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

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Abstract

Invasive ductal carcinomas (IDCs) of the breast are associated with altered expression of hormone receptors (HR), amplification 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 chemotherapy, hormone therapy or HER2 targeted therapy. Using progression tissue microarrays we here demonstrate that the serine/threonine kinase Protein Kinase D3 (PKD3) is highly up-regulated in estrogen receptor (ER)-negative tumors. We identify direct binding of the estrogen receptor 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.

Keywords

invasive breast cancer; estrogen receptor; triple-negative; CRT0066101; protein kinase D

INTRODUCTION

Invasive ductal breast cancers (IDC) 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 ER and 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). The most aggressive cases of IDC are characterized

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by a high proliferation rate, a great propensity to metastasize and their ability to resist to standard chemotherapy, hormone therapy or HER2-directed agents such as trastuzumab (1,2). Particularly, triple-negative breast cancers (TNBC), which represent approximately 20% of all cases, are difficult to treat, because molecular targets are lacking (3,4). Cytotoxic radio- and chemotherapy currently are the only options for patients with TNBC (5). However, the rate of recurrence is high after such treatment (6). The high resistance to therapy has been partially attributed to tumors that show mesenchymal and stem cell features, for which no specific targeted therapies are available (7–9). Thus, identification of tissue biomarkers that may lead to novel therapeutic strategies is of utmost importance.

The Protein Kinase D (PKD) family members PKD1, PKD2 and PKD3 have been implicated in the progression of breast cancer. PKD1 contributes to breast cancer cell proliferation (10), but inhibits the invasive phenotype (11,12). This is mediated by inhibition of epithelial-to-mesenchymal transition (EMT) through phosphorylation of Snail (13–16), down-regulation of the expression of several matrix metalloproteinases (MMPs), and negative-regulation of F-actin reorganization at the leading edge at multiple levels (12,13,17,18). Consequently, *PRKD1* (encoding PKD1) is silenced by hypermethylation in the most aggressive breast cancers including the TNBC subtype (11,19). In contrast to PKD1, the two other isoforms PKD2 and PKD3 in breast cancer cell lines seem to drive all aspects of oncogenic transformation, including cell proliferation, migration, invasion and chemoresistance (20–22). Similar opposing functions in breast cancer have been described for other kinases such as members of the Akt/PKB kinase family (23,24). However, how subtypes of the same kinase family, which recognize the same substrate phosphorylation motif, can have opposite cellular functions remains unclear. Based on recent studies for PKD enzymes it seems that a number of different parameters such as their relative level of expression or activity, their cellular localization and/or their ability to form complexes can differentially influence cellular phenotypes (25).

Using progression tissue microarrays (TMAs), here we demonstrate that a switch towards the isoform PKD3 is associated with the aggressiveness of breast cancer. While PKD1 is down-regulated and PKD2 is expressed homogeneously at low levels in different breast cancer subtypes as well as in normal tissue, PKD3 is highly up-regulated in ER negative 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-negative 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, since it can be given orally, it may be developed for treatment of PKD1-negative breast cancers, including the triple-negative phenotype.

MATERIALS & METHODS

Cell Lines, Antibodies and Reagents

The MDA-MB-231-luc2 cell line was obtained from Perkin Elmer (Waltham, MA) in May, 2009. All other cells lines were obtained from the American Type Culture Collection (Manassas, VA) 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's medium (DMEM) with 10% fetal bovine serum (FBS). MDA-MB-468 and HCC1954 were maintained in RPMI 1640 with 10% FBS. BT20 cells were maintained in Eagle's minimal essential medium with 10% FBS, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids (NEAAs) and 1 mM sodium pyruvate. MCF-10A cells were maintained in DMEM/Ham's F-10 medium (50:50 vol/vol) 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 (Herndon, VA), EGF from PeproTech (Rocky Hill, NJ), insulin and hydrocortisone from Sigma-Aldrich (St Louis, MO). Anti-β-actin antibody was obtained from Sigma-Aldrich, anti-Ki-67 from Dako (Carpinteria, CA), anti-cleaved PARP, anti-cleaved caspase 3 and anti-MMP9 from Cell Signaling Technology (Danvers, MA), anti-COX-2 from Cayman Chemical (Ann Arbor, MI), anti-smooth muscle actin (SMA), anti-GFP and anti-Cyclin D1 from Abcam (Cambridge, MA), anti-Snail from Abgent (San Diego, CA), anti-Vimentin (for Western blotting) from Santa Cruz (Dallas, TX), anti-N-cadherin and anti-Vimentin (for IHC) from Epitomics (Burlingame, CA). The rabbit polyclonal antibody specific for PKD2 was from Upstate Biotechnology (Charlottesville, VA), the rabbit polyclonal antibody specific for PKD3 used for immunoblotting was from Bethyl Laboratories (Montgomery, TX). The mouse monoclonal antibody specific for PKD3 (H00023683-M01) used for immunohistochemistry was from Abnova (Walnut, CA). The mouse monoclonal antibody specific for PKD1 was raised by Creative Biolabs/Creative Dynamics (Shirley, NY) and is further described in (11). Secondary horseradish peroxidase (HRP)-linked antibodies were obtained from Roche Applied Science (Indianapolis, IN). Luciferin was obtained from Gold Biotechnology (St Louis, MO). Fulvestrant and β-Estradiol (E2) were purchased from Sigma Aldrich. The PKD specific inhibitor CRT0066101 was obtained from Cancer Research Technology (Cambridge, UK).

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 ViraPower Lentiviral Expression System (Life Technologies, Carlsbad, CA). 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 (Madison, WI) via Bgl II and Xho I restriction sites, using 5'-TTTTTTGTCCCTTCTGTTTTTGAT-3' and 5'-GACGGAAAGAAATTAGAAAATTTT-3' as primers. The pRL-CMV-renilla luciferase plasmid was from Promega. The ER α (pEGFP-C1-ER α ; #28230) expression plasmid was from Addgene (Cambridge, MA). The pSuper-PKD2-shRNA plasmid was obtained by cloning the oligonucleotides 5'-GATCCCCGTTCCCTGAGTGTGGCTTCTTCAAGAGAGAAGCCACACTCAGGGAAC T TTTTGGAAA-3' and 5'-AGCTTTTCCAAAAAGTTCCTGAGTGTGGCTTCTTCTTGAAGAAGCCACACTCA G GGAACGGG-3' into pSuper. GenJet™ from SignaGen (Rockville, MD) was used for transfection of breast cancer cells.

Cell Lysates and Western Blot Analysis

Cells were washed twice with ice-cold phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2) and lysed with Buffer A (50 mM Tris•HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM 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 ibiTreat μ -Slides (ibidi, Martinsried, Germany) and treated as indicated. Before fixation with 4% paraformaldehyde (20 minutes, 4 °C), cells were washed twice with phosphate-buffered saline (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 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 40x 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 μ g/well; BD Biosciences, San Jose, CA), dried overnight and rehydrated for 1 hour with 40 μ l of tissue culture media. Cells were harvested, washed once with media containing 1% bovine serum albumin (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. 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 (Indianapolis, IN). After attachment, cell migration or invasion (coating of top well with 2 μ g 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, Tissue Microarrays and Immunohistochemistry

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 Institutional Review Board assessed the protocol 09-001642 as minimal risk and waived the need for further consent. All data was analyzed anonymously. Tissue microarrays (TMA) were deparaffinized (1 hour at 60 °C), dewaxed in xylene (five times for 4 minutes), and gradually rehydrated with ethanol (100%, 95% and 75%, twice with each concentration for 3 minutes). The rehydrated TMA sections were rinsed in water and subjected to hematoxylin and eosin (H&E) staining or to antigen retrieval in citrate buffer (pH 6.0) as previously described (26). Slides were treated with 3% hydrogen peroxide (5 minutes) to reduce endogenous peroxidase activity and washed with PBS containing 0.5% Tween 20 (PBS-Tween 20). Proteins of interest were detected using indicated specific antibodies diluted in PBS-Tween 20 and visualized using the EnVision+ Dual Link Labelled Polymer Kit following the manufacturer's instructions (Dako). Images were captured using the Aperio ScanScope slide scanner (Aperio, Vista, CA).

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 non-obese diabetic severe combined immunodeficiency (NOD *scid*) mice were anesthetized, and 500,000 cells washed three times in PBS and mixed with 30 μ l 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 Perkin Elmer; additionally expressing luciferase), MDA-MB-231.Luc stably expressing control shRNA (scr-shRNA) or two different shRNAs specifically-targeting PKD3 (PKD3-shRNA#1 and PKD3-shRNA#2). For studies with CRT0066101, mice were treated orally with 80 mg/kg CRT0066101 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 (Perkin Elmer). At the end point, 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-ERα expression construct (2 µg) in 6 well plates, as indicated. 24 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 min, 4 °C). Assays for luciferase activity were performed according to the Promega Luciferase assay protocol and measured using a Veritas luminometer (Symantec, Cupertino, CA). 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 (ChIP)

ChIP assays were performed using the Imprint® Chromatin Immunoprecipitation Kit from Sigma Aldrich according to the manufacturer's protocol. 5 µg primary antibody (anti-GFP, Abcam) or rabbit IgG control was used for chromatin immunoprecipitations. Immunoprecipitates were analyzed by PCR using the primer sets 5'-TGACAATGCCTGTCAGCTTC-3' and 5'-AAACGCGAATGTGACCCTAC-3' to amplify a 243 bp fragment (ERα site 1) or 5'-TGATAGACACGCTCGCGACT-3' and 5'-TGCCGGGAGCTGTAGTTCCT-3' to amplify a 199 bp fragment (ERα site 2) of the human *PRKD3* promoter.

Bioinformatics

Evaluation of expression of *PRKD3* in annotated breast cancer datasets was performed using the Nextbio server (www.nextbio.com; (27)). KM Plotter data were obtained using the current release of Kaplan Meier Plotter (www.kmplot.com; (28); 2012 version, n=2978), interrogating using Affymetrix ID: "211084_x_at", survival set at distant metastasis-free survival, auto select best cutoff set at checked, follow-up threshold set at all, and array quality control set at exclude biased arrays.

Statistical Analysis

GraphPad Prism version 4.0c software (GraphPad Software, La Jolla, CA) was used for all statistical analyses. Statistical significance (p<0.05) was determined using a two-tailed Student's *t*-test and standard deviations.

RESULTS

PKD3 is highly expressed in ER-negative 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 triple negative breast cancer (TNBC; n=40 samples). Whereas PKD1 is the main isoform expressed in normal breast, TNBC show an isoform switch towards expression of PKD3 (Fig. 1A). PKD2 generally was weakly expressed, but is also slightly down-regulated in TNBC. In order to evaluate if 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 *PRKD3* as compared to triple positive (ER+/PR+/HER2+) biopsies (Table S1). Using The Gene Set Analysis Cell Lines module of GOBO (29,30), we then analyzed a panel of 51 breast cancer cell lines and also found a reverse correlation of *PRKD3* gene expression between basal and luminal BC types (Fig. 1B, left graph). A similar reverse correlation was found between TNBC and HR (hormone receptor) positive cell lines (Fig. 1B, right graph), indicating that loss of hormone receptor expression and increased *PRKD3* expression may be linked. To test this we analyzed 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, Supplemental 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 (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 negative tumors (n=1353) were associated with significantly-decreased distant metastasis-free survival (Fig. 1D).

ER α regulates PKD3 expression levels in breast cancer cells

To determine whether ER α could directly impact *PRKD3* promoter activity we performed luciferase reporter assays using a *PRKD3* promoter-luciferase gene reporter. Reintroduction of ER α 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 triple negative breast cancer cell line. BT20 cells, when transfected with ER α , showed an approximately 75% decrease of *PRKD3* expression and this was further decreased, when media was supplemented with β -estradiol (Fig. 2B). We analyzed the *PRKD3* promoter sequence and identified two potential ER binding sites 780 bases (ER α site 1) or 318 bases (ER α site 2) upstream of the transcription start site (Fig. 2C). To test if ER α directly binds to the PKD3 promoter at these sites we performed chromatin immunoprecipitation (ChIP) and confirmed the direct binding of ER α to both of the two predicted ER-binding sites (Fig. 2D). Next, we determined if presence of ER α translates to a decrease in PKD3 protein expression. Therefore, we reintroduced ER α in triple-negative MDA-MB-231 and BT20 cells, as well as HCC1954 (ER-/PR-/HER2+) cells. As expected, presence of ER α 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 (Supplemental Fig. S2, Supplemental Table S3 and (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 (Figs. 3A, 3B and Supplemental Fig. S3). Similar results were observed in another ER- cell line, HCC1954, which show a similar PKD expression pattern (Supplemental 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 Supplemental Fig. S4), and similar effects were observed for cell invasion through extracellular matrix (Fig. 3D). This confirms that PKD3 is the major isoform 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 impact 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 of female NOD *scid* mice. To exclude that PKD3 effects on cell proliferation affects results on metastasis, end points of the experiment were set for each individual mouse at a primary tumors size of $700 \pm 100 \text{ mm}^3$. At the endpoint, tumors and tissues of potential sites of metastasis were extracted. As a control, primary tumors were analyzed by immunohistochemistry with a monoclonal antibody specific for PKD3 (Supplemental Fig. S5A). As predicted by our *in vitro* experiments primary tumor growth was significantly slower when PKD3 expression was decreased (Supplemental 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 (Supplemental Figs. S5B and S5C). Interestingly, PKD3-shRNA tumors showed reduced local invasion when compared to control (scr-shRNA) tumors (Supplemental 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 to controls (scr-shRNA) (Fig. 3G). Taken together, our data indicates 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. Since PKD3 is upregulated and as such can be targeted in ER- BC 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+ BC. We used the pan-PKD inhibitor CRT0066101, which has been shown to have anti-cancer activity in pancreatic, prostate and colorectal cancer cells (33,34). CRT0066101 induced a significant decrease in cell proliferation in both cell lines (Figs 4A, 4D and Supplemental Fig. S6). In a similar fashion to PKD3 depletion, inhibition of PKD activity with CRT0066101 also blocked directed cell migration (Figs. 4B, 4E and Supplemental Fig. S7) and invasion (Figs. 4C and 4F). 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 (Figs. 4G and H). Overall, data obtained with PKD3 knockdown and CRT0066101 were similar, although both cell lines also express PKD2 which may have

similar functions as PKD3 in regard to regulation of cell migration and invasion (Supplemental 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 negative cancers *in vivo*. Therefore, we orthotopically implanted MDA-MB-231 cells into the mammary fat pad (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 end point (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, Supplemental 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 vivo* were in line with effects observed *in vitro* (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 end point 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 down-regulated 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 negative or triple negative breast cancer biopsies (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, Table S2). This led to the questions if the estrogen receptor could be a direct negative regulator of *PRKD3* expression; or if observed reverse expression between both molecules is correlative. By re-expressing ER in ER-negative cell lines (Fig. 2E) or inhibiting ER expression in ER-positive 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 (Figs. 2C and 2D). Thus, our data demonstrate a direct negative regulation of a gene by this receptor. While 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 (20,22,39,45). 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 implicates that ideal targets to test the efficacy of PKD inhibitors would be ER negative or triple negative, invasive breast cancers, in which upregulation of PKD3 is accompanied by the loss of PKD1 expression. CRT0066101 is a selective and potent PKD inhibitor that targets all three isoforms in a low nanomolar range (i.e. IC₅₀ for PKD3 is 2 nM). In previous work it has been shown to be active *in vivo* in orthotopic animal models for pancreatic cancer and colorectal cancer (33,34). Since 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), they 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 impacted 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 which undergo a switch from PKD1 to PKD3, two strategies are possible, either to re-express PKD1, or to inhibit PKD3 (discussed in (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 re-expressed 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IDC	invasive ductal carcinoma
HR	hormone receptor
PKD	protein kinase D
ER	estrogen receptor
HER2	human epidermal growth factor receptor-2
TNBC	triple-negative breast cancer
EMT	epithelial-to-mesenchymal transition
MMP	matrix metalloproteinase
TMA	tissue microarray
ChIP	chromatin immunoprecipitation

IHC	immunohistochemistry
6K1	S6 kinase 1
mTORC1	mammalian target of rapamycin complex 1 signaling cascade
GIT1	G-protein-coupled receptor kinase-interacting protein 1
ERK	extracellular signal-regulated kinase

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Figure 1. PKD3 is highly expressed in ER-negative breast cancers and correlates with aggressiveness

A: Tissue microarray (TMA) slides containing human triple negative breast cancer (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 is 100 μ m. Statistical analysis of PKD1, PKD2 and PKD3 intensity was performed using Aperio positive pixel count algorithm in the Imagescope software (Aperio, Vista, CA). **B:** Analysis of *PRKD3* gene expression in breast cancer cells 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 graph), or TNBC (n=25) and hormone receptor (HR) positive (n=15) subtypes (right graph). **C:** TMA slides containing histologically-confirmed invasive ductal carcinoma of indicated subtypes were analyzed for PKD3 expression using an isoform-specific antibody. Scale bar is 100 μ m. Statistical analysis of PKD3 intensity in ER+ or ER- groups (n=44 for ER+; n=41 for ER-) was performed using Aperio positive pixel count algorithm in the Imagescope software (Aperio, Vista, CA). **D:** Kaplan-Meier plot depicting the impact of levels of *PRKD3* gene expression on the metastasis-free survival of patients (n=1353) with ER-negative breast cancer. In **A** and **C**, p values were acquired with the student's t-test using Prism v5 software. The asterisk indicates statistical significance. In **A** and **C**, representative pictures from each group are depicted.

Figure 2. ERα directly regulates PKD3 expression in breast cancer cells

A: MDA-MB-231 cells were transfected with *PRKD3* promoter-luciferase, renilla luciferase reporters and vector control or GFP-tagged ERα, as indicated. 48 hours after transfection, reporter gene luciferase assays were performed. The asterisk indicates statistical significance. Cell lysates were also analyzed by Western blot for expression of ERα (anti-GFP), or β-actin (anti-β-actin) as a loading control. **B:** BT20 cells were cultivated in phenol-red free media and transfected with *PRKD3* promoter-luciferase and renilla luciferase reporters and vector control or ERα, as indicated. 48 hours after transfection, BT20 cells were stimulated with 10 nM β-estradiol or vehicle (control) for 6 hours and reporter gene luciferase assays were performed. * indicates statistical significance as compared to unstimulated vector control; # indicates statistical significance as compared to unstimulated ERα-transfected cells. Cell lysates were also analyzed by Western blot for expression of ERα (anti-GFP), or β-actin (anti-β-actin) as a loading control. **C:** Schematic representation of potential ERα binding sites (in red) in the *PRKD3* promoter region -1000-0. **D:** Chromatin IP. MDA-MB-231 cells were transfected with vector control or GFP-tagged ERα. After crosslinking, the ERα/DNA complexes were immunoprecipitated using an anti-GFP antibody. Precipitates were analyzed by PCR for the ERα-bound *PRKD3* promoter. A PCR for the *PRKD3* promoter using the input DNA served as control (input control). **E:** ER-negative cell lines MDA-MB-231, BT20 and HCC1954 were transfected with vector control

or GFP-tagged ER α . 48 hours after transfection, cell lysates were analyzed by Western blot for the expression of PKD3 and ER α (anti-GFP). Staining for β -actin (anti- β -actin) served as a loading control. **F:** The ER-positive cell line T47D was treated with 100 nM fulvestrant or DMSO (control) for 24 hours and cell lysates were analyzed by Western blot for the expression of PKD3 or β -actin (anti- β -actin).

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Figure 3. Knockdown of PKD3 decreases cancer cell proliferation, migration and invasion *in vitro* and *in vivo*

A-E: MDA-MB-231 cells were infected with lentivirus harboring control shRNA (scr-shRNA) or two different shRNAs specifically-targeting PKD3 (PKD3-shRNA#1 and PKD3-shRNA#2). **A:** 48 hours after initial infection, a fraction of the cells was lysed and PKD3 knockdown was verified by Western blot. **B:** Cells were seeded in E-plates and numbers of attached cells were continuously monitored in real-time for 60 hours using an xCELLigence RTCA DP instrument. Error bars (gray) represent three experiments. **C:** Cells were seeded in CIM-plates and (after attachment) directed cell migration was continuously monitored in real-time for 10 hours using an xCELLigence RTCA DP instrument. Error bars (gray) represent three experiments. **D:** Cells were seeded on Matrigel-coated transwell filters and Transwell invasion assays were performed as described in Materials & Methods. **E:** Cells were fixed and stained with DAPI (blue) and phalloidin (red). The bar represents 10 μm. Representative pictures from each group are depicted. The cell area (graph) was measured and analyzed using ImageJ software. In **B-E**, the results presented are the means ± SEM. P values were calculated using a two-tailed Student's t-test. The asterisks indicate statistical significance. **F** and **G:** MDA-MB-231.Luc cells stably expressing control shRNA (scr-shRNA) or two different shRNAs specifically-targeting PKD3 (PKD3-shRNA#1 and PKD3-

shRNA#2) were injected into the mfp of female NOD *scid* mice. In **F**, the presence of metastasis in lymph nodes and lungs was detected by immunostaining with an antibody specific for human vimentin (detects human cancer cells). Representative pictures from each group are depicted. The bars represent 200 μm for lymph nodes; 1 mm for lungs. In **G**, the number of pulmonary metastases per mm^2 , or the average size of pulmonary metastases in μm^2 was quantified from five fields each lung. The asterisks indicate statistical significance.

Figure 4. The PKD inhibitor CRT0066101 decreases the cell proliferation and the invasive phenotype of ER- breast cancer cells

A and D: Indicated cell lines were seeded in E-plates and (after attachment) treated with 2.5 μ M CRT0066101 or DMSO (control). Numbers of attached cells were continuously monitored in real-time for indicated times using an xCELLigence RTCA DP instrument. Error bars (gray) represent three experiments. **B:** MDA-MB-231 cells were seeded in CIM-plates and treated with 2.5 μ M CRT0066101 or DMSO (control). After attachment, cell migration was continuously monitored in real-time for indicated time using an xCELLigence RTCA DP instrument. Error bars (gray) represent three experiments. **C and F:** Indicated cell lines were seeded on Matrigel-coated transwell filters and treated with 2.5 μ M CRT0066101 or DMSO (control). Transwell invasion assays were performed as described in Materials & Methods. **E:** HCC1954 were seeded on transwell filters and treated with 2.5 μ M CRT0066101 or DMSO (control). Transwell migration assays were performed as described in Materials & Methods. **G and H:** Indicated cell lines were treated with 2.5 μ M CRT0066101 or DMSO (control) for 16 hours. Cells were fixed and stained with DAPI (blue) and phalloidin (red). The bar represents 10 μ m. Representative pictures from each group are depicted. The cell area was measured and analyzed using ImageJ software. In **A–H**, the results presented are the means $\pm$ SEM. P values were calculated using a two-tailed Student's t-test. The asterisks indicate statistical significance.

Figure 5. CRT0066101 inhibits tumor growth and local tumor cell invasion *in vivo*

A: MDA-MB-231.Luc cells were injected into the mfp of female NOD *scid* mice. After establishment of primary tumors, at week two mice were treated orally with 80 mg/kg CRT0066101 or vehicle every other day for an additional period of eight weeks. Tumor growth was continuously monitored using the IVIS Spectrum imaging system. Left hand side shows a representative picture of mice from each group (n=6 per group) at week seven. At the end point (week ten), tumor volume and weight was measured. The asterisk indicates statistical significance. **B, C:** Primary tumors of different treatment groups were stained by IHC for the expression of Ki67 (**B**) and cleaved-caspase 3 (**C**). The bars represent 50 μ m. **D:** Samples of primary tumor were stained with haematoxylin and eosin (H&E). The bar represents 200 μ m. Areas where tumors connect with mouse mammary gland tissue were enhanced. **E:** Primary tumors were stained by IHC for the expression of COX2. The bars represent 50 μ m. In **B, C** and **E**, statistical analysis was performed using Aperio positive pixel count algorithm in the Imagescope software. P values were acquired with the student's t-test using Prism v5 software. The asterisk indicates statistical significance. In **B, C, D** and **E**, representative pictures from each group are depicted.

Figure 6. CRT0066101 inhibits metastasis to distant organs

A: Metastasis was continuously monitored using the IVIS Spectrum imaging system *in vivo*. Representative pictures of mice from each treatment group are depicted. **B:** Presence of metastasis in lymph nodes and lungs was analyzed by IHC for human Vimentin. The bar represents 500 μm for lymph nodes; 1 mm for lungs. **C, D:** The number of pulmonary metastases per mm² (**C**), or the average size of pulmonary metastases in μm² (**D**) was quantified from five fields each lung. The asterisks indicate statistical significance. **E:** Lungs were stained with haematoxylin and eosin (H&E) and expression of Ki67 was detected by IHC. The percentage of Ki67-positive cells was determined using Aperio positive pixel count algorithm in the Imagescope software. The bar represents 50 μm. For **B**, **D** and **E**, the values are means ± SEM. P values were acquired with the student's t-test using Prism v5 software. The asterisks indicate statistical significance. In **B** and **E**, representative pictures from each group are depicted.