MINIREVIEW

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Akt as a target for cancer therapy: more is not always better (lessons from studies in mice)

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The PI3K/Akt signalling pathway is one of the most frequently altered signalling networks in human cancers and has become an attractive target in anticancer therapy. Several drugs targeting this pathway are currently in different phases of clinical trials. However, accumulating reports suggest that adverse effects such as hyperglycaemia and hyperinsulinaemia accompany treatment with pan-PI3K and pan-Akt inhibitors. Thus, understanding the consequences of the systemic deletion or inhibition of Akt activity *in vivo* is imperative. Three Akt isoforms may individually affect different cancer cells in culture to varying degrees that could suggest specific targeting of different Akt isoforms for different types of cancer. However, the results obtained in cell culture do not address the consequences of Akt isoform inhibition at the organismal level and consequently fail to predict the feasibility of targeting these isoforms for cancer therapy. This review summarises and discusses the consequences of genetic deletions of Akt isoforms in adult mice and their implications for cancer therapy. Whereas combined Akt1 and Akt2 rapidly induced mortality, hepatic Akt inhibition induced liver injury that promotes hepatocellular carcinoma. These findings may explain some of the side effects exerted by pan-PI3K and pan-Akt inhibitors and suggest that close attention must be paid when targeting all Akt isoforms as a therapeutic intervention.

Before addressing the feasibility of targeting PI3K and Akt for cancer therapy, the evolution of this pathway should be understood. The PI3K/Akt signalling and its downstream effectors, the FOXO transcription factors and mTORC1, constitute an evolutionarily conserved pathway that executes the metabolic response to insulin at the organismal and cellular levels (Kandel and Hay, 1999; Bhaskar and Hay, 2007; Robey and Hay, 2009; Hay, 2011a, b). In mammalian cells, the pathway was first implicated in growth factor-mediated cell survival, and it was proposed as target for cancer therapy long before it was identified to be frequently hyperactivated in cancer (Kennedy *et al*, 1997; Kandel and Hay, 1999). The activation of the PI3K/Akt signalling pathway in cancer cells evolved, at least in part, to employ its conserved function in metabolism to drive cancer cell metabolism and fulfil anabolic demands. As a kinase Akt also affects multiple processes in the cell that contribute to tumourigenesis.

For the past two decades, the PI3K/Akt pathway has been one of the most intensively investigated signalling networks in cancer research. Akt is hyperactivated in cancer cells by multiple mechanisms, including the loss of PTEN, mutations that activate the catalytic subunit of PI3K, p110α, mutations that activate Akt isoforms, the activation of RAS and growth factor receptors and amplification of the genes encoding the catalytic subunit of PI3K and Akt (Figure 1). When PI3K is activated by various signalling events, phosphatidylinositol 3,4,5-trisphosphate (PIP3) is generated from the substrate phosphatidylinositol 3,4-bisphosphate (PIP2). The PIP3 then binds the pleckstrin homology (PH) domain of Akt and translocates Akt to the cell membrane. The recruitment of Akt to the plasma membrane, or other membranes, drives the phosphorylation of Akt at Ser473 within the hydrophobic C-terminal domain by mTORC2, and by the phosphoinositide-dependent kinase 1 (PDK1) at Thr308 within the catalytic domain for full activation (Figure 1). Akt is then able to translocate to other cellular compartments, such as the cytoplasm and nucleus, and phosphorylate various downstream substrates at serine or threonine residues (Manning and Cantley, 2007).

In mammalian cells, the Akt kinase family comprises three members that share a high degree of amino acid identity, Akt1,

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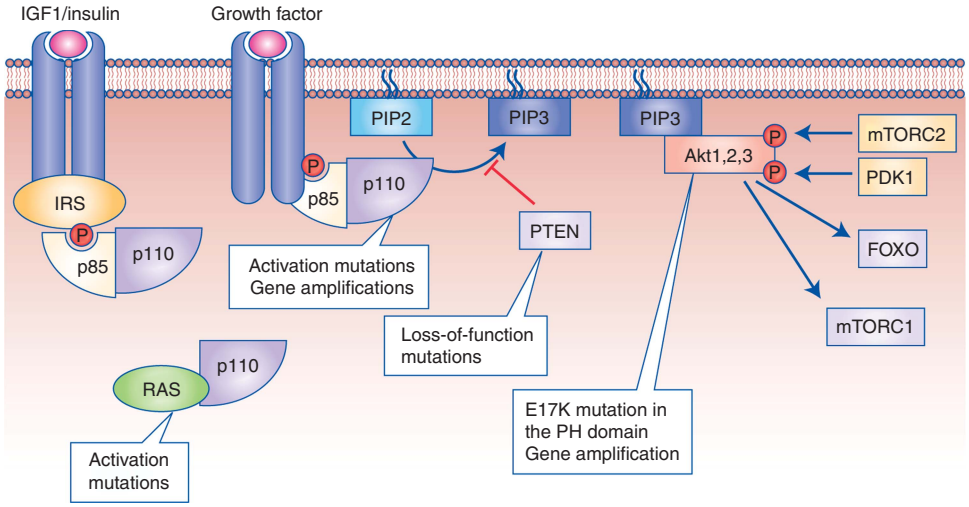

Figure 1. The PI3K/Akt signalling and its activation in cancer. The catalytic subunit of PI3K, p110, is activated by the binding of the regulatory subunit, p85, either to insulin receptor substrate (IRS) proteins downstream of IGF1 or insulin receptor or to the intracellular domain of tyrosine kinase receptors. Activated Ras activates p110 through physical interaction. Lesions that activate the pathway in cancer are indicated (see text for details).

Akt2 and Akt3, that are encoded by distinct genes. Akt1 is highly expressed in the majority of tissues; Akt2 is the predominant isoform in insulin-sensitive tissues, including the liver, skeletal muscle and adipose tissue; and Akt3 expression is highest in the brain and testes. The phenotypes of individual Akt isoform knockout mice correlate with the relative Akt expression levels in the affected tissues or organs. For instance, *Akt2*$^{-/-}$ mice exhibit hyperinsulinaemia and insulin resistance (Cho *et al*, 2001a; Garofalo *et al*, 2003), consistent with the maximised Akt2 expression in insulin-responsive tissues. Moreover, *Akt3*$^{-/-}$ mice exhibit smaller brains (Tschopp *et al*, 2005), consistent with the maximised Akt3 expression observed in the brain. However, the germline deletion of Akt1 did not result in a severe phenotype, and *Akt1*$^{-/-}$ mice live longer than wild-type mice (Chen *et al*, 2001, 2006a; Cho *et al*, 2001b). The phenotypes of combined deletions of different Akt isoforms (reviewed in Hay, 2011a) showed that the deletion of two isoforms could exacerbate the phenotype exerted by the deletion of a single isoform, especially with respect to diabetes. For instance, haploinsufficiency of *Akt1* in *Akt2*$^{-/-}$ mice converts hyperinsulinaemia to hyperglycaemia and hyperactivation of Akt1 in *Akt2*$^{-/-}$ and in Akt1$^{+/-}$; Akt2$^{-/-}$ mice decreased hyperinsulinaemia and hyperglycaemia respectively (Chen *et al*, 2009), suggesting synergetic and overlapping activities of the different Akt isoforms. The diabetic phenotypes of Akt2$^{-/-}$ and Akt1$^{+/-}$; Akt2$^{-/-}$ mice are due in part to lipodystrophy and leptin deficiency (Chen *et al*, 2009). These results exemplify the relevance of studies in mice to humans, as it was reported that a family with inherited mutation in Akt2, which exerts dominant negative activity, developed type II diabetes associated with lipodystrophy (George *et al*, 2004; Tan *et al*, 2007).

Together, these results suggest that the functions of the different Akt isoforms at the organismal level may not be determined by their different substrate specificities but rather by their relative levels of expression. Indeed, the three Akt isoforms exhibited similar substrate specificity towards a generic substrate in a kinase assay *in vitro* (Walker *et al*, 1998). However, multiple reports showed that the different Akt isoforms exhibited specific activities and substrate specificity at the cellular level (Iliopoulos *et al*, 2009; Toker, 2012) that were, at least in part, attributed to differences in the intracellular localisation of the different isoforms (Gonzalez and McGraw, 2009; Cariaga-Martinez *et al*, 2013; Toker and Marmiroli, 2014).

Currently, pan-Akt inhibitors are being used in clinical trials for cancer therapy. Specifically, two types of inhibitors, ATP-competitive inhibitors (GSK690693, GDC0068, AZD5363) and an allosteric inhibitor, MK-2206, are being examined. Some clinical trials have shown promising results, either by treatment with pan-Akt inhibitors alone or in combination with other therapeutic regimens, but several toxicities were also observed. In particular, diarrhoea and hyperglycaemia were observed in many clinical trials (Hudis *et al*, 2013; Yap *et al*, 2014; Ma *et al*, 2015; Ramanathan *et al*, 2015; Jansen *et al*, 2016; Saura *et al*, 2017). Notably, results reported in clinical trials also showed a certain percentage of patients with elevated levels of liver enzymes, suggesting liver injury (https://clinicaltrials.gov/ct2/results?term=akt+inhibitor&show_down=Y).

THE EFFECT OF AKT GENE DELETION ON TUMOURIGENESIS IN MOUSE MODELS OF CANCER

Several mouse models have been used to characterise the function of the different Akt isoforms in tumourigenesis. Akt1 deficiency, and even the haploinsufficiency of Akt1, is sufficient to dramatically inhibit the incidence and development of tumours in *Pten*$^{+/-}$ mice in all tissues tested, including the prostate, endometrium and small intestine (Chen *et al*, 2006b). In contrast, Akt2 deficiency is not sufficient to significantly inhibit the incidence of tumours in these mice, except in the thyroid, where Akt2 is the predominantly expressed isoform (Xu *et al*, 2012). The inability of Akt2 deletion to inhibit tumour development in *Pten*$^{+/-}$ mice was attributed to the high circulating level of insulin as a consequence of Akt2 deletion (Xu *et al*, 2012) that hyperactivates the other Akt isoforms and possibly other oncogenic signalling pathways and may consequently curb the effect of Akt2 deletion on the tumours. Another group also showed that Akt1, but not Akt2, germline deletion prevents lung tumourigenesis in carcinogen-induced or oncogenic K-Ras mouse models, whereas Akt3 deletion increased tumour incidence in the carcinogen-induced model and tumour size in the genetic model (Hollander *et al*, 2011). In a different mouse model of lung cancer, Akt1 germline deletion inhibited tumourigenesis, whereas Akt2 deletion increased it (Linnerth-Petrik *et al*, 2014). In an ErbB2-induced mammary tumourigenesis model, Akt1 deficiency delayed tumour growth and reduced lung metastases (Ju *et al*, 2007). In a different report in both polyoma middle T (PyMT) and ErbB2-driven mammary adenocarcinoma

mouse models, the deletion of Akt1 inhibited, whereas the deletion of Akt2 accelerated, tumour induction (Maroulakou *et al*, 2007).

THE CONSEQUENCES OF SYSTEMIC AKT GENE DELETION IN ADULT MICE

The studies described above employed mice with germline deletions of Akt isoforms and can consequently only address the requirement of Akt isoforms for tumour initiation and development. To emulate drug therapy, we began to systemically delete Akt isoforms in adult mice after tumour detection. We showed that the systemic deletion of Akt1 in $p53^{-/-}$ mice after tumour onset regressed thymic lymphoma and substantially increased the lifespan of the mice without adverse physiological consequences (Yu *et al*, 2015). The effect exerted by systemic Akt1 deletion on $p53^{-/-}$ thymic lymphoma phenocopies the effect of p53 restoration on $p53^{-/-}$ thymic lymphoma (Ventura *et al*, 2007), and the allosteric pan-Akt inhibitor MK2206 recapitulated this effect of systemic Akt1 deletion. However, MK2206 inhibits Akt1 and Akt2 with similar IC50 values.

The germline deletion of both Akt1 and Akt2 or both Akt1 and Akt3 in mice is neonatal lethal and embryonic lethal, respectively (Peng *et al*, 2003; Yang *et al*, 2005). To determine the consequences of their deletion in adult mice, we first systemically deleted Akt1 in either adult $Akt2^{-/-}$ or $Akt3^{-/-}$ mice. Interestingly, unlike the germline deletion, the systemic deletion of Akt1 in $Akt3^{-/-}$ mice was tolerated in adult mice, whereas the systemic deletion of Akt1 in $Akt2^{-/-}$ mice rapidly elicited mortality (Wang *et al*, 2016). Similar results were obtained after the systemic deletion of both Akt1 and Akt2 or after treating the mice with MK2206 at a dose only double the commonly used dose (Wang *et al*, 2016). Mortality was preceded by an increase in circulating glucose and insulin levels, followed by a decrease in glucose to a hypoglycaemic level. The mice lost body weight and body fat, the intestinal villi in the mice were disrupted and crypt cell proliferation was diminished. The intestinal damage observed in mice may explain the high incidence of diarrhoea after treatment with pan-Akt inhibitors in clinical trials. We also observed severe inflammation, as measured by the high level of IL-6 in the blood. This high level of inflammation may be due, at least in part, to infiltrating bacteria resulting from the disrupted intestinal barrier. We speculate that the mice could not absorb food because of the disrupted villi and consequently consumed body fat instead until exhausted, leading to hypoglycaemia and death. It should be noted that systemic deletion of the individual Akt isoforms did not elicit the intestinal phenotype. However, the ability of specifically deleting both Akt1 and Akt2 in the crypt cells to cause the same phenotype remains to be seen. Although these experiments were conducted in mice, their results raise concerns regarding the potential toxicity associated with the use of pan-Akt or pan-PI3K in clinical trials at doses that markedly ablate total Akt activity.

Although the systemic deletion of Akt1 and Akt2 is not tolerated in adult mice, the hepatic deletion of Akt1 in $Akt2^{-/-}$ mice is tolerated. However, unexpectedly, these mice develop early-onset aggressive hepatocellular carcinoma (HCC) (Figure 2). Adult mice in which hepatic deletion of both Akt1 and Akt2 is induced also develop HCC, but with much longer latency period. The loss of Akt1 and Akt2 in hepatocytes resulted in cell apoptosis and consequently elevated the serum level of liver enzymes, resulting in macrophage infiltration and inflammation, as measured by high levels of IL-6 and TNFα. Then, IL-6 activated STAT3 and induced the proliferation of surviving hepatocytes. In our study, we used Ki67 to evaluate cell proliferation and found that most Ki67-positive cells were located inside the tumour, whereas apoptotic cells were located around the tumour, as detected by caspase-3 staining. Notably, liver injury and inflammation is due to the activation FOXO1 in the absence of Akt activity. Activated FOXO1 upregulates some pro-apoptotic genes, such as Fasl and Bcl2l11 (Bid), that are responsible for cell death. The HCC that developed in the absence of Akt1 and Akt2 exhibited the gene signature of aggressive human HCC. Moreover, the dramatic induction of Igf2BP3, which is strongly associated with advanced tumour stage and has been considered a predictor of poor prognosis among patients with HCC (Jeng *et al*, 2008), is especially interesting. Although these results seem counterintuitive, it should be noted that obesity and fatty liver, which inhibit hepatic Akt activity, are also risk factors for HCC (Sun and Karin, 2012). Finally, in diethylnitrosamine (DEN)-treated mice, a model of HCC, the incidence of lung metastasis was markedly increased in $Akt2^{-/-}$ but not $Akt1^{-/-}$ mice. Again, this phenomenon could be attributed to the very high level of insulin in Akt2-deficient mice (Wang *et al*, 2016).

Notably, the hyperactivation of Akt due to the hepatic deletion of PTEN also induces HCC, but with a much longer latency period than that observed in the absence of Akt activity (Horie *et al*, 2004). Interestingly, it was reported the hepatic PTEN deletion also increased liver injury that is attenuated by hepatic deletion of Akt2 (Galicia *et al*, 2010). However, it is likely that total hepatic Akt activity was not markedly decreased because PTEN deficiency hyperactivates Akt1 (hepatocytes do not express Akt3) and the mice likely do not have hyperinsulinaemia. Finally, there are other precedents in which the ablation of pro-oncogenic and survival signalling have been shown to accelerate hepatocarcinogenesis in several examples (Feng, 2012).

CONCLUDING REMARKS

The results obtained in mice suggest the following. First, the complete inhibition of Akt activity in the liver by treatment with

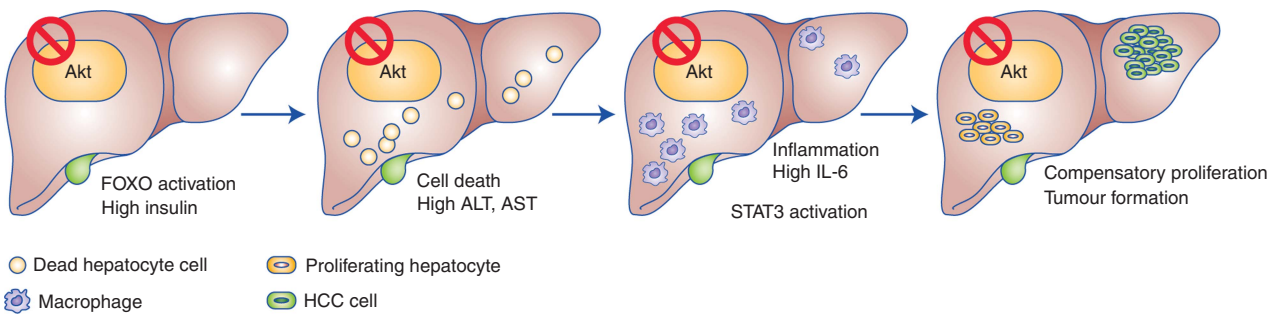

Figure 2. Schematic depicting the stages of HCC development after the ablation of hepatic Akt activity. Deletion of Akt1 and Akt2 in hepatocytes results in cell death, liver damage and inflammation in a FoxO1-dependent manner. Consequently, macrophages (Kupffer cells) are recruited as well as plasma cells that induce inflammatory cytokines such as IL-6. In turn, IL-6 activates STAT3 in the survived hepatocytes and induces proliferation and survival. Proliferating hepatocytes accumulate mutations that eventually results in HCC.

pan-PI3K or pan-Akt inhibitors may increase liver injury and inflammation that are prerequisites for liver cancer. Second, these results suggest that treating obese patients or patients who experienced liver damage with pan-PI3K/Akt inhibitors may exacerbate liver damage and inflammation as well as the risk for liver cancer. Third, close attention should be paid to inflammation and liver injury when pan-PI3K/Akt inhibitors are being used, particularly for HCC therapy.

The effects of pan-PI3K/Akt inhibitors may not have been fully manifested in clinical trials because of the influence of these inhibitors on glucose homeostasis. The systemic inhibition by pan-PI3K/Akt inhibitors may induce hyperinsulinaemia and consequently attenuate the efficacy of the inhibitors. However, it cannot be excluded that a certain dose of the pan-inhibitor could be effective without having a marked effect on glucose homeostasis and insulin level. The side effects on glucose homeostasis and insulin levels may be overcome by combining the treatment with a diabetes drug, such as metformin, that may decrease insulin levels following pan-PI3K/Akt inhibition. As metformin has also been considered for cancer therapy (Chae *et al*, 2016), the combination of metformin and pan-PI3K/Akt inhibitors may be highly beneficial. Alternatively, Akt isoform-specific inhibitors may be employed because early studies that led to the development of MK2206 identified compounds that more selectively inhibit individual Akt isoforms (DeFeo-Jones *et al*, 2005). The use of isoform-specific inhibitors may be more effective if the inhibitors are tailored to the cancer in which the specific Akt isoform is highly expressed or activated. However, one drawback to the use of isoform-specific inhibitors is a potential compensatory response that may lead to the hyperactivation of other Akt isoforms. The results obtained in mice indicate that Akt2-specific inhibition should be avoided if possible because it is the major cause of hyperinsulinaemia and hyperglycaemia. This effect is also observed in humans – a missense mutation in the Akt2 gene has been implicated in insulin resistance and diabetes that phenocopies Akt2 deletion in mice (George *et al*, 2004; Tan *et al*, 2007; Chen *et al*, 2009). Finally, other approaches that exploit the metabolic consequence of Akt activation could be developed to selectively eradicate cancer cells exhibiting Akt hyperactivation (Nogueira *et al*, 2008).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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