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mTOR Complex 2 Is Required for the Development of Prostate Cancer Induced by Pten Loss in Mice

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SUMMARY

mTOR complex 2 (mTORC2) contains the mammalian target of rapamycin (mTOR) kinase and the Rictor regulatory protein and phosphorylates Akt. Whether this function of mTORC2 is critical for cancer progression is unknown. Here, we show that transformed human prostate epithelial cells lacking PTEN require mTORC2 to form tumors when injected into nude mice. Furthermore, we find that Rictor is a haploinsufficient gene and that deleting one copy protects Pten heterozygous mice from prostate cancer. Finally, we show that the development of prostate cancer caused by Pten deletion specifically in prostate epithelium requires mTORC2, but that for normal prostate epithelial cells, mTORC2 activity is nonessential. The selective requirement for mTORC2 in tumor development suggests that mTORC2 inhibitors may be of substantial clinical utility.

INTRODUCTION

The phosphatidylinositol 3-kinase (PI3K) signaling pathway is aberrantly active in many human cancers. The best characterized downstream target of PI3K activation is the Akt kinase, which influences cancer cell metabolism, survival, growth, proliferation, angiogenesis, and migration by phosphorylating a diverse array of substrates (reviewed in Manning and Cantley, 2007). Two common causes of aberrant PI3K activation include loss of the PTEN tumor suppressor and activating mutations in PI3K. Either event results in phosphatidylinositol (3,4,5)P3 accumulation at cell membranes, which serve as a docking site for Akt localization and activation. Akt requires phosphorylation on two sites for full activation (reviewed in Guertin and Sabatini, 2007; Hanada et al., 2004; Manning and Cantley, 2007). Following membrane recruitment, PDK1 phosphorylates Akt at one site (T308 in Akt1) in the kinase domain while mTOR phosphorylates Akt at another site (S473 in Akt1) in a C-terminal hydrophobic motif (reviewed in Guertin and Sabatini, 2007; Hanada et al., 2004; Manning and Cantley, 2007).

The mTOR kinase, which is the mammalian target of the drug rapamycin, assembles into at least two distinct complexes, called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), each of which has unique substrates (reviewed in

SIGNIFICANCE

Small-molecule inhibitors that compromise cancer but not normal cell functions would be valuable anticancer therapeutics. However, identifying intracellular targets for this type of inhibitor is challenging. Here, we present genetic evidence that mTOR complex 2 (mTORC2) is a candidate target for such an inhibitor, as the development of invasive prostate cancer induced by Pten loss in mice requires mTORC2 activity. However, mTORC2 activity is dispensable for the development of normal prostate epithelium in mice and for the proliferation and survival of primary mouse fibroblasts in culture. PTEN loss activates the PI3K signaling pathway, which is inappropriately activated in many human cancers. Our findings suggest that mTORC2 inhibitors could have broad clinical applications.
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Guertin and Sabatini, 2007). In addition to mTOR, mTORC1 contains Raptor, PRAS40, and mLST8/G3L and regulates cell growth by controlling the activity of the S6 kinases and the 4E-BP proteins. mTORC2 contains mLST8/G3L, two unique regulatory proteins named Rictor and SIN1, and a protein of unknown function called PROTOR/P RR5. When assembled into mTORC2, mTOR phosphorylates Akt (Guertin et al., 2006; Jacinto et al., 2006; Sarbassov et al., 2005; Shiotia et al., 2006). Rapamycin, despite being a potent inhibitor of mTORC1, does not appear to be a general inhibitor of mTORC2. In a subset of human cancer cells, rapamycin inhibits mTORC2 by preventing its assembly, but the determinants of this phenomenon are unknown (Sarbassov et al., 2006).

Efforts to develop PI3K inhibitors or inhibitors of downstream effectors like Akt are intensive. One Akt-regulated pathway receiving considerable interest from drug development enterprises is the mTORC1 growth pathway (reviewed in Abraham and Eng, 2008). Akt activates mTORC1 by phosphorylating and inhibiting TSC2, a GTPase-activating protein that negatively regulates mTORC1 activity (reviewed in Manning and Cantley, 2007). Akt also activates mTORC1 directly by phosphorylating the PRAS40 subunit (Vander Haar et al., 2007; Huang and Porter, 2005; Kovacina et al., 2003; Sancak et al., 2007). Other well-known targets of Akt include the FoxO transcription factors and the GSK3 kinase (reviewed in Manning and Cantley, 2007).

Genetic studies in mice have been crucial to understanding the role of PI3K activation in tumorogenesis (Salmena et al., 2008). Pten heterozygous mice spontaneously develop neoplasms in multiple organs, resulting in a shortened life span (Di Cristofano et al., 1998; Freeman et al., 2006; Podsypanina et al., 1999; Suzuki et al., 1998a). Reports demonstrating that mice expressing a hypomorphic allele of Pdk1 or lacking Akt1 (one of three Akt genes in mammals) are protected from tumor development induced by Pten loss highlight the importance of Akt activity (Baycascas et al., 2005; Chen et al., 2006).

Mice expressing conditional alleles of Pten have also been developed to study the role of PTEN in tissue-specific cancers (Backman et al., 2001; Trotman et al., 2003; Wang et al., 2003). Human prostate cancer in particular shows strong association with PTEN loss (Dahia, 2000; Sellers and Sawyers, 2002; Suzuki et al., 1998b). Expression of Cre recombinase in prostate epithelial cells of Pten conditional mice results in Pten deletion and Akt hyperphosphorylation in the prostate epithelium (Wang et al., 2003). These mice develop epithelial hyperplasia, which progresses to murine prostatic intraepithelial neoplasia (mPIN) within 6 weeks of age and to invasive adenocarcinoma and metastasis by 9–12 weeks in a manner that recapitulates the disease progression in humans. Similar to human prostate cancer, tumors in these mice derive from the prostate epithelium, respond to androgen ablation, and exhibit similar gene expression changes, making them a useful model to study the disease.

An ideal target for an anticancer drug is one that, when inhibited, has no effect on normal cells but compromises the proliferation and/or survival of cancer cells. We hypothesized that mTORC2 may be such a target in cancers driven by PI3K activation because previous studies have shown that it is not required for survival of mouse embryonic fibroblasts (MEFs) or development of Drosophila embryos (Guertin et al., 2006; Hietakangas and Cohen, 2007; Jacinto et al., 2006; Shiota et al., 2006). The role of mTORC2 in tumorigenesis cannot be tested pharmacologically because specific inhibitors of mTORC2 are currently unavailable. Therefore, to test our hypothesis, we investigated the role of mTORC2 activity in (1) a human prostate cancer epithelial cell line lacking Pten expression, (2) a spontaneous mouse model of cancer dependent upon Pten loss, and (3) a mouse model of prostate cancer initiated by Pten deletion specifically in prostate epithelial cells.

RESULTS

Rictor Is Required for PC-3 Cells to Form Tumors as Xenografts

Human prostate cancer is associated with loss of the PTEN tumor suppressor (Dahia, 2000; Sellers and Sawyers, 2002; Suzuki et al., 1998b). To test whether Pten-deficient human prostate cancer cells require mTORC2 activity to form tumors, we investigated whether knocking down Rictor in the PC-3 cell line (a human prostate cancer cell line null for PTEN) impairs their ability to form tumors when injected into nude mice. Two independent short hairpin RNAs (shRNAs) targeting Rictor (shRictor1 and shRictor2) or a control shRNA targeting luciferase (shLuc) were delivered by lentivirus and stably expressed in PC-3 cells. We selected hairpins that differentially decrease Rictor expression and showed that, in vitro, shRictor1 robustly decreases total Rictor protein while shRictor2 reduces Rictor to an intermediate degree (Figure 1A). Both Rictor hairpins reduced AktS473 phosphorylation to levels consistent with the level of Rictor knockdown (Figure 1A). In vitro, both shRictor1 and shRictor2 knockdown cells had a proliferation defect, the severity of which also correlated with the level of Rictor knockdown (Figure 1B).

To determine whether Rictor depletion affects the ability of PC-3 cells to form solid tumors in vivo, we injected PC-3 cells expressing shLuc, shRictor1, or shRictor2 subcutaneously into nude mice and monitored tumor formation for 28 days, at which point tumors were removed and measured. PC-3 cells expressing shLuc formed tumors visibly larger than tumors formed by PC-3 cells expressing either shRictor1 or shRictor2 (Figure 1C). Tumors that developed from shLuc-expressing cells grew to an average volume of 312 mm3 (Figure 1D). In contrast, tumors that developed from shRictor1- and shRictor2-expressing cells grew to an average volume of 10.1 mm3 (p = 0.01) and 39.6 mm3 (p = 0.01), respectively, with the difference in tumor volume again corresponding to Rictor knockdown levels (Figure 1D). Our findings indicate that PC-3 cells require mTORC2 activity to form tumors in vivo.

Partial Loss of mTORC2 Activity Extends the Life Span of Pten−/− Mice and Can Protect Mice against Prostate Cancer

Our xenograft study suggested that mTORC2 activity may be important in prostate cancer development when the PTEN tumor suppressor is lost. We next asked whether reducing mTORC1 and/or mTORC2 activity in a genetic model of cancer dependent upon Pten loss could extend life span. Pten−/− mice spontaneously develop tumors in multiple organs, resulting in a shortened life span (Di Cristofano et al., 1998; Freeman et al., 2006; Podsypanina et al., 1999; Suzuki et al., 1998a). We crossed...
Pten−/− mice with mice heterozygous for the mtor, Raptor, mlst8, or Rictor genes, and the offspring were monitored for 52 weeks. Interestingly, Pten−/− mtor+/− (p = 0.023) and Pten−/− mlst8+/− (p = 0.044) mice lived longer than strain-matched Pten−/− controls (Figure 2A). In contrast, Pten−/− Raptor−/− mice showed no difference in life span compared to Pten−/− controls. Pten−/− Rictor−/− mice also tended to live longer than Pten−/− mice, but the effect was less pronounced due to a difference in the Rictor+− mouse strain composition (see Experimental Procedures and Freeman et al., 2006). In developing mouse embryos, only mTORC2 requires mlst8, while mTORC1 requires mtor, Rictor, and Raptor (Guertin et al., 2006). Thus, life-span extension of Pten−/− mice doubly heterozygous for mtor, mlst8, or Rictor likely reflects a reduced capacity for mTORC2 signaling, although this does not rule out the possibility that reducing mTORC1 activity may also contribute to extending life span.

To confirm that deleting one Rictor gene reduces mTORC2 signaling, we examined Akt activity in Rictor heterozygous MEFs. We found that deleting one Rictor gene reduced the serum-stimulated in vitro kinase activity of Akt (Figure 2B) and that this correlated with reduced AktS473 phosphorylation (Figure 2C). To generate MEFs null for Pten and also missing only one Rictor gene, we infected PtenloxP/loxP and PtenloxP/loxP RictorloxP/loxP MEFs with a control adenovirus or adenovirus expressing Cre recombinase (Adeno-Cre) and investigated whether partial loss of Rictor impairs Pten deletion-induced AktS473 phosphorylation. We found that deleting one Rictor gene slightly reduced the increase in AktS473 phosphorylation caused by Pten deletion and prevented insulin from further increasing the phospho-AktS473 signal (Figure 2D). Moreover, loss of one Rictor gene reduced the expected Mendelian ratio at birth (Guertin et al., 2006) and decreased AktS473 phosphorylation in liver tissue (Yang et al., 2006). Thus, Rictor is a haploinsufficient gene, and deleting one copy diminishes mTORC2 activity.

Because we were interested in the role of mTORC2 in prostate cancer, we examined the prostate tissue of offspring born from Pten−/− and Rictor−/− crosses that had survived for 1 year. Five of nine Pten−/− male mice had visible tumors, while only one of ten surviving Pten−/− Rictor−/− mice had a similar phenotype (Figure 2E). No tumors were detectable in any of the wild-type controls (n = 10). A comparison of hematoxylin and eosin (H&E)-stained sections generated from these samples indicated that Pten−/− mice developed severe neoplasia (Figure 2F). Prostates from Pten−/− Rictor−/− mice exhibited signs of hyperplasia (Figure 2F). However, in contrast to the lesions observed in Pten−/− mice, the lesions in the double-heterozygous mice appeared less severe and contained larger cells. There also were fewer proliferating cells in the Pten−/− Rictor−/− samples, as only 2.3% (19 of 836) of the cells in three representative images were positive for the proliferation marker Ki67, compared with 10.7% (203 of 1902) of the cells from Pten−/− samples (p < 0.001) (Figure 2F). Thus, the haploinsufficiency associated with loss of one Rictor gene protects Pten−/− mice from prostate cancer. Because of a difference in genetic background between Rictor−/− mice and mtor, Raptor, and mlst8 heterozygous mice, Pten−/− controls born from crosses between Pten−/− and mtor−/−, Raptor−/−, or mlst8−/− parents are less susceptible to prostate cancer, and this precludes an analysis of the disease in these cohorts (Freeman et al., 2006; Guertin et al., 2006).

**Rictor Is Required for Pten Deletion-Induced Akt Phosphorylation and Transformation of Prostate Epithelial Cells In Vivo**

The observation that partial loss of Rictor protects Pten−/− mice from prostate cancer prompted us to examine this role of
Figure 2. Phenotypes Associated with Pten Heterozygosity Require mTORC2
(A) Pten<sup>+/−</sup> mice lacking one mtor, mls8, or Rictor gene tend to live longer. Survival was monitored for 1 year and is displayed using Kaplan-Meyer plots.
(B and C) Rictor<sup>−/−</sup> mouse embryonic fibroblasts (MEFs) have reduced Akt activity. (B) Akt was isolated from serum-deprived (starved) or stimulated (+FBS) MEFs that were wild-type or deleted for one or both Rictor genes. The ability of immunopurified Akt to incorporate radiolabeled phosphate into a synthetic substrate was measured. Error bars indicate standard error of the mean.
(C) Immunoblot corresponding to (B), showing phospho-Akt<sup>S473</sup> and total Akt levels.
(D) Adeno-Cre was added to MEFs harboring conditional alleles of Pten and Rictor (Pten<sup>L/L</sup> and Pten<sup>L/L</sup>Rictor<sup>L/+</sup>) to generate Pten null MEFs lacking only one Rictor gene. Empty vector was added to generate control cells. Lysates were prepared from serum-deprived cells that were stimulated with 0, 1, 10, or 100 nm insulin for 10 min and probed with the indicated antibodies.
(E and F) Pten<sup>−/−</sup> Rictor<sup>−/−</sup> mice are protected against prostate tumor development.
(E) Genitourinary tracts of wild-type, Pten<sup>+/−</sup>, and Pten<sup>−/−</sup> Rictor<sup>−/−</sup> mice that survived to 1 year were dissected and photographed. Bladder (B) and anterior prostate (AP) are indicated for orientation. An example of a large tumor associated with a Pten<sup>−/−</sup> prostate is circled with a dashed white line.
(F) Pten<sup>−/−</sup> and Pten<sup>−/−</sup> Rictor<sup>−/−</sup> prostate tissue was sectioned and stained with hematoxylin and eosin (H&E, top) or labeled with Ki67 antibody (bottom). Representative images are shown. The asterisk in the upper right image indicates a normal prostate ductule, and the arrow points to a ductule containing large hyperplastic cells. Scale bars = 50 μm (top) and 25 μm (bottom).
mTORC2 more rigorously. Conditional deletion of Pten specifically in the prostate epithelium leads to prostate cancer with short latency and a histological pattern of disease progression modeling that of human patients with prostate cancer (Wang et al., 2003). In this model, expression of Cre recombinase from a modified rat probasin (PB) promoter (Wu et al., 2001) in Pten conditional mice (PtenloxP/loxP-PB-Cre+) induces postnatal deletion of Pten in prostate epithelial cells (Wang et al., 2003; Wu et al., 2001). PtenloxP/loxP-PB-Cre+ mice develop mPIN by 6 weeks of age, which progresses to invasive adenocarcinoma by 9 weeks and then to metastatic cancer by 12 weeks (Wang et al., 2003). To determine the role of mTORC2 in the progression of cancer in this model, we used conditional alleles to delete Rictor in combination with Pten.

To test the efficiency of the double-knockout strategy, primary MEFs derived from PtenloxP/loxP or PtenloxP/loxPRictorloxP/loxP embryos were infected with Adeno-Cre and cultured for 5 days. PTEN and Rictor protein and Akt phosphorylation levels were then measured by western analysis. As expected, infection of PtenloxP/loxP MEFs with Adeno-Cre reduced PTEN expression and elevated both AktS473 and AktT308 phosphorylation in serum-deprived cells (Figure 3A, lanes 1 and 3) and in insulin-stimulated cells (Figure 3A, lanes 2 and 4). In contrast, PtenloxP/loxPRictorloxP/loxP MEFs infected with Adeno-Cre showed no increase in AktS473 phosphorylation despite reduced Pten expression (Figure 3A, lanes 5 and 7). Furthermore, these MEFs exhibited only a small increase in S473 phosphorylation following insulin stimulation (Figure 3A, lanes 6 and 8). This slight increase in S473 phosphorylation in insulin-stimulated PtenloxP/loxPRictorloxP/loxP MEFs likely resulted from a subpopulation of cells that Adeno-Cre failed to infect, as an immunoblot analysis clearly showed that not all Rictor was lost. As expected in MEFs, insulin regulation of AktT308 phosphorylation and S6K1T389 phosphorylation (a reporter for mTORC1 activity) was unaffected by Rictor deletion (Figure 3; Guertin et al., 2006).

We then asked whether conditional deletion of Rictor inhibits the increase in AktS473 phosphorylation induced by Pten deletion in the prostate. PCR analysis of prostate tissue samples from Pten conditional mice harboring either one or two conditional alleles of Rictor and the PB-Cre+ transgene indicated that recombination occurred simultaneously in the prostate epithelium, but not in tail or bladder tissue prepared from the same animals (Figure 3B). Next, we generated protein lysates from dorsolateral prostate tissue of wild-type, PtenloxP/loxP-PB-Cre+, and PtenloxP/loxPRictorloxP/loxP-PB-Cre+ animals. By immunoblot analysis, we found that mTOR protein levels remained unchanged in all three genotypes (Figure 3C). In contrast, total Rictor protein increased in PtenloxP/loxP-PB-Cre+ prostate tissue but was mostly absent in PtenloxP/loxPRictorloxP/loxP-PB-Cre+ tissue (Figure 3C). As expected, Pten loss substantially elevated both AktT308 and AktS473 phosphorylation. Importantly, the increase in phospho-AktS473 was suppressed in PtenloxP/loxPRictorloxP/loxP-PB-Cre+ tissue, indicating that AktS473 phosphorylation caused by Pten deletion requires mTORC2. Interestingly, we found that the increase in T308 phosphorylation induced by Pten loss also requires mTORC2. This is different from what we observed in MEFs (Figure 3A; Guertin et al., 2006) but is more similar to the situation in human cancer cells, wherein phosphorylation at T308 requires mTORC2 activity (Figure 1A; Hresko and Mueckler, 2005; Sarbassov et al., 2005).

Genitourinary tracts were isolated from 7-week-old wild-type, Rictor conditional, Pten conditional, or doubly conditional PB-Cre+ mice. H&E staining and immunohistochemistry (IHC) with a Rictor antibody performed on dorsolateral prostate tissue indicated that, compared to wild-type epithelial cells, RictorloxP/loxP-PB-Cre+ cells are slightly smaller, but the overall architecture of the prostate epithelial cell layer is normal (Figure 3D; see also Figure S1 available online). Consistent with this observation, immortalized Rictor null MEFs were smaller than wild-type MEFs in volume by ~10%, while proliferating at the same rate (Figure S1). We have examined the histology of prostate tissue from RictorloxP/loxP-PB-Cre+ mice as old as 16 weeks and have found no obvious differences compared to age-matched wild-type tissue (data not shown). In both wild-type and RictorloxP/loxP-PB-Cre+ tissue, AktS473 phosphorylation is undetectable (Figure 3D). Thus, Rictor, and by extension mTORC2, is not required to maintain the integrity of normal prostate epithelium.

Consistent with previous studies, PtenloxP/loxP-PB-Cre+ mice developed mPIN, which is characterized by extensive epithelial cell hyperplasia within preexisting ductules (Figure 3D), by 7 weeks of age. In general, the individual prostate ductules were enlarged but remained intact with no signs of a desmoplastic response in the surrounding stromal tissue. The individual atypical epithelial cells were clearly larger than wild-type cells and were positive for AktS473 phosphorylation, with the most intense signal at the cell membrane. Pten deletion also appeared to increase total Rictor protein level, consistent with the immunoblot data in Figure 3C, and suggests possible feedback activation of Rictor gene expression.

In contrast, prostatic ductules from PtenloxP/loxP-RictorloxP/loxP PB-Cre+ mice exhibited a mixed phenotype containing mostly normal, organized epithelial cells and patches of large, disorganized hyperplastic cells (Figure 3D). We suspected that Rictor protein expression and stained positively for phosphorylated AktS473 in T308 expression at the membrane and cytoplasm (Figure 3D). AktS473-negative cells appeared normal. A recent study has suggested that Pten loss downregulates the probasin promoter, which could explain the recombination inefficiency (Jiao et al., 2007; Lei et al., 2006).

By 9–10 weeks of age, prostate adenocarcinoma was detectable in PtenloxP/loxP-PB-Cre+ mice. Nearly all of the dorsolateral lobes at this age contained extensive malignant cells characterized by AktS473 phosphorylation, especially at the membrane (Figure 4A). Compared to wild-type tissue, the diseased glands were enlarged and the ductule boundaries disorganized. Invasive
AktS473-positive cells were also detectable in the surrounding stroma, which was characterized by hypercellularity (Figure 4B). Ki67 staining indicated that the malignant cells were highly proliferative, with proliferating cells concentrating at the stromal interface and invading into the stromal region (Figure 4C). Invasive adenocarcinoma induces a phenomenon called the desmoplastic response, in which collagenous material is deposited in the surrounding stromal tissue. Trichrome stain, which marks collagen blue and fibrin pink, revealed a recruitment of connective tissues to the diseased areas (Figure 4C). Rictor protein was clearly detectable in malignant cells, and notably, some cells at the stromal interface expressed much higher levels of Rictor protein (Figure 4C). In contrast to prostate epithelial cells from PtenLoxP/LoxP PB-Cre+ mice, those from 9- to 10-week-old PtenLoxP/LoxP RictorloxP/loxP PB-Cre+ mice were protected from transformation (Figures 4A and 4B). Individual ductules in many cases were more similar in appearance to wild-type prostate tissue, with little evidence of hyperplasia. Nearly all of these cells were

AktS473-positive cells were also detectable in the surrounding stroma, which was characterized by hypercellularity (Figure 4B). Ki67 staining indicated that the malignant cells were highly proliferative, with proliferating cells concentrating at the stromal inter-

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Figure 3. Pten Deletion-Induced Phosphorylation of AktS473 in MEFs and Prostate Epithelial Cells Requires Rictor
(A) Deleting Rictor in combination with Pten blocks hyperphosphorylation of AktS473 in MEFs. PtenLoxP/LoxP or PtenLoxP/LoxPRictorloxP/loxP MEFs were infected with Adeno-Cre virus. After 5 days, cells were starved and stimulated with 300 nm insulin for 15 min. Protein lysates were prepared and probed with the indicated antibodies. For the Rictor immunoblot, “long” indicates longer exposure.
(B) The PB-Cre transgene induces recombination of Pten and Rictor conditional alleles only in prostate tissue, and not in bladder or tail tissue. A sample set of PCR reactions is shown. “DLV” indicates pooled tissue from dorsal, lateral, and ventral prostate.
(C) Deleting Rictor in combination with Pten inhibits AktS473 phosphorylation and activity toward downstream substrates in vivo. Protein lysates were prepared from dorsolateral prostates dissected from wild-type, PtenLoxP/LoxPPB-Cre+, or PtenLoxP/LoxPRictorloxP/loxPPB-Cre+ mice and probed with the indicated antibodies.
(D and E) Rictor deletion does not affect normal prostate architecture but is required for AktS473 phosphorylation induced by Pten deletion.
(D) Serial sections of 7-week-old prostate tissue stained with H&E or labeled with antibodies to Rictor or phospho-AktS473 are shown. Arrows point to a patch of cells in which inefficient Rictor deletion results in abnormal AktS473-positive cells. Scale bars = 50 μm.
(E) Cells lacking PTEN and phospho-AktS473 appear normal (circled area), consistent with loss of mTORC2 activity blocking transformation. For comparison, this sample includes a nearby section of hyperplastic cells (indicated by asterisk) that stain negative for PTEN and positive for AktS473, indicative of inefficient Rictor deletion. Scale bar = 50 μm.
nonproliferating as determined by Ki67 staining, and there was no desmoplastic response (Figure 4C). Consistent with the inefficient \textit{Rictor} deletion noted above, we again observed patches of AktS473-positive epithelial cells (Figures 4A and 4B). This often resulted in AktS473-positive epithelial cells mixed among many cells that did not stain for AktS473 (Figures 4A and 4B). In some instances, we observed more extensive hyperplasia of large AktS473-positive cells (Figure 4B); however, the phenotype was less severe, as the epithelial-stromal boundaries were intact and invasive cells were not detectable. A proliferation increase was associated with the patches of hyperplastic cells in the double deletion, but not to the extent seen in \textit{Pten}^{IoxP/IoxP-PB-Cre}\textsuperscript{+} tissue (Figure 4C). Furthermore, connective tissue was not recruited to the surrounding stroma (Figure 4C). As expected, the AktS473-positive cells in the double-deletion tissue costained for Rictor protein, although the signal was less pronounced compared to that in \textit{Pten}^{IoxP/IoxP-PB-Cre}\textsuperscript{+} tissue (Figure 4C). Perhaps in these cases, only one allele of \textit{Rictor} was deleted.

A close inspection of Rictor protein expression in \textit{Pten-deficient} prostate epithelium indicated that Rictor was often localized to cell membranes, particularly in the luminal epithelial cells (Figure 4C; Figure S2). This suggests that a fraction of active mTORC2 colocalizes with Akt at cell membranes. Collectively, these results argue that loss of \textit{Rictor} expression does not impair development of the prostate epithelium but protects cells from transformation caused by \textit{Pten} deletion.

**\textit{Rictor} Is Required for Akt Signaling**

Deletion of \textit{Rictor} in MEFs ablates AktS473 phosphorylation, and this partly impairs the ability of Akt to phosphorylate FoxO, but not TSC2 or GSK3\textbeta, suggesting that Akt retains activity in these cells (Guertin et al., 2006; Jacinto et al., 2006). Therefore, we asked whether Akt is still capable of phosphorylating downstream substrates in \textit{Pten}^{IoxP/IoxP-Rictor\textsuperscript{loxP}/IoxP-PB-Cre}\textsuperscript{+} tissue. Akt phosphorylates the FoxO transcription factors, and this inhibits their activity by excluding them from the nucleus. Immunoblot analysis indicated that FoxO1T24 phosphorylation was increased in tissue lysates prepared from \textit{Pten}^{IoxP/IoxP-PB-Cre}\textsuperscript{+} mice, but not \textit{Pten}^{IoxP/IoxP-Rictor\textsuperscript{loxP}/IoxP-PB-Cre}\textsuperscript{+} mice (Figure 3C). We also examined FoxO1 localization. In wild-type prostate epithelial cells, AktS473 phosphorylation was undetectable, and a very faint FoxO1 signal was present in both the nucleus and cytoplasm of these cells (Figure 5A, left). In \textit{Pten}^{IoxP/IoxP-PB-Cre}\textsuperscript{+} prostates, FoxO1 was excluded from the nucleus and concentrated in the cytoplasm (Figure 5A, middle). In contrast to both wild-type and \textit{Pten}^{IoxP/IoxP-PB-Cre}\textsuperscript{+} prostate epithelial cells, we detected strong nuclear localization of FoxO1 in...
AktS473-negative cells in PtenloxP/loxPRictorloxP/loxPPB-Cre+ tissue (Figure 5A, right). Importantly, FoxO1 was excluded from the nucleus in neighboring AktS473-positive cells in the same tissue. Thus, the absence of Akt phosphorylation in PtenloxP/loxP RictorloxP/loxPPB-Cre+ cells reduces FoxO1 phosphorylation and promotes FoxO1 accumulation in the nucleus.

We next examined phosphorylation of the Akt substrates GSK3β and PRAS40 using antibodies to the Akt-dependent S9 or T246 phosphorylation sites, respectively. In prostate tissue lysates from wild-type, PtenloxP/loxP RictorloxP/loxPPB-Cre+ mice, total GSK3β and PRAS40 protein levels were unchanged (Figure 3C). In PtenloxP/loxPPB-Cre+ samples, Pten loss elevated GSK3βS9 and PRAS40T246 phosphorylation. However, the increases in GSK3βS9 and PRAS40T246 phosphorylation were greatly diminished in PtenloxP/loxPRictorloxP/loxPPB-Cre+ tissue samples (Figure 3C). By IHC analysis, GSK3βS9 phosphorylation was below detectable levels in wild-type sections (Figure 5B). Consistent with the immunoblot results, GSK3βS9 phosphorylation, like AktS473 phosphorylation, uniformly increased in all PtenloxP/loxPPB-Cre+ prostate epithelial cells. However, in PtenloxP/loxPRictorloxP/loxPPB-Cre+ prostate tissue, GSK3βS9 phosphorylation was only detectable in the subpopulation of AktS473-positive cells. We were unable to achieve reliable detection of PRAS40T246 phosphorylation by IHC.

Finally, we examined the activity of the mTORC1 pathway. TSC2, which Akt phosphorylates and inhibits, suppresses mTORC1 activity. Phosphorylation of S6S235/236, a target of the mTORC1 substrate S6K1, is commonly used as a reporter for activation of the mTORC1 pathway by IHC. In lysates from wild-type prostate tissue, S6S235/236 phosphorylation was undetectable, while in PtenloxP/loxPPB-Cre+ prostate tissue, phosphorylation increased (Figure 3C). However, total S6 levels also increased, and therefore it was unclear to what extent Pten loss affects the catalytic activity of mTORC1. Both S6S235/236 phosphorylation and total S6 levels in the PtenloxP/loxPPB-Cre+ prostate tissue lysates were comparable to that detected in wild-type lysates. Thus, whatever mechanism is responsible for increasing total S6 protein requires mTORC2. By IHC, S6 phosphorylation was undetectable in wild-type prostate epithelial cells (Figure 5B). Similar to the phospho-AktS473 signal, the phospho-S6S235/236 signal was increased in all cells
in the Pten$^{lox/PB-Cre}$ tissue samples; however, a subset of cells—particularly those near the stromal interface—exhibited a greater increase in phospho-S6 intensity. This variability may reflect the fact that mTORC1 activity is additionally sensitive to nutrient and oxygen availability. Again, consistent with the immunoblots, S6$^{Thr389/396}$ phosphorylation levels were comparable to those of wild-type cells in prostate tissue from Pten$^{lox/PB-Cre}$ Rictor$^{lox/PB-Cre}$ mice, and only in patches of AktS473 positive cells did we detect phospho-S6 signal (Figure 5B). These findings support a model in which Akt requires mTORC2-dependent phosphorylation of AktS473 in order to phosphorylate downstream substrates when Pten is deleted in prostate epithelial cells.

**DISCUSSION**

In this report, we investigate the role of mTORC2 in prostate cancer caused by Pten loss. First, we show that the Pten-deficient PC-3 cell line requires Rictor, an essential component of mTORC2, to form tumors in nude mice. Second, we find that the Rictor gene is haploinsufficient in mice and that Rictor heterozygous mice prone to develop prostate cancer are more resistant to the disease when expressing only one copy of the Rictor gene. Finally, we show that in vivo prostate epithelial cells require Rictor to be transformed by Pten deletion but that Rictor has no significant role by itself in maintaining the integrity of normal prostate epithelium. We propose that cancers driven by PTEN loss or aberrant PI3K activation require mTORC2 signaling and that targeting mTORC2 in these cancers could be a promising therapeutic strategy.

In cultured cells, mTOR, when assembled into mTORC2, is an AktS473 kinase (Sarbassov et al., 2005). Genetic studies in mice confirm that in the developing embryo, mTORC2 is the critical AktS473 kinase complex (Guertin et al., 2006; Jacinto et al., 2006; Shiota et al., 2006). Other AktS473 kinases have been described (reviewed in Bhaskar and Hay, 2007; Bozulic et al., 2008), leaving open the possibility that when cells become transformed, kinases other than mTOR could phosphorylate Akt S473. Our results argue that mTORC2 is the primary kinase that phosphorylates AktS473 in a Pten deletion-dependent model of prostate cancer.

The function of TORC2 as an AktS473 kinase is conserved in Drosophila and Dictyostelium (Hietakangas and Cohen, 2007; Lee et al., 2005; Sarbassov et al., 2005). Unlike developing mouse embryos, Drosophila embryos lacking mTORC2 activity are viable and display only minor growth defects (Guertin et al., 2006; Hietakangas and Cohen, 2007; Shiota et al., 2006). However, tissue overgrowth in the eye caused by dPten loss requires dTORC2 (Hietakangas and Cohen, 2007). Thus, the genetic requirement for TORC2 under conditions of high PI3K activity, but not necessarily under normal conditions, is conserved.

**Inhibition of mTOR in Cancer Therapy**

The most developed mTOR inhibitor for use in oncology is rapamycin. Initial studies suggested that rapamycin is effective against tumors with PTEN loss (Bjornsti and Houghton, 2004; Rowinsky, 2004; Vignot et al., 2005). However, clinical studies have shown variable and unpredictable successes with rapamycin as an anticancer agent (Chiang and Abraham, 2007). In cells, rapamycin binds to its intracellular receptor FKBP12, and together, the rapamycin-FKBP12 complex binds to mTOR adjacent to the kinase domain, inhibiting its in vivo activity toward S6K1 at nanomolar concentrations. Numerous studies show that S6K1$^{Thr389}$ phosphorylation, but not AktS473 phosphorylation, is potently inhibited by rapamycin in most cell types, suggesting that rapamycin is an mTORC1-specific inhibitor.

It is now appreciated that prolonged exposure to rapamycin inhibits both S6K1$^{Thr389}$ and AktS473 phosphorylation in a subset of cell types (Phung et al., 2006; Sarbassov et al., 2006; Zeng et al., 2006). The mechanism of mTORC2 inhibition by rapamycin is under investigation, as rapamycin-FKBP12 binds mTORC1 but not mTORC2 (Sarbassov et al., 2004). However, rapamycin-FKBP12 also binds newly synthesized mTOR, and in some cells this appears to block mTORC2 activity by preventing the assembly of new mTORC2 complexes (Sarbassov et al., 2006). Why this phenomenon is not more ubiquitous is a mystery. Part of the reason could be that in many cells, a negative feedback loop masks this effect. Studies have shown that inhibiting mTORC1 releases PI3K signaling from negative feedback inhibition, resulting in strong Akt activation (Chiang and Abraham, 2007; Manning, 2004). According to this model, rapamycin would inhibit both mTORC1 and mTORC2 but push upstream Akt signaling in two directions: inhibiting Akt by blocking mTORC2 assembly, and activating Akt by releasing PI3K from mTORC1 negative feedback inhibition. This would generate counteractive signals and might result in no net observable difference in Akt phosphorylation levels. There are currently no cancer cell biomarkers predictive of rapamycin sensitivity. Interestingly, rapamycin inhibits mTOR at micromolar concentrations independently of FKBP12 (Shor et al., 2008). This phenomenon, termed the “high-dose effect,” broadens the effectiveness of rapamycin and correlates with inhibition of both mTORC1 and mTORC2.

Next-generation mTOR inhibitors will likely target the catalytic domain of mTOR and thus achieve universal inhibition of both mTOR complexes. It has been suggested that Akt-driven transformation of some cell types preferentially requires mTORC1 activity (Skeen et al., 2006). An mTOR kinase inhibitor would therefore target both an upstream activator and a downstream target of Akt, the major caveat being that such a drug could exhibit considerable toxicity in vivo. In theory, inhibiting the catalytic activity of both complexes might circumvent issues with feedback activation of Akt following mTORC1 inhibition since mTORC2 would also be inactive. However, if mTOR activity is not completely inhibited for technical reasons (i.e., due to a resistant pool of mTORCs) or cannot be inhibited for dose-dependent toxicity reasons, then a negative feedback loop in some cells could still be problematic. Until such a class of drugs is available, we can only speculate on their effectiveness.

**Potential Utility of an mTORC2-Specific Inhibitor**

In this report, we present evidence that an mTORC2-specific inhibitor might be a promising anticancer therapeutic. We believe that mTORC2 is an attractive target for drug development for two reasons. First, targeting only mTORC2 may not result in feedback activation of Akt signaling since there would...
be no direct inhibition of mTORC1. Second, an mTORC2 inhibitor may be well tolerated since mTORC2’s activity is not required in normal prostate epithelial cells (or in cultured MEFs); rather it is required only for prostate epithelial cell transformation when Pten is deleted. This may be evidence for a therapeutic window in which inhibition of mTORC2 could be more deleterious to cancer cells than to normal cells. Because little is known about the physiological roles of mTORC2, it is difficult to predict what effects a theoretical mTORC2 inhibitor could have on other processes. Interestingly, a recent report indicates that muscle-specific deletion of Rictor in mice, while sufficient to block insulin-stimulated AktS473 phosphorylation, results in only a mild impairment in glucose tolerance (Kumar et al., 2008).

The extent to which other cancers require mTORC2 is unclear, although we expect that cancers driven by mutations directly promoting Akt signaling, such as activating mutations in PI3K, may also require mTORC2 activity. A recent study found that in some glioma cell lines, Rictor expression is increased, and this elevates mTORC2 activity and promotes anchorage-independent growth and tumor formation in xenografts (Masri et al., 2007). The increase in mTORC2 activity does not correlate with PTEN status, suggesting that aberrant upregulation of mTORC2 activity might occur independently of PI3K activity. This might define another class of cancers preferentially susceptible to an mTORC2 inhibitor. However, it was not determined whether other mutations activate the PI3K pathway in these cells.

It is currently unclear whether Akt is the only relevant mTORC2 target in cancer. The three mammalian Akt proteins (Akt1, 2, and 3) belong to a family of structurally related kinases called the AGC family, which in addition to Akt includes the S6K, PKC, SGK, and RSK kinases (Hanada et al., 2004). AGC kinases are coregulated by PDK1 phosphorylation and phosphorylation by a hydrophobic motif kinase. Because mTOR phosphorylates the hydrophobic motif sites of Akt (S473) and S6K (T389) when assembled into mTORC2 or mTORC1, respectively, it seems likely that other AGC kinases could be targeted by mTOR. The SGKs are good candidates for mTORC2 regulation in cancer because they are structurally very similar to Akt, are regulated by PI3K signaling, and have cell functions that both overlap with and are distinct from Akt (Tessler and Woodgett, 2006). It was recently shown that mTORC2 controls hydrophobic motif phosphorylation of SGK1, suggesting that tumors with SGK activation might also be good candidates for treatment with mTORC2 inhibitors (Garcia-Martinez and Alessi, 2008). mTORC2 also regulates phosphorylation of the hydrophobic motif site in PKC (S657), but it is not known whether this is a direct effect (Guertin et al., 2006; Sarbassov et al., 2004).

In addition to directly phosphorylating the hydrophobic motif of Akt, mTORC2 is required for the growth-factor-independent phosphorylation of Akt and PKC on the turn motif site, although the exact role of mTORC2 in this event is unclear (Facchinetti et al., 2008; Ikenoue et al., 2008). This constitutive phosphorylation event occurs during or shortly after translation and is important for maintaining protein stability. Interestingly, Hsp90 chaperones prevent Akt from degrading in mTORC2-deficient cells, in which Akt lacks both hydrophobic and turn motif phosphorylation (Facchinetti et al., 2008). These findings suggest that mTORC2 may have a broader role in regulating AGC kinases beyond hydrophobic motif phosphorylation and that combining mTORC2 inhibition with chaperone inhibitors could be an effective therapeutic strategy.

To develop mTORC2 inhibitors, we need detailed knowledge about the structure and assembly of the mTOR complexes. One possible strategy is to disrupt protein-protein interactions required for mTORC2 activity. For instance, dissociation of Rictor, mSin, or mLST8 from mTOR results in disassembly and inactivation of the complex (Frias et al., 2006; Guertin et al., 2006; Jacinto et al., 2006; Sarbassov et al., 2005). Another possible strategy is to inhibit kinase-substrate interactions, for example by disrupting the intracellular localization of the complex. We observed that Rictor concentrates at cell membranes in Pten-deficient prostate epithelial cells. Perhaps disrupting mTORC2 localization might prevent it from phosphorylating Akt.

Coregulation of Akt by PDK1 and mTORC2

One noteworthy finding from genetic studies of mTORC2 is that Akt has different sensitivities to mTORC2 inhibition in normal cells when compared to cancer cells. For example, Akt retains activity in Rictor null MEFs despite the absence of hydrophobic motif phosphorylation on S473 (this study; Guertin et al., 2006; Jacinto et al., 2006; Shiota et al., 2006). Hydrophobic motif phosphorylation is a prerequisite for PDK1 to phosphorylate S6K, SGK, and RSK, but this may not be the case for Akt even though full Akt activity in vitro requires phosphorylation at both sites (Alessi et al., 1996; Biondi et al., 2001; Collins et al., 2003). In MEFs, PDK1-dependent phosphorylation of AktT308 is unaffected by Rictor deletion, supporting the idea that loss of S473 phosphorylation is not sufficient to block Akt activity in some cells (this study; Guertin et al., 2006; Jacinto et al., 2006; Shiota et al., 2006). Interestingly, we found that downstream targets of Akt require mTORC2 activity to be phosphorylated in Pten-deficient prostate epithelial cells. We also found that, unlike in MEFs, mTORC2 is required for both AktS473 and AktT308 phosphorylation when Pten is deleted in prostate epithelial cells. This is similar to what is observed in human cancer cells, in which knocking down Rictor decreases both S473 and T308 phosphorylation (Hresko and Mueckler, 2005; Sarbassov et al., 2005). We are developing AktT308 antibodies for IHC, and our preliminary results are consistent with this finding (see Figure S3). This intriguing observation suggests that coregulation of Akt by mTORC2 and PDK1 is tethered in cancer cells, while in otherwise “normal” cells, these two inputs are uncoupled. This could explain why inhibiting mTORC2 in cancer cells is more deleterious to Akt activity than in normal cells.

EXPERIMENTAL PROCEDURES

RNAi

PC-3 cells stably expressing control or Rictor shRNAs were generated as described previously using a lentiviral system (Sarbassov et al., 2005). PC-3 cell lysates for immunoblot analysis were prepared 6 days postinfection. For proliferation analysis, cells were seeded at equal density onto 12-well cell culture dishes immediately following puromycin selection and counted each day with a Coulter counter. For xenografts, 1 × 106 PC-3 cells were injected in the back of pretrained (4 Gy) Ncr-nu/hu male mice, and tumors were monitored for 28 days.

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MEFs
Rictor-deficient MEFs have been described previously (Guertin et al., 2006). Akt activity was measured in MEFs using the Akt1/PKBζ Immunoprecipitation Kinase Assay Kit (Upstate). To generate Pten null Rictor heterozygous MEFs, we crossed PtenloxP/RictorloxP/ males with PtenloxP/RictorloxP females and prepared MEFs from E14 embryos. Adeno-Cre (Gene Transfer Vector Core, University of Iowa) was added directly to the medium on two consecutive days. Five days after initial infection, cells were starved for 6 hr in serum-free medium, stimulated with insulin for 10 min, and then lysed.

Mice
129/B6 Pten+−/+ mice (Podsypanina et al., 1999) were crossed with mtor+−/−, Raptor+−/−, or mstl+−/− mice of the same strain background or Rictor+−/− mice on a mixed 129/B6/BALB/c background (Guertin et al., 2006). The severity and latency of tumor development in Pten−/− mice varies depending on the strain composition (Freeman et al., 2006). To generate mice with conditional alleles, PtenloxP/RictorloxP/PB-Cre+ mice were crossed with PtenloxP/RictorloxP or PtenloxP/RictorloxP mice (all on a mixed background) (Shiota et al., 2006; Wang et al., 2003; Wu et al., 2001). Cre is expressed in all four lobes of the prostate, with expression being highest in the dorsolateral and ventral lobes. All procedures were approved by the Massachusetts Institute of Technology Division of Comparative Medicine’s Committee on Animal Care and conform to the legal mandates and federal guidelines for the care and maintenance of laboratory animals.

Protein Biochemistry
Cell lysates were prepared using conditions described in Kim et al. (2002). For tissue lysates, frozen samples were homogenized for 8 s in detergent-free tissue lysates, frozen samples were homogenized for 8 s in detergent-free TBST/5% normal goat serum. For primary antibodies, mTOR (#2893), Rictor (#50678), S6K1 (#9202), FoxO1 (#2880), GSK3α/β (#9323). Detection was performed using a DAKO CytoMation ARK kit (#K3954).

Immunohistochemistry
Slides were deparaffinized in three changes of xylene and rehydrated through graded ethanols. Antigen retrieval was performed using 10 mM citrate buffer (pH 6.0). Slides were quenched in 3% hydrogen peroxide and blocked with TBST/5% normal goat serum. Primary antibodies were diluted in TBST/5% normal goat serum (CST #858) or SignalStain Antibody Diluent (CST #8112). Secondary antibodies were detected using diaminobenzidene. IHC for Ki67 (BD Pharmingen #550609) was performed using a DakoCytomation ARK kit (#K3954).

SUPPLEMENTAL DATA
The Supplemental Data include three figures and can be found with this article online at http://www.cancercell.org/supplemental/S1535-6108(08)00436-4.

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