mTORC2 Signaling Drives the Development and Progression of Pancreatic Cancer


Abstract

mTOR signaling controls several critical cellular functions and is deregulated in many cancers, including pancreatic cancer. To date, most efforts have focused on inhibiting the mTORC1 complex. However, clinical trials of mTORC1 inhibitors in pancreatic cancer have failed, raising questions about this therapeutic approach. We employed a genetic approach to delete the obligate mTORC2 subunit Rictor and identified the critical times during which tumorigenesis requires mTORC2 signaling. Rictor deletion resulted in profoundly delayed tumorigenesis. Whereas previous studies showed most pancreatic tumors were insensitive to rapamycin, treatment with a dual mTORC1/2 inhibitor strongly suppressed tumorigenesis. In late-stage tumor-bearing mice, combined mTORC1/2 and PI3K inhibition significantly increased survival. Thus, targeting mTOR may be a potential therapeutic strategy in pancreatic cancer.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) accounts for 85% of cases of pancreatic cancer and is a highly aggressive form of the disease. Indeed, PDAC is predicted to become the second-leading cause of cancer death in the United States by 2030 (2). At diagnosis, the disease is characterized by local invasion and distant metastases, so that even the few patients eligible for resection inevitably develop recurrent or metastatic disease (1). Most systemic therapies are ineffective, thus PDAC is almost universally lethal, with a 5-year survival rate of about 7% (3). Up until the recent adoption of combination chemotherapy regimens, FOLFIRINOX, and gemcitabine-Abraxane, gemcitabine monotherapy had been the standard-of-care for patients with PDAC since 1997 (4). Yet, median survival with FOLFIRINOX or gemcitabine-Abraxane is 11 and 9 months, respectively. Thus, PDAC presents a significant therapeutic challenge and novel, effective therapies are required.

The genetic drivers of pancreatic cancer have been well-studied. Activating mutations in KRAS occur in about 90% of cases and are thought to be the initiating event driving the formation of precursor lesions termed pancreatic intraepithelial neoplasia (PanIN). Accumulation of further mutations in tumor suppressor genes, including CDKN2A, TP53, DPC4, and BRCA2, occurs throughout tumor progression (1). In fact, recent wide-scale genomic studies have revealed that hundreds of genetic aberrations accrue (5). PDAC is also histologically complex, and interactions between tumor cells and the microenvironment contribute to tumor progression. Indeed, the extensive desmoplastic stroma that characterizes PDAC may protect tumor cells from a number of stresses, including chemotherapy, although this is somewhat controversial (6). Therefore, a greater understanding of the signals that drive tumor progression is required.

Activated KRAS drives a complex signaling network, involving multiple downstream effectors, including the MEK/ERK, PI3K/AKT, and RAL pathways (7). The PI3K pathway regulates a number of processes integral to cancer, including cell growth, cell-cycle entry, cell survival, and metabolism (8). Indeed, PI3K signaling has been shown to be a key mediator of the effects of oncogenic KRAS in the pancreas (9). PI3K inhibitors have been developed for clinical trials; however, the pathway interacts with many other signals and there are several feedback loops making it difficult to predict the effects of pathway suppression (10).

One of the major downstream effectors of PI3K signaling is mTOR, a serine/threonine kinase that acts as part of 2 distinct complexes (11). The mTORC1 complex, which includes Raptor, is responsible for several well-studied functions of mTOR, including sensing nutrient stress and regulating translation...
through interaction with p70S6K and 4E-BP1 (11). p70S6K can also phosphorylate IRS1 on an inhibitory serine residue, thus causing negative feedback that inhibits insulin and IGF signaling to PI3K/AKT (12). Inhibition of mTORC1 can also lead to feedback activation of MEK/ERK signaling (13).

The mTORC2 complex, which includes the essential subunit RICTOR, regulates cell growth, metabolism, and survival and organization of the actin cytoskeleton through activation of AKT, SGK, and PKC (14, 15). We previously showed that high levels of phospho-AKTm7S in human PDAC were significantly associated with poor survival (16). More recently, RICTOR amplifications have been described in human lung cancers and melanomas (17, 18). However, as the tumor-promoting properties of mTORC2 signaling have only recently been identified (19, 20), a full understanding of the mechanisms underlying this effect is still developing.

Initial strategies to inhibit mTOR function focused on mTORC1 and the development of analogs of the mTORC1 inhibitor rapamycin. However, clinical trials using these compounds have proved disappointing (21). More recently, small-molecule inhibitors that block mTOR kinase activity and therefore impair both mTORC1 and mTORC2 have been developed. Not only do these inhibitors target both arms of mTOR signaling but also they target 4E-BP1 and thus translational initiation, more efficiently than rapalogs, which primarily inhibit 6S kinase (22). We recently showed that pancreatic tumors in mice bearing mutations in Kras and Pten, a negative regulator of PI3K/mTOR signaling, were exquisitely sensitive to rapamycin, whereas mice wild-type for Pten, but expressing mutant p53, were resistant (23). These findings suggest that mTORC2 signaling may play a critical role downstream of commonly occurring PDAC gene mutations and that inhibition of this complex may enhance therapeutic efficacy in PDAC.

Global deletion of Rictor or Raptor in the mouse embryo results in embryonic lethality (20); however, conditional models of Rictor and Raptor deletion that allow interrogation of tissue-specific roles of mTORC1 and 2 are now available (20). We have utilized pancreas-specific deletion of Rictor to discern the requirement for mTORC2 signaling during pancreatic tumorigenesis. We show that genetic inactivation of mTORC2 significantly blocks PanIN development, impairs progression to invasive carcinoma, and prolongs survival in a PDAC genetically engineered mouse model (GEMM). Moreover, pharmacologic inhibition of mTOR extends survival, even in mice with late-stage disease. Our findings demonstrate an important role for mTORC2 signaling during pancreatic tumorigenesis and support the use of mTOR inhibitors as part of therapeutic strategies in PDAC.

Materials and Methods

Animal experiments

Mice were maintained on a mixed background and given access to standard diet and water ad libitum. Ptf1a-cre, Pdx1-Cre, LSL-KrasG12D, Trp53R172H, and Rictorlox mice have been described previously (24–27). Experiments were approved by UMass Medical School IACUC or performed under UK Home Office regulations and license approved by the local ethics committee. For more information, see Supplementary Data.

Caerulein injections

Caerulein (50 μg/kg, Sigma) or vehicle control (0.05 mol/L NH₄OH in PBS) was injected intraperitoneally into 5- to 7-week-old mice as previously described (28).

In vivo treatment experiments

Mice were randomly assigned to cohorts. Treatments were administered as follows: AZD2014 (AstraZeneca), in 20% w/v captisol in H₂O, at 15 mg/kg by oral gavage (p.o.) daily; gemcitabine (LC labs), in PBS, at 100 mg/kg by intraperitoneal injection twice weekly; and AZD8186 (AstraZeneca), in 0.5% HPMC in H₂O, at 50 mg/kg p.o. twice daily.

Histology

Following necropsy, pancreata were formalin-fixed overnight, embedded in paraffin, and 4-μm to 5-μm sections mounted on positively charged slides. Hematoxylin and eosin (H&E) and quinacrine stainings were done as standard. A licensed pathologist blinded to genotype performed quantification of tissue area. See Supplementary Data for more information.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded (FFPE) sections using standard protocols. For further details and antibodies, see Supplementary Data and Supplementary Table S1.

RNAscope

In situ detection of Rictor transcripts in FFPE mouse PDAC samples was performed using a Rictor-specific RNAscope assay (Advanced Cell Diagnostics, 1:5) according to the manufacturer’s protocol.

Primary PanIN cell culture experiments

Primary PanIN lines (isolated in the laboratory of Bardeesy; ref. 29) were grown on laminin-coated plates as previously described (30) and infected with lentiviruses encoding Rictor or GFP-targeting shRNAs (Supplementary Table S2). Viable cells were counted using trypan blue exclusion.

SA-β-Galactosidase activity was assessed using a standard protocol. See Supplementary Data for more information.

PDAC cell lines

PDAC cell lines were generated in-house from KPC tumors as described previously (23). Testing was performed in-house to confirm that cells were mycoplasma-free. Cells were maintained at 37°C and 5% CO₂ in DMEM (Invitrogen) with 10% FBS (PAA), 2 mmol/L L-glutamine (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). Cells were treated with AZD2014 or AZD8186. Cell viability was measured using the Cell Titer Blue Assay (Promega).

Synergy assay in PDAC cell lines

Mouse PDAC cell line K8484 (established in-house from a KPC tumor) and human PDAC cell line MiaPaca2 [from the European Collection of Cell Cultures, UK, in 2009 (authenticated by STR genotyping)] were treated with AZD2014 and gemcitabine for 96 hours. Cell viability was determined by measuring total protein content using the sulforhodamine B assay. Combenefit software was used to analyze the data for potential synergy (http://www.cruk.cam.ac.uk/research-groups/jodrell-group/combenefit).

Western blotting

Cell lysates were prepared and Western blot analysis was performed using standard protocols. Antibodies are listed in Supplementary Table S3.
Results

Loss of Rictor does not impair pancreas differentiation

To determine the impact of mTORC2 inactivation on pancreas development, we generated mice lacking the essential mTORC2 subunit Rictor, specifically within the pancreas, by crossing mice bearing Ptf1a-cre and RictorloxP alleles (Supplementary Fig. S1A; refs. 24, 25). The absence of Rictor protein was confirmed by immunoblotting of protein lysates isolated from 2-month-old pancreata (Supplementary Fig. S1B). Mice lacking pancreatic Rictor were viable and showed no difference in body weight relative to their wild-type littermates (Supplementary Fig. S1C). However, Rictor-null pancreata weighed significantly less than Rictor-intact pancreata (Supplementary Fig. S1D). Measurement of cell size (Supplementary Fig. S1E) and quantification of the total amount of gDNA as a surrogate measurement for cell number (Supplementary Fig. S1F) demonstrated that the reduced pancreas size likely results from decreased cell numbers. Histologically, Rictor-null pancreata appeared normal, with the relative abundance of acinar, α- and β-cells similar to Rictor-intact pancreata, as determined by immunostaining for the markers amylase, glucagon, and insulin, respectively (Supplementary Fig. S1G). Glucose tolerance tests revealed that mice with Rictor-null pancreata did show impaired glucose metabolism compared with wild-type littermates (Supplementary Fig. S1H). Importantly, however, mice with Rictor-null pancreata were monitored for 500 days and did not exhibit any symptoms of ill health (n = 11).

mTORC2 deficiency impairs development and progression of KRASG12D-driven PanIN lesions

Both 3-phosphoinositide-dependent protein kinase 1 (PDK1) and mTORC2 are required for full activation of AKT and SGK family members. PDK1 is essential for the development of KRAS-driven PanIN lesions and PDAC (9). We therefore hypothesized that mTORC2 is similarly required for KRAS-driven tumorigenesis. The well-established Ptf1a-Cre+/−; LSL-KrasG12D/+;Ptenfl/fl mouse model develops PanIN lesions that mirror the human lesions that can progress to PDAC (31). To investigate the role of mTORC2 signaling in this model, Ptf1a-Cre+/−; LSL-KrasG12D/+; Rictorfl/fl (KC) and Ptf1a-Cre+/−; LSL-KrasG12D/+; Rictorfl/fl (KC, Rictorfl/fl) siblings were produced and their pancreata analyzed at 4 or 8 months of age. Consistent with published studies, significant replacement of acinar cells by PanIN lesions containing abundant reactive stroma was evident in KC mice at 4 months of age (Fig. 1A and B). In contrast, KC Rictorfl/fl pancreata displayed fewer PanIN lesions and significant retention of acinar tissue (Fig. 1A and B). Moreover, while KC pancreata displayed PanIN1B and PanIN2 lesions, precursors lesions observed in KC Rictorfl/fl pancreata were restricted to PanIN1A. Quadruple staining of the mucins produced by PanIN cells and collagen deposited by the reactive stromal cells highlighted the differences between pancreata of the 2 genotypes (Fig. 1A). Importantly, Rictor RNAseq showed that the PanINs arising in KC Rictorfl/fl pancreata lacked Rictor RNA, ruling out the possibility that these cells had escaped recombination (Fig. 1A).

Acinar replacement in KC mice was nearly complete by 8 months, and most PanIN lesions had progressed to PanIN2 (Fig. 1C and D). This KRAS-driven phenotype is significantly impaired in KC Rictorfl/fl pancreata, with retention of acinar tissue, fewer PanINs, and reduced reactive stroma (Fig. 1C and D). Again, the PanINs present still lacked Rictor RNA (Fig. 1C). Moreover, the PanINs present failed to progress beyond PanIN1 (Fig. 1D). Together, these data indicate that Rictor deletion impairs the development of KRAS-driven PanINs and blocks their progression to higher grade lesions.

Rictor-null PanIN lesions display impaired mTORC2 signaling

To determine the status of mTORC2 signaling in PanIN lesions, pancreata from 8-month-old mice were stained for mTORC2 targets. Relative to PanINs present in KC mice, KC Rictorfl/fl PanINs displayed significantly reduced mTORC2 signaling, as measured by phospho-AKT473 and phospho-NDRG1T346 (Fig. 2). The reduced activity was specific to mTORC2 as PDK1-dependent phosphorylation of AKT on Thr308 was unaffected, as was phosphorylation of the mTORC1 target S6Kinase, and the MAPK signaling components ERK1/2 (Fig. 2). Thus, the PanINs present in KC Rictorfl/fl pancreata have not escaped Rictor gene deletion and develop in the absence of active mTORC2 signaling.

Loss of mTORC2 signaling induces proliferative arrest

The lack of compensatory activation of other signaling pathways and the limited progression of KC Rictorfl/fl PanINs indicated fundamental phenotypic differences between KC and KC Rictor+/+ PanINs. We therefore compared the proliferation and apoptosis rates in PanINs of each genotype and found that the percentage of Ki67-positive nuclei in KC Rictor+/+ PanIN1 lesions was significantly lower than in KC PanIN1 lesions, at both 4 and 8 months (Supplementary Fig. S2A and S2B). Apoptosis rates, as assessed by cleaved caspase-3 staining, were very low in both genotypes and not different between the groups (Supplementary Fig. S2C and S2D). Together, these data suggest that mTORC2 inactivation impairs cell-cycle progression in early PanIN lesions.

The cyclin-dependent kinase (CDK) inhibitor p16ink4a has been proposed as a barrier to KRAS-driven PDAC development (32). We therefore assessed p16ink4a expression in KC and KC Rictor+/+ PanIN lesions by immunostaining. We found that the number of cells with nuclear localized p16ink4a was significantly increased in KC Rictor+/+ PanINs, relative to KC PanINs, at both the 4- and 8-month time points (Fig. 3A, Supplementary Fig. S3A). We also evaluated the CDK inhibitors p21cip1 and p27kip1 and found that the number of p21cip1- and p27kip1-positive nuclei was similar between groups at the 4-month time point (Fig. 3B and C; Supplementary Fig. S3B and S3C). However, we observed that while the number of positive nuclei decreased in PanIN lesions in 8-month-old KC mice, they remained elevated in KC Rictor+/+ PanIN lesions (Fig. 3B and C; Supplementary Fig. S3B and S3C). Thus, KC Rictor+/+ PanIN lesions display coordinate elevation of multiple CDK inhibitors.

The concomitant elevation in p16ink4a, p21cip1, and p27kip1 levels suggested the possibility that their expression may be regulated by a common factor. The polycomb repressor group complex 1 protein BMI1 is a negative regulator of the gene deletions in PanIN formation and has been implicated in p16ink4a expression and has been implicated in p21cip1 and p27kip1 downregulation (33). Moreover, BMI1 is required for KRAS-driven PanIN formation and progression (34). We therefore evaluated BMI1 expression in KC and KC Rictor+/+ PanIN1 lesions. We found that the percentage of cells with nuclear BMI1 staining was decreased in KC Rictor+/+ PanIN lesions relative to KC controls at both 4- and 8-month time points (Fig. 3D, Supplementary Fig. S3D).

We next sought to model the effect of mTORC2 inhibition in PanIN cells in an in vitro system. We performed shRNA-mediated knockdown of Rictor in 2 primary PanIN cell lines established...
from KC mice with early PanIN lesions (29). Rictor knockdown significantly reduced proliferation in both cell lines and enhanced the expression of senescence-associated β-galactosidase (Fig. 3E and F; Supplementary Fig. S3E). In contrast to our observations in vivo, however, we did not observe any changes in the expression of the CDK inhibitors p16INK4a, p21Cip1, or p27Kip1, nor did we
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observe changes in BM1 expression (Supplementary Fig. S3F and S3G). It is possible that this difference is due to transformation in culture, given that these cell lines are able to form tumors when transplanted into immunocompromised mice. Taken together, our data suggest that Rictor knockdown impairs PanIN development and progression, at least in part, by robustly blocking cell proliferation.

mTORC2 loss impairs PanIN progression following pancreas injury

Pancreatitis is a major risk factor for the development of PDAC and can be modeled in mice using caerulein-induced pancreatic injury (28). To determine whether Rictor deletion impacted the response to injury, we performed serial caerulein injections in 6-week-old wild-type and RictorA/A mice. Wild-type and RictorA/A mice displayed similar responses with stereotypical histologic changes - including acinar-to-ductal metaplasia (ADM), levels of which were not significantly altered by Rictor deletion, and increased separation of acinar lobules—evident 2 days following injection (Supplementary Fig. S4). As expected, injury was fully resolved by 21 days postinjection in wild-type mice and also in RictorA/A mice (Supplementary Fig. S4). These data suggest that Rictor is not required for pancreatic tissue repair, although it is possible that tissue repair could be delayed at earlier stages postinjury.

Expression of activated Kras enhanced the response to caerulein, with extensive ADM visible 2 days following treatment (Fig. 4A). However, Rictor deletion blunted ADM development (Fig. 4A). Moreover, while KC mice evaluated 21 days following treatment displayed complete replacement of the pancreas parenchyma with PanIN lesions, many of which had progressed to PanIN1B and PanIN2, KC RictorA/A pancreata displayed significant retention of normal acinar tissue and significantly fewer PanIN1B and PanIN2 lesions (Fig. 4A and B).

Evaluation of mTORC2 signaling via immunostaining for p-AKT demonstrated that mTORC2 activity was impaired in KC RictorA/A lesions compared with KC lesions (Fig. 4C). In contrast, p-AKT staining was equivalent between both genotypes (Fig. 4C). Intriguingly, we observed that p-ERK staining was elevated in KC lesions relative to KC RictorA/A lesions, suggesting that mTORC2 signaling is required for activation of the MEK/ERK signaling cascade following injury-mediated PanIN progression (Fig. 4C). To ascertain whether similar mechanisms mediate inhibition of PanIN progression in this model, we assessed the presence of nuclear CDK inhibitors p16Ink4a, p21 and p27. We found that KC RictorA/A lesions had elevated levels of these markers (Fig. 4D–F; Supplementary Fig. S5A–S5C). Moreover, CDK inhibitor levels inversely correlated with nuclear BM1 localization (Fig. 4G and Supplementary Fig. S5D).

Assessment of CD45-positive infiltrating immune cells failed to show differences between KC and KC RictorA/A pancreata (Supplementary Fig. S5E). Further evaluation of the expression levels of inflammation-associated genes using the Nanostring platform demonstrated differences between the pancreata of KC and KC RictorA/A mice injected with PBS (Supplementary Fig. S5F). Notably, macrophage-associated genes, including Ifi6 and Stat3, which have been shown to be involved in macrophage-mediated inflammation in the KC mouse model, were reduced in the KC RictorA/A samples relative to the KC samples (35). However, while these differences remained, they were not further enhanced following caerulein treatment (Supplementary Fig. S5F). Together, these data suggest that the absence of mTORC2 signaling impairs the progression of PanIN lesions following pancreatic injury.

Rictor deletion delays tumor formation in KPC mice

Our data above demonstrated that Rictor gene deletion severely impairs PanIN development and progression. To determine whether Rictor deletion inhibits the progression from PanIN to PDAC in an aggressive model of pancreatic tumorigenesis, we crossed Rictor–/– mice with KPC (Pdx1-Cre;LSL-KrasG12D/wt) mice to generate KC RictorA/A mice. We found that Rictor deletion dramatically delayed tumor formation in the KPC model, with median survival almost doubled in KC RictorA/A mice compared with KPC RictorA/+ mice (Fig. 5A). Furthermore, 8 of 47 KC RictorA/A mice sacrificed because of other pathologies were found to be tumor-free at over 200 days old. Deletion of one copy of Rictor was insufficient to affect tumor development (Fig. 5A). Interestingly, we did not observe any apparent histologic or

Figure 2.
mTORC2, but not mTORC1, is functionally absent in KC RictorA/A PanINs. Immunostaining for pAKT, pNDRG1, pERK, and pSGK1 in pancreata from 8-month-old KC and KC RictorA/A mice (magnification, ×200).

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pathologic differences in the tumors that eventually developed in the KPC RictorΔΔ mice compared with those in KPC Rictor+/+ mice. However, using RNAscope, we confirmed the absence of Rictor RNA demonstrating that the tumors were not derived from cells that failed to delete the Rictor locus (Fig. 5B). These findings are supported by in vitro observations in murine PDAC cell lines in Figure 3. Rictor-deficient PanINs display increased nuclear expression of CDK inhibitors and decreased nuclear expression of BMI1. A-D, Quantification of nuclear staining in PanIN lesions in 4-month-old (left) and 8-month-old (right) KC and KC RictorΔΔ mice. p16Ink4a (A), p21Cip (B), p27Kip1 (C), and BMI1 (D). **, P < 0.01; *** P < 0.001 as determined by the Student t test in individual comparisons of cohorts. E, Doubling time of a PanIN-derived cell line 4 to 6 days following shRNA-mediated Rictor knockdown. P values determined by counter variance analysis of inverse slopes. F, Senescence-associated β-galactosidase positivity in a PanIN-derived cell line 4 days following shRNA-mediated Rictor knockdown. **, P < 0.01; *** P < 0.001 as determined by the Student t test.
which Rictor knockdown impairs cell proliferation (Supplementary Fig. S6A, S6B, S6D, and S6E) and reduces anchorage-independent growth (Supplementary Fig. S6C and S6F).

We next examined the effects of Rictor deletion on mTOR targets by immunohistochemistry. Rictor deletion had no effect on phosphorylation of the mTORC1 signaling targets, S6 and 4E-BP1, but as expected, completely abrogated phosphorylation of the mTORC2 downstream specific targets pAKT<sub>S473</sub> and pNDRG1 (Fig. 5C). We did not observe any differences in proliferation, as determined by Ki67 staining, or in p21 expression, which is perhaps unsurprising in advanced tumors (Fig. 5D). We did however observe slightly elevated nuclear p27 in KPC Rictor<sup>△△</sup> tumors and, interestingly, decreased pERK, suggesting that in this system Akt activation does not result in Raf/MEK/ERK inhibition (Fig. 5D). Taken together, our results show that mTORC2 signaling plays a significant role in pancreatic tumorigenesis and targeting mTORC2 could have therapeutic impact in this disease.
Pharmacological inhibition of mTORC1/2 delays tumor formation in KPC mice

To test this hypothesis, we used vistusertib (AZD2014), a dual mTORC1/2 inhibitor recently investigated in a phase I clinical trial (36). We previously showed that KPC mice were resistant to treatment with rapamycin and therefore not dependent on mTORC1 signaling (23), however, AZD2014 allowed us to evaluate the impact of inhibiting both mTOR signaling complexes using a clinically relevant inhibitor. We first tested AZD2014 in vitro using KPC tumor cell lines and showed that signaling downstream of both mTORC1 and mTORC2, assessed by immunoblotting for pAKT<sup>S473</sup>, pS6, and p4E-BP1, was effectively inhibited (Supplementary Fig. S7A). We next treated cohorts of KPC mice from 10 weeks of age with gemcitabine, AZD2014, or AZD2014 in combination with gemcitabine. This timepoint was selected to mimic surgically resectable disease and to exclude extrapancreatic pathologies. 

Figure 5.
Rictor deletion delays tumor formation in a mouse model of pancreatic cancer. A, Kaplan-Meier survival analysis showing PDAC-free survival in KPC, KPC Rictor<sup>+/+</sup>, and KPC Rictor<sup>-/-</sup> mice. Ticks indicate mice censored because of extrapancreatic pathologies. P values determined by log-rank test comparing individual cohorts separately. B, RNAscope showing absence of Rictor in tumors from KPC Rictor<sup>-/-</sup> (spleen shown as positive control) compared with KPC mice. C, IHC for downstream mTOR signaling pathway components in KPC and KPC Rictor<sup>-/-</sup> tumors as indicated. D, IHC for Ki67, p21, p27, and pERK in KPC and KPC Rictor<sup>-/-</sup> tumors as indicated.
tumor-promoting effects that may not be evident when mice with late-stage disease are treated. In line with previous studies, gemcitabine treatment offered negligible benefit (Fig. 6A). In contrast, AZD2014 treatment significantly delayed tumor progression. Combination of AZD2014 and gemcitabine further prolonged survival, and mice treated with this combination had a median survival of 280 days compared with 147 days for those treated with gemcitabine alone (Fig. 6A). These data were supported by our demonstration that AZD2014 and gemcitabine act synergistically to induce PDAC cell death in vitro (Supplementary Fig. S7B). When we assessed inhibition of mTOR targets by IHC, we found that phosphorylation of both mTORC1 and mTORC2 targets was suppressed, although the data suggested more efficient inhibition of mTORC2 signaling targets (Fig. 6B). Similar to KPC Rictor−/− mice, we did not observe any differences in p21 expression upon AZD2014 treatment; however, we did observe a decrease in proliferation based on Ki67 staining, and as in KPC Rictor−/− tumors, we also observed slightly elevated nuclear p27 and decreased pERK (Fig. 6C). Thus, we conclude that dual mTORC1/2 inhibition can effectively block signaling downstream of mTOR and may hold promise as a novel therapeutic strategy in pancreatic cancer.

Inhibition of mTORC1/2 prolongs survival in late-stage tumors
We also tested the effects of AZD2014 treatment in mice with late-stage tumors. We monitored KPC mice until they exhibited clinical signs of PDAC, including swollen abdomen, cachexia, hunching, and the presence of a palpable tumor. At this stage, mice were randomized onto treatment with vehicle, gemcitabine, AZD2014, or AZD2014 combined with gemcitabine. We found that survival from the commencement of treatment was significantly prolonged by AZD2014 treatment (Fig. 7A). Interestingly, combination of AZD2014 with gemcitabine did not further extend survival (Fig. 7A). When we assessed inhibition of mTOR targets by IHC, we found that phosphorylation of downstream targets of both mTORC1 (S6, 4E-BP1) and mTORC2 (AKT, NDRG1) was suppressed (Fig. 7B), although again, mTORC2 signaling was more efficiently suppressed. Although we saw an impact in a very aggressive late-stage model, mice still rapidly progressed to end-stage disease. Consistent with this, in this late setting in vivo, we did not observe induction of CDK inhibitors, nor did we observe changes in other potential targets of Rictor such as c-Myc (37), showing that as tumors progress in vivo they become less susceptible to mTORC2 inhibition as a single agent.

Inhibition of PI3K cooperates with mTORC1/2 inhibition to prolong survival in late-stage tumor-bearing mice
Recent studies have suggested that cancer cells can escape the requirement for mTOR through uptake of nutrients (38). In addition, mTORC1 inhibition results in feedback activation of the PI3K/AKT axis (39). Therefore, we tested whether inhibiting PI3K signaling could enhance the chemotherapeutic effects of AZD2014. Previous elegant studies have shown that deletion of PI3Kα blocked tumor formation but not tumor growth (9), consistent with our findings with Rictor gene deletion. Moreover, Rac1 deletion strongly suppresses pancreatic carcinogenesis and this was suggested to be downstream of PI3K (40, 41). Given Rac1 can also modify macropinocytosis, inhibiting PI3K may also suppress some of the mechanisms of nutrient uptake when mTOR is depleted. As PI3Kβ is the primary isoform that links PI3K signaling to RAC1 (42), we combined AZD2014 with a PI3Kβ kinase inhibitor, AZD8186 (43). We first investigated whether AZD2014/AZD8186 cotreatment reduced KPC tumor cell viability in vitro (Supplementary Fig. S8A). AZD2014 proved potent in reducing cell viability (as observed previously with Rictor knockdown) and combined treatment with AZD8186 (which alone had little effect) further reduced cell viability (Supplementary Fig. S8A). Interestingly, in this scenario, we were able to detect diminished Myc expression and p27 upregulation in the dual treated cell lines (Supplementary Fig. S8B).

Therefore, we decided to investigate the effects of inhibiting mTORC1/2 and PI3K in mice with late-stage tumors. As previously, we monitored KPC mice until they exhibited symptoms of PDAC and a palpable tumor and then treated with AZD2014 and/or AZD8186. We found that survival was extended by AZD2014 and AZD8186 cotreatment relative to treatment with either compound alone (Fig. 7C). Taken together, our data show that Rictor-mediated signaling plays a crucial role in pancreatic cancer tumorigenesis. Moreover, mTORC2 inhibition, particularly as part of a combinatorial approach, potentially represents a new therapeutic strategy for this disease.

Discussion
Pancreatic cancer is an almost universally lethal disease, and better understanding of the signaling pathways involved in its pathogenesis is required. Cytotoxic chemotherapy has limited efficacy, so the development of targeted therapeutic strategies is an attractive approach. Mutation of KRAS is almost universally the driving force in initiation. Indeed, inducible mouse models have demonstrated that pancreatic tumors retain dependence on activated KRAS signaling (44, 45). Past attempts to directly inhibit KRAS have failed; however, inhibition of key signaling pathways downstream of KRAS may hold promise. Prior studies have shown PI3K signaling to be a key mediator of oncogenic KRAS in the pancreas (9). mTOR is one of the major effectors of PI3K; however, its involvement in pancreatic tumorigenesis has been relatively understudied. Clinical trials aimed at inhibiting mTORC1 have failed, and given the high cost of trials and the high attrition rate for most novel putative therapeutic agents, a greater understanding of how signaling through mTORC1 and mTORC2 contributes to pancreatic tumorigenesis is required.

Here we demonstrate that genetic deletion of the mTORC2 complex component, Rictor, strongly suppresses pancreatic tumorigenesis. This antitumorigenic effect of Rictor deletion was associated with increased expression of CDK inhibitors, reduced proliferation, and features of senescence in vitro. Our findings are in line with recent studies in prostate cancer (19) and melanoma (46) that demonstrated a requirement for mTORC2 signaling. Importantly, while loss of Rictor significantly prolonged tumor latency in KPC mice, tumors ultimately progressed, and the antiproliferative signals observed in preneoplastic lesions were lost in late-stage tumors, perhaps due to loss of p53. Indeed, the presence of wild-type p53 may augment the antiproliferative effects of mTOR inhibition (47). Our findings suggest that mTORC2 signaling plays an important role during pancreatic tumorigenesis. However, it remains possible that mTORC2-independent functions of Rictor contribute to the observed phenotypes. Additional studies utilizing null alleles for other mTORC2 components would address this issue.

Given our prior finding that pharmacologic inhibition of mTORC1 with rapamycin showed no efficacy in the gold-
standard KPC mouse model (23), recapitulating negative results using mTORC1 inhibitors in clinical trials, it was important to determine whether inhibition of both mTOR signaling complexes is an effective strategy. Our data using the mTOR kinase inhibitor AZD2014 demonstrate that blockade of mTOR signaling impairs the progression to end-stage disease and enhanced the benefit of gemcitabine. The mechanism for this cooperation is unclear; however, inhibition of mTOR signaling may reduce expression of the low-density lipoprotein receptor (LDLR; ref. 48), and LDLR depletion can increase the efficacy of gemcitabine in KPC mice.

Figure 6.
Pharmacologic inhibition of mTORC1/2 delays tumor formation in KPC mice. A, Kaplan–Meier survival analysis showing PDAC-free survival in KPC mice treated from 10 weeks with AZD2014, gemcitabine, or AZD2014 + gemcitabine. Ticks indicate mice censored because of extrapancreatic pathologies. P values determined by log-rank test comparing individual cohorts separately. B, IHC for downstream mTOR signaling pathway components in tumors from AZD2014-treated and untreated KPC mice as indicated. C, IHC for Ki67, p21, p27, and pERK in KPC and KPC Rictor−/− tumors as indicated.
It would be interesting in the future to compare directly the effects of clinically relevant mTORC1 inhibitors with dual mTOR inhibitors. Nevertheless, our findings indicate that dual targeting of mTOR may represent a potential treatment strategy in PDAC.

Dual targeting of mTORC1/2 has a number of benefits. mTORC2 signaling is almost completely abrogated in tumors from treated mice, similar to the effects we observed with Rictor deletion. Furthermore, mTORC1 inhibition alone may actually enhance proliferation of activated KRAS-expressing cells in the setting of nutrient deprivation, by increasing the efficiency of utilization of extracellular proteins (38). Considering that PDAC is characterized by a dense stroma that may provide not only physical, but nutritional support for tumor cells, it is perhaps not surprising that inhibition of mTORC1 alone is ineffective. It should also be noted that in all in vitro systems, we found that AZD2014 or Rictor knockdown had profound effects on soft agar growth and tumor cell proliferation, suggesting that the tumor microenvironment might also be the source of support for the late stage aggressive tumors.

Molecularly targeted therapeutics often fail to display expected antitumor effects due to stimulation of feedback mechanisms. Indeed, mTOR inhibition can promote feedback activation of PI3K (39), which, in turn, can specifically activate mTORC2 through release of SIN1-mediated suppression of mTOR by PIP3 binding (50). Therefore, our finding that combined mTOR and PI3Kβ inhibition prolonged survival in mice with end-stage PDAC (49).
disease provides further support for the evaluation of mTOR kinase inhibitors in PDAC where most patients present with disseminated disease.

The pathways downstream of mTORC2 signaling may also represent potential therapeutic targets. For example, in addition to AKT, mTORC2 signaling also regulates SGK1 (51) and PKCζ (51). Increased expression of SGK has been observed in several cancer types, whereas cancer cell lines bearing PIKCA mutations have been shown to be dependent on SGK (52). mTORC2 can also regulate PKCζ and RAC (51, 53), both of which potentially influence tumorigenesis by affecting the spatial control of cell growth. In fact, RAC1 is reported to play an important role in KRAS-driven pancreatic tumorigenesis (40, 41). MYC is also a reported target of mTORC2 (37), and we recently demonstrated that pancreatic tumorigenesis is significantly delayed by Myc deficiency (54). Defining the relative contributions of these downstream molecules is required to determine the mechanisms underlying the requirement for mTORC2 signaling described here. In addition, recent work demonstrated that the temporal sequence of drug delivery can dramatically alter cellular response (55). Therefore, it will be vital to determine the optimal sequence and timing of delivery of mTOR inhibitors (and other targeted drugs) when used in combination with each other or cytotoxic drugs such as gemcitabine.

Disclosure of Potential Conflicts of Interest

D.J. Jodrell reports receiving a commercial research grant and has received speakers bureau honoraria from AstraZeneca. T.R.J. Evans has received other commercial research support from AstraZeneca. O.J. Sansom is a consultant/advisory board member of AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

References


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Acknowledgments

The authors apologize to all those colleagues whose important work was not cited in this article owing to restrictions. The authors would like to thank the CRUK Glasgow Centre and the BSI facilities and Histology Service at the CRUK Beatson Institute. The authors thank Victor Adelanwa for excellent technical assistance and Sabina Cosulich for discussions on this article.

Grant Support

This work was funded by CRUK (C596/A18076, C596/A17196) and NIH grants R01CA155784 to B.C. Lewis and F30CA168063 to B.J. Quattrochi.

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Received March 24, 2016; revised August 31, 2016; accepted September 15, 2016, published OnlineFirst October 6, 2016.

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*Cancer Res* 2016;76:6911-6923. Published OnlineFirst October 6, 2016.

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