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ORIGINAL ARTICLE

PDK1 promotes tumor growth and metastasis in a spontaneous breast cancer model

J Du[1], M Yang[1], S Chen[1,2], D Li[1], Z Chang[3] and Z Dong[1]

Because malignant cells have altered, usually accelerated, energy consumption, targeting metabolic signaling represents a prevailing strategy for tumor therapy. Phosphoinositide-dependent kinase 1 (PDK1) is a proximal signaling molecule of phosphatidylinositol 3-kinase, which is required for metabolic activation. It is still lacking definitive evidence whether inactivation of PDK1 can overwhelm tumorigenesis *in vivo*. Herein we revealed that mammary-specific ablation of PDK1 could delay tumor initiation, progression and metastasis in a spontaneous mouse tumor model. We also demonstrated that inducible deletion of PDK1 could noticeably shrink the growing breast tumors. However, a small portion of PDK1-deficient tumorigenic cells eventually established tumor lesions, albeit at a relatively later phase, most likely owing to compensatory upregulation of extracellular signal–regulated kinase 1/2 (Erk1/2) phosphorylation. Consequently, simultaneous inhibition of PDK1 and Erk1/2 impeded the survival of breast cancer cells. Thus we identify PDK1 as a potential therapeutic target for breast cancer, particularly in combination with an Erk1/2 inhibitor.

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INTRODUCTION

Tumor cells consume excessive energy to support rapid cell division by reprogramming their metabolic pathways.[1,2] Phosphatidylinositol 3-kinase (PI3K) signaling is one of the important signaling pathways that regulate cell growth and metabolism in response to environmental cues. This pathway triggers metabolic signaling mainly through a signaling cascade mediated by protein kinase B (PKB, also called AKT) and downstream mammalian target of rapamycin (mTOR) signaling.[3] mTOR forms two active complexes in the cell, the rapamycin-sensitive mTOR-Raptor (mTORC1) and the rapamycin-insensitive mTOR-Rictor (mTORC2).[4] The PI3K/AKT/mTOR pathway is often activated owing to the high frequency of somatic PI3K mutations in diverse cancers,[5,6] and thus these PI3K-mutated tumor cells are more sensitive to antimetabolism treatment, such as the inhibition of mTORC1 via rapamycin.[7,8] Therefore, numerous efforts have been made to develop drugs that specifically target the PI3K/AKT/mTOR pathway in cancer cell.[9,10]

The activation of the PI3K pathway generates phosphatidylinositol (3, 4, 5)-triphosphate, which recruits pleckstrin homology domain-containing proteins,[11] mainly phosphoinositide-dependent kinase 1 (PDK1) and AKT. PDK1 is a serine/threonine protein kinase, which phosphorylates AKT and several members of the conserved AGC kinase superfamily, including protein kinases A, G, and C.[12] PDK1 exists as an active auto-phosphorylated form, which enables downstream AKT phosphorylation at threonine 308 (T308). The phosphorylated T308 AKT then becomes fully activated after the additional phosphorylation of serine 473 (S473) by mTORC2, also referred as PDK2.[13] PDK1 is also able to directly phosphorylate p70S6K, which is a substrate of mTOR kinase.[14] In clinical data, PDK1 was found to be overexpressed in a large proportion of human breast cancers,[15] which harbored an increased copy number of the gene *PDPK1* that encodes PDK1, due to frequent genomic amplifications.[16] The overexpression of PDK1 could efficiently induce murine mammary cell transformation *in vitro*,[17,18] suggesting that targeting PDK1 may represent a new approach for limiting tumor growth. Several pharmacological inhibitors have been developed, and chemical suppression of PDK1 activity displayed antiproliferative effects on the growth of cancer cell.[19,20] Moreover, knockdown of PDK1 also attenuated the tumorigenesis in xenotransplanted tumor models.[21,22] Despite this, it is not clear whether PDK1 can initiate tumor development and targeting PDK1 can suppress tumor progression and metastasis particularly in a spontaneous tumor model. Thus PDK1 as potential target in anticancer therapy still needs preclinical assessment.

Phosphatase and tensin homolog (PTEN) is a negative regulator of the PI3K pathway, and its dysregulation or mutation is observed in many tumor patients.[23–26] Genetic knockdown of PDK1 alleviated the formation of several types of tumors in heterozygous $PTEN^{+/-}$ mice that only express 10% of the normal PDK1 enzyme,[27] and PDK1 deletion or administration of a PDK1 inhibitor delayed melanoma development and progression.[28] However, interference RNA-based PDK1 inhibition failed to prevent the development of B-cell lymphoma caused by PTEN deficiency.[29] Thus it seems that PDK1 has divergent roles in various tumors. Although PDK1 has been intensely investigated as a target in breast cancer *in vitro*,[30] there is still a need to determine whether targeting PDK1 is suitable for tumor therapy in other *in vivo* models. Genetic deletion of PDK1 will be a promising way to solve this issue.

In this study, transgenic mice expressing polyoma virus middle T antigen (PyMT) under the control of a long terminal repeat of the mouse mammary tumor virus (MMTV) were chosen;[31] the tumors from this model share many characteristics with human breast cancer.[32] We revealed that mammary-specific or inducible

[1]School of Medicine, Tsinghua University, Beijing, China; [2]Tsinghua-Peking Center for Life Sciences, Tsinghua University, Beijing, China and [3]Center of Animal Facility, Tsinghua University, Beijing, China. Correspondence: Dr Z Dong, School of Medicine, Tsinghua University, Biotech Building 4-314, #1, Tsinghuayuan, Haidian District, Beijing 100086, China. E-mail: dongzj@biomed.tsinghua.edu.cn
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deletion of PDK1 could powerfully delay the initiation, progression and metastasis of tumors and demonstrated that targeting PDK1 is a promising way to treat breast cancer, particularly in combination with an extracellular signal–regulated kinase 1/2 (Erk1/2) inhibitor.

RESULTS

Increased PDK1 expression correlates with excessive mTOR activation in spontaneous PyMT-induced breast cancer

Given that PDK1 expression is upregulated in some of human breast cancers, the goal of the study was to investigate whether PDK1 was a potential target suitable for treatment of breast cancer. Because PyMT-induced tumor model shares many characteristics with human breast cancer, we initially examined whether PDK1 expression was also increased in this model. Notably, PDK1 was significantly increased, and the enhanced PDK1 expression correlated with the tumor progression (Figure 1a), which was further consolidated by *in situ* immunohistofluorescence staining (Supplementary Figures S1A and B). Moreover, the mRNA expression of PDK1 was also significantly elevated along with tumor progression, indicating that the increased PDK1 expression was due to the altered transcription efficiency (Supplementary Figure S1D).

In line with this finding, the activation of PI3K signaling was observed. Phosphorylation of AKT on the threonine (T) 308 site was dramatically increased, although total AKT expression was not altered. In contrast, phosphorylation of S473 of AKT was not changed. Consistently, the activation of downstream signaling of AKT/mTORC1, p70S6K and its substrate S6 ribosomal protein, was also significantly increased (Figure 1a). These data indicate that phosphorylation of AKT T308 is probably involved in the tumorigenesis.

Although previous data showed that the aberrant activation of p38 and Erk1/2 signaling might occur in cancer,[33,34] neither of them displayed substantial phosphorylation in the detected model (Supplementary Figure S1C).

To study whether the transgenic expression of PyMT could enhance tumor metabolism, we performed glucose uptake assay. The ability of $PyMT^+$ tumor cells to uptake 2-[N-(7-nitrobenz-2-oxa-1, 3-diaxol-4-yl) amino]-2-deoxyglucose (2-NBDG), a florescent glucose mimic, was much greater than non-tumorigenic control (Figure 1b). As malignant cells usually change the way of energy consumption, we also found the increased level of glycolysis-related enzymes in $PyMT^+$ tumor cells (Figure 1c). Thus PyMT expression leads to the excessive metabolic activation.

Pharmacological or genetic inactivation of PDK1 activity suppresses mammary tumor cell growth *in vitro*

To examine whether blockage of the PDK1-mediated signaling could inhibit the proliferation of breast tumor cells, a series of pharmacological chemical inhibitors with varying specificity, IC-87114, Ly294002, GSK2334470, AKTi, Torin1 and U0126, were then chosen to suppress PI3Kδ, PI3Ks, PDK1, AKT, mTOR and Erk1/2, respectively. As expected, the inhibition of PI3K activity, PDK1 or distal mTOR activity could notably suppress the growth of tumor cells that were prepared from PyMT-induced mammary tumors (Figure 1d), but not normal control cells, albeit with varying efficiency among the inhibitors used (Supplementary Figure S2). Interestingly, GSK2334470 was the strongest chemical that completely inhibited tumor cell proliferation.

On the basis of this screening assay, PDK1 might have the most potential for targeted therapy in breast cancer. To avoid the nonspecificity of GSK2334470, we further evaluated whether genetic inactivation of PDK1 could prevent the proliferation of PyMT-expressing cells *in vitro*. We obtained a series of

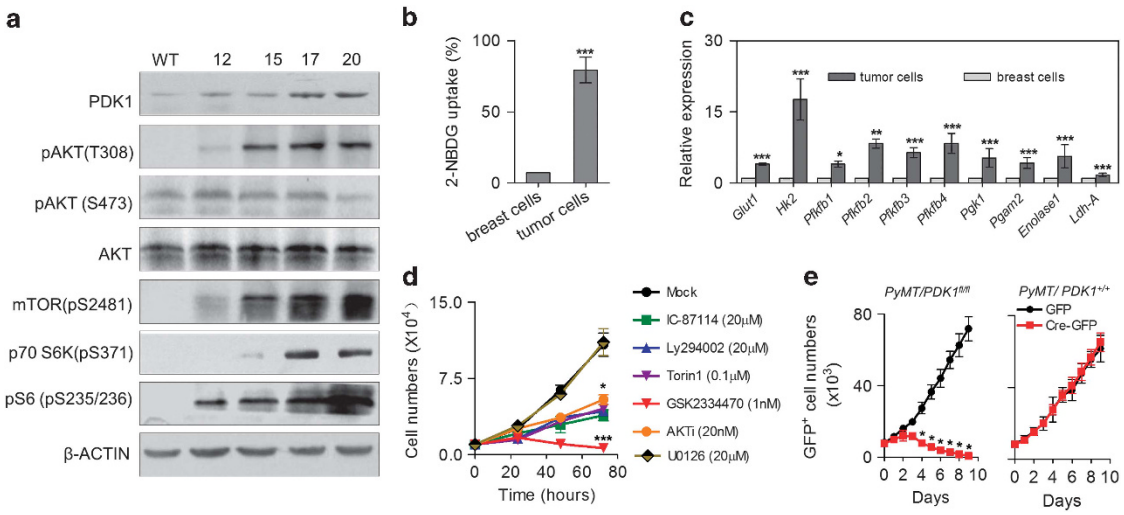

Figure 1. Enhanced PDK1 expression correlates with excessive metabolic activation in spontaneous breast cancer, and pharmacological or genetic inactivation of PDK1 activity suppresses mammary tumor cell growth *in vitro*. (**a**) The activation of PI3K-mTOR signaling in breast tumor tissues from PyMT mice at the indicated ages was analyzed by western blotting, with normal breast tissues from 12-week-old wild-type (WT) mice as control. Data are representative of five independent experiments. (**b**) 2-NBDG uptake by PyMT-induced tumor cells was assayed, with normal breast epithelial cells as control. Data are presented as the means ± s.d. $n = 5$. $^{***}P < 0.005$. (**c**) The mRNA levels of glycolysis-related enzymes were quantified in PyMT-induced tumor cells by real-time PCR, with normal breast epithelial cells as control. *Glut1*, Glucose transporter 1; *Hk2*, Hexokinase 2, *Pfkfb*, 6-phosphofructo-2-kinase/fructose 2, 6-bisphosphatase; *Pgk1*, phosphoglycerate kinase 1; *Pgam2*, phosphoglycerate mutase 2; and Ldh-A, lactate dehydrogenase A. Data are presented as the means ± s.d., $n = 5$. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$. Data are representative of three independent experiments. (**d**) The effect of inhibition of PI3K-mTOR signaling on the growth of cells isolated from primary PyMT tumors. Data are presented as the means ± s.d., $n = 5$. $^{*}P < 0.05$; $^{***}P < 0.005$ vs dimethyl sulfoxide. (**e**) The absolute numbers of GFP^+ cells were counted at the indicated time points after two tumor cell lines obtained from *PyMT/PDK1*$^{fl/fl}$ and *PyMT/PDK1*$^{+/+}$ mice, respectively, were infected with recombinant lentivirus expressing Cre that is fused with GFP (Cre-GFP), with GFP alone as control. Data are presented as the means ± s.d., $n = 5$. $^{*}P < 0.05$ vs GFP.

PyMT-expressing tumor cell lines with or without the floxed PDK1. Two cell lines, *PyMT/PDK1*$^{fl/fl}$ and *PyMT/PDK1*$^{+/+}$, were infected with the recombinant lentivirus encoding the Cre-GFP (Cre-green fluorescent protein), which presumably deletes the floxed PDK1 in *PyMT/PDK1*$^{fl/fl}$, but not in *PyMT/PDK1*$^{+/+}$, cells. As expected, we observed that exogenous expression of Cre-GFP, but not GFP control, dramatically downregulated PDK1 expression and counteracted the phosphorylation of AKT T308 in *PyMT/PDK1*$^{fl/fl}$ cells (Supplementary Figure S3A). As a result, the expression of Cre-GFP significantly decreased the number of *PyMT/PDK1*$^{fl/fl}$, but not *PyMT /PDK1*$^{+/+}$, cells, whereas the expression of GFP alone had no obvious effect either in *PyMT/PDK1*$^{fl/fl}$ or *PyMT /PDK1*$^{+/+}$ cells (Figure 1e). Consistently, the expression of Cre-GFP caused a significant increase in apoptotic *PyMT/PDK1*$^{fl/fl}$ cells (Supplementary Figures S3B and C). Taken together, these data indicate that overexpressed PDK1 might be a potential therapeutic target.

PDK1 is dispensable for the normal development and functions of the mammary gland

PDK1 has been widely reported to have an important role in organogenesis, such as in the heart,[35,36] but the roles that PDK1 have in the development and functions of the mammary gland are unknown to date. Mice with mammary-specific PDK1 deletion (*PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$) were generated by intercrossing *PDK1*$^{fl/fl}$ mice with transgenic mice expressing Cre under the control of long terminal repeat of MMTV. The efficiency of the PDK1 deletion was confirmed to be satisfactory and specific to the breast (Figure 2a). The expression level of PDK1 was low but traceable in the normal mammary epithelial cells by *in situ* immunofluorescence staining, whereas it was undetectable in that of *PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$ mice (Figure 2b). Mammary-specific PDK1 deficiency did not delay the development of normal ductal outgrowth, as evidenced by whole-mount observation and hematoxylin/eosin (H&E) staining (Figures 2c and d). Female *PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$ mice delivered offspring at a typical rate and preserved normal breeding capabilities (Figure 2e). Therefore, mammary-specific PDK1-deleted mice were easily achieved without affecting the normal development and functions of the mammary gland.

PDK1 promotes the initiation and progression of breast cancer

To determine the role of PDK1 in tumorigenesis *in vivo*, *PyMT/PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$mice were generated by intercrossing *PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$ mice with *PyMT* mice, with *PyMT/PDK1*$^{fl/fl}$ genotype as a control. A high efficiency of PDK1 deletion was confirmed in breast tumor tissues (Figures 3a and b). Tumor hyperplasia, which stained as dark clusters, was observed as budding of the ductal tree and of the full gland in whole-mounted inguinal glands of 8-week-old control mice, whereas these characteristics were rarely seen in the PDK1-knockout mice (Figures 3c and d). Compared with tumor hyperplasia, the tumors were visible in the mammary gland of control animals as early as 12–14 weeks after birth, whereas the ablation of PDK1 significantly delayed the tumor initiation to around 17–20 weeks (Figure 3e). Moreover, the tumor progression in *PyMT/PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$mice was much slower than that of control mice (Figure 3f). As a result, the PDK1-deficient mice usually took 7 weeks longer than control mice to be killed owing to the advanced stage of tumor progression (Figure 3g). Tumor burden, justified by tumor weight per mouse at 20 weeks, was also greatly decreased in

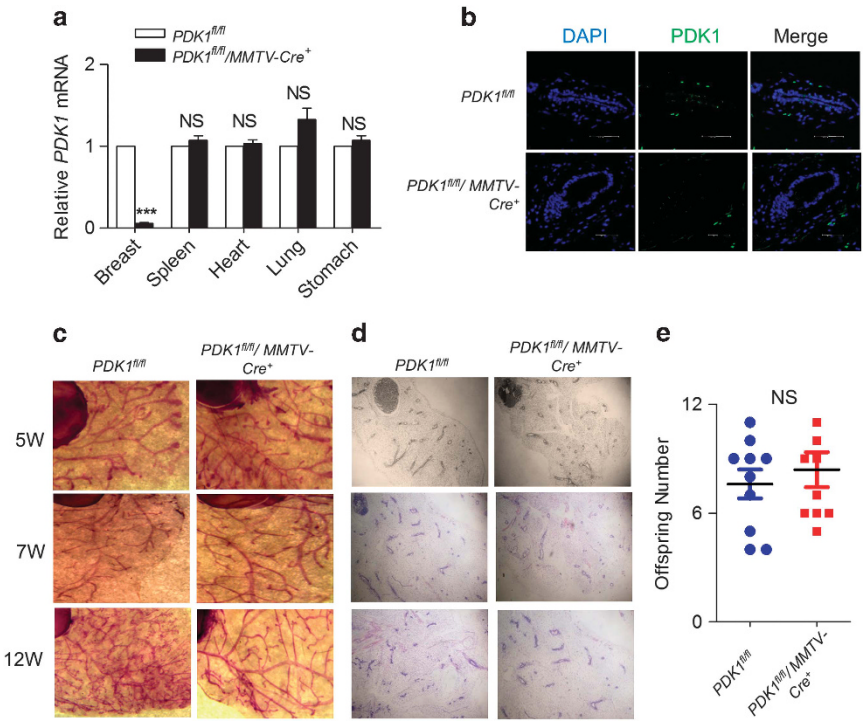

Figure 2. PDK1 is dispensable for the normal development and functions of the mammary gland. (**a**) PDK1 mRNA in the detected organs or tissues from *PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$ and control *PDK1*$^{fl/fl}$ mice was quantified by real-time PCR. Data are presented as the means ± s.d., $n = 5$. ***$P < 0.005$; NS, not significant. Data are representative of three independent experiments. (**b**) Immunohistofluorescence analysis of PDK1 (green) and DAPI (4,6-diamidino-2-phenylindole; blue) in breast tissues from *PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$ and control *PDK1*$^{fl/fl}$ mice. Scale bars, 200 μm. The data are representative of five mice each group. (**c**, **d**) Ductal outgrowth from *PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$ and *PDK1*$^{fl/fl}$ female virgin mice was determined by whole-mount mammary staining with neutral red (**c**) or H&E (**d**). The data are representative of six mice each group. (**e**) The offspring that were fed by female *PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$ was enumerated, with *PDK1*$^{fl/fl}$ mice as control. Each dot represents one mouse. $n = 10$ per group. NS, not significant.

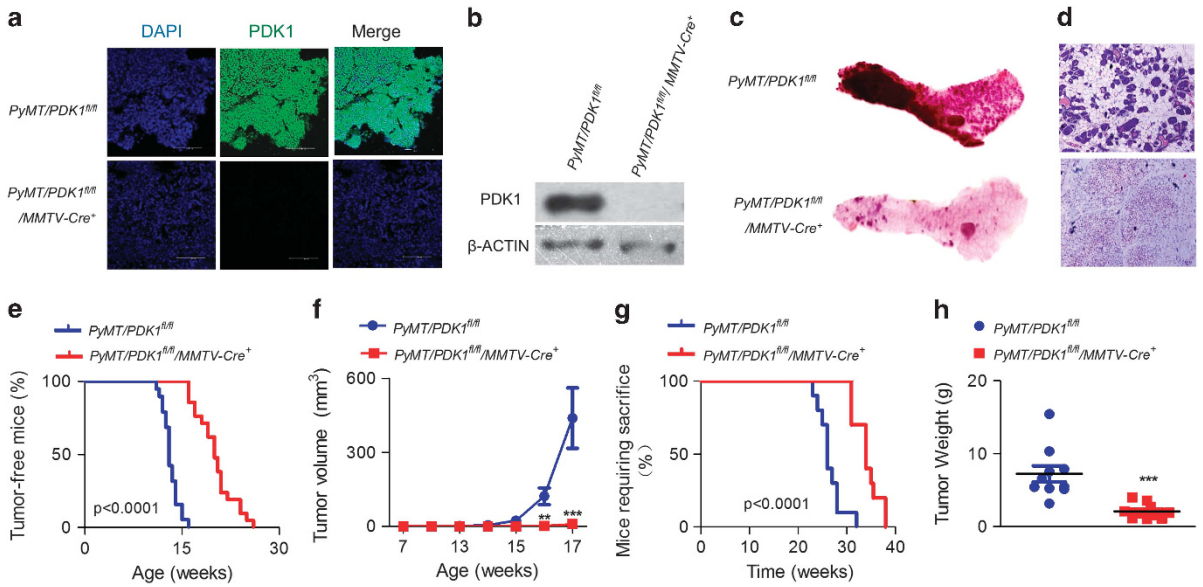

Figure 3. PDK1 deletion delays the initiation and progression of spontaneous breast tumors. (**a**, **b**) PDK1 protein in breast tumors from *PyMT/PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$ and *PyMT/PDK1*$^{fl/fl}$ mice was detected by immunohistofluorescence staining (**a**) or western blotting analysis (**b**). PDK1 (green), DAPI (4,6-diamidino-2-phenylindole; blue), scale bar, 200 μm. Data are representative of at least five mice each group. (**c**, **d**) Whole-mount mammary tissues from 8-week old *PyMT/PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$ and *PyMT/PDK1*$^{fl/fl}$ mice were stained with neutral red (**c**) or H&E (**d**). Representative images of five mice each group. (**e**–**h**) Kaplan–Meier analysis of the percentages of tumor-free mice (**e**), tumor volumes (**f**), the percentage of mice required for killing (**g**) and the tumor weight at 20 weeks of age (**h**) from two genotypes, *PyMT/PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$ and *PyMT/PDK1*$^{fl/fl}$, $n = 20$ (**e**, **f**), $n = 10$ (**g**, **h**). (**f**) Data are means ± s.d. $^{**}P < 0.01$; $^{***}P < 0.005$. (**e**, **g**) The data were analyzed using the log-rank test. $P < 0.0001$. (**h**) Each point shows the tumor weight of each mouse. $^{***}P < 0.005$.

PDK1-ablated mice (Figure 3h). This observed phenotype in *PyMT/PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$mice was not due to the side effect of Cre recombinase on tumor growth (Supplementary Figure S4).

Next we investigated whether the ablation of PDK1 could counteract the enhanced AKT-mTOR activity that was previously observed in the model. Knockout of PDK1 significantly reduced the phosphorylation of AKT at threonine 308 (pAKT T308) but did not alter the serine phosphorylation of AKT on the S473 site (pAKT S473). To determine whether PDK1 deletion could affect distal mTOR activation, p70S6 kinase and eIF4E-binding protein 1 (4E-BP1), two important mTORC1 substrates, were chosen. As expected, phosphorylation of p70S6 kinase on Ser371 site, which is executed by mTORC1,[37] was obviously diminished after PDK1 ablation. However, 4E-BP1 phosphorylation was reproducibly increased by the inactivation of PDK1. PDK1 can activate multiple downstream kinases. In particular, it can directly phosphorylate p70S6 kinase on Thr229 site.[14] In contrast to Ser371 site, PDK1 deletion slightly and reproducibly compromised Thr229 phosphorylation (Figure 4a and Supplementary Figure S5). Consistently, activation of S6 ribosomal protein, which was a substrate of p70S6 kinase, was also significantly inhibited in PDK1-deleted tumor cells (Figure 4a). Therefore, PDK1 regulates tumor growth probably through activating p70S6 kinase, indirectly by mTOR or directly by itself.

We noticed that PDK1 deletion could significantly decrease the consumption of glucose by tumor cells (Figure 4b). Similarly, glycolysis-related enzymes, which highly expressed in PyMT^{+} tumor cells, were also downregulated following PDK1 deletion (Figure 4c). Hence, knockout of PDK1 could inhibit tumor metabolic activation in PyMT-driven tumor cells.

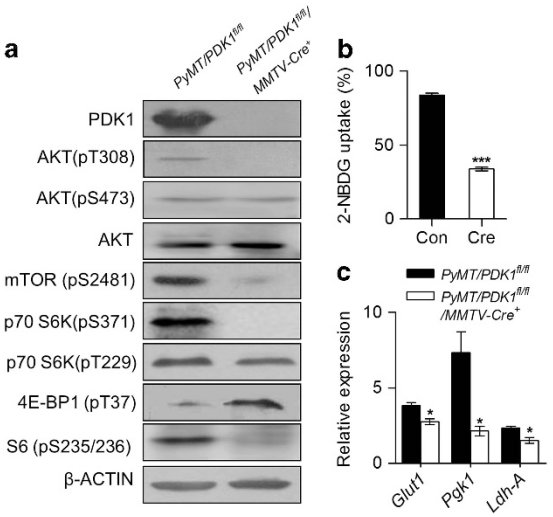

Figure 4. PDK1 ablation diminishes spontaneous activation of PI3K-mTOR signaling in breast cancer. (**a**) The activation of PI3K-mTOR signaling in breast tumor tissues from *PyMT/PDK1*$^{fl/fl}$*/ MMTV-Cre*$^{+}$ and *PyMT/PDK1*$^{fl/fl}$ mice was detected by western blotting. Representative blots are shown of five independent experiments. (**b**) The 2-NBDG uptake by breast tumor cells from the indicated mice was determined. Data are means ± s.d., $n = 5$. $^{***}P < 0.005$. (**c**) The mRNA levels of glycolysis-related enzymes were quantified in the indicated tumor cells by real-time PCR. Data are expressed as the means ± s.d. and are representative of three independent experiments, $n = 5$. $^{*}P < 0.05$.

PDK1 drives tumor metastasis spread to the lungs via regulating epithelial–mesenchymal transition (EMT)

We further studied the role of PDK1 in lung metastasis. The microscopic and macroscopic metastatic lesions were smaller and fewer in number in the lungs of PDK1-ablated mice. When mice reached 17 weeks after birth, the ratio of metastatic lesions in control mice was 30% of the whole lung, which was rarely seen in PDK1-knockout mice (Figure 5a). We attributed the dramatic

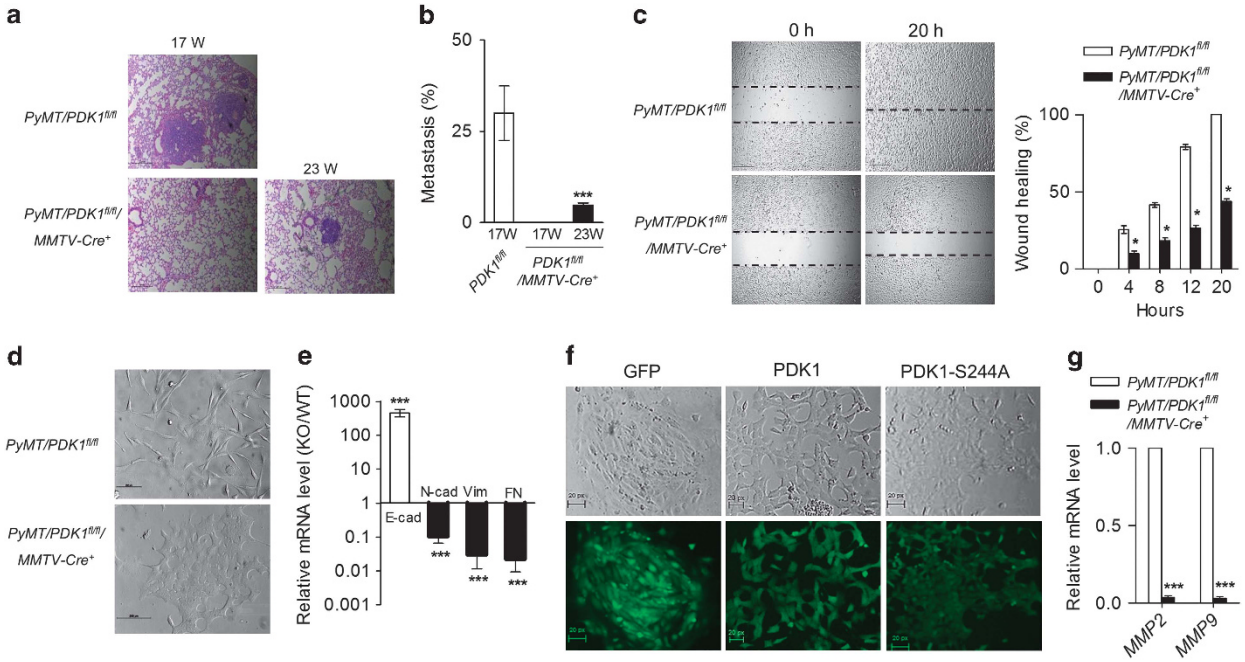

Figure 5. PDK1 promotes tumor cell migration and metastatic spread into the lungs through regulating EMT. (**a**, **b**) Representative H&E staining (upper lane) and quantification of tumor size (lower lane) of lung tissues from *PyMT/PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$ and *PyMT/PDK1*$^{fl/fl}$ mice at the same age of 17 weeks, or 23 weeks, as indicated, when the primary tumor burden is comparable. Scale bars, 100 μm. Representative images of eight mice each group. (**b**) The data are presented as the means ± s.d. $n = 8$ per group. ***$P < 0.005$ vs 17-week aged *PyMT/PDK1*$^{fl/fl}$ mice. (**c**) Wound-healing assay. The representative images at 0 and 20 h time point are shown (left panel), and the ratio of wound healing was quantified at the indicated time points (right panel). The data are presented as the means ± s.d. $n = 5$ per group. *$P < 0.05$. (**d**) The morphology of primary PyMT-induced tumor cells with (*PyMT/PDK1*$^{fl/fl}$) or without PDK1 (*PyMT/PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$). Scale bar, 200 μm. Representative images of five mice each group. (**e**) mRNA expression of EMT-related proteins was detected by real-time PCR. The data are presented as the means ± s.d. $n = 5$ per group. ***$P < 0.005$ vs control tumor tissues from *PyMT/PDK1*$^{fl/fl}$. Data are representative of three independent experiments. (**f**) Representative morphology of *PyMT/PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$ cells after infected with recombinant retrovirus expressing wild-type PDK1 or PDK1 S244A mutant, with GFP alone as negative control. (**g**) The mRNA encoding MMP2 and MMP9 were quantified from the indicated cells by real-time PCR. The data are expressed as the means ± s.d. and representative of three independent experiments. $n = 5$ per group. ***$P < 0.005$.

difference to the variation of initial tumor growth between the two genotypes. To avoid the discrepancy, we chose mice that possessed comparable tumor burden between the two genotypes, such as 23-week-old PDK1-knockout mice and 17-week-old control mice. The ratio of metastatic lesions in the lung was approximately 8% in PDK1-knockout animals and 30% in control mice (Figures 5a and b). Hence, PDK1 is indispensable for controlling primary tumor cell metastasis to the lungs.

To further investigate how PDK1 regulates tumor metastasis, we prepared primary PyMT cell lines from the solid tumor lesions with or without PDK1 for a migration assay. PDK1 expression was further identified before multiple clones were pooled together to avoid clone variation (Supplementary Figure S6A). PDK1-deficient cells displayed much lower motility and took longer to recover a scratched wound (Figure 5c). Hence, PDK1 controls tumor invasion and metastasis most likely by regulating cell migration.

EMT, an early embryonic development program, has a pivotal role during malignant tumor progression and metastasis.[38] We noticed that the PyMT tumor cells from control mice retained an elongated fibroblast-like morphology with a scattered distribution in culture, whereas the PDK1-deleted tumor cells displayed an epithelial and cobblestone-like morphology with tight cell–cell adhesion (Figure 5d). Moreover, the PDK1-deficient tumor cells exhibited the notable downregulation of mesenchymal markers, such as fibronectin, vimentin and N-cadherin; meanwhile, E-cadherin, which is involved in the interface of cell–cell contact, was dramatically upregulated (Figure 5e). Next, we further determined whether the programming was reversible and found that the exogenous reconstitution of PDK1 into PDK1-null breast cancer cells largely re-established the fibroblast-like morphology. However, the expression of PDK1 with a S244A mutation in the catalytic domain resulting in a defect in auto-phosphorylation failed to restore the fibroblast-like morphology (Figure 5f). PDK1 was reported to promote invasion and activation of matrix metalloproteinase (MMP).[39] We also found that PDK1-deficient primary tumor cells had a decreased expression of the mRNA transcripts of *MMP2* and *MMP9* (Figure 5g). Taken together, PDK1 is necessary for tumor-induced EMT, and therefore, the inactivation of PDK1 can significantly prevent tumor metastasis into the lung.

Inducible deletion of PDK1 eliminates established spontaneous breast cancer

To evaluate whether the suppression of PDK1 activity had a therapeutic effect on the established spontaneous breast tumors, mice with inducible expression of Cre under the control of the estrogen receptor (ER) promoter were bred to *PDK1*$^{fl/fl}$ mice. The treatment of tamoxifen, which binds the ER regulatory element, led to a high efficiency of PDK1 knockdown (Supplementary Figure S6B). When the breast tumors from two genotypes, *PyMT/PDK1*$^{fl/fl}$ and *PyMT/PDK1*$^{fl/fl}$*/UBC*^{ER}*-Cre*$^{+}$, reached nearly comparable volumes, tamoxifen treatments dramatically ceased the further growth of the primary tumors in PDK1-inducible knockout mice, whereas it had no visible effect on the control mice (Figure 6a). The temporal removal of PDK1 also overwhelmed the established tumors by inducing the apoptosis of breast tumor cells (Figure 6b). Tamoxifen-induced inactivation of PDK1 also diminished the

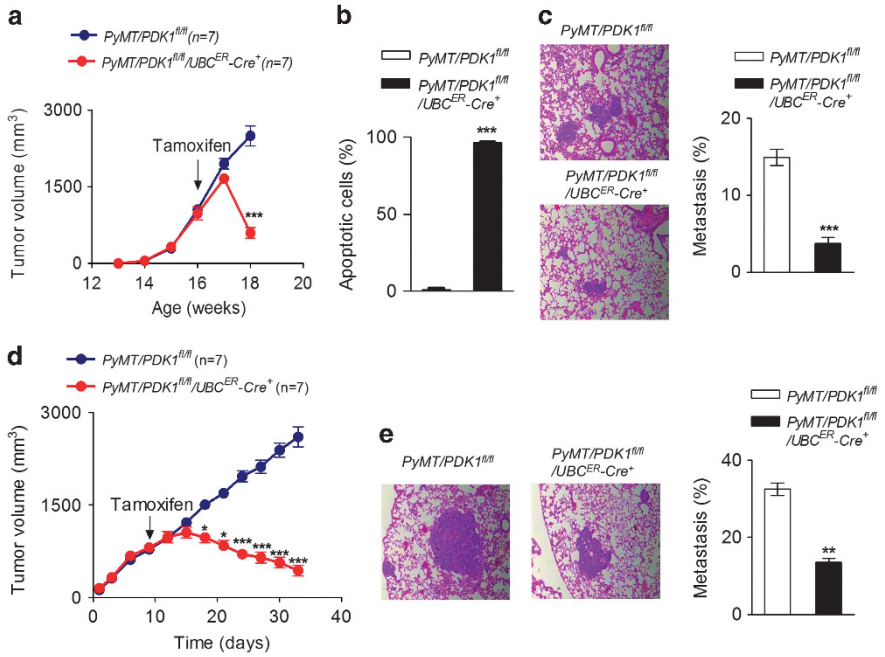

Figure 6. Temporal inactivation of PDK1 shows a considerable therapeutic effect in PyMT-induced tumors. (**a**–**c**) Tumor volumes over time were monitored after mice were injected with tamoxifen at the age of 16 weeks when the tumor burden was comparable. The data are presented as the means ± s.d. $n = 7$ per group. $^{***}P < 0.005$. (**b**) Two days after the last injection of tamoxifen, the tumor cells were isolated from the indicated mice for apoptosis analysis. The apoptotic cells were identified as PI^- Annexin-V^+. The data are presented as the means ± s.d. $n = 5$ per group. $^{***}P < 0.005$. (**c**) The lung tumor metastases were examined 2 weeks after *PyMT/PDK1$^{fl/fl}$/UBC^{ER}-Cre$^+$* and *PyMT/PDK1$^{fl/fl}$* mice were treated with tamoxifen. A representative H&E staining of the lung section (left panels) and the quantification of lung tumor nodules (right panel) are shown. Scale bars, 200 μm. The data are presented as the means ± s.d. $n = 10$ per group. $^{***}P < 0.005$. (**d**, **e**) The primary tumor cells prepared from *PyMT/PDK1$^{fl/fl}$/UBC^{ER}-Cre$^+$* and *PyMT/PDK1$^{fl/fl}$* mice were inoculated to wild-type mice. When the tumor volumes of two groups reached comparable size at day 10, mice were treated with five injections of tamoxifen, and then tumor volume was monitored over time (**d**). Lung tumor nodules were quantified (**e**, right panel) and a representative H&E staining of lung sections are shown (**e**, left panels). The data are presented as the means ± s.d. $n = 7$ per group. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

number of metastatic tumor nodules in the lung tissues under microscopic inspection (Figure 6c).

Because Cre is ubiquitously expressed in the above-mentioned inducible model, tamoxifen treatment could result in mouse lethality in 2 weeks. To strengthen the data, we prepared two $PyMT^+$ tumor cell lines from *PyMT/PDK1$^{fl/fl}$/UBC^{ER}-Cre$^+$* and *PyMT/PDK1$^{fl/fl}$* mice, respectively. When these two lines were subcutaneously inoculated, they apparently displayed comparable growth curves in congenic wild-type B6 mice. When comparable tumor volumes reached at day 10, tamoxifen was administrated for consecutive 5 days. As a result, the treatment dramatically reduced the sizes of transplanted tumor from *PyMT/PDK1$^{fl/fl}$/UBC^{ER}-Cre$^+$*, but not *PyMT/PDK1$^{fl/fl}$* mice (Figure 6d). The treatment also could alleviate the lung metastasis of $PyMT^+$ tumor (Figure 6e).Therefore, the tamoxifen-inducible deletion of PDK1 could noticeably eliminate tumors and alleviate lung metastasis even when tumors were established, indicating that PDK1 is a potential therapeutic target *in vivo*.

Erk1/2 signaling is compensatorily upregulated in the absence of PDK1

Despite the curative effect of PDK1 targeting in breast cancer described above, a population of PDK1-deficient tumorigenic cells existed and eventually established tumor lesions at the age of 20 weeks, albeit markedly later than the control mice (Figure 7a). PyMT induces tumor transformation mainly through two pathways, PI3K and mitogen-activated protein kinases.[40] Because we have confirmed that the later-formed tumors were PDK1-null cells (Figures 3a and b), we speculated that, in the absence of PDK1, the by-passed activation of alternative mitogen-activated protein kinase pathways may act as a compensatory mechanism that rescues the survival of PDK1-null cells. To our surprise, we observed an excessive phosphorylation of Erk1/2 in the tumor cells without PDK1 (Figure 7b), but p38 phosphorylation remained rather unchanged. *In situ* staining of phosphorylated Erk1/2 also confirmed the finding (Figure 7c). Herein, these data imply a new mechanism by which tumor cells adaptively upregulate Erk1/2-mediated survival signaling in the absence of PDK1. As a consequence, PDK1-deficient tumor cells were more sensitive to Erk1/2 inhibition at optimal doses, which failed to induce apoptosis in PDK1-sufficient tumor cells (Figure 7d).

Finally, we sought to evaluate whether a combination of agents targeting both PDK1 and Erk1/2 signaling was more effective for inducing apoptosis in breast cancer. GSK2334470 alone only caused apoptosis in 20% of tumor cells, whereas a low dose of Erk1/2 inhibitor did not interfere with tumor survival. However, the combined inhibition of PDK1 and Erk1/2 dramatically induced apoptosis in nearly half of the cells (Figure 7e), meaning that targeting both the PDK1 and Erk1/2 pathways exhibits an enhanced effect on tumor apoptosis. To strengthen the finding *in vivo*, mice bearing PDK1-deficient tumors were treated with U0126. We noticed that the treatment could greatly suppress the growth of PDK1-deficient tumors. Thus these data provide a preclinical evidence of the potential therapeutic efficacy of the simultaneous inhibition of PDK1 and Erk1/2 signaling (Figure 7f).

DISCUSSION

In the current study, we performed a preclinical assessment of the efficacy of PDK1 as a target in breast cancer treatment.

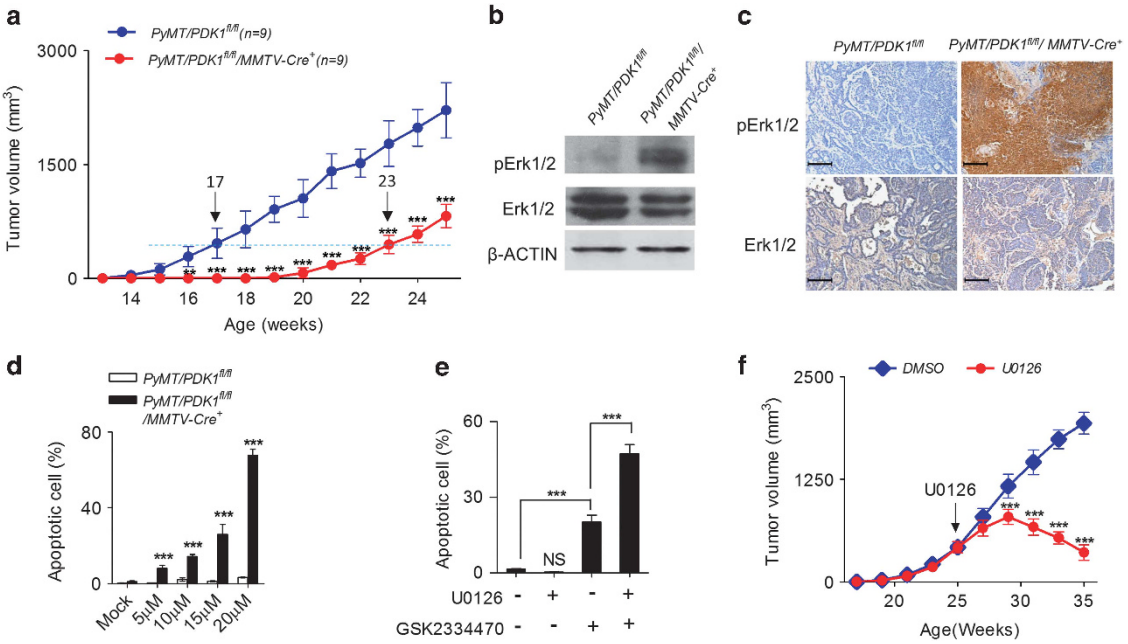

Figure 7. Erk1/2 signaling is compensatorily upregulated in the absence of PDK1. (**a**) Long-term observation of tumor volumes in *PyMT/PDK1*$^{fl/fl}$ and *PyMT/PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$ mice. Data are presented as the means ± s.d. $n = 9$ per group. $^{**}P < 0.01$; $^{***}P < 0.005$. (**b**) The activation of Erk1/2 was analyzed by western blotting. Data are representative of four independent experiments. (**c**) The increased phosphorylation of Erk1/2 was confirmed by *in situ* immunohistofluorescence staining. Data are representative of at least five independent mice. (**d**) The tumor cells with (*PyMT/PDK1*$^{fl/fl}$) or without PDK1 (*PyMT/PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$) were treated by a series of concentrations of U0126 for 24 h. Cell apoptosis was evaluated by co-staining with Annexin V and PI. The data are presented as the means ± s.d. $n = 5$ per group. $^{***}P < 0.005$ vs *PyMT/PDK1*$^{fl/fl}$ cells. (**e**) PDK1-sufficient tumor cells (*PyMT/PDK1*$^{fl/fl}$) were treated for 24 h as indicated. U0126, 20 nM; GSK12334470, 1 nM. Apoptotic cells were quantified as in panel (**d**). The data are presented as the means ± s.d. $n = 5$ per group. $^{***}P < 0.005$; NS, not significant. Data are representative of three independent experiments. (**f**) *PyMT/PDK1*$^{fl/fl}$*/MMTV-Cre*$^{+}$ mice at the age of 25 weeks were intraperitoneally injected with U0126 twice a week, with dimethyl sulfoxide as control. Tumor volume was monitored over time. The data are presented as the means ± s.d. $n = 6$ per group. $^{***}P < 0.005$.

Through two different methods of genetic targeting, we demonstrated that mammary-specific (*MMTV-Cre*) inactivation of PDK1 can efficiently delay the initiation, progression and metastasis of breast cancer, whereas tamoxifen-induced PDK1 deletion (*UBC*^{ER}*-Cre*) can stop the growth of preestablished ongoing tumors and further reduce the tumor burden. We also find that PDK1 deletion could suppress the excessive tumor metabolism, such as glycolysis. Therefore, we believe that PDK1 is a potential therapeutic target for breast cancer through limiting tumor energy consumption.

We first showed that metabolic activation and the phosphorylated Akt and mTOR was highly elevated in the mouse models, which was most likely due to the upregulated PDK1 expression. PDK1 can phosphorylate multiple substrates, including Akt. In human cancer, the increased phosphorylation of Akt correlated with the tumor progression and metastasis.[41] However, chemical inhibition of Akt failed to stop the PDK1-induced tumor progression.[21] Therefore, it is controversial whether PDK1 regulates tumorigenesis by activating Akt. In our current study, we found the minimal phosphorylation of Akt at T308 and mTOR in PDK1-deficient tumor. However, whether Akt was involved in the process needs further validation.

There are several phosphorylation sites that are essential for p70S6K activation or inhibition. Among them, respective phosphorylation of Ser371 and Thr229 by mTOR and PDK1 are required for the full activation of p70S6K. We observed that the both sites were less phosphorylated in PDK1-deleted tumor, so PDK1 blockage delayed the tumor progression probably through two pathways, including direct phosphorylation of p70S6K at Thr229 site and indirect activation of mTORC1 signaling. Because Serine 235/236 phosphorylation on S6 is also the product of the kinase activity of p70S6K, the impairment of serine 235/236 phosphorylation in PDK1-deleted tumor might also be due to the direct effect of PDK1 ablation.

Phosphorylation of p70S6K Thr229 is considered to be solely mediated by PDK1,[14] but the finding was not further proven by genetic evidence. In current study, we failed to observe a complete disappearance of Thr229 phosphorylation in PDK1-deficient tumors, which infers that other kinases most likely phosphorylate Thr229 of p70S6K, which is independent of PDK1.

We also noticed that the phosphorylation of 4E-BP1, the other mTORC1 substrate, was reproducibly increased through an unknown mechanism. Although this finding is contradictory to our conclusion that PDK1 deletion suppresses tumor progression through blocking mTOR pathway, the alternative explanation is that complete inactivation of p70S6K in PDK1-null cells will facilitate the binding of 4E-BP1 to mTORC1, because two mTORC1substrates, p70S6K1 and 4E-BP1, compete each other for the interaction with Raptor, a component of mTORC1.[42] This may present a rescue way of tumor survival in absence of PDK1. Besides, previous data reported that the increased Erk1/2 activation in colon cancer could promote the elongation activity of eIF4E,[43] which is presumably bound and suppressed by 4E-BP1. Phosphorylation of 4EBP1 releases the eukaryotic translation initiation factor eIF4E, which forms part of the eIF4F complex that initiates cap-dependent mRNA translation. Hence, it needs to be further explored whether the increased phosphorylation of 4E-BP1 is due to the altered Erk1/2 activation, which was clearly observed in PDK1-deleted tumor (Figure 7).

The loss of PTEN, which occurs in many patients with cancer, including breast cancer, frequently gives rise to highly activated PI3K and downstream mTOR activation. Drugs targeting PI3K and

mTOR are approved or in advanced clinical trials for tumor therapy.[44] However, there is a debate as to whether the inactivation of PDK1 could dampen tumor growth *in vivo*. Limited *in vivo* studies have been reported, but a consistent conclusion was not reached among the studies. Reducing the expression of PDK1 was reported to markedly protect mice from developing a wide range of tumors in *PTEN*$^{+/-}$ mice,[27,28] whereas another group used a universally applicable interference RNA approach, achieved $>90\%$ PDK1 reduction *in vivo* and found that knocking down PDK1 neither blocked PI3K pathway activation nor prevented tumor formation and progression in a PTEN-null background.[29] The discrepancy obtained by these groups was most likely due to the efficiency of gene targeting by the two approaches, as the residual PDK1 might be sufficient to activate the downstream PI3K pathway and promote tumor formation and progression. In the current study, PDK1 deletion has an undoubted effect on restricting tumor growth, mostly like because a high efficiency of PDK1 inactivation was achieved in our models. The other scenario may be that the PTEN-deficient tumor mouse model did not alter the expression of PDK1, so the tumor cells were not sensitive to PDK1 treatment. In our model, the PDK1 expression level was highly elevated, and downstream Akt and distal mTOR were also activated, which may explain why targeting PDK1 was highly successful in our PyMT model. We believe that targeting PDK1 for breast cancer treatment is most suitable for the patients with a high abundance of PDK1 expression.

We further demonstrated that PDK1 inactivation in the mammary epithelium also inhibited the metastasis of breast cancer. Mechanistically, PDK1 activity is required for tumor cell migration, which requires a program called EMT. The deficiency of PDK1 suppressed EMT induced by PyMT. The requirement of PDK1 for EMT is also tumor cell-intrinsic because exogenous expression of PDK1 could restore the mesenchymal morphology in PDK1-deficient tumor cells, whereas PDK1 mutated at mouse S244, which corresponds to human S241 and is defective in auto-phosphorylation, failed to convert the fibroblast-like morphology. Consistently, the phosphorylation of S241 was frequently seen in human clinical patients with breast cancer.[15,39] Therefore, using the reconstitution assay, we excluded the possibility that this morphological change seen in PDK1-deficient tumor cells was due to a variation of the tumor colony. Although PDK1 deletion could apparently alleviate the activation of mTOR, mainly mTORC1, in tumor cells, the role of mTORC1 in EMT is, to date, controversial. Previously, mTORC1 was reported to promote EMT, accelerating the migration and metastasis of colorectal cancer cells and the non-transformed cells NMuMG.[45,46] However, mTORC1 was also negatively implicated in the process of EMT.[47] Whether PDK1 regulates the EMT program by activating downstream mTOR needs to be further investigated.

In this study, we determined that tamoxifen-induced PDK1 ablation could noticeably shrink tumors and alleviate lung metastasis, suggesting that targeting PDK1 has a therapeutic effect on established tumors, which provides preclinical support for the therapeutic potential of PDK1 inhibitors to treat breast cancers. In addition, we found that PDK1 ablation failed to completely prevent tumor occurrence, as we found that a minority of PDK1-deficient tumor cells indeed exist in the tumor lesions. These tumorigenic cells were adaptively rescued by the upregulation of Erk1/2 phosphorylation. Importantly, simultaneous inhibition of Erk1/2 and PDK1 signaling significantly impeded the survival of breast cancer cells. Data from breast cancer cell lines also indicated that the combination of MEK inhibitors and PI3K inhibitors appeared to be more effective in the basal-like/triple-negative breast cancer subtypes than other breast cancer subtypes.[48] The basal-like breast cancer type occurs in 10–15% of breast cancer and is associated with poor prognosis. This finding is also consistent with a clinical study showing that Erk1/2 phosphorylation was associated with a poor quality of response to hormonal treatment in breast cancer patients.[49] PDK1 is a key signaling molecule for cell proliferation and survival, and the inactivation of PDK1 could have a severe risk of side effects, possibly lethal. In view of the enhancement of therapy effect, the combined usage of inhibitors specific for PDK1 and Erk1/2 at a low dose will help to minimize the side effects of the inhibition of PDK1.

MATERIALS AND METHODS

Mice

Mammary-specific (PDK1$^{fl/fl}$/MMTV-Cre^{+}) or inducible (PDK1$^{fl/fl}$/UBC^{ER}-Cre^{+}) PDK1-deficient mice were generated by crossing PDK1$^{fl/fl}$ mice (a gift from Dario Alessi from University of Dundee) with MMTV-Cre^{+} (Jackson Laboratory, Bar Harbor, ME, USA, stock number 003553) or UBC^{ER}-Cre^{+} mice (Jackson Laboratory, stock number 007001), respectively. PyMT transgenic mice were bought from Jackson Laboratory (stock number 002374) and further backcrossed on C57BL/6 background at least 12 generations. Their male F1 offspring was mated with PDK1$^{fl/fl}$/MMTV-Cre^{+} or PDK1$^{fl/fl}$/ UBC^{ER}-Cre^{+} mice to generate the PyMT/PDK1$^{fl/fl}$/MMTV-Cre^{+} or PyMT/PDK1$^{fl/fl}$/UBC^{ER}-Cre^{+} mice. Only female mice were used for whole study. Mice were bred and maintained in specific pathogen-free animal facility at Tsinghua University. All the procedures involving animals were approved by the Animal Ethics Committee of Tsinghua University.

In vivo tumor analyses

Mice were examined weekly for mammary tumor onset by palpation for nodules in all 10 mammary glands by the person unaware of their genotype. The date of first positive diagnosis was taken as the date the first tumor was found, provided that the diagnosis was confirmed by the following week. Tumor volume was assessed every 3 days by measuring the length and width of individual tumors with a caliper. The volume of each tumor was approximated as the volume of an oblong spheroid of the measured length (l) and width (w), that is V (tumor) $= \pi l w^2/6$. The individual tumor volumes were summed to give the total tumor volume in each mouse. The mice were killed when tumors reached a maximum size of 2 cm^3. The samples' sizes are based on prior observation and experience of tumor progression in PyMT mouse model. Mice were assigned to groups based on genotype, normally the number of groups was 6–10 ($n = 6$–10). For Cre induction, the mice were intraperitoneally injected with tamoxifen (Sigma-Aldrich, St Louis, MO, USA) for 5 consecutive days at a dose of 75 mg/kg body weight/day, with corn oil as vehicle control.

Lung metastasis measurements

For histological examination of the metastases, lungs were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned (1.5 μm), stained with H&E and scanned by light microscopy for metastatic foci. Photomicrographs of H&E-stained slides of lung metastases were analyzed with the Image J software (Bethesda, MD, USA) to assay the metastatic surface/lung.

Cell growth curve

For the cell growth assay, cells (1×10^4 or 1×10^3) were seeded in triplicate in 35-mm wells for 24 h. Cells were then treated with inhibitor or infected with virus for the indicated time before harvesting. The number was counted by a C6 flow cytometer (BD Biosciences, Becton Dickinson, Mountain View, CA, USA).

Reagent

Antibodies against PDK1, AKT, p-Akt (Thr308), p-Akt (Ser473), p-S6 (Ser235/236), p-p38, p38, p-ERK and ERK were purchased from Cell Signaling Technology (Danvers, MA, USA) while antibodies against p-4E-BP1 (Thr37), p-mTOR (S2481) and p-P70S6K (S371) from Epitomics (Cambridge, MA, USA), and p-P70S6K (Thr229) and Erk1/2 antibodies from GeneTex and Millipore (Temecula, CA, USA). β-Actin antibody and anti-rabbit or anti-mouse antibody-peroxidase conjugates were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Torin1 was purchased from Tocris Bioscience (Ellisville, MO, USA), GSK2334470 and U0126 from Sigma-Aldrich, and AKTi, Ly294002 and IC-87114 from EMD Millipore (Temecula, CA, USA).

Reverse transcription PCR

Total RNA was extracted using the TRIZOL Kit (Invitrogen, Carlsbad, MA, USA), and reverse-transcribed using reverse transcription system (Promega, Madison, WI, USA). Quantitative PCR was performed using SYBR Green-based detection. Relative mRNA levels relative to the expression of β-actin were determined using the $2^{(-\Delta\Delta Ct)}$ method. Three independent experiments were performed, with triplicate for each experiment. The primer sequences are provided in Supplementary Data.

Mammary gland whole mount

The right inguinal mammary gland was gently removed from the skin and spread on a glass slide. Mammary whole mounts were prepared by fixing tissues in Carnoy's solution (10% glacial acetic acid/30% chloroform/ 60% absolute ethanol), followed by rehydration and staining with 0.5% neutral red (Sigma-Aldrich).Tissues were then dehydrated, cleared with xylene and mounted.

Western blotting

Proteins were extracted from tissues, and 30–50 μg of protein were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, reacted with primary and secondary antibodies and developed by enhanced chemiluminescence according to standard methods.

Immunohistochemistry/immunofluorescence

Tissues were fixed, embedded, sliced and stained with H&E according to standard protocols. The VECTASTAIN ABC Kit (Vector Laboratories, Burlingame, CA, USA) was used for immunohistochemistry, and the slides were prepared with diaminobenzidine and analyzed with a Leica microscope (Leica Microsystems, Buffalo Grove, IL, USA). For immunofluorescence, tumors were dissected and fixed overnight at 4 °C, dehydrated and embedded in paraffin. Thick sections (5-μm thickness) were then incubated with anti-PDK1 antibody and Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G (Jackson Immunoresearch, West Grove, PA, USA).

Isolation of primary tumor cells from PyMT-induced mammary carcinomas

Tumors were harvested, minced and incubated in 2.4 mg/ml collagenase A in Dulbecco's modified Eagle's medium (DMEM; no fetal bovine serum) at 37 °C for 2 h. Floating cells were washed, pelleted, resuspended and propagated in complete DMEM. Only low-passage number cells (<4 passages total) were used for experiments. All primary cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin and 100 mg/ml streptomycin. Cultured cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

Retrovirus or lentivirus-mediated gene transfer

For *in vitro* deletion of PDK1, PDK1-floxed tumor cells with the PyMT transgene were infected with a pWXLD lentivirus expressing Cre or control GFP. Twenty-four hours later, GFP-positive cells were counted by a C6 flow cytometer (BD Biosciences, Becton Dickinson). For PDK1 reconstitution, PDK1-deficeint PyMT tumor cells were infected twice with MSCV retrovirus expressing wild-type PDK1 or its auto-phosphorylation-defective mutant, S244A.

Wound-healing assay

For wound healing, cells were seeded at a density of 1.5×10^5 in 6-cm plates (BD Biosciences, Becton Dickinson) until a confluent monolayer had developed. Images of the wound were photographed immediately following wounding and 4, 8, 12, 20 hours later. Wound closure was quantified using Image J by following the change in the wound area over time.

Detection of apoptosis

The cells were harvested by trypsinization, washed once with phosphate-buffered saline and resuspended in Annexin binding buffer at approximately 1×10^6 cells/ml. The cells were stained with propidium iodide (PI) and Annexin V according to the manufacturer's protocol (eBiosciences, San Diego, CA, USA) and assayed on a C6 flow cytometer (BD Biosciences, Becton Dickinson).

2-NBDG uptake

Cells were washed twice with modified balanced salt solution (MBSS)[50] and labeled with either 100 μM 2-NBDG dissolved in MBSS or MBSS alone as negative control and incubated for 20 min. The cells were washed three times with MBSS and acquired on a C6 flow cytometer. The uptake ratio was determined by the percentage of cells positive of 2-NBDG fluorescence.

Statistical analysis

The log-rank test was used to compare Kaplan–Meier survival curves and tumor-free mice curves between different groups of mice generated in GraphPad Prism version 5 (San Diego, CA, USA). The two-tailed Student's *t*-test was used to verify statistical significance in the difference between relevant values.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

Juan Du, Meixiang Yang, Shasha Chen and Dan Li performed and analyzed the experiments; Zai Chang provided critical reagents and advice; Juan Du, Meixiang Yang and Zhongjun Dong conceived the research, analyzed data and wrote the manuscript.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)