Signaling by phosphoinositide 3-kinase isoforms downstream of receptor tyrosine kinases in human cancer

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Signaling by Phosphoinositide 3-kinase Isoforms Downstream of Receptor Tyrosine Kinases in Human Cancer

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4. Overview of the presented publications

4.1 Arcaro, A., Volinia, S., Zvelebil, M.J., Stein, R., Gout, I., Layton, M.J., Ahmadi, K., Watton, S.J., Downward, J. and Waterfield, M.D. (1998). Human phosphoinositide 3-kinase C2β, the role of calcium and the C2 domain in enzyme activity. *J. Biol. Chem.*, 273, 33082-33090

4.2 Arcaro, A., Zvelebil, M.J., Wallasch, C., Ullrich, A., Waterfield, M.D. and Domin, J. (2000). Class II phosphoinositide 3-kinases are downstream targets of activated polypeptide growth factor receptors. *Mol. Cell. Biol.,* 20, 3817-3830

4.3 Arcaro, A., Khanzada, U.K., Vanhaesebroeck, B., Tetley, T.D., Waterfield, M.D., and Seckl, M.J. (2002). Two distinct phosphoinositide 3-kinases mediate polypeptide growth factor-stimulated PKB activation. *EMBO J*., 21, 5097-5108

4.4 Katso, R.M., Pardo, O.E., Palamidessi, A., Franz, C., Marinov, M., De Laurentiis, A., Downward, J., Scita, G., Ridley, A.J., Waterfield, M.D., and Arcaro, A. (2006). Phosphoinositide 3-kinase C2β Regulates Cytoskeletal Organization and Cell Migration via Rac-dependent Mechanisms. *Mol. Biol. Cell,* 17, 3729-3744

4.5 Khanzada, U.K., Pardo, O.E., Meier, C., Downward, J., Seckl, M.J., and Arcaro, A. (2006). Potent inhibition of small cell lung cancer cell growth by simvastatin reveals selective functions of Ras isoforms in growth factor signalling. *Oncogene,* 25, 877-887

1. Abbreviations

BCR – B-cell receptor
CREB – cAMP response element-binding protein
DAG - Diacylglycerol
4E-BP – 4E-binding protein
EGFR – Epidermal growth factor receptor
Erk – Extracellular signal-regulated kinase
FGF – Fibroblast growth factor
FOXO – Forkhead box class O transcription factor
FRS2 – Fibroblast growth factor receptor substrate 2
GAP – GTPase activating protein
GPCR – G-protein coupled receptor
GEF – Guanine nucleotide exchange factor
GSK-3 – Glycogen synthase kinase-3
HGF – hepatocyte growth factor
HMG-CoA – 3-Hydroxy-3methylglutaryl CoA
IGF-IR – Insulin-like growth factor-I receptor
IP_3 – Inositol 1,3,5-trisphosphate
IRS – Insulin receptor substrate
JNK – c-Jun N-terminal kinase
MAPK – Mitogen-activated protein kinase
mTOR – Mammalian target of rapamycin
PDGFR – Platelet-derived growth factor receptor
PDK1 – Phosphoinositide-dependent kinase-1
PH – Pleckstrin homology
PI3K – Phosphoinositide 3-kinase
PI – Phosphatidylinositol
PI(4)P – Phosphatidylinositol 4-monophosphate
PI(3)P – Phosphatidylinositol-3-monophosphate
$PI(4,5)P_2$ – Phosphatidylinositol-4,5-bisphosphate
$PI(3,4)P_2$ – Phosphatidylinositol-3,4-bisphosphate

$PI(3,4,5)P_3$ or PIP_3 – Phosphatidylinositol-3,4,5-trisphosphate

PKB – Protein kinase B (also termed Akt)

PKC – Protein kinase C

PLC – Phospholipase C

PTB – Phosphotyrosine-binding

PTEN – Phosphatase and tensin homolog deleted on chromosome 10

RTK – Receptor tyrosine kinase

SCF – Stem cell factor

Shc – Src-homology collagen protein

S6K – Ribosomal protein S6 kinase

SH2 – Src homology region 2

SHIP – SH2-containing inositol-5-phosphatase

TCR – T-cell receptor

TLR – Toll-like receptor

TSC – Tuberous sclerosis complex

2. Summary

The articles presented here report results from studies aimed at investigating the role of phosphoinositide 3-kinase (PI3K) signaling in cellular responses downstream of receptor tyrosine kinases (RTKs) in human cancer cells. The first step was the cloning and functional characterization of a distinct isoform of the PI3K family, termed PI3KC2β (Article I). This enzyme belongs to the Class II of PI3Ks based on sequence homology and substrate specificity *in vitro*. Hallmarks of Class II PI3Ks are a C-terminal phospholipid-binding C2 domain and an *in vitro* substrate specificity restricted to phosphatidylinositol (PI) and phosphatidylinositol 4-phosphate (PI(4)P). PI3KC2β was functionally distinguished from class I and III PI3K isoforms by the fact that it could catalyze phosphate transfer reactions in the presence of Ca^{2+} instead of Mg^{2+}. Moreover, unlike the closely related PI3KC2α it is sensitive to nanomolar concentrations of the PI3K inhibitor wortmannin. In contrast to class I PI3Ks, Ras did not appear to regulate the activity of PI3KC2β. A novel function for the C-terminal C2 domain as a negative regulator of the catalytic activity of PI3KC2β could be established (Article I).

The regulation of the class II PI3KC2α and PI3KC2β was then studied in order to elucidate their activation mechanisms in cells. Both enzymes were rapidly recruited to signaling complexes containing the epidermal growth factor receptor (EGFR) and Erb-B2 upon cell stimulation with EGF (Article II) in human carcinoma-derived A431 cells. It was also found that PI3KC2β associated with the activated platelet-derived growth factor receptor (PDGFR) in fibroblasts. The use of EGFR phosphotyrosine point mutants established that preferentially associated with the Tyr992 and Tyr1068 residues of the EGFR cytoplasmic domain. The N-terminal region of PI3KC2β (residues 1 to 331) was demonstrated to be able to interact with the activated EGFR (Article II).

In a subsequent study, it was shown that different human small cell lung carcinoma (SCLC) cell lines over-express distinct subsets of class I_A and II PI3Ks, which results in striking differences in the signaling cascades activated by stem cell factor (SCF). Over-expression of class I_A p85/p110α in SCLC cells increased SCF-stimulated protein kinase B (PKB) activation and cell growth, but did not affect extracellular signal-regulated kinase (Erk) or glycogen synthase kinase-3 (GSK-3). This effect was selective,

since it was not observed in SCLC cell lines over-expressing p85/p110β or p85/p110δ. c-Kit, the receptor for SCF associated with both class I_A p85 and class II PI3KC2β, and both enzymes contributed to SCF-stimulated PKB activity. A dominant-negative PI3KC2β blocked both PKB activation and SCLC cell growth in response to SCF. (Article III).

In the next report, we demonstrated that class II phosphoinositide 3-kinase C2β (PI3KC2β) associates with the Eps8/Abi1/Sos1 complex and is recruited to the EGFR as part of a multi-protein signaling complex also involving Shc and Grb2. Increased expression of PI3KC2β stimulated Rac activity in A-431 epidermoid carcinoma cells, resulting in enhanced membrane ruffling and migration speed of the cells. Conversely, expression of dominant negative PI3KC2β reduced Rac activity, membrane ruffling, and cell migration. Moreover, PI3KC2β -over-expressing cells were protected from anoikis and displayed enhanced proliferation, independently of Rac function. Taken together, these findings suggest that PI3KC2β regulates the migration and survival of human tumor cells by distinct molecular mechanisms (Article IV).

In the last report, the impact of the 3-hydroxy-3methylglutaryl CoA (HMG-CoA) reductase inhibitor simvastatin on human SCLC cell growth and survival was investigated. Simvastatin profoundly impaired basal and growth factor-stimulated SCLC cell growth *in vitro* and induced apoptosis. SCLC cells treated with simvastatin were sensitized to the effects of the chemotherapeutic agent etoposide. Moreover, SCLC tumour growth in vivo was inhibited by simvastatin. These responses correlated with the inhibition of SCF-stimulated activation of Erk, PKB and ribosomal protein S6 kinase (S6K) by simvastatin. Constitutive activation of the Erk pathway was sufficient to rescue SCLC cell from the effects of simvastatin. The drug did not directly affect activation of c-Kit or its localization to lipid rafts, but in addition to its ability to block Ras membrane localization, it selectively down-regulated H-Ras protein levels at the post-translational level. Down-regulation of either H- or K-Ras by RNA interference (RNAi) did not impair Erk activation by growth factors, whereas an RNAi specific for N-Ras inhibited activation of Erk, PKB and SCLC cell growth. Together our data demonstrate that inhibiting Ras signaling with simvastatin potently disrupts growth and survival in human SCLC cells (Article V).

3. Introduction

3.1 Receptor tyrosine kinases

3.1.1 Discovery of RTKs and mechanism of action

A great variety of membrane-spanning cell surface receptors has been identified in the past 30 years and classified based on ligand preference, their primary structure and the induction of biological responses. Two well characterized types of cell surface receptors are G-protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs). Protein tyrosine kinases (PTKs) are enzymes endowed with an intrinsic protein kinase activity that catalyzes the transfer of γ-phosphate of ATP to tyrosine residues of protein substrates [1]. Protein tyrosine phosphorylation was discovered more than 25 years ago [2] and PTKs, the signaling pathways they activate, as well as the molecular mechanisms underlying their action and regulation have been extensively studied. The RTK class of cell surface receptors is one large family of membrane-spanning PTKs that contain that contain an N-terminal extracellular ligand-binding domain and a C-terminal intracellular tyrosine kinase domain [3, 4]. RTKs are central components of cell signaling networks and play a crucial role in normal physiological processes such as in embryogenesis and development. RTK networks control fundamental cellular activities including cell proliferation and survival, cell cycle control, metabolism, as well as cell shape and movement. They are able to detect, filter, and process a variety of environmental and intercellular factors. The importance of understanding the complexity of RTK signaling networks, their regulation and the RTK interconnections has been highlighted by a large variety of molecular alterations and deregulations found in various human diseases, including cancer.

More than 50 mammalian RTKs have been identified so far including the well known insulin receptor (IR), Fms-like tyrosine kinase 3 (FLT3), c-Kit (the receptor for stem cell factor), epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), colony-stimulating growth factor I receptor (CSF-IR) and insulin-like growth factor I receptor (IGF-IR) [5]. A plethora of ligands such as peptides, proteins, lipids, or carbohydrates bind to and regulate the pleiotropic actions of RTKs. Tight control of the receptor activity is

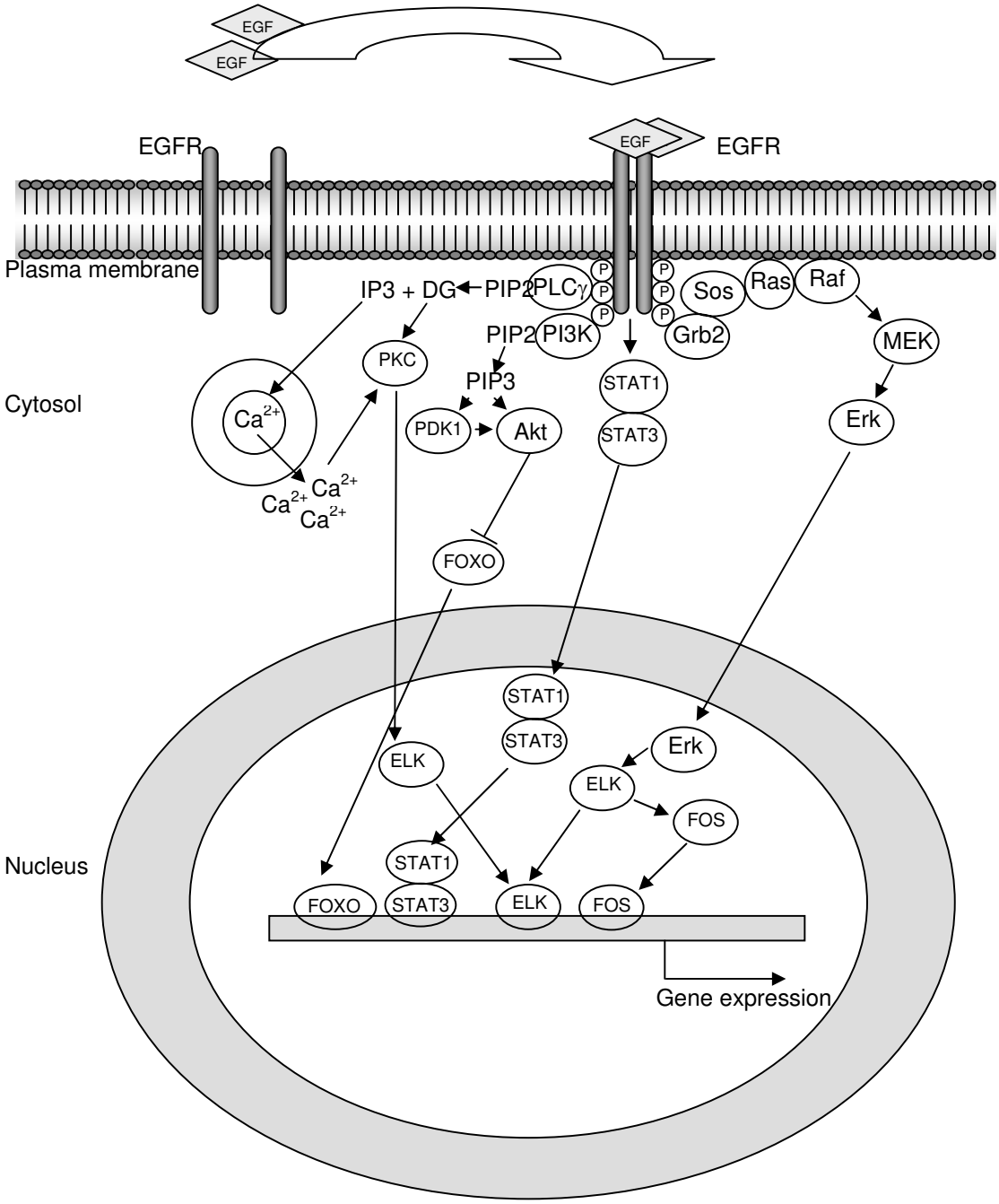
maintained by protein-tyrosine phosphatases, by other serine/threonine kinases, as well as auto-regulatory mechanisms in the receptors. The binding of a specific ligand to the extracellular domain of the RTK induces conformational alterations that are transmitted across the plasma membrane, resulting in the activation of its intracellular tyrosine kinase activity [6]. Most RTKs exist as inactive monomers and form dimers following binding to their ligands, the vast majority of which are soluble peptides. In general, ligand binding induces allosteric interactions causing dimerization of receptor subunits through disulfide-bridges [7] and autophosphorylation of specific tyrosine residues within their cytoplasmatic domains [6, 8]. Dimerization-mediated receptor activation mechanisms have been described for receptors composed of multimeric structures such as the IR, IGF-IR, PDGFR and the EGFR.

3.1.2 RTK signaling

Dimerization of a RTK induces receptor activation and the autophosphorylation of tyrosine residues in its intracellular domain that serve as specific binding sites for proteins that contain Src-homology 2 (SH2) or phosphotyrosine binding (PTB) domains. Src homology-2 (SH2) [9] or phosphotyrosine-binding (PTB) [10] domains are known phosphotyrosine-recognition domains present in various signaling proteins. Proteins recruited to the activated RTK typically fall into two categories: enzymes whose activity is stimulated by the activated receptor (e.g., Src, PLC γ, Shp-2 and PI3K) and adaptors that contain additional protein interaction motifs (e.g., Grb2 and Shc). Many activated RTKs also engage a third group of molecules collectively known as docking proteins (e.g., IRS, FRS and the Gab/Dok proteins). Docking proteins are often recruited to the RTK via an intrinsic PTB domain or through an interaction with the Grb2 adaptor. These proteins also contain a membrane targeting motif (either a pleckstrin homology (PH) domain or a myristoylation site) and multiple tyrosine phosphorylation sites that can interact with additional binding partners. Thus, through the receptor itself, adaptors and docking proteins, numerous signaling components are recruited to the cell surface, thereby activating the repertoire of effector cascades required for a specific response.

Several well characterized signaling pathways downstream of the EGFR involve the Grb2 and the Src-homology collagen protein (Shc) isoforms as adapter molecules. The adapter proteins bind to the phosphotyrosine residues within the juxtamembrane region of the cytoplasmatic receptor and undergo subsequent tyrosine phosphorylation. The Grb2 and Shc adapters recruit the Sos guanine nucleotide exchange factor (GEF) to the plasma membrane. Sos in turn stimulates GDP to GTP exchange on Ras, leading to activation of the Raf/MEK/Erk (p42/44 MAPK) cascade [11] (Figure 1). The phosphoinositide 3-kinase (PI3K)/Akt signaling pathway is activated by binding of the PI3K enzyme to the activated EGFR (Figure 1). The PI3K phosphorylate phosphatidylinositol (PI) on the D-3 position of the inositol ring, producing distinct second messengers such as $PI(3,4,5)P_3$ (PIP_3) [12] (Figure 2). These lipid products activate signal transduction by a cascade of proteins including phosphoinositide-dependent kinase-1 (PDK1), protein kinase B (PKB)/Akt, forkhead transcription factors (FOXO), glycogen synthase kinase-3 (GSK-3), tuberous sclerosis complex 1 and 2 (TSC1, TSC2), BAD, Ras homologue enriched in the brain (Rheb), mammalian target of rapamycin (mTOR), 4E-binding protein (4E-BP) and ribosomal protein S6 kinase (S6K) [12, 13](Figure 3). Other signaling pathways activated by the EGFR include phospholipase Cγ (PLCγ), which generates the second messengers diacylglycerol (DAG) and inositol 1,3,5-trisphosphate (IP_3), activating signaling through protein kinase C (PKC) and intracellular Ca^{2+} (Figure 1). Generally, signals transduced through those signaling cascades have pleiotropic effects on cell behavior controlling cell proliferation, differentiation and cell migration, as well as apoptosis.

Figure 1. Simplified overview of EGFR signaling events



3.2 Signal transduction by the Ras super-family protein of GTP-binding proteins

3.2.1 Ras proteins and their effectors

The Ras GTPase is a key signaling molecule that allows RTKs to signal to the extracellular signal-activated kinase (Erk) cascade. Activation of all RTKs stimulates the exchange of GTP for GDP on Ras, and this nucleotide exchange allows Ras to interact directly with its target effectors, one of which is the initiating kinase of the Erk cascade, Raf [14]. The best-characterized route of Ras activation occurs at the plasma membrane and is mediated by the Sos guanine nucleotide exchange factor [15]. Sos is recruited from the cytosol to the plasma membrane as a result of its constitutive interaction with Grb2, whose SH2 domain can bind phosphotyrosine sites located on one or more of the following molecules: the activated RTK itself, Shc (another adaptor protein that binds activated RTKs via its PTB domain) or one of the membrane-localized docking proteins. The human Ras superfamily consists of three members: H-Ras, K-Ras and N-Ras, whose activity is regulated by cycling between an inactive GDP-bound and an active GTP-bound state [16, 17]. Activated Ras transduces extracellular signals to diverse intracellular pathways, such as the PI3K or MAPK pathways, leading to cell survival, proliferation, differentiation, actin reorganization, vesicular trafficking, and gene expression, depending on cell types [18, 19]. Ras proteins represent some of the most frequently mutated oncogenes in human tumours [20-22]. In cancer, mutated or over-expressed Ras is insensitive to inactivation by GTPase-activating proteins (GAPs) and therefore becomes constitutively activated [23]. This aberrant activation of Ras promotes tumourigenesis by facilitating all aspects of the malignant transformation of cancer cells, including cellular proliferation, transformation, invasion and metastasis [24, 25].

3.2.2 Rho and Rac signaling

Like the majority of Ras superfamily proteins, most Rho/Rac GTPases behave as ‘‘molecular switches’’ that fluctuate between inactive and active states, two conformations that depend on the binding of either GDP or GTP to the GTPases, respectively [26, 27]. Two types of regulatory proteins control this cycling: guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs

promote the exchange of GDP for GTP on Rho/Rac, thereby producing the activation of these proteins during signal transduction [26, 27]. GAPs stimulate the hydrolysis of the bound GTP molecules, thus allowing the transfer of the GTPase back to the inactive state at the end of the stimulation cycle [26, 27]. In the GTP-bound state, these GTPases bind to a large collection of effector molecules that, in turn, lead to the stimulation of signaling cascades that promote general cellular responses, such as cytoskeletal change, microtubule dynamics, vesicle trafficking, cell polarity and cell cycle progression [26, 28].

3.3 Phosphoinositide 3-kinase

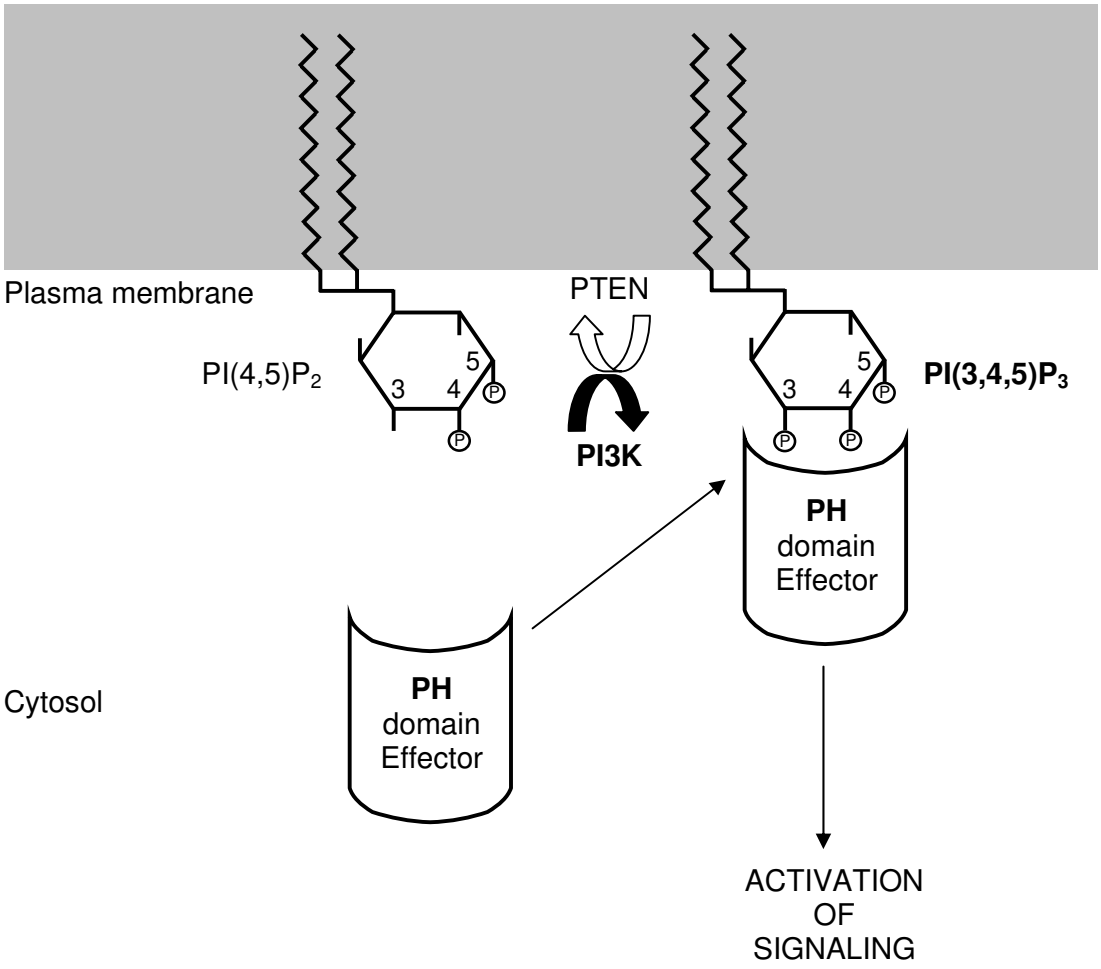
3.3.1 Discovery of PI3K and mechanism of action

The phosphoinositide 3-kinases (PI3K) are a family of evolutionary conserved lipid kinases, playing a crucial role in controlling a wide variety of intracellular signaling events. PI3Ks phosphorylate phosphatidylinositol (PI) on the D-3 position of the inositol ring, producing distinct second messengers such as PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3 (PIP_3) [12] (Figure 2). These lipid products are known to activate diverse target proteins involved in complex signaling cascades, ultimately resulting in the activation of cellular activities comprising cell growth, proliferation, survival and motility.

PI3K was first described 20 years ago as a distinct enzymatic activity associating with activated receptor tyrosine kinases (RTKs), such as the platelet-derived growth factor receptor (PDGFR) or with the polyoma virus middle T protein/pp60(c-src) complex [29-32]. PI3K activity was found to be elevated after cellular transformation by p60(v-src) [33] or abl [34]. After biochemical purification [35], the fist genes encoding the bovine catalytic p110α and regulatory p85α/β subunits of PI3K were cloned [36-39]. PI3K was shown to bind to activated RTKs via interaction of the Src homology-2 (SH2) domains of the p85 subunit to specific phosphotyrosine residues in the cytoplasmic domains of RTKs [37-44]. PI3K was then shown to be recruited to a broad variety of activated RTKs, including c-Met [45-47], c-Kit [48, 49], insulin-like growth factor-I receptor (IGF-IR) [50-52], insulin receptor (IR)/insulin receptor substrate-1 (IRS-1) [53-56], HER2/Neu/ErbB-2 [57], ErbB-3 [58-60], PDGFR [61, 62], Trk [63-65], and Flt3 [66].

Constitutively activated RTKs were found to be associated with PI3K, such as for c-Kit in leukemia [67], Tpr-Met [68] and EGFRvIII [69]. The constitutively activated BCR-ABL tyrosine kinase fusion protein which has been shown to be an essential step in the pathogenesis of Philadelphia chromosome (Ph)-positive leukemias also associates with PI3K [70]. In addition, PI3K interacts with Ras and is directly activated by Ras binding to p110 [25, 71, 72]. PI3K activation by RTKs such as the PDGFR was also reported to be regulated by Ras [73]. It was also shown that p85 contains a GTPase-responsive domain and an inhibitory domain, which together form a molecular switch that regulates PI3K [74]. H-Ras and Rac1 activate PI3K by targeting the GTPase-responsive domain [74]. The stimulatory effect of these molecules, however, is blocked by the inhibitory domain, which functions by binding to tyrosine-phosphorylated molecules and is neutralized by tyrosine phosphorylation [74]. The complementary effects of tyrosine kinases and small GTPases on the p85 molecular switch result in synergy between these two classes of molecules toward the activation of the PI3K/Akt pathway [74]. Another study showed that p85 inhibits p110 activation by Ras [75]. This blockage was released by Tyr kinase stimulation, showing that the classical mechanism of class I_A PI3K stimulation mediated by Tyr kinases also regulates Ras-induced PI3K activation [75]. At the same time as the genes of PI3K were cloned, it was shown that stimulation of cells with polypeptide growth factors such as PDGF induced the synthesis of novel second messengers phosphatidylinositol-3,4,5-trisphosphate (PIP_3) and phosphatidylinositol-3,4-bisphosphate ($PI(3,4)P_2$) [76, 77], which were resistant to the action of phospholipase C (PLC) [78].

Figure 2. Simplified model of the reaction catalyzed by PI3K and the activation of downstream signaling



3.3.2 PI3K isoforms

A family of PI3K isoforms was subsequently cloned and characterized [13, 79, 80]. These enzymes are subdived in three classes (I-III), based on sequence homology and *in vitro* substrate specificity (overview in Table 1). The class I_A of PI3K includes p85α/β and distinct regulatory subunits including mouse $p55^{PIK}$ [81], and splice variants of the p85α gene such as p55α [82], p50α [83], human p55γ [84]. Moreover, in addition to p110α distinct catalytic p110 isoforms were cloned and termed p110β [85] and p110δ [86, 87].

G-protein-coupled receptors (GPCRs) were shown to activate the generation of PIP_3 [88, 89], through the activation of a distinct isoform of PI3K. This distinct class I_B p110 isoform is activated by Gβγ subunits [90] and was termed p110γ [91, 92]. The p110γ isoform associates with an adaptor molecule (p101) contributing to its regulation by Gβγ subunits [92]. It should be also noted that the p110β isoform of class I_A PI3K has also been reported to be regulated by Gβγ [93]. Ras activates p110γ at the level of the membrane, by allosteric modulation and/or reorientation of the p110γ, implying that Ras can activate p110γ without its membrane translocation [94]. This view is supported by structural work that has suggested binding of Ras to p110γ results in a change in the structure of the catalytic pocket [95].

A separate class II of PI3Ks was identified in *Drosophila* and mammalian cells, which is characterized by a C-terminal C2 domain and a substrate specificity restricted to PI and PI(4)P *in vitro* [96-100]. This family includes the human PI3KC2α, PI3KC2β and PI3KC2γ isoforms [101-104].

These class III PI3Ks are homologues of the yeast VPS34 gene product (Vps34p) [105], which forms a complex with theVps15 protein kinase and is essential for protein sorting to the yeast lysosome-like vacuole [106]. The human homologues of Vps34p and Vps15p were subsequently cloned [107, 108], as well as their *Drosophila* counterpart [109]. The human Vps34p was reported to associate with the trans-Golgi network, a key site for the formation of transport vesicles destined for different intracellular compartments [110].

A family of protein kinases with homology to PI3Ks was also cloned and characterized, including yeast target of rapamycin (TOR) proteins [111, 112], and their mammalian homologue FRAP/RAFT1/mTOR [113-116]. This family of protein kinases also includes ATM, the gene product that is mutated in the autosomal recessive disorder ataxia telangiectasia (AT) [117, 118], ATR, and DNA-dependent protein kinase (DNA-PK) [119].

Table 1. Main characteristics of the mammalian PI3K isoforms

Class	Subclass	Catalytic subunit	Adaptor/ Regulatory subunit	Regulation	Tissue distribution	*In vitro* Lipid products	Functions
I	A	p110α	p85α, p85β p55α, p55γ p50α	RTK, Ras	Ubiquitous	PI(3)P PI(3,4)P2 PI(3,4,5)P3	Development Cancer Myocardial contractility
		p110β	p85α, p85β p55α, p55γ p50α	RTK, GPCR Ras	Ubiquitous	PI(3)P PI(3,4)P2 PI(3,4,5)P3	Development Insulin signal. Motility Phagocytosis Thrombosis
		p110δ	p85α, p85β p55α, p55γ p50α	RTK, Ras	Leukocytes	PI(3)P PI(3,4)P2 PI(3,4,5)P3	Immunity Cytokine signal. B-cell dev.+ migr. T-cells dev. BCR+TCR sign. Neutrophil migr. +burst.
	B	p110γ	p101, p84/p87	GPCR	Myeloid	PI(3)P PI(3,4)P2 PI(3,4,5)P3	Thymocyte dev. T cell dev. and migr. Neutrophil migr. +burst. Macrophage migr. Myocardial contractility
II		C2α	clathrin	RTK, GPCR integrin	Ubiquitous	PI(3)P PI(3,4)P2	Insulin signal. Vesicle traf. Cell surv. SM contract.
		C2β	Grb2, clathrin	RTK, GPCR integrin	Ubiquitous	PI(3)P PI(3,4)P2	Vesicle traf. Cell surv. Cell migr. Liver growth
		C2γ	?	?	Hepatic	PI(3)P PI(3,4)P2	Liver regeneration
III		Vps34p	Vps15p	Constitutive TLR	Ubiquitous	PI(3)P	Membrane traffic TLR signal

3.3.3 Class II PI3K isoforms

The class II of PI3Ks was first identified in *Drosophila* and mammalian cells and is characterized by a C-terminal C2 domain and a substrate specificity restricted to PI and PI(4)P *in vitro* [96-100]. This family includes the human PI3KC2α, PI3KC2β and PI3KC2γ isoforms [101-104]. A variety of cell surface receptors have been shown to activate class II PI3Ks, including activated integrins in platelets [120]. It was found that activation of PKBα/Akt in platelets is phosphorylation-dependent and biphasic [121]. The initial phase is PI(3,4,5)P_3-dependent and more efficient, whereas the second phase depends upon PtdIns(3,4)P_2 generated after aggregation [121]. In this system PI(3,4)P_2 is generated by transient formation of PtdIns3P and generation of PI(3,4)P_2, the latter primarily by PI(3)P 4-kinase. This novel pathway and the activation of PKB/Akt are inhibited by the PI3K inhibitor, wortmannin, and the calpain inhibitor, calpeptin, constituting the first evidence that PtdIns(3,4)P_2 can stimulate PKB/Akt in vivo in the absence of PtdIns(3,4,5)P_3 [122]. Integrin-activated generation of the second messenger PtdIns(3,4)P_2 thus depends upon a route distinct from that known to be utilized initially by growth factors.

PI3KC2α was shown to be constitutively associated with phospholipid membranes and that in contrast to the class I_A PI3K enzymes, PI3KC2α could be co-purified with a population of clathrin-coated vesicles (CCVs) [123, 124]. The distribution of PI3KC2α closely paralleled that of markers of the trans-Golgi and the trans-Golgi network (TGN) [123]. Futher work showed that clathrin functions as an adaptor for the PI3KC2α, binding to its N-terminal region and stimulating its catalytic activity, especially toward phosphorylated inositide substrates [125]. Furthermore, it was shown that endogenous PI3KC2α is localized in coated pits and that exogenous expression affects clathrin-mediated endocytosis and sorting in the trans-Golgi network [125]. Another study explored the behavior of novel cytoplasmic clathrin-coated structures containing PI3KC2α and clathrin [126]. These structures were found to exhibit localized, rapid mobility, which is micro-tubule-based [126]. Dynactin was shown to mediate the movement of these clathrin-PI3KC2α structures and an interaction between endogenous PI3KC2α and dynactin subunits was demonstrated [126]. Together, these results reveal a

molecular linkage between PI3KC2α and the microtubule motor machinery, with implications for membrane trafficking in intact cells [126]. Another study demonstrated that the isolated clathrin binding domain of PI3KC2α can drive clathrin lattice assembly and that both it and the lipid kinase activity of the protein can independently modulate clathrin distribution and function when expressed in cells [127]. Together, these results suggest that PI3KC2α employs both protein-protein interaction and localized production of 3-phosphoinositides to affect clathrin dynamics at sites of membrane budding and targeting [127].

The crystal structure of the PI3KC2α C2 domain was determined in [128]. Structural studies reveal that the C2 domain has a typical anti-parallel beta-sandwich fold [128]. The surface of this C2 domain displayed three small, shallow sulfate-binding sites [128]. Site-directed mutagenesis revealed that this C2 domain binds specifically to $PI(3,4)P_2$ and $PI(4,5)P_2$ and that three lysine residues are responsible for the phospholipid binding affinity [128]. The crystal structure of the PI3KC2α PX domain, which binds $PI(4,5)P_2$ was reported in [129]. This PX domain was shown to contain a signature PI-binding site that is optimized for $PI(4,5)P_2$ binding [129]. Furthermore, the PX domain displayed significantly higher $PI(4,5)P_2$ membrane affinity and specificity when compared with the PI3KC2α C2 domain [129].

It was also demonstrated that PI3KC2α has dual cellular localization present in the cytoplasm and in the nucleus [130]. A distinct nuclear localization signal sequence was identified in a stretch of 11 amino acids located within C2 domain of the kinase [130]. Phosphorylation of PI3KC2α was induced by inhibition of RNA polymerase II-dependent transcription and coincides with enlargement and rounding up of the nuclear speckles [130]. The results suggest that phosphorylation of PI3KC2α is inversely linked to mRNA transcription and supported the importance of phosphoinositides for nuclear activity [130]. In another study cell cycle-dependent and genotoxic stress-induced phosphorylation of PI3KC2α was investiagated [131]. It was found that the kinase becomes phosphorylated upon exposure of cells to UV irradiation and in proliferating cells at the G2/M transition of the cell cycle [131]. Stress-dependent and mitotic phosphorylation of PI3KC2α occured on the same serine residue (Ser259) within a

recognition motif for proline-directed kinases [131]. Mitotic phosphorylation of PI3KC2α was attributed to Cdc2 activity, and stress-induced phosphorylation of PI3KC2α was mediated by JNK/SAPK [131]. The protein level of PI3KC2α was regulated by proteolysis in a cell cycle-dependent manner and in response of cells to stress [131]. Phosphorylation appears to be a prerequisite for proteasome-dependent degradation of PI3KC2α and may therefore contribute indirectly to the regulation of the activity of the kinase [131].

Another study investigated the involvement of PI3KC2α in vascular smooth muscle cell migration [132]. PI(3)P and PI(3,4)P_2 were increased upon smooth muscle cell migration but their synthesis was affected only partially by the PI3K inhibitors, wortmannin and LY294002 [132]. Measurement of PI3KC2α demonstrated its activation upon smooth muscle cell migration [132]. Moreover, PI3KC2α was found to be differentially regulated by α(v)β(3) and α(v)β(5) integrin engagement [132].

Another study found that KCl and noradrenaline induced stimulation of PI3KC2α in a Ca^{2+}-dependent manner, but not class I_A PI3K in vascular smooth muscle (VSM) cells [133]. Down-regulation of PI3KC2α expression by siRNA inhibited contraction and phosphorylation of the regulatory subunit of myosin phosphatase (MYPT1) and myosin light chain [133]. Intravenous wortmannin infusion induced sustained hypotension in rats, with inhibition of PI3KC2α activity, GTP-loading of Rho and MYPT1 phosphorylation in the artery [133]. These results indicate the novel role of PI3KC2α in Ca^{2+}-dependent Rho-mediated negative control of myosin phosphatase and thus VSM contraction [133].

Insulin caused a rapid increase in the activity of PI3KC2α in CHO-IR cells, 3T3-L1 adipocytes, and fully differentiated L5L6 myotubes [134]. This suggested that insulin-induced phosphorylation could play a role in regulation of the activity of PI3KC2α [134]. The finding that insulin activates PI3KC2α in cell types known to possess a wide range of responses to insulin suggests that PI3KC2α is a novel component of insulin-stimulated signaling cascades [134]. It was demonstrated that PI3KC2α and PI3KC2β represent two downstream targets of the activated epidermal growth factor (EGF) receptor in human carcinoma-derived A431 cells [135]. Stimulation cells with EGF or PDGF resulted in the rapid recruitment of both enzymes to a phosphotyrosine signaling complex that contained

the activated RTKs [135]. It was thus concluded that class II PI3K enzymes may contribute to the generation of 3' phosphoinositides following the activation of polypeptide growth factor receptors in vivo and thus mediate certain aspects of their biological activity [135]. Proline-rich motifs within the N terminus of PI3KC2β were shown to mediate the association of this enzyme with activated EGFR and that this interaction involves the Grb2 adaptor [136]. Another study showed that different human small cell lung carcinoma (SCLC) cell lines over-express distinct subsets of class I_A and II PI3Ks, which resulted in striking differences in the signaling cascades activated by stem cell factor (SCF) [137]. Over-expression of class I_A p85/p110α in SCLC cells increased SCF-stimulated protein kinase B (PKB) activation and cell growth [137]. c-Kit associated with both class I_A p85 and class II PI3KC2β, and both enzymes contributed to SCF-stimulated Akt activity [137]. A dominant-negative PI3KC2β blocked both Akt activation and SCLC cell growth in response to SCF [137]. It was shown that in renal cells there is a spatial separation of the inositol lipid signaling system between basal-lateral plasma membranes (BLM) and brush-border plasma membranes (BBM), and that HGF causes activation of PLC and PI3K primarily in BLM, which leads to calpain-mediated activation of PI3KC2β in BBM with a concomitant increase in PI(3)P [138]. Another report showed that, in the membrane-depleted liver nuclei during the compensatory liver growth, there is an increase in PI(3)P formation as a result of PI3KC2β activation, which may be a calpain-mediated event [139]. The activity of nuclear PI3KC2β was investigated in HL-60 cells blocked at the G(1)/S boundary and allowed to progress synchronously through the cell cycle [140]. The activity of PI3KC2β in the nuclei and nuclear envelopes showed peak activity at 8 h after release from the G(1)/S block, which correlates with G(2)/M phase of the cell cycle [140]. Further work showed calpain-mediated activation of the nuclear PI3KC2β during G(2)/M phase of the cell cycle in HL-60 cells [140].

It was shown that lysophosphatidic acid (LPA) stimulates the production of PI(3)P through activation of PI3KC2β [141]. Both PI(3)P and PI3KC2β were involved in LPA-mediated cell migration in human cancer cell lines [141]. Another study found that cell motility is increased in cells over-expressing PI3KC2β [142]. In addition,

overexpression of PI3KC2β transiently decreased cell adhesion and expression of α4β1 integrin subunits [142]. It was shown that PI3KC2β is present in lamellipodia of motile cells [142]. Cortical actin staining increased and actin rich lamellipodia and filopodia became evident upon overexpression of PI3KC2β [142]. Overexpression of a FYVE domain fusion protein abolished this response demonstrating that the effect of overexpression of PI3KC2β on the reorganization of actin filaments is dependent upon PI(3)P [142]. Finally, overexpression of PI3KC2β increased GTP loading of Cdc42 [142]. Another study demonstrated that PI3KC2β associates with the Eps8/Abi1/Sos1 complex and is recruited to the EGF receptor as part of a multi-protein signaling complex also involving Shc and Grb2 [143]. Increased expression of PI3KC2β stimulated Rac activity in A-431 epidermoid carcinoma cells, resulting in enhanced membrane ruffling and migration speed of the cells [143]. Conversely, expression of dominant negative PI3KC2β reduced Rac activity, membrane ruffling, and cell migration. Moreover, PI3KC2β -over-expressing cells were protected from anoikis and displayed enhanced proliferation, independently of Rac function [143].

To study the role for PI3KC2β in tissue, transgenic mice over-expressing the enzyme in both suprabasal and basal epidermal layers were generated [144]. These mice lacked epidermal abnormalities [144]. Mice deficient in PI3KC2β were then generated by targeted gene deletion [144]. PI3KC2β knockout mice were viable and fertile and displayed normal epidermal growth, differentiation, barrier function, and wound healing [144]. To exclude compensation by PI3KC2α, RNAi was then used to knock down both PI3KC2α and PI3KC2α in epidermal cells simultaneously [144]. Induction of differentiation markers was unaffected in the absence of PI3KC2α and PI3KC2α [144]. These findings indicated that class II PI3Ks are not essential for epidermal differentiation.

3.3.4 Signal transduction by PI3K

The use of mutant receptors defined for the first time a role for PI3K in PDGF-dependent DNA synthesis, and established PI3K as an independent downstream mediators of PDGF's mitogenic signal [145]. Furthermore, a mutant CSF-1R with a mutation in the

PI3K-binding site had impaired ability to transduce signals controlling changes in morphology and increased cell growth [146]. The PI3K binding sites also appeared both necessary and sufficient for the normal endocytic trafficking of the activated PDGFR [147]. A PDGFR mutant in which both p85-binding sites were mutated failed to stimulate membrane ruffling and chemotaxis, suggesting a role for PI3K in these responses [148, 149].

Pharmacological inhibitors of PI3K were instrumental in elucidating the role of the enzyme in cellular signaling events. These inhibitors include quercetin analogs, the most widely used being LY294002 [150], as well as the microbial product wortmannin [151-153]. In addition to their effects on PI3K isoforms, LY294002 and wortmannin also inhibit the activity of the related kinases such as mTOR [154], ATM and DNA-PK [155]. Studies with wortmannin and LY294002 uncovered a role for PI3K in activation of p70(S6K) by insulin and PDGF [156-159], as well as in the inactivation of glycogen synthase kinase-3 (GSK-3) [160]. Subsequently, PI3K was shown to be essential in the activation of the proto-oncogene Akt (or protein kinase B (PKB)) by PDGF [161] and other growth factors [162, 163]. These results were confirmed by the observation that transfection of membrane-targeted p110 was sufficient to trigger downstream responses characteristic of growth factor action, including the stimulation of p70(S6K) and Akt [164, 165]. The phospholipids products of PI3K, initially $PI(3,4)P_2$, were shown to directly activate Akt by binding to its pleckstrin homology (PH) domain [166, 167]. PH domains were recognized to be modular domains with the ability to specifically bind to the lipid products of PI3K, including PIP_3 [168]. PI3K activity was also required for phosphorylation of both Thr308 and Ser473 activation sites of Akt [169]. The kinase that phosphorylates Akt was then purified, cloned and shown to phosphorylates Akt1 at Thr308 and increases its activity [170-172]. It was found that only $PI(3,4,5)P_3$ or $PI(3,4)P_2$ were effective in potently activating the kinase, which was termed $PI(3,4,5)P_3$-dependent protein kinase-1 (PDK1) [170]. PDK1 is the protein kinase that mediates the activation of Akt/PKB by insulin and growth factors [173]. PDK1 therefore plays a key role in mediating many of the actions of the second messengers produced by PI3K. In response to PDGF, binding of $PI(3,4,5)P_3$ and/or $PI(3,4)P_2$ to the PH domain of PDK-1 causes its translocation to the plasma membrane where it co-localises with Akt/PKB,

significantly contributing to the scale of Akt/PKB activation [173, 174] (Figure 2-3). The identification of the kinase that phosphorylates the Akt Ser473 was achieved only in the last few years [175]. The complex of the mammalian target of rapamycin (mTOR) and Rictor was shown to be essential for this crucial phosphorylation step in Akt by several groups [175, 176].

In addition to its role in Akt activation, PDK1 was also shown to be responsible for the regulation of other protein kinases [177, 178]. PDK1 phosphorylated the activation loop sites of PKCζ and PKCδ *in vitro* and in a PI3K-dependent manner *in vivo* [179, 180]. Several members of the PKC family tested formed complexes with PDK1 [179]. Serum and glucocorticoid-inducible kinase (SGK) was also shown to be a target of PI3K/PDK1 [181, 182]. A regulatory link between p70(S6K) and PDK1 was also described, since PDK1 selectively phosphorylate and activated p70(S6K) *in vitro* and *in vivo* [183, 184].

In addition, PI3K was reported to be involved in the activation of several other protein kinases, including c-Jun N-terminal kinase (JNK) by EGF [185]. Bruton's tyrosine kinase (Btk), which has a PH domain that can bind PIP_3 [186], was described as a downstream target of PI3K (p110γ) [187]. Etk/Bmx a member of the Btk tyrosine kinase family that contains a PH domain is also involved in the PI3K pathway [188]. The Tec family non-receptor tyrosine kinases were shown to be regulated by PIP_3 interacting with its PH domain [189]. Activation of PI3K caused phospholipase C-γ (PLC-γ) PH domain-mediated membrane targeting and PLC-γ activation [190, 191]. Integrin-linked kinase (ILK) was also proposed to be a receptor-proximal effector for the PI3K-dependent, extracellular matrix and growth factor mediated, activation of Akt, and inhibition of GSK-3 [192]. RNA interference (RNAi) as well as conditional knock-out of ILK had no effect on phosphorylation of Akt on Thr-308 but resulted in almost complete inhibition of phosphorylation on Ser-473 and significant inhibition of Akt activity, accompanied by significant stimulation of apoptosis [193]. In addition, Raf-1 activation by Ras was shown to be achieved through a combination of both physical interaction and indirect mechanisms involving the activation of PI3K as a second Ras effector, which directs p21-activated kinase (PAK)-mediated regulatory phosphorylation of Raf-1 [194]. Phosphorylation of Raf-1 on Ser338 through PI3K and Pak was also shown to provide a

co-stimulatory signal which together with Ras leads to strong activation of Raf-1 kinase activity by integrins [195].

A consensus sequence which predicts high-affinity binding of PH domains to PtdIns(3, 4)P_2 and/or PtdIns(3,4,5)P_3 was proposed, and several new PH domain-containing proteins that directly bind PI3K products were identified, including Gab1, Dos, myosinX, and Sbf1 [196], GAP1(m) a member of the GAP1 family of Ras GTPase-activating proteins (GAPs) [197], DAPP1 [198], Tec family tyrosine kinases [199], ARAP3 [200], and P-Rex1, a Rac activator [201].

3.3.5 PI3K /Akt signaling and apoptosis

The involvement of PI3K in prevention of apoptosis by polypeptide growth factor receptors was first described by studies using both wortmannin and LY294002 [202-204]. Experiments with pharmacological inhibitors, as well as expression of wild-type and dominant-inhibitory forms of Akt, demonstrated that Akt mediates PI3K-dependent survival [205-209]. These findings were supported by studies showing that Ras activation of PI3K suppresses c-Myc-induced apoptosis through the activation of Akt but not p70(S6K) [210]. UV-B light-induced-apoptosis was also prevented by IGF-I/PI3K/Akt signaling [211] and interleukin-3-dependent survival of hematopoietic cells required PI3K/Akt signaling [212]. Neuronal survival in the absence of nerve growth factor (NGF) was promoted by PI3K/Akt [213] and it was also shown that Akt can transduce a survival signal for differentiating neuronal cells through a mechanism that is independent of induction of Bcl-2 or Bcl-X_L, or inhibition of JNK activity [214]. PI3K acting through Akt was implicated as a key mediator of the aberrant survival of Ras-transformed epithelial cells in the absence of attachment, and as a mediator of matrix-induced survival of normal epithelial cells [215].

Some of the proposed mechanisms for the antiapoptotic effect of activated Akt include the inhibition of proapoptotic Bcl-2 family proteins, downregulation of death receptors, and enhancement of the glycolytic rate [216]. There exists a large panel of Akt substrates which mediate its effects on cellular responses, including apoptosis, growth and cell cycle regulation [207-209] (Figure 1). The Akt targets identified so far include BAD [217-219], the FOXO (Forkhead Box, subgroup O) family of transcription factors

[220-223] and AFX [224], glycogen synthase kinase-3 (GSK-3) [173, 225, 226], p27(Kip1) [227, 228], Mdm2 [229], endothelial NO synthase (eNOS) [230, 231], cyclic nucleotide phosphodiesterase 3B isoform (PDE3B)[232], Raf [233 , 234], apoptosis signal-regulating kinase 1 (ASK1) [235], androgen receptor (AR)[236], the nuclear factor CREB [237], the p300 transcriptional coactivator [238], E2F [239, 240].

In addition, it was shown that Akt can regulate signaling pathways that lead to induction of the NF-κB family of transcription factors [241-243]. This induction occurred at the level of degradation of the NF-κB inhibitor IκB [241]. PDGF was also shown to activate NF-κB through Ras and PI3K to Akt and the IκB kinase (IKK) [242]. Upon PDGF stimulation, Akt transiently associated *in vivo* with IKK and induced IKK activation [242]. Akt was reported to stimulate NF-κB predominantly by up-regulating of the transactivation potential of the p65 subunit of NF-κB [244]. Survivin, a member of the inhibitors-of-apoptosis gene family, is expressed in a cell-cycle-dependent manner in all the most common cancers but not in normal differentiated adult tissues [245]. Hematopoietic cytokines were reported to exert their antiapoptotic and mitogenic effects, at least in part, by increasing survivin levels, which was dependent on PI3K [245]. It was also shown that both vascular endothelial growth factor (VEGF) and basic fibroblast growth factor significantly reduce the pro-apoptotic potency of chemotherapy on endothelial cells, a response, which PI3K-dependent and could be recapitulated by over-expressing the dominant-active form of Akt [246]. Work by others showed that the anti-apoptotic effects of IL-6 were mediated, at least in part, by Mcl-1 (Bcl-2 family member) expression and that the response occured mainly through the PI3K/ Akt pathway [247]. It was reported that apoptotic cell death of PTEN-deficient prostate cancer cells induced by LY294002 or expression of wild type PTEN can be abrogated by disrupting Fas/Fas ligand (FasL) interactions [248]. These data showed that apoptosis induced by blockade of the PI3K pathway in prostate tumor cells is mediated by an autocrine Fas/FasL apoptotic mechanism and the Fas apoptotic pathway is both necessary and sufficient to mediate apoptosis by PI3K inhibition [248].

3.3.6 PI3K signaling and growth control

Signaling networks that promote cell growth are frequently dysregulated in cancer. One regulatory network, which converges on effectors such as eIF4E-binding proteins-1 (4E-BP1) and p70(S6K), leads to growth by promoting protein synthesis [249]. In particular, a tumor suppressor complex whose function is lost in tuberous sclerosis patients regulates the nutrient signal carried by the critical signaling protein TOR to the effectors 4E-BP1 and p70(S6K)[249].

It was initially demonstrated that the PI3K/Akt signaling pathway, in concert with FRAP/mTOR, induces the phosphorylation and inactivation of the translational repressor, the 4E-BP1 [250] and activation of p70(S6K) [251, 252]. Further work showed that mTOR signals downstream to at least two independent targets, S6K1 and 4E-BP1/eIF4E that function in translational control to regulate mammalian cell size [253]. The tuberous sclerosis complex-2 (TSC2) gene product, tuberin, is as a target of Akt [254-257]. Normal cellular functions of hamartin and tuberin, encoded by the TSC1 and TSC2 tumor suppressor genes, are closely related to their direct interactions. Tuberous sclerosis (TSC) is an autosomal dominant disorder characterized by the formation of hamartomas in a wide range of human tissues [258]. It was demonstrated that, upon activation of PI3K, tuberin is phosphorylated on consensus recognition sites for PI3K-dependent S/T kinases [254]. Moreover, Akt/PKB could phosphorylate tuberin *in vitro* and *in vivo* [254]. It was also shown that S939 and T1462 of tuberin are PI3K-regulated phosphorylation sites and that T1462 is constitutively phosphorylated in *PTEN* (-/-) tumor-derived cell lines [254]. Finally, a tuberin mutant lacking the major PI3K-dependent phosphorylation sites blocked the activation of S6K1, suggesting a means by which the PI3K-Akt pathway regulates S6K1 activity [254]. Another report showed that TSC1-TSC2 inhibits the p70(S6K) and activates the 4E-BP1, which was mediated by inhibition of mTOR [254, 256]. Furthermore, TSC2 was shown to be directly phosphorylated by Akt. TSC2 was inactivated by Akt-dependent phosphorylation, which destabilizes TSC2 and disrupts its interaction with TSC1 [256]. It was shown that TSC1 and TSC2 antagonize the amino acid-TOR signaling pathway [255]. TSC1 and TSC2 could physically associate with TOR and function upstream of TOR genetically. In *Drosophila melanogaster* and mammalian cells, loss of *TSC1* and *TSC2* resulted in a TOR-dependent increase of S6K

activity [259]. Furthermore, although S6K is normally inactivated in animal cells in response to amino acid starvation, loss of *TSC1-TSC2* renders cells resistant to amino acid starvation. It was thus proposed that the TSC1-TSC2 complex antagonizes the TOR-mediated response to amino acid availability [259]. These studies identified TSC1 and TSC2 as regulators of the amino acid-TOR pathway and provide a new paradigm for how proteins involved in nutrient sensing function as tumor suppressors [255, 259]. Work by another group showed that insulin or IGF-I stimulated phosphorylation of tuberin, which was inhibited by the PI3K inhibitor LY294002 [260]. Expression of constitutively active PI3K or active Akt induced tuberin phosphorylation. It was further demonstrated that Akt/PKB associates with hamartin-tuberin complexes, promoting phosphorylation of tuberin and increased degradation of hamartin-tuberin complexes [260]. The ability to form complexes, however, was not blocked. Akt also inhibited tuberin-mediated degradation of p27(KIP1), thereby promoting CDK2 activity and cellular proliferation [260]. These results confirmed that tuberin is a direct physiological substrate of Akt and that phosphorylation of tuberin by PI3K/Akt is a major mechanism controlling hamartin-tuberin function [260].

Further work showed that TSC1/2 is a GAP for the small GTPase Rheb and that insulin-mediated Rheb activation is PI3K-dependent [261-263]. Rheb over-expression induced S6K1 phosphorylation and inhibited Akt phosphorylation, as did loss-of-function mutations in TSC1/2 [261]. Finally, co-expression of a human TSC2 harboring a disease-associated point mutation in the GAP domain, failed to stimulate Rheb GTPase activity or block Rheb activation of S6K1 [261, 263]. A screen for novel regulators of growth identified Rheb (Ras homologue enriched in brain), a member of the Ras superfamily of GTP-binding proteins [262]. Increased levels of Rheb in *Drosophila melanogaster* promoted cell growth and alter cell cycle kinetics in multiple tissues. In mitotic tissues, overexpression of Rheb accelerates passage through G1-S phase without affecting rates of cell division [262]. Genetic and biochemical tests indicated that Rheb functions in the insulin signalling pathway downstream of TSC1-TSC2 and upstream of TOR [262]. In another study, mutations in the *Drosophila melanogaster Rheb* gene were isolated as growth-inhibitors, whereas over-expression of Rheb promoted cell growth [264]. Genetic and biochemical analyses suggest that Rheb functions downstream of the tumour

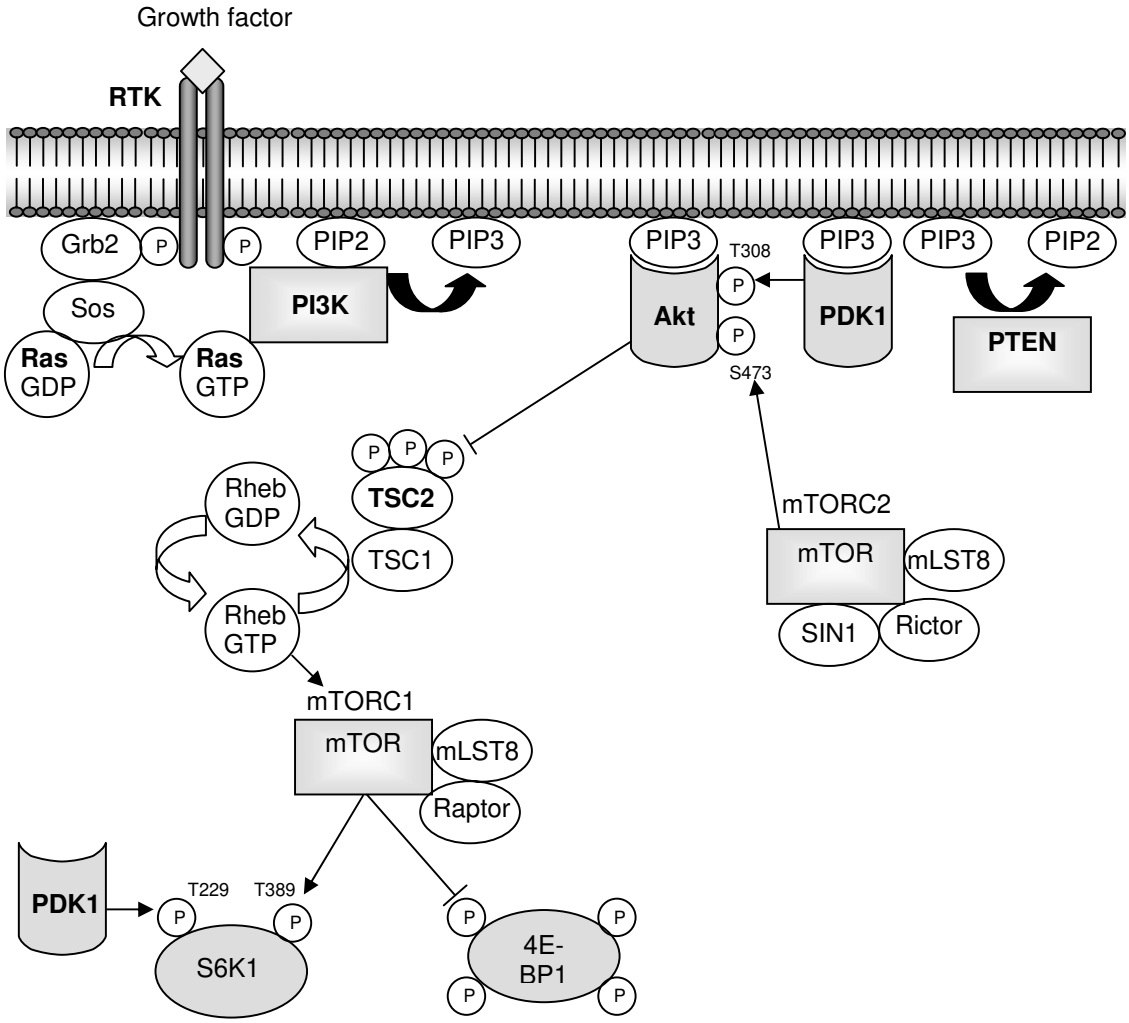
suppressors TSC1-TSC2 in the TOR signalling pathway to control growth, and that a major effector of Rheb function is S6K [264, 265].

It was reported that Akt activation causes proteasomal degradation of substrates that control cell growth and survival [266]. Expression of activated Akt triggered proteasome-dependent declines in the protein levels of the Akt substrates tuberin, FOXO1, and FOXO3a [266]. The addition of proteasome inhibitors stabilized the phosphorylated forms of multiple Akt substrates, including tuberin and FOXO proteins [266]. Activation of Akt also triggered the ubiquitination of several proteins containing phosphorylated Akt substrate motifs [266]. Together the data indicate that activated Akt stimulates proteasomal degradation of its substrates and suggest that Akt-dependent cell growth and survival are induced through the degradation of negative regulators of these processes [266]. It has been shown that FKHR is phosphorylated via insulin or growth factor signaling cascades, resulting in its cytoplasmic retention and the repression of target gene expression. Insulin treatment was shown to decrease endogenous FKHR proteins in HepG2 cells, which was inhibited by proteasome inhibitors [267]. FKHR was ubiquitinated *in vivo* and *in vitro*, and insulin enhances the ubiquitination in the cells [267]. In addition, the signal to FKHR degradation from insulin as shown to be mediated by the PI3K pathway, and the mutation of FKHR at the serine or threonine residues phosphorylated by Akt inhibited the ubiquitination *in vivo* and *in vitro* [267]. Another report showed that FoxO1 expression is constitutively suppressed in chicken embryo fibroblasts transformed by P3k or Akt [268]. In this system, phosphorylation-dependent degradation of FoxO1 by means of proteasomes played a role in oncogenic transformation by P3k and Akt [268].

It was shown that TSC1-TSC2 is required for insulin signaling to PI3K [269, 270]. TSC1-TSC2 was required for insulin signaling to PI3K by restraining the activity of S6K1, which when activated inactivates insulin receptor substrate (IRS) function, via repression of IRS-1 gene expression and via direct phosphorylation of IRS-1 [269]. These results suggested that the low malignant potential of tumors arising from TSC1-2 dysfunction may be explained by the failure of TSC mutant cells to activate PI3K and its downstream effectors [269, 270].

An overview of PI3K/Akt/mTOR signaling pathway is presented in Figure 3.

Figure 3. Simplified overview of the PI3K/Akt/mTOR signaling pathway. Proteins where mutations have been described in human cancer are depicted in bold.



3.3.7 PI3K in cell cycle control

Cell cycle progression is a tightly controlled process. To initiate cell division, mitogens trigger a number of early signals that promote the G(0)-G(1) transition by inducing cell growth and the activation of G(1) cyclins. Activation of cyclin E/cdk2 (cyclin-dependent kinase 2) at the end of G(1) is then required to trigger DNA synthesis (S phase entry). Among the early signals induced by mitogens, activation of PI3K appears essential to induce cell cycle entry, as it regulates cell growth signalling pathways (see previous section), which in turn determine the rate of cell cycle progression. Another mechanisms by which PI3K and its downstream effector Akt regulate cell cycle entry is by inactivation of the FOXO transcription factors, which induce expression of quiescence genes such as those encoding p27(Kip), p130 and cyclin G2 [271]. PI3K/FOXO then work as a complementary switch: when PI3K is active, FOXO transcription factors are inactive [271]. The switch is turned on and off at different phases of the cell cycle, thus regulating cell cycle progression. Akt triggers a network that positively regulates G1/S cell cycle progression through inactivation of GSK3β, leading to increased cyclin D1, and inhibition of Forkhead family transcription factors and the tumor suppressor tuberin (TSC2), leading to reduction of p27(Kip1) [272]. The identification of p21Waf1/Cip1 and p27Kip1 as novel substrates of Akt provided new insights into mechanisms whereby hyperactivation of this lipid signaling pathway may lead to cell cycle deregulation in human cancers [272].

The PI3K/Akt pathway must be activated in G1 to inactivate forkhead transcription factors and allow cell cycle entry. It was subsequently shown that attenuation of the PI3K/Akt pathway is required to allow transcriptional activation of FOXO in G2 [273]. FOXO activity in G2 controls mammalian cell cycle termination, as interference with FOXO transcriptional activation by disrupting PI3K/Akt downregulation, or by expressing a transcriptionally inactive FOXO mutant, induces cell accumulation in G2/M, defective cytokinesis, and delayed transition from M to G1 of the cell cycle [273]. It was demonstrated that FOXO regulate expression of mitotic genes such as cyclin B and polo-like kinase (Plk) [273]. These results supported the important role of forkhead transcription factors in the control of mammalian cell cycle completion,

and suggest that efficient execution of the mitotic programme depends on downregulation of PI3K/Akt and consequent induction of FOXO transcriptional activity [273].

3.3.8 PI3K signaling in migration and invasion

Migration of cancer cells is one of the key factors responsible for cancer metastasis. The elucidation of mechanisms responsible for the highly invasive potential of cancer cells can help to identify specific targets for the treatment of cancer patients. Highly invasive cancers are usually characterized by aberrant activity of specific intra- or extracellular molecules such as protein kinases, phosphatases, transcriptional factors, proteolytic enzymes, and others. Therefore, inhibition of specific target molecules in common signaling pathway(s) responsible for metastatic spread can have potential clinical relevance.

The first identified downstream target of PI3K in PDGF-stimulated membrane ruffling was Rac [274, 275]. Class I_A PI3Ks are implicated in many cellular responses controlled by receptor tyrosine kinases (RTKs), including actin cytoskeletal remodeling. Within this pathway, Rac is a key downstream target/effector of PI3K. One possible candidate for this function is the Rac-activating complex Eps8-Abi1-Sos-1, which possesses Rac-specific guanine nucleotide exchange factor (GEF) activity [276]. It was shown that Abi1 (also known as E3b1) recruits PI3K, via p85, into a multimolecular signaling complex that includes Eps8 and Sos-1 [276]. The recruitment of p85 to the Eps8-Abi1-Sos-1 complex and PIP_3, co-operate to unmask its Rac-GEF activity *in vitro* [276]. Moreover, they are indispensable for the activation of Rac and Rac-dependent actin remodeling *in vivo* [276]. Upon growth factor stimulation, endogenous p85 and Abi1 consistently colocalized into membrane ruffles, and cells lacking p85 failed to support Abi1-dependent Rac activation [276].

Direct PI3K activation was sufficient to disrupt epithelial polarization and induce cell migration and invasion [277]. PI3K inhibition also disrupted actin structures, suggesting that activation of PI3K alters actin organization, leading to increased motility and invasiveness [277]. Integrin-mediated activation of PI3K was shown to promote carcinoma invasion by targeting Rac [278]. Vav, a guanosine diphosphate (GDP)-guanosine triphosphate (GTP) exchange factor (GEF) for Rac that stimulates the

exchange of bound GDP for GTP, bound to and was directly controlled by substrates and products of PI3K [279]. PI3K also acts upstream of Tiam1, an activator of Rac [280]. Akt/PKB potently promoted invasion of highly metastatic cells, by increasing cell motility and matrix metalloproteinase-9 (MMP-9) production, in a manner highly dependent on its kinase activity and membrane-translocating ability [281]. The increase in MMP-9 production was mediated by activation of NF-κB transcriptional activity by Akt/PKB [281]. However, Akt/PKB did not affect the cell-cell or cell-matrix adhesion properties of the cells. These findings thus established Akt/PKB as a major factor in the invasive abilities of cancer cells [281]. Another study showed that PI3K is constitutively active and controls cell motility of highly invasive breast cancer cells by the activation of transcription factor, NF-κB [282]. The urokinase-type plasminogen activator (uPA) promoter contains an NF-κB binding site, and uPA expression in MDA-MB-231 cells was induced by the constitutively active NF-κB [282]. Cell migration was inhibited by overexpression of a dominant negative p85α, as well as by pretreatment of cells with wortmannin and LY294002 [282]. Highly invasive MDA-MB-231 cells constitutively secreted uPA in amounts significantly higher than poorly invasive MCF-7 cells [282]. Furthermore, inhibition of NF-κB markedly attenuated endogenous migration, and inhibition of PI3K and NF-κB reduced secretion of uPA [282]. These data suggest a link between constitutively active PI3K, NF-κB, and secretion of uPA, which is responsible for the migration of highly invasive breast cancer cells [282]. In another study, constitutive activation of Akt was identified in breast cancer cells, while benign breast epithelial cell lines were immortalized through pathways that are independent of the EGF/PI3K/Akt kinase cascade, but this was not associated with invasiveness [283]. Transfection of constitutively active Akt caused accelerated cell division and osteopontin expression [283]. Conversely, dominant-negative Akt kinase slows cell cycle progression and suppresses osteopontin expression [283]. The manipulation of osteopontin expression in this setting by transfection of the gene or its antisense did not affect the growth rate of the cells but altered cell motility and anchorage independence [283]. Therefore, Akt kinase was postulated to activate two distinct genetic programs: the program of growth and survival and the program of invasiveness and anchorage independence, which is mediated by osteopontin [283]. These studies define Akt kinase as a molecular bridge

between cell cycle progression and dissemination. In colorectal cancer, another group investigated the effect of inhibiting the PI3K/Akt/IKKα pathway in regulating the inappropriate constitutive activation of NF-κB and β-catenin [284]. Inducible expression of either dominant-negative IKKα or PTEN strongly inhibited both the constitutive NF-κB- and β-catenin-dependent promoter and endogenous gene activation [284]. Targeted array-based gene expression analysis of this inducible system reveals that many of the genes downregulated upon inhibition of this pathway were involved in tumor angiogenesis and metastasis [284].

3.3.9 Alterations in PI3K signaling in cancer

We will first discuss the evidence describing altered PI3K signaling in human cancer, before more specifically addressing alterations in *PTEN* and *PIK3CA*.

3.3.9.1 Activation of the PI3K/Akt signaling pathway in human cancer

A first study showed that colorectal tumors exhibited enhanced PI3K activity compared with normal colonic mucosa, raising the possibility that PI3K may be a potential target for new strategies for the treatment of colorectal carcinoma [285]. In small cell lung cancer (SCLC) initial reports found high basal constitutive PI3K activity, which results in high basal Akt and ribosomal p70(S6K) activity [286]. Inhibition of PI3K activity markedly inhibited SCLC cell proliferation in liquid culture as a result of stimulating apoptosis and promoting cell cycle delay in G1[286]. Thus, constitutive PI3K activity in SCLC cells was proposed to play an important role in promoting the growth and anchorage independence of SCLC [286]. Another study identified Akt as a constitutively active kinase that promotes survival of NSCLC cells and demonstrated that modulation of Akt activity by pharmacological or genetic approaches alters the cellular responsiveness to therapeutic modalities such as chemotherapy or radiotherapy [287]. Elevated phospho-Akt staining was reported in 65% human malignant mesotheliomas (MM) specimens [288]. In addition, Akt phosphorylation was consistently observed in MMs arising in asbestos-treated mice and in MM cell xenografts [288]. Treatment of a MM cell line with rapamycin resulted in growth arrest in G1 phase, while LY294002 in combination with

cisplatin had greater efficacy in inhibiting cell proliferation and inducing apoptosis than either agent alone [288].

A report showed activation of Akt2 in human primary ovarian cancer and induction of apoptosis by inhibition of PI3K/Akt pathway [289]. The majority of tumors displaying activated Akt2 were high grade and stages III and IV [289]. Immunostaining and Western blot analyses using a phospho-Ser-473 Akt antibody that detects the activated form of Akt2 confirmed the frequent activation of Akt2 in ovarian cancer specimens [289]. Another study determined the frequency of Akt activation in ovarian cancer and found elevated staining (phosphor-Ser473) in 68% ovarian carcinomas [290]. In another report, significantly increased Akt1 kinase activity was detected in primary carcinomas of prostate, breast, and ovary [291]. The majority of Akt1-activated tumors were high grade and stage III/lV [291].

In thyroid cancer, increased levels of phosphorylated total Akt were identified in follicular but not papillary cancers compared with normal tissue [292]. Levels of Akt1 and Akt2 proteins and Akt2 RNA were elevated only in the follicular cancers [292]. In paired samples, Akt 1, 2, 3, and phospho-Akt levels were higher in cancers [292]. These data suggested that Akt activation may play a role in the pathogenesis or progression of sporadic thyroid cancer [292]. In head and neck cancer, a significant association was found between phospho-Akt staining and local recurrence in the patient series. Evaluation of PI3K activation by Akt phosphorylation was thus suggested to be a prognostic marker for response to therapy [293]. Immunohistochemical analyses in breast carcinomas revealed that elevated expression of HER-2/neu was found to correlate with over-expression of Akt2 protein and activation of Akt kinase [294]. HER-2/neu-overexpressing breast cancer cell lines were resistant to apoptosis induced by UV treatment and hypoxia, which was suppressed in the presence of the PI3K inhibitors LY294002 and wortmannin, indicating a link between Akt activation and stress resistance in HER-2/neu-overexpressing cells [294]. In colorectal carcinomas, immunohistochemical analysis showed that 46% of the tumors had a high level of expression of phosphorylated Akt with a close association with Ki-67 proliferative activity and the number of apoptotic bodies [295]. Akt phosphorylation was also correlated with clinicopathologic parameters of the malignancies, including depth of

invasion, infiltration to venous vessels, lymph node metastasis, and clinicopathologic stage [295]. It was concluded that activation of Akt plays an important role during the progression of colorectal carcinomas by helping promote cell growth and rescue cells from apoptosis [295].

Another study suggested that PI3K has a major role in the control of proliferation and apoptosis of growth factor-independent multiple myeloma cell lines [296]. Constitutive activation of this pathway was shown to be a frequent event in the biology of multiple myeloma *in vivo* and may be more frequently observed in primary plasma-cell leukemia [296]. Purified plasmocytes from patients with myeloma or leukemia displayed constitutive phosphorylation of Akt, FKHRL-1 and p70(S6K), which was inhibited by LY294002 and enhanced by IGF-I [296]. Another study demonstrated that Akt is activated in AML blasts and that p70(S6K) and 4EBP-1, downstream mediators of Akt signaling, also are phosphorylated in AML blasts [297]. In a short-term culture system, most AML patient samples showed a dose-dependent decrease in survival after incubation with LY294002 [297]. Incubation of AML blasts with RAD001 induced only a small decrease in survival of the cells [297]. However, when combined with Ara-C, RAD001 enhanced the toxicity of Ara-C [297]. These results demonstrated that constitutive activation of the PI3K pathway is necessary for the survival of AML blasts and that targeting of this pathway with pharmacologic inhibitors may be of clinical benefit in treatment of AML [297]. Another study demonstrated that the overall survival of patients with the Akt phosphoryated on Ser473 was significantly shorter than that of patients without [298]. Thus, the detection of the Akt phosphorylation may provide a new tool for identifying AML patients at high risk of an unfavorable outcome [298].

Based on the observation that melanoma cell lines exhibit constitutive Akt activation, this event was evaluated by immunohistochemistry [299]. Normal and slightly dysplastic nevi exhibited no significant Akt expression, in marked contrast to the dramatic Akt immunoreactivity seen in severely dysplastic nevi and melanomas [299]. It was proposed that activation of Akt may be an early marker for tumor progression in melanoma [299].

In glioma, a study analysed the levels of expression of PI3K pathway members through quantitative Western analysis [300]. Levels of phospho-Akt, and phospho-

p70(S6K) were all found to be inversely associated with cleaved caspase-3 levels, suggesting PI3K pathway activation is associated with reduced levels of apoptosis [300]. Activation of PI3K pathway members was found to be significantly associated with reduced survival times [300].

A study examined the status of activation of Akt in different stages of squamous cell carcinoma development in clinical samples from squamous carcinomas of the head and neck (HNSCC) patients [301]. By immunohistochemical analysis, it was demonstrated that activation of Akt is a frequent event in human HNSCC because active Akt could be detected in these tumors with a pattern of expression and localization correlating with the progression of the lesions [301]. In line with these observations, Akt was constitutively activated in a large fraction of HNSCC-derived cell lines [301].

Gefitinib, a specific epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, was shown to have activity against approximately 10% of unselected non-small-cell lung cancer (NSCLC) patients. An important finding was that patients with phosphorylated Akt-positive tumors who received gefitinib had a better response rate, disease control rate, and time to progression than patients with phosphorylated Akt-negative tumors, suggesting that gefitinib may be most effective in patients with basal Akt activation [302].

3.3.9.2 The tumor suppressor gene *PTEN*

Discovery of PTEN as an antagonist of the PI3K/Akt pathway

The tumor suppressor called phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (or MMAC1) is located on human chromosome 10q23, and was initially described as sharing homology with the protein tyrosine phosphatase family [303, 304]. Germ-line mutations in *PTEN* give rise to several related neoplastic disorders, including Cowden disease [303, 305, 306]. It was demonstrated that over-expression of PTEN reduced insulin-induced PIP_3 production in human cells without effecting insulin-induced PI3K [307]. Purified recombinant PTEN catalyzed dephosphorylation of PIP_3, specifically at position 3 on the inositol ring [307]. These results established the function of PTEN as a phosphoinositide 3-phosphatase by regulating PIP_3 levels [307]. Although more than half of *PTEN* mutations result in protein truncation, a significant fraction of

PTEN mutations are missense mutations. It was shown that the majority of *PTEN* missense mutations (90%) eliminated or reduced phosphatase activity towards inositol 1,3,4,5-tetrakisphosphate and PIP_3 [308]. It was reported that a missense mutation in *PTEN*, PTEN-G129E, which is observed in two Cowden disease kindreds, specifically ablates the ability of PTEN to recognize inositol phospholipids as a substrate, suggesting that loss of the lipid phosphatase activity is responsible for the etiology of the disease [309]. Furthermore, expression of wild-type or substrate-trapping forms of PTEN in mammalian cells altered the levels of the phospholipid products of PI3K and ectopic expression of the phosphatase in PTEN-deficient tumor cell lines resulted in the inhibition of PKB/Akt and regulation of cell survival [309]. It was also shown that glioblastoma cells, in contrast to primary human astrocytes, contain high endogenous Akt activity and high levels of $PI(3,4,5)P_3$ and $PI(3,4)P_2$, the lipid products of PI3K [310]. These glioblastoma cells were shown to express mutant forms of the 3' phospholipid phosphatase PTEN [310]. PTEN antagonized the activation of PKB/Akt by growth factors, by activated PI3K and by PDK1, but did not antagonize the phospholipid-independent activation of PKB/Akt lacking the PH domain [310]. These results confirmed a role for PTEN in regulating the activity of the PI3K pathway in malignant human cells. Another study demonstrated that the acute administration of MMAC/PTEN in glioma cells infected with recombinant adenoviruses resulted in the inhibition of Akt-mediated signaling, growth inhibition, and anoikis [311]. In another report, PTEN inhibited cell growth and/or colony formation in epithelial cell lines [312]. The decrease in cellular proliferation was associated with an induction of apoptosis and an inhibition of signaling through the PI3K pathway [312]. Akt/PKB was able to rescue cells from PTEN-dependent death [312]. PTEN expression potently suppressed the growth and tumorigenicity of human glioblastoma U87MG cells [313]. The growth suppression activity of PTEN was mediated by its ability to block cell cycle progression in the G1 phase [313]. Such an arrest correlated with a significant increase of the cell cycle kinase inhibitor p27(KIP1) and a concomitant decrease in the activities of the G1 cyclin-dependent kinases [313]. PTEN expression also led to the inhibition of Akt/PKB [313]. Further work implicated p27(KIP1) as a critical mediator of PTEN-induced G1 arrest [314]. It was also shown that PTEN protein induces a G1 block when reconstituted in

PTEN-null cells [315]. A PTEN mutant associated with Cowden's disease (PTEN;G129E) has protein phosphatase activity yet is defective in dephosphorylating inositol 1,3,4,5-tetrakisphosphate in vitro and fails to arrest cells in G1[315]. These data suggest a link between induction of a cell-cycle block by PTEN and its ability to dephosphorylate, in *vivo*, PIP_3 [315]. In a parallel study, PTEN impaired activation of endogenous Akt in cells and inhibited phosphorylation of 4E-BP1 [316]. In addition, PTEN/MMAC1 repressed gene expression in a manner that is rescued by Akt but not PI3K [316]. Finally, higher levels of Akt activation are observed in human prostate cancer cell lines and xenografts lacking PTEN/MMAC1 expression when compared with PTEN/MMAC1-positive prostate tumors or normal prostate tissue [316].

Animal models

PTEN-mutant mouse embryos displayed regions of increased proliferation [317]. In contrast, PTEN-deficient immortalized mouse embryonic fibroblasts exhibited decreased sensitivity to cell death in response to a number of apoptotic stimuli, accompanied by constitutively elevated activity and phosphorylation of PKB/Akt [317]. Expression of exogenous PTEN in mutant cells restores both their sensitivity to agonist-induced apoptosis and normal pattern of PKB/Akt phosphorylation. Furthermore, PTEN negatively regulated intracellular levels of PIP_3 in cells and dephosphorylates it *in vitro* [317]. These results showed that PTEN may exert its role as a tumor suppressor by negatively regulating the PI3K/PKB/Akt signaling pathway. The *PTEN* gene was shown to be fundamental for embryonic development in mice, as *PTEN* mutant embryos died by day 9.5 of gestation [318]. Heterozygous mice developed lymphomas associated with loss of heterozygosity of the wild-type *PTEN* allele, and tumor appearance was accelerated by gamma-irradiation [318]. These lymphomas had high levels of activated Akt/PKB [318]. This suggested that tumors associated with *PTEN* loss of heterozygosity may arise as a consequence of an acquired survival advantage [318]. When more than 6 months old, *PTEN* (+/-) mice were shown to develop a range of tumors, partially resembling the spectrum of neoplasia observed in Cowden's syndrome patients [319]. One-half of *PTEN* (+/-) females developed breast tumors, whereas all of the females had endometrial hyperplasia, and there was a high incidence of endometrial cancer [319].

Analysis of prostate cancer progression in transgenic adenocarcinoma of mouse prostate mice bred to *PTEN* (+/-) heterozygous mice, coupled with analysis of the PTEN gene and protein in the resulting tumors, revealed that haploinsufficiency of the *PTEN* gene promotes the progression of prostate cancer in this model system [320]. Using the Cre-loxP system, another group selectively inactivated *PTEN* in skin and prostate in mice [321]. Abnormalities in *PTEN* mutant skin consisted of mild epidermal hyperplasia, whereas prostates from these mice exhibited high-grade prostatic intraepithelial neoplasia that frequently progressed to focally invasive cancer [321]. These data demonstrated that PTEN is an important physiological regulator of growth in the skin and prostate [321]. Further, the early onset of prostatic neoplasia in *PTEN* mutant males implicated *PTEN* mutations in the initiation of prostate cancer. Consistent with high *PTEN* mutation rates in human prostate tumors, these data indicated that PTEN is a critical tumor suppressor in this organ.

Primordial germ cells (PGCs), which are the embryonic precursors of gametes, are the source of testicular teratoma. To elucidate the intracellular signaling mechanisms that underlie germ cell differentiation and proliferation, mice with a PGC-specific deletion of the *PTEN* gene were generated [322]. Male mice that lacked *PTEN* exhibited bilateral testicular teratoma, which resulted from impaired mitotic arrest and outgrowth of cells with immature characters [322]. Experiments with PTEN-null PGCs in culture revealed that these cells had greater proliferative capacity and enhanced pluripotent embryonic germ cell colony formation [322]. PTEN thus appears to be essential for germ cell differentiation and an important factor in testicular germ cell tumor formation [322].

In another study aimed at determining the role of the the PI3K pathway in pancreas development, a pancreas-specific knockout of *PTEN* was generated [323]. Knockout mice displayed progressive replacement of the acinar pancreas with highly proliferative ductal structures that contained abundant mucins and expressed markers of pancreatic progenitor cells [323]. Moreover, a fraction of these mice develop ductal malignancy [323]. Thus, misregulation of the PI3K pathway may contribute to the initiation of pancreatic carcinoma *in vivo* [323].

Functional consequences of PTEN inactivation

A null mutation was introduced into the mouse *PTEN* gene by homologous recombination in embryonic stem (ES) cells [324]. *PTEN* (-/-) ES cells exhibited an increased growth rate and proliferated even in the absence of serum. ES cells lacking PTEN function also displayed advanced entry into S phase [324]. This accelerated G1/S transition was accompanied by down-regulation of p27(KIP1), a major inhibitor for G1 cyclin-dependent kinases [324]. Inactivation of *PTEN* in ES cells and in embryonic fibroblasts resulted in elevated levels of PIP_3. Consequently, *PTEN* deficiency led to dosage-dependent increases in phosphorylation and activation of Akt/PKB and Akt activation increased Bad phosphorylation and promoted *PTEN* (-/-) cell survival [324]. Fas-mediated apoptosis was impaired in PTEN (+/-) mice, and T lymphocytes from these mice show reduced activation-induced cell death and increased proliferation upon activation. PI3K inhibitors restored Fas responsiveness in Pten (+/-) cells [325]. These results indicated that PTEN is an essential mediator of the Fas response and a repressor of autoimmunity and thus implicated PI3K/Akt pathway in Fas-mediated apoptosis [325].

PTEN was shown to suppress breast cancer growth through down-regulating PI3K signaling, which leads to the blockage of cell cycle progression and the induction of cell death in a sequential manner [326]. In breast cancer cells under anchorage-independent conditions, PTEN also induced anoikis, a form of apoptosis that occurs when cells are dissociated from the extracellular matrix, which is enhanced in conjunction with low serum culture conditions [327]. Together, these data suggest that PTEN effects on the PI3K signaling cascade are influenced by the cell stimulatory context, and that depending on the exposure to growth factors and other exogenous stimuli such as integrin ligation, PTEN can induce cell cycle arrest, apoptosis or anoikis in breast cancer cells. The tumour suppressor activities of PTEN were linked to the machinery controlling cell cycle through the modulation of signaling molecules whose final target is the functional inactivation of the retinoblastoma gene product [328]. Expression of wild-type PTEN reduced the expression of cyclin D1 [329]. Cyclin D1 reduction was accompanied by a marked decrease in endogenous retinoblastoma (Rb) protein phosphorylation on cyclin D/CDK4-specific sites, showing an early negative effect of PTEN on Rb inactivation. PTEN expression also prevented cyclin D1 from localizing to the nucleus during the

G(1)- to S-phase cell cycle transition [329]. The PTEN-induced localization defect and the cell growth arrest could be rescued by the expression of a nucleus-persistent mutant form of cyclin D1, indicating that an important effect of PTEN is at the level of nuclear availability of cyclin D1 [329]. Furthermore, in human glioblastoma cells, *PTEN* mutation can cooperate with EGFR activation to increase VEGF mRNA levels by transcriptionally up-regulating the proximal VEGF promoter via the PI3K/Akt pathway [330]. In addition, the ability of PTEN to potently inhibit H-Ras-induced morphological transformation and anchorage-independent growth in NIH3T3 cells was reported [331]. It was also shown that PTEN can regulate prostate cancer cell proliferation and apoptosis through inhibition of IGF-IR synthesis [332].

Clinical findings

Loss of PTEN protein was reported to correlate with pathological markers of poor prognosis in prostate cancer [333]. A relative reciprocity of mutations in *PTEN* and *NRAS* was also reported in melanoma, suggesting that the two genetic changes, in a subset of cutaneous melanomas, are functionally overlapping [334]. In multiple myeloma (MM) it was shown that human lines possessing the highest Akt activity lost PTEN expression [335]. Sequencing analysis demonstrated that the *PTEN* gene contains a deletion. Restoration of PTEN expression suppressed IGF-I-induced Akt activity, suggesting that loss of PTEN is responsible for uncontrolled Akt activity in MM lines [335]. In lymphoma/leukemia-derived cell lines, an inverse relationship between PTEN and phosphorylated Akt was observed in 63% of cell lines [336]. No cell lines showed absence of PTEN expression, whereas 50% of cell lines showed low PTEN expression [336]. Another study demonstrated the phosphorylation of Akt is accompanied by the loss of PTEN in clinical specimens of endometrial carcinomas [337]. Phosphorylation of Akt was accompanied by the loss of PTEN in clinical specimens of endometrial cancers [338]. The survival rate for PTEN-positive patients was significantly higher than that for PTEN-negative or -heterogeneous staining patients, and thus PTEN-positive staining was proposed to be a significant prognostic indicator of favorable survival for patients with advanced endometrial cancer [338]. In glioma, a strong inverse correlation was described between PTEN levels and both phosphorylated Akt expression and Akt activity [339]. A

significant association was evident between PTEN expression level and histology. PTEN levels were highest in normal brain, lowest in GBM tumors, and intermediate in grade II oligoastrocytomas [339]. Another study demonstrated that loss PTEN was highly correlated with activation of the main PI3K effector Akt *in vivo* [340]. It was also shown that Akt activation is significantly correlated with mTOR, the family of forkhead transcription factors (FOXO1, FOXO3a, and FOXO4) and the S6 protein [340]. Expression of the mutant EGFRvIII was also tightly correlated with phosphorylation of these effectors, demonstrating an additional route to PI3K pathway activation in glioblastomas *in vivo* [340]. PTEN expression correlated significantly with survival time within the entire cohort and was associated with survival within the subgroup of GBM tumors [339]. Thus, reduced PTEN expression is ubiquitous among GBM tumors and may play a role in the development of low-grade gliomas. PTEN inactivation in gliomas portends a particularly aggressive clinical behavior [339].

Therapeutic implications

It was shown that transformed cells of PTEN (+/-) mice have elevated levels of phosphorylated Akt and activated p70(S6K) associated with an increase in proliferation. Pharmacological inactivation of mTOR/RAFT/FRAP reduced neoplastic proliferation, tumor size, and p70(S6K) activity, but did not affect the status of Akt [341]. These data suggesed that p70(S6K) and possibly other targets of mTOR contribute significantly to tumor development and that inhibition of these proteins may be therapeutic for cancer patients with deranged PI3K signaling [341]. *In vitro* and *in viv*o studies of isogenic PTEN(+/+) and PTEN(-/-) mouse cells as well as human cancer cells with defined PTEN status confirmed that the growth of PTEN null cells was blocked preferentially by pharmacologic FRAP/mTOR inhibition [342]. Enhanced tumor growth caused by constitutive activation of Akt in PTEN (+/+) cells also was reversed by CCI-779 (rapamycin derivative) treatment, indicating that mTOR functions downstream of Akt in tumorigenesis [342]. Loss of *PTEN* correlated with increased S6 kinase activity and phosphorylation of ribosomal S6 protein, providing evidence for activation of the FRAP/mTOR pathway in these cells [342]. Another study aimed to determine the effects of *PTEN* status and treatment with rapamycin in the response of prostate cancer cell lines

to doxorubicin [343]. The PTEN-positive cells were significantly more susceptible to the anti-proliferative effects of doxorubicin as compared with the PTEN-negative cells. Transfection of PTEN into the PTEN-negative decreased the activation of Akt and the downstream p70(S6K) and reversed the resistance to doxorubicin in these cells, indicating that changes in PTEN status/Akt activation modulate the cellular response to doxorubicin [343]. Treatment of PC-3 PTEN-negative cells with rapamycin inhibited p70(S6K) and increased the proliferative response of these cells to doxorubicin [343]. Furthermore, treatment of mice bearing the PTEN-negative prostate cancer xenografts with CCI-779, an ester of rapamycin combined with doxorubicin, inhibited the growth of the doxorubicin-resistant tumors confirming the observations *in vitro* [343]. Thus, rapamycin and CCI-779, by interacting with downstream intermediates in the PI3K/Akt signaling pathway, reverse the resistance to doxorubicin conferred by PTEN mutation/Akt activation [343]. Another study examined the possible mechanisms of resistance to the EGFR inhibitor ZD1839 (Iressa) in tumor cells with variable levels of EGFR [344]. The results suggested that loss of *PTEN*, by permitting a high level of Akt activity independent of RTK inputs, can temporally dissociate the inhibition of the EGFR with that of Akt induced by EGFR inhibitors [344]. Thus, it was suggested that in EGFR-expressing tumor cells with concomitant amplification(s) of PI3K/Akt signaling, combined blockade of the EGFR tyrosine kinase and Akt should be considered as a therapeutic approach [344].In another study, sensitivity to ZD1839 required intact growth factor receptor-stimulated Akt signaling activity. PTEN loss leads to uncoupling of this signaling pathway and results in ZD1839 resistance, which could be reversed with reintroduction of PTEN or pharmacologic down-regulation of constitutive PI3K/Akt pathway activity [345].

In myeloma, PI3K inhibitors preferentially suppressed PTEN-null myeloma growth to those expressing PTEN, indicating that PI3K activation is more critical for growth and survival of those lines with PTEN mutations than others expressing a functional PTEN gene [346]. Expression of an active Akt, reversed wortmannin- and dexamethasone-induced apoptosis and growth inhibition in PTEN-null myeloma lines, suggesting that Akt lies downstream of PI3K for PTEN-null myeloma survival and dexamethasone resistance [346].

The ErbB2-targeting antibody, trastuzumab (Herceptin), has remarkable therapeutic efficacy in certain patients with ErbB2-overexpressing tumors. The overall trastuzumab response rate for reasons that ae not completely understood. It was reported that PTEN activation contributes to trastuzumab's antitumor activity. Reducing PTEN in breast cancer cells by antisense oligonucleotides conferred trastuzumab resistance *in vitro* and *in vivo*[347]. Patients with PTEN-deficient breast cancers had significantly poorer responses to trastuzumab-based therapy than those with normal PTEN [347]. Additionally, PI3K inhibitors rescued PTEN loss-induced trastuzumab resistance [347]. Thus, PTEN deficiency was proposed to be a predictor for trastuzumab resistance [347].

Forkhead family of transcription factors (FOXO1a, FOXO3a, FOXO4) are downstream targets of the PI3K/PTEN/Akt pathway. In *PTEN* null cells, FOXO1a is inactivated by PI3K-dependent phosphorylation and mislocalization to the cytoplasm, yet still undergoes nucleocytoplasmic shuttling. Since forcible localization of FOXO1a to the nucleus can reverse tumorigenicity of *PTEN* null cells, a high-content, chemical genetic screen for inhibitors of FOXO1a nuclear export was performed [348]. The compounds detected in the primary screen were retested in secondary assays, and structure-function relationships were identified. Novel general export inhibitors were found that react with CRM1 as well as a number of compounds that inhibit PI3K/Akt signaling, among which are included multiple antagonists of calmodulin signaling [348].

Interactions with other tumor suppressor pathways

The PTEN tumor suppressor protein inhibits PI3K/Akt signaling that promotes translocation of Mdm2 into the nucleus [229, 349]. When restricted to the cytoplasm, Mdm2 is degraded [349]. The ability of PTEN to inhibit the nuclear entry of Mdm2 increases the cellular content and transactivation of the p53 tumor suppressor protein. Retroviral transduction of PTEN into *PTEN* null glioblastoma cells increases p53 activity and expression of p53 target genes and induces cell cycle arrest [350]. U87MG/PTEN glioblastoma cells were more sensitive than U87MG/PTEN null cells to death induced by etoposide, a chemotherapeutic agent that induces DNA damage [350]. These results established a direct connection between the activities of two major tumor suppressors and

show that they act together to respond to stresses and malignancies. PTEN protects p53 from survival signals, permitting p53 to function as a guardian of the genome [349, 350].

3.3.9.3 The human *PIK3CA* oncogene

Increases in PIK3CA copy numbers

Studies using comparative genomic hybridization (CGH) revealed several regions of recurrent, abnormal, DNA sequence copy number that may encode genes involved in the genesis or progression of ovarian cancer [351]. One region at 3q26 found to be increased in copy number in approximately 40% of ovarian and others cancers contains *PIK3CA*, which encodes the p110α catalytic subunit of PI3K [351]. *PIK3CA* was shown to be frequently increased in copy number in ovarian cancers, and the increased copy number is associated with increased *PIK3CA* transcription, p110α protein expression and PI3K activity and the treatment with the PI3K inhibitor LY294002 decreased proliferation and increases apoptosis [351]. These observations suggested *PIK3CA* is an oncogene that has an important role in ovarian cancer [351]. In a subsequent study, *PIK3CA* mRNA was detected in 66.6% of stage I and 93.9% of advanced stage ovarian cancer specimens and in all ovarian cancer cell lines [352]. *PIK3CA* mRNA levels were significantly higher in invasive carcinomas compared with benign and low malignant potential neoplasms [352]. Strong expression of immunoreactive p110α was detected in tumor cells and/or stroma endothelium [352]. *PIK3CA* expression *in vivo* positively correlated, both at the mRNA and the protein level, with the expression of VEGF as well as with the extent of microvascular development [352]. Furthermore, *PIK3CA* mRNA over-expression positively correlated with increased proliferation and decreased apoptosis of tumor cells *in vivo* [352]. *In vitro*, *PIK3CA* expression positively correlated with the expression of VEGF in ovarian cancer cells, whereas LY294002 reduced both the constitutive and inducible expression of HIF-1α at the mRNA and protein levels and abrogated VEGF up-regulation by glucose starvation [352]. Furthermore, LY294002 suppressed cell proliferation and, at higher doses, induced marked apoptosis in ovarian cancer cells. Collectively, these data strongly indicate that *PIK3CA* supports ovarian cancer growth through multiple and independent pathways affecting cell proliferation, apoptosis and angiogenesis, and plays an important role in ovarian cancer progression [352].

The results of CGH also showed that the 3q26.3 amplification was the most consistent chromosomal aberration in primary tissues of cervical carcinoma, and a positive correlation between an increased copy number of *PIK3CA* and 3q26.3 amplification was found in tumor tissues and in cervical cancer cell lines [353]. In cervical cancer cell lines harboring amplified *PIK3CA*, the expression of p110α was increased, and was subsequently associated with high kinase activity [353]. These evidences supported that *PIK3CA* is an oncogene in cervical cancer and *PIK3CA* amplification may be linked to cervical tumorigenesis [353]. In low-grade head and neck squamous cell carcinomas *PIK3CA* was over-represented, as analyzed by CGH or fluorescence in situ hybridization [354]. These results indicated that *PIK3CA* may participate to the progression of head and neck tumors [354].

In another study, *PIK3CA* was identified as an oncogene involved in squamous cell carcinomas [355]. Simultaneous abnormalities in both pathways were rare in primary tumors, suggesting that amplification of *PIK3CA* and mutation of p53 are mutually exclusive events and either event is able to promote a malignant phenotype [355]. Moreover, the negative effect of p53 induction on cell survival involved the transcriptional inhibition of *PIK3CA* that was independent of PTEN activity, as PTEN was not expressed in the primary tumors [355]. Conversely, constitutive activation of *PIK3CA* resulted in resistance to p53-related apoptosis in PTEN deficient cells [355]. Thus, p53 regulates cell survival by inhibiting the PI3K/AKT prosurvival signal independent of PTEN in epithelial tumors. This inhibition is required for p53-mediated apoptosis in malignant cells [355].

Array CGH was used to identify genomic abnormalities at loci encoding genes that may contribute to lung cancer transformation and progression in squamous carcinomas (SqCas) and adenocarcinomas (AdCas) [356]. The most noticeable differences between SqCas and AdCas were gain of chromosome 3q22-q26 and loss of chromosome 3p [356]. These occurred almost exclusively in SqCas and the region of recurrent increase contained *PIK3CA* [356]. The activity of Akt was higher in SqCas than in AdCas and was correlated with *PIK3CA* copy number, suggesting that these copy number increases contribute to activation of PI3K signaling in SqCas of the lung [356]. In head and neck squamous cell carcinoma (HNSCC) a study suggested that 3q26 copy

number gain and amplification represent early genomic aberrations in HNSCC carcinogenesis [357]. In addition, p110α mRNA and protein expression in HNSCC may be regulated by these genomic aberrations as well as by epigenetic events [357].

Discovery of somatic mutations in PIK3CA

To determine if PI3Ks are genetically altered in tumorigenesis, the PI3K genes were sequenced in human cancers and corresponding normal tissue [358]. Eight PI3K and eight PI3K-like genes, including two uncharacterized genes, were identified in the human genome [358]. The sequences of 117 exons that encode the predicted kinase domains of these genes were examined in 35 colorectal cancers [358]. *PIK3CA* was the only gene with somatic (i.e., tumor-specific) mutations [358]. Subsequent sequence analysis of all coding exons of *PIK3CA* in 199 additional colorectal cancers revealed mutations in a total of 74 tumors (32%). The authors also evaluated 76 premalignant colorectal tumors and found only two mutations [358]. Thus, *PIK3CA* mutations generally arise late in tumorigenesis, just before or coincident with invasion. Mutations in *PIK3CA* were also identified in glioblastoma, gastric cancer, breast cancer and lung cancer [358]. In total, 92 mutations were observed, all of which were determined to be somatic in the cancers that could be assessed [358]. No truncating mutations were observed and >75% of alterations occurred in two small clusters in the helical and kinase domains [358]. The affected residues within these clusters are highly conserved evolutionarily. The lipid kinase activity of wild-type p110α or a "hot-spot" mutants (H1047R) were measured. Expression of mutant p110α conferred more lipid kinase activity than expression of wild-type protein [358]. These data suggested that mutant *PIK3CA* was likely to function as an oncogene in human cancers [358, 359]. This idea is consistent with previously reported alterations of members of the PI3K pathway, particularly inactivation of the *PTEN* tumor suppressor [359]. This study found no evidence of *PIK3CA* gene amplification in 96 colorectal cancers, suggesting that amplification is not a common mechanism of activation in this tumor type [358]. A subsequent study analysed 340 genes coding for serine/threonine kinases in colorectal tumours [360]. A total of 23 changes, including a majority (20) of non-synonymous point mutations were identified [360]. The gene mutations affected eight different proteins, including PDK1 (3 mutations out of which

two mutations affected the same residue in the kinase domain) and Akt2 (2 mutations) [360]. Eighteen of the 23 somatic mutations occurred at evolutionarily conserved residues in the genes encoding the serine/threonine kinases [360].

Clinical findings related to PIK3CA mutations

A subsequent report showed that 25% of breast cancers contain somatic mutations in *PIK3CA*, with the majority of mutations located in the kinase domain [358]. These results demonstrate that *PIK3CA* is the most mutated oncogene in breast cancer and support a role for *PIK3CA* in epithelial carcinogenesis [358]. A large-scale mutational analysis of the helical and catalytic domains of *PIK3CA* was performed in brain tumors [361]. A total of 13 mutations of *PIK3CA* within these specific domains were identified in anaplastic oligodendrogliomas, anaplastic astrocytomas, glioblastoma multiforme, and medulloblastomas, whereas no mutations were identified in ependymomas or low-grade astrocytomas [361]. These observations implicated *PIK3CA* as an oncogene in a wider spectrum of adult and pediatric brain tumors and suggested that *PIK3CA* may be a useful diagnostic marker or a therapeutic target in these cancers [361].

Another group screened a large panel of primary human tumors for mutations in all coding exons of *PIK3CA* [362]. A strong proportion of primary breast cancers (40%) harbored mutations in *PIK3CA* [362]. Mutations were not associated with histologic subtype, estrogen receptor status, grade or presence of tumor in lymph nodes [362]. Among the primary epithelial ovarian cancers only 6.6% contain somatic mutations, but there was a clear histologic subtype bias in their distribution [362]. Only a minority (2.3%) of serous carcinomas had *PIK3CA* mutations compared with 20.0% of endometrioid and clear cell cancers. In contrast, *PIK3CA* gene amplification (>7-fold) was common among all histologic subtypes and was inversely associated with the presence of mutations [362]. Overall, *PIK3CA* mutation or gene amplification was detected in 30.5% of all ovarian cancers and 45% of the endometrioid and clear cell subtypes [362]. In advanced ovarian carcinomas, activating *PIK3CA* missense mutations were found in only about 4% of the cases [363]. Somatic missense mutations in *PIK3CA* were observed in 12% of ovarian carcinomas, and in 18% breast carcinomas in [364].

Another study reported *PIK3CA* somatic mutations in hepatocellular carcinomas (35.6%), breast carcinomas (26.9%), gastric carcinomas (6.5%), acute leukemias (1.1%) and non-small-cell lung cancers (1.3%) [365]. Some of the *PIK3CA* mutations were detected in the early lesions of breast cancer carcinoma, hepatocellular carcinoma, and gastric carcinomas, suggesting that *PIK3CA* mutation may occur independent of stage of the tumors [365]. *PIK3CA* mutations were identified in 26% of human breast tumor samples and cell lines at about equal frequency in tumor stages I to IV in another study [366]. A highly significant association between *PIK3CA* mutations and retention of PTEN protein expression was observed. In addition, *PIK3CA* mutations were associated with expression of estrogen and progesterone receptors, lymph node metastasis, and ErbB2 overexpression. The fact that *PIK3CA* mutations and *PTEN* loss were nearly mutually exclusive implied that deregulated PI3K signaling is critical for tumorigenesis in breast cancers and that loss of either *PIK3CA* or *PTEN* abrogates the selective pressure for targeting of the other gene. However, a subsequent study found that *PIK3CA* and *PTEN* mutations in breat cancer were not mutually exclusive and correlated with similar prognostic factors [367]. Intriguingly, *PIK3CA* mutations predicted for longer local recurrence-free survival [367]. In thyroid cancer, non-synonymous somatic mutations in *PIK3CA* were found in anaplastic thyroid carcinomas (23%) [368]. In endometrial carcinoma, *PIK3CA* mutations occurred at high frequency (36%) as did the coexistence of *PIK3CA/PTEN* mutations (26%) [369]. *PIK3CA* mutations were more common in tumors with *PTEN* mutations (46%) compared with those without *PTEN* mutations (24%) [369]. In head and neck squamous cell carcinoma, mutations in *PIK3CA* were reported in 11% of the cases analysed [370]. Three of the four mutations (H1047R, E542K, E545K) had been previously reported as hotspot mutations [370]. In pancreatic cancer a recent study described *PIK3CA* mutations in a fraction (11%) of the specimens analysed and some of the somatic mutations were novel [371].

Validation of PIK3CA as an oncogene

The oncogenic potential of three of the most commonly observed *PIK3CA* mutations (E542K, E545K, H1047R) was investigated in chicken embryo fibroblasts [372]. All three mutants induced oncogenic transformation with high efficiency [372]. The mutant-

transformed cells displayed constitutive phosphorylation of Akt, of p70(S6K), and of the 4E-BP1 [372]. Rapamycin strongly suppressed cellular transformation induced by the PI3K mutants, suggesting that TOR and its downstream targets are essential components of the transformation process [372]. It was also shown that three prevalent mutants of p110α are oncogenic *in vivo* [373]. The mutants induced tumors in the chorioallantoic membrane of the chicken embryo and cause hemangiosarcomas in the animal [373]. These tumors were marked by increased angiogenesis and an activation of the Akt pathway [373]. The TOR inhibitor RAD001 blocked tumor growth induced by the H1047R p110α mutant [373]. In a subsequent study, all of eight *PIK3CA* mutations examined were shown increased p110α lipid kinase activity compared with the wild-type enzyme [374]. All the mutants strongly activated Akt and p70(S6K) and also induced morphologic changes, loss of contact inhibition, and anchorage-independent growth of NIH3T3 cells [374]. The hotspot mutations E542K, E545K, and H1047R, all had high enzymatic and transforming activities [374]. These results showed that almost all the colon cancer-associated *PIK3CA* mutations are functionally active so that they are likely to be involved in carcinogenesis [374]. In a recent report, a panel of rare *PIK3CA* mutants was evalulated in chicken fibroblast tansformation assays, which also revealed their oncogenic potential and mechanism of activation [375].

To evaluate the consequences of *PIK3CA* alterations, the two most common mutations were inactivated by gene targeting in colorectal cancer cells [376]. Biochemical analyses of these cells showed that mutant *PIK3CA* selectively regulated the phosphorylation of Akt and of forkhead transcription factors [376]. *PIK3CA* mutations had little effect on growth under standard conditions, but reduced cellular dependence on growth factors [376]. *PIK3CA* mutations resulted in attenuation of apoptosis and facilitated tumor invasion [376]. Importantly, treatment with LY294002 abrogated *PIK3CA* signaling and preferentially inhibited growth of *PIK3CA* mutant cells [376]. A recent study also showed that *PIK3CA* mutant colon cancer cell lines have increased metastatic potential in an orthotopic model [377]. Two *PIK3CA* mutants observed in breast cancer (E545K and H1047R) were also tested in the MCF-10A immortalized breast epithelial cell line [378]. Both variants displayed higher PI3K activity than wild-type p110α yet remained sensitive to pharmacologic PI3K inhibition [378]. In addition,

expression of p110α mutants in mammary epithelial cells induced multiple phenotypic alterations characteristic of breast tumor cells, including anchorage-independent proliferation in soft agar, growth factor-independent proliferation, and protection from anoikis [378]. Expression of these mutant p110α isoforms also confered increased resistance to paclitaxel and induced abnormal mammary acinar morphogenesis in three-dimensional basement membrane cultures [378].

Another study compared the biochemical activity and transforming potential of mutant forms of p110α and p110β in a human mammary epithelial cell system [379]. The two most common tumor-derived alleles of p110α (H1047R and E545K) potently activated PI3K signaling [379]. Human mammary epithelial cells expressing these alleles grew efficiently in soft agar and as orthotopic tumors in nude mice [379]. A mutation in p110β homologous to the E545K allele of p110α was then constructed, but the resulting p110β mutant was only weakly activated and induced minimal soft-agar growth [379]. However, a gene fusion of p110β with a membrane anchor was highly active and transforming in both soft-agar and orthotopic nude mouse assays [379]. Work by another group investigated the oncogenic potential of all 4 catalytic class I PI3K isoforms [380]. At physiological levels of expression, the wild-type p110α isoform lacked oncogenic potential, but gain-of-function mutations and overexpression of p110α were correlated with oncogenicity [380]. The p110β, p110γ and p110δ isoforms induced transformation of cultured cells as wild-type proteins, which could be suppressed by rapamycin [380]. The p110δ isoform constitutively activated the Akt signaling pathway, but p110γ activated Akt only in the presence of serum [380]. The p110 isoforms also differed in their requirements for upstream (Ras) signaling [380].

3.4 Lung cancer

Lung cancer is the main cause of neoplastic death in the world and accounts for approximately 26% of all cancer deaths. Lung cancer causes the death of 85% of newly diagnosed patients within five years [381, 382]. Worldwide over 1 million people die annually in consequence of lung cancer [383]. Approximately 85 % of these cases are non-small cell lung cancer (NSCLC) with the rest being small-cell lung cancer (SCLC)

[384]. NSCLC is composed of three main types: squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. The development of lung cancer is associated with tobacco smoking and exposure to asbestos, radon and other potential carcinogens. Current treatments of lung cancer include surgery, radiotherapy and chemotherapy. Chemotherapy improves the survival rate of NSCLC patients although the median 5-year survival rate is still only 15% [385]. SCLC is correlated with tobacco smoking in 98% of cases and patients have a very poor outcome with a 5-year survival of less than 5% [386].

In the past years, the molecular mechanisms responsible for cancer development have been extensively studied and the contribution of abnormal Ras and PI3K signaling to tumor growth, maintenance and chemoresistance has been well documented [387]. A growing body of evidence suggests that lung tumors selectively up-regulate different signaling pathways implicated in cell proliferation, survival and angiogenesis, in order to become invasive and chemoresistant [388]. It has also become clear that deregulated RTKs and their downstream effectors possess a high oncogenic capacity. Recently, an interesting model termed “oncogene addiction” has been proposed for tumors which develop a dependency on a single oncogenic signaling pathway and there is growing experimental evidence that this mechanism is indeed very important for tumor cell survival [389, 390]. Even if unexpected in its theoretical simplicity, the specific targeting of activated oncoproteins can lead to tumor growth arrest and cause apoptosis [390]. One of the most prominent examples so far is the successful treatment of chronic myelogenous leukemia (CML) patients with imatinib (Gleevec) an inhibitor of Bcr-Abl [391]. A beneficial response to imatinib has also been reported in gastrointestinal stromal tumors (GISTs) patient with mutationally activated c-Kit [392]. Moreover, the improved outcome of NSCLC patients with mutationally activated or amplified EGFR in response to treatment with either gefitinib or erlotinib [393-395], further supports this model.

The PI3K/Akt/mTOR pathway and its implication in human cancer have been extensively reviewed in the past years [387, 396, 397]. mTOR is a serine/threonine kinase which has emerged in the past 5 years as one of the most important intracellular signaling enzyme regulating cell growth, survival and motility in cancer cells [398]. mTOR has been identified as a downstream target of both the PI3K [399, 400] and Ras [401-404] signaling pathways. The discovery of the highly specific and potent mTOR inhibitor

rapamycin and its derivatives (RAD001, CCI-779, and AP23573) further boosted the interest of the scientific community in elucidating its biological function [405, 406]. Below, we will summarize the current knowledge about the contribution and implication of the PI3K/Akt/mTOR pathway in lung cancer development and maintenance.

3.4.1 Non-small cell lung cancer (NSCLC)

Many lines of evidence point to the importance of deregulated PI3K/Akt/mTOR signaling in lung cancer. The PI3K/Akt pathway was reported to mediate the effects of several tyrosine kinase receptors on proliferation and survival in small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [388]. These receptors include c-Met [407, 408], EGFR [409, 410], c-Kit [137] and IGF-IR [411]. Over-expression of both the regulatory p85α and the catalytic p110α subunits of PI3K was reported in primary human lung carcinomas [12]. Genomic amplification of the *PIK3CA* gene was also documented in several lung cancers and in pre-invasive bronchial lesions, suggesting an implication of the PI3K pathway in lung cancer development [412]. The up-regulations of PI3K may be linked to tumor-cell transformation in lung cancer. Most lung tumors also demonstrate activation of downstream mediators of PI3K such as Akt or mTOR. Several groups have reported that Akt can be activated by amplification or mutations of upstream components in the PI3K pathway. Notably, mutated EGFR has been reported to have higher expression levels compared to the wild type protein and furthermore to induce a higher activation of Akt and signal transducer and activator of transcription (STAT), but not of the Erk signaling pathway [393, 409, 413]. Akt activation can also occur through direct over-expression or activation of the Akt protein itself. Brognard *et al*. reported PI3K-dependent Akt phosphorylation in a panel of NSCLC cell lines, which was correlated with resistance to chemotherapy. The PI3K inhibitor LY294002 alone or in combination with chemotherapy treatment rendered NSCLC cell lines with highly activated Akt more responsive to apoptosis and growth inhibition [287, 407]. Increased phosphorylation of Akt was also reported in pre-malignant and malignant human bronchial epithelial cells, but not in normal bronchial cells [414]. Moreover, the ability of a novel pro-apoptotic drug to induce apoptosis in these cells correlated with increased Akt activation levels [414]. Furthermore, immunohistochemical analysis detected high levels of

phosphorylated Akt in tumor tissues from NSCLC patients and revealed the implication of activated PI3K/Akt pathway in disease progression [412, 415-417]. Activation of Akt was also observed in primary NSCLC tumors, supporting a role of activated Akt in the conversion of a precursor lung lesion to malignant cancer [415, 416, 418]. Moreover, phosphorylation or over-expression of Akt correlated with poor prognosis in NSCLC patients with stage I disease or with primary tumors [417, 419]. Another study identified somatic mutations in the Akt gene, which could contribute to oncogenesis in a subset of lung carcinomas [420]. It has also been shown that the over-expression of PI3K or Akt was frequently associated with low expression of PTEN in NSCLC [421-424]. The tumor suppressor gene *PTEN* negatively regulates the PI3K/Akt pathway and cancer cells in which the *PTEN* gene is deleted or its expression down-regulated display constitutively activated PI3K signaling, which can contribute to lung carcinogenesis [425, 426].

Concerning mTOR, it was shown that this protein is phosphorylated in several lung cancer cell lines [418, 427]. Importantly, mTOR activation was more frequent in tumors with gene alterations such as EGFR mutations or PI3K/Akt over-expression [428]. Additionally, expression of transgenic K-RasV12 in mice induced the transformation of normal alveolar epithelial cells, which was dependent on mTOR signaling [429]. Indeed, mTOR phosphorylation increased with the malignant progression and blocking mTOR with the rapamycin derivative CCI-779 was sufficient to block lung tumor progression [429]. Recently, mTOR has been implicated in the development of metastasis in NSCLC. Phillips et al. reported that the metastatic potential of NSCLC cells is increased under conditions of low oxygen tension and chemokine receptor CXCR4 and EGFR overexpression, and that this process could be blocked by rapamycin [430]. Furthermore, the simultaneous inhibition of the PI3K/Akt and c-Jun NH2-terminal kinase (JNK) pathways has been demonstrated to increase apoptosis in NSCLC, both *in vitro* and *in vivo* [431]. Moreover, over-expression of a dominant-negative mutant of the regulatory PI3K p85α subunit induced apoptosis in NSCLC cells [432]. The same effect was observed after inhibition of Akt by expression of a dominant-negative mutant of this protein in NSCLC cell lines [287].

Nicotine, which is an important component of cigarette smoke, was shown to activate growth-promoting pathways, particularly the PI3K/Akt/mTOR pathway [433, 434] and

was associated with NF-κB-dependent resistance to chemotherapeutic drugs [433]. This activation was accompanied with endogenous acetylcholine release, which may serve as an autocrine or paracrine growth factor and may promote the development of lung cancer [433, 434]. The inhibition of PI3K or Akt blocked lung cancer cells growth triggered by nicotine, suggesting that the activation of the PI3K/Akt pathway is involved in nicotine-induced cell survival [435, 436]. Furthermore, it has been found that BAD is inactivated as a result of activation of the PI3K/Akt pathway in response to nicotine exposure, thus leading to a cell growth advantage. Nicotine-dependent Akt activation also effectively led to increased phosphorylation of Bax, another member of the Bcl-2 protein family and thus abrogated its pro-apoptotic function [435]. The correlation between cigarette smoking and NSCLC development has been further supported by the findings that 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco-specific carcinogen, activates both the Erk and Akt signaling pathways in association with increased survival of normal non-immortalized lung epithelial cells [437].

In addition, activation of the PI3K pathway has been shown to increase resistance to chemo- or radiotherapy [287]. Indeed, NSCLC cells resistant to gefitinib demonstrated increased PI3K/Akt activation [422, 438]. The inhibition of PI3K led to sensitization of lung cancer cells to radiotherapy [439], or chemotherapy [411]. Accordingly, RAD001 (Everolimus) sensitized NSCLC cells to the DNA-damaging agent cisplatin by downregulation of p21, which is required for DNA repair [440]. Based on these data, inhibition of the PI3K/AKT/mTOR pathway has been widely proposed to represent a promising therapeutic approach for lung cancer. Taken together, these data also indicate that the activation of PI3K/Akt/mTOR pathway plays a significant role in the development, growth and chemoresistance in NSCLC.

3.4.2 Small cell lung cancer (SCLC)

Small cell lung cancer (SCLC) represents 10-15% of all lung cancer cases and is strongly associated with tobacco smoking. This type of lung cancer is initially very sensitive to chemotherapy and radiotherapy. However, the initial therapeutic responsiveness is followed by disease recurrence and therefore the outcome is still very poor [386, 441]. Although the understanding of the biology of this cancer has greatly increased in the past

20 years, no obvious survival improvement has been achieved in the clinic so far. A plethora of genetic and molecular alterations have been reported, including autocrine signaling loops, oncogene activation and loss of tumor-suppressor genes [384]. Constitutive activation of the PI3K/Akt/mTOR pathway was related to the transformed phenotype in SCLC cells in the late 1990s [286]. Differential over-expression for several PI3K isoforms and their contribution to Akt activation were demonstrated in a panel of SCLC cell lines [137]. Moreover, several autocrine loops have been described in SCLC cells, including SCF/c-Kit, IGF-I/IGF-IR and hepatocyte growth factor (HGF)/c-Met. Inhibition of the PI3K/Akt pathway was able to reverse the mitogenic effects of these autocrine loops [411]. In addition, immunohistochemical analysis detected high levels (70%) of phosphorylated Akt in tumor tissues from SCLC patients and revealed the implication of the activated pathway in disease progression [412, 442]. This observation is difficult to explain solely through mutations in the *PTEN* gene, since this event has been found only in 15% of SCLC tumors [443-445]. Moreover, the PI3K pathway was also shown to be activated through integrins in response to adhesion on extracellular matrix (ECM) molecules and this resulted in resistance of SCLC cells to various therapies and protection from apoptosis [446, 447].

Further studies have shown that inhibition of the PI3K/Akt/mTOR pathway with LY294002 or rapamycin led to apoptosis and decreased cell growth in SCLC cells [446]. The same effect was observed after inhibition of Akt by expression of a dominant-negative mutant of this protein in SCLC cells [411]. Adherent-growing SCLC cells, which are those thought to initially survive chemotherapy, have been reported to activate Akt and thus to increase chemo- and radiotherapy resistance by mechanisms involving over-expression of anti-apoptotic proteins [448]. One possible mechanism by which SCLC cells can escape the effects of cytotoxic drugs was discovered in experiments elucidating SCLC responses to cisplatin, a DNA-damaging agent. Surprisingly, treatment with cisplatin up-regulated Akt activation and contributed to the expression of pro-survival proteins in SCLC cells [449]. Additionally, it has been found that a rapamycin derivative (CCI-779) sensitized cisplatin-resistant SCLC cells to the effects of the cytotoxic drug [450]. Unfortunately CCI-779 did not improve patient survival, when administrated as a single agent in a Phase II clinical trial for SCLC patients after

chemotherapy induction [451]. Collectively, these data demonstrate a significant role for the PI3K/Akt/mTOR pathway in the biology of SCLC. However, the first line targeted therapy was disappointing in the clinic. Very recent pre-clinical studies with vascular endothelial growth factor receptor (VEGFR) inhibitors and other multi-targeted drugs showed promising result [452-454] and further multi-targeted clinical trials are expected with interest.

For SCLC, there exist so far no pre-clinical studies investigating the potential therapeutical benefit of the use of rapamycin derivatives in combination with chemotherapy or other targeted inhibitors. Pandya et al. reported the failure of CCI-779 to improve SCLC patient survival in a Phase II clinical trial [451]. This disappointing result should first lead to studies aimed at gaining a better understanding of the molecular deregulations in the PI3K/Akt/mTOR axis in SCLC. Based on established findings it will then be possible to choose the optimal therapeutic strategy for selected patient groups, which are more likely to benefit from targeted treatment. In fact, CCI-779 showed activity in Phase II clinical trials in patients with renal cell carcinoma and glioblastoma [455-457]. However, it has been found that a small population of glioblastoma patients with highly elevated phospho-S6K levels mainly benefited from CCI-779 treatment [456]. Interestingly, elevated protein expression and phosphorylation of S6K1 and its recently identified homologue S6K2 have been reported in different SCLC cell lines, as compared to normal pneumocytes [286, 458]. Surprisingly, S6K2 was activated in a PI3K-independent manner upon fibroblast growth factor-2 (FGF-2) stimulation. S6K2 activity could be blocked by a mitogen-activated Erk kinase (MEK) inhibitor and only high concentrations of rapamycin were able to partially inhibit its activity [458]. Recent studies have elucidated the molecular signaling pathway involving S6K2 and provide a possible explanation why rapamycin or its analogues fail to show any anti-tumor activity in SCLC patient, when administered as a single agent. Pardo *et al*. described a novel multi-protein complex comprising protein kinase Cε (PKCε), B-Raf and S6K2. FGF-2-dependent S6K2 activation or a constitutively active mutant of S6K2 were sufficient to elevate the expression of anti-apoptotic proteins and render SCLC cells resistant to chemotherapy [459]. Furthermore, the authors found that elevated S6K2 protein levels in SCLC and NSCLC tumor specimens correlated with chemoresistance. Thus, S6K2

represents a novel target, decoupled from mTOR signaling which can promote pro-survival signaling in SCLC tumors. In this context, increased FGF-2 serum levels were correlated with poor survival and active angiogenesis in SCLC patients [460]. Furthermore, it has been found that FGF-2 renders SCLC cells more resistant to etoposide-induced apoptosis by up-regulation of pro-survival pathways [461, 462]. In pre-clinical studies the multitargeted inhibitor SU6668, known to bind and inactivate the fibroblast growth factor receptor (FGFR), vascular endothelial growth factor receptor (VEGFR), and platelet-derived growth factor receptor (PDGFR), showed a high efficacy in a panel of human tumor xenografts, including lung cancer. High anti-proliferative and anti-angiogenic activity has been reported for this compound [463]. The inhibitory effect was found to be mainly restricted to vascular endothelial cells in the tumor bed and inhibition of their pro-angiogenic capacity [464]. These findings are of particular interest for SCLC. Recently, a high vascularization status of SCLC tumors has been reported [465]. Furthermore, high levels of VEGF found in SCLC patient plasma were related to poor prognosis [466, 467]. Indeed, pre-clinical studies testing antibodies against the VEGFR or multi-targeted inhibitors targeting the VEGFR, FGFR and c-Kit have described very promising results [452-454, 468, 469]. The molecular events triggering angiogenesis require the coordinated cross-talk between VEGF, FGF, PDGF and other angiogenic factors, in order to activate endothelial cell proliferation and to facilitate neovascularisation. Interestingly, all these growth factors signal through the PI3K/Akt/mTOR pathway in cancer cells [470]. Thus, it can be hypothesized that effective therapy of SCLC tumors will require combinations of targeted drugs with anti-angiogenic properties in combination with mTOR inhibitors, in order to inhibit a large panel of SCLC molecular dysfunctions and to prevent the development of acquired secondary resistance.

In SCLC, multiple receptor tyrosine kinases are over-expressed and each is capable of activating an overlapping spectrum of critical downstream signaling pathways. One of these activated downstream signaling events is the Ras pathway. Moreover, Ras activation has been shown to be an important mediator of lung carcinogenesis [471, 472]. Indeed, mutations or over-expression of Ras, predominantly of the K-Ras isoform, have been detected in lung tumours, particularly in adenocarcinoma [473-475]. These

mutations were correlated with a shorter survival of patients with NSCLC [476, 477]. In contrast, Ras mutations were found in less than 1% of SCLC tumours [478, 479].

However, despite the lack of known activating mutations of Ras in SCLC, Ras proteins are involved in the growth of this cancer. Indeed, our recent work has shown that inhibiting Ras signalling potently disrupts proliferation and survival of SCLC cell lines and sensitizes SCLC to the effects of chemotherapy [480]. This study showed that the 3-Hydroxy-3methylglutaryl CoA (HMG-CoA) reductase inhibitor simvastatin inhibits the function of Ras proteins and the activation of the MAPK and Akt signalling pathways [480]. Simvastatin may represent a potential new drug to inhibit SCLC proliferation. Similarly, another drug, gefitinib, has been shown to inhibit the Ras-MAPK pathway in three different SCLC cell lines [481]. Thus, targeting Ras pathway might be clinically effective against SCLC.

Additionally, anti-cancer therapies can be directed against regulators of Ras activity. Ras-GTPase activating protein (RasGAP) is the main regulator of Ras. It controls the conversion of the active form of Ras to inactive Ras-GDP, by accelerating GTP hydrolysis. SCLC cell lines express lower levels of RasGAP proteins [482]. This altered expression of RasGAP is involved in oncogenesis [483]. It has also been shown that RasGAP can be cleaved into an anti-apoptotic fragment [484], which might contribute to the highly malignant and terminally therapy-resistant phenotype of SCLC [482].

Moreover, Ras signalling requires the attachment of Ras protein to the plasma membrane, a process initiated by the enzyme: farnesyl protein transferase. Farnesyl transferase inhibitors (FTIs) specifically prevent farnesylation and have been reported to have anti-cancer effects [485]. SCLC cell lines have demonstrated sensitivity to FTIs in preclinical studies. Indeed, FTI L-744,832 inhibited the activation of Ras and cell growth in three human SCLC cell lines [486]. Unfortunately, this inhibitor demonstrated no significant clinical anti-tumour activity as a single agent, in patients with sensitive relapse SCLC [487]. Furthermore, the effects of another inhibitor of Ras localization, zoledronic acid (ZOL) have been investigated *in vitro* and *in vivo* in SCLC [488]. This compound prevents both farnesylation and geranylgeranylation of Ras proteins. Geranylgeranylation is another modification required by Ras to become functional, especially when farnesyl

transferase is inhibited. These properties suggest that ZOL may have stronger anti-cancer activity than FTIs. Indeed, ZOL showed an anti-proliferative effect and increased the effects of several chemotherapeutic drugs in different SCLC cell lines [488]. It also significantly inhibited the growth of SCLC tumours subcutaneously transplanted into nude mice [488]. Moreover, it has been reported that ZOL could be a possible radiosensitizing agent in SCLC [489], and that it could also be significantly effective for treatment of bone metastasis of this cancer [490]. Thus, the use of ZOL may be a promising therapeutic strategy and its efficacy should be verified in early phase clinical trials. In conclusion, blockade of Ras activation or binding to the plasma membrane may represent a promising therapeutic approach for SCLC.

4. Overview of the presented publications

4.1 Arcaro, A., Volinia, S., Zvelebil, M.J., Stein, R., Gout, I., Layton, M.J., Ahmadi, K., Watton, S.J., Downward, J. and Waterfield, M.D. (1998). Human phosphoinositide 3-kinase C2β, the role of calcium and the C2 domain in enzyme activity. *J. Biol. Chem.*, 273, 33082-33090

Impact factor: 5.808; citations: 54

AIM: The aim of the study was the cloning and functional characterization of a novel member of the PI3K family of enzymes.

RESULTS: The cDNA for a human Class II phosphoinositide 3-kinase (PI3KC2β) with a C2 domain was cloned from a U937 monocyte cDNA library and the enzyme expressed in mammalian and insect cells. Like other Class II PI 3-kinases *in vitro*, PI3KC2β utilizes phosphatidylinositol (PI) and PI 4-monophosphate but not PI 4,5-biphosphate as substrates in the presence of Mg^{2+}. Remarkably, and unlike other PI3Ks, the enzyme can use either Mg-ATP or Ca-ATP to generate PI 3-monophosphate. PI3KC2β, like the Class I PI 3-kinases, but unlike PI3KC2α, is sensitive to low nanomolar levels of the inhibitor wortmannin. The enzyme is not regulated by the small GTP-binding protein Ras. The C2 domain of the enzyme bound anionic phospholipids such as PI and phosphatidylserine *in vitro*, but did not co-operatively bind Ca^{2+} and phospholipids. Deletion of the C2 domain increased the lipid kinase activity suggesting that it functions as a negative regulator of the catalytic domain.

MAIN CONCLUSION: Although presently it is not known whether PI3KC2β is regulated by Ca^{2+} *in vivo*, our results suggest a novel role for Ca^{2+} ions in phosphate transfer reactions.

4.2 Arcaro, A., Zvelebil, M.J., Wallasch, C., Ullrich, A., Waterfield, M.D. and Domin, J. (2000). Class II phosphoinositide 3-kinases are downstream targets of activated polypeptide growth factor receptors. *Mol. Cell. Biol.,* 20, 3817-3830

Impact factor: 6.773; citations: 54

AIM: The aim of this study was to investigate the regulation of the class II PI3K enzymes in the context of polypeptide growth factor receptors.

RESULTS: In this study, we demonstrate that PI3K-C2α and PI3K-C2β represent two downstream targets of the activated epidermal growth factor (EGF) receptor in human carcinoma-derived A431 cells. Stimulation of quiescent cultures with EGF resulted in the rapid recruitment of both enzymes to a phosphotyrosine signaling complex that contained the EGF receptor and ErbB2. To examine this interaction in greater detail, PI3KC2β was chosen for further investigation. EGF and platelet-derived growth factor also stimulated the association of PI3KC2β with their respective receptors in other cells, including epithelial cells and fibroblasts. The use of EGF receptor mutants and phosphopeptides derived from the EGF receptor and ErbB2 demonstrated that the interaction with recombinant PI3K-C2β occurs through E(p)YL/I phosphotyrosine motifs. The N-terminal region of PI3KC2β was found to selectively interact with the EGF receptor *in vitro*, suggesting that it mediates the association of this PI3K with the receptor. However, the mechanism of this interaction remains unclear.

MAIN CONCLUSION: We conclude that class II PI3K enzymes may contribute to the generation of 3' phosphoinositides following the activation of polypeptide growth factor receptors in vivo and thus mediate certain aspects of their biological activity.

4.3 Arcaro, A., Khanzada, U.K., Vanhaesebroeck, B., Tetley, T.D., Waterfield, M.D., and Seckl, M.J. (2002). Two distinct phosphoinositide 3-kinases mediate polypeptide growth factor-stimulated PKB activation. *EMBO J*., 21, 5097-5108

Impact factor: 10.086; citations: 20

AIM: The aim of this study was to investigate the expression pattern and individual functions of class I_A and II PI3Ks in human small cell lung cancer (SCLC) cells.

RESULTS: Here, we show that different human SCLC cell lines over-express distinct subsets of class I_A and II PI3Ks, which results in striking differences in the signaling cascades activated by stem cell factor (SCF). Over-expression of class I_A p85/p110α in SCLC cells increased SCF-stimulated protein kinase B (PKB) activation and cell growth, but did not affect extracellular signal-regulated kinase (Erk) or glycogen synthase kinase-3 (GSK-3). This effect was selective, since it was not observed in SCLC cell lines over-expressing p85/p110β or p85/p110δ. The SCF receptor associated with both class I_A p85 and class II PI3KC2β, and both enzymes contributed to SCF-stimulated PKB activity. A dominant-negative PI3KC2β blocked both PKB activation and SCLC cell growth in response to SCF.

MAIN CONCLUSION: Together our data provide novel insights into the specificity and functional significance of PI3K signaling in human cancer. Both the class I_A p110α isoform and the class II PI3KC2β play an important role in c-Kit signaling in SCLC cells.

4.4 Katso, R.M., Pardo, O.E., Palamidessi, A., Franz, C., Marinov, M., De Laurentiis, A., Downward, J., Scita, G., Ridley, A.J., Waterfield, M.D., and Arcaro, A. (2006). Phosphoinositide 3-kinase C2β Regulates Cytoskeletal Organization and Cell Migration via Rac-dependent Mechanisms. *Mol. Biol. Cell,* 17, 3729-3744

Impact factor: 6.562; citations: 2

AIM : The aim of this study was to further study the role of PI3KC2β in cytoskeletal rearrangements, cell migration and survival in carcinoma cells over-expressing the epidermal growth factor receptor (EGFR).

RESULTS: In this report, we demonstrate that class II phosphoinositide 3-kinase C2β (PI3KC2β) associates with the Eps8/Abi1/Sos1 complex and is recruited to the EGF receptor as part of a multi-protein signaling complex also involving Shc and Grb2. Increased expression of PI3KC2β stimulated Rac activity in A-431 epidermoid carcinoma cells, resulting in enhanced membrane ruffling and migration speed of the cells. Conversely, expression of dominant negative PI3KC2β reduced Rac activity, membrane ruffling, and cell migration. Moreover, PI3KC2β -over-expressing cells were protected from anoikis and displayed enhanced proliferation, independently of Rac function.

MAIN CONCLUSION: Taken together, these findings suggest that PI3KC2β regulates the migration and survival of human epidermoid carcinoma cells by distinct molecular mechanisms.

4.5 Khanzada, U.K., Pardo, O.E., Meier, C., Downward, J., Seckl, M.J., and Arcaro, A. (2006). Potent inhibition of small cell lung cancer cell growth by simvastatin reveals selective functions of Ras isoforms in growth factor signalling. *Oncogene,* 25, 877-887

Impact factor: 6.582; citations: 11

AIM: The aim of this study was to investigate the impact of the 3-hydroxy-3methylglutaryl CoA (HMG-CoA) reductase inhibitor simvastatin on human small-cell lung cancer (SCLC) cell growth and survival.

RESULTS: Simvastatin profoundly impaired basal and growth factor-stimulated SCLC cell growth *in vitro* and induced apoptosis. SCLC cells treated with simvastatin were sensitized to the effects of the chemotherapeutic agent etoposide. Moreover, SCLC tumor growth in vivo was inhibited by simvastatin. These responses correlated with the inhibition of stem cell factor (SCF)-stimulated activation of extracellular signal-regulated kinase (Erk), protein kinase B (PKB) and ribosomal S6 kinase by simvastatin. Constitutive activation of the Erk pathway was sufficient to rescue SCLC cell from the effects of simvastatin. The drug did not directly affect activation of c-Kit or its localization to lipid rafts, but in addition to its ability to block Ras membrane localization, it selectively down-regulated H-Ras protein levels at the post-translational level. Down-regulation of either H- or K-Ras by RNA interference (RNAi) did not impair Erk activation by growth factors, whereas an RNAi specific for N-Ras inhibited activation of Erk, PKB and SCLC cell growth.

MAIN CONCLUSION: Together our data demonstrate that inhibiting Ras signaling with simvastatin potently disrupts growth and survival in human SCLC cells.

5. Discussion

5.1 PI3K signaling in human cancer

Almost twenty years after the discovery of PI3K, this family of enzymes has become the focus of intensive research aimed at developing new drugs for a wide variety of diseases, including cancer, inflammation and allergy. The discovery of the many genetic alterations affecting the PI3K/PTEN/Akt/mTOR pathway in human cancer has clearly placed some of the enzymes involved, including p110α, mTOR and Akt, on the top list of druggable targets in the field on oncology research. Moreover, the recognition that some of the molecular alterations, such as increased Akt phosphorylation or *PTEN* mutations correlate with clinical parameters, such as outcome, in cancer patients will possibly result in the development of new diagnostic protocols in specific types of cancer. What are the challenges ahead? The generic PI3K inhibitors LY294002 ad wortmannin have greatly facilitated the deciphering of the biological responses and signaling pathways controlled by PI3K and have even proved to be successful in a variety of pre-clinical models of human cancer. The new generations of pharmacological inhibitors with increased specificity for one or a restricted number of PI3K isoforms and related kinases are entering clinical trials. It has been demonstrated that combining these agents with receptor tyrosine kinase inhibitors, which have already been evaluated in clinical trials, can produce synergistic effects in stopping tumor growth in a variety of pre-clinical models. One can thus reasonably hope that these drug combinations will result in improved survival benefits for cancer patients. However, there remain many open questions related to the mechanisms governing sensitivity or resistance of tumors to these targeted agents. The best examples of such open questions are rapamycin and its analogs. There are now several reports describing potential novel mechanisms of resistance to these agents and there may be even more to be discovered in the future. A central question in the field of isoform-specific PI3K inhibitors is the question of specificity. The class I_A PI3K catalytic isoforms have a high degree of sequence homology in their catalytic domains and demonstrating the specificity of the cellular effects of these agents remains problematic, since there are no specific readouts that discriminate betwenn these three enzymes. Moreover, assessing the exact expression pattern of all enzymes of the PI3K family in a given tumor sample remains problematic, in view of the lack of

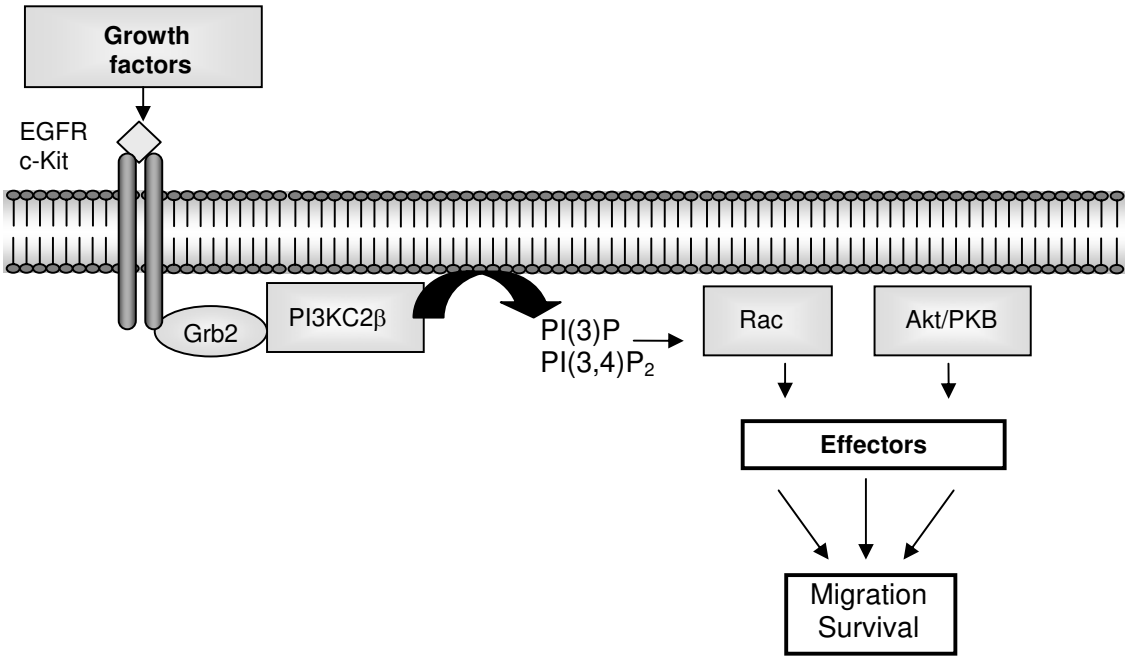
availability of isoform-specific antibodies which can be used in immunohistochemistry. Such information is in our opinion of great importance, since there exist, in addition to the well-studied class I PI3K isoforms, three class II enzymes, with still poorly understood cellular functions, at least in human cancer. In addition, the class III PI3K Vps34p should also be taken into account when designing future therapies based on targeting PI3K isoforms, in view of its roles in autophagy and intracellular trafficking events.

5.2 The class II PI3K family of signaling enzymes

It is presently 10 years since the class II PI3K enzymes were cloned by different groups, including ours. However, the exact biological functions of these 3 enzymes (PI3KC2α, PI3KC2β and PI3KC2γ) remain somewhat elusive [491]. The most recent published data indicate a role for PI3KC2α in clathrin-mediated intracellular sorting events and ATP-dependent priming of neurosecretory granule exocytosis [125-127, 492]. Concerning PI3KC2γ, its restricted tissue distribution (i.e. liver) suggests a function in hepatic functions and liver regeneration [104]. The most recent papers addressing the biological functions of PI3KC2β have described a role for this enzyme in cell migration [141-143]. Rac is a downstream target of this isoform in A-431 epidermal carcinoma cells, which provides a possible explanation for the observation by several groups that PI3KC2β regulates cell migration in various cell types [143]. It should be noted, however, that most of the published work was performed on cancer cell lines and that, in contrast to the results obtained in cell lines, the PI3KC2β knock-out mice displayed no impairment in wound healing *in vivo* [144]. Moreover, no obvious phenotype was observed in the PI3KC2β knock-out mice, in a recent paper analyzing mostly the process of epidermal differentiation [144]. This was in contrast to a previous report in *Drosophila melanogaster*, where ectopic expression of the class II PI3K_68D could modulate the process of differentiation in the wing [493]. A possible explanation for these discrepancies is a certain degree of redundancy in signaling by the various classes of PI3Ks, since only 3 enzymes (one of each class) exist in *Drosophila melanogaster*, while there are 8 known isoforms in mammals. Our observation that the expression of PI3KC2β is enhanced in SCLC, compared to normal lung epithelial cells [137], indicates

that this enzyme could play a more important role in the responses of certain cancer cells, as compared to normal cells.

Figure 4. A simplified model of the main findings presented in papers I-IV



5.3 The development of targeted therapies for small cell lung cancer

A significant increase in the understanding of the importance of RTK signaling in both NSCLC and SCLC biology has been achieved in the past years. In the case of NSCLC, targeted compounds inhibiting the EGFR have successfully achieved the transition from the bench to the bedside. However, the number of patients benefiting from these new drugs is still not as large as initially expected. The causes for this apparent lack of efficacy are multiple, but recent research has made a significant progress in better defining the conditions of optimal responsiveness to these compounds in lung cancer. The PI3K/Akt/mTOR pathway plays a crucial role in many aspects of tumor cell responses downstream of activated RTKs. Although rapamycin derivatives have not shown significant efficacy as single agents in either NSCLC or SCLC, their potential in combination with cytotoxic agents of RTK inhibitors has clearly emerged in the past years. In view of the multiple genetic and epigenetic aberrations found in solid tumors such as lung cancer and the variability in the spectrum of these alterations between patients, it is certainly not surprising that targeted drugs have not achieved a great therapeutic success so far as single agents. Combinatorial therapeutic interventions have a far greater potential of success, but this also depends on establishing reliable protocols to assess the molecular status of individual tumors with respect to the pathways that are being targeted.

5.4 Future perspectives

A significant effort has to be invested if one is to more definitely understand the individual biological functions of class II PI3Ks in normal cells, as well as in cancer cells. This will involve the generation of additional animal models (mouse knock-outs etc.) for each class II PI3K isoform, as well as the development of new isoform-specific pharmacological inhibitors. In collaboration with PIramed Ltd (Slough, United Kingdom), we have completed a first pre-clinical study of two isoform-specific inhibitors of PI3KC2β in a panel of human cancer cell lines, including leukemia (AML), neuroendocrine cancers (SCLC and neuroblastoma) and brain tumors (glioblastoma and medulloblastoma). These findings have been submitted for publication (Boller *et al*., Manuscript submitted).

Concerning our results with simvastatin in SCLC [480], future research will involve evaluating statins in clinical trials in SCLC patients. Indeed, a Phase III clinical trial has been initiated by Prof. M.J. Seckl in the United Kingdom, which is funded by Cancer Research UK (LungStar). This trial aims to recruit 1300 SCLC patients and investigate the clinical response to pravastatin in combination with chemotherapy. The LungStar trial was started in January 2007 and is planned to last until 2010.

In collaboration with Prof. M.J. Seckl and other colleagues in the United Kingdom and Switzerland, we have also completed a pre-clinical study of the mTOR inhibitor RAD001 (Everolimus, Novartis) in SCLC. The results of this study have been submitted for publication (Marinov *et al*., Manuscript submitted). It should be noted, however, the another rapamycin derivative CCI-779 showed no activity as a single agent in a recent Phase I clinical trial in SCLC, the results of which were presented at the ASCO 2006 Meeting. Clearly, there is a need to better understand the molecular mechanisms controlling sensitivity/resistance of SCLC cells to these agents.

Our group has also recently made some new contributions to the field of RTK and PI3K signaling in human cancer. In SCLC, we have shown that the Src family of non-receptor tyrosine kinases couples to PI3K signaling and is required for optimal Akt activation and cell survival, downstream of c-Kit [494]. In neuroblastoma, we have evaluated the potential of a novel IGF-IR inhibitor (NVP-AEW541, Novartis) as an anti-proliferative and cytostatic agent [495]. In this study, we have also characterized Akt as a major contributor to resistance to IGF-IR inhibitors in neuroblastoma [495]. In AML, we have, together with another group, characterized an autocrine signaling loop involving IGF-I and the IGF-IR in proliferation, survival and chemoresistance [496, 497]. Finally, in the atypical teratoid/rhabdoid tumor of the central nervous system, we have described a novel autocrine signaling loop involving insulin and the insulin receptor, which via the PI3K isoform p110α, contributes to proliferation and survival of the tumor cells [498].

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7. Publications