Pak1 Kinase Links ErbB2 to β-Catenin in Transformation of Breast Epithelial Cells

Luis E. Arias-Romero1, Olga Villamar-Cruz1, Min Huang2, Klaus P. Hoeflich3, and Jonathan Chernoff1

Abstract

p21-Activated kinase-1 (Pak1) is frequently upregulated in human breast cancer and is required for transformation of mammary epithelial cells by ErbB2. Here, we show that loss of Pak1, but not the closely related Pak2, leads to diminished expression of β-catenin and its target genes. In MMTV-ErbB2 transgenic mice, loss of Pak1 prolonged survival, and mammary tissues of such mice showed loss of β-catenin. Expression of a β-catenin mutant bearing a phospho-mimetic mutation at Ser 675, a specific Pak1 phosphorylation site, restored transformation to ErbB2-positive, Pak1-deficient mammary epithelial cells. Mice bearing xenografts of ErbB2-positive breast cancer cells showed tumor regression when treated with small-molecule inhibitors of Pak or β-catenin, and combined inhibition by both agents was synergistic. These data delineate a signaling pathway from ErbB2 to Pak to β-catenin that is required for efficient transformation of mammary epithelial cells, and suggest new therapeutic strategies in ErbB2-positive breast cancer. Cancer Res; 73(12); 1–12. ©2013 AACR.

Introduction

The ErbB2 oncogene is amplified in approximately 30% of human breast cancers and represents a clinically useful therapeutic target (1). ErbB2 proteins frequently heterodimerize with ErbB1 or ErbB3, and these heterodimers activate a signaling program that drives cell proliferation, resistance to apoptosis, loss of polarity, and increased motility and invasiveness (2). Key signaling pathways that emanate from ErbB2 include, but are not limited to, the phosphoinositide 3-kinase (PI3K)/Akt/mTOR, Ras/ERK, and Src/Fak networks (3).

p21-activated kinases (Pak) are Cdc42/Rac–activated serine-threonine protein kinases that regulate the PI3K/Akt, Ras/ERK, and Src/Fak signaling pathways (4, 5). Pak1 is required for activation of Akt, perhaps due to a scaffolding function that links Pdk1 to Akt (6, 7). In Ras/ERK signaling, Pak1 phosphorylates c-Raf at S338 and Mek1 at S298, sites that are required for full activation of these proteins in some cell types (8, 9). Pak1 also acts downstream in the Src/Fak pathway, as an effector for the small GTPase Rac1 (10, 11). Loss of Pak1, induced by siRNA, expression of dominant-negative alleles, gene disruption, or small-molecule inhibitors, has been shown to block transformation in vitro by oncogenic forms of Kras, ErbB2, and KSHV (9, 12–16). In addition, Pak1 is frequently overexpressed in human breast, ovary, bladder, uterine, and brain cancer, due to amplification of the PAK1 gene in an 11q13 amplicon (9), and has oncogenic properties when expressed in mouse breast epithelial cells and tissues (17, 18). However, the role of Pak1 in tumorigenesis in vivo, and the particular signaling pathways affected, is not defined. In addition, the apparent special role of Pak1, versus the closely related and broadly expressed Pak2, is not understood.

In this work, we examined the distinct roles of Pak1 and Pak2 in cellular and animal models of ErbB2-driven breast cancer. We found that inhibition of Pak1, but not Pak2, impedes transformation by ErbB2 in a 3-dimensional (3D) cell culture system, but that loss of either Pak1 or Pak2 causes loss of both ERK and Akt activation. A phospho-proteomic screen revealed that Pak1-deficient, but not Pak2-deficient, ErbB2 cells showed almost total loss of β-catenin expression, and that exogenous expression of a stabilized β-catenin or a mutant form bearing a phosphomimetic residue at a known site of Pak1 phosphorylation (S675E), restored the ability of ErbB2 to transform Pak1-deficient cells. Finally, we showed that small-molecule inhibitors of Pak or β-catenin blocked transformation by ErbB2 in 3D culture and tumorigenesis by ErbB2 in mouse xenografts. Combined use of anti-Pak and anti-β-catenin agents was synergistic. These findings establish Pak1 as a new target in ErbB2-driven breast cancer and define a new mechanism of action primarily through the β-catenin, but not the ERK or Akt, signaling pathways.

Materials and Methods

Generation of transgenic mice and tumor measurement

All animal experiments were approved by the Fox Chase Cancer Center Institutional Animal Care and Use Committee (IACUC) and carried out according to NIH-approved protocols in compliance with the guide for the Care and Use of Laboratory Animals. Detailed methods are contained in Supplementary Materials and Methods.
Cell lines and 3D cell culture

Tumors from MMTV-Neu/Pak1+/− and MMTV-Neu/Pak1−/− animals were dissected, rinsed with PBS and minced, then treated with 0.2% collagenase for 2 hours at 37°C. Cells were washed several times with serum-free Dulbecco’s Modified Eagle Medium (DMEM), and finally with low calcium medium supplemented with 5% horse serum. Tissues kept in low calcium medium were transferred to T-25 flasks coated with 0.1% gelatin and incubated in a 37°C incubator overnight. The next day, supernatants containing floating cells were transferred and seeded into new flasks and maintained with regular media changes until confluence after 6 to 8 weeks.

Fibroblast-free cell populations were derived typically after 6 to 8 weeks. In vitro proliferation was measured by seeded approximately 1 × 105 cells on 0.1% gelatin-coated T25 flasks. At specific time points, cells were trypsinized and counted using Trypan blue exclusion analysis. All analyses used cells passaged less than 6 times.

10A.ErbB2 cells (MCF-10A cells expressing a chimeric form of ErbB2; ref. 19) were maintained in DMEM/F12 (Gibco BRL) supplemented with 5% donor horse serum, 20 ng/mL EGF (Harlan Bioproducts), 10 μg/mL insulin (Sigma), 1 ng/mL cholera toxin (Sigma), 100 μg/mL hydrocortisone (Sigma), 50 U/mL penicillin, and 50 μg/mL streptomycin. For 3D cultures, approximately 5,000 cells were plated atop reconstituted basement membrane (rBM) in 8-well slide chambers as described (19). To activate chimeric ErbB proteins, 1 μmol/L AP1510 was added to the growth medium. MCF-7, MDA-MB-231, BT-474, and SK-BR3 were obtained from American Type Culture Collection, MCF-7 and MDA-MB-231 cells were grown in DMEM supplemented with 10% FBS, BT-474 cells were grown in RPMI supplemented with 10% FBS and SK-BR3 were grown in McCoy’s 5A supplemented with 10% FBS. BT-474R cells were a kind gift from Dr. Jose Baselga (Massachusetts General Hospital, Boston, MA).

Tissue preparation, histology, immunohistochemistry, and immunoblotting

All tumor samples and control tissues were fixed overnight in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Hematoxylin and eosin (H&E)-stained sections were used for diagnostic purposes and unstained sections for immunohistochemical studies. Protein concentration was determined, and equal amounts of total proteins were separated on SDS-PAGE. A detailed list of antibodies used is contained in Supplementary Fig. S1. All tumor samples and control tissues were assayed for proliferation and apoptosis. Upon doxycycline induction, the Pak1 and Pak2 shRNA-infected cells showed specific loss of their intended target (Fig. 1A). Loss of Pak1, but not Pak2, impeded the proliferation (Fig. 1B) and survival (Fig. 1C) of 10A.ErbB2 cells, similar results were observed in BT-474, SK-BR3, MCF-7, and MDA-MB-231 (Supplementary Fig. S1B–S1D). These data suggest that only Pak1 is required for ErbB2 effects on 2 of the cardinal features of transformation: cell division and apoptosis.

Pak1 knockdown restores normal acinar morphology in 3D cell culture

To assess the effects of Pak1 and Pak2 on acinar development and architecture, we plated the shRNA-expressing 10A.ErbB2 cells in a 3D Matrigel matrix. Under such conditions, induction of ErbB2 induces a striking phenotype characterized by formation of multilobular acini (Fig. 1D, a and b), loss of central apoptosis (Fig. 1D, c and d), and increase in proliferation (Fig. 1D, e and f). Expression of Pak1-directed shRNA had little effect on noninduced 10A.ErbB2 cells (Fig. 1D, g, i, and k), but strongly suppressed the transformed phenotype of induced cells (Fig. 1D, h, j, and l). In contrast, Pak2-directed shRNA had little effect on acinar structure irrespective of ErbB2 expression (Fig. 1D, m–r).

As Pak1 and Pak2 have similar catalytic properties, we sought to uncover differences in their behavior in breast epithelial cells. We and others had earlier established that Pak1 can be driven into the nucleus upon EGF receptor activation in mammary epithelial cells (20, 21); we therefore asked whether Pak2 responded in a similar manner. 10A.ErbB2 cells, stably transduced with GFP-Pak1 or GFP-Pak2, were stimulated with EGF or AP1510 for 10 minutes and the distribution of Pak1 and Pak2 determined by immunofluorescence (Supplementary Fig. S2A–S2C) and by subcellular fractionation (Supplementary Fig. S2D). As expected, a substantial fraction of Pak1 redistributed from the cytoplasm to the nucleus upon EGF or AP1510 stimulation. In contrast, Pak2 was never detected in the nucleus.

Molecular pathways affected by Pak1 and Pak2

Paks have been implicated in regulating ERK signaling downstream of Ras via phosphorylation of Mek1 and c-Raf (5, 12, 22, 23), and Pak1 has also been implicated in activating Akt via a scaffolding interaction with PDK1 (6, 13). Given that loss of Pak1, but not Pak2, impeded ErbB2 oncogenic signaling, we asked whether these 2 Pak isoforms affected different pathways in breast epithelial cells.
Figure 1. Pak1, but not Pak2, is required for ErbB2-mediated transformation of MCF-10A cells. A, Western blot analysis shows specific loss of Pak1 and Pak2 in shRNA-infected cells. B, proliferation of Pak1 and Pak2 10A.ErbB2-deficient cells upon ErbB2 stimulation. Cells were seeded, harvested, and counted at 0, 24, 48, 72, and 96 hours. The data are representative of 3 independent experiments. Points, mean; bars, SD. C, apoptosis of Pak1 and Pak2 10A.ErbB2-deficient cells. Apoptosis was measured calculating the percent of positive Annexin V-phycoerythrin cells by flow cytometry. The data are representative of 3 independent experiments. Bars, SD. D, Pak1 and Pak2 in shRNA-infected 10A.ErbB2 cells were plated atop reconstituted basement membrane. Cells treated with doxycycline and stimulated with vehicle or 1 μmol/L AP1510 on day 3 and fixed on day 12 were stained with Oregon green-phalloidin, Ki-67, or anti-cleaved caspase-3. Percentage of unilamellar acini, Ki-67–positive, and anti-cleaved caspase-3–positive acini were scored based on assessment of 50 to 60 acini per well. Bar, 50 μm.
signaling pathways. We examined the activation of ERK and Akt in 10A.ErbB2 cells bearing Pak1- or Pak2-specific shRNA. Interestingly, reduction of either Pak1 or Pak2 by shRNA had a profound negative effect on both ERK and Akt activation (Fig. 2A). In contrast, expression of a constitutively active form of Pak1 in a panel of breast cancer cell lines had only a modest effect in ERK and Akt activation (Supplementary Fig. S3A). This result was expected, as Pak1 is generally thought to be necessary, but not sufficient, for activation of these pathways (4).

As ERK and Akt activation was lost in cells depleted of either Pak1 or Pak2, but only Pak1 depletion affected transformation by ErbB2, we used a phospho-antibody array to examine additional signaling pathways that might be differentially regulated by these 2 Pak isoforms. An array containing several dozen ErbB2-relevant phospho-protein-specific antibodies was probed with lysates from control and Pak1 knockdown 10A.ErbB2 cells. This experiment showed that phosphorylation of a number of known Pak1 direct and indirect substrates, including ERK, c-Raf, BAD, and Akt, were strongly reduced in Pak1 knockdown cells (Fig. 2B). The protein showing the greatest loss of phosphorylation was β-catenin, a protein that has recently been shown to be stabilized by Pak1-mediated phosphorylation (24, 25).

Immuno-fluorescence and immunoblot analyses showed a severe reduction in total β-catenin (Fig. 2C and D). Similar effects were noted in SK-BR-3 and BT-474 cells, in which expression of a dominant-negative form of Pak1, or treatment with the Pak inhibitor PF-3758309 (26) induced loss of

Figure 2. Effects of Pak depletion on proliferation and survival signaling pathways in MCF-10A cells. 10A.ErbB2 cells stably expressing inducible Pak1 or Pak2 shRNAs were treated with doxycycline and stimulated with vehicle or 1 μmol/L AP1510. A, the activities of Mek, ERK, and Akt were assessed by immunoblot using total and phospho-specific antibodies. B, the activities of a panel of signaling proteins were assessed using a phospho-antibody array. Results are presented as changes in phosphorylation between control and Pak1-depleted cells (C) β-catenin levels in control and Pak1-depleted 10A.ErbB2 cells. D, Pak1 depletion in 10A.ErbB2 cells negatively affects the transcription of β-catenin target genes. E, effects of Pak1 depletion on cyclin D and c-Myc expression, as assessed by immunoblot.
Loss of Pak1 impedes ErbB2-mediated carcinogenesis and is associated with reduced levels of β-catenin in vivo

To determine whether the observed effects of Pak1 in ErbB2 signaling were relevant in vivo, we crossed MMTV-Neu mice with WT and Pak1−/− mice and followed the natural history of NeuPak1+/+ and NeuPak1−/− female mice over the course of 2 years. Pak1 deletion was well tolerated in mice, with no effects on general health, longevity, or fertility (30). Consistent with prior reports (31), half the MMTV-Neu mice developed palpable breast tumors by 9 months of age (Fig. 3A). In contrast, the MMTV-Neu/Pak1−/− mice showed a much longer latency to tumor formation and tumor growth, with half the mice showing detectable disease by 16 months. This result shows that Pak1 negatively affects the progression of ErbB2-Neu-initiated breast cancer in this mouse model.

Immunohistochemical staining of tumor tissue revealed strong activity for ErbB2, ERK, Akt, β-catenin, and Pak in NeuPak1+/+ mice, and almost absent staining for active ERK, Akt, and β-catenin in NeuPak1−/− mice. Immunohistochemical staining also revealed that Pak1−/− tumor cells expressed high levels of phospho-ERK and phospho-Akt, compared with NeuPak1+/+ tumor cells, indicating that Pak1−/− tumor cells have reduced tumor growth.

Figure 3. Pak1 deficiency delays tumorigenesis and impacts proliferation, survival, migration and invasion of ErbB2/neu-expressing tumor cells. A, Kaplan–Meier curve (top) indicates significant increase in latency of tumor formation in MMTV-Neu/Pak1+/− (n = 22) versus MMTV-Neu/Pak1−/− (n = 21) mice (P = 0.0015). Reduced tumor burden (bottom) in MMTV-Neu/Pak1−/− mice (P = 0.0001). B, representative example of MMTV-Neu/Pak1+/+ and MMTV-Neu/Pak1−/− breast cancer specimens stained for ErbB2, phospho-ERK, phospho-Akt, β-catenin, and phospho-Pak. C, proliferation of MMTV-Neu/Pak1+/+ and MMTV-Neu/Pak1−/− tumor-derived cells. Cells were seeded, harvested, and counted at 0, 24, 48, 72, and 96 h. The data are representative of 3 independent experiments. D, apoptosis of MMTV-Neu/Pak1+/+ and MMTV-Neu/Pak1−/− tumor-derived cells. Apoptosis was measured calculating the percent of positive Annexin V-PE cells by flow cytometry. The data are representative of 3 independent experiments. E, in vitro scratch assay using MMTV-Neu/Pak1+/+ and MMTV-Neu/Pak1−/− tumor-derived cells. F, Matrigel invasion assay using MMTV-Neu/Pak1+/+ and MMTV-Neu/Pak1−/− tumor-derived cells. The data are representative of 3 independent experiments. Bars, SD.
Akt, β-catenin, and Pak in NeuPak1−/− mice (Fig. 3B). These results show that, as in mammary epithelial cell lines (Fig. 2 and Supplementary Fig. S3), Pak1 is required for the activation of ERK, Akt, and β-catenin downstream of ErbB2 in vivo. These results also suggest that other Pak isoforms (e.g., Pak2 and Pak3) are not redundant for Pak1 in ErbB2 signaling in mammary epithelial cells.

We derived epithelial cell lines from mammary tumors from Neu/Pak1+/+ and Neu/Pak1−/− mice and assessed their growth and signaling properties. Neu/Pak1+/+ cells grew faster than Neu/Pak1−/− cells (Fig. 3C), showed greater viability following treatment with actinomycin D (Fig. 3D), had greater motility (Fig. 3E, Supplementary Movies S1 and S2), and were more invasive (Fig. 3F). Moreover, Neu/Pak1−/− cells displayed a defective cell cycle and a unilobular morphology when plated in a 3D Matrigel matrix and compared with Neu/Pak1+/+ and other breast cancer cell lines (Supplementary Fig. S5 and S6). Thus, many of the hallmark features of transformation were impeded in mouse-derived ErbB2 mammary epithelial cells lacking Pak1. As in 10A.ErbB2 cells, basal and EGF-stimulated levels of phospho-ERK, phospho-Akt, and total β-catenin were decreased in mammary epithelial cells derived from Neu/Pak1−/− mice (Supplementary Fig. S7). Phosphorylation of β-catenin at a destabilizing site (S33) was augmented in Pak1-catalyzed sites (S675), was diminished, consistent with the overall reduction in β-catenin expression noted in these cells. Phosphorylation of glycogen synthase kinase 3β at an inhibitory site (S9) was also decreased in Pak1−/− cells, as might be expected in cells with reduced Akt activity. These data suggest that Pak1 is required for β-catenin stabilization in mammary epithelial cells derived from Neu mice.

Role of β-catenin in ErbB2-mediated signaling

As Pak1 was required for β-catenin expression in mammary epithelial cells as well as for ErbB2-mediated oncogenesis, we asked whether activators or inhibitors of Wnt signaling affected ErbB2 signaling. We first tested the effects of overexpressing wild-type or a stabilized form of β-catenin (S33Y), as well as a phosphomimetic mutant at the putative Pak1 phosphorylation site (S675E) in 10A.ErbB2 cells (24, 32). All these forms of β-catenin induced multilobular acini even in the absence of ErbB2 stimulation (Fig. 4A–C). Conversely, expression of β-catenin S675A, which lacks the Pak1 phosphorylation site, did not on its own induce this multilobular phenotype and blocked the ability of ErbB2 to confer this phenotype. Finally, treatment of cells with a small-molecule inhibitor of Pak or β-catenin blocked formation of aberrant acini in ErbB2-stimulated cells, but the Pak inhibitor did not affect β-catenin S675E-mediated transformation (Fig. 4E–H).

In vitro synergy between Pak and β-catenin inhibitors

As Pak1 and β-catenin seem to act in a linked pathway downstream of ErbB2 (Fig. 5A), we next tested the effects of small-molecule inhibitors of Pak1 and β-catenin, alone and together, on the survival of ErbB2-driven breast cancer cells. These compounds included PF-3758309, which potently suppresses both group A and B Paks (26), iCRT14 and iCRT3, which...
inhibit β-catenin interaction with T-cell factor (TCF)-4 (33), and JW55, a tankyrase inhibitor that suppresses the activity of the PARP domain of TNKS1/2, leading to the stabilization of AXIN2 followed by increased degradation of β-catenin (34).

BT-474 and SK-BR3 cells were treated with varying concentrations of these inhibitors and the effect on cell survival was assessed following 3 days of treatment. Each compound alone, except JW55 (IC\textsubscript{50} values of 7.3 μmol/L and 6.6 μmol/L in

Figure 5. Synergistic interactions between Pak and β-catenin inhibitors. A, network of Pak1 signaling. Graphical representation of some of the molecular pathways affected by Pak1 loss in 10A.ErbB2 cells. Pink lines represent connections based on experimental evidence; green and blue lines represent connections based on databases and text mining respectively. B, effect of Pak and β-catenin inhibitors on survival of BT-474 and SK-BR3 cells. Cells were treated with the indicated amounts of PF-3758309 and/or iCRT14, iCRT3, or JW55 for 4 days; cell viability was determined by Trypan blue exclusion. C, combination of PF-3758309 and/or iCRT14 or iCRT3 treatment increases apoptosis of BT-474 and SK-BR3 cells. Cells were treated with the indicated amounts of PF-3758309 and/or iCRT14 or iCRT3 for 4 days, collected, and apoptosis was measured calculating the percent of positive Annexin V-phycoerythrin cells by flow cytometry. The data are representative of 3 independent experiments. Bars, SD.
BT-474 and SK-BR3, respectively), had a nearly identical effect on cell growth. The IC_{50} values for iCRT14 in BT-474 and SK-BR3 cells were 71 and 107 nmol/L, respectively; the IC_{50} values for iCRT3 were 52.3 and 82 nmol/L, respectively; whereas the IC_{50} values for PF-3758309 were 62.5 and 85.7 nmol/L, respectively (Fig. 5B). When the compounds were coadministered, however, a marked synergistic effect was noted [combination index (CI) < 0.5; Fig. 5B and Table 1]. Coadministration of PF-3758309 and iCRT14 yielded CI values of 13.3 and 20.4 nmol/L respectively, and coadministration of PF-3758309 and iCRT3 yielded CI values of 19.3 and 31 nmol/L respectively, indicating a high degree of synergy. Interestingly, synergistic effects were not seen in cells treated with the tankyrase inhibitor JW55 plus PF-3758309, most likely because β-catenin, once stabilized by phosphorylation at S675, is not readily susceptible to destruction by cleavage. However, the combination of Pak and either of the iCRT β-catenin–targeting agents, which block interactions of β-catenin with TCF, did not merely produce cytostasis, but also resulted in cell death, increasing the frequency of apoptosis by nearly a factor of 2 (Fig. 5C).

### Table 1. Synergistic effects of Pak plus β-catenin inhibitors

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Inhibitors</th>
<th>Molar Ratio</th>
<th>ED50 (μM)</th>
<th>ED75 (μM)</th>
<th>ED95 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT-474</td>
<td>PF-3758309</td>
<td>iCRT14</td>
<td>1:1</td>
<td>0.368 ± 0.025</td>
<td>0.416 ± 0.026</td>
</tr>
<tr>
<td></td>
<td>PF-3758309</td>
<td>iCRT3</td>
<td>1:1</td>
<td>0.396 ± 0.037</td>
<td>0.429 ± 0.032</td>
</tr>
<tr>
<td></td>
<td>PF-3758309</td>
<td>JW55</td>
<td>1:1</td>
<td>0.982 ± 0.015</td>
<td>1.002 ± 0.013</td>
</tr>
<tr>
<td>SK-BR3</td>
<td>PF-3758309</td>
<td>iCRT14</td>
<td>1:1</td>
<td>0.314 ± 0.002</td>
<td>0.330 ± 0.114</td>
</tr>
<tr>
<td></td>
<td>PF-3758309</td>
<td>iCRT3</td>
<td>1:1</td>
<td>0.402 ± 0.096</td>
<td>0.472 ± 0.103</td>
</tr>
<tr>
<td></td>
<td>PF-3758309</td>
<td>JW55</td>
<td>1:1</td>
<td>0.928 ± 0.016</td>
<td>0.982 ± 0.031</td>
</tr>
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**NOTE:** Summary results of drug interactions calculated as Chou-Talalay CI based on CellTiter-Blue viability determinations. CI values <1 indicate synergy and <0.5 (in bold) strong synergy between the two agents in producing cytotoxic effect.

Sensitization of trastuzumab-resistant breast cancer cells by Pak and β-catenin inhibitors

As Pak1 and β-catenin seem to act in a linked pathway downstream of ErbB2, we assessed whether blocking these proteins would augment growth inhibition by an anti-ErbB2 agent. For these experiments, we chose the cell line BT-474R, known to be resistant to trastuzumab in vitro (35). As with parental BT-474 and SK-BR3 cells, we observed a synergistic inhibition of survival when BT-474R cells were treated with Pak plus β-catenin inhibitors (Fig. 7A). As expected, addition of trastuzumab alone had little effect on these resistant cells (Fig. 7B). Addition of either the Pak or the β-catenin inhibitor slightly increased apoptosis in BT-474R cells; however, in combination with trastuzumab, these inhibitors increased the number of apoptotic cells to more than 30% and approximately 40%, respectively. When combined, the Pak and β-catenin inhibitors increased apoptosis in BT-474R cells to approximately 15%; this effect was greatly accentuated by the addition of trastuzumab (Fig. 7B). These results suggest that inhibiting downstream components of ErbB2 signaling such as the Pak/β-catenin signaling axis, can sensitize trastuzumab-resistant cells to ErbB2-targeted therapeutic antibodies.

### Discussion

Pak1 has previously been implicated in breast cancer for the following reasons: the PAK1 gene is frequently amplified in human breast cancer; PAK1 amplification is associated with resistance to tamoxifen; transgenic expression of an activated...
PAK1 allele induces transformation of mammary epithelial cells in culture and induces breast cancer in mice; and expression of dominant-negative alleles, shRNAs, or treatment with Pak inhibitors, impede the growth and/or normalize the morphology of various breast cancer cell lines in tissue culture (13, 15–18). However, none of these studies examined the particular roles of the 2 most highly expressed group A Pak alleles, Pak1 and Pak2, nor did they use clinically relevant inhibitors, nor establish key oncogenic signaling pathways activated by Pak in mouse breast cancer models. Here, we show that (i) blockade of Pak1, but not Pak2 expression or activity in vitro inhibits ErbB2 function; (ii) loss of either Pak1 or Pak2 causes loss of both ERK and Akt activation, but loss of Pak1 uniquely causes destabilization of β-catenin; (iii) loss of Pak1 in vivo delays oncogenesis and progression in MMTV-Neu mice; (iv) restoring β-catenin expression by means of a stabilized β-catenin mutant or a Pak1 phospho-site mimic of β-catenin overcomes the effects of Pak inhibition; and (v) small-molecule inhibitors of Pak or β-catenin block transformation by ErbB2 in cells and in mice. These results, coupled with recent work showing that Pak1 can phosphorylate β-catenin and establish a ErbB2-Pak-β-catenin linkage that is essential for transformation of mammary epithelial cells by ErbB2.

Of the 6 Pak isoforms, Pak1 in particular has garnered much attention with respect to tumorigenesis, as amplification of the PAK1 gene, with concomitant overexpression of the Pak1 protein, is commonly observed in human cancers of the breast, ovary, and bladder (9). Unlike other oncogenic serine/threonine protein kinases such as BRAF, activating point mutations, or deletions of PAK1 have not been found in human cancers, despite the ability of activated PAK1 mutants to transform cells in vitro and in vivo. PAK1 gene amplification seems to be particularly relevant in human breast cancer, as it has been shown that such amplification is associated with resistance
In addition, the effects of gene deletion are quite different for Pak1 and Pak2; deletion of the former is associated with a mild phenotype, whereas deletion of the latter results in early embryonic lethality (8). Why Pak1, but not Pak2, affects β-catenin levels in mammary epithelial cells is unknown, and it will be of interest to determine whether these different behaviors reside in different intrinsic substrate selection or differences in the noncatalytic, N-terminal–regulatory domains of these kinases.

The role of the Wnt/β-catenin pathway in ErbB2 signaling in breast cancer is incompletely understood. In general, ErbB2 positivity in breast cancer is associated with a luminal phenotype, whereas Wnt activation is more commonly associated with a basal phenotype (41). However, multiple lines of evidence suggest important links between ErbB2 and β-catenin signaling. First, ErbB2 physically associates with β-catenin, and such complexes are associated with human infiltrating ductal breast and also with MMTV-c-Neu and MMTV-Wnt-1 mouse models of breast cancer (28). Second, ErbB2 phosphorylates β-catenin at Tyr 654, leading to dissociation of the E-cadherin–β-catenin membrane complex and increased signaling to Wnt target genes such as cyclin D1 (29). In addition, ErbB2 has been reported to transcriptionally activate expression of the Wnt pathway target gene Jab1 via an Akt/β-catenin pathway in breast cancer cells (42). These observations are supported by recent publications showing a positive correlation between HER2/neu expression and nucleocytoplasmic (i.e., non–plasma membrane) β-catenin in node-positive carcinomas (P = 0.02) and in HER2/neu-induced mouse mammary tumors, with activation of Wnt pathway genes (27), as well as eradication of breast tumor-initiating cells by pharmacologic inhibitors of Wnt/β-catenin signaling in this mouse model (43).

Recently, it has been reported that Pak1 has a direct role in β-catenin stabilization in colon cancer cells, via phosphorylation of β-catenin at Ser 675 (24, 44) and Ser 663/675 (45). As we have previously shown that ErbB2 activates Pak1 (16), we here propose that Pak1 is a required signaling element linking ErbB2 activation to β-catenin in mammary epithelial cells, and that such activation is necessary for oncogenesis. This view is supported by the loss of β-catenin and target gene activation in Pak1-depleted mammary epithelial cells or Pak1-deleted mammary tissue, and the blockade of tumor growth by Pak or β-catenin inhibition seen in animals xenografted with ErbB2-positive tumor cells.

Small-molecule Pak inhibitors have recently entered clinical trials and it should soon be possible to evaluate the potential of such agents in human cancer. We suggest that such agents may be useful in 2 general settings: cancers that have 11q13 amplification, such as a large fraction of breast, ovarian,
Disclosure of Potential Conflicts of Interest
No potential conflict of interest were disclosed.

Authors’ Contributions
Conception and design: L.E. Arias-Romero, J. Chernoff
Methodology: L.E. Arias-Romero, K.P. Hoeßl, J. Chernoff
Acquisition of data (provided specimens, acquired and managed patients, provided facilities, etc.): L.E. Arias-Romero, O. Villamar-Cruz, M. Huang, J. Chernoff
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.E. Arias-Romero, J. Chernoff
Writing, review, and/or revision of the manuscript: L.E. Arias-Romero, J. Chernoff

References
27. Khalli S, Tan GA, Girl DD, Zhou XK, Howe LR. Activation status of Wnt/beta-catenin signaling in normal and neoplastic breast tissues:...


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