CLK2 Is an Oncogenic Kinase and Splicing Regulator in Breast Cancer

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Abstract

Genetically activated kinases have been attractive therapeutic targets in cancer due to the relative ease of developing tumor-specific treatment strategies for them. To discover novel putative oncogenic kinases, we identified 26 genes commonly amplified and overexpressed in breast cancer and subjected them to a lentiviral shRNA cell viability screen in a panel of breast cancer cell lines. Here, we report that CLK2, a kinase that phosphorylates SR proteins involved in splicing, acts as an oncogene in breast cancer. Deregulated alternative splicing patterns are commonly observed in human cancers but the underlying mechanisms and functional relevance are still largely unknown. CLK2 is amplified and overexpressed in a significant fraction of breast tumors. Downregulation of CLK2 inhibits breast cancer growth in cell culture and in xenograft models and it enhances cell migration and invasion. Loss of CLK2 in luminal breast cancer cells leads to the upregulation of epithelial-to-mesenchymal transition (EMT)-related genes and a switch to mesenchymal splice variants of several genes, including ENAH (MENA). These results imply that therapeutic targeting of CLK2 may be used to modulate EMT splicing patterns and to inhibit breast tumor growth. Cancer Res; 75(7). 1–11. ©2015 AACR.

Introduction

Breast cancer is the second most commonly diagnosed cancer and is the main cause of cancer-related mortality in women worldwide (1). Breast tumors are highly heterogeneous and are classified on the basis of the expression of estrogen and progesterone receptors and HER2 into ER+ HER2−, and ER−PR−HER2− (triple-negative breast cancer, TNBC) disease. On the basis of gene expression profiles, breast tumors are characterized as luminal or basal (2–4). HER2+ and ER+ tumors typically have luminal features, whereas TNBCs show significant but not complete overlap with basal-like subtype. The categorization of breast tumors based on hormone receptor and HER2 status and the use of endocrine and HER2-targeted therapy, respectively, are some of the first examples of a molecular-based classification and personalized cancer treatment leading to a meaningful improvement in cancer clinical outcomes (5–9). Unfortunately, therapeutic resistance is common and a significant fraction of patients is inherently resistant to treatment or acquires resistance during disease progression (10).

The successful development of effective cancer therapies requires the identification of druggable disease-specific molecular pathways, the targeting of which leads to therapeutic response, and the choice of appropriate patient populations likely to realize clinical benefit from the therapeutic intervention. An elusory goal in oncology has been to improve the identification of such targets and patient populations before empirical clinical testing. As cancer is a genetic disease, the identification of genetic alterations playing a pivotal role in tumor initiation and progression has been key toward achieving this goal. Therapeutic inhibition of oncogenic protein kinases has been one of the most successful forms of targeted therapies as demonstrated by the treatment of BCR-ABL–mutant CML with imatinib and EGFR-mutant lung cancers with erlotinib.

In the past decade, systematic sequencing of putative therapeutic targets (e.g., kinases) has revealed several previously unknown but frequently mutated genes (e.g., PIK3CA) in breast and other cancer types (11). However, subsequent large-scale sequencing of breast cancer genomes in the past few years has somewhat disappointed initial expectations and identified relatively few recurrent mutations that could be explored for therapy (12, 13). This is especially the case in TNBCs where, aside from the already known cancer genes TP53, PTEN, and PIK3CA, very few
new genetic alterations were found (14–18). In addition, most of the mutations were detected only in a subset of tumors and at a low frequency, making it difficult to determine whether they are true drivers of tumorigenesis or just happen to be there as “passengers.” Here, we describe the identification of amplified kinases required for breast cancer cell growth using functional genomics and the further characterization of CLK2 in breast cancer.

**Materials and Methods**

**Cell culture**

Breast cancer cell lines were purchased from the ATCC or provided by Dr. S. Ethier, University of Michigan, Ann Arbor, MI (SUM series) and Dr. Fred Miller, Karmanos Cancer Institute, Detroit, MI (MCF10DCIS.com cells). All cell lines were cultured in the medium recommended by the provider at 37°C with 5% CO₂. The identity of the cell lines was verified on the basis of SNP array data. Three-dimensional Matrigel on top cultures were performed as previously described (19). Briefly, 96-well plates were coated with Matrigel (BD Biosciences) and allowed to solidify for 15 to 30 minutes at 37°C followed by plating 1 x 10⁴ cells per well. After the attachment of cells to the Matrigel coating, 10% Matrigel-containing medium was added and maintained with medium changes in every 2 to 3 days. Cell viability was determined using CellTitre-Glo (Promega) 8 days after shRNA infection. For colony formation assays, 96-well plates were coated with 0.66% agarose gel, layered with 0.33% agarose gel–containing cells, and topped with medium. Colonies were stained by MTT for imaging and counting as performed using GelCount (Oxford Optronix).

**siRNAs, shRNA plasmids, and lentivirus production**

siGENOME SMARTpool for negative control, CLK2, RBFOX2, and SF2/ASF were purchased from Thermo Scientific. pKO shRNA vectors for control GFP (clone 437) and CLK2 (clones 572, 1640, 1870, and 1969) were obtained from the Broad Institute RNAi consortium (TRC). To express dosycycline-inducible shRNAs, annealed oligos (LacZ: 5’-CCGATGCAGTTAATCACCAGCAGT-3’, CLK2: 5’-CATCCGATCCATCTGATGA-3’) were cloned into plKO-tet-on lentiviral vector (kindly provided by Dr. Alex Toker, Beth-Israe Deaconess Medical Center, Boston, MA). For expression of CLK2, full-length cDNA was inserted into plentiviral vector (Life Technologies) using Gateway recombination reaction (LifeTechnologies). To produce lentiviral supernatants, HEK293T cells were cotransfected with shRNA vectors, VSVG, and pDG8.91 using Fugene 6 (Roche). The target-supernatants, HEK293T cells were cotransfected with shRNA plasmids, and lentivirus production was conducted as previously described (24). Briefly, for primary screen, 11 breast cancer cell lines were infected with lentiviral shRNAs against the 26 kinases. Infected cells were selected by puromycin, and cultured for 6 days, followed by cell viability assay using CellTitre-Glo (Promega). To identify hits, Z-score representing the effect on cell viability was calculated by normalizing the viability score of each well to the plate mean viability score and SD of plate viability score: (X–μ)/σ (X = assay value, μ = mean plate viability score, σ = SD of plate viability score). Hits showing the lowest 10% of average of Z-score were selected for secondary screening. In secondary shRNA screen, shRNAs for the 10 selected genes were assayed in 17 breast cancer and 2 immortalized mammary epithelial cell lines. Hits were identified using ≥30% decrease in cell viability compared with control shRNAs on day 6 after infection.

**Cell proliferation, apoptosis, and cell-cycle assay**

To determine viable cell numbers, Trypan blue–negative cells were counted at days 0, 3, 6, and 9 after infection. Cells were assayed 4 days after virus infection for apoptosis using Apo-ONE Homogeneous Caspase-3/7 Assay (Promega) and cell-cycle analysis. Cell-cycle analysis was performed as follows, and cells were harvested, washed in PBS, and fixed in ice-cold 70% ethanol for 1 hour. Fixed cells were resuspended in a solution containing 100 µg/ml RNase, 40 µg/ml propidium iodide (PI, Sigma), and incubated for 30 minutes on ice. Finally, the cells were suspended in 20 µg/ml PI solution. The DNA content of 10,000 cells was determined with FACScan (BD Biosciences).

**Cell migration and invasion assays**

Cell migration assay and invasion assays were performed as described (25). Briefly, cells (5 x 10⁴ and 2.5 x 10⁵) were seeded onto top chambers of BioCoat Control and Matrigel invasion chambers (8.0 µm pore size, BD Biosciences), respectively. Top chambers (culture inserts) were filled with serum-free medium and bottom chambers were filled with medium containing 20% FBS as chemoattractant. The number of cells that migrated or invaded to bottom surfaces of the membranes was counted after 24 hours of incubation followed by staining with Giemsa solution.

**RT-PCR and qRT-PCR analysis**

Total RNA from cultured cells or tumor specimens was prepared using RNeasy Mini Kit (Qiagen). Reverse transcription was carried out using Super Script II Reverse Transcriptase (Life Technologies). For RT-PCR, synthesized cDNA was amplified and run in 2% agarose gel. For qRT-PCR, the quantities of DNA amplified with SYBRgreen PCR Master Mix or TaqMan PCR Master Mix (Applied Biosystems) were measured by ABI7500 or ABI7900, respectively (Applied Biosystems). Assessment of changes in gene expression was conducted by comparison of ΔΔCt values for each capture using CytoVysion Imaging System (Applied Imaging Pittsburgh).

**SNP array data analysis and shRNA screen**

SNP array data (22) were analyzed to define ARI (aberration amplitude and recurrence index) and AFI (aberration focality index) scores essentially as described (23). ARI is a measure of the copy number gain and the recurrence of such copy numbers across the samples. AFI weights the focality of the observed copy number changes. Lentiviral shRNA screen was conducted as previously described (24). Briefly, for primary screen, 11 breast cancer cell lines were infected with lentiviral shRNAs against the 26 kinases. Infected cells were selected by puromycin, and cultured for 6 days, followed by cell viability assay using CellTitre-Glo (Promega). To identify hits, Z-score representing the effect on cell viability was calculated by normalizing the viability score of each well to the plate mean viability score and SD of plate viability score: (X–μ)/σ (X = assay value, μ = mean plate viability score, σ = SD of plate viability score). Hits showing the lowest 10% of average of Z-score were selected for secondary screening. In secondary shRNA screen, shRNAs for the 10 selected genes were assayed in 17 breast cancer and 2 immortalized mammary epithelial cell lines. Hits were identified using ≥30% decrease in cell viability compared with control shRNAs on day 6 after infection.
sample. Primer sequences used in RT-PCR are as follows: GAPDH:5'-GGGCTGCTTGCTGCAACC-3' (forward), 5'-GAGGGGCTACAACTGCTTC-3' (reverse), ENAH:5'-TGCTGGCCACAGGAGAAGAT-3' (forward), 5'-ACTGGGCTGGTATGCGGTGGTC-3' (reverse). The primers of CDH1, CDH2, VIM, SNAI1, SNAI2, ZEB1, TWIST1, and FN1 used for qRT-PCR were designed as previously described (20). The primer sequences of GAPDH and CLK2 for qRT-PCR were as follows: GAPDH:5'-CGAGATCCCTCAGAAATC-3' (forward), 5'-GTCCTGGGTTGCGATGAT-3' (reverse). CLK2:5'-AAATTTTCTACCAGGCTGTCG-3' (forward), 5'-AGCCCGTACCAGGTTTCTACT-3' (reverse). TaqMan probes used are GAPDH (Hs99999905_m1), CLK2 (Hs00241874_m1), ESRP1 (Hs002241472_m1), ESRP2 (Hs002277840_m1), RBOX1 (Hs01125659_m1), RBOX2 (Hs00204814_m1), and RBOX3 (Hs01370653_m1).

Immunoblotting, immunoprecipitation, and immunohistochemical analyses

Cells were lysed in radioimmunoprecipitation assay buffer with Halt protease inhibitor Cocktail (Thermo Scientific). Lysates mixed with sample buffer were separated by electrophoresis on 20% gels and transferred to membranes. Membranes were blocked with 5% skim milk in TBST, followed by incubations with primary antibodies. After washing with TBS-0.05% Tween, membranes were incubated with horseradish peroxidase–conjugated anti-mouse or anti-rabbit IgGs, and visualized with Immobilon Western Detection Reagent (Millipore). Signals were analyzed and quantified using NIH image software. Anti-CLK2 (cat# ab53082), RBOX2 (cat# ab51361), and SRSF1 (cat# ab38017) polyclonal antibodies were purchased from Abcam. Anti-phospho-STAT3, anti-phospho-ERK protein monoclonal antibody (1H4, cat# MABE50) was purchased from Millipore. Anti-β-actin monoclonal antibody was purchased from Sigma-Aldrich (cat#A2228). For immunoprecipitation assays, cell lysates were incubated with anti-CLK2 antibody (AP7530, ABGENT) overnight followed by incubation with protein G beads (Life Technologies) for 2 hours. Immunoprecipitates were applied for immunoblot analysis and membranes were incubated with anti-phospho-serine (4A4, Millipore) or anti-CLK2 antibodies. Signal was visualized with Immobilon Western Detection Reagent. Immunohistochemical analyses for SMA, Ki67, and TUNEL assay were performed as recently described (26).

Mouse xenograft assays

Female NCRNU-F mice (6-week-old) were purchased from Taconic and maintained in pathogen-free conditions. Mice were injected subcutaneously with 1 × 10^5 MCF10DCIS.com cells in media with 50% Matrigel. Control and treatment groups received water supplemented with 2% sucrose in the absence or presence of 1 mg/mL doxycycline. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Dana-Faber Cancer Institute (Boston, MA) under protocol #11-023.

RNA-seq and splicing analysis

MCF7 cells were infected with lentiviruses expressing shGFP or shCLK2 and subject to 4 days of puromycin selection. Total RNA was isolated using RNeasy Mini Kit (Qiagen), and applied for μMACS mRNA isolation kit (Milltenyi Biotec). RNA-seq libraries were prepared using mRNA Sequencing Sample Preparation Kit (Illumina) with slight modifications. The sequencing library was enriched with 18 cycles of PCR reaction and size-fractionated into 250–400 bp by running on a polyacrylamide gel. After validation on 2100 Bioanalyzer (Agilent Technologies), samples were subject to 50 bp pair-end sequencing on Hi-seq 2000 (Illumina). Sequence reads were aligned to human reference genome (hg19) using TopHat software (27). The mapping results were visualized using the Integrative Genomics Viewer (IGV) genome browser (28). Differentially spliced genes between the basal and luminal A subtype breast cancers were identified using level 3 data from TCGA BRCA dataset (downloaded from https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm) using a previous described method (29).

Results

Novel amplified kinases required for breast cancer growth

In search of novel oncogenic kinases in breast cancer, we identified 52 genes encoding for protein kinases with common copy number gain based on SNP array analysis of breast tumors and breast cancer cell lines (Fig. 1A, and Supplementary Fig. S1A; Supplementary Table S1; ref. 22), which we also confirmed by FISH and qPCR for selected genes (Fig. 1B; Supplementary Tables S2 and S3). We also analyzed the expression of the most commonly amplified genes in breast tumors and breast cancer cell lines by qRT-PCR (Supplementary Tables S2 and S3). We selected 26 genes that have not been extensively characterized in breast cancer for follow-up functional studies due to their frequency and amplitude of copy number gain and overexpression.

To determine whether the amplification and overexpression of these 26 kinases reflect potential oncogenic functions in breast cancer, we performed a targeted lentiviral shRNA screen using cellular viability as endpoint. The shRNA screen was performed in two phases. In the primary screen, we tested all 26 genes (4–5 shRNAs each) in 11 breast cancer lines of different subtypes and defined shRNA hits showing the lowest 10% of average of Z-score representing their effects on cellular viability (Fig. 2A). The top 10 hits in the primary screen were selected for a secondary screen on an extended panel of 17 breast cancer lines of different subtypes and two immortalized mammary epithelial lines (Fig. 2B). In the secondary screen, we considered shRNAs to have "scored" in a cell line if they decreased viability by ≥ 30% compared with control (after averaging effects with and without puromycin). RIGER analysis (30) of the shRNA data to assess potential tumor subtype specificity of the hits revealed the preferential requirement for CLK2 in luminal and RIOK2 and AKT3 in basal cell lines. We selected CLK2 for further study due to its high frequency of amplification and overexpression in breast cancer and because it is a relatively poorly characterized kinase.

Expression of CLK2 in breast cancer

To investigate the potential role of CLK2 in breast cancer cell growth, we first analyzed its expression in a panel of breast cancer cell lines by qRT-PCR and immunoblot assays. We found that CLK2 mRNA and protein levels were in general higher in luminal and HER2- breast cancer cells although the EGFR-amplified MDA-MB-468 and EGF-dependent MCF10DCIS TNBC cell lines also expressed fairly high levels (Fig. 3A–C). Analysis of CLK2 immunoprecipitated by phospho-serine immunoblot showed slightly higher phospho-CLK2 levels in luminal cell lines (Fig. 3D). In the TCGA breast cancer dataset (31), we found a good correlation between CLK2 gene copy number and mRNA levels (Supplementary Fig. S1B). We also analyzed CLK2 protein levels based on TCGA RPPA (reverse phase protein array) data and categorized tumors into
CLK2 low, intermediate, and high classes (Supplementary Fig. S1C). To explore signaling pathways that may be differentially activated depending on CLK2 protein levels, we identified 21 proteins that showed significant differences between CLK2-low and CLK2-high tumors (Supplementary Fig. S1D). Tumors with high CLK2 expression seem to be more proliferative based on high levels of cyclin B1, CDK1, phospho-Rb, and also display activation of the hippo signaling pathway reflected by high YAP/TAZ levels. Finally, we explored potential associations between CLK2 expression and clinical outcome in breast cancer by comparing the survival of patients with CLK2-low and CLK2-high tumors. Although there was a trend for shorter survival associated with high CLK2 protein levels, this did not reach significance (Supplementary Fig. S1E) possibly due to relatively small cohort size.

CLK2 regulates breast cancer cell growth and morphology

To validate our shRNA screen results, we first confirmed the efficacy and specificity of the CLK2-targeting shRNAs by immunoblot analyses and by rescue experiments using a shRNA-resistant CLK2 expression construct (Supplementary Fig. S2A–S2C). Next, we assessed the proliferation of MCF7 and T-47D luminal breast cancer cell lines following the downregulation of CLK2 by counting viable cell numbers (short-term effect) and performing colony growth assays (long-term effects). Downregulation of CLK2 by two different shRNAs significantly suppressed the growth of both cell lines that was accompanied by a slight increase in apoptosis and arrest in the G1 phase of the cell cycle (Fig. 3E and F and Supplementary Fig. S2D and S2E). Interestingly overexpression of CLK2 in MCF7 cells also decreased cell proliferation (data not shown) implying the requirement for tightly controlled CLK2 levels for optimal breast cancer cell growth.

During the cell proliferation assays, we noticed that the morphology of shCLK2-infected MCF7 cells became noticeably more fibroblastoid implying potential epithelial-to-mesenchymal transition (EMT) (Supplementary Fig. S3A). To test this hypothesis, we analyzed the migration and invasion of MCF7 cells and found that downregulation of CLK2 significantly increased both cell migration and invasion (Fig. 3G and H). Similar results were obtained in T-47D, BT-474, and MCF10DCIS breast cancer cells as well (Supplementary Fig. S3B and data not shown).

To analyze the effect of CLK2 downregulation on breast cancer cell proliferation and invasive behavior in more physiologic growth conditions, we assessed the growth of MCF10DCIS cells infected with control or CLK2-targeting shRNAs in three-
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dimensional Matrigel cultures. Downregulation of CLK2 significantly decreased both the number and size of MCF10DCIS spheroids in these conditions, but did not make them more invasive (Fig. 4A and B).

Finally, we analyzed the role of CLK2 in breast tumorigenesis in xenograft assays using MCF10DCIS cells. MCF10DCIS cells form tumors that resemble human ductal carcinoma in situ (DCIS) that spontaneously progress to invasive tumors with time (32, 33). Thus, the use of this cell line allowed us to explore the potential role for CLK2 in breast tumor growth and invasive progression. Downregulation of CLK2 on day 1 or day 14 after injection using doxycycline-inducible shRNAs significantly decreased the growth of MCF10DCIS xenografts (Fig. 4C and D). Analysis of xenograft histology and the expression of smooth muscle actin (SMA), a myoepithelial cell marker, revealed that shCLK2-expressing tumors maintained DCIS histology even at time points when the control tumors have already lost the myoepithelial cell layer and were all invasive (Fig. 4E). The apparent delay of invasive progression by downregulation of CLK2 may be in part due to the slower growth of shCLK2-expressing xenografts as demonstrated by lower Ki67 index (Fig. 4E) and also potentially due to the upregulation of EMT-related signaling pathways following CLK2 loss that promote the differentiation of myoepithelial cells (33). Correlating with this, a higher fraction of cells were vimentin positive and E-cadherin negative in shCLK2-expressing xenografts (Fig. 4E), although the differences in histology and the known luminal (E-cadherin) and myoepithelial (vimentin) cell type specificity of these markers confound the interpretation of these results.

CLK2-mediated changes in gene expression and splicing patterns

To explore the molecular basis of CLK2 loss-induced changes in cell growth and morphology, we analyzed the gene expression profiles of MCF7 cells by RNA-seq four days after infection with lentiviruses expressing control and CLK2-targeting shRNAs. Downregulation of CLK2 led to significant changes in the expression of many genes; almost 4,000 transcripts had significantly (Gfold ≥ 2; ref. 34) increased or decreased levels in shCLK2-expressing cells implying global reprogramming of the transcriptome (Supplementary Table S4). Many of the most significantly downregulated genes encode for proteins with known roles in epithelial cell differentiation such as SPRR1A, SPRR1B, S100A7, S100A9. In contrast, many genes upregulated after CLK2 loss encode for extracellular matrix–related proteins and proteases, including ADAM2, TIMP3, and KLK11. Analysis of the gene expression data for pathway and network enrichment using the Metacore suite (35) revealed the activation of EMT and TGFβ signaling pathways following downregulation of CLK2 (Supplementary Table S5). To confirm the induction of EMT following CLK2 loss, we analyzed the expression of known EMT regulators by qRT-PCR in MCF7, T-47D, MCF10DCIS, and BT-474 breast cancer cell lines. We found significant upregulation of VM (vimentin) after shCLK2 in all luminal cell lines tested, whereas the EMT-inducing transcription factors such as SNAI1, SNAI2, TWIST1, and ZEB1 were upregulated at variable levels (Fig. 5A and Supplementary Fig. S3C and S3D, and data not shown)

One of the few known substrates of CLK2 is the SRSF1 protein that plays key roles in splicing (36). Phosphorylation of SRSF1 is required for nuclear localization and, thus, for its splicing functions (37). To investigate whether downregulation of CLK2 may modulate SRSF1 and via this potentially influence splicing, we first analyzed the expression and phosphorylation of SRSF1 in MCF7 cells expressing control or CLK2-targeting siRNAs. Downregulation of CLK2 did not affect overall SRSF1 protein levels, but it significantly decreased its phosphorylation (Fig. 5B).

Next, we explored potential changes in alternative splicing patterns in our RNA-seq data. We processed RNA-seq results after tophat and cuffdiff runs and focused on the top 275 genes with more than 30% change in splice variants (Supplementary Table S6). Interestingly, ENAH (MENA), a gene implicated in breast tumor invasion and metastasis (38, 39), was one of the genes that showed the most significant changes in splicing patterns in shCLK2-expressing cells. Exon 11a of ENAH is subject to alternative splicing in a cell type and epithelial differentiation state-dependent manner: it is included in the ENAH transcript in luminal but not in basal cells (40). Our RNA-seq data suggested that the levels of the longer ENAH transcript that includes exon11a are decreased after downregulation of CLK2 (Fig. 5C). We confirmed this change by RT-PCR (Fig. 5D and Supplementary Fig. S3E) and also verified that luminal (including HER2+) breast cancer cell lines expressed the longer, exon11a-containing transcripts, whereas basal ones did not with the exception of MCF10DCIS cells that expressed both variants at about the same levels (Fig. 5E). Interestingly, no alteration of ENAH splice variant was observed following downregulation of SRSF1, suggesting that regulation of ENAH splicing might be via other substrates of CLK2 or via indirect mechanisms (Fig. 5D). The splicing of ENAH has been shown to be regulated by ESRI2/1/2 proteins (41–44) and RBFOX family members (45, 46). We investigated whether the expression of these genes may be affected by CLK2 expression. On the basis of our RNA-seq data we found that ESRI2/1/2 are not differentially expressed in MCF7 cells expressing control and CLK2-targeting shRNAs, whereas the expression of RBFOX family members was very low in MCF7 cells; consistent with their lower expression in luminal compared with mesenchymal cells (45, 46). We have also performed qRT-PCR and Western blot analysis of these proteins in MCF7 cells after CLK2 downregulation and found that the expression of RBFOX2 was approximately 50% decreased after CLK2 knockdown (Fig. 5F and G). Subsequently, we tested whether downregulation of RBFOX2 may influence the expression of CLK2 and ENAH splice variants. We found no effect on CLK2 mRNA levels after RBFOX2 downregulation, whereas ENAH splicing switched to the basal pattern, similar to what was observed after shCLK2 (Fig. 5H and I). These results imply that RBFOX2 may act downstream of CLK2 and may mediate its effect on splicing.

Figure 2.
shRNA screen results. Blue, pink, red, and orange colors indicate breast cancer subtypes (luminal, TNBC, and HER2+), and immortalized cells, respectively, in all panels. A, primary screen results. Average Z-scores ([average viability – plate median average viability]/[plate median absolute deviation of average viability]) from primary shRNA screen of 26 amplified kinases in breast cancer are shown. shRNA clones highlighted in blue, pink, and red colors with the lowest 10% Z-score were identified as hits. B, results of the secondary shRNA screen for 10 kinases in 17 breast cell lines. Numbers indicate percent cell viability compared with control. Shading indicates shRNAs that showed ≥30% decrease in viability compared with control shRNAs.
We also investigated whether the expression of CLK2 is associated with ENAH exon 11a usage in the TCGA breast cancer RNA-seq dataset. This revealed a good correlation of CLK2 expression to ENAH exon 11a usage in normal breast tissues (Fig. 5I) and Supplementary Fig. S3F), which confirm results from our siCLK2 studies. However, this correlation is completely lost in breast tumors, irrespective of breast tumor subtypes (Supplementary Fig. S3F).

Finally, to identify genes that show common EMT-related and breast tumor subtype-specific splicing patterns, we explored potential overlaps between splice variants differentially expressed in MCF7 cells before and after shCLK2 expression and in HMLE mammary epithelial cells undergoing EMT induced by the overexpression of the TWIST transcription factor, and differentially expressed between luminal and basal breast tumors in the TCGA data (Supplementary Fig. S4A). ENAH was the only gene that showed differential splicing due to EMT in both MCF7 shCLK2 and HMLE–TWIST cells. In contrast, the splice variants of 11 genes (e.g., CASZ1, CD47, GREB1, PMP22, and PTK2) were significantly different in MCF7 cells after CLK2 downregulation and also between basal and luminal breast tumors (Supplementary Fig. S4B). The limited overlap between EMT-related splicing changes
between two cell lines is not surprising as HMLE and MCF7 cells are very different and overexpression of TWIST and downregulation of CLK2 may activate different downstream pathways. However, differences in experimental conditions and data analysis may also contribute to the limited overlaps.

Discussion

We describe here a functional genomics screen and the identification of several novel putative oncogenic kinases in breast cancer that may be explored as therapeutic targets. We have previously reported the detailed characterization of two of these kinases IKKBE (47) and AKT3 (48) that promote tumorigenesis via activating the NF-κB (49) and PI3K/AKT/mTOR signaling pathways, respectively. Interestingly, several of the kinases that we identified as hits in our shRNA screen are involved in the AMPK–mTOR–AKT signaling pathway implying a central role for this pathway in breast cancer cell growth and survival. ULK1 is a substrate of AMPK and a key regulator of autophagy (50) and was recently reported to promote the growth of HER2+ breast cancer cells (51). NUAK2 is an AMPK-related kinase and a putative oncogene in melanoma (52, 53). RIOK2 regulates glioblastoma growth and survival via regulating AKT (54).

In this study, we focused on the more detailed characterization of CLK2, a dual specificity kinase implicated in the regulation of splicing due to its ability to phosphorylate SR proteins (55–57). Besides splicing, CLK2 has also been shown to play a role in hepatic gluconeogenesis and fatty acid oxidation by directly phosphorylating PGC-1α and decreasing its transcriptional activity (58, 59). CLK2 was also shown to phosphorylate and activate PTP-1B and influence tumor growth via this action (60). Finally, CLK2 is a negative regulator of AKT activity via phosphorylating and activating PP2A that leads to dephosphorylation of AKT (59, 61). At the same time, CLK2 itself is activated by phosphorylation by AKT following ionizing radiation (62). Thus, CLK2 is both upstream and downstream of AKT forming a self-regulatory loop. However, some of these signaling pathways may be cell type–specific as in human breast cancer cells, we did not find conclusive evidence for the regulation of AKT by CLK2 (data not shown).
CLK2 as a Therapeutic Target in Breast Cancer

We found that CLK2 is amplified and overexpressed in a significant fraction of human breast tumors and its downregulation inhibits breast cancer cell growth in cell culture models and tumorigenesis in xenograft assays. Importantly, we show that decreased CLK2 levels induce EMT and EMT-related changes in alternative splicing patterns in luminal breast cancer cells. In line with our findings, a recent study described that changes in alternative splicing by themselves can lead to EMT-related changes in cellular phenotypes (40). Similarly SRSF1, a splicing factor and target of CLK2, regulates mammary epithelial cell transformation (63) and an oncogenic splice variant of KLF6 induces EMT and promotes breast cancer metastasis (64). One of the genes whose splice variants are changed following downregulation of CLK2 is ENAH (MENA). ENAH is an actin-binding protein that promotes cancer cell invasion and metastasis and its expression in primary breast tumors is associated with poor outcome (38, 39). The switch from EMT variant of ENAH after downregulation of CLK2 in primary breast tumors is associated with poor outcome (38, 39).

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in MCF7 cells enhances mesenchymal-type ENAH splicing implying that CLK2's effects on ENAH splicing might be mediated via RBFOX2. However, the function and cell type specificity of these splicing factors is complex, thus, further studies are required to delineate how CLK2 may regulate alternative splicing.

In summary, our results should encourage the further preclinical validation of CLK2 and the other putative oncogenic kinases we identified as therapeutic targets in breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: C. Brennans, K. Polyaak

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Yoshida, J. H. Kim

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Yoshida, K. Carver, Y. Su, S. Weremowicz, L. Mulvey, S. Yamamoto, C. Brennans, S. Mei, H. Long, J. Yao

Writing, review, and/or revision of the manuscript: T. Yoshida, K. Carver, Y. Su, S. Weremowicz, L. Mulvey, S. Yamamoto, K. Polyaak

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Carver, Y. Su

Study supervision: K. Polyaak

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References


39. Warzecha CC, Shen S, Xing Y, Carstens RP. The epithelial splicing factors
42. Warzecha CC, Shen S, Xing Y, Carstens RP. The epithelial splicing factors
49. Moselein FM, Myers MP, Landreth GE. The CLK family kinases, CLK1 and CLK2, phosphorylate and activate the tyrosine phosphatase, PTP-1B. J Biol Chem 1999;274:26697–704
# CLK2 Is an Oncogenic Kinase and Splicing Regulator in Breast Cancer

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