

## Review

# The Akt isoforms, their unique functions and potential as anticancer therapeutic targets

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## Abstract

Akt (also known as protein kinase B or PKB) is the major downstream nodal point of the PI3K signaling pathway. This pathway is a promising anticancer therapeutic target, because constitutive activation of the PI3K-Akt pathway is correlated with tumor development, progression, poor prognosis, and resistance to cancer therapies. The Akt serine/threonine kinase regulates diverse cellular functions including cell growth, proliferation, glucose metabolism, and survival. Although all three known Akt isoforms (Akt1–3) are encoded by separate genes, their amino acid sequences show a high degree of similarity. For this and other reasons, it has long been assumed that all three Akt isoforms are activated in the same way, and their functions largely overlap. However, accumulating lines of evidence now suggest that the three Akt isoforms might have unique modes of activation and many distinct functions. In particular, it has recently been found that the Akt isoforms are localized at different subcellular compartments in both adipocytes and cancer cells. In this review, we highlight the unique roles of each Akt isoform by introducing published data obtained from both *in vitro* and *in vivo* studies. We also discuss the significant potential of the Akt isoforms as effective anticancer therapeutic targets.

**Keywords:** Akt isoforms; anticancer target; cancer therapeutics; PI3K; signaling pathway.

## Introduction

The phosphatidylinositol-3 kinase (PI3K)-protein kinase B (PKB/Akt) pathway regulates many cellular functions in mammalian cells, making it one of the most extensively studied protein kinase signaling pathways (1–4). Signaling through this axis regulates the delicate balance between the life and death of the cell by controlling numerous processes, including cellular metabolism, cell growth and proliferation, protein synthesis, and cell death through apoptosis and autophagy (5–7). Furthermore, the PI3K-Akt pathway is often deregulated in many different types of cancer (4, 8). Thus, the PI3K-Akt signaling pathway becomes the subject of intense research interest as an attractive target for cancer therapeutics.

The Akt serine/threonine kinase family comprises three isoforms, Akt1 (PKB $\alpha$ ), Akt2 (PKB $\beta$ ), and Akt3 (PKB $\gamma$ ), which show a high degree of amino acid sequence similarity. In spite of the high sequence similarity, the three Akt isoforms appear to have many unique functions. Unfortunately, the functional differences among the isoforms are often obscured by the fact that data presented in many publications were generated without distinguishing each isoform. For example, researchers often study Akt activities using PI3K inhibitors such as LY294002 and wortmannin under the assumption that the Akt isoforms are functionally redundant. Furthermore, pan-Akt antibodies have been widely used for a long time in studying Akt-specific functions, mainly owing to the lack of isoform-specific antibodies. As a result, the majority of published data do not delineate the unique function of each Akt isoform. Therefore, some of the known Akt functions can be applicable to one isoform but not others, although the three Akt isoforms apparently share some functional redundancy.

Fortunately, the development of Akt isoform-specific antibodies and the availability of isoform-specific siRNA now allow researchers to study the unique functions of each Akt isoform. Using these new tools, we have recently found that the Akt isoforms are localized in distinct subcellular compartments (9). The unique function of each Akt isoform has also been elucidated using transgenic mice whose Akt isoforms have been selectively ablated. This review focuses on the unique functions of each Akt isoform by analyzing data obtained from *in vitro* and *in vivo* models. Furthermore, we also discuss the significant potential of the Akt isoforms as effective anticancer targets.

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## Discovery of the Akt isoforms

Akt1, which was initially named AKT8, was identified and cloned from a T cell lymphoma (10, 11). The amino acid sequence of Akt shows similarity to those of protein kinases A and C (5, 7, 12). Thus, Akt1 is often called PKB $\alpha$  and RAC-PK $\alpha$  (for related to PKA and PKC) (13, 14). Akt2/PKB $\beta$  was the second Akt isoform identified and cloned, which was subsequently found to overexpress in ovarian cancer (15). The Akt3/PKB $\gamma$  isoform was cloned much later, independently by three different groups (16–18).

## Gene and amino acid sequences of the Akt isoforms

Genes encoding the Akt1–3 isoforms have been mapped to 14q32, 19q13.1–q13.2, and 1q43–44, respectively (12, 17). Although encoded by separate genes, the Akt isoforms show similar overall protein structure and amino acid sequence.

All three isoforms comprise an N-terminal pleckstrin homology (PH) domain, a catalytic or kinase domain in the middle, and a C-terminal regulatory domain/extension region (Figure 1). The amino acid sequence of the Akt isoforms shows approximately 80%, 90%, and 70% identity within their PH, catalytic, and C-terminal domains, respectively (7, 22) (Figure 1). Sequence divergence is present within the linker region (LR) that joins the PH and catalytic domains (22). The catalytic/kinase domain includes a regulatory threonine phosphorylation site (Thr308, Thr309, and Thr305 for Akt1–3, respectively), which is required for the activation of Akt. All of the three Akt isoforms also contain a second regulatory phosphorylation site (Ser473, Ser474, and Ser472 for Akt1–3, respectively) within the C-terminal domain (Figure 1).

Unlike the Akt1 and Akt2 isoforms, a splice variant has been reported for Akt3, which has been termed Akt3-v1 (23). The amino acid sequence of Akt3-v1 is almost identical with Akt3, except that it does not contain the C-terminal extension region including the Ser472 regulatory residue. Thus, it is

Akt1	MSDVAIVKEGWLHKGREYIKTWRPRYFLLKNDGTFIGYKERPQVDVDQREAPLNNSVAQC	60
Akt2	MNEVSVIKEGWLHKGREYIKTWRPRYFLLKSDGSFIGYKERPEAPDQTLPLNNSVAEC	60
Akt3	MSDVTIVKEGWVQKRGREYIKNWRPRYFLLKTDGSGFIGYKEKQPVDVLP-YPLNNSVAKC	59
	* * **** *	
Akt1	QLMKTERPRPNTFIIIRCLQWTTVIERTFHVETPEEREWTTAIQTIVADGLKKQE--EEM	118
Akt2	QLMKTERPRPNTFVIRCLQWTTVIERTFHVDSPDEREEMRAIQMVANSKQRAPGEDPM	120
Akt3	QLMKTERPKPNTFIIIRCLQWTTVIERTFHVDTPEEREWTEAIQAVADRLQRQE--EERM	117
	***** *	
Akt1	DFRSGSPSDNSGAEEMEVSLAKPKHRVTMNEFEYLKLLGKGTFGKVIIVKKEKATGRYYAM	178
Akt2	DYKCGSPSDSSTTEEMEVAVSKARAKVTMNDFDYLKLLGKGTFGKVIIVREKATGRYYAM	180
Akt3	NCSPTSQIDNIGEEEMDASTTHHK-RKTMNDFDYLKLLGKGTFGKVIIVREKASGKYYAM	176
	* *	
Akt1	KILKKEVIVAKDEVAHTLTENRVLQNSRHPFLTALKYSFQTHDRLCFVMEYANGGELFFH	238
Akt2	KILRKEVIAKDEVAHTVTSRVLQNRHPFLTALKYAFQTHDRLCFVMEYANGGELFFH	240
Akt3	KILKKEVIAKDEVAHTLTESRVLKNRHPFLTSLKYSFQTKDRLCFVMEYVNGGELFFH	236
	*** *	
Akt1	LSRERVFSEDRARFYGAEIVSALDYLHSEKNVYRDLEKLENLMLDKDGHKIKITDFGLCKE	298
Akt2	LSRERVFTTEERARFYGAEIVSALEYLHSD-VVYRDIKLENLMLDKDGHKIKITDFGLCKE	299
Akt3	LSRERVFSEDRTRFYGAEIVSALDYLHSGK-IVYRDLKLENLMLDKDGHKIKITDFGLCKE	295
	***** *	
Akt1	GIKDGATMKTFCGTPPEYLAPEVLEDNDYGRAVDWVWGLGVVYEMMCGRLPFYVQDHEKLE	358
Akt2	GISDGATMKTFCGTPPEYLAPEVLEDNDYGRAVDWVWGLGVVYEMMCGRLPFYVQDHERLE	359
Akt3	GITDAATMKTFCGTPPEYLAPEVLEDNDYGRAVDWVWGLGVVYEMMCGRLPFYVQDHEKLE	355
	** *	
Akt1	ELILMEEIRFPRTLGPPEAKSLLSGLLKDPKQRLGGGSEDAKEMQHRRFFAGIVWQHVVYE	418
Akt2	ELILMEEIRFPRTLSPPEAKSLLAGLLKDPKQRLGGGSPDAKEMHRFFLSINWQDVVQ	419
Akt3	ELILMEDIKFPRTLSSDAKSLLSGLLIKDPKRLGGGPDDAKEMRHSFFSGVNWQDVVD	415
	***** *	
Akt1	KKLSPFPKQVTSSETDTRYFDEEFTAQMITITPPD--QDDSMCEVDSERRPHFPQFSYSA	476
Akt2	KKLLPFPKQVTSVDTRYFDDEFTAQSITITPPD--RYDSLGLLELDQTHFPQFSYSA	477
Akt3	KKLVFPFPKQVTSSETDTRYFDEEFTAQTITITPPEKYDEGDMDMCDNERRPHFPQFSYSA	475
	*** *	
Akt1	SGTA	480
Akt2	SIRE	481
Akt3	SGRE	479
Akt3	SGRE	479
	*	

**Figure 1** The alignment of Akt1 (19), Akt2 (20), and Akt3 (20) amino acid sequences is shown.

Alignment was carried out using the Clustal W software (21). Highlighted boxes in gray, yellow, and blue denote the PH domain, kinase domain, and the C-terminal hydrophobic motif (FXXFSY among AGC kinases), respectively. Highlighted boxes in orange denote the ATP binding motif, and threonine and serine phosphorylation sites (from the N- to C-termini).

conceivable that Akt3-v1 could have a specific regulatory function. As expected, Brodbeck et al. (23) demonstrated that Akt3-v1 has a lower kinase activity than the full-length Akt3. Interestingly, the Akt3-v1 kinase was activated by pervanadate, but not by other stimuli that normally activate PI3K-Akt, including insulin. A study using transgenic mice showed that overexpression of Akt3-v1 was cardioprotective in a short-term, but eventually resulted in cardiomyopathy (24). The recently published crystallization data of the Akt2 kinase domain and the protein structure of Akt1 bound to an allosteric inhibitor (Akt1/2 inhibitor VIII) will further facilitate our understanding of the isoform-specific Akt function (25, 26).

## Regulation of the Akt isoforms

The PI3K-Akt signaling pathway could be activated through the stimulation of receptor tyrosine kinases or G-protein-coupled receptors. Activated PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate [PI(4,5)P<sub>2</sub>] to generate phosphatidylinositol-3,4,5-triphosphate [PI(3,4,5)P<sub>3</sub>]. PIP<sub>3</sub>, in turn, binds to Akt in the cytoplasm and translocates it to the plasma membrane. The localization of Akt at the plasma membrane inner leaflet prompts membrane-localized PDK1 (phosphoinositide-dependent kinase-1) to phosphorylate Akt at Thr308 within the T-loop/activation loop (5). Although Akt phosphorylated at the Thr308 residue is active, full activation requires an additional phosphorylation at the Ser473 by PDK2. mTORC2 (a complex formed by mTor and Rictor) appears to be the long-sought PDK2; however, other protein kinases including ILK (integrin-linked kinase) have been suggested as potential PDK2 (2, 27–30). The phosphorylation at Thr308 more potently activates Akt (approx. 100-fold), compared to Ser473 phosphorylation (approx. 10-fold) (31). The Akt activation process is countered by the tumor suppressor PTEN, which dephosphorylates PIP<sub>3</sub>. Brognard et al. (32) recently found that PHLPP (PH-domain leucine-rich repeat protein) can also downregulate Akt by dephosphorylating Ser473.

## Isoform-specific signaling to downstream substrates

Although Akt isoform-specific signaling pathways have started to gain more attention in recent years, most experiments carried out prior to the year 2003 did not rigorously examine isoform-specific functions as they simply used either pan-Akt antibodies or Akt1-specific substrates in their experiments (Table 1). Data obtained from these studies using a variety of cell lines identified numerous Akt1-specific substrates including FOXO1 (43, 44), FOXO3A (45), FOXO4 (46), Casp9 (47), TSC2 (48, 49), GSK3 $\alpha$  (50), AS160 (51), BAD (52), eNOS (53, 54), ASK1 (55), IKK $\alpha$  (56), p21 (57), p27 (58–60), PFK2 (61), and Palladin (35). One potential caveat is that data described in these early studies were obtained using Akt1-fusion plasmid constructs

and/or pan-Akt antibodies to delineate isoform-specific functions. Akt2-specific substrates, which have been described mostly in recent years include HIF2 $\alpha$  (36), PGC-1 $\alpha$  (37), ER $\alpha$  (41), and B23 (38). B-Raf (42) and HIF1 $\alpha$  have been found to be Akt3-specific (36). The X-linked inhibitor of apoptosis protein (XIAP) is regulated by both Akt1 and Akt2 (39) (Table 1).

One of the most significant extensions to our understanding of isoform-specific Akt signaling was the identification of Skp2 as a direct target of Akt1 (33, 34). The Akt1-mediated phosphorylation of Skp2, a component of the SCF complex E3 ligase, ensures cell cycle progression by degradation of p27, a potent inhibitor of Cdks. Although a substantial number of downstream substrates for each Akt isoform have been identified, considerably more isoform-specific substrates have to be characterized to gain comprehensive understanding on the Akt isoform-specific signaling cascades.

## Characterization of the Akt isoforms using transgenic mice with isoform-specific knockouts

### Akt1 knockouts

The unique function of each Akt isoform has been studied using transgenic knockout mice, where one or two isoforms have been selectively deleted. Both Chen et al. (62) and Cho et al. (63) found that Akt1-knockout mice were not only smaller than the Akt1 intact control but also displayed apoptosis in thymus and defects in spermatogenesis. However, the Akt1<sup>-/-</sup> mice did not display any alterations in glucose or insulin regulation. Interestingly, Chen et al. (62) found that Akt1 mice were viable, but Cho et al. (63) observed that some Akt1<sup>-/-</sup> pups died within 3 days post-parturition. The latter group also found that the surviving mice developed into adulthood, but were much smaller in size than the controls (63). Akt1<sup>-/-</sup> mice showed defective in vascular endothelial growth factor-mediated angiogenesis and ischemia effects, which were not observed in Akt2<sup>-/-</sup> mice (64).

### Akt2 knockouts

Unlike Akt1-knockout mice, Akt2<sup>-/-</sup> mice displayed pathological defects in insulin regulation and glucose homeostasis (65). The Akt2<sup>-/-</sup> transgenic mice showed hyperglycemia and resistance to insulin (65). These phenotypic alterations were specific to Akt2 and were not compensated by wild-type Akt1 and/or Akt3 (65). Garafalo et al. (66) confirmed the earlier findings by Cho et al. (63). However, unlike Cho et al. (63), these authors noted that the Akt2<sup>-/-</sup> mice also displayed a growth defect, similarly to Akt1<sup>-/-</sup> mice (66).

### Akt3 knockouts

Akt3-knockout mice displayed a decrease in brain size by approximately 20%, compared with the Akt3 intact control (67). Akt1<sup>-/-</sup> mice also showed a small brain size; however, this was mainly owing to the decrease in overall body size. Interestingly, the decrease in brain size in the Akt1<sup>-/-</sup> mice

**Table 1** Summary of Akt targets and downstream effectors.

Targets/effectors	Isoforms	Direct target? <sup>a</sup>	Cells used for experiments	References
SKP2	AKT1	Yes	MEF, HEK293T, PC-3, COS-1, IMR90, HeLa	(33, 34)
Palladin	AKT1	Yes	HeLa, SKBR3, 231	(35)
HIF2 $\alpha$	AKT2	No	Renal cell carcinoma (RCC4)	(36)
PGC-1 $\alpha$	AKT2	No	Akt2 <sup>-/-</sup> mice, liver extracts	(37)
Nucleophosmin (NPM/B23)	AKT2	No	MEF Akt2 <sup>-/-</sup> (2KO), HEK293	(38)
XIAP	AKT1, 2	Yes	HEK293 and ovarian (A2780S)	(39)
ASK1	AKT2	Yes	HEK293	(40)
ER $\alpha$	AKT2	Yes	MCF7	(41)
B-Raf	AKT3	Yes	Melanocytes (NHEM), WM35, UACC 903, SK-MEL-24	(42)
HIF1 $\alpha$	AKT3	No	Renal cell carcinoma (RCC4)	(36)
Akt1-specific targets identified prior to 2003				
FOXO1	AKT1	Yes	HEK293	(43, 44)
FOXO3A	AKT1	Yes	HEK293T, CCL39 fibroblasts	(45)
FOXO4	AKT1	Yes	NIH3T3	(46)
Casp9	AKT1	Yes	267 cells, NIH3T3	(47)
TSC2	AKT1	Yes	HEK293	(48, 49)
GSK3 $\alpha$	AKT1	Yes	L6 myotubes	(50)
AS160	AKT1	Yes	NIH3T3-L1 adipocytes	(51)
BAD	AKT1	Yes	NIH3T3	(52)
eNOS	AKT1	Yes	COS cells, human umbilical vein endothelial cells (HUVEC)	(53, 54)
ASK1	AKT1	Yes	HEK293, L929, MCF-7, and HeLa	(55)
IKK $\alpha$	AKT1	Yes	NIH3T3, HEK293	(56)
P21	AKT1	Yes	NIH3T3, 3T3, MDA-MB453, MEF, and HEK293T	(57)
P27	AKT1	Yes	MCF-7, WM35, WM239	(58, 59, 60)
PFK2	AKT1	Yes	HEK293	(61)

<sup>a</sup>“Yes” denotes that these substrates are phosphorylated directly by the Akt isoforms. “No” denotes that signaling transduction to these proteins is dependent on an Akt isoform; however, direct phosphorylation by the particular Akt isoform has not yet been demonstrated.

was as a result of the decrease in overall cell number, whereas that in the Akt3<sup>-/-</sup> mice was caused by reduction in both cell size and number (67). The authors suggested that these decreases could be as a result of the decrease in mTOR signaling in the Akt3<sup>-/-</sup> mice (67). Similar to the Akt1-knockout mice, Akt3<sup>-/-</sup> mice also showed normal glucose metabolism (67), lending credence to the specific role of glucose regulation by Akt2. Data from the Hemming group also confirmed these defects in the Akt3<sup>-/-</sup> mice (68). Taken together, data obtained from mice with single Akt-isoform knockouts delineate Akt1 as being associated with whole body growth, whereas Akt2 and Akt3 are associated with organ-specific functions.

### Akt1 and Akt2 double knockouts

A double deletion of two of the three Akt isoforms was mostly lethal either at the embryonic stage, or a few days after birth. Even when surviving, they displayed extreme growth deficiencies. Akt1 and Akt2 double knockouts were 50% smaller than age-matched controls; displayed decreased cell proliferation, resulting in extremely thin and translucent skin; and muscle atrophy and abnormal bone development (69).

In addition, Akt1 and Akt2 double knockouts displayed a decrease in adipogenesis. This abnormality was the result of defect in PPAR $\gamma$ -1 $\alpha$  (peroxisome-proliferation-activated receptor- $\gamma$ ), which is attributed to the inactivation of Forkhead transcription factors (69). These mice died within a few hours of birth, most probably owing to some type of respiratory failure (69).

### Akt1 and Akt3 double knockouts

The Akt1 and Akt3 double knockouts were lethal at embryonic days E11–E12. These mice displayed developmental defects in the heart and nervous system (70). Mice with a complete Akt1 deletion but with a single Akt3 allele (Akt1<sup>-/-</sup>, Akt3<sup>+/-</sup>) also died shortly after birth. However, double knockout mice left with a single Akt1 allele (Akt1<sup>+/-</sup>, Akt3<sup>-/-</sup>) survived. Together, these data suggest that Akt1 plays an important role in embryonic development.

### Akt2 and Akt3 double knockouts

The Akt2 and Akt3 double knockouts were viable, but displayed defects in glucose metabolism (as observed in the



Akt2 single knockout). The double knockout mice also displayed reduced brain size, similarly to a single Akt3 knockout mouse (71). The Akt2 and Akt3 double knockout in the background of single Akt1 allele (Akt1<sup>+/+</sup>, Akt2<sup>-/-</sup>, Akt3<sup>-/-</sup>) survived, although they displayed growth defects. Collectively, data obtained from experiments with double knockout mice suggests that Akt1 and Akt3 are required for growth and development, whereas Akt2 is involved in the regulation of glucose metabolism.

### **Akt isoforms and glucose metabolism in the context of diabetes**

Despite specific defects shown by deletion of each Akt isoform in transgenic mice, the question of whether the Akt isoforms are functionally redundant is still the subject of intense debate (72). One of the most interesting aspects is the isoform-specific role of Akt1 and Akt2 in glucose metabolism. There are convincing data that Akt2 has an essential role in glucose uptake and transport (73). However, Akt1 also plays a role in the glucose metabolism. It was shown previously that Akt1 could partially compensate for the Akt2 function in glucose metabolism when Akt2 was compromised (74). These authors found that both Akt1- and Akt2-ablated adipocytes with isoform-specific siRNA displayed decreases in glucose uptake. However, the magnitude of the effect was much lower in the Akt1-ablated cells (approx. 20% reduction), compared with Akt2 ablation (approx. 50% reduction) (74). The glucose uptake was reduced by approximately 80% when both Akt1 and Akt2 were ablated, suggesting that both the Akt isoforms played a role in glucose transport. Similarly, Katome et al. (75) found that both Akt1 and Akt2 played a role in glucose metabolism, although Akt2 was the predominant isoform involved in glucose uptake and transport. Bae et al. (76) carried out a similar experiment using Akt2 knockout mice. Data from this experiment demonstrated that Akt2-ablated murine embryonic fibroblasts (MEFs) and adipocytes were decreased in glucose uptake and Glut4 translocation. Their data also indicated that Akt1 could not compensate for Akt2, as only re-introduction of Akt2 in the Akt2-ablated cells could restore the defects in insulin signaling (76). The knockout of Akt2 did not eliminate all glucose uptake in these cells, suggesting that other gene products (such as Akt1) are also involved in the glucose metabolism. The authors found that the Akt1-ablated cells were unable to differentiate into adipocytes, whereas the Akt2-knockout cells were (76). Taken together, Akt1 appears to regulate fibroblast differentiation into adipocytes (adipogenesis), and Akt2 primarily regulates glucose transport and uptake.

### **Akt isoforms and cancer: data from *in vitro* and human tumor studies**

The role of each Akt isoform in cancer development has been studied by analyzing genetic alterations; such as, gene

amplification, mutations in nucleotide sequence, constitutive activation, and abnormally high or low levels of proteins. Mende et al. (77) noted that overexpression of any of the Akt isoforms is sufficient to cause cell transformation and tumor progression. A study of 20 human tumor cell lines revealed that the protein levels of the Akt isoforms were largely cell line-dependent (78). The authors also found that ablation of Akt with isoform-specific siRNA could induce apoptosis; however, the induction was again cell line-dependent. This data is consistent with the idea that each Akt isoform plays a unique role in the regulation of cell survival, although the role might not be the same in the different genetic background. This concept is largely supported by published data (79, 80) (also see below).

### **Akt1 mutations and cancer**

The majority of published Akt-related data were obtained using pan-Akt antibodies that preferentially interact with Akt1. Therefore, many of these 'Akt' data can actually be more relevant to Akt1 functions; however, this tentative conclusion requires a careful and thorough analysis of those data.

Although a substantial number of gene amplifications and other mutations have been found upstream of the PI3K pathway (e.g., PI3KCA encoding p110 $\alpha$ ), a relatively small proportion of malignant tumors contain Akt1 mutations (4, 8, 81). An exception is gastric adenocarcinoma, where 20% of tumors contained Akt1 gene amplification (4, 11). Carpten et al. (82) found Akt1 E17K mutations from human breast, ovarian, and colon tumors. Akt1 E17K is constitutively activated by docking the Akt1 to the plasma membrane, thereby causing uncontrolled Akt signaling and, eventually, cell transformation (82). The same point mutations in Akt2 and Akt3 did not transform cells *in vitro* (82), although another group found an Akt3 E17K mutation in malignant melanoma cells (83).

### **Activation and upregulation of Akt1 in cancer**

There is evidence supporting that the Akt1 isoform is involved in the regulation of cell proliferation and transformation, and tumor metastases. For example, Yun et al. (84) found that Akt1, but not Akt2, was crucial for the G1/S transition. Using a soft agar-based colony formation assay, Liu et al. (85) found that the ablation of Akt1 with isoform-specific antisense RNA resulted in a decrease in transformation ability and induction of apoptosis in several different types of cancer cell lines, including MiaPaCa-2, H460, HCT-15, and HT1080. Consistent with this finding, Sun et al. (86) reported that Akt1 overexpression promoted the proliferation of cancer cells in soft agar. Furthermore, tumors formed when cells overexpressing Akt1 were injected into nude mice (86). Constitutive activation of Akt1 was found in breast, prostate, and ovarian cancers (86). Interestingly, overexpression of Akt1 is associated with a high level of Her2 expression in breast cancer (87). Taken together, these data suggest

that Akt1 might be involved in tumorigenesis and, thus, could be an excellent target for cancer therapeutics.

### Gene amplification and upregulation of Akt2 in cancer

Gene amplification and upregulation of Akt2 proteins have been reported in a variety of different tumors or tumor cell lines. One early study reported the gene amplification and protein overexpression of Akt2 in breast (2.1–3.0%) and ovarian (12.1–14.1%) carcinomas (4, 88). Akt2 gene amplification was also found in primary pancreatic tumors (20%) and cancer cell lines (4, 41, 89–93). Particularly high frequencies (30%) of Akt2 gene amplification were found in primary squamous tumors isolated from head and neck cancer patients (4, 94).

Analysis of tumor samples suggests that there are positive correlations not only between the levels of Akt2 and Her2/neu expression (95) but also between those of Akt2 and the estrogen receptor  $\alpha$  (ER $\alpha$ ) (41). Data reported by Sun et al. (41) suggested that estrogen signaling could be regulated by Akt2, as ER $\alpha$  was phosphorylated by Akt2 at Ser167. Data obtained from hepatocellular carcinoma showed that the elevated level of Akt2 was associated with poor prognosis in hepatocellular carcinoma (96). Single-strand conformation polymorphism assays showed that somatic mutations in the Akt2 kinase domain existed in stomach and lung tumors, although the mutation rates were relatively low (97).

### The role of Akt2 in cancer

Arboleda et al. (98) found that the overexpression of Akt2 in both breast and ovarian cancer cells was associated with an increase in metastases and invasion. The authors also found that tumor cells with an elevated level of Akt2 upregulated the level of  $\beta$ 1 integrin. Data obtained from xenografts with MDA-MB435 breast cancer cells revealed that Akt2 overexpression was associated with an increase in tumor formation and metastasis (98). A study conducted by Jin and Woodgett (99) revealed that a membrane-targeted Akt2 by myristoylation promoted morphological and behavioral changes in the Hek293 human embryonic kidney cells. These changes apparently contributed to cell transformation, because these cells showed increases in cell proliferation, multinucleation, and cell fusion (99). The implication of this finding is that the high level of Akt2 can increase the aggressiveness of metastatic breast cancer.

Because Akt2 upregulation has been associated with cell transformation and cancer progression, its downregulation would have the opposite effect. Consistent with this hypothesis, the knockdown of Akt2 with siRNA resulted in a decrease in proliferation of ovarian cancer cells (100). Similarly, the ablation of Akt2 in lung adenocarcinoma cells with siRNA resulted in a decrease in both cell migration and invasion. By contrast, the downregulation of Akt1 with siRNA did not show a notable effect on lung cancer cells (101). Interestingly, cell migration and invasion were partially inhibited by Akt3 ablation; however, the degree of inhibition

was much lower than that caused by Akt2 ablation (101). Mure et al. (102) found that ablation of both Akt2 and Akt3 induced apoptosis in human malignant glioma cells.

### Akt3 and melanoma

A cDNA microarray showed that Akt3 mRNA was elevated in malignant melanoma, suggesting that Akt3 could play a role in the development or maintenance of the tumor (103). Stahl et al. (104) found that the high expression of Akt3 was directly correlated with the high level of overall Akt activity as well as the advancement and aggressiveness of malignant melanoma. Ablation of Akt3 with isoform-specific siRNA effectively downregulated the overall Akt activity in their melanoma model. Interestingly, however, ablation of neither Akt1 nor Akt2 resulted in a decrease in the overall Akt activity. Furthermore, the increase in Akt3 activity in their melanoma model was associated with both Akt3 gene amplification and mutations in PTEN (104). Subsequently, data from the Robertson group (42, 105) demonstrated a direct link between tumor progression in melanoma cells with Akt3-dependent phosphorylation of B-RAF.

### Akt3 deregulation in other types of cancer

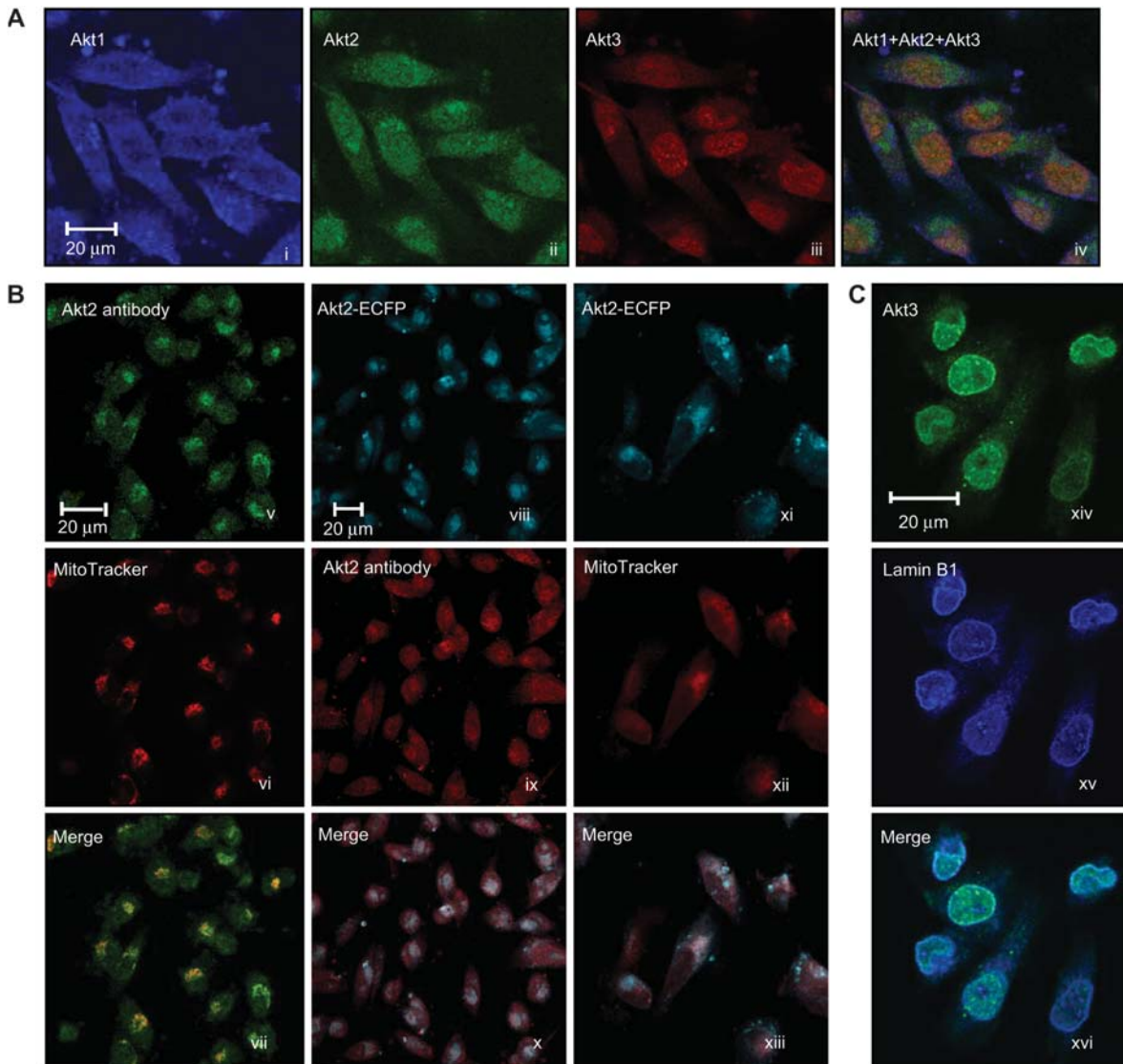
In addition to melanoma, deregulation of the Akt3 activity has also been reported in other types of cancer including ovarian and breast tumors. Cristiano et al. (106) reported upregulation of Akt3 in 20% of ovarian tumors. Their data showed that ablation of Akt3 with shRNA resulted in decreased proliferation of ovarian tumor cells by arresting cell cycle progression at G2/M (106). Faridi et al. (107) showed that the estrogen-dependent MCF7 cells could form estrogen-independent tumors in mice, if Akt3 is constitutively activated. Furthermore, MCF7 cells could develop resistance to tamoxifen when Akt3 was overexpressed. In this case, tamoxifen treatment actually stimulated tumor growth (107, 108). The elevated level of Akt3 activity was also linked to increased aggressiveness in breast cancer and prostate cancer (109).

Collectively, published data suggest that abnormally elevated Akt can increase cell proliferation and transformation as well as tumor progression and metastasis, although a single Akt isoform might not be involved in all of these different stages. Among the three isoforms, Akt1 has been most extensively studied. However, the obscurity of published data on its unique function, particularly those published prior to 2003, make it difficult to accurately delineate the truly unique function for Akt1. Regarding the role in tumor development and progression, Akt2 appears to be the most relevant isoform because it is involved in the regulation of cell transformation and tumor invasion. In addition, Akt2 also plays an essential role in cell proliferation because its activity is dramatically increased upon stimulation with a variety of growth factors including EGF, IGF-I, and PDGF (110). However, the exact mechanism as to how Akt contributes to cancer development and progress is yet to be elucidated.

### The Akt isoforms: does the distinct subcellular localization of the Akt isoforms underlie their unique functions?

According to the currently accepted model, the cytoplasmic Akt bound by PIP<sub>3</sub> is translocated to the inner leaflet of the plasma membrane where it is phosphorylated by PDK1 and PDK2. This model makes sense only if all of the Akt isoforms are normally localized in the cytoplasm. However, we previously found that the Akt isoforms exhibit a differential subcellular localization in most of the cancer cell lines examined: Akt1 was localized to the cytoplasm, whereas Akt2 and

Akt3 were localized to the mitochondria and the nucleus/nuclear membrane, respectively (Figure 2) (9). These distinct subcellular localizations of the Akt isoforms did not change in response to external stimuli such as ionizing radiation or epidermal growth factor (9). Furthermore, siRNA-mediated ablation of one or two Akt isoforms did not result in the translocation of the remaining Akt isoform(s) (9). Taken together, we can make the following two conclusions: (i) the currently accepted model might be applicable to the activation of Akt1, but not to Akt2 and Akt3, because these two isoforms do not localize in the cytoplasm; and (ii) one Akt isoform might not compensate for the function of other iso-



**Figure 2** The Akt isoforms are localized in different subcellular compartments.

(A) Triple staining of MDA-MB231 cells showed distinct subcellular localization patterns. Cells were immunostained with antibodies against Akt1 (blue, panel i), Akt2 (green, panel ii) and Akt3 (red, panel iii). Note that Akt1–3 are localized in the cytoplasm, mitochondria, and nucleus, respectively. (B) Akt2 was confirmed to colocalize with the mitochondria. Colocalization of Akt2 with MitoTracker Red is shown in panels v–vii. Ectopically expressed recombinant Akt2 proteins (Akt2-ECFP, panels viii–x; Akt2-ECFP, panels xi–xiii) were also localized at mitochondria. (C) Akt3 is localized in the nucleus. Lamin B1 was used as a nuclear marker. The data were originally published in (9) and are reprinted here with permission.



form(s) in the majority of cases, because the subcellular localization of each isoform does not alter even when one or more isoform(s) are ablated.

The finding that both activated and non-activated Akt2 localized to the mitochondria is intriguing, because both the Akt and the mitochondria are intimately involved in cell survival and death (9). Although pan-Akt and phospho-pan-Akt antibodies were used in their experiments, Bijur and Jope (111) found that Akt was associated with the mitochondrial matrix and the inner/outer mitochondrial membranes. The authors hypothesized that activated Akt could undergo 'selective' uptake into the mitochondria, because mitochondrially localized Akt was able to phosphorylate known Akt substrates such as GSK-3 $\beta$ . In light of the data presented by Santi and Lee who used isoform-specific antibodies and siRNA in their experiment (9), the Akt associated with the mitochondria described by Bijur and Jope is probably Akt2, not a selective uptake of (any) Akt. Consistent with this interpretation, Finocchietto et al. (112) found that phosphorylated Akt2 translocated to the inner membrane of the mitochondria in response to insulin and phosphorylated nNOS.

Knockdown of Akt2 with isoform-specific siRNA resulted in initially a cell cycle arrest and an increase in the mitochondrial volume, probably by upregulating PGC-1 $\alpha$  (peroxisome proliferator-activated receptor-coactivator 1 $\alpha$ ), a key regulator of mitochondrial biogenesis (Santi and Lee, unpublished data). Prolonged inhibition of Akt2 eventually caused pathological autophagy of mitochondria in the MDA-MB231 breast cancer cells (Santi and Lee, unpublished data). This data suggests that Akt2 is intimately involved in the mitochondrial homeostasis.

Two different groups reported that Akt2 is associated with Glut4 transporters in adipocytes, supporting the role of Akt2 in glucose transport (73, 113). Gonzalez and McGraw (114) found that the stimulation of a cell with insulin caused Akt2 to accumulate at the plasma membrane promoting the translocation of Glut4. However, the Akt1 E17K mutant that constitutively localized to the plasma membrane could also regulate Glut4 activation, suggesting that the subcellular localization of Akt, not a specific isoform, is important for the regulation of this pathway (114).

## Akt inhibitors as cancer therapeutics

Compelling lines of evidence now suggest that deregulation of the PI3K-Akt signaling pathway is involved in cell transformation and tumor progression, which make this pathway an attractive target for anticancer therapeutics. Therefore, it is no wonder that PI3K and Akt have become an intense research interest by many researchers in both academia and industry. Some of the PI3K inhibitors such as LY294002 and wortmannin effectively inhibit the entire PI3K-Akt pathway. However, these upstream inhibitors often show high side effects (81, 115, 116). It is probable that targeting nodal proteins immediately downstream of crucial genetic alterations within a signaling pathway would be more specific and effective. Thus, Akt could be a better target for anticancer

therapeutics than PI3K, if deregulation occurs at receptor tyrosine kinase or PI3K. Indeed, many pharmaceutical companies are in the process of developing effective Akt inhibitors, some of which are in clinical trials (4, 81, 117–122).

One of the most studied Akt inhibitors at the clinical level is octadecyl-(1,1-dimethyl-4-piperidyl) phosphate (perifosine, AEterna Zentaris Pharmaceuticals, Warren, NJ, USA), a lipophilic orally bioavailable synthetic acetylphospholipid molecule. Data obtained from phase II trials with perifosine on many different tumor types showed mixed results [reviewed in (117)]. Perifosine appears to be not very effective as a single anticancer agent for clinically advanced cancers, including sarcoma (120), metastatic melanoma [(122), and prostate cancer (123)]. However, encouraging results have been obtained when perifosine is used in combination with other anticancer agents, including bortezomib and dexamethason for multiple myeloma and capecitabine for colorectal cancer [reviewed in (117)]. Although perifosine is known as an Akt inhibitor, it also inhibits protein kinase C, MAP kinase and SAPK/JNK (124–126).

Akti (MK-2206, Merck & Co., Rahway, NJ, USA) is an allosteric Akt inhibitor that shows promising clinical results. Cherrin et al. (118) recently found that weekly subcutaneous dosing of Akti resulted in the dose-dependent suppression of LNCap prostate cancer xenografts (an AR-dependent tumor with PTEN deletion and constitutively active Akt). Their data showed complete tumor growth inhibition at 200 mg/kg body weight, a dose that maintained inhibition of Akt1 and Akt2 of >80% and 50%, respectively. As expected, Akti treatment caused a significant increase in blood glucose and insulin levels. However, the increase was transient and reversible, suggesting that appropriate dosing and treatment schedules can overcome the hyperglycemia problem (118). Akti (MK-2206) inhibits all three Akt isoforms, although it inhibits Akt1 preferentially.

Clinical data generated by isoform-specific Akt inhibitor is presently unavailable. The only exception is the Akt1-specific RX-0201 (an antisense oligonucleotide to Akt1 mRNA), which showed a promising result by a preliminary phase I study [reviewed in (117)]. Presently, developing isoform-specific pharmacological Akt inhibitors remains a challenge.

## Expert opinion

One problem of developing signal inhibitors as anticancer agents is the presence of feedback loops and crosstalk within or between signaling pathways. It is now well-known that the inhibition of mTOR, a major downstream nodal protein in the PI3K-Akt pathway, can elevate Akt activities and further promotes tumor growth. This is because the mTOR-p70S6K signaling pathway has a negative feedback loop to downregulate the Akt signaling pathway (127, 128). Blocking the PI3K pathway in the context of certain genetic alterations can also be a problem owing to a crosstalk regulation. For example, tumor cells could contain mutations in the receptor tyrosine kinase or RAS that can activate both RAF-



MAPK and PI3K pathways. Inhibition of PI3K in these cells can actually upregulate the RAF-MAPK signaling pathway and, thus, promote cancer cell proliferation. This is because these two pathways have cross-inhibitory effects (129). One additional problem is that the PI3K-Akt and other signaling pathways can be substantially different between different tumors, making it necessary to take different approaches to treat different tumors (9).

To overcome some of these problems, the following two strategies are currently being considered. One approach is to use combinations of targeting two (or more) different nodal points in the same or different pathway(s) (4, 130). For example, PI-103, a potent inhibitor for both PI3K and mTOR, could effectively block the growth of aggressive glioma cells (131). Although not mutually exclusive with the combinational approach, the second option is to specifically block a key Akt isoform for the treatment of certain types of tumors. For example, we have recently found that the ablation of Akt2 with isoform-specific siRNA effectively killed MDA-MB231 breast cancer cells through the blocking of both Akt2 and p70S6K (Santi and Lee, unpublished data). This approach can be effective because specific Akt2 inhibition can circumvent the feedback loop. Theoretically, it is also possible that Akt isoform-specific blocking might not activate other signaling pathways by crosstalk. Despite advantages gained by hypothetically targeting the individual Akt isoforms, targeted specificity of the Akt isoforms with pharmacological inhibitors in cancer treatment remains elusive owing to the shared homology between the isoforms. Furthermore, a thorough understanding of activation mechanism for each Akt isoform-specific signaling cascade is essential to develop isoform-specific inhibitors. Data obtained from subcellular localization experiments by several different laboratories cast serious doubt whether the currently accepted Akt activation mechanism is applicable to the activation of Akt2 and Akt3. Future research will undoubtedly focus on this aspect.

## Outlook

Akt isoform-specific blocking (with or without the combination of other inhibitors) appears to be an excellent strategy in developing effective and safe cancer therapeutics. However, Akt isoform-specific signal cascades are still poorly understood, hampering the development of effective blockers for each isoform. One encouraging development is that Akt isoform-specific siRNA and antibodies are now readily available (9). Using these and other tools, researchers are now in a good position to 'map' the isoform-specific signaling cascade in the context of different genetic backgrounds. Future research should also include biomarker studies to correlate Akt isoform-specific signal circuits and different genetic backgrounds. Developing Akt isoform-specific pharmacological agents is still very challenging, mainly owing to the extensive amino acid similarity between the Akt isoforms. Therefore, it is also imperative to determine the protein structures of all three Akt isoforms, which will help to develop

isoform-specific (allosteric) small molecular inhibitors. Nevertheless, effective treatments of cancer (and other diseases such as diabetes) based on the inhibition of each Akt isoform are likely to be developed in the near future.

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