PKCα Attenuates Jagged-1–Mediated Notch Signaling in ErbB-2–Positive Breast Cancer to Reverse Trastuzumab Resistance

Kinnari Pandya1, Debra Wyatt2, Brian Gallagher2, Deep Shah3, Andrew Baker4, Jeffrey Bloodworth1, Andrei Zlobin2, Antonio Pannuti5, Andrew Green6, Ian O. Ellis6, Aleksandra Filipovic7, Jason Sagert8, Ajay Rana3, Kathy S. Albain9, Lucio Miele5, Mitchell F. Denning2,10, and Clodia Osipo2,10,11

Abstract

Purpose: Breast cancer is the second leading cause of cancer mortality among women worldwide. The major problem with current treatments is tumor resistance, recurrence, and disease progression. ErbB-2–positive breast tumors are aggressive and frequently become resistant to trastuzumab or lapatinib. We showed previously that Notch-1 is required for trastuzumab resistance in ErbB-2–positive breast cancer.

Experimental Design: Here, we sought to elucidate mechanisms by which ErbB-2 attenuates Notch signaling and how this is reversed by trastuzumab or lapatinib.

Results: The current study elucidates a novel Notch inhibitory mechanism by which PKCα downstream of ErbB-2 (i) restricts the availability of Jagged-1 at the cell surface to transactivate Notch, (ii) restricts the critical interaction between Jagged-1 and Mindbomb-1, an E3 ligase that is required for Jagged-1 ubiquitylation and subsequent Notch activation, (iii) reverses trastuzumab resistance in vivo, and (iv) predicts better outcome in women with ErbB-2–positive breast cancer.

Conclusions: The clinical impact of these studies is PKCα is potentially a good prognostic marker for low Notch activity and increased trastuzumab sensitivity in ErbB-2–positive breast cancer. Moreover, women with ErbB-2–positive breast tumors expressing high Notch activation and low PKCα expression could be the best candidates for anti-Notch therapy. Clin Cancer Res; 1–12. ©2015 AACR.
Breast cancer is the second leading cause of cancer mortality among women worldwide. The major problem with current treatments is tumor resistance, recurrence, and disease progression. The current study elucidates a novel mechanism by which PKCδ attenuates Jagged-1-mediated Notch signaling in ErbB-2-positive breast cancer. The clinical impact of these studies is that PKCδ is a potential therapeutic target in low Notch activity and increased trastuzumab sensitivity in ErbB-2-positive breast cancer. Moreover, women with ErbB-2-positive breast tumors expressing high Notch activation and low PKCδ expression could be the best candidates for anti-Notch therapy. These studies provide a preclinical proof of concept for future clinical trials using combinations of trastuzumab/lapatinib plus an anti-Jagged-1–targeted therapy for trastuzumab-resistant, ErbB-2-positive breast cancer expressing low levels of PKCδ.

Recently, PKCδ was shown to mediate breast cancer stem cell survival (16) and is a therapeutic target in triple-negative breast cancer (17). However, the role of PKCδ in the ErbB-2-positive breast cancer and its significance for anti-ErbB-2–targeted therapy remain unclear.

Notch ligands (Delta-like 1, 3, and 4 and Jagged-1 and -2) and receptors (Notch-1, -2, -3, and -4) are implicated in breast cancer development and drug resistance (18). They require cell–cell contact for engagement and subsequent cleavage of membrane-bound receptors to intracellular transcriptional activators. The ligand-induced Notch activation is regulated by E3 ubiquitin ligases, Mindbomb-1 (18), and Neuralized (19). Co-expression of Notch-1 and Jagged-1 predicts for the poorest overall survival (20). Notch-1, -3, and -4 are breast oncogenes and potent regulators of cell differentiation, proliferation, and apoptosis (21). High Notch-1 and -4 expression correlates to poor prognosis (22) and Notch-3 promotes ErbB-2–negative breast cancer cell proliferation (23). Notch receptor–ligand interactions are important for mammary stem cell differentiation (24) and tumor-initiating cells (25).

We showed that trastuzumab or a dual EGFR/ErbB-2 inhibitor increased Notch-1 activity, and trastuzumab resistance was reversed by Notch-1 knockdown or a γ-secretase inhibitor (GSI; ref. 26). Subsequently, we demonstrated that dual targeting of ErbB-2 and Notch prevented recurrence and partially reversed resistance to trastuzumab (27). Here, we demonstrate a novel mechanism of action: PKCδ attenuates Mib-1–mediated Jagged-1–Notch activation to predict sensitivity to trastuzumab. Furthermore, breast cancers expressing low PKCδ might be more sensitive to anti-ErbB-2 agents. Importantly, anti-Jagged-1 therapy could reverse resistance to trastuzumab by attenuating Jagged-1–mediated Notch activity.

Materials and Methods

Cell culture and reagents

MDA-MB-453, BT474, and HCC1954 breast cancer cells were purchased from ATCC within the last 6 years. BT474 trastuzumab-resistant (BT474 Resistant) cells were generated by treating parental BT474 cells with increasing concentrations of trastuzumab for 6 months (26). All cell lines were authenticated using short tandem repeat (STR) allelic profiling (DCC Medical).

Biotinylation assay

MDA-MB-453 cells were seeded in 10-cm plates at a density of 40% to 50% confluency. After 48 hours, cells were treated with either mouse IgG (20 μg/mL in PBS) or trastuzumab (20 μg/mL in PBS) for 48 hours. The cells were then washed 3 times in PBS, and a cell-impermeable biotinylation reagent EZ-Link Sulfo-NHS-Biotin Reagent (Pierce Chemicals) as previously described (28) was added to cells to label cell surface proteins at 4°C under constant shaking. Cells were scraped, centrifuged at 1,000 rpm for 1 minute, washed twice with PBS, and lysed in RIPA lysis buffer. The biotinylated cell surface proteins were precipitated from the total protein lysate using 30 μL of Immobilized Neutavidin Protein (Cat. 29200, Pierce Chemicals). The beads were washed 4 times with PBS and the protein was eluted with 4 × SDS Laemmli buffer and then heated at 95°C for denaturation. Western blotting was performed to detect Jagged-1 and ErbB-2 as described in the Supplementary Section.

Confocal immunofluorescence microscopy

MDA-MB-453, BT474HS, or BT474HR cells were either transfected with control siRNA, PKCδ siRNA, or ErbB-2 siRNA or transfected with LRZS-linker alone or LRZS-PKCδ and subsequently plated into chamber slides or untransfected cells were treated with PBS or 20 μg/mL trastuzumab directly in the well. Cells were then fixed in the chamber with 3.7% paraformaldehyde, permeabilized with 0.1% saponin, and nonspecific interactions were blocked with 1% BSA. The staining protocol for specific proteins of interest is provided in the Supplementary Section.

Co-immunoprecipitation

Jagged-1 (Jagged-1 2H28 rabbit monoclonal antibody, Cat. 2620, Cell Signaling) was immunoprecipitated under treatment conditions as described in the figure legends. Lysates at a concentration of 3 to 5 mg/mL were incubated with 6 μg of antibody for the specific protein of interest or rabbit isotype control IgG (Cat. SC-2027, Santa Cruz) overnight with gentle rocking at 4°C. Thirty microliters of protein A-plus beads (sc-2002, Santa Cruz) were added to the immune complexes for 2 hours, the beads were washed with lysis buffer, and the proteins attached to the pelleted beads were eluted with 20 μL of 2 × Laemmli sample buffer plus β-mercaptoethanol and heated for 10 minutes at 95°C while vigorously shaking. Immunoblots are described in Supplementary Section.

Coculture assay

MDA-MB-453 cells were plated at a density of 3 × 10⁶ cells in a 10-cm plate. Mouse fibroblast (LTK) cells expressing no ligand or overexpressing Jagged-1 were added in equal parts (1:1) to previously plated MDA-MB-453 cells. The cells were then treated with PBS or 20 μg/mL trastuzumab for 18 hours. Cells were then stained with 10 μL of phycoerythrin (PE)-conjugated human ErbB-2 antibody (Cat. 340552, Becton Dickinson). Stained cells were then sorted for ErbB-2 expression at the cell surface by FACS. Total RNA was extracted from sorted cells, reverse transcribed to total cDNA, and real-time PCR was performed as previously described. The PCR primers that were used for detection of specific
transcripts were Hes-1 and Deltex-1 with RPL13a as a loading control.

**Trastuzumab-resistant xenografts**

Five million BT474 trastuzumab-resistant cells were injected bilaterally into mammary fat pads of ovariectomized FoxN1 nu/nu athymic nude mice (Harlan Sprague-Dawley) followed by implantation of a 17β-estradiol–containing silastic capsule of 0.3 cm in length with a constant release providing 83 to 100 µg/mL as described previously (29). The identity of each mouse and tumor was tracked by an ear tag. Once tumors grew to a mean cross sectional area (CRA) of 0.30 to 0.50 cm², mice were randomized to four treatment groups: (i) vehicle control (PBS), (ii) trastuzumab (10 mg/kg in a total volume of 100 µL sterile PBS, intraperitoneally once weekly), (iii) CTX-033 (5 mg/kg, intraperitoneally once weekly), or (iv) trastuzumab plus CTX-033. In case of BT474HR cells retrovirally transduced with the LRZS-linker or LRZS-PKCa, these tumors were randomized to two treatment groups each: (i) PBS or (ii) trastuzumab. Tumor area ([l × w] was measured weekly using Vernier calipers and cross sectional area ([l × w]×π)/4 was calculated and graphed. All animal study protocols were approved by Loyola University’s Institutional Animal Care and Use Committee.

**Flow cytometry**

MDA-MB-453 cells were treated with indicated dose of lapatinib/trastuzumab for 48 hours. DMSO and IgG were used as negative controls. Cells were harvested using Cellstripper (Cat. 25–056-CI Corning Cellgro) and were stained with fluorescein isothiocyanate (FITC)–conjugated Jagged-1 antibody as per the manufacturer's protocol. The cells were then resuspended in Flow Cytometry Staining Buffer (Cat. FC001 R&D Systems) and analyzed with BD FACS Canto II. Data acquisition was done using BD FACSDiva software and data analysis was performed using FlowJo software.

**Proliferation assay**

MDA-MB-453 cells were seeded into 10-cm dishes at 5 × 10⁶ cells. HCC-1954 cells were seeded into 10-cm dishes at 3.5 × 10⁶ cells. The cells were transfected as described in the RNA interference of Methods. After completion of the siRNA transfection, the cells were seeded in triplicate in 6-well plates at a density of 50,000 for MDA-MB-453 cells or HCC-1954 cells. BT474/HS sensitive cells were seeded into 6-well plates at a density of 60,000 cells. BT474HR resistant cells were seeded into 6-well plates at a density of 60,000 cells. After plating into the 6-well plates, cells were treated with PBS or 20 µg/mL trastuzumab on a daily basis. Cells were counted at days 2, 4, 6, and 8 posttreatment and cells were trypsinized into single-cell suspensions and counted using the Countess Automated Cell Counter (Cat. C10310, Life Technologies). The live cell number was then used to calculate the total number of live cells over the number of live cells plated.

**Immunohistochemical staining of human ErbB-2–positive breast tumors**

Tissue microarray (TMA) sections were placed in a 58 °C to 60 °C oven overnight for tissue to adhere. The sections were deparaffinized in xylene, rehydrated through graded ethanol, and washed with PBS before being treated with 1 × Reveal in a Decloaking Chamber (Biocare Medical) for antigen retrieval following the manufacturer's protocol. After rinsed in PBS for 15 minutes, the sections were soaked in 3% H₂O₂ in PBS for 20 minutes to quench endogenous peroxidase activity. Sections were incubated for 60 minutes in 3% normal rabbit serum (Vector Laboratories) in PBS at room temperature to block nonspecific binding sites and then probed with primary antibodies (PKCa antibody was Santa Cruz C20 at 1 µg/mL), with nonimmune IgG used as controls. The details of the staining protocol are in the Supplementary Section.

**Statistical analysis**

Most experiments were performed at least three times. Means ± SDs were calculated on at least n = 3 experiments. A two-tailed Student t test was performed on results with two comparisons. ANOVA was performed on results with multiple comparisons. Linear regression analysis was performed on tumor growth studies, as each mouse was tagged with a number and each tumor was measured independently followed by ANOVA for multiple comparisons. A Kaplan–Meier curve for recurrence-free survival or overall survival of human patients was generated by the Kaplan–Meier Plotter software (30) or generated by GraphPad Prism software and analyzed with a log-rank (Mantel—Cox) test.

**Supplementary Materials and Methods**

More detailed methods are provided in the Supplementary Section.

**Results**

**Jagged-1 is required for trastuzumab-induced Notch activation**

We demonstrated that inhibiting ErbB-2 increased NICD1 expression, CBF-1–driven reporter activity, Deltex-1 and Hey-1 transcripts, and thus most likely canonical Notch activation (26). Jagged-1 predicts poor outcome in women with breast cancer (31). Here, we sought to understand whether ErbB-2 attenuates Notch signaling by regulating Jagged-1 expression and/or cellular localization in breast cancer cells. Figure 1A confirms that ErbB-2 inhibits Notch transcriptional activity. Conversely, ErbB-2 inhibition using a kinase-dead mutant, ErbB-2 siRNA, or lapatinib increased RNA transcripts of Notch gene targets, Hes-1, Deltex-1, and/or Hey-1 (Fig. 1A). Figure 1B shows that trastuzumab or lapatinib increased Jagged-1 protein expression on the cell surface by flow cytometry. Furthermore, this increase in Jagged-1 protein expression by trastuzumab treatment is restricted to the cell surface as measured by a cell surface–specific biotinylation assay (Fig. 1C). These results indicate that ErbB-2 inhibition promoted the accumulation of Jagged-1 at the cell surface.

To confirm the supporting cell surface biochemical assays from Fig. 1B and C that ErbB-2 overexpression may restrict cell surface expression of Jagged-1, we performed confocal fluorescence microscopy to visualize Jagged-1 cellular localization. The IgG control cells showed that a portion of Jagged-1 colocalized with early endosomal antigen-1 (EEA-1; Fig. 1D). In contrast, trastuzumab-treated cells showed that Jagged-1 was no longer retained in EEA-1–positive endosomes and primarily accumulated at the cell surface (Fig. 1D). Furthermore, Notch-1 colocalized with Jagged-1 in control cells (Fig. 1D). However, trastuzumab treatment induced accumulation of Notch-1 throughout the cell and Jagged-1 at sites of cell–cell contacts (Fig. 1D). These results indicate that ErbB-2 overexpression possibly traps both Jagged-1 and Notch-1 in subplasma membrane compartments, including
early endosomes. However, anti-ErbB-2 treatment with trastuzumab disrupts the Jagged-1–Notch-1 colocalization. If Jagged-1–mediated transactivation of Notch is attenuated by ErbB-2, we predicted that supplying the Notch-expressing breast cancer cells with stromal cells expressing abundant surface Jagged-1 would restore Notch activation. We cocultured ErbB-2–expressing MDA-MB-453 breast cancer cells with mouse fibroblasts expressing no ligand (LTK-P) or overexpressing Jagged-1 (LTK-JAG1) and then treated with vehicle or trastuzumab. Trastuzumab increased both Hes-1 (4-fold; Fig. 1E right) and Deltex-1 mRNAs (7-fold; Fig. 1E, left) in ErbB-2+ cells when cocultured with LTK-P cells. Providing MDA-MB-453 cells with abundant Jagged-1 in trans increased Hes-1 mRNA by 3-fold (Fig. 1E, right) and Deltex-1 mRNA by more than 2-fold (Fig. 1E, left) in the presence of or absence of trastuzumab. Interestingly, trastuzumab treatment in the presence of LTK-JAG1 did not increase Deltex-1 levels compared with LTK-P cells. It is possible that there are cis-inhibitory mechanisms within the breast cancer cells that limited the activation of the Notch gene target. These results indicate that ErbB-2 most likely restricts Jagged-1 cell surface localization and limits transactivation of Notch.

Figure 1.
ErbB2 inhibits Notch signaling and Jagged-1 cell surface expression. A, ErbB2 expression and activity inhibits Notch activation. Left, MDA-MB-453 cells were stably transfected with vector alone or kinase dead ErbB2 (ErbB2KD) in an expression plasmid. Middle, MDA-MB-453 cells were transfected with a scrambled control siRNA (SCBi) or an ErbB2 siRNA. Right, MDA-MB-453 cells were treated with DMSO or lapatinib (LAP) for 6 days. Total protein from cell lysates were subjected to SDS-PAGE followed by Western blotting to detect tyrosine phosphorylated ErbB2 (PY-ErbB2), total ErbB2, and actin proteins. Bottom, similar studies were performed as described in A, and real-time PCR was performed to detect RNA transcript levels for Hes-1, Hey-1, and/or Deltex-1. Bar graphs, means ± SDs of three independent experiments. *, statistical significance P < 0.05 using a nonpaired Student t test. B, trastuzumab (Trast) or lapatinib increases cell surface Jagged-1 protein expression. MDA-MB-453 cells were treated with IgG control, DMSO, or increasing concentrations of trastuzumab or lapatinib for 48 hours. Flow cytometry was performed on live cells to detect cell surface expression of Jagged-1 protein. The results are representative of at least three independent studies. C, inhibition of ErbB2-2 increases cell surface Jagged-1 expression. MDA-MB-453 cells were treated with IgG or trastuzumab for 48 hours. Cell surface proteins were biotinylated as described in experimental procedures followed by streptavidin precipitation and Western blotting to detect the following proteins: Jagged-1 (JAG1) and total ErbB-2 in both precipitates and total lysates. D, trastuzumab promotes Jagged-1 surface localization and Notch-1 activation as shown by confocal immunofluorescence. Top, scale bars = 20 μm; bottom, scale bars = 10 μm. E, Jagged-1 mediated transactivation of Notch. MDA-MB-453 cells were cocultured with mouse fibroblasts expressing no ligand (LTK-Parental) or overexpressing Jagged-1 (LTK-JAG1) in a 1:1 ratio and then treated with PBS or trastuzumab for 18 hours. Cells were sorted by flow cytometry for ErbB-2–positive cells. RNA was extracted and real-time PCR was performed to detect relative transcript levels: Hes-1 (right) or Deltex-1 (left). Western blotting was performed on LTK cells to detect levels of Jagged-1 protein (middle). ***, P < 0.05 between PBS and trastuzumab; ***, statistical significance between LTK-P and LTK-JAG1; and ****, statistical significance between LTK-P and LTK-JAG1 in response to trastuzumab using an ANOVA.
To determine whether Jagged-1 is necessary for trastuzumab-induced Notch activation, we used a genetic approach to downregulate Jagged-1 in two ErbB-2–positive breast cancer cell lines. Different Jagged-1 siRNAs (JAG1iA or JAG1iNew) decreased Jagged-1 protein expression (Figs. 2A, 2B; Supplementary Fig. S1A) and significantly inhibited the trastuzumab-induced increase of Notch gene targets, Hes-1, Deltex-1, Hey-1, and/or Notch-4 transcripts (Figs. 2A and 2B; Supplementary Fig. S1A). These results show that Notch activation mediated by ErbB-2 inhibition is most likely Jagged-1–dependent.

**Dual blockade of ErbB-2 and Jagged-1 inhibits proliferation in vitro and reverses resistance in vivo**

We tested whether decreasing Jagged-1 by siRNA would enhance the effectiveness of trastuzumab in a panel of ErbB-2–positive breast cancer cell lines. Different Jagged-1 siRNAs (JAG1iA or JAG1iNew) decreased Jagged-1 protein expression (Figs. 2A, 2B; Supplementary Fig. S1A) and significantly inhibited the trastuzumab-induced increase of Notch gene targets, Hes-1, Deltex-1, Hey-1, and/or Notch-4 transcripts (Figs. 2A and 2B; Supplementary Fig. S1A). These results show that Notch activation mediated by ErbB-2 inhibition is most likely Jagged-1–dependent.

**ErbB-2 limits Mib-1–mediated ubiquitinylation of Jagged-1 and subsequent transactivation of Notch**

Because ubiquitinylation of Jagged-1 by Mib-1 is required to activate Notch (35, 36), we hypothesized that ErbB-2 attenuates expression of Mib-1 and/or its association with Jagged-1 to inhibit Notch activity. To test this hypothesis, Jagged-1 was immunoprecipitated from MDA-MB-453 cells treated with trastuzumab or lapatinib, and Western blotting was performed to detect Jagged-1 and Mib-1 proteins. Co-immunoprecipitation (co-IP) demonstrated that trastuzumab or lapatinib increased the amount of Mib-1–bound Jagged-1 (Fig. S1B). These results indicate that Jagged-1 is required for trastuzumab resistance.

Previously, Pandya and colleagues demonstrated partial restoration of trastuzumab sensitivity and prevention of ErbB-2–positive breast tumor recurrence using a combination of trastuzumab plus a GSI (27). GSIs are pan-Notch inhibitors and might not specifically target Notch-1. To determine whether Jagged-1 is required for trastuzumab resistance in vivo, we chose a novel format of an anti-Jagged antibody, termed a Probody therapeutic (CTX-033; refs. 32, 33). The anti-Jagged Probody, CTX-033, has demonstrated antitumor activity in a mouse xenograft model of pancreatic cancer (34). Figure 3D showed that xenograft breast tumors generated from trastuzumab-resistant cells were resistant to trastuzumab following 4 weeks of treatment. Treatment with the CTX-033 inhibited tumor growth when used alone. However, trastuzumab plus CTX-033 significantly decreased tumor growth compared with CTX-033 or trastuzumab alone (Fig. 3D). These data suggest that using a combination of trastuzumab plus CTX-033 can partially reverse trastuzumab resistance in this model.

**ErbB-2–mediated PKCα signaling regulates Notch to prevent trastuzumab resistance**

PKCα is a key mediator of ErbB-2 signaling and is required for the maintenance of breast cancer cell viability and tumorigenicity (35). Here, we showed that PKCα regulated Notch to prevent trastuzumab resistance. PKCα inhibition decreased Notch activity, which was associated with decreased expression of Notch target genes and reduced Notch signaling in breast cancer cells. These findings suggest that PKCα is a critical regulator of Notch activity in breast cancer cells and that targeting PKCα may improve the efficacy of trastuzumab in breast cancer treatment.

**Figure 2.**

Jagged-1 is required to promote trastuzumab-induced Notch activation. A, Jagged-1 is required for trastuzumab-induced increase of Notch gene targets. MDA-MB-453 cells were transfected with control siRNA (SCBi) or two distinct Jagged-1 siRNAs (JAG1iA or JAG1iNew) and then treated with PBS or trastuzumab. Protein expression of Jagged-1 or actin was determined by Western blotting (top). Transcript levels of Notch gene targets, Deltex-1, Hey-1, and Notch-4 were measured by real-time PCR (bottom). The results are means ± SDs of three independent experiments. B, BT474 cells were transfected with SCBi or JAG1i siRNA and then treated with PBS or trastuzumab for 48 hours. Western blotting was performed to detect efficiency of Jagged-1 knockdown (top) and real-time PCR was performed to detect transcripts of Notch gene targets: Hes-1 and Deltex-1 (bottom). The results are means ± SDs of three independent experiments. * and **, Statistical significance of $P < 0.05$ as determined by an ANOVA.
Mib-1 in a complex with Jagged-1 (Fig. 4A). From these results, we conclude that ErbB-2 possibly limits the interaction between Jagged-1 and Mib-1.

To investigate the role of Mib-1 on Notch-1 activity, the effect of a Mib-1 siRNA on the expression of Hes-1 protein levels was measured. Western blot analysis confirmed that the kinase inhibitors inhibited their targets (Supplementary Fig. S3A). These results suggest that ErbB-2 restricts the interaction of Mib-1 to Jagged-1 and its ubiquitinylation possibly through a different kinase downstream of ErbB-2.

ErbB-2 promotes an association between Jagged-1 and PKCα

To determine the mechanism by which ErbB-2 attenuates the association between Jagged-1 and Mib-1, the protein sequences of Jagged-1 and Mib-1 were scanned using Prosite Expasy and several high scoring putative PKC phosphorylation sites were identified. ErbB-2 activates PKCα in breast cancer cells via PLCγ (37) or by upregulating c-Src (37). Co-IP demonstrated that Jagged-1 was associated with PKCα when ErbB-2 was active but failed to bind PKCα when cells were treated with lapatinib (Fig. 4C). Down-regulation of PKCα using an siRNA facilitated the association of Mib-1 with Jagged-1 and subsequent ubiquitinylation of Jagged-1.
similarly to lapatinib treatment (Fig. 4D). Control Western blot analyses were performed to detect protein levels of active ErbB-2 (PY), total ErbB-2, and PKCα to confirm the efficiency of the PKCα siRNA and that it had little effect on the expression and activity of ErbB-2 (Fig. 4D).

Jagged-1 and PKCα colocalize in ErbB-2–positive breast cancer cells and dissociate upon trastuzumab treatment

We performed confocal fluorescence microscopy to explore whether Jagged-1 and PKCα colocalize in ErbB-2–overexpressing breast cancer cells. Jagged-1 colocalizes with PKCα (Fig. 4E, left) in PBS-treated cells. In contrast, Jagged-1 was no longer colocalized with PKCα (Fig. 4E, left) in response to trastuzumab. Similar, results were observed in trastuzumab-sensitive BT474 cells (Fig. 4E, right). Interestingly, trastuzumab-resistant BT474 cells express less PKCα and Jagged-1 is primarily near the cell surface (Fig. 4E, right, top). PKCα knockdown in BT474 sensitive cells disrupts the Jagged-1–PKCα colocalization similar to what we observed in resistant cells (Fig. 4E, right, bottom).

PKCα inhibits Jagged-1–mediated Notch activation and restores trastuzumab sensitivity in vivo

To determine whether PKCα is sufficient to restrict Notch activation, we tested whether a constitutive active PKCα

Figure 4.
ErbB-2 or PKCα attenuates Mib1-mediated ubiquitinylation of Jagged-1. A, trastuzumab or lapatinib increases the interaction between Mib1 and Jagged-1. MDA-MB-453 cells were treated with controls (IgG or DMSO) or trastuzumab or lapatinib, respectively, for 30 minutes. An IgG antibody was used to immunoprecipitate Jagged-1 from treated cell lysates. An IgG antibody was used as a control for nonspecific immunoprecipitation. Western blotting was performed to detect co-immunoprecipitated proteins such as Mib-1 (Mib1). Jagged-1 (JAG1) protein was detected to determine specificity of the immunoprecipitation. Western blotting was also performed on total lysates to detect levels of total Mib1 and JAG1. B, lapatinib-induced Jagged-1 ubiquitinylation is independent of PI3K and MAPK signaling. Cells were treated with DMSO, lapatinib, LY294002, or U0126 and then lysed. Jagged-1 was immunoprecipitated and Western blotting was performed to detect ubiquitinylation (Ub). Mib1, and JAG1. C, recruitment of PKCα to Jagged-1 is inhibited by lapatinib. MDA-MB-453 cells were transfected with the FLAG-tagged Ub for 48 hours and treated with DMSO or lapatinib for 30 minutes. JAG1 was immunoprecipitated from cell lysates followed by Western blotting to detect Ub, Mib1, PKCα, and JAG1. D, PKCα inhibits ubiquitinylation of Mib1 and Jagged-1 ubiquitinylation. MDA-MB-453 cells were transfected with SCBi or PKCα siRNA for 72 hours. Cells were then treated with DMSO or lapatinib for 30 minutes followed by Jagged-1 immunoprecipitation and Western blotting to detect Ub, Mib1, and JAG1. Western blotting was also performed on total lysates to detect PY-ErbB2, ErbB2, PKCα, and actin. E, Jagged-1 and PKCα colocalize in ErbB-2–positive breast cancer cells. MDA-MB-453 cells were treated with PBS or trastuzumab for 48 hours. Trastuzumab-sensitive (Sens) and -resistant (Res) BT474 cells were transfected with SCBi or PKCα siRNA for 72 hours. Cells were fixed and stained for JAG1 (green) and PKCα (red) and visualized using confocal immunofluorescence microscopy. The results are representative of three independent experiments. Left two, scale bars = 10 μm; right, scale bars = 20 μm. ImageJ software was used to measure densitometry of protein bands on Western blot analyses. Ratios of proteins are presented below images.
(Δ22–28) would prevent the lapatinib-induced Mib-1 and Jagged-1 interaction, Jagged-1 ubiquitylation, and increase in Notch gene targets. Figure 5A showed that lapatinib increased recruitment of Mib-1 to a Jagged-1 complex and increased Jagged-1 ubiquitylation in cells transduced with control LRZS-linker. In contrast, lapatinib treatment of cells transduced with LRZS-PKCαΔ22–28, which increased PKC activation as shown in Fig. 5A, right, failed to increase Mib-1 recruitment to Jagged-1 and ubiquitylation (Fig. 5A). Furthermore, Fig. 5C showed that lapatinib significantly increased 3 of 5 Notch gene targets (Deltex-1, Hey-1, and Notch-4) in cells transduced with LRZS-linker but not in cells expressing PKCαΔ22–28. In contrast, knockdown of PKCα increased expression of Hes-1 and Hey-1 proteins (Fig. 5B, left) and Deltex-1 transcripts (Fig. 5B, right), respectively. Taken together, these results indicate that ErbB-2 facilitates a PKCα–Jagged-1 association to limit Mib-1–mediated ubiquitylation of Jagged-1 and activation of Notch.

To determine whether PKCα expression limits Notch-1 nuclear localization, we performed confocal immunofluorescence. The results showed that cellular localization of Notch-1 and Jagged-1 was relatively unchanged in cells expressing LRZS-linker alone or LRZS-PKCα upon vehicle treatment (Fig. 5D, top). In contrast, trastuzumab treatment of cells transduced with the LRZS-linker induced nuclear localization of a proportion of the Notch-1 protein, whereas Jagged-1 accumulated in the cytoplasm or near the cell surface (Fig. 5D, bottom left). PKCα overexpression...
PKCα activation by trastuzumab or lapatinib is required for ErbB-2 blocks the interaction of Mib1 with Jagged-1, subsequently limiting ubiquitinylation of Jagged-1 to restrict Notch activation. Jagged-1 expression (top), high expression (middle), and moderate expression (lower). C, model summarizes conclusion of current study. ErbB-2 through PKCα

Figure 6. PKCα overexpression reverses trastuzumab resistance and predicts better survival in women with ErbB-2–positive human breast cancer. A, overexpression of PKCα inhibits growth of trastuzumab-resistant BT474 tumor xenografts. Five million trastuzumab-resistant BT474 cells retrovirally transduced with LRZS-linker or LRZS-PKCα were injected into mammary fat pads of 20 female nude athymic mice, respectively. Tumors were allowed to grow to a mean cross-sectional area of approximately 10 to 20 mm² and then mice were randomized to PBS or trastuzumab, injected intraperitoneally once weekly for up to 10 weeks. Statistical significance was calculated by a two-way ANOVA. B, low PKCα protein predicts poorer overall survival in women with ErbB-2–positive breast cancer. The Nottingham cohort of 100 ErbB-2–positive breast tumor tissues was stained for PKCα protein and scored 0.00 for negative staining or 1.00 for positive staining. Overall survival follow-up results were collected prospectively up to 400 months. Kaplan–Meier analysis for overall survival was performed on negative and positive expression of PKCα. Statistical significance was calculated using log-rank (Mantel–Cox) test. Immunohistochemistry was performed to detect PKCα protein in ErbB-2–positive breast cancer tissue. The three panels are representative of negative expression (top), high expression (middle), and moderate expression (lower). C, model summarizes conclusion of current study. ErbB-2 through PKCα blocks the interaction of Mib1 with Jagged-1, subsequently limiting ubiquitinylation of Jagged-1 to restrict Notch activation. Jagged-1–mediated Notch activation by trastuzumab or lapatinib is required for ErbB-2–positive breast cancer survival and resistance.

almost completely prevented Notch-1 nuclear localization (Fig. 5D, bottom right). These results indicate that PKCα is sufficient to prevent Notch-1 nuclear localization and activation in ErbB-2–positive breast cancer cells.

On the basis of results shown so far, we hypothesized that trastuzumab-resistant cells that have high Notch-1 activation and require Notch-1 for their survival (26) should have lower PKCα protein levels or activity than their sensitive parental cells. The results show that trastuzumab-resistant BT474 cells express higher PKCα protein than trastuzumab-resistant BT474 cells and that trastuzumab treatment of both sensitive and resistant cells decreased levels of PKCα protein and kinase activity as measured by detection of phosphorylated Serine-specific PKC substrates (Supplementary Fig. S3B). We hypothesized that overexpression of PKCα might restore trastuzumab sensitivity. To address this, we transduced the trastuzumab-resistant BT474 breast cancer cell line with LRZS-linker or LRZS-PKCα and injected these cells (Supplementary Fig. S3B) into female nude mice to determine their sensitivity to trastuzumab treatment in vivo. The results demonstrated that trastuzumab-resistant BT474 cells expressing LRZS-linker are resistant to trastuzumab (Fig. 6A). However, PKCα overexpression significantly decreased BT474 breast tumor growth compared with LRZS-linker. This result is not surprising, as we have shown previously that Notch-1 knockdown alone in trastuzumab-resistant BT474 cells was necessary to inhibit cell proliferation in vitro in the absence or presence of trastuzumab (26). These current results would suggest that PKCα overexpression could also be necessary to inhibit ErbB-2–positive breast tumor growth after acquired resistance to trastuzumab possibly by suppressing Notch signaling. PKCα overexpression partially restored trastuzumab sensitivity, as the tumors almost completely regressed upon trastuzumab treatment (Fig. 6A). These results demonstrate that PKCα could limit Notch-1 activation and thus possibly trastuzumab resistance in ErbB-2–positive breast cancer cells.

PKCα and Hey-1 inversely predict ErbB-2–positive breast cancer recurrence and survival

These results suggest that ErbB-2–positive breast cancer patients with high PKCα expression should have better outcomes, as PKCα can attenuate Notch signaling to promote sensitivity to anti-ErbB-2 therapy. To address this, we used survival analysis via the Kaplan–Meier Plotter software, version 2014 (30) and searched for expression of PRKCA and Hey-1 transcripts in a
cohort of 208 patients with ErbB-2–positive breast cancer to predict recurrence-free survival (RFS) outcome. Results showed that high PRKCA transcript expression based on an average of four probes predicted better RFS outcome compared with low with an HR of 0.64 (log-rank \( P = 0.037 \)). In contrast, high expression of a Notch gene target, Hey-1, predicted poor RFS outcome (HR, 1.72; log-rank \( P = 0.011 \)) using an average of two Hey-1 probes (Supplementary Fig. S3). Interestingly, when we used the multigene analyzer and asked whether the same breast cancer samples that express high Hey-1 and low PRKCA predict worse RFS outcomes compared with the individual genes, the results showed a slightly worse outcome with \( HR = 1.77 \) and log-rank \( P = 0.0074 \) (Supplementary Fig. S3).

We examined another cohort, the Nottingham primary breast cancer series (the Tenovus cohort) prepared as TMA. Outcome data were collected on a prospective basis. These include breast cancer–specific survival (BCSS) defined as the time in months from the date of surgery to the breast cancer-related death (36, 39). One hundred nine ErbB-2–positive breast cancer tissues were stained for PKC \( \alpha \) protein by immunohistochemistry, blindly scored for staining intensity (0–3). Samples were classified into two groups: Group 1.0 with high PKC \( \alpha \) (staining intensity, 2–3) and group 0.0 with low or negative PKC \( \alpha \) (staining intensity, 0–1) and labeled as “0.00.” The results demonstrated that high PKC \( \alpha \) protein expression predicted better BCSS compared with low expressing patients (log-rank \( P = 0.019 \); Fig. 6B). Figure 6B showed a sample of the immunohistochemistry for PKC \( \alpha \) protein stained as negative (top), positive sample #1 (middle), and positive sample #2 (bottom). Therefore, on the basis of the results of our current study, we propose a model where PKC\( \alpha \) attenuates the association between Jagged-1 and Mib-1 to restrict Jagged-1 ubiquitylation and subsequent transactivation of Notch-1 to prevent Notch-1–driven resistance to ErbB-2–targeted therapies (summarized in Fig. 6C).

**Discussion**

ErbB-2–positive breast cancer is currently treated with trastuzumab, lapatinib, pertuzumab, or trastuzumab emtansine. Many patients will not initially respond to these drugs and, among responders, more than 25% develop resistance within the first year of therapy (40). Thus, resistance remains a clinical problem that requires identification of new targets for future therapeutic strategies and novel prognostic biomarkers. Our results reveal that PKC\( \alpha \) downstream of ErbB-2 limits an association between Mib-1 and Jagged-1 to attenuate Notch-1 activation. The PKC\( \alpha \)-mediated attenuation of Notch-1 signaling is necessary and sufficient to enhance sensitivity to trastuzumab and possibly prevent resistance to potentially improve survival for women with ErbB-2–positive breast cancer.

Notch signaling promotes breast cancer progression (41, 42) and is critical for survival and self-renewal of breast cancer stem cells (24, 25). These cells are hypothesized to be the dormant or slowly self-replicating cell population resistant to standard therapies. We showed that Notch-1 is required for trastuzumab resistance (26) and ErbB-2 breast tumor recurrence (27). Others have shown that Notch cooperates with ErbB-2 to promote a more invasive phenotype in ductal carcinoma in situ (DCIS; ref. 43). Furthermore, Notch expression and activity have been implicated in ErbB-2–driven cancer stem cells (44). Recently, Abravanel and colleagues showed that Notch promotes recurrence of breast tumor cells after anti-ErbB-2 therapy (45). Thus, Notch could promote breast tumor development during early stages of breast tumorigenesis, and at later a stage, its activity and function is attenuated as other pathways become dominant. From our studies, we conclude that ErbB-2 attenuates Notch signaling via PKC\( \alpha \) under conditions whereby ErbB-2 is the primary growth driver. However, when ErbB-2 is inhibited by trastuzumab or lapatinib, PKC\( \alpha \) is inhibited and Jagged-1 is made competent to transactivate Notch to promote a compensatory survival signal.

The role of PKC\( \alpha \) in breast cancer remains controversial, as it is not clear whether PKC\( \alpha \) is a tumor promoter or a suppressor. PKC\( \alpha \) is implicated in human breast cancer progression (46). However, there are conflicting data, as some have shown PKC\( \alpha \) is decreased in human breast tissue (47). Ectopic expression of PKC\( \alpha \) in cell lines promotes a more aggressive phenotype. For example, PKC\( \alpha \) overexpression induces tamoxifen resistance in ER-positive cell lines (14). Furthermore, PKC\( \alpha \) is required for invasion of breast cancer cells (48). In addition, PKC\( \alpha \) activates Notch-4 expression in ER-positive breast cancer cells to promote tamoxifen resistance (49). PKC\( \alpha \) is also a mediator of breast cancer stem cell expansion (16), regulates recycling of ErbB2 in breast cancer cells (50), and a therapeutic target in triple-negative breast cancer (17). One possible explanation for the conflicting results could be that the activation status of PKC\( \alpha \), its subcellular localization, and its immediate substrates in breast cancer remain unclear. As breast cancer is a heterogeneous disease, it would not be surprising that PKC\( \alpha \) could have pleiotropic functions in different subtypes of breast cancer. For example, patients with ErbB-2–positive tumors expressing low PKC\( \alpha \) have poorer RFS than high expressors. These data included both ER-positive and -negative tumors. Ideally, if we could stratify and separate these data based on ER expression, results could determine whether ER status changes the prediction. However, because of the low number of ErbB-2− patients from the Kaplan–Meier Plotter dataset and the Nottingham cohort, this could not be done. Increasing the number of retrospective samples and/or a prospective clinical trial will help increase power and answer some of these critical questions. Results from clinical trials using PKC inhibitors have been disappointing. The ISIS 3521 (aprinocarsen or LY900003) trial failed to show a response and this could be due to multifactorial roles of PKCs. More than 20 trials from phase I to III have been conducted using various PKC inhibitors in many solid tumors (51). However, results of trials have been disappointing in women with metastatic breast cancer (52), suggesting that targeting PKC\( \alpha \) might depend on the cell context.

The mechanism by which PKC\( \alpha \) attenuates Jagged-1 vesicular trafficking and recruitment of Mib-1 to Jagged-1 is under investigation. Both Jagged-1 and Mib-1 contain putative PKC phosphorylation sites. Therefore, Jagged-1 and/or Mib-1 could be direct substrates of PKC\( \alpha \). Phosphorylation events regulate recruitment of E3 ligases and thus PKC\( \alpha \) could directly phosphorylate Jagged-1 and/or Mib-1 to prevent their interaction. Alternatively, PKC\( \alpha \) could regulate vesicular trafficking of Jagged-1 possibly sequestering Jagged-1 in endosomes targeted for recycling. Classical PKCs such as \( \alpha \) and \( \beta I \) regulate proteins that are normally recycled by sequestering them in the pericentriolar (53). Our results are consistent with this as we showed that Jagged-1 is localized to endosomes when ErbB-2 was hyperactive.
but accumulated at the cell surface upon ErbB-2 blockade. Thus, PKCθ might limit the cell surface availability of Jagged-1 by facilitating sequestration of Jagged-1 in recycling endosomes.

We describe a critical function for PKCθ downstream of ErbB-2, which is to attenuate the competency of Jagged-1 to activate Notch-1. Notch ligands are made competent by Mib-1 and subsequent ubiquitinylation that drives endocytosis. We show that Jagged-1 is required for trastuzumab resistance in breast cancer (Fig. 3) providing the first preclinical proof of concept for the use of an anti-Jagged-1, such as the Probody CTX-033, targeted therapy for prevention or reversal of resistance possibly in breast cancers expressing low PKCθ levels (Fig. 6) and/or high Hey-1 levels (Supplementary Fig. S3). An anti-Jagged Probody therapeutic may be well positioned for such a strategy given that it demonstrates less systemic toxicity compared with other Notch targeting approaches because it is only activated within the tumor microenvironment (34). The human data from two cohorts of ErbB-2-positive breast tumors predict that women with breast tumors expressing low PKCθ mRNA (Supplementary Fig. S4) or protein (Fig. 6B) have poorer RFS or BCSS, respectively. Conversely, high Hey-1 expression predicts poor RFS outcome in women with ErbB-2-positive breast cancer (Supplementary Fig. S4). To determine whether the same tumors that express low PKCθ also express high Hey-1 would be better targets for anti-Notch therapy, a prospective clinical trial should be conducted using the Jagged-1 Probody or a Notch-specific inhibitor in combination with trastuzumab. These data are consistent with the overall model where the ErbB-2–PKCθ axis attenuates Notch activation and this subset of tumors could predict better sensitivity to anti-ErbB-2–targeted agents. Conversely, low PKCθ tumors could require concomitant Notch inhibition to prevent or reverse trastuzumab resistance. These results could have clinical impact for the treatment of women with ErbB-2–positive breast cancer. Future clinical trials need to be designed to test whether PKCθ protein levels will predict for trastuzumab sensitivity.

Disclosure of Potential Conflicts of Interest
J. Sager has ownership interest in CytoMx. L. Miele is a consultant/advisory board member for CytoMx. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: K. Pandya, D. Wyatt, D. Shah, A. Baker, A. Rana, M.F. Denning, C. Osipo
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Pandya, D. Wyatt, B. Gallagher, D. Shah, J. Bloodworth, A. Green, I.O. Ellis
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Pandya, D. Wyatt, D. Shah, A. Zlobin, A. Panzutti, A. Green, I.O. Ellis, A. Filipovic, A. Rana, L. Miele, M.F. Denning
Writing, review, and/or revision of the manuscript: K. Pandya, D. Wyatt, D. Shah, A. Baker, A. Green, I.O. Ellis, J. Sager, A. Rana, K.S. Alibain, L. Miele, M.F. Denning, C. Osipo
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Pandya, D. Wyatt, B. Gallagher, D. Shah, J. Bloodworth, A. Rana
Study supervision: C. Osipo
Other (development of a molecule that was used in the study): J. Sager

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PKCα Attenuates Jagged-1–Mediated Notch Signaling in ErbB-2–Positive Breast Cancer to Reverse Trastuzumab Resistance

Kinnari Pandya, Debra Wyatt, Brian Gallagher, et al.

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