Effective Targeting of Estrogen Receptor-Negative Breast Cancers with the Protein Kinase D Inhibitor CRT0066101

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Abstract

Invasive ductal carcinomas (IDC) of the breast are among the most aggressive types of cancer affecting women. IDCs with the worst outcome are associated either with loss of expression of hormone receptors (HR), amplification of amplified or overexpression of HER2, or a triple-negative phenotype. The most aggressive cases of IDC are characterized by a high proliferation rate, a great propensity to metastasize, and their ability to resist to standard chemotheraphy, hormone therapy, or HER2-targeted therapy. Using progression tissue microarrays, we here demonstrate that the serine/threonine kinase protein kinase D3 (PKD3) is highly upregulated in estrogen receptor (ER)-negative (ER-) tumors. We identify direct binding of the ER to the PRKD3 gene promoter as a mechanism of inhibition of PKD3 expression. Loss of ER results in upregulation of PKD3, leading to all hallmarks of aggressive IDC, including increased cell proliferation, migration, and invasion. This identifies ER-negative breast cancers as ideal for treatment with the PKD inhibitor CRT0066101. We show that similar to a knockdown of PKD3, treatment with this inhibitor targets all tumorigenic processes in vitro and decreases growth of primary tumors and metastasis in vivo. Our data strongly support the development of PKD inhibitors for clinical use for ER-negative breast cancers, including the triple-negative phenotype. Mol Cancer Ther; 14(6); 1306–16. ©2015 AACR.

Introduction

Invasive ductal carcinomas (IDC) of the breast are among the most aggressive types of cancer affecting women. IDCs with the worst outcome are associated either with loss of expression of hormone receptors, estrogen receptor (ER) and progesterone receptor (PR), overexpression, or amplification of the human epidermal growth factor receptor-2 (HER2/ErbB2), or a triple-negative phenotype [lack of expression of HER2 and both hormone receptors (HR)]. The most aggressive cases of IDC are characterized by a high proliferation rate, a great propensity to metastasize, and their ability to resist to standard chemotherapy, hormone therapy, or HER2-directed therapy. Using progression tissue microarrays, we here demonstrate that the serine/threonine kinase protein kinase D3 (PKD3) is highly upregulated in estrogen receptor (ER)-negative (ER-) tumors. We identify direct binding of the ER to the PRKD3 gene promoter as a mechanism of inhibition of PKD3 expression. Loss of ER results in upregulation of PKD3, leading to all hallmarks of aggressive IDC, including increased cell proliferation, migration, and invasion. This identifies ER-negative breast cancers as ideal for treatment with the PKD inhibitor CRT0066101. We show that similar to a knockdown of PKD3, treatment with this inhibitor targets all tumorigenic processes in vitro and decreases growth of primary tumors and metastasis in vivo. Our data strongly support the development of PKD inhibitors for clinical use for ER-negative breast cancers, including the triple-negative phenotype. Mol Cancer Ther; 14(6); 1306–16. ©2015 AACR.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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levels in different breast cancer subtypes as well as in normal tissue, PKD3 is highly upregulated in ER-negative (ER-) tumors. We identify estrogen-dependent signaling as the mechanism of inhibition of PKD3 expression in ER-expressing ductal cancer cells. Loss of ER results in upregulation of PKD3, leading to increased cell proliferation, migration, and invasion. These data identify ER- breast cancers as ideal cancers for treatment with the PKD inhibitor CRT0066101, because they express little or no PKD1 and high levels of PKD3. We show that, similar to a knockdown of PKD3, treatment with this inhibitor targets most tumorigenic processes in vitro, and also decreases growth of primary tumors and prevents metastasis in vivo. Thus, because it can be given orally, it may be developed for treatment of primary tumors and prevents metastasis.

Materials and Methods

Cell lines, antibodies, and reagents

The MDA-MB-231-luc2 cell line was obtained from PerkinElmer in May 2009. All other cell lines were obtained from the American Type Culture Collection in August 2008. All cell lines were not further authenticated. MCF-7, MDA-MB-231, and T47D cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids (NEAA), and 1 mmol/L sodium pyruvate. MCF-10A cells were maintained in DMEM/Ham’s F-10 medium (50:50 v/v) with 5% horse serum, 20 ng/mL EGF, 0.5 µg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 µg/mL insulin, and 1% penicillin/streptomycin. NEAAs were obtained from Mediatech, EGF from PeproTech, and insulin and hydrocortisone from Sigma-Aldrich. Anti-β-actin antibody was obtained from Sigma-Aldrich; anti-Ki67 from Dako; anti-cleaved PARP, anti-cleaved caspase-3, and anti-MMP9 from Cell Signaling Technology; anti-COX-2 from Cayman Chemical; anti-smooth muscle actin (SMA), anti-GFP, and anti-cyclin D1 from Abcam; anti-Snail from Abgent; anti-vimentin (for Western blotting) from Santa Cruz Biotechnology; and anti-N-cadherin and anti-vimentin [for immunohistochemistry (IHC)] from Epitomics. The rabbit polyclonal antibody specific for PKD2 was from Upstate Biotechnology and the rabbit polyclonal antibody specific for PKD3 used for immunoblotting was from Bethyl Laboratories. The mouse monoclonal antibody specific for PKD1 was raised by Creative Biolabs/Creative Dynamics and is further described in ref. (11). Secondary horseradish peroxidase (HRP)–linked antibodies were obtained from Roche Applied Science. Luciferin was obtained from Gold Biotechnology. Fulvestrant and β-estradiol (E2) were purchased from Sigma-Aldrich. The PKD-specific inhibitor CRT0066101 was obtained from Cancer Research Technology.

Lentiviral shRNA expression and shRNA constructs

Specific lentiviral expression constructs for short hairpin RNA (shRNA) targeting human PKD3 were purchased from Sigma-Aldrich (MISSION shRNA Plasmid DNA). Constructs used were NM_005813.x:3393s1c1 (labeled as PKD3-shRNA#1) and NM_005813.x:2494s1c1 (labeled as PKD3-shRNA#2). Lentivirus was produced in HEK293FT cells using the ViralPower Lentiviral Expression System (Life Technologies). MDA-MB-231 cells were infected with PKD3-shRNA lentivirus to generate stable cell lines. After infection, cell pools were selected using puromycin (1 µg/mL) for 15 days.

Plasmids and transfections

To generate a PKD3 promoter-luciferase reporter, the human PRKD3 promoter region (−1000 to +3) was cloned in pGL3 plasmid from Promega via BglII and Xhol restriction sites, using 5′-TTTTTTTTTCTCTTTTGTAT-3′ and 5′-GACGGAAAGAAAATTAGAAAATTTT-3′ as primers. The pRL-CMV-Renilla luciferase plasmid was from Promega. The ERα (pEGFP-C1-ERα; #28230) expression plasmid was from Addgene. The pSuper-PKD2-shRNA plasmid was obtained by cloning the oligonucleotides 5′-GATGGCCGGTTCCCTGAGTGTGGCTTCTCTCTTGAAGAAGCCACACTCAGGGA- TCGAGGAGCCTTITTTTGAAA-3′ and 5′-AGCCTTTTCCCCAAGAGTTG- CCTTGAGTGTGGCCCTTGCTTCITGTAAGAGACCGACCACTAGCAGGGAGCCTTITTTTGAAAA-3′ into pSuper. GenJet from SigmaGen was used for transfection of breast cancer cells.

Cell lyses and Western blot analysis

Cells were washed twice with ice-cold phosphate-buffered saline (PBS; 140 mmol/L NaCl, 2.7 mmol/L KCl, 8 mmol/L Na2HPO4, 1.5 mmol/L KH2PO4, pH 7.2) and lysed with Buffer A (50 mmol/L Tris–HCl, pH 7.4, 1% Triton X-100, 150 mmol/L NaCl, 5 mmol/L EDTA, pH 7.4) plus Protease Inhibitor Cocktail (Sigma-Aldrich). Lysates were used for Western blot analysis as described previously (12).

Immunofluorescence

Cells were seeded in 8-well ibiSlides (ibidi) and treated as indicated. Before fixation with 4% paraformaldehyde (20 minutes, 4°C), cells were washed twice with PBS. Fixed cells were washed three times in PBS, permeabilized with 0.1% Triton X-100 in PBS (2 minutes, room temperature), and then blocked with blocking solution [3% bovine serum albumin (BSA) and 0.05% Tween 20 in PBS] for 30 minutes at room temperature. F-actin was stained with Alexa Fluor 633–Phalloidin (Life Technologies), nuclei with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich) in blocking solution. After extensive washes with PBS, cells were mounted in ibidi mounting medium (ibidi). Samples were examined using an IX81 DSU Spinning Disc Confocal from Olympus with a 40× objective.

Proliferation, migration, and invasion assays

Transwell migration and invasion assays were performed as described previously (12). Briefly, Transwell chambers were left uncoated (migration assay) or coated with Matrigel (2 µg/well; BD Biosciences), dried overnight, and rehydrated for 1 hour with 40 µL of tissue culture media. Cells were harvested, washed once with media containing 1% BSA, and resuspended in media containing 0.1% BSA. Then, 100,000 cells were seeded per Transwell insert. NIH-3T3 conditioned medium served as a chemoattractant in the lower chamber. Remaining cells were used to control the expression of genes of interest by Western blot analysis. After 16 hours, cells on top of the Transwell insert were removed and cells that had migrated/invaded to the lower surface of the filters were fixed in 4% paraformaldehyde, stained with DAPI, and counted. For impedance-based real-time chemotactic assays, cells were seeded onto an E-Plate (for proliferation assays)
or a CIM-Plate 16 Transwell (for migration/invasion assays) from Roche Applied Science. After attachment, cell migration or invasion (coating of top well with 2 µg of Matrigel) toward NIH-3T3 conditioned media was continuously monitored in real time for the indicated times using the xCELLigence RTCA DP Instrument from Roche Applied Science.

Patient samples, TMAs, and IHC

Tissue samples were initially collected with the approval of the Mayo Clinic Institutional Review Board (IRB) under protocol MC0033. Written informed consent for the use of these tissues in research was obtained from all participants. Generation of the TMA was performed under protocol 09-001642. Therefore, all unique patient identifiers and confidential data were removed and tissue samples were de-identified. The Mayo Clinic IRB assessed the protocol 09-001642 as minimal risk and waived the need for further consent. All data were analyzed anonymously.

Orthotopic tumor models and treatment

Animal experiments were performed under protocols A43213 and A17313 approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC). Female nonobese diabetic severe combined immunodeficiency (NOD scid) mice were anesthetized, and 500,000 cells washed three times in PBS and mixed with 30 µL of complete Matrigel (BD Biosciences) were injected into the fourth mammary gland on the right side of each animal. As indicated, cell lines used were MDA-MB-231. Luc (MDA-MB-231-luc2 from PerkinElmer; additionally expressing luciferase), MDA-MB-231.Luc stably expressing control shRNA (scr-shRNA), or two different shRNAs specific for PKD3 (PKD3-shRNA#1 and PKD3-shRNA#2). For studies with CRTC0066101, mice were treated orally with 80 mg/kg CRTC0066101 diluted in a 5% dextrose saline solution (Sigma-Aldrich) or 5% dextrose saline solution alone (control) every other day starting 14 days after cell injection. Body weight and tumor volume (caliper measurement) were determined once per week. The presence of metastases was detected using the IVIS Spectrum Imaging System (PerkinElmer). At the endpoint, primary tumors and sites of metastasis were removed and analyzed as indicated.

 Luciferase reporter assay

Cells were transfected with PRKD3 promoter-luciferase reporter (2 µg), Renilla luciferase reporter (0.1 µg), and pEGFP-Er¢ expression construct (2 µg) in 6-well plates, as indicated. Twenty-four hours after transfection, cells were washed twice with ice-cold PBS, scraped in 250-µL Passive Lysis Buffer (Promega), and centrifuged (13,000 rpm, 10 minutes, 4°C). Assays for luciferase activity were performed according to the Promega Luciferase assay protocol and measured using a Veritas luminesimeter (Synamtec). Luciferase activity of the PRKD3 promoter-luciferase reporter was normalized to Renilla luciferase activity. Expression of proteins was controlled by Western blot analysis.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed using the Imprint Chromatin Immunoprecipitation Kit from Sigma-Aldrich according to the manufacturer’s protocol. Five micrograms of primary antibody (anti-GFP, Abcam) or rabbit IgG control was used for ChIPs. Immunoprecipitates were analyzed by PCR using the primer sets 5′-TGAATGCTG-CAGCTTC-3′ and 5′-AAAGGGAAATGTGACCGCTAC-3′ to amplify a 243 bp fragment (Er¢ site 1) or 5′-TGATAGACAGGTGGG-GACT-3′ and 5′-GGCCGGGACGCTAGTGTCC-3′ to amplify a 199-bp fragment (Er¢ site 2) of the human PRKD3 promoter.

Statistical analysis

GraphPad Prism version 4.0c software (GraphPad Software) was used for all statistical analyses. Statistical significance (P < 0.05) was determined using a two-tailed Student t test and standard deviations.

Results

PKD3 is highly expressed in ER+/PR– breast cancers and correlates with aggressiveness

Loss of gene expression of PRKD1 (encoding for PKD1) is a marker for aggressive breast cancer (11, 17). Using isoform-specific antibodies, we determined the expression pattern of PKD1 and the two oncogenic versions of this kinase family, PKD2 and PKD3, in normal breast (n = 60 samples) and TNBC (n = 40 samples). Whereas PKD1 is the main isoform expressed in normal breast, TNBC show an isoform switch toward expression of PKD3 (Fig. 1A). PKD2 generally was weakly expressed, but is also slightly downregulated in TNBC. In order to evaluate whether increased PKD3 expression is indeed linked to the triple-negative phenotype, we evaluated annotated clinical breast cancer datasets from 13 different studies. In all studies, triple-negative biopsy samples showed significantly increased expression of PKD3 as compared with triple-positive (ER+ /PR+/HER2–) biopsies (Supplementary Table S1). Using The Gene Set Analysis Cell Lines module of GOBO (29, 30), we found a reverse correlation of PRKD3 gene expression between basal and luminal breast cancer types (Fig. 1B, left). A similar reverse correlation was found between TNBC and HR-positive
Targeting of ER− Breast Cancer with CRT0066101

PKD3 is highly expressed in ER− breast cancers and correlates with aggressiveness. A, TMA slides containing human TNBC (n = 40) and normal human breast tissue samples (n = 60) were analyzed for PKD1, PKD2, and PKD3 expression using isoform-specific antibodies. Scale bar, 100 μm. Statistical analysis of PKD1, PKD2, and PKD3 intensity was performed using Aperio positive pixel count algorithm in the Imagescope software (Aperio). B, analysis of PRKD3 gene expression in breast cancer cell lines using the Gene Set Analysis Cell Lines module of GOBO. Cell lines were grouped into Basal A (n = 12), Basal B (n = 14), or Luminal (n = 25) subtypes (left), or TNBC (n = 25) and HR-positive (n = 15) subtypes (right). C, TMA slides containing histologically confirmed IDC of indicated subtypes were analyzed for PKD3 expression using an isoform-specific antibody. Scale bar, 100 μm. Statistical analysis of PKD3 intensity in ER+ and ER− groups (n = 44 for ER+; n = 41 for ER−) was performed using Aperio positive pixel count algorithm in the Imagescope software (Aperio). D, the Kaplan–Meier plot depicting the impact of levels of PRKD3 gene expression on the metastasis-free survival of patients (n = 1,353) with ER− breast cancer. In A and C, P values were acquired with the Student t test using Prism v5 software. *, P < 0.05, statistical significance. In A and C, representative pictures from each group are depicted.

cell lines (Fig. 1B, right), indicating that loss of HR expression and increased PRKD3 expression may be linked. To test this, we analyzed ER-positive (ER+; n = 44) and ER− (n = 41) patient tumors for PKD3 expression and confirmed that increased PKD3 expression is linked to loss of ER expression (Fig. 1C and Supplementary Fig. S1). The negative correlation between ER and PKD3 was bolstered by meta-analysis of 24 published studies in which we found that PRKD3 shows significantly higher expression in ER− versus ER+ breast cancers (Supplementary Table S2). We next assessed the association of PRKD3 expression with prognosis in breast cancer patients, and found that elevated PRKD3 expression levels in ER− tumors (n = 1,353) were associated with significantly decreased distant metastasis-free survival (Fig. 1D).

ERx regulates PKD3 expression levels in breast cancer cells

To determine whether ERx could directly affect PRKD3 promoter activity, we performed luciferase reporter assays using a PRKD3 promoter-luciferase gene reporter. Reintroduction of ERx into MDA-MB-231 (ER+/PR−/HER2−) cells led to a significant decrease (approximately 40%) of PRKD3 gene promoter activity (Fig. 2A). These data were confirmed with BT20, another TNBC cell line. BT20 cells, when transfected with ERx, showed an approximately 75% decrease of PRKD3 expression and this was further decreased, when media were supplemented with E2 (Fig. 2B). We analyzed the PRKD3 promoter sequence and identified two potential ER-binding sites, 780 bases (ERx site 1) or 318 bases (ERx site 2), upstream of the transcription start site (Fig. 2C). To test whether ERx directly
binds to the PKD3 promoter at these sites, we performed ChIP and confirmed the direct binding of ERα to both of the two predicted ER-binding sites (Fig. 2D). Next, we determined whether presence of ERs translates to a decrease in PKD3 protein expression. Therefore, we reintroduced ERα in triple-negative MDA-MB-231 and BT20 cells, as well as HCC1954
(ER⁺/PR⁺/HER2⁻) cells. As expected, the presence of ERα led to a significant decrease in PKD3 expression, and this was independent of the HER2 amplification status (Fig. 2E). Eventually, we treated the ER⁺ cell line T47D with fulvestrant, a compound that leads to nuclear export and degradation of ER (31, 32). As expected, treatment of T47D cells with fulvestrant increased PKD3 expression further indicating a role for ER as a regulator of PKD3 expression (Fig. 2F).

The knockdown of PKD3 decreases cancer cell proliferation, migration, and invasion in vitro and in vivo

To test the impact of PKD3 on breast cancer cell behavior, we used the invasive breast cancer cells MDA-MB-231 (ER⁺, PR⁺, HER2⁻), which express high levels of PKD3, low levels of PKD2, and no PKD1 (Supplementary Fig. S2; Supplementary Table S3; ref. 11). A knockdown of basal PKD3 expression using two different PKD3-specific shRNA sequences significantly decreased MDA-MB-231 cell numbers over a time period of 60 hours (Fig. 3A and B and Supplementary Fig. S3). Similar results were observed in another ER⁺ cell line, HCC1954, which show a similar PKD expression pattern (Supplementary Fig. S2; data not shown). Next, we tested the role of PKD3 on the invasive phenotype. The knockdown of PKD3 in MDA-MB-231 cells led to a dramatic decrease in directed cell migration (Fig. 3C and Supplementary Fig. S4), and similar effects were observed for cell invasion through extracellular matrix (Fig. 3D). This confirms
that PKD3 is the major isofrom driving motility (and proliferation) in these cells. Interestingly, along with a decreased motility, we noticed a dramatic increase in cell spreading and altered F-actin organization when PKD3 was knocked down (Fig. 3E). To test whether the knockdown of PKD3 can affect breast tumor metastasis in vivo, we orthotopically implanted MDA-MB-231.Luc cells either stably expressing scrambled shRNA control or two different specific shRNA sequences for PKD3 into the mammary fat pad (mfp) of female NOD scid mice. To exclude that PKD3 effects on cell proliferation affect results on metastasis, endpoints of the experiment were set for each individual mouse at a primary tumors size of 700 ± 100 mm³. At the endpoint, tumors and tissues of potential sites of metastasis were extracted. As a control, primary tumors were analyzed by IHC with a monoclonal antibody specific for PKD3 (Supplementary Fig. S5A). As predicted by our in vitro experiments, primary tumor growth was significantly slower when PKD3 expression was decreased (Supplementary Fig. S5A). Analysis of expression of Ki67 and cleaved caspase-3 staining indicated that this was due to a decrease in cell proliferation, as well as an increase in cell death (Supplementary Fig. S5B and S5C). Interestingly, PKD3-shRNA tumors showed reduced local invasion when compared with control (sc-shRNA) tumors (Supplementary Fig. S5D). Next, we determined metastasis to distant organs by immunohistochemical staining for human vimentin as a marker for human cancer cells. We found that PKD3 downregulation dramatically decreased cancer cells infiltration to lymph nodes and lungs (Fig. 3F). Furthermore, mice implanted with PKD3-shRNA cells had significantly fewer and smaller metastases to their lungs compared with controls (sc-shRNA; Fig. 3G). Taken together, our data indicate that PKD3 plays an important role in breast tumor growth, progression, and metastasis.

The PKD inhibitor CRT0066101 decreases cancer cell proliferation, migration, and invasion in vitro

Next, we tested the impact of PKD3 inhibition on cell proliferation and the invasive phenotype. Because PKD3 is upregulated and as such can be targeted in ER⁺ breast cancer independently of the HER2 status, we decided to test two different cell lines, MDA-MB-231 as a model for TNBC and HCC1954 as a model for ER⁺, HER2⁺ breast cancer. We used the pan-PKD inhibitor CRT0066101, which has been shown to have antican-cer activity in pancreatic, prostate, and colorectal cancer cells (33, 34). CRT0066101 induced a significant decrease in cell proliferation in both cell lines (Fig. 4A and D and Supplementary Fig. S6). In a similar fashion to PKD3 depletion, inhibition of PKD activity with CRT0066101 also blocked directed cell migration (Fig. 4B and E and Supplementary Fig. S7) and invasion (Fig. 4C and F). Similar as observed with the PKD3 knockdown in Fig. 3E, along with a decreased motility, we noticed increased spreading and altered F-actin organization of cells that were treated with CRT0066101 (Fig. 4G and H). Overall, data obtained with PKD3 knockdown and CRT0066101 were similar, although both cell lines also express PKD2 that may have similar functions as PKD3 in regard to regulation of cell migration and invasion (Supplementary Fig. S8).

CRT0066101 decreases primary tumor size, local invasiveness, and metastasis in vivo

Next, we tested whether CRT0066101 can be used as an efficient strategy for the treatment of tumor growth and metastasis of ER⁺ cancers in vivo. Therefore, we orthotopically implanted MDA-MB-231 cells into the mfp of female NOD scid mice. After establishment of primary tumors (day 14 after cell implantation), mice were treated with 80 mg/kg CRT0066101 (oral administration, every other day) or vehicle control. At the endpoint (10 weeks after cell implantation), primary tumors and tissues of potential sites of metastasis were extracted. As in a previous study modeling pancreatic cancer (33), no toxicity was detected at this dosage of CRT0066101 and no significant changes in body weight or damage in tissue was observed (not shown). Treatment of mice with CRT0066101 did result in a significant decrease of primary tumor size and weight (Fig. 5A and Supplementary Fig. S9), associated with an approximately 50% decrease in tumor cell proliferation (Fig. 5B), and an increase of apoptosis (Fig. 5C). Of note, the effects of CRT0066101 on tumor cell viability and proliferation that were observed in vitro were in line with effects observed in vivo (Fig. 4). Additional analysis of tumor edges as well as the connection of tumor cells to the normal adjacent mouse mammary tissue showed a reduced local invasion in tumors treated with the PKD inhibitor (Fig. 5D). This decrease in invasiveness correlated with decreased expression of COX-2 (Fig. 5E), which previously was associated with local invasion of breast cancer cells, as well as metastasis to the lungs (35). Indeed, IVIS imaging of animals indicated that CRT0066101 may affect metastasis to distant organs (Fig. 6A). Immunohistochemical analysis for human cancer cells (IHC for anti-human vimentin) at the endpoint indicated a dramatic decrease of infiltration of tumor cells into lymph nodes in CRT0066101-treated mice (Fig. 6B). Similarly, lung metastases were fewer in numbers and smaller in size (Fig. 6B–D). Metastases in the lungs of the treated mice showed a significant lower expression of Ki67, indicating that the decrease in average size of metastases may be due to CRT0066101 effects on the ability of tumor cells to proliferate in their new environment (Fig. 6E).

Discussion

Silencing of PKD1 and increased expression of the oncogenic versions of this kinase family, PKD2 and PKD3, has been described to contribute to progression of several epithelial cancers, including breast cancer (11, 13, 16, 17, 20–22), gastric cancer (36), pancreatic cancer (37, 38), colorectal cancer (34), and prostate cancer (14, 39–42). We here show that the switch from PKD1 to PKD3 expression defines the transition to an aggressive breast tumor phenotype. PKD1 previously had been shown to maintain the epithelial phenotype by preventing EMT (13, 14, 16) and to negatively regulate cell migration (12), invasion (17), and metastatic progression of breast cancer (11, 19). Consequently, in invasive breast cancers, PKD1 expression is downregulated by promoter hypermethylation (11, 36). The signaling mechanisms by which other PKD isoforms are (up)regulated at the transcriptional levels have not been identified so far.

Analysis of cell lines, TMAs from patient samples (Fig. 1), and annotated clinical breast cancer datasets showed significantly higher PRKD3 gene expression in ER⁺ or TNBC biopsies (Supplementary Table S1). A detailed analysis suggested that increased PKD3 expression is mainly due to loss of ER expression and not dependent on the HER2 status of tumors (Fig. 1C and Supplementary Table S2). This led to the questions whether the ER could be a direct negative regulator of PRKD3 expression; or whether observed reverse expression between both molecules is
correlative. By reexpressing ER in ER− cell lines (Fig. 2E) or inhibiting ER expression in ER+ T47D cells (Fig. 2F), we clearly demonstrate that PKD3 repression depends on ER activity. As a mechanism of regulation, we demonstrate that ER decreases PKD3 expression through direct binding to the PRKD3 promoter at two different ER-binding sites (Fig. 2C and D). Thus, our data demonstrate a direct negative regulation of a gene by this receptor. Although ER is mostly known for its positive effect on gene transcription, some studies have demonstrated that it can also act as a repressor of gene expression (43, 44). For example, ER can repress a cytochrome P450-encoding gene (CYP1A1) by targeting Dnmt3B DNA methyltransferase (44).

PKD3 has been implicated in all aspects of tumor formation and progression, such as mediating proliferation, survival, and invasiveness, in different cancers (20, 22, 23, 39, 45). However, relatively little is known about the molecular mechanisms by which PKD3 may drive carcinogenesis. PKD3 previously has been shown to mediate activation of Akt, leading to prolonged activation of extracellular signal-regulated kinase (ERK) 1/2 (39). Furthermore, S6 kinase 1 (S6K1), a member of the mammalian target of rapamycin complex 1 signaling cascade (mTORC1), was identified as a downstream target of PKD3 to mediate its effect on cell proliferation in TNBC cell lines (22). Another target involved may be the G-protein–coupled receptor kinase-interacting protein 1 (GIT1), a key mediator of PKD3-induced cell spreading and proliferation (46).

Besides describing a previously unknown regulation of PKD3 expression that can be linked to aggressiveness of breast cancers, we also tested the use of PKD inhibitors in breast cancers that mainly express PKD3. Our data and previous work implicate that CRT0066101 or DMSO (control) (22). Another target involved may be the G-protein–coupled receptor kinase-interacting protein 1 (GIT1), a key mediator of PKD3-induced cell spreading and proliferation (46).
all three isoforms in a low nanomolar range (i.e., IC50 for PKD3 is 2 nmol/L). In a previous work, it has been shown to be active in vivo in orthotopic animal models for pancreatic cancer and colorectal cancer (33, 34). Because it can be orally administered and has no side effects in mice, when used at doses that inhibit PKD (33, 34), it is an inhibitor that could be developed for clinical use.

Our data not only show that CRT0066101 can block all aspects of the tumor phenotype in PKD1-negative/PKD3-positive breast cancer cells in vitro (Fig. 4), but also demonstrate in vivo relevance by showing that CRT0066101 significantly inhibits primary tumor growth, local invasion, and metastasis to distant organs in vivo (Figs. 5 and 6). It is also important to note that the same events were obtained with specific knockdown of PKD3 showing that this kinase is the main target in ER− cancer cells (Fig. 3). Although our in vitro data using shRNA or CRT0066101 clearly demonstrate effects on cell proliferation, migration, and invasion, it is possible that additional tumorigenic functions are affected by knockdown or inhibition of PKD3 in vivo. For example, PKD signaling previously has been implicated in angiogenesis (38, 47, 48) and it is very possible that the decrease in size of the primary tumors that we observed in response to CRT0066101 treatment is partly due to blocking of angiogenesis.

It should be noted that for breast cancers that undergo a switch from PKD1 to PKD3, two strategies are possible, either to reexpress PKD1, or to inhibit PKD3 (discussed in ref. 49). We recently have tested the first strategy, and shown that reverting the epigenetic silencing of PKD1 with the DNA methyltransferase inhibitor decitabine can dramatically reduce the invasive and metastatic potential of triple-negative orthotopic tumors in vivo (11, 19). What is still ill-defined is how PKD1, once it is reexpressed in invasive breast cancers, can exert a protective effect and antagonize PKD3 functions. Because the ESR1 gene promoters also can be silenced by DNA methylation (50), the simplest explanation for this may be that decitabine treatment also upregulates ER, which then decreases PKD3 expression.
In conclusion, our study provides a rationale that supports the use of PKD inhibitors such as CRT0066101 for treatment of patients diagnosed with ER− or TNBC. Key for treatment with PKD inhibitors is a downregulation of PKD1 and upregulation of the oncogenic version PKD3 (or PKD2). This requires developing reliable techniques that could be used in clinical settings to determine PKD1, 2, and 3 expression status before treatment decisions are made.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: S. Borges, E.A. Perez, E.A. Thompson, P. Storz
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E.A. Perez, E.A. Thompson, X.J. Geiger
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Borges, E.A. Perez, E.A. Thompson, D.C. Radisky, P. Storz
Writing, review, and/or revision of the manuscript: S. Borges, E.A. Perez, D.C. Radisky, X.J. Geiger, P. Storz

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