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Research Article

Oncology

Many breast cancers exhibit a degree of dependence on estrogen for tumor growth. Although several therapies have been developed to treat individuals with estrogen-dependent breast cancers, some tumors show de novo or acquired resistance, rendering them particularly elusive to current therapeutic strategies. Understanding the mechanisms by which these cancers develop resistance would enable the development of new and effective therapeutics. In order to determine mechanisms of escape from hormone dependence in estrogen receptor–positive (ER-positive) breast cancer, we established 4 human breast cancer cell lines after long-term estrogen deprivation (LTED). LTED cells showed variable changes in ER levels and sensitivity to  $17\beta$ -estradiol. Proteomic profiling of LTED cells revealed increased phosphorylation of the mammalian target of rapamycin (mTOR) substrates p70S6 kinase and p85S6 kinase as well as the PI3K substrate AKT. Inhibition of PI3K and mTOR induced LTED cell apoptosis and prevented the emergence of hormone-independent cells. Using reverse-phase protein microarrays, we identified a breast tumor protein signature of PI3K pathway activation that predicted poor outcome after adjuvant endocrine therapy in patients. Our data suggest that upon adaptation to hormone deprivation, breast cancer cells rely heavily on PI3K signaling. Our findings also imply that acquired resistance to endocrine therapy in breast cancer may be abrogated by combination therapies targeting both ER and PI3K pathways.

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# Hyperactivation of phosphatidylinositol-3 kinase promotes escape from hormone dependence in estrogen receptor–positive human breast cancer

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**Many breast cancers exhibit a degree of dependence on estrogen for tumor growth. Although several therapies have been developed to treat individuals with estrogen-dependent breast cancers, some tumors show de novo or acquired resistance, rendering them particularly elusive to current therapeutic strategies. Understanding the mechanisms by which these cancers develop resistance would enable the development of new and effective therapeutics. In order to determine mechanisms of escape from hormone dependence in estrogen receptor–positive (ER-positive) breast cancer, we established 4 human breast cancer cell lines after long-term estrogen deprivation (LTED). LTED cells showed variable changes in ER levels and sensitivity to 17 $\beta$ -estradiol. Proteomic profiling of LTED cells revealed increased phosphorylation of the mammalian target of rapamycin (mTOR) substrates p70S6 kinase and p85S6 kinase as well as the PI3K substrate AKT. Inhibition of PI3K and mTOR induced LTED cell apoptosis and prevented the emergence of hormone-independent cells. Using reverse-phase protein microarrays, we identified a breast tumor protein signature of PI3K pathway activation that predicted poor outcome after adjuvant endocrine therapy in patients. Our data suggest that upon adaptation to hormone deprivation, breast cancer cells rely heavily on PI3K signaling. Our findings also imply that acquired resistance to endocrine therapy in breast cancer may be abrogated by combination therapies targeting both ER and PI3K pathways.**

## Introduction

Two-thirds of breast cancers express estrogen receptor  $\alpha$  (ER) and/or progesterone receptor, which typically indicate a degree of estrogen dependence for growth. Treatment options for such patients include endocrine therapies that inhibit ER signaling, either by antagonizing ligand binding to ER (tamoxifen), downregulating ER (fulvestrant), or blocking estrogen biosynthesis (aromatase inhibitors [AIs]). Although endocrine therapies have changed the natural history of hormone-dependent breast cancer, many tumors exhibit de novo or acquired resistance (1). The only mechanism of resistance to endocrine therapy for which clinical data exist is overexpression of the *HER2* protooncogene (also known as *ErbB2*; refs. 2–4). Because less than 10% of hormone receptor–positive breast cancers express high *HER2* levels (2), mechanisms of endocrine resistance remain to be discovered for the majority of ER-positive breast cancers.

Models of AI-resistant breast cancer have been based primarily on MCF-7 human breast cancer cells (5–7). To mimic the low estrogen levels seen in AI-treated patients (8) and gain a broad perspective of mechanisms of acquired hormone-independent growth common across several model systems, we generated long-term estrogen-deprived (LTED) derivatives from a panel of 4 ER-positive

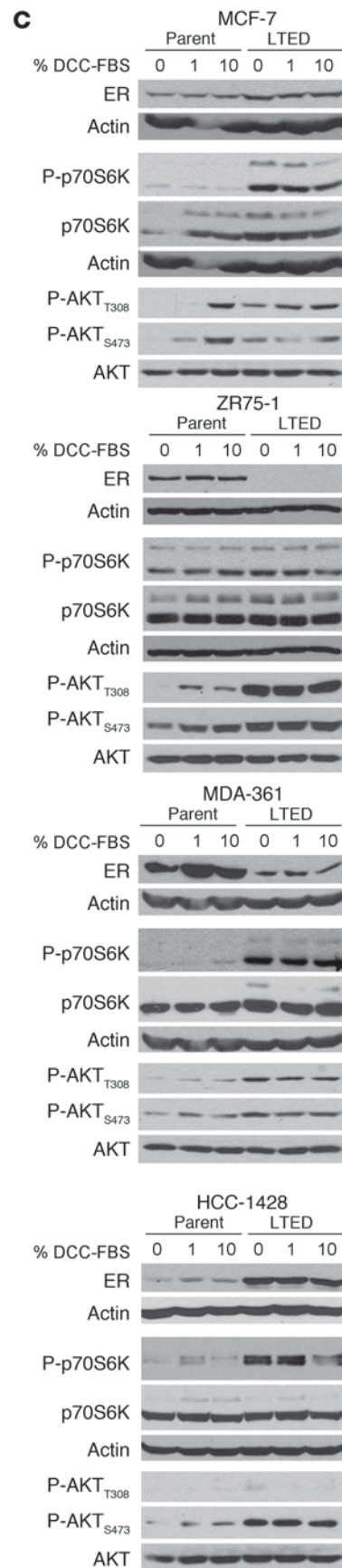
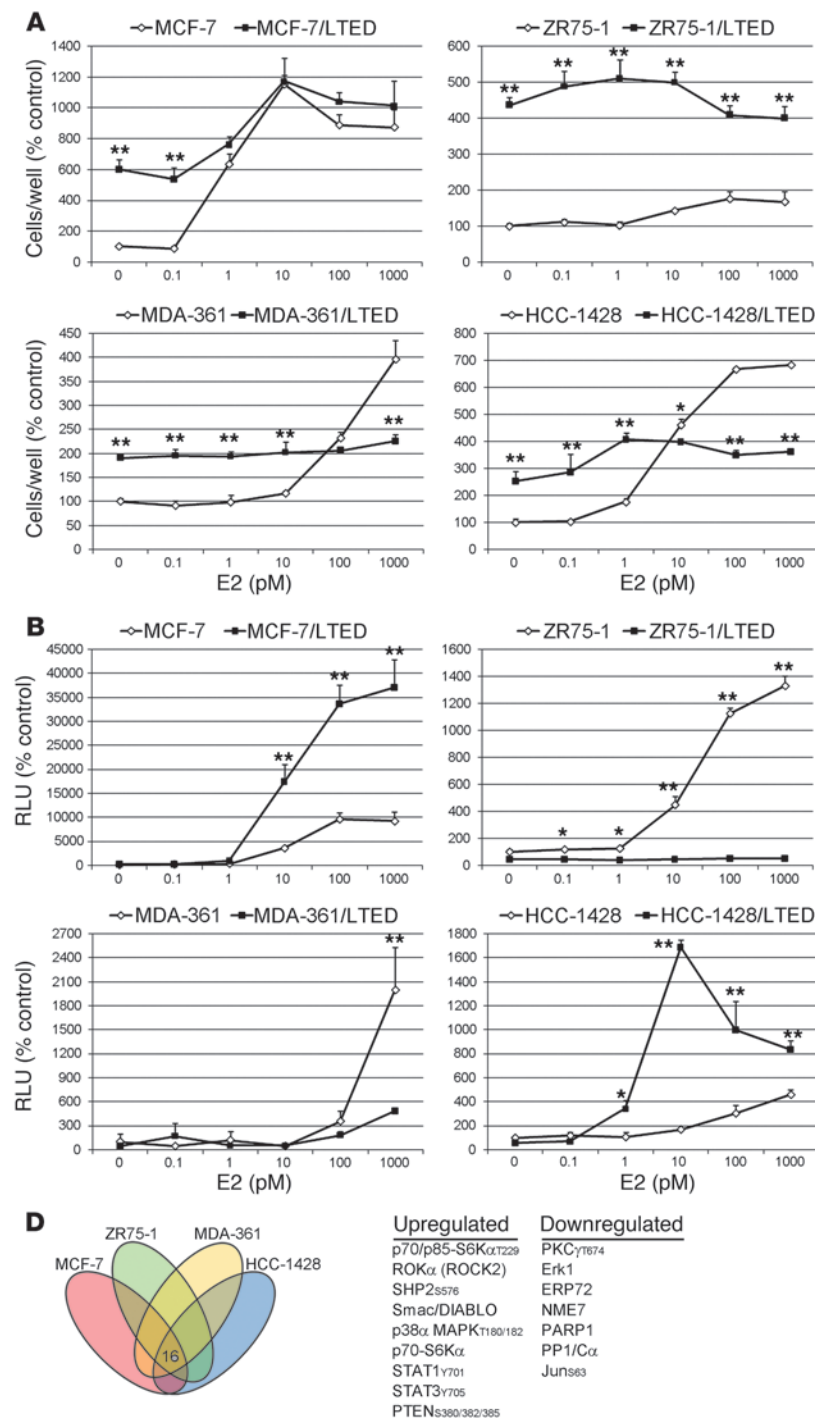
breast cancer cell lines. Proteomic and gene expression profiling identified amplification of the PI3K/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling pathway in LTED cells. Studies with kinase inhibitors suggested that PI3K is required for the adaptation of ER-positive cells to hormone deprivation. Furthermore, PI3K pathway activation, as measured by reverse-phase protein microarray analysis (RPPA) of primary tumors, predicted poor recurrence-free survival after adjuvant endocrine therapy.

## Results

*Variable changes in estrogen sensitivity accompany acquired hormone-independent cell growth.* We generated MCF-7, HCC1428, MDA-361, and ZR75-1 hormone-independent breast cancer cells after LTED in culture. LTED cells outgrew their respective parental counterparts under hormone-depleted conditions (10% dextran-charcoal-treated FBS [DCC-FBS]; Figure 1A). Prior reports on MCF-7/LTED cells suggest that hormone-independent growth is driven by increased ER expression and estrogen hypersensitivity (6, 9). We found that MCF-7/LTED cells showed 17 $\beta$ -estradiol (E2) sensitivity similar to that of parental controls in cell proliferation assays (Figure 1A). HCC-1428/LTED cells showed decreased sensitivity to exogenous E2 compared with parental controls, whereas ZR75-1/LTED and MDA-361/LTED cells were essentially insensitive to E2. We then determined whether such changes were reflected in ER transcrip-

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**Figure 1**

LTED cells exhibit PI3K/mTOR pathway hyperactivation and variable response to E2. (A) Cells were treated with 10% DCC-FBS with or without 0–1,000 pM E2. Media and drugs were replenished every 2–3 days. Adherent cells were counted after 5–10 days. (B) Cells transfected with luciferase reporter plasmids were treated as in A. Luciferase activities were measured after 16–20 hours. Data are presented as percent parental control (RLU, firefly/*Renilla*), mean of triplicates  $\pm$  SD, and were analyzed using 2-way ANOVA. \* $P$  < 0.05, \*\* $P$  < 0.01 versus parental cells, Bonferroni post-hoc test corrected for multiple comparisons. (C) Lysates from cells treated with 0%–10% DCC-FBS for 24 hours were analyzed by immunoblotting with the indicated antibodies. (D) Lysates from cells treated with 1% DCC-FBS for 24 hours were analyzed using antibody microarrays. Proteins/phosphoproteins altered at least 1.4-fold in LTED/parental comparisons were compared for overlap, which revealed 16 proteins/phosphoproteins commonly altered across all LTED lines.



tional activity. MCF-7/LTED and HCC-1428/LTED cells were hyperresponsive and/or hypersensitive to E2 in ER transcriptional reporter assays, whereas ZR75-1/LTED and MDA-361/LTED cells showed the opposite (Figure 1B). Such changes in ER transcriptional activity were paralleled by changes in ER protein (Figure 1C) and mRNA levels (data not shown). These results suggest that the acquired hormone-independent growth of LTED cells may not be consistently caused by estrogen hypersensitivity.

*LTED cells exhibit increased PI3K/mTOR pathway activation.* To explore mechanisms driving acquired hormone-independent cell growth, we interrogated lysates from LTED and parental cells using microarrays containing 652 antibodies against signaling proteins/phosphoproteins. The levels of 16 proteins/phosphoproteins were commonly altered 1.4-fold or greater across all 4 LTED lines compared with respective parental controls (Figure 1D). Levels of p70/p85 ribosomal protein S6 kinase (referred to herein as p70S6K) phosphorylated at amino acid T229, a site phosphorylated by 3'-phosphoinositide-dependent kinase 1 (PDK1), were increased in all LTED lines. PDK1 is activated by PI3K, and p70S6K phosphorylation at T229 requires prior phosphorylation at T389 by target of rapamycin complex 1 (TORC1; ref. 10), which is also downstream of PI3K (11). These data suggested that PI3K/mTOR signaling is increased in LTED cells. Indeed, immunoblot analysis revealed that MCF-7/LTED, MDA-361/LTED, and HCC-1428/LTED cells had increased levels of P-p70S6K<sub>T389</sub>, and all LTED lines had increased AKT phosphorylation at T308 and/or S473, the PDK1 and TORC2 sites, respectively (Figure 1C).

We also used gene expression microarray analysis to identify signaling pathways dysregulated in LTED cells. Sets of genes with expression significantly altered between each pair of LTED and parental cells ( $\geq 1.3$ -fold, FDR-adjusted  $P \leq 0.05$ ) were compared. Genes commonly dysregulated in all 4 LTED/parental comparisons were filtered for platform compatibility, and the resulting 35-gene set was used to query the Connectivity Map, a collection of gene signatures induced by the treatment of cells with a panel of 1,309 small molecules (12). Inhibition of PI3K or mTOR using wortmannin or sirolimus, respectively, induced gene signatures that were diametrically opposed to the 35-gene signature (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI41680DS1), which suggests that LTED cells exhibit increased PI3K/mTOR signaling.

PI3K is commonly activated by receptor tyrosine kinase (RTK) signaling. To explore which RTKs activate PI3K in LTED cells, we applied cell lysates to immunocapture arrays that included antibodies against 42 RTKs. Overall, cells showed predominant tyrosine phosphorylation of ErbB family members (EGFR, HER2, HER3, and HER4; Figure 2A) and receptors for insulin (InsR) and insulin-like growth factor-I (IGF-IR). Notably, MCF-7/LTED and ZR75-1/LTED cells showed increased P-IGF-IR and P-InsR, and MDA-361/LTED cells showed increased P-EGFR, P-HER2, P-HER3, and P-InsR. In combination with the data shown in Figure 1, these findings suggest that acquired hormone-independent growth is associated with hyperactivation of the IGF-IR/InsR/PI3K/mTOR pathway. IGF-IR hyperactivation has also been linked with tamoxifen resistance in MCF-7 cells (13). Pathway analysis of gene expression microarray profiles identified dysregulated IGF-IR/InsR signaling in LTED cells. Sets of genes with expression significantly altered between each pair of LTED and parental cells ( $\geq 1.5$ -fold, FDR-adjusted  $P \leq 0.05$ ) were evaluated using Ingenuity Pathways Analysis to group gene products by biological function.

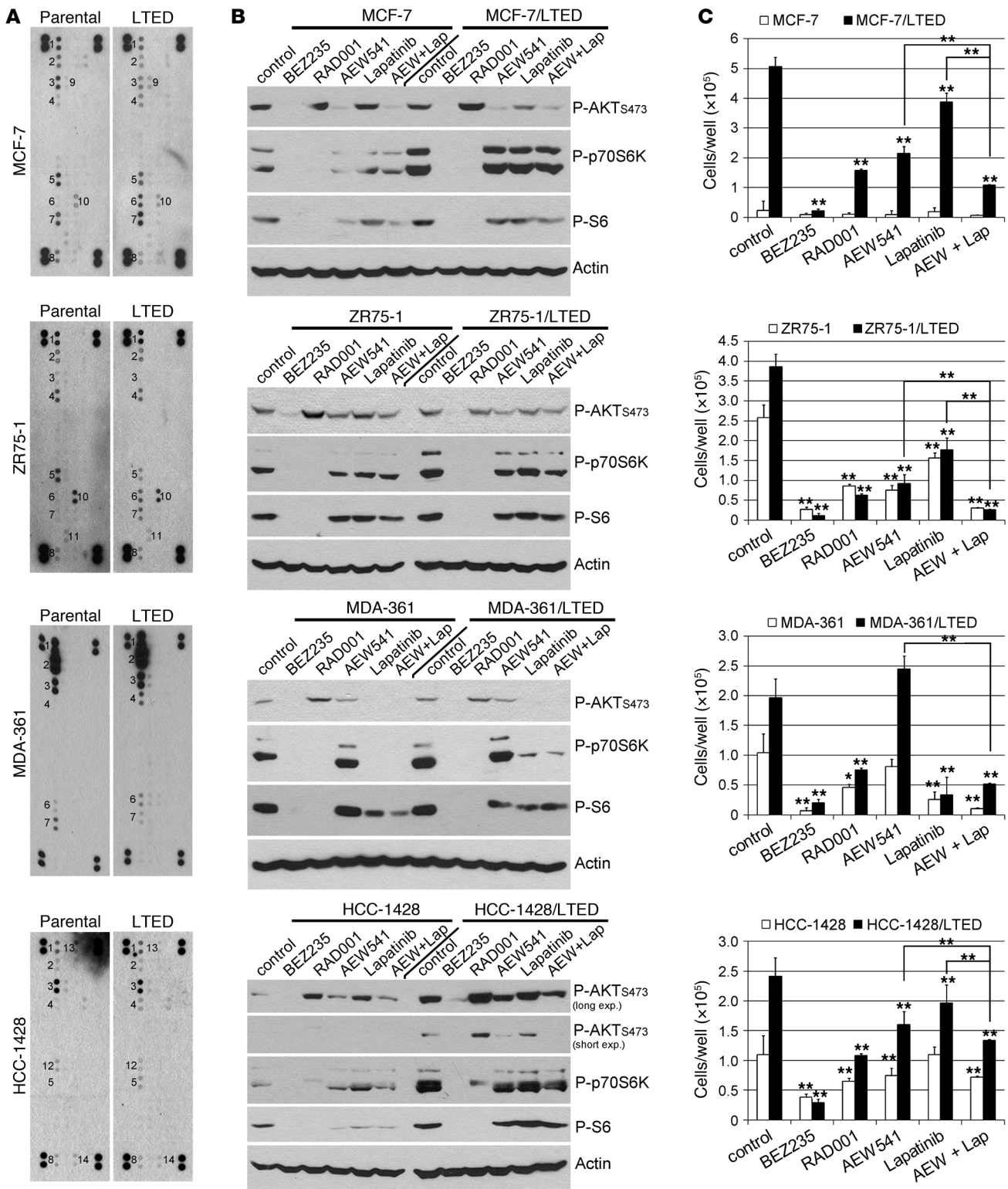
Profiles from 3 LTED/parental comparisons showed significant enrichment for genes encoding proteins implicated in IGF signaling (Supplemental Figure 2).

The requirement of IGF-IR and InsR signaling for LTED cell growth was confirmed using siRNA. Knockdown of either receptor inhibited MCF-7, MCF-7/LTED, and ZR75-1/LTED cell growth, and IGF-IR knockdown inhibited HCC-1428/LTED cell growth (Supplemental Figure 3). We further determined that IGF-IR activation in primary human breast tumors was linked with resistance to endocrine therapy. Through analysis of 64 ER-positive tumors by RPPA for levels of phosphorylated and total IGF-IR $\beta$ , we observed a significant correlation between P-IGF-IR $\beta$ /IGF-IR $\beta$  ratio and disease recurrence following endocrine therapy (median follow-up, 290 days; Cox proportional hazards [PH] model,  $P = 0.036$ ; hazard ratio, 1.705; 95% CI, 1.0073–2.8851). This P-IGF-IR $\beta$  antibody crossreacts with P-InsR $\beta$ , so we cannot rule out a role for InsR activation in this correlation. Patients were then dichotomized based on P-IGF-IR $\beta$ /IGF-IR $\beta$  ratio. A high P-IGF-IR $\beta$ /IGF-IR $\beta$  ratio correlated with poor patient outcome ( $P = 0.053$ , log-rank test; Supplemental Figure 3C), in agreement with previous findings linking IGF-IR activation to poor survival in breast cancer (14).

Using a loss-of-function approach with pharmacological inhibitors, we assessed which RTKs correlated with PI3K/mTOR pathway activation, measuring P-AKT<sub>S473</sub>, P-p70S6K<sub>T389</sub>, and P-S6. Treatment with the IGF-IR/InsR kinase inhibitor AEW541 (Supplemental Figure 4A and ref. 15) decreased P-AKT<sub>S473</sub> levels in MCF-7, MCF-7/LTED, ZR75-1/LTED, and HCC-1428/LTED cells (Figure 2B), which suggests that PI3K is mainly driven by IGF-IR and/or InsR signaling in these cells. Treatment with the EGFR/HER2 kinase inhibitor lapatinib (which blocks EGFR- and HER2-induced phosphorylation of HER3; Supplemental Figure 4B) decreased P-AKT<sub>S473</sub> levels in MCF-7/LTED, MDA-361, and MDA-361/LTED cells. Although treatment with RTK inhibitors modestly decreased levels of P-p70S6K<sub>T389</sub> and P-S6, treatment with the dual PI3K/mTOR inhibitor BEZ235 (16) drastically suppressed P-AKT<sub>S473</sub>, P-p70S6K<sub>T389</sub>, and P-S6 in all lines. Treatment with the mTOR inhibitor RAD001 (17) decreased P-p70S6K<sub>T389</sub> and P-S6, but increased P-AKT<sub>S473</sub>, likely as a result of feedback activation of PI3K (18). Therefore, concomitant inhibition of PI3K/mTOR appears to more effectively suppress pathway signaling than does blocking individual upstream RTKs or TORC1.

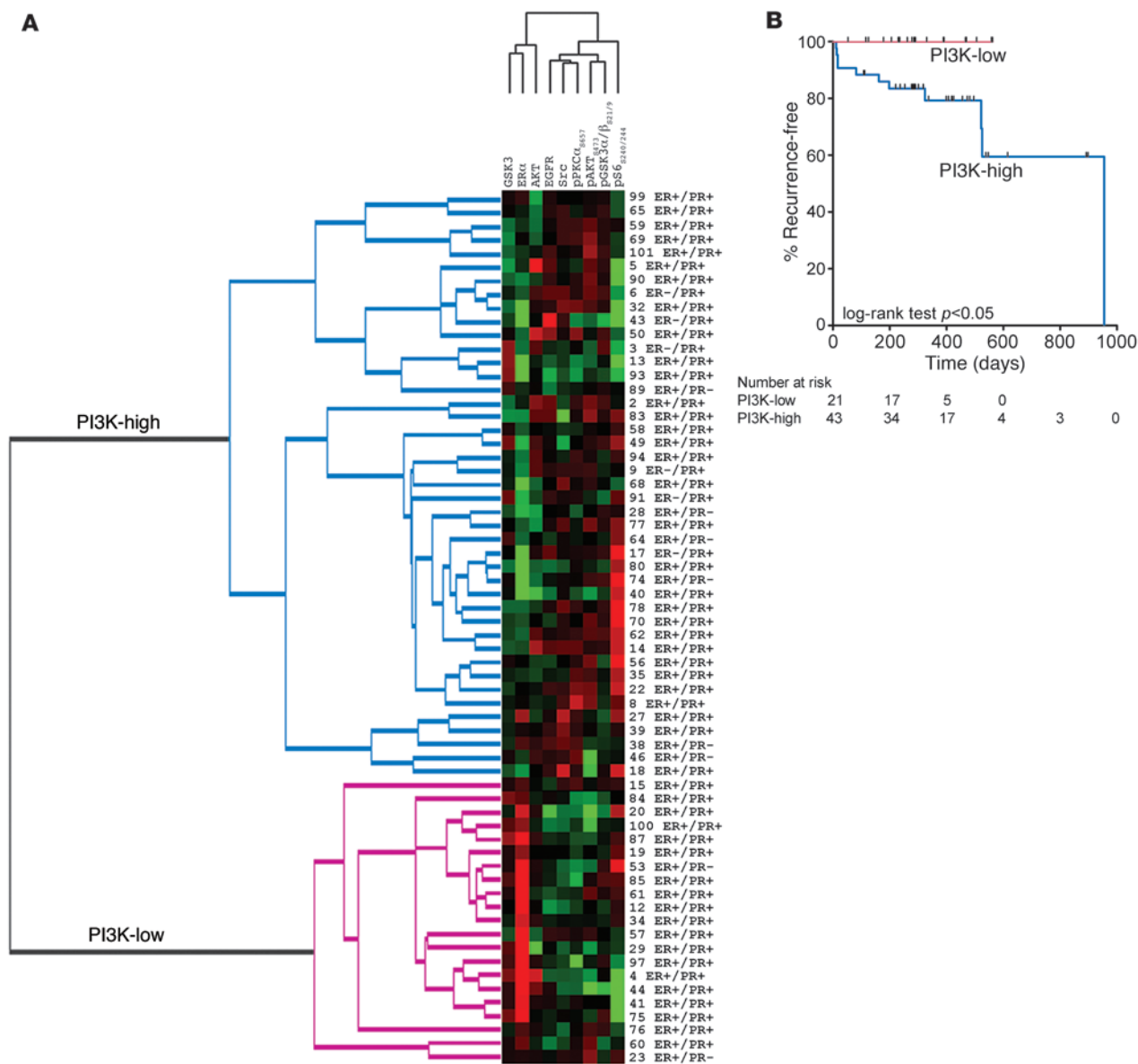
*Acquired hormone-independent cell growth requires PI3K signaling.* We next analyzed the effects of pathway inhibition on LTED cell growth and the acquisition of hormone independence. Treatment with BEZ235 potently suppressed the growth of both LTED and parental cells, whereas RAD001 treatment was modestly inhibitory (Figure 2C). Treatment with RTK inhibitors reduced LTED cell growth, and this effect correlated with the magnitude of inhibition of P-AKT<sub>S473</sub>. AEW541 treatment inhibited the hormone-independent growth of LTED and parental MCF-7, ZR75-1, and HCC-1428 cells, whereas lapatinib inhibited the growth of MDA-361 and MDA-361/LTED cells but was less effective against other cell lines. The combination of AEW541 and lapatinib more effectively inhibited growth of MCF-7/LTED, ZR75-1/LTED, and HCC-1428/LTED lines than did either agent alone, which suggests that ErbB and IGF-IR/InsR RTKs promote hormone-independent cell growth. However, inhibition of both PI3K (P-AKT<sub>S473</sub>) and mTOR (P-p70S6K<sub>T389</sub> and P-S6) using BEZ235 (Figure 2B) most effectively suppressed growth (Figure 2C), whereas inhibition of either PI3K (using RTK inhibitors) or





**Figure 2**

PI3K pathway inhibition suppresses hormone-independent cell growth. (A) Phospho-RTK arrays were probed with lysates from cells treated with 10% DCC-FBS for 24 hours. Signal indicates tyrosine phosphorylation. Blots from each pair of lines are exposure-matched. Blots are labeled as follows: 1, EGFR; 2, HER2; 3, HER3; 4, HER4; 5, FGFR4; 6, InsR; 7, IGF-IR; 8, Dtk; 9, MSP-R; 10, EphA1; 11, ROR2; 12, FGFR3; 13, Tie-2; 14, EphA4. Corner spots are positive controls. (B) Immunoblot analysis of lysates from cells treated for 24 hours with 10% DCC-FBS and the indicated kinase inhibitors using the indicated antibodies. (C) Cells were treated as in B. Media and drugs were replenished every 2–3 days. Adherent cells were counted after 5–10 days. Data are presented as mean of triplicates ± SD and were analyzed using 2-way ANOVA. \**P* < 0.05, \*\**P* < 0.01 versus control (or as indicated by brackets), Bonferroni post-hoc test corrected for multiple comparisons.



**Figure 3**  
 A tumor protein signature of PI3K activation predicts poor disease outcome following adjuvant endocrine therapy. **(A)** Lysates from 64 primary human breast tumors were analyzed by RPPA using antibodies against P-AKT<sub>S473</sub>, AKT, P-S6<sub>S240/244</sub>, P-GSK3 $\alpha/\beta$ <sub>S21/9</sub>, GSK3 $\alpha/\beta$ , EGFR, ER, Src, and P-PKC $\alpha$ <sub>S657</sub>. Log<sub>2</sub> mean-centered values were hierarchically clustered, which revealed PI3K-high (blue) and -low (red) tumor clusters. **(B)** Kaplan-Meier recurrence curves of the clusters in **A** were compared by log-rank test. The number of patients at risk of recurrence at different time points is noted below.

mTOR (using RAD001) was generally less effective. Similar phenomena were observed in anchorage-independent colony formation assays (Supplemental Figure 5). These data suggest that inhibition of both PI3K and mTOR is required for maximal inhibition of hormone-independent cell growth.

To determine whether PI3K signaling is required for the acquisition of hormone independence, parental cells were seeded at low density and reselected for hormone-independent growth in the presence of kinase inhibitors. BEZ235 treatment blocked the emergence of hormone-independent cells in all lines (Supplemental Figure 6A). RAD001 treatment partially suppressed cell

growth, but residual adherent cells suggested a cytostatic effect. Importantly, treatment with BEZ235 induced apoptosis in LTED and parental MCF-7, ZR75-1, and MDA-361 cells, while RAD001 treatment induced apoptosis only in ZR75-1/LTED cells (Supplemental Figure 6B). These results suggest that PI3K inhibition prevents the emergence of hormone-independent cells by inducing cell death. Inhibiting PI3K activation with the RTK inhibitors AEW541 or lapatinib suppressed the emergence of hormone-independent cells less effectively than did inhibiting PI3K directly with BEZ235 (Supplemental Figure 6A), in parallel with the ability of RTK inhibitors to partially block PI3K signaling (Figure 2B).



*A baseline protein signature of PI3K activation predicts poor outcome after adjuvant endocrine therapy.* Because hormone-independent cell growth required PI3K signaling (Figure 2 and Supplemental Figures 5 and 6), and LTED cells showed PI3K pathway hyperactivation (Figure 1), we sought to determine whether the degree of PI3K pathway activation is predictive of disease outcome following endocrine therapy. To comprehensively represent the activation status of the PI3K pathway, we analyzed lysates from 64 hormone receptor-positive primary breast tumors (same cohort as in Supplemental Figure 2C) by RPPA using antibodies against known PI3K pathway markers (P-AKT<sub>S473</sub>, AKT, P-S6<sub>S240/244</sub>, P-GSK3 $\alpha$ / $\beta$ <sub>S21/9</sub>, GSK3 $\alpha$ / $\beta$ , EGFR, ER, Src, and P-PKC $\alpha$ <sub>S657</sub>). Hierarchical clustering of tumors revealed 2 major clusters with relatively high and low levels of PI3K pathway markers (Figure 3A). Notably, levels of PI3K pathway markers and ER were inversely correlated. We then generated Kaplan-Meier recurrence curves of the 2 patient clusters. Whereas 0 of 21 patients with PI3K-low tumors relapsed within the time of follow-up, 11 of 43 (26%) patients with PI3K-high tumors showed disease recurrence ( $P < 0.05$ , log-rank test; Figure 3B). In the univariate Cox PH model, the PI3K cluster effect was significantly associated with recurrence-free survival ( $P < 0.01$ ). Therefore, this protein signature of PI3K pathway activation is predictive of poor disease outcome following adjuvant endocrine therapy. In a multivariate analysis, clinical stage accounted for much of the information provided by the PI3K cluster effect. Shorter recurrence-free survival was independently associated with increased clinical stage ( $P < 0.05$ ), in agreement with prior reports correlating PI3K pathway alterations (i.e., *PTEN* loss and *HER2* overexpression) with tumor stage (19, 20). There was no association between recurrence-free survival and type of endocrine therapy, use of adjuvant chemotherapy, age, or nuclear grade by multivariate analysis.

## Discussion

Herein, we identified PI3K as a critical hub in the emergence and maintenance of hormone-independent, ER-positive breast cancer cells. We found that acquired hormone-independent (i.e., LTED) breast cancer cell growth was associated with hyperactivation of the IGF-IR/InsR/PI3K/mTOR pathway. Direct inhibition of PI3K effectively suppressed the hormone-independent growth of both estrogen-independent and -dependent cells, whereas inhibition of nodes upstream (RTKs) and downstream (mTOR) of PI3K only partially blocked cell growth. In support of these findings, we identified a protein signature of PI3K pathway activation that was predictive of poor disease outcome following adjuvant endocrine therapy in breast cancer patients.

Although prior reports indicate that MCF-7 cells selected for hormone independence exhibit hypersensitivity to exogenous E2 (6, 9), we found no such change in MCF-7/LTED cells. The 3 other LTED lines showed an attenuated response to E2 in growth assays (Figure 1). In ER transcriptional reporter assays, MCF-7/LTED and HCC-1428/LTED cells showed hyperresponsiveness to E2, whereas the other 2 LTED lines showed the opposite. Such changes in ER transcriptional activity were paralleled by changes in ER levels, which suggests that ER content may be a marker of estrogen sensitivity. Indeed, increased ER levels in breast cancer cells have previously been linked with improved patient outcome following endocrine therapy (21–23). Additionally, decreased ER levels following therapy with tamoxifen or AIs have been shown to predict shorter time to recurrence (24, 25). Importantly, 3 of 4 LTED lines remained ER positive and sensitive to nanomolar

concentrations of E2 (Figure 1A), which suggests that cessation of estrogen deprivation with an AI in patients who progress on this therapy may not always be warranted. Discontinuation of an AI would result in resumption of estrogen production, stimulation of hormone-independent but still hormone-responsive cancer, and protection from an alternative therapy.

We and others previously found that activation of the PI3K pathway (by *PTEN* knockdown or *HER2* overexpression) promotes antiestrogen resistance and hormone independence in ER-positive breast cancer cells (26, 27). Herein, we report that intrinsic PI3K signaling was required for the acquisition and maintenance of hormone independence. These findings corroborate the observation that mice bearing MCF-7/aromatase xenografts resistant to the AI letrozole showed modestly inhibited tumor growth when treated with the PI3K inhibitor wortmannin (28). Moreover, treatment of MCF-7 and MCF-7/LTED cells with the Ras inhibitor farnesylthiosalicylic acid decreased mTOR signaling and hormone-independent growth (29). We found that RAD001 treatment blocked TORC1, but feedback activation of PI3K/AKT (Figure 2B) likely hampered its ability to completely inhibit hormone-independent cell growth (Figure 2C and Supplemental Figures 5 and 6). In support of these findings, neoadjuvant treatment of patients bearing ER-positive tumors with letrozole and RAD001 decreased the fraction of Ki67-positive tumor cells and induced clinical responses more effectively than did letrozole alone (30). Interestingly, neoadjuvant therapy with letrozole decreased tumor levels of PI3K, P-AKT<sub>S473</sub>, and P-mTOR<sub>S2448</sub>. Because reductions in P-AKT<sub>S473</sub> and P-mTOR<sub>S2448</sub> correlate with better response rates and outcome (31), we speculate that estrogen deprivation may suppress cell growth in part by decreasing PI3K signaling.

Treatment with the PI3K/mTOR inhibitor BEZ235 inhibited growth of all cell lines and induced apoptosis in 3 of 4 LTED and 3 of 4 parental cell lines, thereby preventing the emergence of hormone-independent cells (Figure 2C and Supplemental Figures 5 and 6). Notably, the 3 parental/LTED cell line pairs most sensitive to BEZ235 were MCF-7, MDA-361, and ZR75-1, which harbor mutations in *PIK3CA* (MCF-7 and MDA-361) or *PTEN* (ZR75-1; ref. 32) or gene amplification of *HER2* (MDA-361). These mutations result in hyperactivation of the PI3K pathway (33) and confer sensitivity to PI3K and AKT inhibitors (34–36). HCC-1428 cells, which were growth arrested by BEZ235 but did not undergo apoptosis, have no known PI3K pathway mutations. These data suggest that PI3K pathway-activating mutations may generate dependence on this pathway for cell survival. However, *PIK3CA* mutations and disease outcome following endocrine therapy are not consistently correlated (30, 37–39), which suggests that alternative measurements of PI3K pathway activation as shown herein (Figure 3) may provide additional predictive information.

In summary, our findings suggest that patients with hormone receptor-positive tumors exhibiting a high degree of PI3K signaling, and patients who relapse on endocrine therapy, may benefit from therapeutics targeting both the ER and the PI3K pathways. Although directly targeting PI3K and mTOR maximally inhibited hormone-independent cell growth and induced apoptosis, inhibition of signaling kinases upstream (IGF-IR/InsR/ErbBs) and downstream (mTOR) of PI3K also had partial inhibitory effects. Additionally, inhibition of PI3K prevented the emergence of hormone-independent cells, which suggests that early intervention with combined endocrine and PI3K-directed therapies could limit escape from antiestrogens in patients with ER-positive breast cancer.





**Methods**

**Cell lines.** MCF-7, ZR75-1, MDA-361, and HCC-1428 cells (ATCC) were maintained in improved modified Eagle medium (IMEM) with 10% FBS (Invitrogen). LTED cells were generated through culture in phenol red-free IMEM with 10% DCC-FBS (Hyclone; contains <0.0367 pM E2) for 3, 3, 7, and 7 months, respectively. Cells were defined as LTED when sustainable cell populations emerged that outgrew parental cells under hormone-depleted conditions.

**Cell proliferation assays.** Cells seeded in triplicate in 12-well plates ( $2.5 \times 10^4$  cells/well for MCF-7 lines;  $4 \times 10^4$  cells/well for other lines) were treated with 10% DCC-FBS with or without 0–1 nM E2, 200 nM BEZ235 (16), 1  $\mu$ M AEW541 (15), 20 nM RAD001 (ref. 17, all provided by Novartis), or 1  $\mu$ M lapatinib ditosylate (GW-572016; LC Laboratories). Media and inhibitors were replenished every 2–3 days, and after 5–10 days, cells were trypsinized and counted using a Coulter counter.

**ER transcriptional reporter assay.** Cells were plated as above and transfected with pGLB-MERE (encodes estrogen response element-regulated *Firefly* luciferase) and pCMV-Renilla (encodes CMV-driven *Renilla* luciferase; Promega) plasmids. Cells were then treated as above, and luciferase activities were measured 16–20 hours later as described previously (40).

**Antibody microarray analysis.** Cells were treated for 24 hours with 1% DCC-FBS. Protein lysates were quantitated using BCA assay (Pierce). Lysates from LTED and parental cell line pairs were labeled with different fluorescent dyes, mixed at a 1:1 ratio, and used to probe immunocapture microarrays containing antibodies against 652 antigens in duplicate (see Supplemental Methods for details). LTED<sub>mean</sub> protein levels were considered different from parental<sub>mean</sub> levels when fold change was 1.4 or greater.

**Immunoblotting and RTK arrays.** Cells were treated as indicated, then lysed in NP-40 buffer plus protease and phosphatase inhibitors, sonicated for 10 seconds, and centrifuged at 18,000 g for 10 minutes. Protein was quantitated as described above. Lysates were analyzed by immunoblotting using antibodies against ER $\alpha$ , p70S6K (Santa Cruz Biotechnology), AKT, P-AKT<sub>S473</sub>, P-AKT<sub>T308</sub>, P-p70S6K<sub>T389</sub>, P-S6<sub>S240/244</sub> (Cell Signaling), and actin (Sigma-Aldrich). Phospho-RTK arrays were performed according to the manufacturer’s protocol (R&D Systems).

**RPPA.** We obtained 64 hormone receptor-positive primary breast tumor samples, collected under IRB-approved protocols, from the M.D. Anderson Cancer Center Breast Tissue Frozen Tumor Bank. Patients prospectively provided informed consent for the study before tissue collection. Details

of patient therapy and tumor protein analysis using antibodies against P-IGF-IR $\beta_{Y1135/1136}$  (cross-reacts with P-InsR $\beta_{Y1131}$ ), IGF-IR $\beta$ , P-AKT<sub>S473</sub>, AKT, P-S6<sub>S240/244</sub>, P-GSK3 $\alpha/\beta_{S21/9}$  (Cell Signaling), GSK3 $\alpha/\beta$ , EGFR (Santa Cruz), ER (Lab Vision), Src, and P-PKC $\alpha_{S657}$  (Upstate) are provided in Supplemental Methods and ref. 41. Data were hierarchically clustered, and Kaplan-Meier recurrence curves for each patient cluster were compared using log-rank testing. Cox PH univariate regression was used to assess the association of cluster effect with recurrence-free survival. Multivariate analysis included clinical stage, type of endocrine therapy, adjuvant chemotherapy, age, and nuclear grade.

**Statistics.** In cell proliferation and ER transcriptional reporter assays, significant differences were determined by 2-way ANOVA and Bonferroni post-hoc tests adjusted for multiple comparisons. A *P* value less than 0.05 was considered significant.

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- Forbes JF, et al. Arimidex, Tamoxifen, Alone or in Combination (ATAC) Trialists’ Group. Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 100-month analysis of the ATAC trial. *Lancet Oncol.* 2008;9(1):45–53.
- Ellis MJ, et al. Estrogen-independent proliferation is present in estrogen-receptor HER2-positive primary breast cancer after neoadjuvant letrozole. *J Clin Oncol.* 2006;24(19):3019–3025.
- Arpino G, et al. HER-2 amplification, HER-1 expression, and tamoxifen response in estrogen receptor-positive metastatic breast cancer: a southwest oncology group study. *Clin Cancer Res.* 2004;10(17):5670–5676.
- De Laurentiis M, et al. A meta-analysis on the interaction between HER-2 expression and response to endocrine treatment in advanced breast cancer. *Clin Cancer Res.* 2005;11(13):4741–4748.
- Jelovac D, Sabnis G, Long BJ, Macedo L, Goloubeva OG, Brodie AM. Activation of mitogen-activated protein kinase in xenografts and cells during prolonged treatment with aromatase inhibitor letrozole. *Cancer Res.* 2005;65(12):5380–5389.
- Chan CM, Martin LA, Johnston SR, Ali S, Dowsett M. Molecular changes associated with the acquisition of oestrogen hypersensitivity in MCF-7 breast cancer cells on long-term oestrogen deprivation. *J Steroid Biochem Mol Biol.* 2002;81(4–5):333–341.
- Jeng MH, et al. Estrogen receptor expression and function in long-term estrogen-deprived human breast cancer cells. *Endocrinology.* 1998;139(10):4164–4174.
- Dixon JM, et al. Letrozole suppresses plasma estradiol and estrone sulphate more completely than anastrozole in postmenopausal women with breast cancer. *J Clin Oncol.* 2008;26(10):1671–1676.
- Yue W, Wang JP, Conaway MR, Li Y, Santen RJ. Adaptive hypersensitivity following long-term estrogen deprivation: involvement of multiple signal pathways. *J Steroid Biochem Mol Biol.* 2003;86(3–5):265–274.
- Pullen N, et al. Phosphorylation and activation of p70s6k by PDK1. *Science.* 1998;279(5351):707–710.
- Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. *Cancer Cell.* 2007;12(1):9–22.
- Lamb J, et al. The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. *Science.* 2006;313(5795):1929–1935.
- Massarweh S, et al. Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function. *Cancer Res.* 2008;68(3):826–833.
- Law JH, et al. Phosphorylated insulin-like growth factor-1/insulin receptor is present in all breast cancer subtypes and is related to poor survival. *Cancer Res.* 2008;68(24):10238–10246.
- Garcia-Echeverria C, et al. In vivo antitumor activity of NVP-AEW541-A novel, potent, and selective inhibitor of the IGF-IR kinase. *Cancer Cell.* 2004;5(3):231–239.
- Maira SM, et al. Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. *Mol Cancer Ther.* 2008;7(7):1851–1863.
- Schuler W, et al. SDZ RAD, a new rapamycin derivative: pharmacological properties in vitro and in vivo. *Transplantation.* 1997;64(1):36–42.
- O’Reilly KE, et al. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res.* 2006;66(3):1500–1508.
- Borg A, et al. HER-2/neu amplification predicts poor survival in node-positive breast cancer. *Cancer Res.* 1990;50(14):4332–4337.
- Pandolfi PP. Breast cancer—loss of PTEN predicts resistance to treatment. *N Engl J Med.* 2004;351(22):2337–2338.
- Ellis MJ, et al. Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1- and/or ErbB-2-positive, estrogen receptor-positive primary breast cancer: evidence from a phase III ran-





- domized trial. *J Clin Oncol*. 2001;19(18):3808–3816.
22. Viale G, et al. Prognostic and predictive value of centrally reviewed expression of estrogen and progesterone receptors in a randomized trial comparing letrozole and tamoxifen adjuvant therapy for postmenopausal early breast cancer: BIG 1-98. *J Clin Oncol*. 2007;25(25):3846–3852.
23. Dowsett M, et al. Relationship between quantitative estrogen and progesterone receptor expression and human epidermal growth factor receptor 2 (HER-2) status with recurrence in the Arimidex, Tamoxifen, Alone or in Combination trial. *J Clin Oncol*. 2008;26(7):1059–1065.
24. Johnston SR, et al. Changes in estrogen receptor, progesterone receptor, and pS2 expression in tamoxifen-resistant human breast cancer. *Cancer Res*. 1995;55(15):3331–3338.
25. Ellis MJ, et al. Outcome prediction for estrogen receptor-positive breast cancer based on postneoadjuvant endocrine therapy tumor characteristics. *J Natl Cancer Inst*. 2008;100(19):1380–1388.
26. Miller TW, et al. 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. *Cancer Res*. 2009;69(10):4192–4201.
27. Shou J, et al. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. *J Natl Cancer Inst*. 2004;96(12):926–935.
28. Sabnis G, Goloubeva O, Jelovac D, Schayowitz A, Brodie A. Inhibition of the phosphatidylinositol 3-kinase/Akt pathway improves response of long-term estrogen-deprived breast cancer xenografts to antiestrogens. *Clin Cancer Res*. 2007;13(9):2751–2757.
29. Yue W, Wang J, Li Y, Fan P, Santen RJ. Farnesylthiosalicylic acid blocks mammalian target of rapamycin signaling in breast cancer cells. *Int J Cancer*. 2005;117(5):746–754.
30. Baselga J, et al. Phase II randomized study of neoadjuvant everolimus plus letrozole compared with placebo plus letrozole in patients with estrogen receptor-positive breast cancer. *J Clin Oncol*. 2009;27(16):2630–2637.
31. Generali D, et al. Down-regulation of phosphatidylinositol 3'-kinase/AKT/molecular target of rapamycin metabolic pathway by primary letrozole-based therapy in human breast cancer. *Clin Cancer Res*. 2008;14(9):2673–2680.
32. Hollestelle A, Elstrodt F, Nagel JH, Kallemeijn WW, Schutte M. Phosphatidylinositol-3-OH kinase or RAS pathway mutations in human breast cancer cell lines. *Mol Cancer Res*. 2007;5(2):195–201.
33. Kang S, Bader AG, Vogt PK. Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. *Proc Natl Acad Sci U S A*. 2005;102(3):802–807.
34. Ihle NT, et al. Mutations in the phosphatidylinositol-3-kinase pathway predict for antitumor activity of the inhibitor PX-866 whereas oncogenic Ras is a dominant predictor for resistance. *Cancer Res*. 2009;69(1):143–150.
35. She QB, et al. Breast tumor cells with PI3K mutation or HER2 amplification are selectively addicted to Akt signaling. *PLoS ONE*. 2008;3(8):e3065.
36. Brachmann SM, et al. Specific apoptosis induction by the dual PI3K/mTor inhibitor NVP-BE235 in HER2 amplified and PIK3CA mutant breast cancer cells. *Proc Natl Acad Sci U S A*. 2009;106(52):22299–22304.
37. Stemke-Hale K, et al. An integrative genomic and proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer. *Cancer Res*. 2008;68(15):6084–6091.
38. Kalinsky K, et al. PIK3CA mutation associates with improved outcome in breast cancer. *Clin Cancer Res*. 2009;15(16):5049–5059.
39. Ellis MJ, et al. Phosphatidylinositol-3-kinase alpha catalytic subunit mutation and response to neoadjuvant endocrine therapy for estrogen receptor positive breast cancer. *Breast Cancer Res Treat*. 2010;119(2):379–390.
40. Kurokawa H, et al. Inhibition of HER2/neu (erbB-2) and mitogen-activated protein kinases enhances tamoxifen action against HER2-overexpressing, tamoxifen-resistant breast cancer cells. *Cancer Res*. 2000;60(20):5887–5894.
41. Tibes R, et al. Reverse phase protein array: validation of a novel proteomic technology and utility for analysis of primary leukemia specimens and hematopoietic stem cells. *Mol Cancer Ther*. 2006;5(10):2512–2521.