PTEN-Deficient Tumors Depend on AKT2 for Maintenance and Survival

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INTRODUCTION

The PI3K signaling pathway is frequently deregulated in virtually all human solid tumors (1). Upon activation by growth factors, class IA PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) at the plasma membrane to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3) (2). PIP3 fulfills an essential second messenger role by recruiting inactive signaling proteins to the plasma membrane, resulting in the activation of numerous pathways that traverse the signal to a plethora of cellular processes (3). The intracellular levels of PIP3 are tightly regulated by the opposing activities of PI3K and PTEN, a PIP3 3′-phosphatase that dephosphorylates PIP3 back to PIP2 (4, 5). The p110α catalytic subunit of PI3K, encoded by the PIK3CA gene, is frequently activated by somatic mutation in many epithelial cancers, including breast, endometrial, and colon cancers (3). In contrast, PIK3CA mutations are rare in highly aggressive metastatic prostate cancer. Instead, loss of PTEN due to LOH or inactivating mutations is the predominant mechanism driving PI3K pathway activation in prostate tumors (6, 7). The critical role of the PI3K pathway in tumorigenesis has led to the development of numerous small-molecule inhibitors that translocate to distinct subcellular compartments where it phosphorylates numerous substrates, many of which are oncogenes or tumor suppressors (13). The essential role of AKT in tumorigenesis has led to the development of a number of first-generation pan-AKT inhibitors currently in clinical trials (14). Although the three AKT isoforms share a high degree of sequence identity and are regulated by similar mechanisms, studies have highlighted distinct functions of AKT isoforms in cancer progression (reviewed in ref. 15). For example, whereas AKT2 promotes breast cancer cell migration and metastatic dissemination, AKT1 can actually function as a metastasis suppressor (9, 16–19). These and other studies suggest that AKT isoform–selective inhibitors might provide more optimal therapeutic responses in tumor-specific contexts.

A critical role for AKT in PTEN-deficient tumors is evident from a number of studies. PTEN heterozygous mice develop tumors spontaneously in multiple organs, concomitant with hyperphosphorylated AKT (20–22). Prostate tumor development induced by PTEN loss requires functional mTORC2 (23). Similarly, mice lacking AKT1 are protected from tumorigenesis induced by PTEN loss (24). Curiously, a more recent study indicated that inactivation of AKT2 has little or no consequence on prostate neoplasia, explained in part by the relatively minimal impact of AKT2 loss on total AKT activity and also an increase in blood insulin levels (25). In contrast, AKT2 is required for proliferation and invasive migration of PTEN-deficient glioblastoma (26, 27). In late-stage colorectal cancer, AKT2 is highly expressed and functions synergistically with...
PTEN loss to promote metastasis (28). It has also been demonstrated that deletion of AKT2 in PTEN-null mice attenuates hepatic injury, thereby delaying liver tumor development (29).

Although the contribution of AKT in tumor initiation in the context of PTEN inactivation has been determined, the role of AKT isoforms in the maintenance of established PTEN-null tumors is unknown. Moreover, it is unclear whether PTEN loss to promote metastasis (28). It has also been demonstrated that deletion of AKT2 in PTEN-null mice attenuates hepatic injury, thereby delaying liver tumor development (29).

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The role of AKT in the initiation of PTEN-deficient prostate tumors has been studied (24, 25). However, the function of AKT isoforms in modulating cancer cell survival in this context has not been determined. To assess the roles of AKT isoforms in tumor initiation as well as maintenance, we generated isoform-specific shRNAs in a doxycycline (dox)-regulated system. Because of the high frequency of PTEN inactivation (up to 50%) observed in human prostate cancers (6), we first introduced shRNAs in the PTEN-deficient prostate LNCaP line. Addition of doxycycline to LNCaP cells results in highly selective silencing of AKT1 and AKT2 (LNCaP cells do not express AKT3; Fig. 1A). We next performed 3D spheroid assays with Matrigel, because growth of tumor cells in 3D more accurately recapitulates the morphology of tumors grown in three-dimensional (3D) culture. We find that in PTEN-deficient tumor cells depletion of AKT2 induces apoptosis and leads to regression of established prostate xenografts. In contrast, downregulation of AKT1 or AKT3 has no effect on the integrity of tumor spheroids. We also demonstrate that p21 is a downstream effector of AKT2 in modulating tumor-cell survival. These data identify AKT2 as the critical isoform for driving maintenance of established PTEN-deficient cancers.

**RESULTS**

**AKT2 Silencing Induces Prostate Tumor Spheroid Disintegration**

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AKT2 and prostate tumor maintenance

AKT2, but Not AKT1, Regulates Survival of PTEN-Deficient Prostate Tumor Cells

To determine whether AKT2 depletion induces spheroid disintegration by promoting apoptosis, we stained spheroids for active caspase-3 followed by analysis with confocal microscopy. AKT1 silencing has no effect on the levels of active caspase-3, despite a dramatic downregulation of AKT1 in tumor spheroids (Fig. 2A). In contrast, there is a robust induction of active caspase-3 upon AKT2 depletion (Fig. 2B). In addition, condensed apoptotic nuclei and marked disruption of cell morphology are observed in AKT2-depleted, but not AKT1-depleted, spheroids. Whereas a minimal number of apoptotic cells are observed in AKT1-silenced spheroids, more than 50% of cells in AKT2-depleted spheroids display active caspase-3 and fragmented nuclei (Fig. 2C).

To avoid potential shRNA off-target effects, we used a cDNA-rescue strategy. shRNA-resistant doxycycline-inducible AKT1 and AKT2 sequences were generated and reintroduced into AKT2-depleted LNCaP cells. Apoptosis was assessed by staining cells with the apoptotic marker annexin V followed by FACS analysis. Whereas expression of AKT2 completely rescues apoptosis induced by AKT2 silencing, expression of AKT1 does not (Fig. 2D). In addition, robust apoptosis is induced when AKT2 is knocked down by an independent shRNA targeting a distinct region of the AKT2 transcript (Fig. 2E). The exclusive function of AKT2 in regulating survival in 3D was also observed in a distinct PTEN-null prostate cell line, PC3 (Fig. 2F). We also evaluated the activity of AKT small-molecule inhibitors in PTEN-deficient prostate cancer survival. As expected, treatment of LNCaP spheroids with an AKT2-selective inhibitor (EMD Millipore; IC50 = 0.8 μmol/L for AKT2) inhibits spheroid growth in a dose-dependent manner (Fig. 2G). These data demonstrate that AKT2, but not AKT1, plays a critical and exclusive role in the maintenance of PTEN-deficient prostate cancer spheroids.

Next, we evaluated the consequence of AKT isoform depletion in conventional two-dimensional (2D) culture. Silencing AKT1 or AKT2 significantly inhibits LNCaP cell proliferation to a similar extent (Supplementary Fig. S1A), consistent with the essential role of AKT1 and AKT2 in proliferation that is observed in 3D. Silencing of AKT1 or AKT2 also suppresses cell migration in Transwell migration assays (Supplementary Fig. S1B), indicating that both AKT1 and AKT2 are promigratory in LNCaP cells. However, depletion of AKT2 in cells grown in 2D does not induce apoptosis after 5 days of doxycycline treatment, whereas the same cells grown as 3D spheroids undergo robust apoptosis upon AKT2 silencing (Fig. 3A). When cells in 2D culture were allowed to grow longer, apoptosis is observed after 7 days of doxycycline treatment, although the effect is much more modest compared with AKT2-depleted 3D spheroids (Fig. 3B). We then addressed whether AKT1 or AKT2 depletion acts synergistically with chemotherapeutic agents to induce apoptosis in tumor cells in 2D culture. Apoptosis was assessed 3 days after doxycycline treatment, when AKT1 or AKT2 knockdown alone has minimal effect on cell death (Fig. 3C). Depletion of AKT1 or AKT2 functions synergistically with doxorubicin to induce apoptosis of LNCaP cells. Taken together, these findings further demonstrate that AKT2 is both necessary and sufficient to regulate survival of PTEN-deficient prostate cancer cells in a manner that is robustly observed in 3D, but not 2D, culture.

PTEN-Deficient Tumor Spheroids Depend on AKT2 for Survival

Because both LNCaP and PC3 cells are PTEN-deficient, we next investigated whether AKT2 has an exclusive function in survival phenotypes in other solid tumors with high frequencies of PTEN inactivation, including glioblastoma and breast carcinoma. Similar to what is observed in prostate cancer cells, silencing of AKT1, AKT2, or AKT3 in PTEN-deficient MDA-MB-468 breast cancer cells attenuates proliferation in monolayer culture as well as inhibits spheroid growth in 3D (Supplementary Fig. S2A and S2B). However, when MDA-MB-468 spheroids were allowed to form before doxycycline administration, silencing of AKT2, but not AKT1 or AKT3, leads to spheroid disintegration and caspase-3 activation (Fig. 4A and B). Similar results are observed for a distinct PTEN-deficient breast cancer line, BT-549 (Fig. 4C). PTEN-deficient U87-MG glioblastoma cells form highly invasive spheroids in 3D culture. Whereas knockdown of AKT1 or AKT3 has no effect on spheroid phenotypes, AKT2 depletion results in regression of invasive spheroids and robust cell death (Fig. 4D). Furthermore, six other cancer lines that express wild-type PTEN (T47D, MCF10DCIS, SKBR3, H1703, SKOV3, and DU145) were analyzed and the data show that AKT1 or AKT2 silencing in these lines has no effect on spheroid integrity (Supplementary Fig. S3A–S3G).

To more directly test the specific antiapoptotic function of AKT2 in PTEN-deficient tumor spheroids, we used a molecular genetic approach in the prostate cell line Crw22-RV-1 that is PTEN wild-type. Upon knockdown of AKT1 or AKT2, robust spheroid disintegration is observed (Fig. 4E). Conversely, AKT3 silencing has no effect on spheroid growth or phenotypes. Strikingly, whereas disintegration is observed in AKT2-depleted spheroids regardless of PTEN expression or silencing, knockdown of PTEN completely rescues spheroid disintegration mediated by AKT1 silencing (Fig. 4F). Collectively, these data indicate that PTEN-deficient tumor spheroids are addicted to AKT2 for survival signaling and maintenance, at least in vitro.

Identification of AKT2-Specific Targets: Role of p21 in AKT2-Mediated Spheroid Maintenance

We next determined the molecular mechanisms that account for the exclusive function of AKT2 in tumor spheroid
Figure 2. AKT2, but not AKT1, is required for regulating survival of PTEN-deficient prostate tumor spheroids. **A**, LNCaP cells expressing tet-on AKT1 shRNA were grown in 3D culture for 7 days, followed by doxycycline (dox) treatment for 5 days. Immunofluorescence was performed using active caspase-3 (active Casp3) and AKT1 antibodies followed by confocal microscopy. Cell nuclei and actin were labeled with 4,6-diamidino-2-phenylindole (DAPI) and Alexa Fluor 488–conjugated phalloidin, respectively. **B**, LNCaP cells expressing tet-on AKT2 shRNA were cultured and stained as in **A**. AKT2 knockdown was confirmed by staining cells with AKT2 antibody. **C**, percentage of cells containing active caspase-3 and fragmented nuclei was quantified and depicted in the bar graph. Error bars, mean ± SEM; ***P < 0.001 (Student t test; n = 5). **D**, LNCaP cells expressing tet-on AKT1 or AKT2 or pTRIPZ control vector were infected with tet-on AKT2 shRNA. Cells were cultured in 3D for 6 days, followed by doxycycline for 8 days. Apoptosis was assessed by staining cells with annexin V and 7-AAD, followed by FACS analysis. Relative apoptosis was determined by calculating percentage of annexin V–positive, apoptotic cells in doxycycline–treated cells relative to the percentage in mock-treated cells of the same infection. The basal apoptosis levels of pTRIPZ vector–, AKT1 pTRIPZ–, and AKT2 pTRIPZ–infected cells are 25%, 23%, and 18%, respectively. Error bars, mean ± SEM; *, P < 0.05; **, P < 0.01 (Student t test; n = 3). Whole-cell lysates were subjected to immunoblotting. **E**, FACS analysis for assessing the effect of an independent AKT2 shRNA (AKT2 #2) on the maintenance of LNCaP spheroids. The basal apoptosis levels of cells expressing tet-on AKT1 and AKT2 shRNA are 33% and 30%, respectively. **F**, PC3 cells expressing tet-on AKT1 or AKT2 shRNA were grown in 3D culture for 6 days, followed by doxycycline treatment for 7 days. Immunofluorescence was performed using active caspase-3 antibody followed by confocal microscopy. Cell nuclei and actin were labeled with DAPI and Alexa Fluor 647–conjugated phalloidin, respectively. **G**, size of LNCaP spheroids grown in 3D culture for 7 days was measured in pixel area using ImageJ. The spheroids were then treated with the indicated dose of AKT2-selective inhibitor for 48 hours. Size of the same spheroids after treatment was measured, and percentage increase in spheroid size relative to pretreated spheroids was depicted in a bar graph (n = 8). Morphology of DMSO and AKT2-selective inhibitor–treated spheroids are shown in the phase-contrast images. Results are representative of at least three independent experiments.
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Figure 3. AKT2 depletion has a more profound effect on apoptosis in 3D LNCaP spheroids than in 2D monolayer. A, LNCaP cells expressing Tet-on AKT1 or AKT2 shRNA were grown in 2D monolayer or 3D culture for 7 days, followed by doxycycline (dox) for 5 days. Apoptosis was assessed by staining cells with annexin V and 7-AAD, followed by FACS analysis. B, LNCaP cells grown in 2D were treated with doxycycline for 3, 5, or 7 days, followed by annexin V staining and FACS analysis. C, LNCaP cells expressing Tet-on AKT1 or AKT2 shRNA grown in 2D were treated with doxycycline for 3 days and/or doxorubicin (0.5 μmol/L) for 24 hours followed by assessment of apoptosis (n ≥ 3). The basal apoptosis levels of cells expressing Tet-on AKT1 and AKT2 shRNA are 9% and 18%, respectively. Results are representative of at least three independent experiments.

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Maintenance. Quantitative RT-PCR analysis shows that the expression levels of AKT1 and AKT2 in LNCaP cells are comparable (Supplementary Fig. S4A). To examine the activation status of AKT1 and AKT2 in 3D cultures, we tested the effect of AKT isoform knockdown on the phosphorylation level of total AKT as well as classical downstream pan-AKT substrates. AKT1 and AKT2 depletion results in a 70% and 20% reduction of pAKT Ser473, respectively (Supplementary Fig. S4B). Interestingly, AKT1 plays a predominant role in the phosphorylation of GSK3β in LNCaP cells. Conversely, AKT2 knockdown has a more profound effect on the phosphorylation of PRAS40 compared with AKT1 knockdown. Taken together, these results suggest that both AKT1 and AKT2 are active in cells growing in 3D, and that the exclusive function of AKT2 cannot be accounted for by gross differences between the expression and activation status of individual AKT isoforms. To explore isoform-specific signaling, we used quantitative RT-PCR to assess the expression levels of proapoptotic proteins. Silencing of AKT1 or AKT2 in LNCaP spheroids growing in 3D results in upregulation of p53 (TP53) and Puma (BBC3) message levels (Fig. 3A). Surprisingly, whereas the mRNA levels of two targets of p53, p21 and BAX, are not affected by AKT1 depletion, knockdown of AKT2 significantly enhances p21 (CDKN1A) and BAX messages. Immunoblot analysis shows that AKT2 silencing also leads to increased p21 and BAX protein expression when cells are cultured in both 2D and 3D (Fig. 3B). Upregulation of p21 and BAX by AKT2 silencing is also observed in two distinct PTEN-deficient lines, PC3 and U87-MG (Fig. 3C). Interestingly, reduced p21 protein levels are observed in AKT1-depleted LNCaP and PC3 cells, suggesting a general function for the AKT pathway in regulating the stability of p21, at least in prostate cancer (Fig. 3C).

To explore this further and evaluate whether p21 is a downstream target of AKT2 in tumor maintenance, we used p21 shRNA in rescue experiments. Because it is established that p21 can modulate AKT-mediated cell-cycle progression, we first assessed the effect of p21 knockdown on the cell-cycle profile of AKT2-depleted cells. As expected, silencing of AKT2 leads to cell-cycle arrest, and silencing of p21 restores cell-cycle progression in AKT2-depleted cells (Supplementary Fig. S5). We then evaluated the contribution of p21 in AKT2-mediated spheroid maintenance. Whereas knockdown of p21 alone has no effect on cell death, apoptosis mediated by AKT2 depletion is quantitatively rescued by p21 silencing (Fig. 3D), indicating that p21 is at least one downstream target of AKT2 that regulates apoptotic response. We have also examined whether the proapoptotic protein BAX is a mediator of AKT2 in regulating prostate spheroid maintenance. Knockdown of BAX results in a small but statistically significant reduction in apoptosis (Supplementary Fig. S6). However, BAX depletion does not rescue apoptosis induced by AKT2 knockdown, suggesting that BAX does not play a role in AKT2-mediated spheroid maintenance.

Inhibition of AKT results in the relief of negative feedback prosurvival pathways, which in turn lead to upregulation of growth factor receptor tyrosine kinases (30). Consistent with this model, treatment of LNCaP spheroids with the pan-AKT inhibitor GSK690693 reduces phosphorylation of the AKT substrate PRAS40, yet leads to increased insulin-like growth factor–1 receptor β (IGF1Rβ) expression (Fig. 3E). Conversely,
**Figure 4.** Dependence of PTEN-deficient tumor spheroids on AKT2 for survival. **A,** MDA-MB-468 cells containing tet-on AKT isoform shRNA were grown in 3D culture for 6 days, followed by doxycycline (dox) treatment for 5 days. Representative phase-contrast images are shown. **B,** MDA-MB-468 spheroids were treated with doxycycline for 6 days, followed by staining with active caspase-3 (Casp3) and confocal microscopy to assess apoptosis. **C,** BT-549 cells were cultured and stained as in B. **D,** U87-MG cells were cultured in 3D for 7 days, followed by doxycycline treatment for 5 days. Representative phase-contrast images are shown. **E,** CWr22-RV-1 cells containing tet-on AKT isoform shRNA were grown in 3D culture for 7 days, followed by doxycycline treatment for 8 days. **F,** CWr22-RV-1 cells expressing tet-on AKT isoform shRNA were infected with PTEN shRNA. Cells were cultured in 3D for 8 days, followed by doxycycline for 6 days. Percentage of disintegrated spheroids was quantified and depicted in the bar graph (n ≥ 29). Results are representative of at least three independent experiments.
the levels of p21 do not change with pan-AKT inhibitor treatment, consistent with the opposing effects of AKT1 and AKT2 on p21 expression. Moreover, silencing of AKT1 in 2D as well as 3D leads to elevated IGF1R mRNA, but this does not translate into changes in IGF1R total protein (Fig. 5B and F). Conversely, AKT2 depletion in 3D, but not 2D, dramatically reduces IGF1R protein as well as mRNA levels (Fig. 5B and F). Inhibition of IGF1R with NVP AEW541 does not induce apoptosis in LNCaP tumor spheroids (Fig. 5G). Interestingly, whereas concomitant inhibition of IGF1R and AKT1 depletion has no effect on spheroid maintenance, NVP AEW541 acts synergistically with AKT2 knockdown in potentiating apoptosis. The concomitant induction of proapoptotic proteins and decrease of prosurvival proteins in tumor spheroids by AKT2 depletion may explain the more robust apoptotic phenotype observed in 3D cultures when compared with 2D.

**AKT2 Depletion Induces Prostate Tumor Regression in a Xenograft Model**

Finally, using inducible AKT isoform shRNA, we investigated the relative contribution of AKT1 and AKT2 in the maintenance of established tumors in vivo. LNCaP cells
inhibiting AKT2 signaling induces tumor regression. exclusive addiction to AKT2 for tumor maintenance, and that data demonstrate that prostate tumors deficient in PTEN are significantly induces p21 expression (Fig. 6B). Taken together, these tumor spheroids, depletion of AKT2, but not AKT1, significantly reduces tumor size (Fig. 6A and Supplementary Fig. S7). AKT1 silencing of PTEN-null breast and glioblastoma spheroids. Of particular clinical importance, we also find that breast, prostate, lung, and ovarian tumor lines that express wild-type PTEN, but do not harbor PTEN null breast and glioblastoma spheroids, depletion of AKT2, but not AKT1, significantly induces p21 expression (Fig. 6B). Taken together, these data demonstrate that prostate tumors deficient in PTEN are exclusively addicted to AKT2 for tumor maintenance, and that inhibiting AKT2 signaling induces tumor regression.

**DISCUSSION**

PTEN inactivation is a common event in prostate cancer, with frequencies ranging from 30% to 60%, and is associated with aggressive tumor progression and poor prognosis (31). However, to date, PTEN has proved to be an intractable target for drug development due to the therapeutic challenges associated with reactivation of a tumor suppressor. Although numerous studies have established the functional importance of AKT activation in PTEN-deficient tumors, the specific contributions of AKT isoforms in mediating the proliferative and survival signals necessary for tumor initiation and maintenance have not been explored. The present work expands on previous studies that have concluded that both AKT1 and AKT2 function to modulate prostate cancer cell proliferation (32). However, our studies point to a critical and exclusive function for AKT2 in mediating the signals necessary for PTEN-deficient tumor cell maintenance and survival. One important aspect that previously had not been considered is the assessment of survival in 3D spheroids, whereby induction of apoptosis is observed only upon AKT2, but not AKT1 or AKT3, silencing. The function of AKT2 in PTEN-driven tumor maintenance is also corroborated by depletion of PTEN in PTEN wild-type prostate cancer cells, which is sufficient to render them exclusively AKT2-dependent. Importantly, the addiction of PTEN-deficient tumors to AKT2 signaling is observed not only in prostate cancer, but also in PTEN-null breast and glioblastoma spheroids. Of particular clinical importance, we also find that breast, prostate, lung, and ovarian tumor lines that express wild-type PTEN, but display hyperactive AKT signaling due to other pathway lesions, such as oncogenic PIK3CA or receptor amplification, do not depend exclusively on AKT2 activity for survival. These findings advocate for the development of AKT2-selective inhibitors and also suggest that PTEN deficiency could be used as a patient tailoring strategy for therapeutic benefit.

Several potential mechanisms may contribute to the addiction of AKT2 in PTEN-deficient but not in PIK3CA-mutant tumor cells. It is well established that PTEN antagonizes the...
**PI3K-AKT pathway by acting as a PIP3 phosphatase.** More recent studies have shown that nuclear PTEN functions as an essential tumor suppressor and regulates chromosome stability as well as cell-cycle arrest (33, 34). It is possible that nuclear PTEN specifically antagonizes AKT2 activity in the nucleus, such that PTEN inactivation results in the accumulation of hyperactive nuclear AKT2 and affords a dominant effect of AKT2 on tumor maintenance. It has also been shown that in certain tumor contexts, p110β, but not p110α, plays a critical role in PTEN-deficient prostate tumorigenesis (7, 35). It would be interesting to examine whether p110β cooperates with AKT2 to mediate survival signaling in PTEN-null tumor spheroids.

Previous studies have shown that AKT1 depletion markedly reduces the incidence of prostate tumors in PTEN heterozygous mice (24), whereas AKT2 depletion has little or no effect (25). These results suggest that AKT1, but not AKT2, plays a critical role in prostate tumor initiation. In contrast, our studies demonstrate that AKT2, but not AKT1, is necessary for tumor maintenance and survival, because AKT2 depletion alone induces regression of prostate xenografts. This finding has significant clinical implications, because studies have shown that inhibition of a number of oncogenic proteins in the PI3K pathway, including p110α and mTOR, have cytostatic effects only in xenograft models (36, 37). However, regression of established tumors is not commonly observed, possibly due to the relief of feedback suppression mechanisms. In this context, inhibition of AKT relieves feedback suppression by inducing the expression of multiple receptor tyrosine kinases (30). This is consistent with our finding that inhibiting AKT activity in prostate cancer cells induces expression of IGF1R. Interestingly, silencing AKT2, but not AKT1, leads to reduced IGF1R expression in tumor spheroids. These data further point to an unmet need for the development of AKT2-selective inhibitors for PTEN-null tumors.

A separate line of evidence in support of the development of AKT2-selective inhibitors comes from findings of the control of tumor invasion and metastasis that are differentially regulated by AKT1 and AKT2 (9). Considering other potential isoform-specific functions of AKT, such as metabolism and stem cell maintenance, one prediction is that isoform-selective inhibitors would offer less toxicity and other unwanted on-target effects. Indeed, PI3K isoform-specific inhibitors (e.g., CAL-101 for PI3Kδ) offer clear-cut examples of powerful single agents in treating selected cancer types and patient populations (38).

The potent regression of prostate xenografts achieved by AKT2 inhibition is accompanied by a robust induction of p21. Using cDNA rescue experiments, we show that p21 is one critical downstream effector of AKT2 in the apoptotic response, and as such could serve as a potential biomarker used for screening AKT2 activity in vivo. In addition to its classical role as a cyclin-dependent kinase inhibitor, p21 has been shown to act as a proapoptotic or antiapoptotic protein, depending on the tumor context as well as intracellular localization (39). Overexpression of p21 in glioma and ovarian cancer cells enhances apoptosis induced by chemotherapeutic agents (40, 41). Moreover, adenoviral gene transfer of p21 induces apoptosis in established cervical xenografts (42). Similarly, a chimeric peptide comprising the carboxyl terminus of p21 conjugated to a pentapeptide induces apoptosis in lymphoma cells (39). The mechanism by which p21 induces apoptosis has not been well studied. In hepatoma cells, p21 expression promotes ceramide-induced apoptosis through the proapoptotic protein BAX (43). Our analysis also reveals a concomitant induction of p21 and BAX in a variety of conditions, indicating that these proteins may play a synergistic role in mediating the apoptotic response.
of AKT2-depleted PTEN-deficient cancer cell lines. However, rescue experiments indicate that BAX does not play an essential role in AKT2-mediated tumor maintenance.

We further show that AKT2 regulates p21 at the mRNA level. However, the precise mechanism is not known. p21 is a direct target of p53 (44); however, we have been unable to detect a robust alterations of p53 activity upon AKT2 inhibition or silencing. Moreover, p21 expression is also regulated by a variety of other transcription factors, including Ap2, STATs, C/EBPs, and MyoD (45). Interestingly, AKT2 has a distinct function in modulating the transactivation activity of MyoD-MEF2 (46). Whether AKT2 modulates p21 expression via MyoD in PTEN-deficient cells remains to be determined.

One important concept that has emerged from our studies is the profound difference observed in the ability of AKT2 and AKT1 to mediate survival and maintenance of PTEN-deficient tumor cells in 3D culture versus growth on 2D. The altered molecular behavior of cells in 3D compared with growth in 2D has been reported by many groups and so it has been proposed that growth in 3D serves as a better model for the responsiveness of tumors to therapy in patients. For example, in triple-negative breast cancer, increased sensitivity to MEK inhibitors is observed in 3D cultures when compared with monolayer models (47). It has also been shown that in HER2-overexpressing breast cancers, cells grown in 3D have enhanced activation of HER2 as compared with growth in 2D, resulting in an increased response to trastuzumab (48). One report indicates that mitochondrial DNA depletion in prostate epithelial cells enhances PI3K–AKT2 activation, which in turn promotes anoikis resistance (49). We therefore examined whether the response of tumor cells to AKT2 silencing could be due to the enhanced activation of this isoform when cells are grown in 3D. However, our data show that the differential effects on apoptosis cannot be explained by differences in AKT2 activation levels compared with AKT1, at least when AKT1 and AKT2 phosphorylation and activity are analyzed in whole-cell lysates. One possibility is that a distinct, perhaps cellular localization of AKT2, especially evident when cells are grown in 3D culture, offers the appropriate platform for protein–protein interactions and accessibility to specific AKT2 substrates and targets, such as p21, that in turn mediate the survival response in PTEN-deficient tumor cells. Whether this model accounts for the exclusive function of AKT2 in mediating tumor maintenance of PTEN-deficient tumors remains to be determined.

Taken together, our findings demonstrate that AKT2 and its downstream targets are responsible for driving PTEN-null tumor progression, and identify AKT2 as an important target for the effective treatment of PTEN-deficient solid tumors. They also further advocate for the development of AKT isoform-specific inhibitors, analogous to the selective PI3K p110 isoform-selective inhibitors currently in preclinical development.

**METHODS**

**Cell Culture**

MDA-MB-468, U87-MG, and HEK293T cells were obtained from ATCC and maintained in DMEM (CellGro) supplemented with 10% tet system-approved FBS (Clontech). LNCaP, PC3, BT-549, and CW2-RV-1 cells were cultured in RPMI-1640 medium (Cambrex) supplemented with 10% FBS. All cell lines obtained from the cell banks listed above are tested for authentication using short tandem repeat (STR) profiling and passaged for fewer than 6 months, and routinely assayed for Mycoplasma contamination.

**Plasmids**

For doxycycline-inducible overexpression of AKT1 and AKT2, HA-AKT1/pTRIPZ and HA-AKT2/pTRIPZ were constructed. HA-AKT1 and HA-AKT2 cDNAs were amplified by PCR from HA-AKT1/pcDNA3

**3D Cultures**

3D cultures were prepared as previously described (50). Briefly, chamber slides were coated with growth factor–reduced Matrigel (BD Biosciences) and allowed to solidify for 30 minutes. Cells (2,000–4,000) in assay medium were seeded on coated chamber slides. Assay medium contained DMEM or RPMI-1640 supplemented with 10% FBS and 2% Matrigel. Assay medium for U87-MG cells contained RPMI-1640 medium supplemented with 2% FBS and 5% Matrigel. The assay medium was replaced every 4 days. Doxycycline (100 ng/mL) was added every 2 or 3 days. For annexin V apoptosis assays and Western blot analysis, 3D cultures were set up in ultra-low adherent 6-well plates (Corning). Cells were seeded in 1.5 mL assay medium containing DMEM or RPMI-1640 supplemented with 10% FBS and 2% Matrigel. Assay medium (1.5 mL) was added every 4 days. Morphology of spheroids grown in ultra-low adherent plates is similar to those grown in Matrigel coated on chamber slides.

**Antibodies**

Anti-AKT1, anti-AKT2, anti-AKT3, anti-phospho-AKT S473 (pAKT), anti-phospho-AKT1 S473 (pAKT1), anti-phospho-AKT2 S474 (pAKT2), anti-BAX, anti-p53, anti-PUMA, anti-IGF1Rβ, anti-pGSK3β, anti-GSK3β, anti-p21, anti-PRAS40, anti-p-PRAS40, anti-NAH, and anti-active caspase-3 antibodies were obtained from Cell Signaling Technology. Anti-β-actin antibody was purchased from Sigma-Aldrich. Anti-p85 polyclonal antibody was generated in-house and has been described previously (51). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibody was purchased from Chemicon.

**RNA Interference**

For doxycycline-inducible shRNA-mediated knockdown of AKT isoforms, a set of single-stranded oligonucleotides encoding the AKTI, AKT2, and AKT3 target shRNA and its complement were synthesized. The hairpin sequences have been validated (17). AKTI: sense, 5′-CCGCGAGGTGTGAGTACGTCAAACTCTCGAGGTCTTGATGTATTCACCACGCTTTTT-3′; AKT2: sense, 5′-CCGGCAATTGTGGAGTTGACGAGCTTTCTTCAGGG-3′; AKT3: sense, 5′-CCCGGCTGCTGGGACTATCACATCTCTTCAGGAGAAGTTTG-3′. The oligonucleotide sense and antisense pair were annealed and inserted into tet-on pLKO. To produce lentiviral supernatants, 293T cells were cotransfected with control or shRNA-containing tet-on pLKO vectors, VSVG and psPAX2 for 48 hours. p21 and BAX shRNA sequences (p21: sense, 5′-CCGGACACAGAAGAGACACCATTGCCTGAGACATGGTCTCTCTCTGCTGCTTTTTG-3′; BAX: sense, 5′-CCGGAAAGTGGCCGAACATGGACGTACGTCAACGATGATCCCGACCTTCTTCAGGAGGATCTGATGTC-3′; CCAGGCACCTTTTCTTTTGC-3′) have been validated (52, 53) and were cloned into the tet-on pLKO lentiviral expression system as described above. Cells stably expressing doxycycline-inducible shRNA were cultured in medium containing puromycin (0.5–2 μg/mL). Gene knockdown was induced by incubating cells with 100 ng/mL doxycycline for 48 to 72 hours.
and HA-AKT2/pcDNA3, respectively. The resulting PCR product was digested with restriction enzymes AgeI and CldI, followed by insertion into pTRIPZ lentivector (Thermo Scientific). shRNA-resistant variants of AKT were constructed by site-directed mutagenesis with the following primers: 

**AKT1**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5′-GCACCGGCCTACTGAGGAGAAG-3′</td>
</tr>
<tr>
<td>Reverse</td>
<td>5′-ATGCGCTAATG-3′</td>
</tr>
</tbody>
</table>

**AKT2**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5′-CGCCGAGAGCGGGCTAATG-3′</td>
</tr>
<tr>
<td>Reverse</td>
<td>5′-GGCTCATGGTGAATGTCGTC-3′</td>
</tr>
</tbody>
</table>

Cells were then blocked with primary antibodies for overnight at 4°C. Cells were washed three times with PBS followed by incubation with biotinylated secondary antibodies for 1 hour. The slides were washed and developed using the diaminobenzidine (DAB) metal enhanced kit (Vector Lab) and counterstained with hematoxylin.

**Transwell Migration Assays**

Cells (1 × 10^6) in serum-free medium containing 0.1% BSA were added to upper Transwell chambers in triplicate. NIH 3T3-cell-conditioned medium was added to the lower chambers. After 2 to 16 hours of incubation at 37°C, nonmigrated cells on Transwell filters (8-μm pore size; Corning) were removed. Cells that had migrated to the bottom of the filters were fixed and stained using the Hema-3 stain set (Protocol).

**Statistical Real-Time PCR**

Total RNA was isolated with the RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. Reverse transcription was performed using random hexamers and multiscribe reverse transcriptase (Applied Biosystems). Quantitative real-time PCR was performed using an ABI Prism 7700 sequence detection system. AKT1 primer: sense, 5′-CAAGCCCAAGCACGC-3′, antisense, 5′-GGATACCTCTATTCCACGGCCG-3′; AKT2 primer: sense, 5′-GCAACCAGGACGCGTAAAG-3′, antisense, 5′-CCGCACCAAGATGCTGT-3′; p21 primer: sense, 5′-GCAGGACTGTGATGGGTTGTAAGGCT-3′, antisense, 5′-TGGCAGCTTGATGGGTTGTAAGGCT-3′; anti-sense, 5′-TGGCAGCTTGATGGGTTGTAAGGCT-3′; Puma primer: sense, 5′-GGGACCCATGGATGCTGCTGTG-3′, antisense, 5′-GGGACCCATGGATGCTGCTGTG-3′; GAPDH primer: sense, 5′-CAGAAGGCTATCCATGTGAGAAGG-3′, antisense, 5′-GGGACCCATGGATGCTGCTGTG-3′.

**Statistical Analysis**

Statistical significance between conditions was assessed by Student t test. In all figures, data are presented as mean ± standard error of the mean (SEM) for one representative experiment. Statistical significance between conditions is denoted as *, P < 0.05; **, P < 0.01; and ***, P < 0.001.
For Transwell migration analyses, at least 5 random images were counted and averaged per well, for 3 wells, for each condition. At least three independent experiments were performed for each condition for verification of the emphasized trends in *in vitro* studies. For xenograft studies, at least seven tumors in each condition were analyzed.

**Disclosure of Potential Conflicts of Interest**

A. Toker reports receiving a commercial research grant from Eli Lilly and Company. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: Y.R. Chin, A. Toker
Development of methodology: Y.R. Chin, A. Toker
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.R. Chin, S.P. Balk
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.R. Chin, X. Yuan, S.P. Balk, A. Toker
Writing, review, and/or revision of the manuscript: Y.R. Chin, A. Toker
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Toker
Study supervision: Y.R. Chin, X. Yuan, A. Toker

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