Pten-Null Tumors Cohabiting the Same Lung Display Differential AKT Activation and Sensitivity to Dietary Restriction

ABSTRACT

PTEN loss is considered a biomarker for activated phosphoinositide 3-kinase (PI3K)/AKT, a pathway frequently mutated in cancer, and was recently shown to confer resistance to dietary restriction. Here, we show that Pten loss is not sufficient to drive AKT activation and resistance to dietary restriction in tumors with low growth factor receptor levels. We describe a murine Pten-null Kras-driven lung cancer model that harbors both dietary restriction-resistant, higher-grade, bronchiolar tumors with high AKT activity, and dietary restriction-sensitive, lower-grade, alveolar tumors with low AKT activity. We find that this phenotype is cell autonomous and that normal bronchiolar cells express higher levels of insulin-like growth factor-I receptor (IGF-IR) and of ectonucleoside triphosphate diphosphohydrolase 5 (ENTPD5), an endoplasmic reticulum enzyme known to modulate growth factor receptor levels. Suppression of ENTPD5 is sufficient to decrease IGF-IR levels and sensitize bronchiolar tumor cells to serum in vitro and to dietary restriction in vivo. Furthermore, we find that a significant percentage of human non–small cell lung carcinomas (NSCLC) have low AKT activity despite PTEN loss.

SIGNIFICANCE: Our studies point to a heterogeneity of AKT activation in the same murine Pten-null lung tissue and in human NSCLC, further underscoring the challenges of personalizing cancer therapy based solely on cancer genotype. Our findings therefore indicate that the tumor response to anticancer therapies, including dietary restriction, needs to be based on PI3K/AKT activity per se, rather than on genetic alterations in the PTEN/PI3K pathway. Cancer Discov; 3(8): 908–21. © 2013 AACR.

INTRODUCTION

Dietary restriction has long been known to have antitumorigenic effects (1). We recently showed, however, that tumors with an activated phosphoinositol 3-kinase (PI3K) pathway are resistant to dietary restriction (2). PI3K is a lipid kinase whose activation through the binding of insulin/insulin-like growth factor-I (IGF-I) to receptor tyrosine kinases leads to the recruitment and activation of AKT (3). In turn, AKT, a serine/threonine kinase that is aberrantly activated in a multitude of cancers, enhances cellular growth and inhibits apoptosis. This signaling cascade is antagonized by the lipid phosphatase activity of the tumor suppressor PTEN (4, 5). Genetic loss of PTEN has been considered a predictive biomarker of AKT activation and response to therapies in different cancers (6, 7).

Using various human xenograft and genetically engineered mouse models of cancers of different tissues, we have previously shown that tumors with PI3K activation resulting from either activating mutations in the PI3K catalytic subunit or PTEN loss are resistant to dietary restriction (2). However, the question of whether PI3K- versus non–PI3K-driven tumors of the same tissue, in an otherwise genetically identical host, would display differential sensitivities to dietary restriction remains unanswered. Here, we investigate the response of lung tumors to dietary restriction, using two Kras-driven mouse models of lung cancer with either loss of the tumor suppressor Trp53 or, alternatively, loss of Pten. We show that adjacent tumors cohabiting the same lung can have differential AKT activities and sensitivities to dietary restriction, despite Pten loss. Such heterogeneity is contingent upon the expression of the endoplasmic reticulum (ER) enzyme ectonucleoside triphosphate diphosphohydrolase 5 (ENTPD5), which modulates growth factor receptor levels in cells with active AKT (8).

RESULTS

Adjacent Pten-Null, Kras-Driven Lung Tumors Display Differential AKT Activation and Sensitivity to Dietary Restriction

To investigate the responses of PI3K- and non–PI3K-driven tumors of the same tissue to dietary restriction, we used two conditional genetically engineered mouse models of lung cancer: LSL-KrasG12D; Trp53fl/fl (9, 10) and LSL-KrasG12D;Ptenfl/fl (refs. 9, 11; hereafter referred to as the Kp53 and KPTEN models, respectively). Upon administration of adenoviral Cre recombinase through nasal inhalation, both models develop lung cancer that is driven, respectively, by KRAS activation plus loss of the tumor suppressor Trp53 (Kp53 mice), or by KRAS activation and Pten loss (KPTEN mice). Although both Kp53 and KPTEN mice develop adenomas at full penetrance following Cre-mediated recombination, KPTEN (12) tumors...
grow at a higher rate than Kp53 (13) tumors, causing death at an earlier time point. Therefore, we waited either 1 week (KPTEN mice) or 6 weeks (Kp53 mice) postinfection in order to reach a comparable tumor burden before subjecting these mice to a dietary regimen.

Both mouse models were subjected to ad libitum feeding or 40% dietary restriction (2) for 3 weeks. Then, the tumor response to the different diets was analyzed. All mice experienced similar decreases in body weight (25%) and lower plasma insulin and IGF-I levels at the end of the restriction period, independent of genotype (Supplementary Fig. S1A–S1C). Unexpectedly, however, the tumor burden upon dietary restriction was decreased, to a similar extent, in both KPTEN and Kp53 mice (3.6- and 4.5-fold, respectively; Fig. 1A and Supplementary Fig. S1D). Because the KPTEN tumors did not display resistance to dietary restriction, we further investigated whether AKT was indeed activated in these tumors. Careful histologic and immunohistochemical examination of lung sections from these mice (Fig. 2A and B and Supplementary Fig. S2A) revealed the presence of two adjacent types of tumors in both the KPTEN and the Kp53 models: lower-grade adenomas (hereafter referred to as alveolar tumors) that express the alveolar marker surfactant protein C (SPC; ref. 14) and higher-grade, atypical papillary tumors, upon dietary restriction was decreased, to a similar extent, in both KPTEN and Kp53 mice (3.6- and 4.5-fold, respectively; Fig. 1A and Supplementary Fig. S1D). Because the KPTEN tumors did not display resistance to dietary restriction, we further investigated whether AKT was indeed activated in these tumors. Careful histologic and immunohistochemical examination of lung sections from these mice (Fig. 2A and B and Supplementary Fig. S2A) revealed the presence of two adjacent types of tumors in both the KPTEN and the Kp53 models: lower-grade adenomas (hereafter referred to as alveolar tumors) that express the alveolar marker surfactant protein C (SPC; ref. 14) and higher-grade, atypical papillary tumors,
Differential AKT Activation in Adjacent Pten-Null Lung Tumors

Both the alveolar and the bronchiolar tumors of the Kp53 mouse, similar to wild-type cells, had low to undetectable AKT activity levels (Fig. 2C, middle and right), which correlated with PTEN expression (Fig. 2D, middle and right). Interestingly, however, the KPTEN mouse displayed differential AKT activation in the different types of tumors: bronchiolar tumors had significant AKT activation, whereas adjacent alveolar tumors maintained low to undetectable AKT activity (Fig. 2C, left). To investigate the possibility that Pten was differentially lost in the bronchiolar but not the alveolar tumors of the KPTEN mouse, we conducted immunohistochemical analysis on sequential lung sections and found that Pten was indeed deleted in both types of tumors despite their differential AKT activation (Fig. 2D, left). These results were confirmed at the genetic level using laser capture microdissection (data not shown). Indeed, compared with the Kp53 lungs where PTEN presence was noted in all cells, the KPTEN lungs displayed PTEN expression only in normal tissue, endothelial cells, and CD68-positive macrophages that were dispersed within the tumors (Fig. 2D; Supplementary Figs. S2B, S2C, S3A, and S3B).

We then investigated the cellular localization, in both Kp53 and KPTEN tumors, of the transcription factor FOXO1, whose phosphorylation by active AKT results in its exclusion from the nucleus (15). Consistent with differential AKT activity in the different tumors, we found that FOXO1 was

Figure 2. Pten-null bronchiolar but not alveolar tumors display high AKT activation and express high levels of ENTPD5 and IGF-IR. A–F, H&E staining (A) and immunohistochemical analyses of SPC (B), phospho-S473 AKT (C), PTEN (D), ENTPD5 (E), and IGF-IRβ (F) in sequential sections of lungs from KPTEN mice under ad libitum feeding (AL) or dietary restriction (DR) conditions and from an ad libitum–fed wild-type mouse (WT). Arrows indicate alveolar (alv) or bronchiolar (bro) tumors. All pictures were captured under the same magnification; scale bars, 40 μm. Framed insets in A are a fourfold magnification of representative alveolar (AL–fed panels) or bronchiolar (DR panels) tumor areas of corresponding H&E larger images. The black line in A marks the outside layer of tumor-enclosing (KPTEN, Kp53) or normal (WT) bronchioles. H&E, hematoxylin and eosin.
cytoplasmic in the bronchiolar KPTEN tumors, independent of diet (Supplementary Fig. S4A, right), but could be detected in both the nucleus and cytoplasm of the KPTEN alveolar tumors and in all Kp53 tumors under ad libitum feeding and dietary restriction conditions (Supplementary Fig. S4A, left and S4B).

The observed differential AKT activation in the KPTEN mice led us to reinvestigate the tumor burden response to dietary restriction, taking into consideration the two types of tumors. We found that, in response to dietary restriction, both the alveolar and bronchiolar Kp53 tumors displayed a significant decrease in total burden (~3.5-fold; Fig. 1B). Such a decrease was represented by decreases in both the average size and number of individual alveolar tumors (56% and 57%, respectively) and bronchiolar tumors (70% and 40%, respectively) in the Kp53 lungs (Fig. 1C). In the KPTEN mice, however, we observed a differential tumor response to dietary restriction: whereas alveolar tumors displayed pronounced sensitivity to dietary restriction (7.4-fold decrease in total tumor burden, Fig. 1D) corresponding to 79% and 63% decreases in the average size and number of individual tumors, respectively (Fig. 1E), bronchiolar tumors were strongly dietary restriction–resistant, showing no decreases in tumor burden (Fig. 1D and E) in restricted mice. Consistently, the Kp53 alveolar and bronchiolar tumors displayed a 40% to 50% decrease in tumor cell proliferation upon dietary restriction, as quantified by bromodeoxyuridine (BrdUrd) labeling of mitotic cells. Whereas cellular proliferation in the KPTEN alveolar tumors was similarly decreased (40%) under dietary restriction conditions, that of KPTEN bronchiolar tumors was not altered (Fig. 1F). Furthermore, no apoptosis (caspase-3 cleavage) was detected in tumors of either mouse model under ad libitum feeding or dietary restriction conditions (data not shown).

The Alveolar Tumor Phenotype of Low AKT Activity Despite Pten Loss Is Cell Autonomous

The observed phenotype of differential AKT activation and sensitivity to dietary restriction, despite Pten loss, in adjacent tumors coexisting in the lungs of the same host was quite surprising. We reasoned that the underlying mechanism could be either the result of a tumor–environment interaction or, alternatively, a cell-autonomous characteristic of the alveolar but not bronchiolar cells, which prevented enhanced AKT activation upon Pten loss. To test these hypotheses, we decided to carry out a syngeneic transplant experiment and ask whether this phenotype would be maintained upon changing the environment of tumors harvested from the lungs of a donor mouse to the subcutaneous region of a recipient mouse of the same strain. To accomplish this experiment, deletion of Trp53 in the KPTEN alveolar tumors was necessary to overcome oncogene-induced senescence (16, 17) and to allow subcutaneous tumor growth in the recipient mice. To that end, we incorporated the Trp53<sup>fl/fl</sup> allele into KPTEN mice and generated KPTENp53 mice (LSL-Kras<sup>G12D</sup>Pten<sup>fl/fl</sup>, Trp53<sup>fl/fl</sup>), in which Trp53 would be concomitantly lost upon tumor induction. The phenotype of differential AKT activation and sensitivity to dietary restriction in adjacent alveolar and bronchiolar tumors was recapitulated in KPTENp53 mice (Supplementary Fig. S5A–S5E). Dissociated tumor cell mixtures (10<sup>6</sup> cells) from donor KPTENp53 mice were able to grow and form Pten-null tumors (~550 mm<sup>3</sup> in volume) in 5 weeks following their injection under the skin of a recipient mouse of the same strain (Fig. 3A and B). Pten-positive stroma was noted in Pten-null recipient tumors (Fig. 3B). We also noticed that the predominant cell population in the recipient mouse tumors was SPC-negative (bronchiolar). Importantly, however, the SPC-positive (alveolar)/low AKT activity versus the SPC-negative (bronchiolar)/high AKT activity phenotype of the KPTEN tumors was conserved upon transplantation and despite alteration of the tumor environment from donor to recipient mice (Fig. 3). As a control, subcutaneous transplantation of Kp53 alveolar/bronchiolar tumor mixtures in mice of the same strain showed maintenance of low AKT activity in the recipient mice (Supplementary Fig. S6A and S6B). These results implied the existence of an intrinsic characteristic of the alveolar tumor cell type, resulting in low AKT activity despite Pten loss.

Entpd5 Expression Modulates IGF-IR Levels and Sensitivity to Dietary Restriction in Murine Pten-Null Lung Tumors

The cell-autonomous nature of this phenotype led us to ask whether proteins involved in the Pten/Pi3K/AKT pathway are differentially expressed in the alveolar and bronchiolar tumors of the KPTEN lungs. We found that Entpd5 is expressed at significantly higher levels in the bronchioles as compared with the alveoli of normal mouse lung (Fig. 2E, right). ENTPD5 is an ER UDPase (18) whose activity promotes N-glycosylation and folding of proteins in the ER, including receptor tyrosine kinases (8). ENTPD5 knockdown in Pten-null cells results in ER stress, loss of growth factor receptors [e.g., IGF-IR, HER-2/ERBB-2, and EGF receptor (EGFR)], and induction of apoptosis (8). Furthermore, ENTPD5 is a target of the PI3K/AKT pathway; its expression can be suppressed by FOXO1–4 transcription factors (8), which are negatively regulated by AKT (15). We found that, compared with normal alveolar cells, alveolar tumors expressed moderate levels of ENTPD5, as detected by immunohistochemistry in KPTEN and Kp53 mice. However, a consistent, markedly higher level of ENTPD5 expression was observed in bronchiolar versus alveolar tumors in both mouse models (Fig. 2E, left and middle). Similarly, we found that the IGF-1 receptor (IGF-IR) was differentially expressed in the lung, with high expression and membranous localization in normal bronchioles, as well as KPTEN and Kp53 bronchiolar tumors, but low to undetectable levels in alveolar cells (Fig. 2F). Because AKT is not equally activated in alveolar and bronchiolar tumors of KPTEN lungs despite Pten loss, we hypothesized that loss of this major PI3K antagonist was not sufficient to drive AKT activation, but that additional positive upstream pathway activation was required. Differential growth factor receptor expression in the different types of cells, possibly resulting from differential Entpd5 expression (8), might explain such a phenotype.

To investigate this possibility, we first attempted to generate alveolar and bronchiolar tumor cell lines for in vitro manipulation. Because specific alveolar/bronchiolar antibodies did not allow for successful fluorescence-activated cell sorting (FACS) of tumor cell mixtures, we resorted to colony isolation in tissue culture dishes. Dissociated tumor cells from KPTENp53 lungs were seeded at very low dilution, and distinct colonies with different epithelial morphologies (Fig. 4A)
Figure 3. The alveolar tumor phenotype of low AKT activity despite Pten loss is maintained upon transplantation from donor to recipient mice. A and B, H&E staining and immunohistochemical analyses of SPC, PTEN, and phospho-S473 AKT in the lungs of a KPTENpS3 donor mouse (A) and in subcutaneous tumors of a recipient KPTENpS3 mouse, 5 weeks following injection of the donor tumor cells (B). Arrows indicate alveolar (alv) or bronchiolar (bro) tumors. All pictures were captured under the same magnification; scale bars, 40 μm. Framed insets in A are a threefold magnification of a representative alveolar (bottom left) or bronchiolar (top right) tumor area of the H&E larger image. The black line in A [left] marks the outside layer of a tumor-enclosing bronchiole. H&E, hematoxylin and eosin.
**Figure 4.** ENTPD5 modulates growth factor receptor levels and AKT activity in Pten-null tumor cells. **A,** representative pictures of KPTENp53 tumor cell colonies (10× magnification). **B,** SPC, PTEN, and GAPDH protein levels in KPTENp53 cells compared with NSCLC PC-9 cells. **C,** N-glycosylation levels in bronchiolar KPTENp53 cell lines with stable knockdown of control GFP (shGFP) or ENTPD5 (shE5-1), as assessed by PHA-E lectin blotting. GAPDH protein levels are represented as a loading control. **D,** levels of ENTPD5, IGF-IRβ, phospho-S473 AKT, total AKT, and GAPDH in shGFP and shE5-1 cells grown for 24 hours in media supplemented with 10% serum (S) or with different concentrations of IGF-I (25 or 50 ng/mL) or insulin (10, 50, or 100 ng/mL), in the absence of serum. **E,** ENTPD5, IGF-IRβ, phospho-S473 AKT, total AKT, phospho-T24/T32 FOXO1/3a (p-FOXO1/3a, low and high exposure levels), total FOXO1, BiP, CHOP, cleaved caspase-3, and GAPDH protein levels in shGFP and shE5-1 cells described in **C,** grown in the presence of 10%, 3%, or 1.5% serum for 72 hours. **F,** graphs representing quantified changes (obtained by ImageJ) in p-AKT/AKT, p-FOXO1/FOXO1 or ENTPD5, IGF-IRβ, BiP, CHOP, cleaved caspase-3, over GAPDH protein levels assayed for in **E.** C–F, representative of at least three independent experiments. In **D** and **E,** separated lanes of individual blots were acquired from a single electrophoresis gel. BiP, heat shock protein HSPA5; CHOP, c/EBP-homologous protein 10; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PHA, phytohemagglutinin.
were individually trypsinized using cloning cylinders and grown separately as cell lines. PTEN was confirmed to be completely lost in all isolated tumor cells, but was expressed in a control non–small cell lung carcinoma (NSCLC) cell line (PC-9; Fig. 4B). Expression of the alveolar marker SPC (14) was then used to identify SPC-high (alveolar) and SPC-low (bronchiolar) tumor cells (Fig. 4B). The epithelial nature of the alveolar and bronchiolar tumor cells was further confirmed by FACS analysis using an antibody against the pan-epithelial marker epithelial cell adhesion molecule (EpCAM). We noted significant epithelial enrichment in both alveolar and bronchiolar cell lines, where more than 88% of cells expressed high levels of EpCAM (EpCAMhi) and only 0.3% to 1.1% expressed low EpCAM levels (EpCAMlo). This is in contrast to the parental mixture, where only 11% of cells were EpCAMlo and the majority (~85%) of cells were EpCAMlo (Supplementary Fig. S7). Unfortunately, growth of alveolar tumor cells in culture resulted in higher ENTPD5 expression levels than those observed in bronchiolar cells, enhanced AKT activation, and high phosphorylation levels of the AKT targets FOXO1 and FOXO3a, which were maintained upon culture under low-serum conditions (Supplementary Fig. S8A and S8B). Consistently, the in vitro proliferation of alveolar cells, similar to that of bronchiolar cells, was not decreased when the cells were grown in low-serum (3% and 1.5%), compared with high-serum (6% and 10%), concentrations for 1 week (Supplementary Fig. S8C). Knockdown of Entpd5 in alveolar cells did not restore sensitivity to serum (data not shown), indicating that in vitro culture of alveolar cells resulted in the activation of at least two distinct compensatory pathways leading to increased ENTPD5 expression, an enhancement of AKT activity and resistance to serum. We therefore resorted to the use of KPTENp53 bronchiolar tumor cells to investigate the role of Entpd5 expression levels in modulating the sensitivity of Pten-null tumors to dietary restriction.

To that end, we generated stable KPTENp53 bronchiolar tumor cell lines with Entpd5 (shE5-1 and shE5-2) or control GFP (shGFP) knockdown. Suppression of Entpd5 resulted in a significant decrease in cellular N-glycosylation levels (Fig. 4C) and a decrease in IGF-IR levels that were accompanied by lower phosphorylation levels of AKT in cells cultured for 24 hours in media supplemented with insulin or IGF-1 (Fig. 4D). We then asked whether a decrease in ENTPD5 levels is sufficient to convert a dietary restriction–resistant Pten-null tumor to one that is dietary restriction–sensitive. We had previously shown that tumor cells with in vivo dietary restriction resistance exhibit in vitro constitutive AKT activation, despite serum starvation, and also proliferate in a growth factor–independent manner (2). Indeed, shGFP control bronchiolar tumor cells maintained constitutive activation of AKT when grown in vitro under low-serum (3% and 1.5%), compared with high-serum (10%) concentrations. Consistently, these cells displayed FOXO1 and FOXO3a phosphorylation levels that were maintained under low-serum culture conditions (Fig. 4E and F).

Intriguingly, however, compared with shGFP cells, the phosphorylation of AKT on S473 was higher in shE5-1 and shE5-2 cells grown in media supplemented with 10% serum (Fig. 4E and F and Supplementary Fig. S9A). The latter could be attributed to activation of compensatory pathways aimed at increasing AKT activity despite decreased ENTPD5 levels, although the precise mechanisms for such compensation remain unknown. Nevertheless, knockdown of Entpd5 resulted in decreased IGF-IRβ levels independent of serum, and a consistent and significant decrease in AKT phosphorylation under low-serum conditions (3% and 1.5%). The AKT activity was mirrored by the phosphorylation pattern of its targets, FOXO1/3a. Consistent with a recent report (8), we noticed induction of the ER stress markers heat shock protein HSPA5 (or BIP/GRP78) and c/EBP-homologous protein 10 (CHOP/GADD153), as well as increased caspase-3 cleavage upon Entpd5 knockdown (Fig. 4E and F and Supplementary Fig. S9A). Moreover, proliferation of shE5-1 and shE5-2, but not that of shGFP cells, was severely decreased when the cells were grown in low-serum (3% and 1.5%), compared with high-serum (6% and 10%) concentrations for 1 week (Figs. 5A and B and Supplementary Fig. S9B). Hence, knockdown of Entpd5 in PTEN-null bronchiolar cells resulted in decreased IGF-IR levels and enhanced sensitivity of the tumor cells to low-serum conditions in vitro. Similarly, knockdown of Igf1r in these cells resulted in decreased AKT activity, the induction of ER stress markers (albeit without inducing caspase-3 cleavage; Supplementary Fig. S10A and S10B), and decreased cellular proliferation in response to low-serum conditions (Supplementary Fig. S10C).

When transplanted subcutaneously in mice of the same strain (syngeneic transplants), shE5-1 tumors displayed larger volumes under ad libitum feeding conditions as compared with shGFP controls (Fig. 5C). However, upon dietary restriction, which was initiated 1 to 2 days following tumor injection and maintained for 2 weeks, shE5-1 bronchiolar tumors, but not shGFP control tumors, displayed a marked (twofold) decrease in tumor volume (Fig. 5C). This was accompanied by a significant (25%) decrease in tumor cell proliferation in shE5-1 tumors, but not shGFP tumors, as measured by BrdUrd labeling (Fig. 5D). Consistent with the tissue culture results, higher levels of apoptosis (caspase-3 cleavage) were noted in shE5-1 tumors compared with shGFP tumors, independent of feeding regimen (Fig. 5E). Histologic and immunohistochemical analyses of shGFP and shE5-1 transplanted tumor sections revealed marked infiltration of tumor cells with PTEN-positive stroma, characterized by lower levels of ENTPD5 expression and AKT phosphorylation (S473), compared with adjacent PTEN-null tumor cells. Nevertheless, it was evident that the high AKT activity levels observed in Pten-null tumor cells were significantly decreased upon Entpd5 knockdown under both ad libitum feeding and dietary restriction conditions (Fig. 5F). Suppression of Entpd5 expression in the bronchiolar tumor cells therefore resulted in the conversion of Pten-null tumors from dietary restriction–resistance to dietary restriction–sensitivity in vivo.

**Entpd5 Expression Modulates AKT Activity and Sensitivity to Dietary Restriction in NSCLC**

Our findings imply that loss of Pten in a tumor model with low growth factor receptor levels does not necessarily result in AKT activation. To investigate this further in human samples, we analyzed, by immunohistochemistry, the levels of PTEN, phospho-S473 AKT, ENTPD5, and EGFR in 130 cases of lung adenocarcinoma, the most prevalent...
Figure 5. ENTPD5 modulates Pten-null tumor sensitivity to serum in vitro and dietary restriction (DR) in vivo. A and B, proliferation curves of bronchial KPTENp53 cell lines with stable knockdown of control GFP (shGFP) or ENTPD5 (shE5-1), grown in the presence of 10%, 6%, 3%, or 1.5% serum for 7 days (n = 6). C, volumes of shGFP or shE5-1 bronchial KPTENp53 subcutaneous tumor transplants in ad libitum-fed (AL) or dietary restriction mice, as estimated by the ellipsoid formula (ref. 30; n = 10–12). D, proliferative indices of tumors measured by BrdUrd labeling quantification in shGFP and shE5-1 tumor transplants described in C. E, apoptotic indices represented as apoptotic area/total tumor area in shGFP and shE5-1 tumor transplants described in C and assessed by immunohistochemical analysis of cleaved caspase-3 (n = 4 mice/condition). F, H&E staining and immunohistochemical analyses of PTEN, ENTPD5, and phospho-S473 AKT in sequential sections of tumor transplants described in C. All pictures were captured under the same magnification; scale bars, 40 μm. In A–E, data are mean ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 (ANOVA/Bonferroni tests for differences between 10%, 3%, and 1.5% serum at day 5 in A and B or Newman-Keuls test in C–E). A and B are representative of at least three and C–F of two independent experiments, respectively. The black line in F (left) separates predominantly stromal “S” areas from tumor “T” areas. Arrowheads in F point to PTEN-positive stroma within the tumor area. H&E, hematoxylin and eosin.
Differential AKT Activation in Adjacent Pten-Null Lung Tumors

Cases of human NSCLC

<table>
<thead>
<tr>
<th>PTEN low</th>
<th>PTEN low/p-AKT low</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases of human NSCLC</td>
</tr>
<tr>
<td></td>
<td>PTEN low</td>
</tr>
<tr>
<td></td>
<td>PTEN low/p-AKT low</td>
</tr>
<tr>
<td></td>
<td>ENTPD5 High</td>
</tr>
<tr>
<td></td>
<td>p-AKT Low</td>
</tr>
<tr>
<td></td>
<td>ENTPD5 Low</td>
</tr>
<tr>
<td></td>
<td>p-AKT High</td>
</tr>
</tbody>
</table>

**Figure 6.** ENTPD5 expression correlates with EGFR and activated AKT levels in human NSCLC. A, percentages of PTEN-low lung adenocarcinoma cases with at least one core scoring either high or low for p-S473 AKT; Fisher test illustrating the correlation between ENTPD5 and p-AKT (P < 0.0011). B and C, immunohistochemistry of PTEN-low adenocarcinoma cases with high (B) or low (C) p-S473 AKT, ENTPD5, and EGFR levels. All pictures were captured under the same magnification; scale bars, 40 μm.

Histologic subtype of NSCLC are rare in NSCLC (20). However, decreased PTEN protein levels, partly attributed to promoter methylation (21), have been reported in 24% of cases. Consistently, we found that out of 130 cases of human lung adenocarcinoma, 21% displayed a low PTEN expression level. Interestingly, of these, 33% also harbored low p-S473 AKT activity levels. In all cases, phosphorylation of AKT on S473 positively correlated with ENTPD5 expression (P = 0.0011; Fisher Exact Test), as well as levels of EGFR (P = 0.0013; Spearman r = 0.247; Fig. 6A–C).

A limited number of established NSCLC cell lines express low levels of PTEN, including NCI-H23, which harbors a nonsense mutation in exon 7 (22). We therefore characterized the sensitivity to serum in vitro of Pten-low NCI-H23 cells, compared with that of A549 cells, which express high levels of PTEN (Fig. 7A–C). Unlike A549 cells, which displayed significantly decreased AKT activity upon serum starvation for 1 or 24 hours (compared with 10% serum conditions), NCI-H23 cells maintained similar AKT activity levels in the presence or absence of serum (Fig. 7A and B). Moreover, A549 cells displayed a differential proliferation pattern when grown for 1 week in increasing concentrations of insulin (1–1,000 ng/mL) or IGF-I (0.5–100 ng/mL), whereas NCI-H23 cells proliferated to the same extent independent of insulin or IGF-I levels (Fig. 7C). Knockdown of ENTPD5 (shE5-a and shE5-b) in NCI-H23 cells resulted in a significant decrease in AKT activity when the cells were grown in media deprived of serum for 1 or 24 hours, compared with 10% serum conditions (Fig. 7D and E and Supplementary Fig. S11A and S11B). A similar effect was observed in A549 cells with stable knockdown of ENTPD5 (Supplementary Fig. S11C and S11D), although significantly lower IGF-IR (but not EGFR) levels were observed only under low-serum (1.5%) conditions in both cell lines with ENTPD5 knockdown (Fig. 7D and E and Supplementary Fig. S11A–S11D). Nevertheless, the proliferation of NCI-H23 cells with ENTPD5 knockdown was significantly decreased when the cells were grown for 1 week under low-insulin or low-IGF-I conditions compared with control NCI-H23-shGFP cells (Fig. 7F).

**DISCUSSION**

Our results point to a heterogeneity of AKT activation in Pten-null tumors of the same tissue. Furthermore, they confirm our previous findings that PI3K/AKT–activated tumors are resistant to dietary restriction. It is noteworthy that the alveolar and bronchiolar tumors that we studied in the KPTEN and KPTENp53 mice represent earlier tumor stages and that a distinct phenotype of AKT signaling and response to dietary restriction might prevail in later, more advanced stages. However, the heterogeneity in Pten expression and AKT activity that we describe in the mouse can also be found in humans, within different cores of the same, relatively advanced, NSCLC adenocarcinoma cases (Fig. 6B and C). This is reminiscent of the recently reported intratumoral heterogeneity detected by exome sequencing of clear cell carcinoma (23), and further underscores the challenges facing personalized medicine, including diagnosis and therapy. Studying the effect of ENTPD5 expression on cellular AKT activity and sensitivity to dietary restriction in NSCLC was limited by the low availability of established NSCLC cell lines that are Pten-null or express very low levels of PTEN (22), despite the previous report (21) and the evidence herein confirming that PTEN protein loss occurs in a significant portion (21%–24%) of NSCLC cases. Although IGF-IR, but not EGFR, levels were decreased only upon Enptd5 knockdown in the NCI-H23 NSCLC cell line under low-serum conditions (Fig. 7D and E and Supplementary Fig. S11A and S11B), it is possible that the levels of other growth factor receptors were also affected in these cells. Nevertheless, we find that EGFR levels do correlate in a positive manner with AKT phosphorylation and ENTPD5 expression in NSCLC adenocarcinoma cases (Fig. 6B and C).
Figure 7. Suppression of ENTPD5 in human NSCLC cells results in decreased AKT activity and decreased proliferation under low-serum conditions. 

A and D, levels of ENTPD5, IGF-IRβ, phospho-S473 AKT (p-AKT, high and low exposures), total AKT, PTEN, and GAPDH in A549 and NCI-H23 human NSCLC cell lines (A), or in NCI-H23 cells with stable knockdown of GFP or ENTPD5 (shE5-a; D), grown in media supplemented with 10% serum or 0% serum for 1 or 24 hours. B and E, graphs representing quantified changes (obtained by ImageJ) in ENTPD5, IGF-IRβ or PTEN, over GAPDH protein levels or p-AKT/AKT, assayed for in A or D, respectively. C and F, proliferation curves of A549 and NCI-H23 (C), described in A or NCI-H23-shGFP, NCI-H23-shE5-a cell lines (F), described in D, grown in the presence of 0.1% serum supplemented with increasing concentrations of insulin or IGF-I for 7 days (n = 6). **, P < 0.01; ****, P < 0.0001 (ANOVA/Bonferroni tests for differences between 1 ng/mL insulin or 0.5 ng/mL IGF-I and all other conditions at day 7 in C and F). In A, separated lanes of the ENTPD5 blot were acquired from a single electrophoresis gel. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Differential AKT Activation in Adjacent Pten-Null Lung Tumors

Studying the KPTEN mouse has led us to describe a useful model and research tool, where adjacent tumors in the same tissue display differential signaling outputs and respond differently to metabolic status, despite sharing an identical genetic background. Either loss of Pten alone (KPTEN alveolar cells) or high expression levels of ENTPD5 and IGF-IR (Kp53 and wild-type bronchiolar tumors) are not sufficient to drive high AKT activation. It is rather the concomitant loss of the PI3K antagonist (Pten) and the presence of upstream positive activation of PI3K (through increased expression of ENTPD5 and IGF-IR) that resulted in AKT activation in the KPTEN bronchiolar tumor cells.

Unexpectedly, Entpd5 knockdown resulted in increased rather than decreased AKT activity in KPTENp53 bronchiolar cell lines grown in high-serum (10%) conditions, compared with shGFP control cells (Fig. 4E and F and Supplementary Fig. S9A). This was also observed in NCI-H23 NSCLC cells (Supplementary Fig. S11A and S11B). It is possible that the generation of cells lines with stable Entpd5 knockdown may have selected for cells with, or alternatively led to the compensatory induction of, AKT activation under high-serum conditions. Nevertheless, Entpd5 suppression resulted in the conversion of the serum-resistant and dietary restriction-resistant KPTENp53 bronchiolar cells and NCI-H23 NSCLC cells to sensitivity to lower serum, insulin, or IGF-I conditions in vitro (Figs. 5A and B) and in vivo (Fig. 5C). The induction of compensatory pathways favoring tumor cell growth upon loss of Entpd5 was consistent with the observation that transplanted KPTENp53 tumor cells with Entpd5 knockdown yielded tumors with larger volumes, compared with shGFP control tumors, when grown in ad libitum-fed mice (Fig. 5C). It is noteworthy that an attempt to increase IGF-IR protein levels (through lentiviral infection) in cells with Entpd5 knockdown was unsuccessful, despite significantly increasing IGF-IR mRNA levels. In comparison, both protein and mRNA levels were increased in shGFP control cells stably overexpressing IGF-IR (data not shown). It is possible that these results underscore a key role for ENTPD5 in promoting the N-glycosylation, proper folding and stability of IGF-IR proteins (8).

The intriguing observation that ENTPD5 and IGF-IR are expressed at markedly higher levels in normal bronchiolar versus alveolar cells (Fig. 2E, right) may be dependent upon the cell of origin of these two cell populations, and it remains to be determined what specific mechanisms underlie such differential ENTPD5 and IGF-IR expression in the same lung epithelial tissue. It is also possible that the alveolar and bronchiolar tumor cells described in the KPTEN and KPTENp53 mouse are derived from different cells of origin in the lung. Bronchiolar tumors have indeed been found in a different mouse model of lung cancer to be more resistant to therapy (24). It is also interesting that Pten loss in the mouse lung has been associated with not only an acceleration of Kras-driven tumorigenesis (12) but also an increase in the bronchoalveolar stem cell (BASC) pool (25, 26). Indeed, Yanagi and colleagues (25) reported a larger number of BASCs in an inducible mouse model of Pten loss in SPC-expressing cells, leading to alveolar and bronchiolar hyperplasia and susceptibility of developing spontaneous lung adenocarcinomas. Although AKT was shown to be activated in whole lysates of the tumor-bearing lungs, immunohistochemical staining was not conducted, which would have potentially allowed for the detection of differential AKT activation in Pten-null alveolar and bronchiolar tumor cells.

In sum, our studies point to a heterogeneity of AKT activation in the same Pten-null tissue. Predicting the tumor response to systemic metabolic status, such as dietary restriction, or to antitumor therapies targeting Pten/Pik3, needs therefore to be based on AKT activity per se, rather than the genetic alterations in the PTEN/PI3K pathway.

METHODS

Mouse Studies

All animal studies and procedures were approved by the Animal Care and Use Committee at Massachusetts Institute of Technology (Cambridge, MA) and Boston Children's Hospital (Boston, MA). Kp53 and KPTEN (129 sv) mice were crossed by generating LSL-KrasG12D (9) to either Trp53fl/fl (10) or Ptenfl/fl (11) mice, respectively. Kp53 and KPTEN progeny were intercrossed to generate KPTENp53 mice. Four- to 8-week-old mice were infected by a single 67.5 µL intranasal instillation of 3 x 10⁸ infectious particles of adenovirus-Cre (University of Iowa, Iowa City, IA), following isoflurane anesthesia (27). In syngeneic transplant experiments, 5- to 7-week-old donor KPTENp53 male mice were infected with adenovirus-Cre, and tumors were allowed to grow for 4 weeks. Tumors were dissociated from donor lungs following euthanasia and subcutaneously injected (10⁶ cells) in 4- to 5-month-old recipient KPTENp53 mice that were not infected with adenovirus-Cre. Recipient tumors were harvested 5 weeks following tumor cell injection. For bronchiolar tumor cell transplants, 10- to 14-week-old male KPTENp53 mice were injected with 10⁶ tumor cells per injection. All subcutaneous cell injections were delivered in 100 µL Hank's Balanced Salt Solution (HBSS; Invitrogen) with 15% phenol red-free, growth factor-reduced Matrigel (BD Biosciences). For BrdUrd labeling experiments, BrdUrd (Sigma) was injected intraperitoneally (30 mg/kg) 24 hours before euthanasia.

Dietary Restriction

Dietary restriction was conducted as previously described (2). Mice were individually caged for at least 4 days before subdividing them into an ad libitum-fed group (Prolab RMH 3000, 3P76) and a 40% dietary restriction group (LabDiet, 5B6V). Weekly body weights and food intake were recorded.

Necropsy and Plasma Analyses

Mice were euthanized at the beginning of the light cycle after retro-orbital blood withdrawal, and plasma was prepared as described previously (28). Plasma insulin and IGF-I were assayed using kits from CrystalChem and Diagnostic Systems Laboratories, respectively. Lungs and subcutaneous tumors were harvested and lung lobes were separated in ice-cold PBS. Tissues were immediately fixed in formalin for later processing.

Cell Dissociation and Culture

Tumors from donor KPTENp53 mice (5 weeks following infection with adenovirus-Cre) were dissociated in HBSS (Invitrogen; calcium/magnesium free) containing 0.025% trypsin–EDTA (Invitrogen) and 1 mg/mL collagenase IV (Worthington Biochemicals). Following a 2-hour incubation with rotation at 37°C, the samples were quenched with a solution containing Leibovitz’s L15 medium without phenol red (Invitrogen), 10 mmol/L HEPES pH 7.4 (Invitrogen), 1 mg/mL bovine serum albumin (Sigma), 75 U/mL DNase1 (Roche), and 100 U/mL penicillin/streptomycin (Invitrogen). The cells were
then pelleted, resuspended in 1 ml quench solution, and filtered through 40-μm cell strainers (BD Falcon). Cells were then either immediately injected subcutaneously into recipient mice or seeded (10,000–40,000 cells/well) in six-well plates for colony isolation. Using cloning cylinders (Corning), individual colonies were trypsinized from the parental cells and grown separately as either alveolar or bronchiolar cell lines depending on their expression of the alveolar marker SPC. Compared with parental cells, alveolar and bronchiolar cell lines were confirmed to be highly enriched in epithelial cells (EpCAMP) by flow cytometry analysis using an allopseudocyanin (APC)-conjugated CD326 (EpCAM) antibody (eBioscience, G8.8). For generation of mouse bronchiolar or human cells with stable EntPD5 and control GFP knockdown, lentiviral supernatants produced from pLKO.1 plasmids encoding the corresponding hairpins were used, and infected cells were selected for at least 7 days with 4 μl/l KO.1 plasmids encoding the corresponding hairpins were used, and control cell lines were confirmed to be highly enriched in epithelial cells (EpCAMP) by flow cytometry analysis using an allopseudocyanin (APC)-conjugated CD326 (EpCAM) antibody (eBioscience, G8.8).

Cloning cylinders (Corning), individual colonies were trypsinized (10,000–40,000 cells/well) in six-well plates for colony isolation. Using immediately injected subcutaneously into recipient mice or seeded (1:100), IGF-IR stained according to the manufacturers’ protocols using antibodies (DNA profiling). All cells were grown in RPMI supplemented with antibiotics (1% penicillin, streptomycin, and 10% FBS). Mouse Entpd5 hairpins: shE5-1 (GTCCACATCTTGGAAGAATAA GCTT) and shE5-2 (GGGTTGCAGAGATGCGCTTT; GCCGAGTTGTTTGGAGAT); control GFP hairpins: shGFP (CTACACAGCCACACAGCTCTT and TCTCGGCAATGAGGCTGTA). Human non–small cancer cell lines A549 and NCI-H23 were obtained from the American Type Culture Collection and NCI-H23 were obtained from the American Type Culture Collection (ATCC; in 2006 and 2013, respectively); the PC-9 cell line was a generous gift from Dr. Nabeel Bardeesy at Massachusetts General Hospital/Harvard Medical School (Boston, MA; 2011). No authentication of A549 or PC-9 cells was done by the authors. ATCC cell lines are routinely authenticated by short-tandem repeat analysis (DNA profiling). All cells were grown in RPMI supplemented with 10% FBS.

**Cell Proliferation Assay**

The assay was conducted as previously described (2), using the proliferation kit II (XTT; Roche).

**Immunoblotting and PHA Blotting**

Cells were rinsed once in ice-cold PBS and collected in lysis buffer containing 50 mmol/L HEPES, pH 7.4, 40 mmol/L NaCl, 2 mmol/L EDTA, 50 mmol/L NaF, 10 mmol/L pyrophosphate, 10 mmol/L glycophosphate, EDTA-free protease inhibitors (Roche), and 1% Triton X-100. Proteins were resolved by 4% to 12% or 16% SDS-PAGE, and analyzed by immunoblotting as described previously (29) using antibodies for AKT (#4061), caspase-3 (9626), BiP (#3177), CHOP (#5554), FOXO1 (2880), IGF-I (#3027), PTEN (#9552), phospho-S473 AKT (#4058) and phospho-T24/T32 FOXO1 (9464; 1:1,000; Cell Signaling Technology); EntPD5 (#2997-1; 1:50; Epitomics), SPC (#WRAB-9337; 1:5,000; Seven Hills Bioreagents), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sc-25778; 1:5,000; Santa Cruz Biotechnology). For N-glycosylation assessment, protein lysates were resolved by 10% SDS-PAGE and analyzed by blotting using horseradish peroxidase-conjugated phytahemagglutinin (PHA)-E lectin (United States Biological; #P3370-25).

**Immunohistochemistry**

Formalin-fixed lung lobes were bisected and embedded in paraffin. Paraffin-embedded lung or tumor sections were immunostained according to the manufacturers’ protocols using antibodies for cleaved caspase-3 (#9664; 1:200), EGFR (#4267; 1:50), FOXO1 (#2880; 1:100), IGF-I (#3027; 1:600), PTEN (#9559; 1:100), and phospho-S473 AKT (#4060; 1:50), from Cell Signaling Technology; SPC (#WRAB-9337; 1:15,000; Seven Hills Bioreagents); EntPD5 (#2997-1; 1:500; Epitomics), and BrdUrd (#ab6326; 1:40; Abcam). For human samples, a pathologist scored, in a blinded fashion, the intensity of PTEN and p-S473 AKT staining in a first set of 130 lung adenocarcinoma cases with two to eight cores/case. Scores for each tumor core were estimated using the following formula: intensity score = 0 (%) area of tumor with weak staining) + 1 (%) tumor area with moderate staining) + 2 (%) tumor area with strong staining), where 200 would be the highest score. These scores were then converted to a binary system where values above “100” were converted to a score of “1” (high) and scores below 100 were converted to “0” (low). Use of human samples for immunostaining was approved by the Institutional Review Board at Massachusetts General Hospital (Protocol Number 2009P001838).

**Tumor Burden and BrdUrd Quantification**

Subcutaneous tumors were collected, their dimensions were measured with a caliper, and tumor volume was estimated according to the ellipsoid formula (30). Lung tumor burden and BrdUrd labeling were quantified using cellsens software in either hematoxylin and eosin (H&E)-stained or BrdUrd-stained, transversely sectioned lung lobes, respectively. Total bronchiolar or alveolar tumor area (distinguished by morphology) in each bisected lobe was measured and normalized to the corresponding lobe area. Values represent averages of whole lung tumor burden from at least three mice. The number of BrdUrd-positive cells per tumor area was quantified using cellSens software and averaged from six to 10 alveolar or bronchiolar tumors/ lung of three mice, or from three to seven areas (0.36 mm2)/subcutaneous tumor in three mice.

**Statistical Analysis**

Data are presented as mean ± SEM. In comparing two groups, a two-tailed unpaired Student’s t test was conducted. For three or more groups, one-way ANOVA was conducted, followed by a post hoc Student–Newman–Keuls or Bonferroni test. Nonparametric two-tailed Fisher exact test and Spearman correlation were conducted for analysis of stain intensity scores. P ≤ 0.05 (unless otherwise stated) was considered significant.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

**Conception and design:** D.M. Sabatini, N.Y. Kalaany

**Development of methodology:** N.L. Curry, T.G. Oliver, D.M. Sabatini, N.Y. Kalaany

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** N.L. Curry, M. Mino-Kenudson, T.G. Oliver, O.H. Yilmaz, J.Y. Moon, T. Jacks

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** N.L. Curry, M. Mino-Kenudson, N.Y. Kalaany

**Writing, review, and/or revision of the manuscript:** N.L. Curry, M. Mino-Kenudson, T.G. Oliver, D.M. Sabatini, N.Y. Kalaany

**Administrative, technical, or material support:** D.M. Sabatini, T.G. Oliver, D.M. Sabatini, N.Y. Kalaany

**Study supervision:** D.M. Sabatini, N.Y. Kalaany

**Acknowledgments**

The authors thank F. Reinhardt, V. Gunduz, and T. Zaytouni for assistance with experiments; H. Wu for the Ptenfl/fl mice and A. Bens for the Tpr53fl/fl mice; X. Wang for ENTPD5 constructs; R. Weinberg, members of the Sabatini and Kalaany laboratories, and members of the Endocrinology Division at Boston Children’s Hospital for support and discussions; R. Bronson for histologic analysis; the Histology Core Facility at the Koch Institute for Integrative Cancer Research; and H. Wu for the Ptenfl/fl mice. This work was supported by grants from the National Institutes of Health, the American Society for Clinical Research, and the American Cancer Society.
Differential AKT Activation in Adjacent Pten-Null Lung Tumors

Research (Cambridge, MA); and the Rodent Histopathology Core Facility at the Dana-Farber/Harvard Cancer Center (Boston, MA) for assistance with tissue sectioning and H&E staining.

Grant Support
This research was supported by the Anna Fuller Fund fellowship (to N.Y. Kalaany); Boston Children’s Hospital (to N.Y. Kalaany, N.L. Curry, and V.O. Yilmaz); the Alexander and Margaret Stewart Trust Award (to D.M. Sabatini); the David H. Koch Cancer Research Award (to D.M. Sabatini); and NIH grants R01 AI047389, R01 CA129105 (to D.M. Sabatini), and Z-F30 CA114051 (to D.M. Sabatini, T. Jacks, and T.G. Oliver). D.M. Sabatini is an investigator of the Howard Hughes Medical Institute.

Received November 2, 2012; revised May 22, 2013; accepted May 23, 2013; published OnlineFirst May 29, 2013.

REFERENCES