant differences were determined by two-tailed *t* test. In reverse phase protein lysate microarray analysis, relative units for PTEN, P-IGF-IRβ, and IGF-IRβ levels were converted to logarithms, and the relationship between PTEN and ratio of P-IGF-IRβ/IGF-IRβ was analyzed using two-tailed *t* test and Pearson correlation. *P* value of <0.05 was considered significant.

Results

PTEN loss results in hormone-independent growth and antiestrogen resistance. We stably knocked down *PTEN* in MCF-7, T47D, and MDA-361 ER+ breast cancer cells using shMM. In shPTEN cell lines, PTEN protein was undetectable and AKT phosphorylation at Ser_{473} (P-AKT) was up-regulated compared with shMM controls (Fig. 1*A*). All three of these cell lines harbor

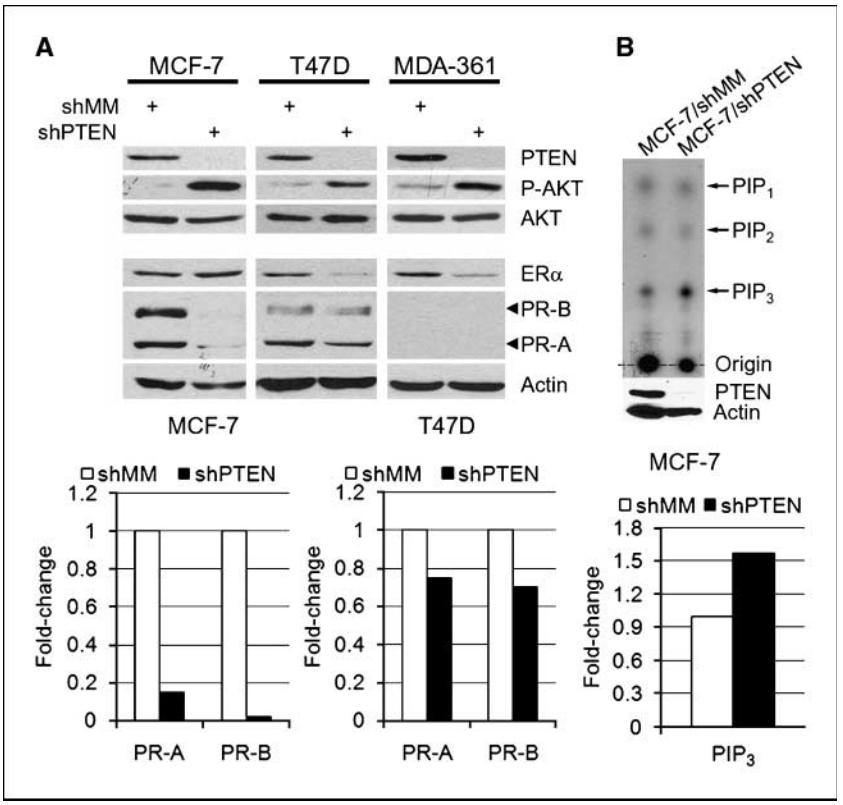

Figure 1. PTEN loss increases PIP_3 and P-AKT, and alters ER and PR levels. *A,* cells were treated with medium containing DCC-FBS (MCF-7, 2%; T47D and MDA-361, 0.5%) ×24 h, and lysates were analyzed by immunoblotting with the indicated antibodies. PR was not detected in MDA-361 cells. *B,* MCF-7 lines were metabolically labeled with ^{32}P-orthophosphatase in 10% dialyzed FBS ×16 h. Lipids were extracted, resolved by TLC, and ^{32}P-PIP species were detected by autoradiography (*arrows*). Origin of spotting is indicated. Cell lysates were also used for immunoblotting to confirm PTEN status. Fold changes in PR isoforms (*A*) and PIP_3 (*B*) normalized to actin were determined by densitometry analysis (*bar graphs*).

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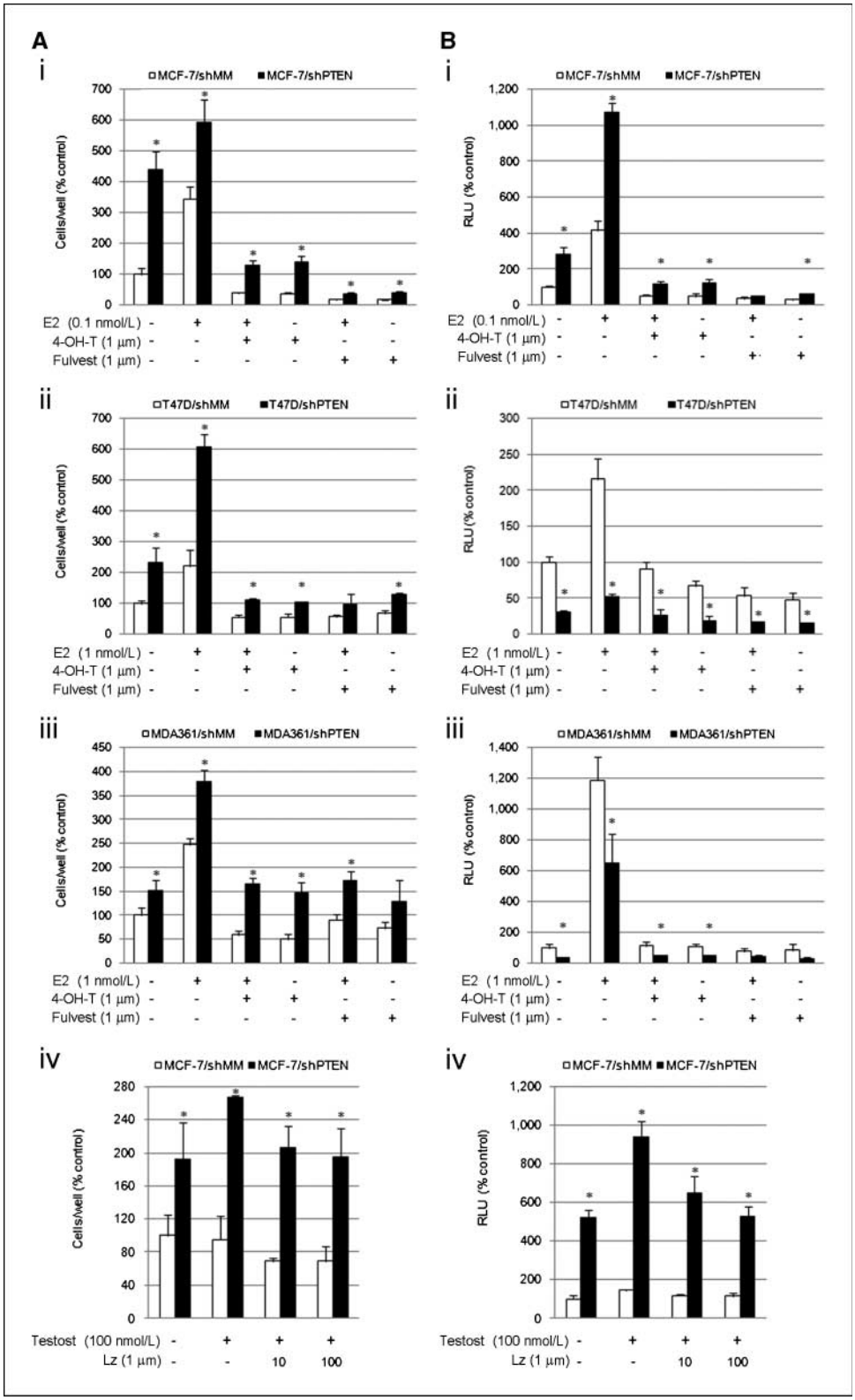

Figure 2. PTEN loss increases hormone-independent growth and antiestrogen resistance. *A,* cell proliferation assays. MCF-7 (*i* and *iv*), T47D (*ii*), and MDA-361 (*iii*) lines were treated with medium containing DCC-FBS (MCF-7, 2%; T47D and MDA-361, 0.5%) with the indicated compounds (E2; 4-OH-T; *Fulvest,* Fulvestrant; *Lz,* letrozole). Media and drugs were refreshed every 2 to 3 d. Adherent cells were counted after 5 to 8 d. *B,* ER transcriptional reporter assays. MCF-7 (*i* and *iv*), T47D (*ii*), and MDA-361 (*iii*) lines were transfected with luciferase reporter plasmids. Cells were treated as in (*A*), and luciferase activities were measured after 16 to 20 h. *RLU,* relative light units (firefly/*Renilla*). All data are presented as % untreated shMM control; *columns,* mean of triplicates; *bars,* SD. *, $P < 0.05$ by *t* test comparing shPTEN to shMM under each condition.

activating mutations in *PIK3CA*, which encodes the PI3K p110α catalytic subunit (2). In addition, PTEN loss increased levels of the PI3K product PIP_3 (Fig. 1*B*). ER levels were unchanged by PTEN loss in MCF-7 cells but were decreased in T47D and MDA-361 cells. PTEN loss reduced progesterone receptor (PR) levels in MCF-7 and T47D cells, which is consistent with the negative correlation between PTEN and PR levels observed in human breast cancers (2).

In cell proliferation assays, shPTEN cells significantly outgrew shMM controls under hormone-depleted and E2-induced conditions ($P < 0.05$; Supplementary Fig. S1; Fig. 2*A*). In 5 of 5 nude mice,

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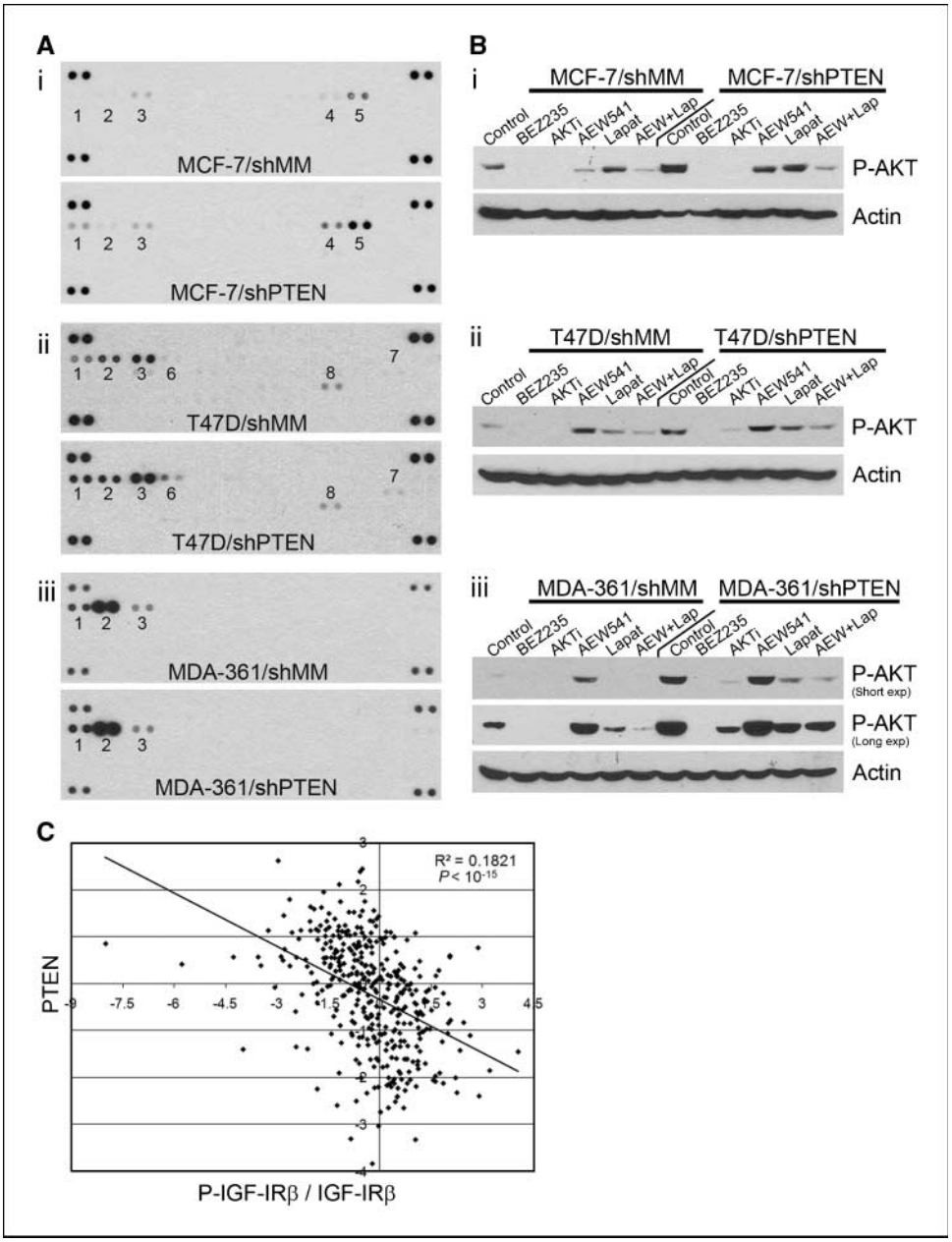

Figure 3. PTEN loss activates RTKs upstream of PI3K. *A,* lysates from MCF-7 (*i*), T47D (*ii*), and MDA-361 (*iii*) lines treated with medium containing DCC-FBS (MCF-7, 2%; T47D and MDA-361, 0.5%) overnight were used to probe phospho-RTK arrays. Antibodies against 42 RTKs are spotted in duplicate on a membrane. Membranes are incubated with cell lysates followed by probing with a P-tyrosine antibody. A positive signal is indicative of receptor phosphorylation. Blots from each pair of lines are exposure-matched. Tyrosine-phosphorylated RTKs are labeled as follows: *1,* EGFR; *2,* HER2; *3,* HER3; *4,* InsRβ; *5,* IGF-IRβ; *6,* ErbB4; *7,* ROR2; *8,* EphA1. Positive controls are spotted at corners. *B,* lysates from MCF-7 (*i*), T47D (*ii*), and MDA-361 (*iii*) lines treated as in *A* overnight ± the indicated kinase inhibitors were used for immunoblotting with the indicated antibodies. Short and long exposures (*exp*) are shown in *iii. C,* tumor lysates from 383 hormone receptor-positive breast cancers were analyzed by reverse phase protein lysate microarray analysis to quantify relative levels of PTEN, P-IGF-IR$\beta_{Y1135/1136}$, and IGF-IRβ. Shown is a scatterplot of PTEN versus ratio of P-IGF-IRβ/IGF-IRβ (indicative of the fraction of activated IGF-IRβ). This relationship was analyzed using two-tailed *t* test and Pearson correlation.

MCF-7/shPTEN cells formed palpable tumors (≥3 mm in diameter) versus only 1 of 5 animals injected with MCF-7/shMM cells (Supplementary Fig. S1). Although all lines were sensitive to the inhibitory effects of the selective ER modulator 4-OH-T and the ER down-regulator fulvestrant, shPTEN cells exhibited significantly increased growth compared with shMM controls ($P < 0.05$; Fig. 2*A*), indicative of relative antiestrogen resistance. The hormone-independent proliferation of MDA-361/shMM cells, but not MDA-361/shPTEN cells, was inhibited by 4-OH-T and fulvestrant. Furthermore, testosterone induced the growth of MCF-7/shPTEN but not MCF-7/shMM cells ($P < 0.05$; Fig. 2*A, iv*). This response was blocked by the aromatase inhibitor letrozole, suggesting that *PTEN* knockdown up-regulated the cellular response to E2 produced by the aromatization of testosterone. Aromatase mRNA levels were unchanged by *PTEN* knockdown (determined by microarray analysis; data not shown). Aromatase protein was undetectable by immunoblot analysis of MCF-7 lines, but low levels of aromatase are expressed in MCF-7 cells (32).

PI3K/AKT pathway activation has been associated with increased ER transcriptional activity and reduced ER expression in MCF-7 cells (14, 33). Here, MCF-7/shPTEN cells showed increased basal, E2-induced, and testosterone-induced ER transcriptional reporter activity compared with shMM cells (Supplementary Fig. S2; Fig. 2*B, i* and *iv*). However, PTEN loss decreased basal and E2-stimulated ER transcriptional activity in T47D and MDA-361 cells (Supplementary Fig. S2; Fig. 2*B, ii* and *iii*), reflective of the reduced ER levels in these cells (Fig. 1). We also observed variable effects of PTEN loss on the expression of the E2-inducible genes *AREG* and *EGR3* (18). In MCF-7 and T47D cells, *PTEN* knockdown increased the basal and E2-induced mRNA levels of *AREG* and *EGR3* compared with shMM controls (Supplementary Fig. S3). Opposite effects were observed in MDA-361 cells.

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Treatment with 4-OH-T or fulvestrant did not consistently suppress the effects of *PTEN* knockdown on *AREG* or *EGR3* expression, suggesting that PTEN loss alters their expression by both ER-dependent and ER-independent mechanisms. Although treatment with 4-OH-T, fulvestrant, or letrozole did not abrogate the growth advantage of shPTEN cell lines (Fig. 2*A*), these inhibitors markedly suppressed E2- and testosterone-induced ER transcriptional activity in all lines (Supplementary Fig. S4; Fig. 2*B*). Overall, these data suggest that the increased hormone-independent growth and relative resistance to 4-OH-T and fulvestrant conferred by PTEN loss cannot be solely explained by increased ER transcriptional activity.

PTEN loss increases IGF-IR– and ErbB3-mediated activation of PI3K. We next examined mechanisms that can activate PI3K in PTEN-deficient cells. Using phospho-RTK array analysis, we detected predominant tyrosine phosphorylation of IGF-IRβ, ErbB3/HER3, insulin receptor-β (InsRβ), and EGFR in MCF-7 cells (Fig. 3*A, i*), and of EGFR, HER2, and HER3 in T47D and MDA-361 cells (Fig. 3*A*, *ii* and *iii*). PTEN loss up-regulated tyrosine phosphorylation of EGFR, InsRβ, and IGF-IRβ in MCF-7 cells, and of EGFR, HER3, ErbB4/HER4, and ROR2 in T47D cells. We next used a loss-of-function approach to assess which RTKs activated PI3K, measuring P-AKT$_{S473}$ as a surrogate of PI3K activity. The IGF-IR tyrosine kinase inhibitor AEW541 reduced P-AKT in MCF-7 cells (Fig. 3*B, i*), whereas the EGFR/HER2 tyrosine kinase inhibitor lapatinib reduced P-AKT in T47D and MDA-361 cells (Fig. 3*B, ii* and *iii*). Treatment with lapatinib decreased P-HER3 in all three cell lines (Supplementary Fig. S4), suggesting that HER3 activation is EGFR/HER2-dependent in these cells, and that HER3 is the predominant activator of PI3K in T47D and MDA-361 cells. In all shPTEN lines, the combination of AEW541 plus lapatinib inhibited P-AKT more effectively than either drug alone. AKTi and the PI3K/mammalian target of rapamycin inhibitor BEZ235 decreased P-AKT in all lines.

To ascertain whether PTEN loss is linked with IGF-IR activation in primary breast cancers, we analyzed 383 hormone receptor–positive tumors by reverse phase protein lysate microarray for levels of PTEN, tyrosine-phosphorylated IGF-IRβ (P-IGF-IRβ), and total IGF-IRβ. We observed a statistically significant inverse correlation between PTEN levels and P-IGF-IRβ/IGF-IRβ ratio ($R^2 = 0.182$; $P < 10^{-15}$; Fig. 3*C*), consistent with the results observed upon *PTEN* knockdown in MCF-7 cells (Fig. 3*A*). Because P-IGF-IRβ antibodies may cross-react with P-InsRβ, we cannot rule out that P-InsRβ may also contribute to this correlation.

Growth factor receptors activate PI3K by phosphorylating adaptor proteins such as GAB1, GAB2, IRS-1, IRS-2, and HER3. Tyrosine-phosphorylated adaptors engage the N-SH2 domain of the PI3K regulatory subunit p85, relieving the inhibition of the p110 catalytic subunit by p85, and recruiting the p85-p110 heterodimer to its substrate phosphatidylinositol-4,5-bisphosphate at the plasma membrane (34). p110 phosphorylates phosphatidylinositol-4,5-bisphosphate to produce PIP_3. The interaction between p85 and tyrosine-phosphorylated adaptors permits the identification of PI3K activators by their coprecipitation with p85

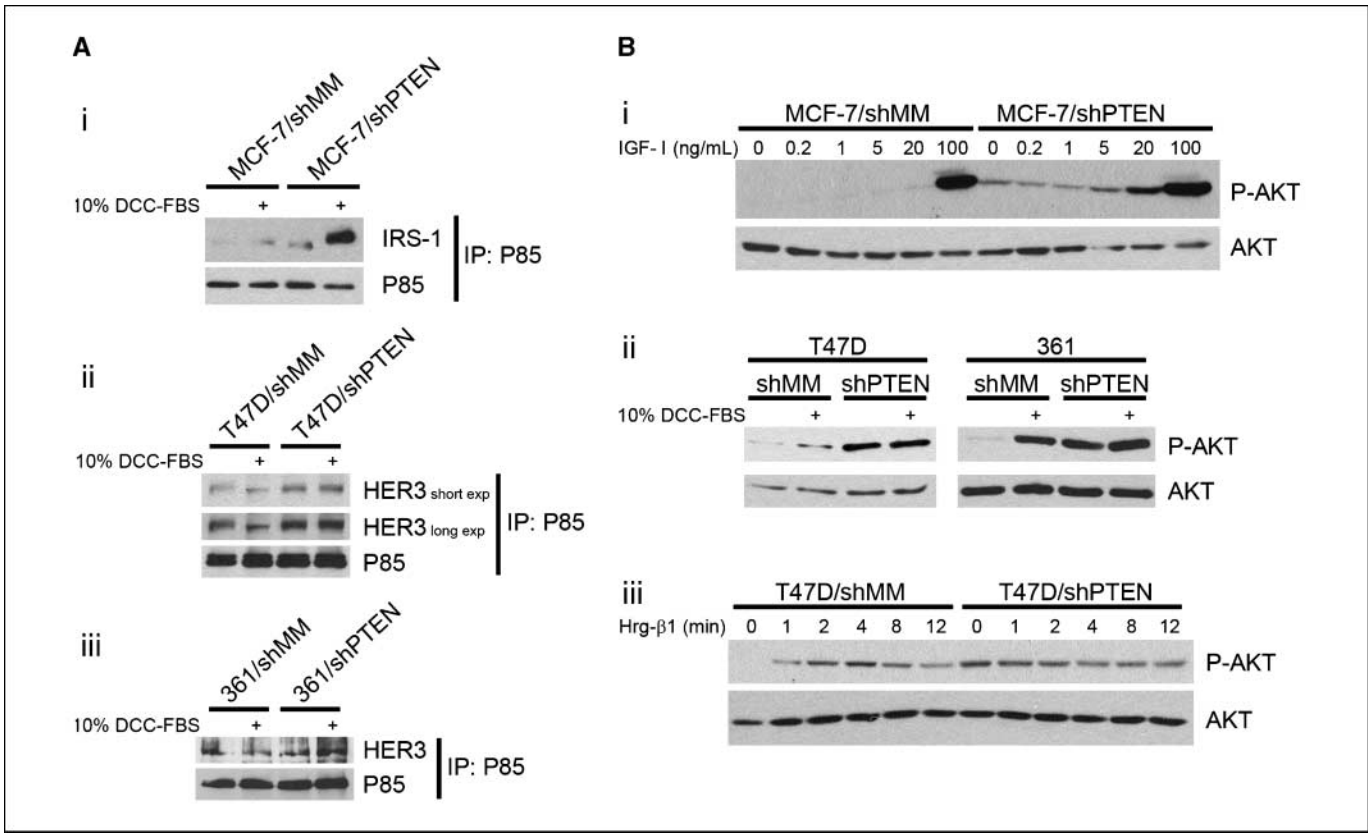

Figure 4. PTEN loss increases PI3K activation and sensitivity to RTK ligands. *A,* p85 was immunoprecipitated from cell lysates of MCF-7 (*i*), T47D (*ii*), and MDA-361 (*iii*) lines treated overnight ± 10% DCC-FBS. Short and long exposures are shown in *ii. B,* immunoblotting with the indicated antibodies of (*i*) lysates from MCF-7 cells serum-starved overnight, then treated with IGF-I (0–100 ng/mL × 15 min.); (*ii*) lysates from T47D and MDA-361 lines treated overnight ± 10% DCC-FBS; (*iii*) lysates from T47D cells serum-starved overnight, then treated with heregulin-β1 (20 ng/mL × 0–12 min).

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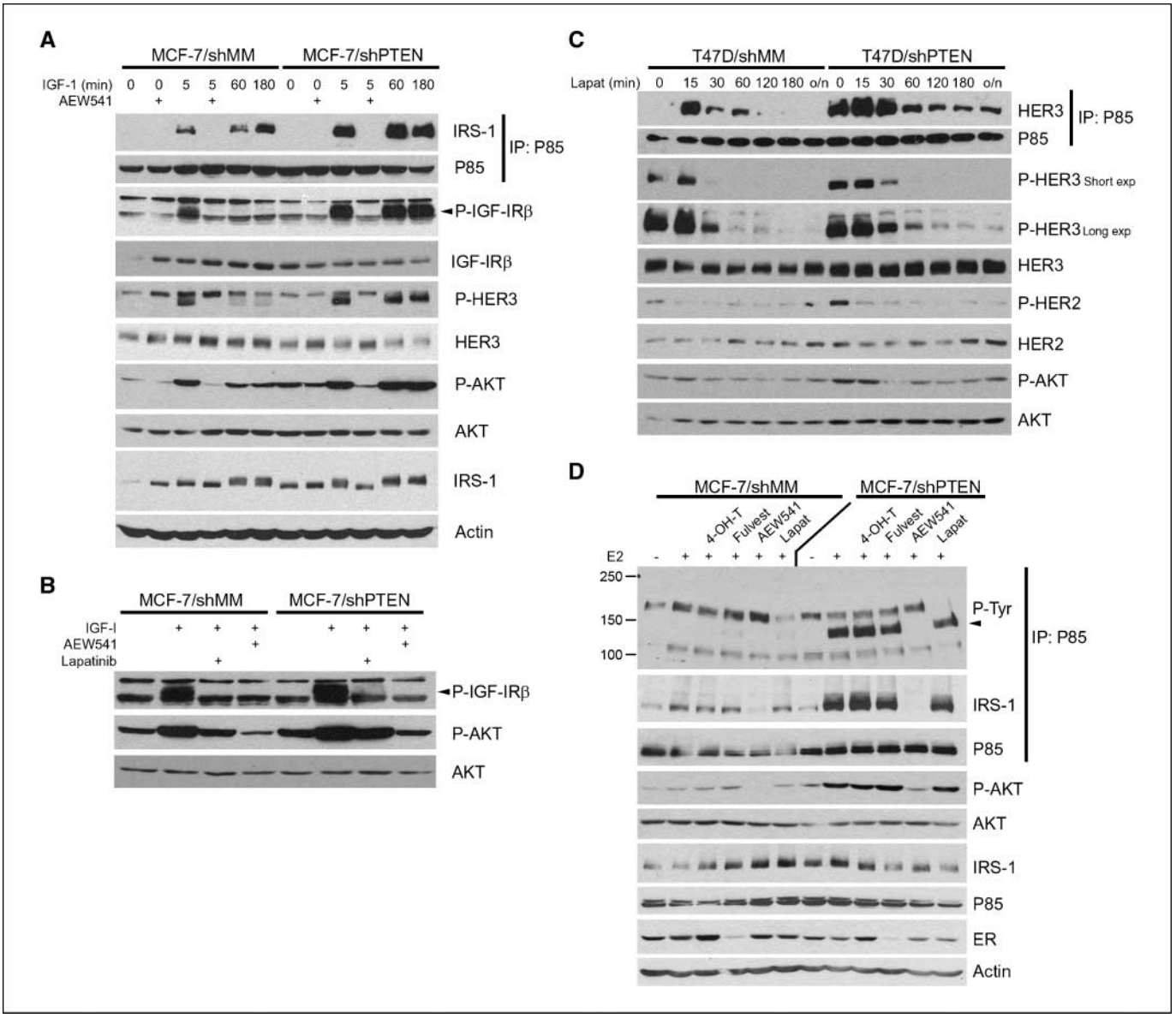

Figure 5. PTEN loss prolongs IGF-IR and HER3 signaling, and increases E2-induced nongenomic signaling through IGF-IR. *A,* p85 was immunoprecipitated (*IP*) from lysates of MCF-7 cells that had been pretreated overnight with serum-free medium ± AEW541 (1 μmol/L), then stimulated ± 100 ng/mL IGF-I ± AEW541 × 5, 60, or 180 min. *Arrowhead,* P-IGF-IRβ. *B,* lysates from MCF-7 cells pretreated as in *A* ± AEW541 (1 μmol/L) or lapatinib (1 μmol/L), then stimulated ± IGF-I (100 ng/mL) ± inhibitors ×15 min. *C,* p85 was immunoprecipitated from lysates of T47D cells treated with 0.5% DCC-FBS ± lapatinib [1 μmol/L) × 15, 30, 60, 120, or 180 min, or overnight (*o/n*)]. Short and long exposures for P-HER3 are shown. *D,* p85 was immunoprecipitated from lysates of MCF-7 cells pretreated overnight with 10% DCC-FBS ± 1 μmol/L 4-OH-T, 1 μmol/L fulvestrant, 1 μmol/L AEW541, or 1 μmol/L lapatinib, and then stimulated ± 1 nmol/L E2 ± inhibitors ×20 min. *Arrowhead,* tyrosine-phosphorylated IRS-1 (~150 kDa). All immunoprecipitates and cell lysates were analyzed by immunoblotting with the indicated antibodies.

antibodies. Immunoprecipitation of p85 followed by immunoblot analysis showed increased p85-IRS-1 binding under basal and serum-stimulated conditions in MCF-7/shPTEN cells compared with shMM control (Fig. 4*A*). Similarly, T47D/shPTEN cells showed increased p85-HER3 association compared with shMM cells. PTEN loss did not significantly alter the high basal p85-HER3 association in MDA-361 cells. Additionally, MCF-7/shPTEN cells showed increased PI3K/AKT pathway sensitivity to IGF-I ligand compared with shMM control (Fig. 4*B, i*). In contrast, PTEN loss maximally activated PI3K under basal conditions in T47D and MDA-361 cells, whereas shMM cells showed increased P-AKT upon stimulation with serum or the HER3 ligand heregulin-β1 (Fig. 4*B*, *ii* and *iii*).

A time course in IGF-I–stimulated MCF-7 cell lines showed that the association of p85 with IRS-1 upon ligand addition was enhanced by PTEN loss (Fig. 5*A*). P-IGF-IRβ was detectable after 5 min. of IGF-I stimulation and returned to baseline within 1 hour in MCF-7/shMM cells. In contrast, P-IGF-IRβ and the increased p85-IRS-1 association remained detectable for ≥3 hours after IGF-I stimulation in MCF-7/shPTEN cells. Therefore, PTEN loss increased and prolonged the activation of IGF-IR and IRS-1. Prior work has shown that PI3K pathway activation suppresses IRS-1 expression (35). Indeed, extended IGF-I stimulation for 24 hours modestly decreased IRS-1 levels in MCF-7/shPTEN but not MCF-7/shMM cells (Supplementary Fig. S5). Therefore, PTEN loss may promote IRS-1 down-regulation due to increased negative feedback from PI3K signaling.

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Additionally, we detected IGF-I–induced tyrosine phosphorylation of HER3 (Fig. 5*A*), suggestive of crosstalk between IGF-IR and ErbB receptors. This phosphorylation was more robust and sustained in MCF-7/shPTEN compared with MCF-7/shMM cells and was inhibited by AEW541. IGF-I–induced activation of IGF-IR, HER3, and AKT was inhibited by either AEW541 or lapatinib, suggesting that EGFR/HER2 tyrosine kinase activity was required for HER3 activation in response to IGF-I (Supplementary Fig. S7; Fig. 5*B*).

PTEN loss induced maximal activation of PI3K/AKT in T47D cells (Fig. 4*B*). Lapatinib inhibited PI3K in these cells, suggesting that PI3K activation was EGFR/HER2/HER3 dependent (Supplementary Fig. S5; Figs. 3*A–B* and 4*A*). Therefore, we examined the temporal effect of lapatinib on HER3 inactivation and p85-HER3 interaction. T47D/shPTEN cells exhibited constitutive association between p85 and HER3, and higher levels of P-HER3 and P-AKT compared with shMM control (Supplementary Fig. S7; Fig. 5*C*). In T47D/shPTEN cells, p85-HER3 association, P-HER3, and P-AKT remained detectable after overnight treatment with lapatinib, compared with near-complete inhibition after 2 to 3 hours in T47D/shMM cells. These data suggest that, like for IGF-IR, *PTEN* knockdown increases and prolongs the activation of HER3 and PI3K. PTEN loss did not alter the ability of lapatinib to suppress P-HER3 and p85-HER3 association in MDA-361 cells (not shown).

PTEN loss increases nongenomic estrogen signaling through IGF-IR. Nongenomic estrogen signaling to activate PI3K and MAPK has been proposed as a mechanism of resistance to hormonal therapy (22). We found that PTEN loss enhances an E2-induced increase in P-AKT and p85–IRS-1 interaction in MCF-7 cells (Fig. 5*D*). This signaling was unaffected by pretreatment with 4-OH-T or fulvestrant, although these compounds respectively increased and decreased ER protein levels. AEW541 but not lapatinib blocked p85-IRS-1 binding and P-AKT, suggesting that IGF-IR permits E2-induced PI3K activation in these cells. E2 did not increase P-AKT in T47D or MDA-361 cells (data not shown).

Combined blockade of IGF-IR and ErbB signaling inhibits PTEN-deficient cell growth. Treatment with AKTi, BEZ235, or AEW541 inhibited the growth of MCF-7/shPTEN cells in hormone-depleted medium and in the presence of tamoxifen or fulvestrant (Fig. 6*A*). In contrast, lapatinib was effective mainly when combined with 4-OH-T or fulvestrant. MCF-7/shPTEN cells showed

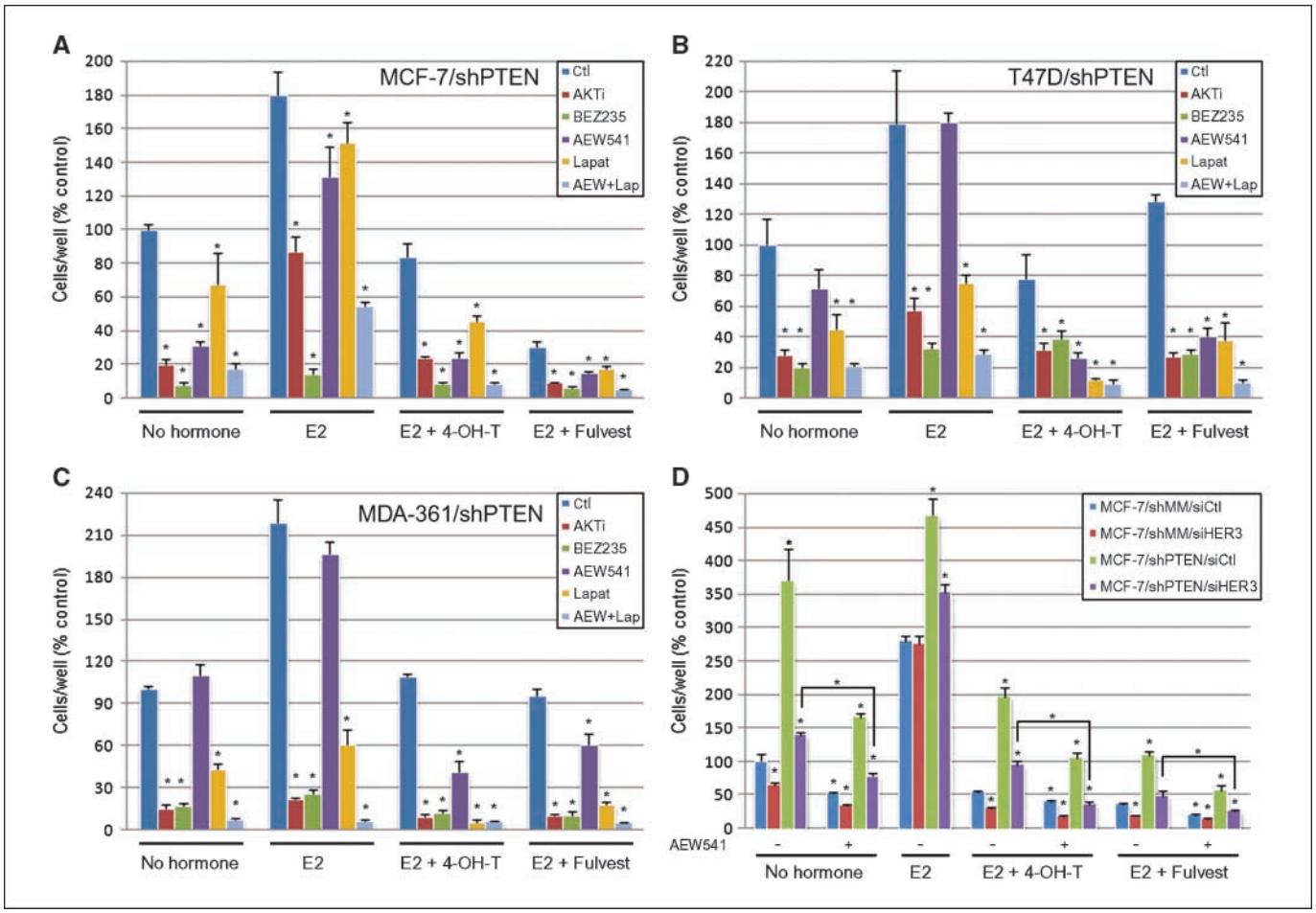

Figure 6. Combined inhibition of IGF-IR and EGFR/HER2 synergizes with 4-OH-T, fulvestrant, and hormone deprivation to block cell proliferation. *A,* MCF-7/shPTEN, (*B*) T47D/shPTEN, and (*C*) MDA-361/shPTEN cells were treated with medium containing DCC-FBS (MCF-7, 2%; T47D and MDA-361, 0.5%) as indicated [0.1 nmol/L E2 (MCF-7), 1 nmol/L E2 (T47D, MDA-361), 1 μmol/L 4-OH-T, 1 μmol/L fulvestrant, 1 μmol/L AKTi, 200 nmol/L BEZ235, 1 μmol/L AEW541, 1 μmol/L lapatinib]. Media and drugs were refreshed every 2 to 3 d. Adherent cells were counted after 5 to 8 d. Data are presented as % untreated shMM control; *columns,* mean of triplicates; *bars,* SD. *, $P < 0.05$ by *t* test comparing kinase inhibitor-treated cells to control (*Ctl*) cells within each group. *D,* MCF-7/shPTEN and/shMM cells transfected with siRNA against *HER3* or control (*siCtl*) were treated and analyzed as in *A*. *, $P < 0.05$ by *t* test compared with untreated shMM/siCtl within each group.

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increased sensitivity to AEW541 and the PI3K inhibitor LY294002 compared with shMM control (Supplementary Fig. S7), suggesting that PTEN loss increases dependence upon IGF-IR and PI3K. Similarly, AKTi, BEZ235, and lapatinib significantly inhibited T47D/shPTEN (Fig. 6*B*) and MDA-361/shPTEN (Fig. 6*C*) cell growth. In these cells, AEW541 was only effective when combined with 4-OH-T or fulvestrant.

In all shPTEN lines, the combination of AEW541 plus lapatinib was significantly more inhibitory than either agent alone (Fig. 6). These results imply that in MCF-7 cells, IGF-IR mainly drives PI3K/AKT (Figs. 3–5), but EGFR/HER2/HER3 may also contribute to hormone-independent growth and antiestrogen resistance (Fig. 6*A*). Indeed, siRNA-mediated knockdown of *HER3* (verified in Supplementary Fig. S8) inhibited the growth of MCF-7/shPTEN and/shMM cells in hormone-depleted medium, and sensitized them to 4-OH-T and fulvestrant (Fig. 6*D*). The inhibitory effect of *HER3* knockdown was augmented by AEW541 treatment, implicating both HER3 and IGF-IR in the modulation of MCF-7/shPTEN cell growth. Similarly, HER3 drives PI3K in T47D and MDA-361 cells (Figs. 3–5), but the synergistic effect of lapatinib plus AEW541 also implicates IGF-IR in the modulation of growth of T47D/shPTEN and MDA-361/shPTEN cells (Fig. 6*B*–*C*). Because these kinase inhibitors synergized with 4-OH-T, fulvestrant, and hormone-deprivation to block cell growth, PTEN-deficient, ER+ breast cancer patients may be effectively treated with drug combinations targeting ER and RTK pathways that activate PI3K.

Discussion

Herein, we show that shRNA-mediated knockdown of *PTEN* in three ER+ breast cancer cell lines resulted in antiestrogen resistance and hormone-independent growth by both genomic and nongenomic mechanisms. PTEN loss up-regulated PI3K/AKT and enhanced IGF-IR/IRS-1 and HER3 signaling, implicating PTEN in the modulation of RTK signaling upstream of PI3K. Notably, all cell lines used herein harbor activating mutations in *PIK3CA*, the gene encoding p110α (2). These results suggest that (*a*) different signaling outputs result from PTEN loss versus *PIK3CA* mutations, and (*b*) PTEN loss is a more transforming event than *PIK3CA* mutations. This is consistent with reports that *PIK3CA* mutations and PTEN loss coexist in 5% to 14% of primary breast cancers (2, 23, 24). Cross-talk between IGF-IR and EGFR/HER2 was also enhanced by PTEN loss, as IGF-I–induced HER3 phosphorylation was inhibited by lapatinib (Supplementary Fig. S7; Fig. 5*B*). Combined inhibition of these RTKs with tyrosine kinase inhibitors and/or RNAi most effectively inhibited PI3K activation (Fig. 3*B*) and cell growth (Fig. 6).

The lipid phosphatase activity of PTEN mediates its tumor suppressive function through dephosphorylation of PIP_3. However, evidence also suggests a tumor suppressor role for PTEN protein phosphatase activity. First, a $PTEN_{G129E}$ mutation was found in two kindreds of Cowden's disease, a cancer predisposition disorder. $PTEN_{G129E}$ lacks lipid phosphatase activity but retains protein phosphatase activity (36), inhibits cell migration (5) and epithelial-to-mesenchymal transition (37), and promotes G_1 cell cycle arrest (38). Second, we found that PTEN loss dysregulates kinase signaling upstream of PI3K. PTEN directly modulates tyrosine phosphorylation of EGFR and platelet-derived growth factor receptor (6), and regulates IRS-1 activation and InsR-IRS-1 binding (39). We showed that PTEN loss increases and prolongs IGF-IR and HER3 tyrosine-phosphorylation, p85-IRS-1 and p85-HER3 binding, and E2-induced, IGF-IR/IRS-1–dependent activation of PI3K/AKT (Supplementary Fig. S7; Figs. 3–5). Furthermore, PTEN levels were inversely correlated with P-IGF-IRβ in hormone receptor–positive breast cancers (Fig. 3*C*).

These findings collectively suggest that PTEN modulates RTKs and adaptors that activate PI3K, implicating PTEN in regulatory processes both upstream and downstream of PI3K. Because PTEN loss increases RTK activation and sensitivity to RTK ligands (Figs. 3–5), we speculate that other RTK-initiated signaling pathways besides PI3K are likely to also be activated in PTEN-deficient cells. Although the mechanism(s) by which PTEN regulates IGF-IR and HER3 remains unclear, possibilities include genomic effects, feedback signaling to RTKs or their adaptors (39), modulation of RTK ligand production, or PTEN binding and/or dephosphorylation of RTKs or adaptor proteins such as focal adhesion kinase (5). Notably, focal adhesion kinase is a PTEN substrate (5), and focal adhesion kinase phosphorylation has been implicated in IGF-IR (40), HER2/HER3 (41, 42), EGFR, and platelet-derived growth factor receptor signaling (43). To explore changes in RTK ligand production upon PTEN loss in MCF-7 cells, we used reverse transcription-PCR and ELISA assays for IGF-I and IGF-II, but we found no change in mRNA or protein levels (data not shown).

We show that IGF-IR/ErbB cross-talk is enhanced by PTEN loss. IGF-I stimulation increased P-HER3 in direct correlation with P-IGF-IRβ, and these effects were prolonged in PTEN-deficient cells (Fig. 5*A*). These responses were blocked by lapatinib or AEW541 (Supplementary Fig. S7; Fig. 5*B*), suggesting that IGF-IR and EGFR/HER2 kinases were required. IGF-IR has been shown to complex with P-HER2 upon stimulation with IGF-I or heregulin (44). IGF-IR has also been shown to activate EGFR (45). Interestingly, lapatinib suppressed IGF-I–induced IGF-IR phosphorylation (Supplementary Fig. S7; Fig. 5*B*), suggesting that EGFR and/or HER2 kinases are also permissive of IGF-IR activation. This RTK cross-talk supports the synergistic inhibitory effect of AEW541 plus either lapatinib or *HER3* knockdown on shPTEN cell growth (Fig. 6) and PI3K activation (Fig. 3*B*). We speculate that PTEN-deficient cancer cells use an alternative RTK pathway(s) to drive growth and survival when the primary PI3K-activating RTK is blocked. Dissecting the roles of IGF-IR and ErbB RTKs in heteromeric complex formation, stability, and signaling requires further evaluation, but these data suggest that inhibition of one RTK type (EGFR/HER2) may disrupt signaling of another RTK type (IGF-IR), and that combinations of tyrosine kinase inhibitors should be considered for the treatment of PTEN-deficient cancers.

Breast cancer cells may maintain a balance between ER and RTK pathways. Upon treatment with 4-OH-T or hormone deprivation, breast cancer cells adapt by up-regulating RTK network components (e.g., P-AKT, P-MAPK, RTK ligands; ref. 46). In turn, RTK pathway activation modulates ER function. For example, activation of the Src tyrosine kinase can induce ER degradation (47). Prior findings with MCF-7 cells suggest that PI3K activation suppresses ER expression while increasing transcriptional activity (14, 33). However, we found that PTEN loss decreased both ER protein levels and transcriptional activity in T47D and MDA-361 cells (Supplementary Fig. S2; Fig. 1*A* and 2*B*), indicating that the increase in ER transcriptional activity observed upon PTEN loss in MCF-7 cells (Supplementary Fig. S2; Fig. 2*B*) is not widely applicable to all hormone-dependent breast cancers. The decreased levels of PR seen upon *PTEN* knockdown in MCF-7 and T47D cells (Fig. 1*A*) may be due to increased PR degradation or decreased

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gene expression (determined by microarray analysis; data not shown). PI3K pathway activation has been shown to increase the expression of the E2-inducible gene *Cyclin D1* while suppressing *PgR* expression (48). Furthermore, heregulin-β1 stimulation of T47D cells increased PR phosphorylation at Ser_{294} (49), which, in turn, promotes PR degradation (50). Therefore, PI3K activation as a result of PTEN loss may up-regulate or down-regulate transcription of ER target genes, as well as modulate their gene products posttranslationally.

In summary, we report that induced PTEN loss in hormone-dependent human breast cancer cells resulted in (*a*) hormone-independent growth and resistance to 4-OH-T and fulvestrant, (*b*) variable alterations in ER transcriptional activity, and (*c*) up-regulation of kinase signaling upstream and downstream of PI3K. Furthermore, we showed that *PTEN* knockdown in three *PIK3CA*-mutant breast cancer cell lines confers gain-of-function effects, indicating different signaling outputs as a result of PTEN loss versus *PIK3CA* mutation. The use of three cell lines allowed us to discover pathways commonly dysregulated by PTEN loss in different systems, and we found that PTEN loss up-regulates intact pathways upstream of PI3K. Inhibition of IGF-IR and ErbB signaling synergized with 4-OH-T, fulvestrant, and hormone-deprivation to overcome the growth advantage conferred by PTEN loss. These data hold promise for the treatment of PTEN-deficient, ER+ breast cancer patients with combinations of drugs targeting both ER and RTK/PI3K pathways.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Loss of *Phosphatase and Tensin Homologue Deleted on Chromosome 10* Engages ErbB3 and Insulin-Like Growth Factor-I Receptor Signaling to Promote Antiestrogen Resistance in Breast Cancer

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