HER3 Is Required for HER2-Induced Preneoplastic Changes to the Breast Epithelium and Tumor Formation

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Abstract

Increasing evidence suggests that HER2-amplified breast cancer cells use HER3/ErbB3 to drive therapeutic resistance to HER2 inhibitors. However, the role of ErbB3 in the earliest events of breast epithelial transformation remains unknown. Using mouse mammary specific models of Cre-mediated ErbB3 ablation, we show that ErbB3 loss prevents the progressive transformation of HER2-overexpressing mammary epithelium. Decreased proliferation and increased apoptosis were seen in MMTV-HER2 and MMTV-Neu mammary glands lacking ErbB3, thus inhibiting premalignant HER2-induced hyperplasia. Using a transgenic model in which HER2 and Cre are expressed from a single polycistronic transcript, we showed that palpable tumor penetrance decreased from 93.3% to 6.7% upon ErbB3 ablation. Penetration of ductal carcinomas in situ was also decreased. In addition, loss of ErbB3 impaired Akt and p44/42 phosphorylation in preneoplastic HER2-overexpressing mammary glands and in tumors, decreased growth of preexisting HER2-overexpressing tumors, and improved tumor response to the HER2 tyrosine kinase inhibitor lapatinib. These events were rescued by reexpression of ErbB3, but were only partially rescued by ErbB36F, an ErbB3 mutant harboring six tyrosine-to-phenylalanine mutations that block its interaction with phosphatidylinositol 3-kinase. Taken together, our findings suggest that ErbB3 promotes HER2-induced changes in the breast epithelium before, during, and after tumor formation. These results may have important translational implications for the treatment and prevention of HER2-amplified breast tumors through ErbB3 inhibition.

Introduction

Aberrant regulation of ErbB receptor tyrosine kinases is common in human cancers (1). This family of 4 related members, EGFR (epidermal growth factor receptor), HER2/ErbB2/Neu, HER3/ErbB3, and HER4/ErbB4 (2) exhibit dimerization-induced tyrosine phosphorylation and catalytic activity, with the exception of ErbB3, which has only weak intrinsic kinase activity, and therefore relies on heterodimeric transphosphorylation for signal transduction (3). Nonetheless, ErbB2/ErbB3 heterodimers are powerful oncogenic units, in part, because P-ErbB3 amplifies signaling through phosphatidylinositol 3-kinase (PI3K; refs. 4–6). The p85 regulatory subunit of PI3K interacts directly with P-ErbB3 at 6 consensus P-Tyr p85-binding motifs within ErbB3 (3). In contrast, ErbB2 cannot directly engage p85. Therefore, it is thought that ErbB3 functions primarily to drive ErbB2-mediated PI3K signaling (7, 8). This is of critical therapeutic importance, as currently approved ErbB2 inhibitors (e.g., trastuzumab and the dual EGFR/ErbB2 TK inhibitor lapatinib) decrease P-ErbB3/PI3K signaling (9–11). However, many metastatic ERBB2/HER2-amplified breast cancers do not respond to, or eventually escape, trastuzumab and lapatinib, often with recovery of P-ErbB3/PI3K signaling (12). These studies would predict that therapeutic combinations targeting ErbB2 and ErbB3 would improve outcome in ERBB2/HER2-amplified breast cancers.

It is unclear how ErbB3 contributes to earliest events in ErbB2-mediated transformation of the breast epithelium, although ErbB3 is expressed in ErbB2-overexpressing hyperplasias in mice (13, 14). Tumorigenesis is a multistep process proceeding first through hyperplasia, to noninvasive ductal carcinoma in situ (DCIS), invasive adenocarcinoma, and finally to the formation of distant metastases. Although the expression of ErbB3 in DCIS lesions has not been reported, DCIS frequently overexpress ErbB2 (15, 16). It is important to understand what supports the transition of ErbB2þ DCIS to invasive, metastatic breast cancers.

To study the role of ErbB3 in all stages of ErbB2-mediated breast transformation ranging from premalignant hyperplasia...
to therapeutic response, we used genetically engineered mouse models to impair ErbB3 expression within ErbB2+ mammary epithelial cells (MEC; ref. 17). These studies revealed that the formation of ErbB2-mediated hyperplasias, DCIS, adenocarcinomas, and distant metastatic lesions were substantially decreased upon ErbB3 ablation. Inducible ErbB3 ablation in preexisting ErbB2-overexpressing tumors improved lapatinib-mediated growth inhibition. Genetic ablation of ErbB3 reduced cell proliferation and survival, and interfered with PI3K-Akt signaling. These signaling events were rescued by ErbB3 reexpression, but only partially rescued by ErbB3<sup>6F</sup>, a mutant ErbB3 isoform incapable of interacting with p85 (4). These findings support a role for ErbB3 throughout the continuum of ErbB2-mediated tumorigenesis. Strategies targeting ErbB3 expression may prove useful in combination with ErbB2 inhibitors to improve the clinical outcome of ErbB2/HER2-amplified breast cancer patients. Furthermore, these studies suggest that ErbB3 targeting may be a novel prevention strategy to limit premalignant ErbB2-induced changes to the breast epithelium.

Materials and Methods

Histologic analysis

Mammary glands were whole mounted on glass slides and stained with hematoxylin and eosin (H&E), with the ApopTag kit (Millipore), or by immunohistochemistry (IHC) using the following antibodies as described previously: ErbB3 (C-17), ErbB2 (F-11), and Ki67 (Santa Cruz Biotechnology, Inc.), S473 P-Akt (Cell Signaling Technology, Inc.; ref. 17). Immunodetection was done using the Vectastain kit (Vector Laboratories) according to the manufacturer’s directions. Specificity of the anti-ErbB3 antibody was determined using ErbB3-null tissues described herein. The specificity of the ErbB2 and P-Akt antibodies has been described previously (19).

Mice

All mice were generated on, or crossed more than 10 generations into, an FVB inbred background. ErbB3<sup>FL/FL</sup>, MMTV-Cre, MMTV-rtTA, TetOp-Cre, MMTV-Neu, MMTV-HER2, and MMTV-NIC mice have been previously described (9, 19–22). ErbB3<sup>FL/FL</sup> X MMTV-Cre is referred to herein as ErbB3<sup>MMTV-KO</sup>, ErbB3<sup>6F/6F</sup> X MMTV-rtTA X TetOp-Cre as ErbB3<sup>6F/6F</sup>-NIC, and ErbB3<sup>6F/6F</sup> X MMTV-Neu as NiB3. Where indicated, mice were treated with doxycycline (DOX, 2 mg/ml in 4% sucrose in drinking water; Sigma-Aldrich), or lapatinib (100 mg/kg daily for 4 weeks via orogastric gavage, I.C Laboratories). Mammary glands were harvested from age-matched virgin siblings. Where indicated, tumor cells (1 x 10<sup>6</sup>) were injected into left and right inguinal fat pads of 6-week-old female nu/nu (Balb/C) mice. Mice were examined twice weekly for palpable tumor formation, and tumors measured twice weekly with calipers. Tumor volume was calculated as (length x width<sup>2</sup>/2).

Cell culture

Primary tumor cells were harvested from collagenase-digested NiB3 tumors, and cultured as previously described (19). Cells were infected with retroviral particles generated in 293T cells using pMSCV-ErbB3<sup>WT</sup>, pMSCV-ErbB3<sup>6F</sup>, or empty pMSCV. After viral infection, cells were selected in 1 μg/ml puromycin. Pooled puromycin-resistant clones were used for analysis. For colony forming assays, cells were seeded in 6-well dishes (10<sup>4</sup> cells per dish), and cultured 10 to 21 days in Dulbecco’s modified Eagle’s medium/F12 supplemented with 10% FBS (Atlanta Biologicals). Cells were crystal violet stained, and imaged on a flatbed scanner. For cell growth assays, cells were cultured in 10% serum ± lapatinib (1 μg/ml). Viable cells were counted using trypan blue exclusion with a TC-10 automated cell counter (Bio-Rad).

Western blotting

Cells and tissues were homogenized as described previously (19). The following primary antibodies were used: ErbB3 (C-17, Santa Cruz Biotechnology; 1:2,000), α-actin (Sigma-Aldrich; 1:5,000), Y1197 P-ErbB3, p85, AKT, and S473 P-Akt (Cell Signaling Technology; 1:1,000, 1:2,000, and 1:500, respectively). Total RNA (1 μg), harvested from tumors using RNeasy (Qiagen), was used for cDNA synthesis (High Capacity cDNA RT; Applied Biosystems) followed by qPCR (iQ SYBR Green Supermix (Bio-Rad) using the following primers: ErbB3 (5'-GGGCAACTCTCACGGCATATGT-3' and 5'-CGGATCTCATGAGGAAAGAAGA-3') and Rplp0 (5'-TCTTATAAAGGCCACACGCGGC-3' and 5'-AGACAGTGTCATCTCCAAGGAGGACG-3'). C<sub>t</sub> values were calculated as (C<sub>tErbB3</sub> - C<sub>tRplp0</sub>)<sup>-ΔΔCt</sup>. Values are presented as mean ± SEM of at least three biological replicates.

Quantitative reverse transcriptase

Results

HER2-driven hyperplasia of the mammary epithelium requires ErbB3

The MMTV-HER2 transgenic mouse model expresses HER2, the human homolog of mouse ErbB2, in the mammary epithelium, resulting in mammary adenocarcinoma formation preceded by a prolonged period of diffuse epithelial hyperplasia (21). Little is known about the molecular events promoting ErbB2/HER2-driven mammary hyperplasia, or what causes hyperplastic lesions to convert to invasive malignant cancers. To determine whether ErbB3 affects early events in the continuum of ErbB2-driven tumor formation, we used transgenic mammary specific Cre recombinase (MMTV-Cre; ref. 22) to drive recombination at floxed ERBB3 alleles (ErbB3<sup>F/F</sup>) in genetically engineered mice (17, 21) referred to hereafter as ErbB3<sup>F/F</sup> X MMTV-KO mice. Whole-mount hematoxylin staining of mammary glands harvested from 12-week-old virgin female mice revealed that ErbB3 loss impaired HER2-driven hyperplasia (Fig. 1A). Decreased hyperplasia was not due to changes in human HER2 expression levels (Fig. 1A, bottom panels). Because ErbB2/HER2 potently activates PI3K signaling through ErbB3, we examined Akt, a primary downstream PI3K effector (23). P-Akt was not seen in ErbB3<sup>F/F</sup> X MMTV-KO samples (Fig. 1B).

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P-Akt signaling activates antiapoptotic pathways in numerous cancer cell types. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) analysis showed increased cell death in ErbB3-deficient samples (Fig. 1B). Similar results were obtained using the MMTV-Neu transgenic model of breast cancer, which expresses Neu, the rat ErbB2/HER2 homolog, in the mammary epithelium (20), in which ErbB3<sup>MMTV-KO</sup> X MMTV-Neu mammary glands displayed decreased hyperplasia (Supplementary Fig. S1A), cell proliferation, and increased cell death (Supplementary Fig. S1B).

ErbB2/HER2-expressing cells escaping Cre-mediated ErbB3 ablation give rise to ErbB3-expressing tumors in ErbB3<sup>MMTV-KO</sup> mice

Inhibition of ErbB2/HER2-driven hyperplasia was maintained in ErbB3<sup>MMTV-KO</sup> X MMTV-HER2 mammary glands through 27 weeks of age (Fig. 2A). However, focal neoplasias were present in ErbB3<sup>MMTV-KO</sup> X MMTV-HER2 mammary glands, even in the context of suppressed hyperplasia. Average tumor latency was statistically similar in ErbB3<sup>MMTV-KO</sup> X MMTV-HER2 mice and ErbB3<sup>F<sup>-</sup></sup> X MMTV-HER2 mice (P > 0.05, log-rank test; Fig. 2B). Similar findings were seen in ErbB3<sup>MMTV-KO</sup> X MMTV-Neu mice versus ErbB3<sup>F<sup>-</sup></sup> X MMTV-Neu (P > 0.05, log-rank test; Fig. 2C).

These results suggested 2 scenarios, one in which ErbB3 is lost but is not required for ErbB2-mediated focal tumor formation, or another in which some cells escape Cre-mediated recombination, leaving ErbB3 intact to support ErbB2-induced tumor formation. Immunohistochemical analysis of tumor sections revealed that ErbB3 expression was maintained in 100% of ErbB3<sup>MMTV-KO</sup> X MMTV-HER2 tumors (N = 13), suggesting that tumors arise from epithelial cells escaping Cre-mediated genomic recombination (Fig. 2D). ErbB3 was not detected in normal adjacent mammary epithelium of ErbB3<sup>MMTV-KO</sup> X MMTV-HER2 mice (Fig. 2D, magnified inset). These data suggested that ErbB3 loss continues to suppress ErbB2/HER2-mediated growth of adjacent epithelial structures.

Nonhomogenous expression of Cre recombinase from the MMTV promoter has been previously reported (24–26) and may contribute to ErbB3 retention in this model. We assessed Cre activity using Rosa26 transgenic mice, which irreversibly express β-galactosidase upon Cre-mediated recombination (27). Whole-mount β-galactosidase staining revealed numerous ErbB3<sup>MMTV-KO</sup> X MMTV-HER2 X Rosa26 MECs lacking β-galactosidase (Fig. 2E). Furthermore, we detected numerous ErbB3<sup>MMTV-KO</sup> X MMTV-HER2 epithelial structures with only partial ErbB3 ablation (Supplementary Fig. S2), although the average number of MMTV-HER2 tumors per mouse was not different between ErbB3<sup>MMTV-KO</sup> and ErbB3<sup>F<sup>-</sup></sup> mice, the tumor distribution was (Fig. 2F). Ninety percent of all ErbB3<sup>F<sup>-</sup></sup> X MMTV-HER2 tumors occurred in thoracic mammary glands (9 of 10 mice), but occurred at a lesser frequency in ErbB3<sup>MMTV-KO</sup> X MMTV-HER2 (5 of 13), but cervical and inguinal mammary tumors occurred with higher frequency. These data underscored the nonoverlapping expression patterns of MMTV-Cre and MMTV-HER2.
transgenes. Because ErbB3 expression is maintained in this model, an alternate model would be required to determine whether ErbB3 is required for malignant transformation of the breast epithelium by ErbB2.

**Polycistronic expression of ErbB2 and Cre reveals ErbB3 is required for ErbB2-induced hyperplasia**

We employed an alternative transgenic approach, in which a single polycistronic transcript encodes both Neu
(the rat ErbB2 homolog) and Cre recombinase, separated by an internal ribosomal entry site (IRES). Mammary specific expression of the MMTV-Neu-IRESCre (NIC) transgene results in mammary tumor formation (28, 29). Mammary glands harvested from 12-week-old virgin female NIC mice revealed ErbB2-induced diffuse hyperplasia and focal neoplasia in ErbB3<sup>FL/+</sup> X NIC samples, but not ErbB3<sup>FL/+</sup> X NIC (Fig. 3A). Cell proliferation in ErbB3<sup>FL/FL</sup> X NIC mammary glands was decreased as compared with ErbB3<sup>FL/+</sup> X NIC, whereas cell death was increased. Real-time reverse transcriptase PCR experiments revealed that ErbB3 mRNA was eliminated in ErbB3<sup>FL/FL</sup> samples.

**ErbB3 promotes ErbB2-induced DCIS and adenocarcinoma**

Female NIC mice were examined by manual palpation twice weekly through 180 days of age for tumor formation. Palpable tumors were not detected in ErbB3<sup>FL/FL</sup> X NIC mice. However, 2 of 15 ErbB3<sup>FL/FL</sup> X NIC mice developed DCIS that were too small for detection by manual palpation, but were detected at necropsy at 180 days. Analysis of these 2 DCIS revealed decreased cell proliferation (as seen by Ki67 IHC) and increased cell death (as seen by TUNEL analysis) as compared with similar DCIS lesions found in ErbB3<sup>FL/+</sup> X NIC mice (Fig. 3B). Western blot analysis showed loss of ErbB3 expression in ErbB3<sup>FL/+</sup> X NIC whole tissue lysates, which did not affect Akt phosphorylation at S473, but drastically reduced phosphorylation of p44/42 mitogen-activated protein kinase (MAPK). Therefore, ErbB3 loss profoundly decreased premalignant changes in the ErbB2-overexpressing breast epithelium, including hyperplastic lesions and DCIS. Palpable mammary tumors were evident in 7 of 8 ErbB3<sup>+/+</sup> X NIC mice, and in 7 of 8 ErbB3<sup>FL/+</sup> X NIC mice, occurring with similar average tumor latencies (Fig. 3C, 165 days vs. 145 days, respectively, P > 0.05, log-rank test). Average total tumor burden per mouse was decreased in ErbB3<sup>FL/FL</sup> X NIC mice as compared with ErbB3<sup>+/+</sup> X NIC and ErbB3<sup>FL/+</sup> X NIC (Fig. 3D), as was the number of lung metastases per mouse (Fig. 3E). Therefore, ErbB3 ablation greatly decreases progression of ErbB2<sup>+</sup> premalignant lesions to metastatic breast cancers.

**Inducible Cre-mediated ErbB3 ablation decreases growth of preexisting HER2-overexpressing mammary tumors**

To determine the potential use of therapeutic ErbB3 targeting, we developed a transgenic model system in which mammary specific Cre expression could be temporally regulated by doxycycline resulting in doxycycline-inducible ErbB3 ablation (ErbB3<sup>DOX-KO</sup>) as described previously (19). Treatment of 12-week-old virgin female ErbB3<sup>DOX-KO</sup> X MMTV-Neu mice with doxycycline for 14 days decreased ErbB3 expression and ErbB2-induced hyperplastic structures (Fig. 4A). Next, we maintained ErbB3<sup>DOX-KO</sup> X MMTV-Neu naive to doxycycline until their tumors surpassed 200 mm<sup>3</sup>, at which point mice were treated ±doxycycline for 4 weeks. Doxycycline-treated tumors grew more slowly than untreated tumors (Fig. 4B), and after 4 weeks harbored decreased tumor cellularity (Fig. 4C), IHC confirmed decreased ErbB3. P-Akt, Ki67, and cell survival after 7 days of doxycycline (Fig. 4D and E).

**Inhibition of ErbB3 expression or ErbB3-to-PI3K signaling improves response of ErbB2-overexpressing breast cancers to lapatinib**

We treated ErbB3<sup>DOX-KO</sup> X MMTV-Neu mice bearing tumors more than 200 mm<sup>3</sup> for 3 weeks with control vehicles, doxycycline, lapatinib (100 mg/kg once daily), or doxycycline + lapatinib. ErbB3<sup>DOX-KO</sup> tumors treated with either lapatinib or doxycycline grew more slowly than untreated tumors (Fig. 5A). Tumor growth was reduced to a greater extent when treated with doxycycline and lapatinib together, suggesting that ErbB3 inhibition improves therapeutic response of ErbB2-overexpressing breast cancers to lapatinib.

ErbB3 is a powerful activator of PI3K/Akt signaling (23). To understand the role of ErbB3 in total PI3K signaling output in ErbB2-overexpressing breast cancer cells, we eliminated endogenous ErbB3 expression in primary NiB3 cells (ErbB3<sup>FL/FL</sup> X MMTV-Neu) using adenoviral Cre (Ad.Cre), thus generating an ErbB3-deficient background for adding back wild-type (WT) ErbB3 or a mutant ErbB3 isoform unable to engage PI3K. Ad.Cre-infected NiB3 cells grew more slowly than Ad.LacZ-infected cells, and exhibited greater lapatinib-mediated growth inhibition (Fig. 5B), suggesting that these primary tumor cells mirror the phenotype of the tumors from which they were derived.

Next, NiB3 cells (with endogenous ErbB3 still intact) were infected with pMSCV-based retroviral particles encoding WT rat ErbB3 (ErbB3<sup>WT</sup>), or mutant rat ErbB3 in which tyrosine-to-phenylalanine mutations were introduced into the 6 PI3K interaction motifs of ErbB3 (ErbB3<sup>6F</sup>; ref. 4). Pools of puromycin-resistant cells were infected with Ad.Cre to remove endogenous ErbB3, leaving expression of ErbB3<sup>WT</sup> or ErbB3<sup>6F</sup> (Fig. 5C). P-ErbB3 and total ErbB3 expression were rescued in Ad.Cre-infected NiB3-ErbB3<sup>WT</sup> cells as compared with vector controls. ErbB3 expression, but not Tyr1197 phosphorylation (a PI3K-interaction site), was rescued in Ad.Cre-infected NiB3-ErbB3<sup>6F</sup> cells. Akt phosphorylation was also substantially reduced but not eliminated in NiB3 vector (i.e., ErbB3 deficient) and NiB3-ErbB3<sup>6F</sup> cells, consistent with the idea that ErbB3 facilitates PI3K activation in ErbB2-overexpressing cells, but that ErbB2 retains alternative mechanisms to activate the PI3K/Akt signaling pathway outside of ErbB3, as previously reported (3). Interestingly, we found that Erk1/2 MAPK phosphorylation was lost in cells lacking ErbB3, but was restored in cells expressing either ErbB3<sup>WT</sup> or ErbB3<sup>6F</sup>, suggesting that ErbB3 is required for ErbB2-induced MAPK activation through mechanisms that do not involve tyrosine phosphorylation at these 6 residues.

Immunoprecipitation of ErbB3 from whole-cell lysates confirmed that Ad.Cre infection eliminated endogenous ErbB3 (Fig. 5D). Total tyrosine phosphorylation of ErbB3<sup>6F</sup> was detected, consistent with the presence of remaining phosphorylated tyrosine residues in the ErbB3 C-terminal tail that are not involved in p85 interaction. Importantly, coprecipitation of p85 with ErbB3 was eliminated in ErbB3<sup>6F</sup>-expressing cells.

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Figure 3. Decreased cell growth, cell survival, hyperplasia, and tumor formation in MMTV-NIC mice, expressing ErbB2 and Cre from the same polycistronic transcript. A, histologic analysis of mammary glands harvested from 12-week-old virgin female mice by whole-mount hematoxylin staining, IHC detection of Ki67, and TUNEL analysis revealed that loss of ErbB3 in ErbB3FL/FL X NIC mice decreased diffuse hyperplasia and focal neoplasia, epithelial proliferation, and cell survival. RNA harvested from ErbB3+/+ NIC (n = 8), ErbB3+/Fl NIC (n = 7), and ErbB3FL/FL NIC (n = 7) was used to assess ErbB3 mRNA expression levels. Values are expressed as mean fold differences (± SE) in comparison with ErbB3+/+ NIC, and normalized to the Rplp0 housekeeping gene. B, DCIS too small for detection by manual palpation became evident in 2/15 ErbB3FL/FL X NIC mice by 6 months of age. Analysis of these 2 DCIS revealed decreased cell proliferation (as seen by Ki67 IHC) and increased cell death (as seen by TUNEL analysis). Comparable DCIS lesions were evident in ErbB3FL/+ X NIC mice, as were frank adenocarcinomas, but DCIS lesions found in control samples displayed profoundly elevated cell proliferation and very low levels of cell death. Higher power magnifications of boxed areas are shown at the right. Bottom, Western analysis of whole mammary lysates using the antibodies indicated at the right of each panel. Genotype for ErbB3 is indicated as +/+ (ErbB3+/+ X NIC), FL/+ (ErbB3FL/+ X NIC) and FL/FL (ErbB3FL/FL X NIC). C, Kaplan–Meier analysis of tumor latency in MMTV-NIC mice. Average tumor latency could not be determined (n.d.) in ErbB3FL/FL mice, as the majority of these mice did not develop tumors within the course of this study. Loss of a single ErbB3 allele (ErbB3FL/+ X NIC) did not affect tumor latency as compared with ErbB3+/+. D, average tumor burden per mouse, as determined by the average number of tumors per mouse (top) or total tumor volume (bottom) at 180 days of age, was measured. P value calculated using Student t test. E, lung metastases were identified upon histologic examination. Average number of lung metastases per mouse is shown, N = 8 (ErbB3+/+), 7 (ErbB3FL/FL), and 15 (ErbB3FL/FL). P value, Student t test. Representative lung sections are shown.
Despite the presence of tyrosine phosphorylated ErbB3, confirming that ErbB3-PI3K interactions were disrupted.

In cell growth assays, acute loss of ErbB3 expression by Ad.Cre infection of NiB3-vector cells decreased cell number nearly 10-fold, which was rescued by exogenous ErbB3WT expression (Fig. 5E). NiB3-ErbB36F cells grew more slowly than NiB3-ErbB3WT, but more rapidly than NiB3-vector cells, suggesting ErbB36F maintains some mitogenic properties, despite loss of ErbB3-PI3K interactions. In long-term colony assays, Ad.Cre-infected NiB3 cells expressing ErbB36F were more sensitive to lapatinib-induced growth inhibition than ErbB3WT-expressing cells (Fig. 5F), suggesting that ErbB3-induced PI3K activity is a critical barrier to achieving full response to lapatinib. However, complete loss of ErbB3 improved response to lapatinib over ErbB36F-expressing cells. In addition, treatment of cells with the pan-PI3K inhibitor BKM120 decreased colony formation in lapatinib-treated NiB3-ErbB3WT cells and in NiB3-ErbB36F cells.

We carried out orthotopic transplant of NiB3-ErbB3WT and NiB3-ErbB36F into contralateral mammary fat pads of mice, using a protocol in which Ad.Cre eliminated endogenous ErbB3 ex vivo, 7 days before tumor inoculation. Tumor latency was similar for each group, occurring within 10 days after tumor cell injection. Tumor-bearing mice were treated daily for

Figure 4. Inducible Cre-mediated ErbB3 ablation decreases growth of ErbB2-overexpressing mammary tumors. A, treatment of 12-week-old virgin female ErbB2Dox−/− X MMTV-Neu mice with doxycycline for 14 days decreased mammary epithelial ErbB3 expression and ErbB2-induced lobuloalveolar density. B, ErbB2Dox−/− X MMTV-Neu female mice remained without doxycycline until tumors surpassed 200 mm3, at which point mice were treated with doxycycline (2 mg/mL in drinking water). Tumor volume was measured twice weekly. Values shown represent the average ± SD. P value calculated using Student’s t test, N = 6. C, histologic analysis of tumors treated 26 days with doxycycline show decreased tumor cellularity and increased acellular debris. D, ErbB3, P-Akt, Ki67, and cell survival were decreased in doxycycline-treated samples. TUNEL analysis revealed increased cell death in doxycycline-treated tumors. E and F, values shown indicate the average number of Ki67+ cells (E) or TUNEL+ cells (F) per total tumor cells. N = 4 per group, 5 fields per sample. P values calculated using Student’s t test. DOX, doxycycline.
Figure 5. Inhibition of ErbB3 expression or ErbB3-to-Pi3K signaling improves response of ErbB2-overexpressing breast cancers to lapatinib. A, ErbB3DOX−/ko mice bearing tumors more than 200 mm³ were randomized into groups treated with doxycycline (2 mg/mL in drinking water) and lapatinib (100 mg/kg once daily). Tumor volume was measured twice weekly. Left, average tumor volume ± SE. Right, fold-increase in tumor volume calculated individually for each tumor, then used to calculate average fold increase in tumor volume for each group. *P values, Student t test. N = 8. B, primary NiB3 cells were infected with Ad.Cre or Ad.LacZ. Cells (1 × 10⁴) were cultured in 10% serum with lapatinib (1 μg/mL) or dimethyl sulfoxide (DMSO) for 96 hours. Viable cells were counted (Bio-Rad TC-10). Values represent average cell number ± SD. *P < 0.01. C and D, NiB3 cells were infected with pBMN retrovirus expressing the empty vector, ErbB3WT, or ErbB36F. Cells were infected with Ad.Cre or Ad.LacZ. Ten days after infection, cell lysates were used for Western blot analysis (C) or ErbB3 immunoprecipitation followed by Western blot analysis (D). E, 24 hours after infection with Ad.Cre or Ad.LacZ, NiB3 cells (1 × 10⁴) were cultured in 10% serum for 96 hours. Viable cells were counted. Values shown represent the average ± SD. N = 3 per group analyzed in duplicate. *P < 0.01, Student t test. F, 24 hours after infection with Ad.Cre, NiB3 cells (1 × 10⁴) were cultured with lapatinib (1 μmol/L), BKM120 (2 μmol/L), or DMSO for 21 days, stained with crystal violet and imaged. G, NiB3 tumors cells stably expressing ErbB3WT or ErbB36F were infected with Ad.Cre and cultured for 7 days. Cells (10⁶) were injected into the left (NiB3-ErbB3WT) and right (NiB3-ErbB36F) inguinal mammary fat pads. Tumor bearing mice were treated daily 5 days with lapatinib or BKM120. TUNEL analysis of tumors collected 1 hour after final treatment was quantitated as average number of apoptotic bodies per 400 field (N = 9–15 per group). *P values were calculated using Student t test. H, representative photomicrograph of TUNEL-stained NiB3 tumors. DOX, doxycycline.
5 days with lapatinib or BKM120 (20 mg/kg). Relative levels of cell proliferation (as measured by mitotic index) in tumors harvested 1 hour after the final treatment were not statistically different in NiB3-ErbB36F and NiB3-ErbB3WT tumors (Supplementary Fig. S3A). However, basal and BKM120-induced rates of cell death were upregulated in NiB3-ErbB36F tumors (Fig. 5G and H). More cell death was also observed in lapatinib-treated NiB3-ErbB36F tumors as compared with lapatinib-treated NiB3-ErbB3WT tumors (Supplementary Fig. S3B), but was not greater than basal cell death levels in NiB3-ErbB6F tumors (Supplementary Fig. S3C). Therefore, ErbB2-driven tumors can activate PI3K signaling pathways in the absence of canonical ErbB3-p85 interactions, although loss of this interaction leaves cells more susceptible to PI3K inhibition. Importantly, these studies highlighted the importance of ErbB3-induced signaling beyond PI3K.

Discussion

Nearly 25% of all breast cancers overexpress HER2/ErbB2. Therapeutic strategies targeting ErbB2 have improved breast cancer mortality for patients with ERBB2/HER2-amplified breast cancers. However, clinical success of ErbB2 inhibitors is limited by tumor resistance to these inhibitors (11). It is important to understand the signaling mechanisms driving anti-ErbB2 resistance, which is often associated with recovery of PI3K signaling (12, 19, 30, 31). ErbB2 inhibitors initially decrease P-ErbB3/PI3K signaling (10, 30, 31), but resurgent ErbB3 signaling after sustained lapatinib exposure increases PI3K signaling, and is the rationale for clinical trials of pertuzumab–trastuzumab combinations in advanced ErbB2-overexpressing breast cancers, which have seen clinical success (32). We report herein that ErbB3, a heterodimeric partner of ErbB2, drives the response of ErbB2-overexpressing breast cancers to ErbB2 inhibitors. Our results suggest that complete ablation of ErbB3 decreases growth and survival of ErbB2-expressing mammary tumors in mice. Importantly, our results expand the role of ErbB3 in ErbB2-expressing breast cancers beyond PI3K activation, and highlight the critical importance of PI3K-independent pathways in driving tumor cell growth and survival.

Although genetic ErbB3 ablation decreased tumor growth as a single therapeutic strategy (Fig. 4), treatment of BT474 xenografts with U3-1287 did provide any benefit as a single-agent therapy (10, 12). The reasons for this apparent discrepancy remain unclear, but may include model differences (xenografts vs. endogenous tumors, human cells vs. mouse cells). Also, exposure of tumor cells to the targeting agent may have been a contributing factor, as ErbB3 loss in the genetic model was tumor cell-intrinsic upon continuous exposure to doxycycline, as opposed to pulsatile treatment with exogenous antibodies twice weekly as previously reported. Finally, it is possible that U3-1287 may leave ErbB3 intact at the cell surface or in recycling endosomes, allowing low levels of ErbB3 signaling. In contrast, genetic ablation of ErbB3 removes all ErbB3. The observation that genetic ErbB3 ablation effectively limits tumor cell growth as a single-agent strategy is consistent with previous reports that siRNA sequences targeting ErbB3 decreased growth of ERBB2/HER2-amplified breast cell lines (8) and the hormone-independent growth of MCF-7 cells (33). Furthermore, genetic ablation of ErbB3, or treatment of mice with EZN-2920, an antisense oligonucleotide that was used to knock down ErbB3 in vivo, decreased PI3K signaling and tumor growth in the MMTV-PyMT model of breast cancer (19).

It is also important to understand the underlying signaling pathways contributing to the earliest events in ERBB2/HER2-amplified breast cancer formation, to eventually develop strategies to prevent ErbB2-overexpressing breast cancers. We report herein that ErbB3 drives ErbB2-mediated preneoplastic hyperplasia, the transition of DCIS to malignant adenocarcinoma, and the response of ErbB2-overexpressing breast cancers to ErbB2 inhibitors. Large phase III clinical trials have identified selective estrogen receptor (ER) modulators (tamoxifen, raloxifene, and lasofoxifene) and aromatase inhibitors (exemestane) that reduce the risk of breast cancer in moderate-to-high risk women without prior breast cancer (34, 35). Although these drugs only prevent ER-positive tumors, their success is precedence for prevention of other types of breast cancers. Given that ErbB2 is overexpressed in up to 45% of premalignant DCIS (36, 37), lapatinib or other ErB family inhibitors may have potential for breast cancer prevention. Lapatinib suppressed ER-negative mammary tumor development in MMTV-Neu mice through decreased epithelial cell proliferation without affecting cell death (38). We show herein that ErbB3 loss prevented formation of ErbB2-overexpressing breast cancers in mice, even at earliest stages of ErbB2-induced epithelial hyperplasia. ErbB3 loss decreased cell proliferation, but unlike lapatinib, also increased cell death, suggesting that drugs targeting ErbB3, including monoclonal antibodies U3-1287 and MM-121 (targeting ErbB3 extracellular domain), or pertuzumab (preventing ErbB2-ErbB3 heterodimerization), may be effective pharmacologic strategies to prevent malignant conversion of ErbB2+ DCIS.

In summary, ErbB3 is expressed in preneoplastic ErbB2-overexpressing breast epithelium and is required for cell growth and malignant progression of ErbB2-driven hyperplasias. Furthermore, targeted inhibition of ErbB3 decreased growth of preexisting ErbB2-driven tumors and improved response to the ErbB2 inhibitor lapatinib. These data support future studies examining novel ErbB3-targeting agents in prevention and treatment of ErbB2+ breast cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
ErbB3 Promotes Each Stage of HER2/ErbB2-Driven Breast Cancer

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