Combined deletion of Pten and p53 in mammary epithelium accelerates triple-negative breast cancer with dependency on eEF2K

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Abstract

The tumor suppressors Pten and p53 are frequently lost in breast cancer, yet the consequences of their combined inactivation are poorly understood. Here, we show that mammary-specific deletion of Pten via WAP-Cre, which targets alveolar progenitors, induced tumors with shortened latency compared to those induced by MMTV-Cre, which targets basal/luminal progenitors. Combined Pten-p53 mutations accelerated formation of claudin-low, triple-negative-like breast cancer (TNBC) that exhibited hyper-activated AKT signaling and more mesenchymal features relative to Pten or p53 single-mutant tumors. Twenty-four genes that were significantly and differentially expressed between WAP-Cre:Pten/p53 and MMTV-Cre:Pten/p53 tumors predicted poor survival for claudin-low patients. Kinome screens identified eukaryotic elongation factor-2 kinase (eEF2K) inhibitors as more potent than PI3K/AKT/mTOR inhibitors on both mouse and human Pten/p53-deficient TNBC cells. Sensitivity to eEF2K inhibition correlated with AKT pathway activity. eEF2K monotherapy suppressed growth of Pten/p53-deficient TNBC xenografts in vivo and cooperated with doxorubicin to efficiently kill tumor cells in vitro. Our results identify a prognostic signature for claudin-low patients and provide a rationale for using eEF2K inhibitors for treatment of TNBC with elevated AKT signaling.

Keywords: eEF2K; p53; prognosis; Pten; triple-negative breast cancer

Introduction

Breast cancer (BC) is a heterogeneous disease that can be classified into estrogen receptor-α-positive (ERα⁺) and HER2⁺ tumors as well as triple-negative (TN) tumors, which do not express high levels of these or the progesterone receptors (Prat & Perou, 2011). TNBCs include two major subtypes: basal-like, expressing basal-cell markers such as cytokeratin 14, and claudin-low/mesenchymal-like, expressing low levels of tight junction proteins including certain claudins and E-cadherin, and high levels of genes associated with epithelial-to-mesenchymal transition (EMT) (Prat et al., 2010; Lehmann et al., 2011; Timmerman et al., 2013). Interest in the latter tumors is driven by observations that following conventional therapy, residual tumors exhibit features of cancer stem cells and EMT (Mani et al., 2008; Creighton et al., 2009; Guo et al., 2012). Moreover, TNBCs often resist therapy, and metastatic disease is virtually incurable (Carey et al., 2007; Irshad et al., 2011). While specific treatments have been developed for ERα⁺ BC (tamoxifen, aromatase inhibitors) and HER2⁺ BC (trastuzumab), the only option for most TNBC patients is cytotoxic chemotherapy such as anthracyclines (doxorubicin), which leads to significant morbidity.
In TNBC, p53 is deleted or mutated in 60–80% of cases (Holstege et al., 2010; Koboldt et al., 2012; Shah et al., 2012), whereas the Phosphatase and TENsin (Pten) homolog deleted in chromosome 10 (Li et al., 1997; Steck et al., 1997) is lost in 25–30% of cases primarily through promoter silencing or microRNA-mediated suppression (Salmena et al., 2008; Korkaya et al., 2009; Koboldt et al., 2012). The protein, Pten, regulates cell growth by converting phosphatidylinositol-3,4,5-trisphosphate (PIP3) into phosphatidylinositol-4,5-disphosphate (PIP2), thereby antagonizing phosphatidylinositol-3 kinase (PI3K) pathway activation (Stambolic et al., 1998; Cully et al., 2006; Adams et al., 2011). Dysregulation of the PI3K pathway induces AKT/PKB, leading to increased cell motility, proliferation and survival, as well as increased protein translation via mTOR. Pten and p53 were shown to regulate EMT and cell migration (Leslie et al., 2007; Jiang et al., 2011), and interact with each other at several levels (Stambolic et al., 2001; Kawase et al., 2009).

While p53 loss is not actionable, activation of the PI3K pathway can be targeted by PI3K pathway antagonists such as PI3K, AKT or mTOR inhibitors (Janku et al., 2012; Kim et al., 2012). However, as the PI3K pathway is subject to tight autoregulation, such inhibitors often have modest or transient effects (Gordon & Banerji, 2013). There is therefore an urgent need to identify new therapeutic targets that may be useful for treatment of Pten/p53-deficient TNBC. The effects of mutations in p53 or Pten on the mammary epithelium have been documented (Stambolic et al., 2000; Li et al., 2002; Herschkowitz et al., 2012; Knight et al., 2013). The impact of combined inactivation of these tumor suppressors, which frequently occurs in breast cancer, is poorly understood. Here, we disrupted Pten and p53 in mammary epithelium either alone or in combination and determined the effect on tumor formation, tumor-initiating cells, prognosis, PI3K/AKT pathway activation and response to therapeutic drugs. We found that Pten/p53 deficiency induces TNBCs, which are distinct from Pten or p53 single-mutant tumors with more mesenchymal features and poor clinical outcome. A non-biased screen revealed that while PI3K/AKT/mTOR inhibitors efficiently kill Pten/p53-deficient tumors, the most potent drugs target JNK, which was previously linked to Pten-deficient cancer, and eEF2K, a kinase that controls protein translation downstream of mTOR. Sensitivity to eEF2K was proportional to AKT pathway activity and was demonstrated both in vitro and in xenografts of mouse and human Pten/p53-deficient TNBC. Our results should encourage development of effective eEF2K inhibitors for treatment of TNBC with elevated AKT signaling.

**Results**

**Combined deletion of Pten and p53 induces spindle-/mesenchymal-like mammary tumors**

To model the effect of Pten loss on BC, we used a floxed allele (Ptenfl/fl) (Suzuki et al., 1998) and the deleter lines WAP-Cre (which preferentially targets pregnancy-identified alveolar progenitors) and MMTV-CreERT2 (which targets basal and luminal progenitors) (Wagner et al., 2002; Jiang et al., 2010). MMTV-Cre:Ptenfl/fl mice developed mammary tumors after a long latency of 26.4 months with incomplete penetrance (70%) (Fig 1A). WAP-Cre:Ptenfl/fl females developed tumors with shorter latency (15.2 months) and almost complete penetrance; by 18 months, nearly all mice had succumbed to cancer. In both cases, pregnancy accelerated tumor formation. Tumors from both models were heterogeneous, consisting primarily of adenomyoepithelioma (~70%) or adenosquamous carcinoma (20–25%) (Fig 1B and C). In addition, a small fraction of tumors was classified as acinar or poorly differentiated adenocarcinoma (4–7%), or spindle-cell/adenosarcoma (3–4%). Marker expression analysis of the dominant tumor subtypes revealed mixed expression of smooth muscle actin (SMA) and cytokeratin 5 (K5), K6, K14 (basal markers), K18 (luminal marker), vimentin, ERα, as well as nuclear co-localization of β-catenin and cyclin D2 (Supplementary Fig S1), a pattern often found in other tumor models of mixed lineages such as MMTV-WNT1 (Li et al., 2003).

Next, we determined the effect of concurrent loss of Pten and p53, which are frequently inactivated in TNBC. MMTV-Cre:Ptenfl/fl, p53fl/fl and WAP-Cre:Ptenfl/fl,p53fl/fl double-mutant females developed tumors with a reduced latency of 11.3 and 9.8 months, respectively, compared with 26.4, 15.2 and 16.9 months for single-mutant MMTV-Cre:Ptenfl/fl, WAP-Cre:Ptenfl/fl and MMTV-Cre:p53fl/fl mice (Fig 1D). Deletion of the Ptenfl/fl and p53fl/fl alleles in these tumors was confirmed by PCR (Fig 1E). In contrast to the heterogeneity of Ptenfl/fl tumors and small percentage of adenosarcomas, approximately 70% of Ptenfl/fl;p53fl/fl lesions were histologically classified as adeno-sarcomatoid/spindle-cell/mesenchymal-like BC. The rest exhibited mixed mesenchymal plus adenocarcinomas or differentiated adenocarcinomas (Fig 1F). In comparison, only 30% of p53fl/fl tumors were sarcomatoid. The Pten/p53-deficient adeno-sarcomatoid-like tumors expressed the mesenchymal markers vimentin, SMA and desmin but not ERα (Supplementary Fig S2).

**Pten/p53-deficient mouse tumors cluster with human claudin-low TNBC**

To molecularly classify the Pten/p53-deficient tumors, we compared them to other mouse models and human BC subtypes using an extended intrinsic BC signature and unsupervised hierarchical clustering (Herschkowitz et al., 2007) (Supplementary Table S1A). Expression across platforms was combined and integrated using the distance weighted discrimination (DWD) algorithm (Benito et al., 2004). Three MMTV-Her2/Neu tumors were included as internal control. Cluster analysis grouped them with published MMTV-Her2/Neu tumors (Fig 2A), thus validating our normalization process. Most (10/16) Ptenfl/fl tumors clustered with “normal”-like BCs. Importantly, the majority of MMTV-Cre:Ptenfl/fl,p53fl/fl and WAP-Cre: Ptenfl/fl,p53fl/fl tumors (12/15) clustered with mouse spindle-like mammary tumors and human claudin-low BC. In contrast, only half (3/6) of MMTV-Cre:p53fl/fl tumors clustered with Ptenfl/fl,p53fl/fl tumors/claudin-low BC.

We next used a claudin-low signature developed by Prat and Perou to classify our Ptenfl/fl,p53fl/fl tumors with human BC samples (Prat et al., 2010) (Fig 2B; Supplementary Table S1B). All but one Ptenfl/fl,p53fl/fl tumors clustered with claudin-low BC. Accordingly, expression of claudin 3, 4 and 7 was very low in 14 of 15 MMTV-Cre:Ptenfl/fl,p53fl/fl and WAP-Cre:Ptenfl/fl,p53fl/fl tumors (Fig 2C). In contrast, only 3 of 6 MMTV-Cre:p53fl/fl tumors expressed low levels of claudin genes. The mouse Ptenfl/fl,p53fl/fl tumors and most human claudin-low BC samples, but only 1 of 6 p53fl/fl tumors, expressed high levels of the EMT inducers Twist1/2, Snail1/2 and Zeb1/2
Figure 1. Pten plus p53 mutations cooperate to accelerate sarcomatoid/mesenchymal-like mammary tumors.

A Kaplan–Meier mammary tumor-free curves for WAP-Cre:Pten\(^{ff}\) and MMTV-Cre:Pten\(^{ff}\) mice. Dashed lines represent nulliparous or multiparous females and solid line the average for all mice. Tumor latency (average) for the two models was significantly different (\(P = 3.47 \times 10^{-17}\), Wilcoxon method).

B Histology of four major tumor types in WAP-Cre:Pten\(^{ff}\) and MMTV-Cre:Pten\(^{ff}\) mice.

C Distribution of tumor types (%) in WAP-Cre:Pten\(^{ff}\) (left) and MMTV-Cre:Pten\(^{ff}\) (right) mice. Statistical significance by Wilcoxon method. p53 versus Pten, \(P = 0.00158\); Pten/p53 versus p53, \(P = 0.0329\); Pten/p53 versus Pten, \(P = 4.71 \times 10^{-14}\).

D Kaplan–Meier mammary tumor-free curves for WAP-Cre:Pten\(^{ff}\), p53\(^{ff}\), MMTV-Cre:p53\(^{ff}\) and MMTV-Cre:p53\(^{ff}\) versus (average) WAP-Cre:Pten\(^{ff}\) and MMTV-Cre:Pten\(^{ff}\) mice. Statistical significance by Wilcoxon method. p53 versus Pten, \(P = 0.00158\); Pten/p53 versus p53, \(P = 0.0329\); Pten/p53 versus Pten, \(P = 4.71 \times 10^{-14}\).

E Detection of Pten and p53 gene deletion by PCR using primers specific for Cre-excised Pten\(^{ff}\) and p53\(^{ff}\) alleles.

F Histology of indicated tumors and distribution (%) of tumor types.
Figure 2. Dependency of Pten/p53-deficient TNBC on eEF2K.
We also asked whether the 24-gene set was predictive of clinical outcome using a cohort of 96 claudin-low BC patients with metastatic-free survival (MFS) data. Remarkably, this gene set, which we termed WAP-Cre Claudin-Low Signature (WCLS, Supplementary Table S1G), could stratify claudin-low patients into high and low risk groups with a hazard ratio of 2.24 (P = 0.0124; Fig 3C). Comparing to a recently reported signature for Basal-Like Breast Cancer (BLBC) (Hallett et al, 2012), WCLS was specific for the claudin-low tumors, whereas BLBC was specific for basal-like BC with HR = 1.96 (P = 0.0287; Fig 3C). Both signatures were not informative for HER2+, luminal A or luminal B BC (Supplementary Fig S4B).

To assess the possibility that WCLS is predictive by chance alone, we generated 1,000 random signatures with the same composition (i.e. 7 up-, 17 down-regulated genes) and analyzed their predictive power against the same patient cohort, as previously described (Liu et al, 2013). We found that 4.6% of the random signatures were significant (P < 0.05), of which 2% had HR > 1 (Fig 3D). Importantly, WCLS ranked 3rd with HR of 2.8 (P = 0.03), indicating that its prognostic power is statistically significant. In contrast, BLBC ranked 242 with insignificant P-value. For basal-like BC patients, BLBC, but not WCLS, ranked high (2nd) compared to 1,000 random signatures of similar composition (Supplementary Fig S4C).

The better prognostic of WCLS-negative versus WCLS-positive patients suggests that overt activation of EMT/mesenchymal pathways may improve outcome by blocking mesenchymal-to-epithelial transition (MET), which is required for metastatic growth at distal sites (Ocanà et al, 2012; Tsai et al, 2012). In this case, an EMT signature should also not be associated with worse outcome. To test this prediction, we determined whether a core EMT/mesenchymal signature developed by Taube et al (2010) could predict clinical outcome, using the same claudin-low patient cohorts. We found that claudin-low patients expressing the Taube/Mani EMT signature did not show a poorer prognosis than signature-negative patients. In fact, there was a trend, albeit not statistically significant, toward better outcome (Fig 3C). Taken together, our analysis shows that despite their similarity, there is a small number of genes that is significantly and differentially expressed between WAP-Cre:PtenΔf:p53Δf and MMTV-Cre:Ptenf/f:p53f/f tumors and that this small gene set can predict clinical outcome for claudin-low BC patients.

**Figure 2.** Pten/p53-deficient mammary tumors cluster with claudin-low TNBC.

A Cluster analysis of Pten/p53-deficient tumors using an intrinsic gene signature (Supplementary Table S1A and B) in comparison with human (solid boxes; basal, CL: claudin-low, lumA: luminal A, lumB: luminal B, HER2 and N: normal-like) and mouse (open boxes; Snd: spindle, M: mammary glands, Nu: MMTV-Neu, MC: Myc-derived; V: MMTV-PyVT, In: MMTV-ini3, T: tag-derived, Wnt-Brca-p53; MMTV-Wnt1, Brca1-deficient, p53-deficient) BC samples.

B Cluster analysis of Pten/p53-deficient mammary tumors with human claudin-low (green) and basal-like (blue) BC using the Prat/Perou claudin-low signature. Mouse Pten/p53-deficient tumors clustered with human claudin-low—not with basal-like BC—on the far right. Non-claudin-low human and mouse tumors clustered together on the left.

C Expression of EMT genes in indicated mouse tumors and in human claudin-low versus basal-like BC.

D Expression of hypoxia signature genes in indicated mouse tumors and in human claudin-low versus basal-like BC.

We next asked whether the 24-gene set was predictive of clinical outcome using a cohort of 96 claudin-low BC patients with metastatic-free survival (MFS) data. Remarkably, this gene set, which we termed WAP-Cre Claudin-Low Signature (WCLS, Supplementary Table S1G), could stratify claudin-low patients into high and low risk groups with a hazard ratio of 2.24 (P = 0.0124; Fig 3C). Comparing to a recently reported signature for Basal-Like Breast Cancer (BLBC) (Hallett et al, 2012), WCLS was specific for the claudin-low tumors, whereas BLBC was specific for basal-like BC with HR = 1.96 (P = 0.0287; Fig 3C). Both signatures were not informative for HER2+, luminal A or luminal B BC (Supplementary Fig S4B).

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**Unique and frequent tumor-initiating cells in Pten/p53-deficient claudin-low-like mammary tumors**

To determine the impact of combined Pten/p53 loss relative to p53 deletion alone, we analyzed cancer stem cell (CSC) populations in these tumors. CSCs represent a subset of tumor cells that is capable of sustaining tumorigenesis as well as giving rise to...
24 differentially regulated genes between WAP-Cre:Pten$^{f/f}$ and MMTV-Cre:Pten$^{f/f}$ tumors predict clinical outcome for claudin-low BC patients.

A List of 24 genes that are significantly (FDR q-value < 0.05) and differentially (> twofold) regulated between WAP-Cre:Pten$^{f/f}$ and MMTV-Cre:Pten$^{f/f}$ tumors, including 7 up-regulated and 17 down-regulated (Supplementary Table S1G).

B Selected pathways that are significantly associated with WCLS from GSEA analysis of WAP-Cre:Pten$^{f/f}$ (red) versus MMTV-Cre:Pten$^{f/f}$ (blue) tumors. Green lines connect overlapping pathways. Circle size corresponds to levels of enrichment and thickness of lines to degree of overlap. The full GSEA map and association with WCLS are shown in Supplementary Fig S4A.

C Kaplan–Meier metastasis-free survival (% MFS) curve for claudin-low and basal-like BC patients with WCLS, BLBC and the Taube/Mani EMT signature.

D Comparison of WCLS and BLBC relative to 1,000 random sets of signatures, generated from atmosphere background noise (random.org), with the same gene composition (7 up-regulated, 17 down-regulated for WCLS; 9 up-regulated, 5 down-regulated for BLBC) on claudin-low BC patients. The percentage of signatures with significant HR > 1.0 is listed at the bottom. WCLS ranked 3rd for claudin-low BC, while BLBC ranked #242. For similar analysis on basal-like BC, see Supplementary Fig S4C.

Figure 3.
the tumor bulk, which is derived from CSCs but has lost its tumorigenic potential through epigenetic alterations (Kreso & Dick, 2014). CSCs are functionally defined as tumor-initiating cells (TICs) through their ability to seed new tumors following transplantation into recipient mice and to grow as spheres under non-adherent conditions (Liu et al., 2007; Deng et al., 2014). In the mouse, many mammary TICs are defined on the basis of CD49f (α6 integrin) and CD24 (a luminal marker) expression (Liu et al., 2007). Interestingly, in contrast to PtenΔf, many mammary TICs are defined on the basis of CD49f & CD24 double-positive fractions (Fig 4B and C).

To test whether PtenΔf/p53Δf tumors exhibit increased cell proliferation and reduced apoptosis, as predicted from the pathway analysis, we stained tumor sections for cell proliferation using Ki67 and for cell death using TUNEL, which detects cleaved/nicked DNA, the hallmark of apoptosis. The ratio of cell proliferation to apoptosis was significantly higher in PtenΔf/p53Δf tumors (n = 10) relative to PtenΔf, p53Δf, Wnt1 and Neu tumors (n = 3–5 for each; Fig 5B). Supplementary Figure S5E and F shows examples of staining for these markers and statistical analysis. To assess cellular senescence, primary tumors were dissociated, lineage-depleted, seeded at similar densities onto collagen-coated cover slides and, 3 days later, stained for senescence-associated β-galactosidase activity (Debacq-Chainiaux et al., 2009). This revealed much reduced cellular senescence and increased cellularity in PtenΔf/p53Δf compared to PtenΔf or Her2/Neu tumor cells (Fig SC). Thus, relative to PtenΔf or p53Δf single mutant, PtenΔf/p53Δf double-mutant claudin-low-like tumors exhibit multiple hallmarks of aggressive cancer.

Low Pten-expression/p53-pathway activity identifies TNBC patients with poor clinical outcome

To evaluate the effect of combined loss of Pten and p53 in TNBC, we used bioinformatics to identify Pten/p53-deficient patients with clinical data. Pten is often deregulated in BC through promoter methylation and microRNA-mediated silencing (Salmena et al., 2008; Koboldt et al., 2012), and its mRNA expression is the primary determinant of Pten protein levels in BC (Saal et al., 2007). We therefore assessed Pten RNA level from publicly available microarray expression data sets. For p53, we used a p53 pathway activity signature developed by Gatza et al. (2010) (Supplementary Table S1N and O). We then took advantage of a BC cohort (GSE4922) with known p53 status to normalize pathway-activation values, using as a reference the median (0.15) of p53-mutant tumors (Fig 6A). With these conditions, we determined Pten expression and p53 pathway activity for 2,179 patients including 471 TNBC, combined from 13 cohorts, six of which also had clinical information. Intrinsic BC subtypes were classified using PAM50 (Parker et al., 2009) (Supplementary Table S1P), and claudin-low TNBCs were identified using the Prat/Perou claudin-low signature (Supplementary Fig S6A). We found that 24.4% of TNBCs were Pten-low, 65.6% were p53-activity-low, and 18.7% were both Pten-low and p53-pathway-activity-low (Fig 6B). This frequency of...
**Figure 4.**

A. Flow cytometry plots showing CD49f-PE and CD24-FITC expression in MMTV-Cre:Pten^ff and MMTV-Cre:p53^ff cells.

B. TFU Ratio (%) graph comparing CD49f^+ vs CD49f^- cells.

C. Tumorspheres images with 50 μm scale bar.

D. Table summarizing TIC counts for CD24^-:CD49f-, CD24^-:CD49f^+, and CD24^+:CD49f^+ cell populations.

E. Flow cytometry plots for primary and secondary cells from CD24^-:CD49f^- and CD24^-:CD49f^+ populations.

F. Primary and secondary tissue sections showing CD24^-:CD49f^- and CD24^-:CD49f^+ cells.
Pten-low/p53-low tumors in TNBC was significantly higher than in all other BC subtypes ($P \leq 5 \times 10^{-6}$). In addition, only in TNBCs, there was a statistically significant correlation (0.11) between low Pten-expression and low p53-activity ($P = 0.02$; Fig 6B). This positive yet low correlation is likely because in TNBC, p53 is often lost with other tumor suppressors, for example, INPP4B and RB, whereas Pten is...
Figure 6.
often lost together with Brca1. Nevertheless, our results reveal that a substantial % of TNBC tumors (18.7%) is driven by combined loss of Pten and p53.

Importantly, patients harboring TNBC with low Pten expression and low p53-pathway activity had significantly worse metastasis-free survival (MFS) compared to those with normal expression of both tumor suppressors, with hazard ratio (HR) of 1.75 (P = 0.034; Fig 6C). Tumors with only one of these tumor suppressors lost exhibited intermediate MFS curves that were not significantly different than those for Pten/p53-low or Pten/p53-normal tumors (not shown). Thus, both in mouse and in human, Pten/p53 deficiency leads to aggressive TNBC.

AKT pathway activation occurs in mouse PtenΔf:p53Δf tumors and a subset of human Pten/p53-deficient TNBC

We next assessed the impact of Pten/p53 deletion on PI3K/AKT signaling by calculating pathway activation for AKT, PI3K and p53 (Gatza et al, 2010). As expected, p53-pathway activity in mouse p53Δf tumors was completely negative (Fig 6D, p53 lane), thus validating the p53-pathway analysis. Interestingly, AKT pathway activity was only modestly elevated in the Pten-only or p53-deficient tumors relative to MMTV-Neu, but strongly induced in Pten/p53 double-mutant tumors (Fig 6D and E), indicating that loss of Pten alone does not fully dysregulate the PI3K/AKT pathway. Indeed, a strong negative correlation between AKT- and p53-pathway activities was found in Pten/p53-deficient (−0.68; P = 0.005), but not in Pten or p53 single-mutant tumors. A negative correlation between the Pten and p53 pathways was also seen in Pten/p53-deficient mammary tumors (−0.51; P = 0.049).

Importantly, a box plot analysis revealed significantly elevated AKT signaling in mouse Pten/p53 claudin-low tumors (0.69) compared to p53Δf, PtenΔf or Neu tumors (P < 0.04 by Kruskal–Wallis; Fig 6E, Supplementary Table S1N and O). Consistent with this, we observed elevated AKT phosphorylation at Ser473 in Pten/p53-deficient tumors (Supplementary Fig S6B). Analysis of 15 additional signaling pathways (Gatza et al, 2010) revealed that MYC, E2F1 and β-catenin pathway activities were also induced in Pten/p53-deficient tumors as compared to other subtypes and that similar induction was seen in human TNBC (Supplementary Fig S6C, Supplementary Table S1N and O).

In human BC, we found a modest negative correlation between AKT versus p53 pathways, and PI3K versus p53 pathways (−0.27, P = 0.001; −0.23; P = 0.005, respectively) in claudin-low but not in basal-like TNBC (Fig 6D). When examining AKT signaling, only Pten/p53-low claudin-low tumors showed a trend toward elevated AKT pathway activation (Fig 6E). This trend was not sufficiently significant (P = 0.268), possibly because in human TNBC, AKT signaling is induced through both Pten-dependent and Pten-independent mechanisms. Together, this analysis demonstrates the existence of a subgroup of Pten/p53-deficient TNBC (18.7%) and that even within this subgroup, there is great variability in the level of AKT pathway activity, likely due to different cooperating oncogenic networks.

To determine whether AKT-pathway-high/p53-pathway-low activity could predict clinical outcome, we used the top 30% high AKT pathway activity as “cut-off” level. Patients with AKT-pathway-high/p53-pathway-low TNBCs had poorer prognosis than those with AKT-pathway-low/p53-pathway-high (HR = 1.78; P = 0.044; Fig 6F). Thus, TNBC patients with high AKT signaling and/or low Pten expression plus p53 loss have poor clinical outcome and should be prioritized for aggressive or new therapy.

Pten/p53-deficient claudin-low TNBC with elevated AKT signaling is susceptible to eEF2K inhibitors

To identify drugs that can target Pten/p53-deficient TNBCs with high AKT pathway activity, we performed a kinome drug screen (238 compounds targeting 154 different kinases; 3 μM; alamar blue assay) on four PtenΔf:p53Δf tumor cultures, each established from a distinct MMTV-Cre:PtenΔf:p53Δf mammary tumor. We also screened two human TNBC lines, HCC1937 and BT549, which harbor mutations in both tumor suppressors (Neve et al, 2006; Helledest et al, 2007; Blick et al, 2008). Top inhibitors from both screens were eukaryotic elongation factor-2 kinase (eEF2K: TX-1918; NH125) and c-Jun N-terminal kinase (JNK; BI78D3) (Fig 7A, Supplementary Fig S7A). Multiple PI3K, AKT and PI3K/mTOR inhibitors such as PIK-75, A-443654 and NVP-BEZ235 were also identified (Fig 7A, Supplementary Fig S7A), hence validating our screen, but they were not as efficient as the eEF2K or JNK inhibitors. Dose-response curves for TX-1918 and NH125 using
Figure 7.
MTT assays revealed IC_{50} of approximately 0.2 μM for mouse Pten^{−/−}:p53^{−/−} tumors cells versus 1.1–1.8 μM for immortalized HC11 mammary epithelial cells (Fig 7B). BI78D3 had IC_{50} of approximately 0.57 and 1.44 μM for Pten^{−/−}:p53^{−/−} tumors and HC11 cells, respectively (Supplementary Fig S7B). Western blot analysis confirmed inhibition of eEF2 phosphorylation on Thr56 following treatment of mouse and human Pten/p53-mutant TNBC cells with the eEF2K inhibitor (Fig 7C).

Interestingly, protein translation including eEF2 was one of the modulated pathways in Pten/p53 versus p53 tumors (brown circle, Fig 5A, Supplementary Table S1M). eEF2K is required for growth and is elevated in many types of cancer (Silvera et al., 2010). Our non-biased screen of six different lines is the first to identify this kinase as one of the most potent targets for Pten/p53-deficient TNBC. Recently, eEF2K was shown to maintain survival of brain tumor cells under nutrient deprivation (Leprivier et al., 2013). In contrast, we identified eEF2K inhibitors under normal serum conditions. To further examine these results, we knocked down eEF2K using RNA interference (Dharmacon). This led to incomplete (≤50%) reduction in eEF2K protein expression, yet suppressed cell growth twofold under normal (nutrient abundant) conditions (P < 0.005) (Fig 7D).

Both eEF2K and JNK are downstream targets of the PI3K pathway (Vivanco et al., 2007; Py et al., 2009; Hubner et al., 2012; Leprivier et al., 2013), and both have been implicated in autophagy (Zhang et al., 2008; Wu et al., 2009; Cheng et al., 2010). In accordance, suppression of autophagy by chloroquine (CQ), an inhibitor of autophagosome–lysosome fusion (Klionsky et al., 2012), revealed high autophagy flux (LC3-II accumulation) and efficient killing of both mouse and human Pten/p53-deficient tumor cells as compared to immortalized mammary epithelial cells or human luminal BC cells (Fig 7E and F). However, pharmacological inhibition of eEF2K with or without CQ did not have a discernable effect on the LC3-II/LC3-I ratio under non-starving conditions (Fig 7E), suggesting that eEF2K does not sustain growth by modulating autophagy in Pten/p53-deficient tumor cells. Instead, flow cytometry analysis for Annexin V, a marker for apoptosis, revealed that low concentrations of TX-1918 (1 μM) induced apoptotic cell death in both mouse and human TNBC cells but not in the HER2+ BC line HCC1954 (Fig 7G).

Understanding the link between genetic alterations in cancer and response to therapy is crucial for stratifying patients for therapy. We therefore determined whether sensitivity of six human TNBC cell lines (BT549, HCC1937, MDAMB157, MDAMB436, MDAMB468) to eEF2K inhibitors was proportional to any of the 18 pathway signatures defined by Gatza et al. (2010) (Supplementary Table S1Q). Remarkably, only the AKT pathway signature significantly correlated with sensitivity of TNBC cells to TX-1918 and NH125 (r = –0.85, P = 0.034; r = –0.91, P = 0.01, respectively; Fig 7H). We then extended the analysis to seven independent experiments, each in duplicates, and plotted IC_{50} against pathway activity in the different cell lines. Linear regression using meta-analysis revealed that the sensitivity of TNBC cells to TX-1918 had a correlation coefficient of –0.70 (P < 0.0001; Fig 7I; Supplementary Fig S7C). In contrast, response to doxorubicin was reduced with increased AKT signaling (correlation coefficient = 0.42, P < 0.02). Thus, AKT pathway signaling may be used as a predictor for patient response to eEF2K inhibitors.

**eEF2K and JNK inhibitors suppress xenograft growth of Pten/p53-deficient claudin-low TNBC**

TX-1918 contains a reactive side chain that is predicted to interact with glutathione in the blood and diminish half-life. Thus, to determine the effect of eEF2K inhibition on xenograft growth, we used NH125, which is active in vivo (Arora et al., 2003). Following orthotopic injection of mouse or human Pten/p53-mutant TNBC cells, mice were treated with tolerable doses of NH125 (intraperitoneal, 1 mg/kg/day for 1 week followed by 1 mg/kg every second day). Both mouse tumor volume and human tumor volume were significantly inhibited (P < 0.0001; Fig 8A). For the treated human BT549 xenograft, relapse occurred before the end point. Switching back to daily treatment halted further growth (Fig 8A; center, 3rd arrow).

To test for the effect of JNK inhibitors on xenograft growth, we used SP600125, which unlike BI78D3 identified in our screen is stable in vivo (Ennis et al., 2005). Administration of this inhibitor also attenuated xenograft growth of mouse Pten/p53-mutant tumor cells in vivo (P < 0.0001; Fig 8A, right).
Current treatment of TNBC patients involves cytotoxic drugs such as doxorubicin, which have serious adverse side effects. Targeted drugs that can cooperate with doxorubicin to kill TNBC may reduce toxicity and improve outcome. We therefore tested for cooperation between TX-1918 (eEF2K), BI78D3 (JNK) or NVP-BEZ235 (PI3K/mTOR) and doxorubicin. Using Compusyn software...
to assess level of synergy for drug combinations, we found that TX-1918 and BI78D3 had additive effects with doxorubicin (Fig 8B–D). Notably, although similar trends were observed, responses to TX-1918 or BI78D3 alone or together with doxorubicin were stronger than to NVP-BEZ235. Together, these results suggest that while patients carrying TNBC with high AKT pathway activity have poor prognosis, they would benefit from anti-eEF2K (as well as anti-JNK) therapy in combination with doxorubicin, thus encouraging rapid development of effective eEF2K inhibitors (Fig 8E).

Discussion

TNBCs represent heterogeneous types of tumors that are highly aggressive and difficult to treat; metastatic disease is common and lethal. We found that the tumor suppressors Pten and p53 are lost together in over 18% of TNBC. Moreover, we showed that a subset of patients carrying Pten/p53-deficient TNBC have the worst prognosis compared to other TNBCs with normal level of these tumor suppressors. Using a kinase screen on primary mouse PtenΔf:p53Δf tumors and Pten/p53 mutant TNBC lines, we identified eEF2K as well as JNK as potent therapeutic targets. Inhibitors of these targets were significantly more effective than PI3K, AKT or PI3K/mTOR antagonists, some of which are currently tested in the clinic on TNBC patients. Our results therefore identify both eEF2K and JNK as promising therapeutic targets for Pten/p53-deficient TNBC.

We disrupted Pten and/or p53 with two different deleter lines: WAP-Cre (which preferentially targets CD24−, pregnancy-identified luminal/alveolar progenitors) and MMTV-CreNLST (which targets both the CD49flo/Cd24− and CD24− compartments) (Wagner et al., 2002; Jiang et al., 2010). Although tumor latency was shorter when Pten and p53 were deleted via WAP-Cre relative to MMTV-Cre, histology and cluster analysis revealed that tumors driven by these two promoter-Cre lines were indistinguishable. This is somewhat unexpected because BC subtypes have been linked to the cell-of-origin within the mammary epithelial cell hierarchy; claudin-low tumors are thought to arise from transformation of mammary stem cells, basal-like tumors from bi-potent/early luminal progenitors, HER2+ BC from more committed luminal progenitors, etc. (Lim et al., 2009; Prat & Perou, 2009). As WAP-Cre is expressed in pregnancy-identified CD24− alveolar progenitors, our observation that WAP-Cre:PtenΔf mice develop diverse types of mammary tumors whereas nearly all WAP-Cre: PtenΔf:p53Δf (and MMTV-Cre:PtenΔf:p53Δf) mice give rise to claudin-low-like tumors supports a model whereby tumor subtype is dictated by both the cell-of-origin and the oncogenic/tumor suppressor networks that drive neoplastic transformation. Likewise, the fact that both WAP-Cre:PtenΔf and MMTV-Cre:PtenΔf mice gave rise to myoepithelioma, which is thought to originate from a myoepithelial cell of origin, suggests that combined deletion of Rb and p53 in luminal cells may induce dedifferentiation or transdifferentiation into myoepithelial cells. Two recent reports using other approaches or Cre drivers reached similar conclusions (Kim et al., 2014; Melchior et al., 2014).

Strikingly, despite the similarity between WAP-Cre:PtenΔf:p53Δf and MMTV-Cre:PtenΔf:p53Δf tumors, we identified 24 genes that were significantly and differentially expressed between the two models. This 24-gene set (WCLS) could stratify claudin-low patients into two groups with different clinical outcomes. WCLS marked low EMT/senescent, suggesting that tumors that originate from alveolar progenitors, as in WAP-Cre-driven tumors, are not locked in the EMT state as those driven by MMTV-Cre and are therefore more aggressive. Interestingly, in the prostate gland, a signature derived from luminal cells is also more predictive of poor patient outcome than a signature derived from tumors that originate from basal cells (Wang et al., 2013). Taken together, our results identify a novel predictor for claudin-low BC and support the idea that overcommitment to EMT diminishes metastasis.

Our genetic analysis of PtenΔf:p53Δf tumors revealed several levels of cooperation between these two tumor suppressors. First, Pten/p53 tumors are induced faster than p53 or Pten single-mutant tumors and exhibit an increase in pathways associated with proliferation and motility and reduction in pathways associated with cell death and immune response. In addition, while only some p53-deficient tumors are sarcomatoid, nearly all Pten/p53-deficient tumors share this histology and cluster with human claudin-low TNBC. Finally, Pten/p53-deficient tumors had a prominent CD24−:CD49f− fraction where most TICs reside, whereas both Pten- and p53-single knockout tumor cells are primarily CD24+:CD49f+ . The absence of CD24 expression, a luminal marker, on PtenΔf:p53Δf TICs underscores the highly mesenchymal nature of these tumors.

Second, we found that while AKT pathway activity was elevated in mouse Pten/p53-deficient mammary tumors, it was not consistently induced in human Pten/p53-low TNBC, likely because mutations in other components of the PI3K pathway, for example, Pik3ca, INPP4B, activate the pathway independently of Pten loss. Notably, Pten and PIK3CA are co-mutated in some human TNBCs (Yuan & Cantley, 2008; Lehmann et al., 2011), suggesting that mutations in more than one gene on the PI3K pathway may be required to fully activate the pathway, which is tightly autoregulated in normal cells (Cully et al., 2006). As a consequence, only a fraction of human Pten/p53-deficient TNBCs show high PI3K/AKT signaling and become sensitive to antagonists of this pathway. This model has direct implications for cancer therapy as it suggests that to guide therapy, patients should be screened for PI3K/AKT pathway activation or for multiple mutations along the pathway rather than for a single gene mutation (Janku et al., 2012; Rodon et al., 2013).

Our screen identified two eEF2K inhibitors as the most potent drugs against Pten/p53-deficient claudin-low TNBC cell lines. eEF2K is phosphorylated and inactivated by S6K1, downstream of mTORC1, leading to activation of eEF2 and mRNA translation elongation. eEF2K is also regulated by other signaling pathways including ERK and AMPK (Leprivier et al., 2013). Inhibition of eEF2K is thought to increase protein translation to unsustainable rate under nutrient deprivation, leading to cell demise. eEF2K has also been implicated in autophagy (Wu et al., 2009; Cheng et al., 2010). Interestingly, JNK is also linked to this process and, consistent with this, we found that Pten/p53-deficient tumor cells exhibit elevated autophagy flux and high sensitivity to the autophagosome-lysosome inhibitor CQ. However, inhibition of eEF2K (or JNK) did not affect autophagy under non-starving conditions. Instead, we found that eEF2K inhibition triggered apoptotic cell death even in nutrient-rich media through a mechanism that is not yet fully understood. Notably, TNBCs in general and as we show here Pten/p53-deficient...
tumors in particular are highly hypoxic. Hypoxia, similarly to nutrient deprivation, inhibits mTOR and protein translation in part by activating eEF2K and, therefore, eEF2K inhibitors may be exceptionally useful in treating hypoxic TNBC.

Available eEF2K inhibitors used herein have short half-life or off target effects (Arora et al., 2004). The essential role of eEF2K in Pten/p53-deficient TNBC (this study) and in brain cancer (Leprivier et al., 2013) should encourage development of specific and effective eEF2K inhibitors. These inhibitors may be used, as we showed here, as monotherapy, in combination with standard anthracycline therapy or with other drugs, such as a recently identified PLK4 inhibitor, which show strong anti-tumor activity against Pten-deficient BC (Mason et al., 2014). Moreover, we demonstrated that AKT pathway activity could predict response of TNBC to eEF2K inhibitors. Thus, development of a simple surrogate assay (e.g. immunostaining) for AKT pathway activation would simplify identification of TNBC patients who would benefit from anti-eEF2K-based therapy.

Materials and Methods

Animals

Mice used in this study were on mixed background (FvB, C57BL/6 and 129/sv): WAP-Cre mice were kindly received from Dr. Lothar Hennighausen, NIH, p53f/f mice were obtained from the NCI Mouse Repository, and Ptenf/f mice were generated as described (Suzuki et al., 1998). For transplantation assays, we used immunocompromised Rag1−/− females as recipients (JAX). Mice were housed in ventilated cages in our pathogen-free facility and monitored for mammary tumors as indicated in Figs 1, 4 and 8. For this study, we used approximately 580 female mice for tumor analysis and transplantation assays. All experimental protocols were approved by the Toronto General Research Institute—UHN Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care (AUP#10.50 and AUP#803).

Bioinformatics

Microarray analysis with mouse tumor models was carried out using Affymetrix Mouse Gene 1.0 ST with 500 ng of total RNA isolated by double TRIzol extractions (Centre for Applied Genomics, using Affymetrix Mouse Gene 1.0 ST with 500 ng of total RNA Microarray analysis with mouse tumor models was carried out in accordance with the guidelines of the Canadian Council on Animal Care (AUP#10.50 and AUP#803). Gene set enrichment analysis was performed using GSEA (Subramanian et al., 2005) with parameters set to 2,000 gene set permutations and gene sets size between 8 and 500. Gene sets were obtained from KEGG, MSigDB-c2, NCI, Biocarta, IOB, Netpath, Human Cyc, Reactome and the Gene Ontology (GO) databases.

For generating prognostic signature for claudin-low breast cancer (WCLS), ANOVA with FDR correction was performed between WAP-Cre: Pten−/−; p53f/f and MMTV-Cre: Pten−/−; p53f/f tumors to identify significantly (FDR q-value < 0.05) and differentially (> twofold) expressed genes. Kaplan–Meier and survival analysis were performed with PAST program (P.D. Ryan and Ø. Hammer, University of Oslo), and P-value was calculated by Wilcoxon method. Hazard ratios were obtained using the COX proportional hazards survival regression method. Heatmaps and dendrograms were generated by JAVA tree view.

Gene set enrichment analysis was performed using GSEA (Subramanian et al., 2005) with parameters set to 2,000 gene set permutations and gene sets size between 8 and 500. Gene sets were obtained from KEGG, MSigDB-c2, NCI, Biocarta, IOB, Netpath, Human Cyc, Reactome and the Gene Ontology (GO) databases.

The paper explained

Problem

Triple-negative breast cancer (TNBC) is a devastating subtype that affects approximately 10–20% of breast cancer patients. The tumor suppressors p53 and Pten are often inactivated in TNBC, but the consequences of combined mutations in these genes on tumorigenesis and response to therapy are largely unknown.

Results

We used Pten/p53-deficient mice and human TNBC cell lines to investigate how these two tumor suppressors cooperate to induce aggressive TNBC. We show that combined inactivation of Pten plus p53 via WAP-Cre or MMTV-Cre deleter lines induced claudin-low-like TNBC. We found 24 genes (WCLS) that are differentially and significantly expressed between MMTV-Cre:Ptenf/f; p53f/f and WAP-Cre:Ptenf/f; p53f/f double-mutant tumors and demonstrated that they can predict clinical outcome in claudin-low TNBC patients. Through non-biased kinome screens of mouse and human Pten/p53-deficient TNBC cells, we identified eEF2K as a potent inhibitor for TNBC with elevated AKT pathway activity.

Impact

WCLS-positive claudin-low TNBC patients should be prioritized for aggressive therapy. TNBC patients with elevated AKT pathway activity may benefit from anti-eEF2K therapy.

Kinase inhibitor screening, IC50 and MTT assay

238 compounds targeting 154 different kinases were screened using a Biomek FX liquid handler equipped with a pin tool for automated compound dispensing. Assays were carried out in a 384-well format with 300 cells/well.

Xenograft assays

Pten:p53-mutant mouse (200,000 cells/injection) or human BT549 (1 million cells/injection) tumor cells were resuspended in 20 µl media/matricegr mixture (1:1) and injected into 4 mammary glands of NOD/SCID females (9 mice per group). Tumor-bearing mice were randomized and then treated intraperitoneally with NH125 (1 mg/kg, dissolved in PBS with 2% DMSO) or SP600125 (60 mg/kg and 30 mg/kg, dissolved in DMSO). Control mice were injected with vehicle alone at the same weight/volume ratio.

Supplementary information for this article is available online: http://embomolmed.embopress.org

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Author contributions
JCL, VV, SW, DYW and DU conducted experiments and analyzed the data. AD, RA, SE, GDB, MT and TWJM supervised and interpreted the data. RAJ provided some tumor samples. JCL and EZ conceived and supervised the study, analyzed and interpreted the data, and wrote the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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Dependency of Pten/p53-deficient TNBC on eEF2K

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