How moderate changes in Akt T-loop phosphorylation impact on tumorigenesis and insulin resistance

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SUMMARY
The Akt signalling pathway plays vital roles in controlling cellular responses to insulin as well as in proliferation and survival. Inhibition of Akt signalling leads to insulin resistance and type 2 diabetes, whereas hyperactivation of Akt promotes tumorigenesis. In this study, we investigate how modest changes in the activity of the Akt signalling pathway, to an extent that might be achieved by drug treatment, would impact on insulin resistance and tumorigenesis. Using insulin-resistant PDK1K465E/K465E PH domain knock-in mice, we found that introducing the PTEN+/– mutation to slightly stimulate Akt restored normal insulin sensitivity. Introducing the PDK1K465E/K465E PH domain knock-in mutation into cancer-prone PTEN−/– mice, lowered Akt activity only by about 50%, but led to a delay in tumour onset of ~4 months in a broad range of tumours. This was also accompanied by slower growth of B cell follicular lymphomas, as monitored by magnetic resonance imaging. Our findings imply that signal transduction inhibitors that lead to a modest reduction in Akt activity would not only delay onset of tumours possessing elevated phosphoinositide 3-kinase pathway activity but would also reduce the growth rate of developed tumours.

INTRODUCTION
The phosphoinositide 3-kinase (PI3K)-Akt signalling pathway plays essential roles in regulating diverse biological responses, including metabolic actions of insulin as well as growth and proliferation (Taniguchi et al., 2006). Insufficient activation of the PI3K-Akt pathway is a hallmark of insulin resistance and can lead to type 2 diabetes. A high fat diet and mutations in signalling components that suppress the PI3K-Akt signalling pathway invariably lead to insulin resistance (Whiteman et al., 2002). By contrast, many cancer-driving mutations stimulate cell proliferation and survival by promoting activation of the PI3K-Akt pathway (Alessi et al., 2009; Manning and Cantley, 2007; Wullschleger et al., 2006). One of the most commonly mutated genes in human cancer is PTEN, which encodes a lipid phosphatase that breaks down phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)]P3, a second messenger produced downstream of PI3K activation (Salmena et al., 2008). Loss of PTEN results in increased levels of PtdIns(3,4,5)P3, thereby promoting the activation of Akt via the upstream 3-phosphoinositide-dependent protein kinase 1 (PDK1) and mTOR complex 2 kinases (Pearce et al., 2010). Furthermore, heterozygous PTEN+/− mice have been shown to spontaneously develop a variety of tumours (Di Cristofano et al., 1998; Haas-Kogan et al., 1998; Maehama and Dixon, 1998; Myers et al., 1998; Podsypanina et al., 1999; Stambolic et al., 1998; Suzuki et al., 1998).

The activation of Akt is mediated by phosphorylation of the T-loop (T308) by PDK1 and requires the interaction of Akt and PDK1 with PtdIns(3,4,5)P3 via their Pleckstrin homology (PH) domains (Pearce et al., 2010). PDK1K465E/K465E PH domain knock-in mice express a form of PDK1 (with lysine 465 exchanged for glutamic acid) that is normally active, but unable to bind PtdIns(3,4,5)P3. These mice display a small size phenotype and are insulin resistant (Bayascas et al., 2008). The insulin resistance in the PDK1K465E/K465E animals is thought to result from partial inhibition of insulin-induced activation of Akt isoforms. Akt binds to the AGC kinase family, and PDK1 activates other members of this family including S6K and SGK isoforms. The activation of these kinases is not dependent on PDK1-binding to PtdIns(3,4,5)P3 and therefore these enzymes are normally activated in the PDK1K465E/K465E PH domain knock-in mice (Bayascas et al., 2008). The physiological roles of the Akt signalling pathway have been studied using knockout mouse models (Dummier and Hemmings, 2007); however, the PDK1K465E/K465E PH domain knock-in mice represent a unique mouse model in which Akt isoform activity is not ablated, but only moderately reduced.

There is much effort to develop drugs that inhibit the Akt pathway for the treatment of cancer (Liu et al., 2009). Because the Akt pathway plays vital roles in controlling many diverse essential cellular processes, it is likely that drugs would only be tolerated if they modestly inhibit the Akt-signalling network. In this study, we exploit the PDK1K465E/K465E mouse model to investigate how small changes in the activity of the Akt pathway impact on insulin resistance and tumorigenesis. Our data indicate that drugs that moderately reduce Akt pathway activity would have therapeutic benefit in delaying the onset as well as growth of a broad spectrum of tumours driven by elevated PI3K pathway signalling.

RESULTS
Generation of PDK1K465E/K465EPTEN−/− mice
Employing the breeding strategy described in Fig. 1A, we generated littermate strains of mice expressing wild-type PDK1 and wild-type
PTEN (PDK1<sup>+/+</sup> PTEN<sup>+/+</sup>), PH domain mutant PDK1 and wild-type PTEN (PDK1<sup>K465E/K465E</sup> PTEN<sup>+/+</sup>), wild-type PDK1 and deficient PTEN (PDK1<sup>+/+</sup> PTEN<sup>−/−</sup>), and PH domain mutant PDK1 and deficient PTEN (PDK1<sup>K465E/K465E</sup> PTEN<sup>−/−</sup>). Apart from the PDK1<sup>K465E/K465E</sup> PTEN<sup>+/+</sup> mice that were born at ~70% of the expected number, the other mouse strains were recovered at around the expected Mendelian frequency (Fig. 1A, Cross 2). Consistent with previous work (Bayascas et al., 2008), PDK1<sup>K465E/K465E</sup> PTEN<sup>+/+</sup> mice were ~35% smaller than wild-type PDK1<sup>+/+</sup> PTEN<sup>+/+</sup> mice (Fig. 1B), which is probably a result of reduced Akt pathway activity during development. The PDK1<sup>K465E/K465E</sup> PTEN<sup>−/−</sup> mice were of an intermediate size and only ~20% smaller than wild-type PDK1<sup>+/+</sup> PTEN<sup>+/+</sup> mice, suggesting that deficiency of PTEN can partially restore normal Akt pathway activity during development and therefore rescue the small size phenotype.

**Analysis of Akt1 and Akt2 activity and phosphorylation**

To establish the effect of PTEN heterozygosity on Akt activation in PDK1<sup>K465E/K465E</sup> animals, mice were starved overnight and then injected intravenously with a physiological dose of insulin (1 mU/g) for 10 minutes. Insulin-sensitive tissues, including liver (Fig. 1C),
heart (Fig. 1D) and skeletal muscle (Fig. 1E) were removed and extracts generated. In the DPK1K465E/K465EPTEN+/− or DPK1+/−PTEN+/− mice, the level of the PTEN protein in liver, heart, and skeletal muscle was reduced by half compared with tissues derived from DPK1K465E/K465EPTEN+/− or DPK1+/−PTEN+/− mice (Fig. 1C-E). As reported previously, DPK1 expression in all tissues examined was not affected by the K465E knock-in mutation (Bayascas et al., 2008) (Fig. 1C-E). Akt1 (all tissues) and Akt2 (liver) protein kinase activity was measured following immunoprecipitation. We also assessed Akt phosphorylation at the T-loop PDK1 site (T308-Akt1) and phosphorylation of the hydrophobic motif mTOR complex 2 site (S473-Akt1) by immunoblotting with phosphospecific antibodies. Consistent with previous work, insulin induced a 30-50% lower activation of Akt1 and Akt2 in DPK1K465E/K465EPTEN+/− or DPK1+/−PTEN+/− animals (Fig. 1C-E). However, a modest increase in Akt activity and phosphorylation was observed in DPK1K465E/K465EPTEN+/− or DPK1+/−PTEN+/− animals compared with DPK1K465E/K465EPTEN+/− or DPK1+/−PTEN+/− mice (Fig. 1C-E). We also monitored phosphorylation of various Akt substrates, including PRAS40, FOXO1 and GSK3 using phosphospecific antibodies against Akt phosphorylation sites of these proteins. We detected a decrease in the phosphorylation of GSK3 in heart, comparing wild-type DPK1+/−PTEN+/− mice with DPK1K465E/K465EPTEN+/− animals (Fig. 1D). Reduced GSK3 phosphorylation was partially rescued in heart lysates from DPK1K465E/K465EPTEN+/− mice compared with DPK1K465E/K465EPTEN+/− mice (Fig. 1D). Similarly, phosphorylation of PRAS40 was reduced in muscle extracts from DPK1K465E/K465EPTEN+/− mice compared with DPK1+/−PTEN+/− animals (Fig. 1E). PRAS40 phosphorylation in muscle extracts was restored to wild-type DPK1+/−PTEN+/− levels in DPK1+/−PTEN+/− animals (Fig. 1E). We did not detect significant changes in the phosphorylation status of FOXO1 in any of the tissues examined, nor did we observe any significant effects in the liver of phosphorylation of Akt substrates in the DPK1K465E/K465EPTEN+/− knock-in mice (Fig. 1C). This indicates that, in some tissues, a moderate decrease in Akt activity does not affect phosphorylation of Akt substrates induced by acute insulin stimulation.

Enhanced insulin sensitivity of DPK1K465E/K465EPTEN+/− mice

To investigate whether the modestly enhanced Akt activity observed in the DPK1K465E/K465EPTEN+/− compared with the DPK1K465E/K465EPTEN+/− mice would be sufficient to counteract the insulin resistance, we performed a glucose tolerance test on these animals. Mice were starved overnight and injected with an intraperitoneal bolus of glucose, and blood glucose levels monitored over a 120-minute period. As reported previously (Bayascas et al., 2008), DPK1K465E/K465EPTEN+/− animals displayed significant glucose intolerance because their blood glucose levels were markedly higher at all time points compared with DPK1+/−PTEN+/− wild-type mice (Fig. 2A). The insulin resistance in the DPK1K465E/K465EPTEN+/− animals was also emphasised by the markedly higher levels of plasma insulin compared with DPK1+/−PTEN+/− mice (Fig. 2B). Strikingly, however, the DPK1K465E/K465EPTEN+/− mice showed markedly improved glucose tolerance compared with DPK1+/−PTEN+/− animals. The
rises in blood glucose levels at all time points were similar to the wild-type \( PDK1^{+/+} \) PTEN\(^{+/+} \) mice in the glucose tolerance test (Fig. 2A). In addition, plasma insulin levels were not elevated in the \( PDK1^{K465E/K465E} \) PTEN\(^{+/+} \) animals and were similar to those of wild-type mice (Fig. 2B). As reported previously (Wong et al., 2007), we also observed that the \( PDK1^{+/+} \) PTEN\(^{+/+} \) mice displayed enhanced insulin sensitivity compared with wild-type \( PDK1^{+/+} \) PTEN\(^{+/+} \) animals (Fig. 2A). The \( PDK1^{+/+} \) PTEN\(^{+/–} \) mice also possessed lower plasma insulin levels than wild-type mice (Fig. 2B). As reported previously (Wong et al., 2007), we also observed that the \( PDK1^{+/+} \) PTEN\(^{+/–} \) mice displayed enhanced insulin sensitivity compared with wild-type \( PDK1^{+/+} \) PTEN\(^{+/+} \) animals (Fig. 2A). The \( PDK1^{+/+} \) PTEN\(^{+/–} \) mice also possessed lower plasma insulin levels than wild-type mice (Fig. 2B). These data indicate that lowering the expression of PTEN by only 50% is sufficient to rescue the insulin resistance phenotype observed in \( PDK1^{K465E/K465E} \) PTEN\(^{+/+} \) animals.

**Delayed tumour onset in \( PDK1^{K465E/K465E} \) PTEN\(^{+/–} \) mice**

We next sought to define how a modest reduction in Akt signalling, which results from introducing the PDK1-PH domain K465E mutation, impinges on tumour development in PTEN\(^{+/–} \) animals. We monitored groups of ~25 \( PDK1^{+/+} \) PTEN\(^{+/+} \), \( PDK1^{K465E/K465E} \) PTEN\(^{+/+} \), \( PDK1^{+/+} \) PTEN\(^{+/–} \) and \( PDK1^{K465E/K465E} \) PTEN\(^{+/–} \) animals for tumour development. Animals were sacrificed when they exhibited large externally palpable tumours, reduced body weight, became unwell, or reached 14 months of age. A necropsy was performed, the tissues fixed in 10% formalin and subjected to a detailed histopathological examination. By 14 months of age, wild-type \( PDK1^{+/+} \) PTEN\(^{+/+} \) and \( PDK1^{K465E/K465E} \) PTEN\(^{+/+} \) mice displayed no significant tumours (Fig. 3A). Consistent with previous work (Podsypanina et al., 1999), tumour development in \( PDK1^{+/+} \) PTEN\(^{+/–} \) mice was observed at ~5 months of age, and by 10 months of age ~90% had one or more tumours. Strikingly, the \( PDK1^{K465E/K465E} \) PTEN\(^{+/–} \) mice developed tumours significantly later, commencing at 7 months of age. At 10 months of age, only ~35% of the \( PDK1^{K465E/K465E} \) PTEN\(^{+/–} \) mice had
developed tumours (Fig. 3A). These data demonstrate that tumour initiation in \( \text{PDK1}^{+/+}/\text{PTEN}^{+/-} \) was delayed by 3-4 months compared with \( \text{PDK1}^{+/+}/\text{PTEN}^{+/-} \) mice. The types and morphology of tumours arising in \( \text{PTEN}^{+/-} \) mice with either wild-type PDK1 or PDK1-PH domain mutation were similar (Table 1). Comparing the incidence of detected tumours in much younger \( \text{PDK1}^{+/+}/\text{PTEN}^{+/-} \) mice with older \( \text{PDK1}^{+/+}/\text{PTEN}^{+/-} \) animals did not suggest a major difference in the frequency of different tumours. We detected a significant difference in tumour formation in B cell follicular lymphomas, which were found at ~25% lower incidence in the older \( \text{PDK1}^{+/+}/\text{PTEN}^{+/-} \) mice compared with younger \( \text{PDK1}^{+/+}/\text{PTEN}^{+/-} \) animals (Table 1). We also found a lower incidence rate for the formation of intestinal polyps, phaeochromocytoma and prostate carcinoma in \( \text{PDK1}^{+/+}/\text{PTEN}^{+/-} \) mice compared with \( \text{PDK1}^{+/+}/\text{PTEN}^{+/-} \) mice (Table 1), indicating that inhibition of Akt activity might have a broad spectrum of action in tumour formation.

Representative histopathological sections of the tumours found in \( \text{PDK1}^{+/+}/\text{PTEN}^{+/-} \) and \( \text{PDK1}^{+/+}/\text{PTEN}^{+/-} \) mice are shown in Fig. 3B,C. Lymphomas in both genotypes had a follicular architecture and showed extension beyond the lymph node capsule (Fig. 3B, low magnification images). The neoplastic follicles were composed of centrocytes and centroblasts (Fig. 3B, high magnification images). Reactivity for B220 and CD79a by immunohistochemistry confirmed that the lymphomas were all B cell follicular lymphomas. Occasional CD3-reactive T cells were seen mostly in interfollicular areas (Fig. 3B). Intestinal polyps could be seen at low magnification, projecting into the bowel lumen (Fig. 3C). High magnification examination showed the composition of these polyps to include mucin secreting epithelium and a smooth muscle cell rich stroma (Fig. 3C). Phaeochromocytoma of the adrenal medulla was seen in both genotypes. There was no evidence of invasion beyond the adrenal medulla (Fig. 3C). Prostate carcinoma in both genotypes consisted of microacinar or cribriform adenocarcinoma, with focal cell death. Microinvasion or frank carcinoma in both genotypes consisted of microacinar or cribriform architecture and showed extension beyond the lymph node capsule (Fig. 3B, low magnification images). Lymphomas in both genotypes had a follicular morphology arising in the two different genotypes were similar.

Tumour growth is reduced but Akt pathway is unaltered in tumours from \( \text{PDK1}^{+/+}/\text{PTEN}^{+/-} \) mice

To assess the growth rate of tumours formed in \( \text{PDK1}^{+/+}/\text{PTEN}^{+/-} \) and \( \text{PDK1}^{+/+}/\text{PTEN}^{+/-} \) mice, the volumes of B cell follicular lymphomas present in the animals were measured at 3-week intervals over a 6-week period by magnetic resonance imaging (MRI). For these experiments, littermate mice of the same age and possessing tumours were selected. We observed that the volume of B cell follicular lymphomas in \( \text{PDK1}^{+/+}/\text{PTEN}^{+/-} \) mice increased 1.7-fold over this period of analysis (Fig. 4A). By contrast, the size of tumours observed in \( \text{PDK1}^{+/+}\text{K465E}/\text{PTEN}^{+/-} \) mice did not appreciably increase over 6 weeks. After this period, total cell extracts were generated from the B cell follicular lymphomas studied by MRI. Phospho-immunoblotting revealed that Akt phosphorylation at T308, but not S473, was significantly reduced in the B cell follicular lymphomas derived from \( \text{PDK1}^{+/+}\text{K465E}/\text{PTEN}^{+/-} \) mice compared with \( \text{PDK1}^{+/+}/\text{PTEN}^{+/-} \) animals (Fig. 4B). We did not detect significant differences in the phosphorylation of Akt substrates, such as PRAS40, FOXO1 and GSK3, comparing lymphomas from \( \text{PDK1}^{+/+}/\text{PTEN}^{+/-} \) with those from \( \text{PDK1}^{+/+}\text{K465E}/\text{PTEN}^{+/-} \) mice. Similarly, there was no significant difference in the phosphorylation of other PDK1 substrates, 56k and SGK, assessed by phosphorylation of its substrate NDRG1 (Garcia-Martinez and Alessi, 2008), in tumours derived from \( \text{PDK1}^{+/+}/\text{PTEN}^{+/-} \) as compared with \( \text{PDK1}^{+/+}\text{K465E}/\text{PTEN}^{+/-} \) mice. To further assess the properties of the tumours formed in \( \text{PDK1}^{+/+}/\text{PTEN}^{+/-} \) and \( \text{PDK1}^{+/+}\text{K465E}/\text{PTEN}^{+/-} \) mice, sections of B cell follicular lymphomas were analysed by immunohistochemistry. PTEN staining of the lymphoma was detected, which indicates that these lymphomas have not lost all expression of PTEN. We also investigated the subcellular localisation of the FOXO1 transcription factor, which is localised in the cytoplasm after phosphorylation by Akt. In both centroblasts and smaller centrocytes, FOXO1 staining was predominantly cytoplasmic, irrespective of genotype, indicating that Akt was not activated. Strong cytoplasmic staining for phosphorylation of S6 in centroblasts was observed, suggesting that S6K was active in tumours of either genotype. There was no significant difference between the genotypes in the proportion of the proliferative marker Ki67. These data indicate that although Akt phosphorylation at T308 was reduced in tumours from \( \text{PDK1}^{+/+}\text{K465E}/\text{PTEN}^{+/-} \) mice, once tumours had formed there was no significant difference in the signalling pathway downstream of Akt, which is consistent with previous studies (Bayascas et al., 2005).

Table 1. Tumour development in \( \text{PTEN}^{+/-} \) mice

<table>
<thead>
<tr>
<th>Tumour</th>
<th>Incidence ( \text{PDK1}^{+/+}/\text{PTEN}^{+/-} )</th>
<th>Incidence ( \text{PDK1}^{+/+}\text{K465E}/\text{PTEN}^{+/-} )</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lympoma</td>
<td>26</td>
<td>96</td>
<td>73</td>
</tr>
<tr>
<td>Intestinal polyps</td>
<td>14</td>
<td>52</td>
<td>8</td>
</tr>
<tr>
<td>Phaeochromocytoma</td>
<td>10</td>
<td>37</td>
<td>5</td>
</tr>
<tr>
<td>Prostate carcinoma (male)</td>
<td>12</td>
<td>86</td>
<td>9</td>
</tr>
<tr>
<td>Breast carcinoma (female)</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Number of mice displaying tumours</td>
<td>27</td>
<td>100</td>
<td>24</td>
</tr>
</tbody>
</table>

*Sample size: 27 (14 males, 13 females); mean age (months): 8.9 (males), 6.9 (females). **Sample size: 26 (12 males, 14 females); mean age (months): 11.4 (males), 11.6 (females).

\( P \)-values were calculated using Fisher’s exact test to compare the incidence of tumours in mice expressing either wild-type or mutant PDK1.
Mutation of PDK1-PH domain suppresses PTEN-mediated tumorigenesis. We found that lowering the expression of PTEN in insulin-resistant PDK1K465E/K465E mice resulted in a significant improvement in the insulin sensitivity (Figs 1, 2). We also observed that a reduction in Akt activity in PDK1K465E/K465E PTEN+/– animals lead to a considerable delay in the onset and growth rate of tumours (Figs 3, 4).

Earlier studies from our laboratory indicated that lowering the expression of PDK1 has a pronounced effect on tumorigenesis of PTEN+/– mice (Bayascas et al., 2005). Reduction in levels of PDK1 expression is likely to impact on the activity of multiple downstream targets of PDK1. However, the only major signalling defect we observed in the PDK1K465E/K465E PTEN+/– mice was a moderate reduction of Akt T308 phosphorylation, which reduces Akt isoform activity. All other AGC kinases we have studied, including S6K1 and SGK activity (as judged by phosphorylation of NDRG1), are not affected in the tumours that develop in the PDK1K465E/K465E PTEN+/– mice. This indicates that a moderate reduction in Akt isoform activity is sufficient to delay tumour onset and development. The mechanism by which reduction in Akt activity delays tumour onset and development requires further investigation because phosphorylation of the Akt substrates we have investigated is not markedly inhibited in tumours derived from PDK1K465E/K465E PTEN+/– animals. It cannot be ruled out that there are other Akt substrates that we have not investigated whose phosphorylation will be more significantly affected in tumours derived from PDK1K465E/K465E PTEN+/– mice and which could play a key role in regulating tumour initiation and development.

Previous studies have demonstrated that genetic ablation of PI3K (Jia et al., 2008), Akt isoforms (Chen et al., 2006) and mTOR regulatory subunits such as Rictor (Guertin et al., 2009), when combined with loss of PTEN, markedly suppress tumorigenesis. It is unclear whether these mouse models represent the clinical situation of a human cancer patient taking a dose of a drug that might only partially suppress Akt pathway activity. Because the Akt network controls many essential physiological roles, it is likely that inhibitors would only be tolerated at doses that moderately lower Akt activity. The PDK1K465E/K465E mouse model is unique in that it only leads to a slight reduction in the activity of Akt isoforms in
all tissues, without affecting the activity of other related kinases such as S6K and SGK (Bayascas et al., 2008). We argue that the PDK1\textsuperscript{K465E/K465E} mice mimic the situation of a patient treated with an Akt inhibitor better than the complete knockout mouse models that have been generated previously. The observation that tumour development and tumour growth is delayed in PDK1\textsuperscript{K465E/K465E}-PTEN\textsuperscript{+/–} mice is encouraging and suggests that a drug causing partial inhibition of the Akt pathway would have some efficacy in slowing tumour growth. This could be relevant to the treatment of human tumours because diagnosis is normally made once a tumour has formed. In addition, there is the concern that inhibition of the PI3K signalling pathway would promote insulin resistance and result in overt diabetes. However, as the PDK1\textsuperscript{K465E/K465E} mice display only a mild diabetic phenotype, it is probable that any insulin resistance that develops in human cancer patients treated with an Akt inhibitor could be managed in the clinic. Indeed, measuring increased plasma insulin levels as a marker of insulin resistance in patients administered with drugs that suppress Akt activity is probably an effective biomarker strategy for assessing the efficacy with which such compounds inhibit Akt in patients. Although our data suggest that a moderate inhibition of PTEN would effectively counteract insulin resistance, it is unlikely that drugs that inhibit PTEN will ever be utilised for the treatment of diabetes because recent genetic analysis has elegantly demonstrated that even a 20% reduction in PTEN expression is sufficient to cause significant neoplastic lesions (Alimonti et al., 2010).

Our study demonstrates the importance of the PI3K-Akt signalling pathway in the development of diseases such as diabetes and cancer, and provides insights into how targeting this pathway might be useful for treatment of cancer. Crucially, our study suggests that the inhibition of Akt activity by either an Akt, PDK1, PI3K or mTOR inhibitor would be effective in not only delaying onset of tumours driven by PTEN inactivation or other mutations that stimulate the Akt pathway, but also by suppressing the growth of these tumours once formed. Importantly, although our study focuses on the formation of B cell follicular lymphomas, our results indicate that drugs that suppress Akt might be effective for the treatment of a broader range of common tumour types, such as intestinal polyps, phaeochromocytoma and prostate carcinoma (Table 1). It will be interesting to see how efficacious Akt-pathway inhibitors (now in early stage clinical trials) are at suppressing tumorigenesis of different tumour types (Liu et al., 2009). Finally, this study also suggests that compounds that inhibit the PH domain of PDK1 binding to PtdIns(3,4,5)P\textsubscript{3} could be used as an alternative approach to the development of anticancer agents that suppress the Akt pathway. Perhaps these agents could be employed in combination with Akt kinase domain inhibitors or with other drugs that target components of the PI3K signal transduction pathway.

METHODS

Materials and general buffers

Protein G-Sepharose and 32P-ATP were purchased from Amersham Pharmacia Biotech. Human insulin from Novo-Nordisk was obtained from Ninewells Pharmacy, Dundee. Lysis buffer was 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% (w/v) Triton-X 100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol and ‘Complete’ protease inhibitor cocktail (Roche).

Antibodies

The following antibodies were raised in sheep and affinity-purified on the appropriate antigen. The total antibody used for immunoprecipitation and immunoblotting of Akt1 (S742B, 3rd bleed) was raised against full-length His-Akt1 expressed in Esherichia coli. Akt2 antibody (S069C, 2nd bleed) was raised against the sequence RYDSDLPLEDQRT\textsubscript{H}, which corresponds to residues 455-469 of mouse Akt2. The PDK1 antibody (S48A, 3rd bleed) was raised against the sequence RIKIQEVRWRQYQSNPDAAVQ, which corresponds to residues 540-559 of mouse PDK1. The S6K1 antibody (S417B, 2nd bleed) was raised against the sequence AGVFDDLQPDAGSEDEL, corresponding to residues 25-44 of human S6K1. The NDRG1 antibody (S276, 2nd bleed) was raised against full length GST-NDRG1. An antibody that recognizes NDRG1 phosphorylated at T346, T356 and T366 (S911B, 2nd bleed, termed NDRG1 3' x p- T) was raised against the peptide CRRSRHPTSEG, whose sequence is common to all three sites. The PRAS40 antibody (S115B, 1st bleed) was raised against the sequence DLPRPRLNTDFKQLKRY, which corresponds to residues 238-256 of human PRAS40. An antibody that recognizes PRAS40 phosphorylated at T246 (S114B, 2nd bleed) was raised against the peptide CRPRNLNFSQK. The FOXO1 antibody (S457, 3rd bleed) was raised against GST-FOXO1, comprising residues 2-655 of human FOXO1. The Akt1 p-T308 (#9275), Akt1 p-S473 (#9271), FOXO1 p-T24 (#9464), GSK3\textbeta\ p-S21/p-S9 (#9331), S6K1 pT389 (#9205), PTK (p-T559) and the pan-PDK1 site antibody (#9379) that recognise the phosphorylated T-loop of S6K were purchased from Cell Signaling Technology. The GSK3\textalpha\ antibody (#44-610) was purchased from Biosource. Secondary antibodies coupled to horseradish peroxidase were from Pierce.

Mice breeding, genotype analysis and tumour analysis

All animal studies and breeding was approved by the University of Dundee Ethical Committee and performed under a UK Home Office project licence. The generation and genotyping of the PTEN\textsuperscript{+/–} mice (Podsypanina et al., 1999) and the PDK1\textsuperscript{K465E/K465E} knock-in mice (Bayascas et al., 2008) have been described previously. The parental PTEN\textsuperscript{+/–} and PDK1\textsuperscript{K465E/K465E} mice used for these experiments had been backcrossed with C57BL/6 for over five generations before initiating the crosses for the present study. Littermates with the different genotypes of PTEN and PDK1 were derived as described in Fig. 1A and were maintained under standard husbandry conditions for a period of up to 14 months. The onset of tumour formation was monitored weekly by palpation of the major lymph nodes. According to our UK Home Office licence, animals displaying a tumour of over 1.44 cm\textsuperscript{2}, showing signs of sickness, or having lost over 20% of body weight were culled. The tissues were fixed in 10% formalin and subjected to necropsy and pathological analysis. Tumour slices were generated and analysed as described previously (Bayascas et al., 2005).

Tissue microarray construction

The use of tissue microarrays allows all samples to be simultaneously stained, thus reducing variability between antibody
runs. A tissue microarray (TMA) was constructed from the representative lymphoma areas from the original mouse pathology wax blocks. A manual tissue arrayer (Beecher Instruments) was used to bore cores of tissue from the areas of the paraffin blocks marked by the pathologist and transfer them into a recipient paraffin block. Either two or three cores, each 0.6 mm in diameter, were donated from each lymphoma and eight cases of lymphomas from each of the PDK1+/–, PTEN+/– or PDK1+/+;PTEN−/− genotypes were analysed.

**IHC staining**

Primary antibodies were used to detect B220/CD45R (RA3-6B2, BD Pharmingen), CD79ac (H57, Dako), CD3 (F7.2.38, Dako) and Ki67 (VP-K452, Vector Laboratories). Antibodies against PTEN protein (#9188), FOXO1 (#9462) and S6 p-S235/S236 (#4857) were purchased from Cell Signaling Technology. Antibody binding was visualised using Vectastain reagents and protocols performed on a Dako immunostainer. Sections were viewed on a Nikon Eclipse E600 microscope, and digital images captured on a Nikon DXM 1200 digital camera.

**Preparation of tissue lysates, immunoblotting and kinase assays**

Following an overnight fast, a bolus of insulin (1 mM U/g body weight) was intravenously injected through the inferior vena cava to mice that had been terminally anaesthetised by pentobarbital (86 µg/g body weight, intraperitoneally injected). At 10 minutes after injection, tissues were extracted, frozen in liquid nitrogen and stored at −80°C. Tissues were homogenised on ice in a tenfold mass excess of ice-cold lysis buffer using a Kinematica Polytron. Tissue lysates were centrifuged at 18,000 g for 15 minutes at 4°C and the supernatant snap-frozen and stored at −80°C. The activity of Akt1 was assessed either by immunoblotting of tissue lysate (20 µg) or by kinase activity assays. Briefly, Akt1 or Akt2 was immunoprecipitated from 1 mg of tissue lysate and the kinase activity measured using the Crosstide peptide (GRPRTSSFAEG) as described previously (Williams et al., 2000).

**Glucose tolerance test**

Mice were deprived of food overnight and then basal blood glucose levels determined using the Ascensia Breeze 2 blood glucose monitoring system (Bayer) following tail incision. Mice were injected intraperitoneally with 2 mg glucose/g body weight. Blood glucose levels were measured 15, 30, 45, 60 and 120 minutes after glucose injection. The area under the curve was calculated using GraphPad PRISM software.

**Plasma insulin measurement**

Blood was collected from mice following tail incision and using Na-heparinised capillary tubes (Hawksley). The blood was centrifuged at 3000 g for 15 minutes and the supernatant collected. Plasma insulin levels were determined using a rat/mouse insulin ELISA kit from Millipore (EZRMI-13K) according to the instructions of the manufacturer. Rat insulin of 0–10 ng/ml was used as a standard.

**Micro-magnetic resonance imaging of tumours**

Micro-magnetic resonance imaging (micro-MRI) data were acquired on a Bruker Avance FT NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) with a wide-bore 7.1-Tesla vertical magnet resonating at 300.15 MHz for 1H, fitted with Bruker micro-imaging magnetic field gradients. A MicroMouse birdcage radio-frequency resonator with an internal diameter of 30 mm was used. Three dimensional (3D) 128×128×128 rapid acquisition relaxation enhanced (RARE) MRI experiments were performed with RARE factor of 4, taken from the Paravision library. The recycle time (TR) was 250 milliseconds and echo time (TE) was 10 milliseconds. One acquisition sequence was acquired to minimise the experimental time, which was 17 minutes. The field of view was 30 mm, the isotropic image spatial resolution was 234 µm per pixel. Mice were anaesthetised with isoflurane (1.5% in 100% oxygen) using a nose cone with scavenging. The mice were monitored during imaging and recovery, and then returned to their

**Mutation of PDK1-PH domain suppresses PTEN-mediated tumorigenesis**

In this paper, the authors define the effect of a moderate ~twofold inhibition on the activity of Akt or on the initiation and growth of PTEN-deficient tumours. The mouse model they developed carries a mutation in the PH domain of PDK1 that prevents an interaction between PDK1 and the PtdIns(3,4,5)P3 second messenger, thereby reducing the activation of all Akt isoforms in vivo. The authors argue that, compared with previous knockout mouse models, in which Akt activity is completely ablated, the partial inhibition of Akt in the PDK1-PH domain mutant mouse more accurately mimics the effect observed in a cancer patient treated with an Akt inhibitor. The authors demonstrate that introducing the PDK1-PH domain mutation into PTEN+/– mice reduced the activity of Akt isoforms ~twofold and markedly delayed the development of a wide range of spontaneous tumours by ~4 months. Moreover, the PDK1-PH domain mutation markedly slowed the growth of B-cell follicular lymphomas that developed in PTEN−/− mice. These data emphasise the dependence of PTEN-deficient tumours on intrinsic Akt kinase activity and suggest that the moderate reduction in this activity can significantly delay tumour initiation and development.

**Clinical issue**

Most human cancers possess mutations in genes that lead to hyperactivation of the Akt signalling pathway, which promotes cellular growth and proliferation, such as the lipid phosphatase PTEN. Studies of various animal models have shown that knocking out components of the Akt signalling pathway suppresses tumorigenesis in the context of PTEN loss or other cancer-causing mutations that activate the Akt pathway. These exciting results have stimulated pharmaceutical companies to develop small-molecule inhibitors that suppress the Akt pathway as anticancer agents. However, treatment of patients with drugs that target the Akt pathway at well-tolerated doses will only partially reduce Akt activity and not ablate it, as in experimental mouse knockout models. It is therefore unclear how relevant previous studies using mouse knockout models are in validating the efficacy of Akt-pathway inhibitors as anticancer agents.

**Results**

In this paper, the authors define the effect of a moderate ~twofold inhibition on the activity of Akt or on the initiation and growth of PTEN-deficient tumours. The mouse model they developed carries a mutation in the PH domain of PDK1 that prevents an interaction between PDK1 and the PtdIns(3,4,5)P3 second messenger, thereby reducing the activation of all Akt isoforms in vivo. The authors argue that, compared with previous knockout mouse models, in which Akt activity is completely ablated, the partial inhibition of Akt in the PDK1-PH domain mutant mouse more accurately mimics the effect observed in a cancer patient treated with an Akt inhibitor. The authors demonstrate that introducing the PDK1-PH domain mutation into PTEN+/– mice reduced the activity of Akt isoforms ~twofold and markedly delayed the development of a wide range of spontaneous tumours by ~4 months. Moreover, the PDK1-PH domain mutation markedly slowed the growth of B-cell follicular lymphomas that developed in PTEN−/− mice. These data emphasise the dependence of PTEN-deficient tumours on intrinsic Akt kinase activity and suggest that the moderate reduction in this activity can significantly delay tumour initiation and development.

**Implications and future directions**

This study will provide encouragement to pharmaceutical companies that are currently elaborating Akt-pathway inhibitors, because it suggests that even agents that modestly reduce Akt activation might have significant therapeutic benefit on a broad range of common human tumours. It also suggests that drugs that inhibit the binding of PDK1 to PtdIns(3,4,5)P3 could be developed for use either alone or in combination with other Akt-pathway suppressors to treat cancer. Further work is required to establish the key downstream pathways by which Akt mediates effects on tumour initiation and development observed in this study.

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cage. The MRI data were Fourier-transformed and visualised using Amira PC-based software (Visage Imaging, San Diego, CA). The tumours were digitally segmented and then 3D finite element meshes generated and surface rendered to produce 3D surface representations. The volumes of the tumours were calculated from these images.

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COMPETING INTERESTS
The authors declare no competing interests.

AUTHOR CONTRIBUTIONS
S.W. and D.R.A. conceived of the project. All authors were involved in planning and analysing the experimental data. S.W. undertook most of the experimentation. K.S. helped with insulin studies, S.D. undertook the MRI analysis and S.F. and L.J. helped with insulin studies, S.D. undertook the MRI analysis and S.F. and L.J. helped with insulin studies. All authors were involved in planning and writing the manuscript. D.R.A. supervised the project.

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