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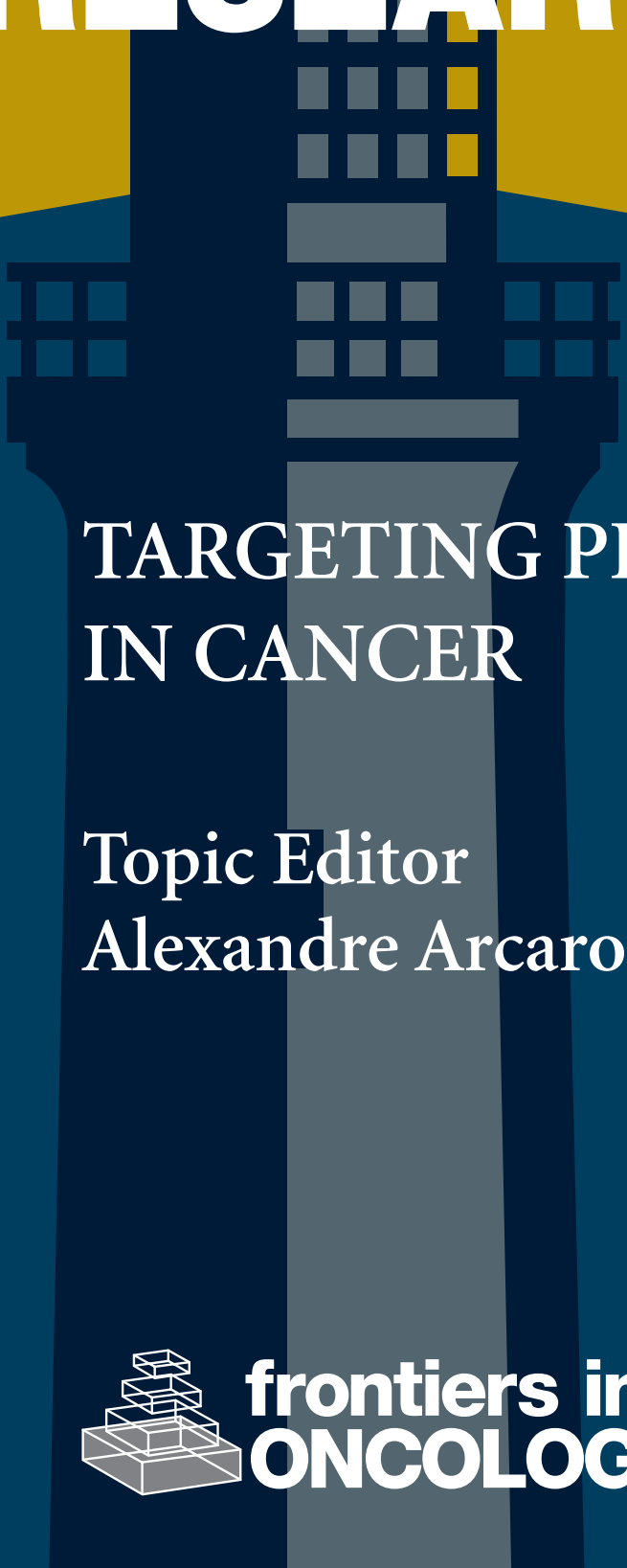

TARGETING PI3K/MTOR SIGNALING IN CANCER

Topic Editor
Alexandre Arcaro

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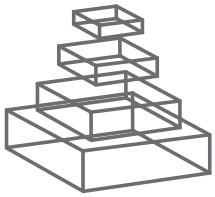

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ISSN 1664-8714
ISBN 978-2-88919-244-1
DOI 10.3389/978-2-88919-244-1

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TARGETING PI3K/MTOR SIGNALING IN CANCER

Topic Editor:
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The phosphatidylinositol 3-kinase (PI3K)/mTOR pathway integrates signals from growth factors with nutrient signals and other conditions and controls multiple cell responses, including proliferation, survival and metabolism. Deregulation of the PI3K pathway has been extensively investigated in connection to cancer. Somatic or inherited mutations frequently occur in tumor suppressor genes (PTEN, TSC1/2, LKB1) and oncogenes (PIK3CA, PIK3R1, AKT) in the PI3K/mTOR pathway. The fact that the PI3K/mTOR pathway is deregulated in a large number of human malignancies, and its importance for different cellular responses, makes it an attractive drug target. Pharmacological PI3K inhibitors have played a very important role in studying cellular responses involving these enzymes. Currently, a wide range of selective PI3K inhibitors have been tested in preclinical studies and some have entered clinical trials in oncology. Rapamycin and its analogs targeting mTOR are effective in many preclinical cancer models. Although rapalogs are approved for the treatment of some cancers, their efficacy in clinical trials remains the subject of debate. Due to the complexity of the PI3K/mTOR signaling pathway, developing an effective anti-cancer therapy remains a challenge. The biggest challenge in curing cancer patients with various signaling pathway abnormalities is to target multiple components of different signal transduction pathways with mechanism-based combinatorial treatments.

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EDITORIAL
published: 22 April 2014
doi: 10.3389/fonc.2014.00084

Targeting PI3K/mTOR signaling in cancer

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Keywords: Akt, cancer, clinical trials, mTOR, phosphoinositide 3-kinase

The phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway is very frequently activated in human cancer by a variety of genetic and epigenetic events. This pathway is thought to contribute to many of the hallmarks of cancer and a large array of agents targeting its key components are currently undergoing clinical testing in cancer patients. In addition to rapamycin analogs ("rapalogs"), which are approved for the treatment of multiple cancers, PI3K inhibitors are likely to be soon approved for B-cell malignancies (1, 2).

In this research topic, we have assembled a collection of articles describing recent key aspects of the role of the PI3K/mTOR pathway in cancer and the development of targeted therapies.

Martini et al. review the role of the different classes of PI3K isoforms as targets in oncology (3). Tzenaki and Papakonstanti focus on the role of the PI3K isoform p110δ in cancer (4). The role of the PI3K/mTOR pathway in cell cycle progression and metabolism is discussed by Vadlakonda and colleagues (5–7). Pardo and Seckl present an overview of S6K2, the p70 ribosomal S6 kinase homolog (8).

Porta and colleagues present an up to date overview of the development of selective inhibitors of Akt, mTOR, and PI3K with a focus on the latest clinical trials (9). Weigelt and Downward review the genetic determinants of response to these targeted agents (10). Fox et al. discuss the potential of co-targeting PI3K and the estrogen receptor (ER) in breast cancer (11).

ACKNOWLEDGMENTS

Work in the author's laboratory is supported by grants from the European Union FP7 (ASSET, project number: 259348 and LUNGTARGET, project number: 259770), the Swiss National Science Foundation (Grant 31003A-146464), the Fondation FORCE, the Novartis Stiftung für Medizinisch-Biologische Forschung, the Jubiläumsstiftung der Schweizerischen Mobiliar Genossenschaft, the Stiftung zur Krebsbekämpfung, the Huggenberger-Bischoff Stiftung zur Krebsforschung, the UniBern Forschungsstiftung, the Stiftung für klinisch-experimentelle Tumorforschung, Bern and the Berner Stiftung für krebskranke Kinder und Jugendliche.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 19 March 2014; accepted: 05 April 2014; published online: 22 April 2014.
*Citation: Arcaro A (2014) Targeting PI3K/mTOR signaling in cancer. Front. Oncol. **4**:84. doi: 10.3389/fonc.2014.00084*
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology.
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REVIEW ARTICLE
published: 31 August 2012
doi: 10.3389/fonc.2012.00109

Genomic determinants of PI3K pathway inhibitor response in cancer

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The phosphoinositide 3-kinase (PI3K) pathway is frequently activated in cancer as a result of genetic (e.g., amplifications, mutations, deletions) and epigenetic (e.g., methylation, regulation by non-coding RNAs) aberrations targeting its key components. Several lines of evidence demonstrate that tumors from different anatomical sites depend on the continued activation of this pathway for the maintenance of their malignant phenotype. The PI3K pathway therefore is an attractive candidate for therapeutic intervention, and inhibitors targeting different components of this pathway are in various stages of clinical development. Burgeoning data suggest that the genomic features of a given tumor determine its response to targeted small molecule inhibitors. Importantly, alterations of different components of the PI3K pathway may result in distinct types of dependencies and response to specific therapeutic agents. In this review, we will focus on the genomic determinants of response to PI3K, dual PI3K/mechanistic target of rapamycin (mTOR), mTOR, and AKT inhibitors in cancer identified in preclinical models and clinical trials to date, and the development of molecular tools for the stratification of cancer patients.

Keywords: PI3K pathway inhibitors, drug response, genetic determinant, cancer

INTRODUCTION

The phosphoinositide 3-kinase (PI3K) signaling pathway regulates numerous processes in the normal cell such as growth, proliferation, survival, motility, and metabolism (Engelman et al., 2006). In human cancer, the PI3K pathway is one of the most frequently activated signal transduction pathways, and its prominent role is highlighted by the array of mechanisms targeting several of its key components (**Figure 1**). Mutations and/or amplifications of genes encoding receptor tyrosine kinases (RTKs) upstream of class I PI3Ks (glossary box), including the human epidermal growth factor receptors EGFR (*ERBB1*) and HER2 (*ERBB2*), of the PI3K catalytic subunits p110α (*PIK3CA*) and p110β (*PIK3CB*), the PI3K regulatory subunits p85α (*PIK3R1*) and p85β (*PIK3R2*), the PI3K effector AKT (*AKT1*), and of the PI3K activator KRAS are frequently observed in cancer [Catalog Of Somatic Mutations In Cancer (COSMIC), http://www.sanger.ac.uk/cosmic; Forbes et al., 2011], as is loss of function of the tumor suppressors phosphatase and tensin homolog (PTEN) and inositol polyphosphate 4-phosphatase-II (INPP4B), negative regulators of PI3K signaling, through mutations, deletions, or epigenetic mechanisms (Gewinner et al., 2009; Fedele et al., 2010; Hollander et al., 2011).

Given that the PI3K pathway is frequently activated in cancers, that tumorigenesis and/or maintenance of the malignant phenotype of different tumor types is driven by its continued activation (Bader et al., 2005; Hollander et al., 2011), and that kinases are amendable to pharmacological intervention, it is not surprising that there has been great interest in the development of allosteric and ATP-competitive small molecule inhibitors targeting different components of this pathway downstream of RTKs (Liu et al., 2009). These targeted agents include PI3K inhibitors, either isoform specific [i.e., class I isoforms p110α, p110β, p110γ, p110δ; (glossary box)] or pan-class I PI3K inhibitors, dual PI3K/mechanistic target of rapamycin (mTOR) inhibitors, mTOR inhibitors, and AKT inhibitors, which are all currently in various stages of clinical development (**Table 1**).

Over the past years it has become apparent that irrespective of the cancer type and small molecule inhibitor or antibody used, kinase inhibitor response is limited to those tumors whose proliferation and survival are reliant on the activation of the targeted oncogenic kinase (Sharma and Settleman, 2007; Janne et al., 2009). Bernard Weinstein coined the term "oncogene addiction" to describe this phenomenon (Weinstein, 2002), which has important implications for the targeting of kinases: given the incredibly diverse repertoire of genetic and epigenetic aberrations observed within a given cancer type, only the subset of tumors "addicted" to the continued activation of the oncogenic kinase targeted will prove vulnerable to the therapeutic intervention. Consistent with this "oncogene addiction" concept, strong associations between a tumor's genotype and its response to small molecule kinase inhibitors or antibodies targeting kinases have been identified. For example, melanomas harboring $BRAF^{V600E}$ mutations are selectively sensitive to the BRAF inhibitor Vemurafenib (Flaherty et al., 2010), non-small cell lung cancers (NSCLCs) harboring *EGFR* mutations to the EGFR inhibitors Gefitinib or Erlotinib (Pallis et al., 2011), *HER2* amplified breast and gastric cancers to the HER2 targeting agents Trastuzumab or Lapatinib (Stern, 2012), and *KIT* and *PDGFRA* mutant gastrointestinal stromal tumors to Imanitib Mesylate and other small molecule inhibitors targeting mutant KIT and PDGFRα (Antonescu, 2011). Importantly, however, cancers harboring only wild-type copies of

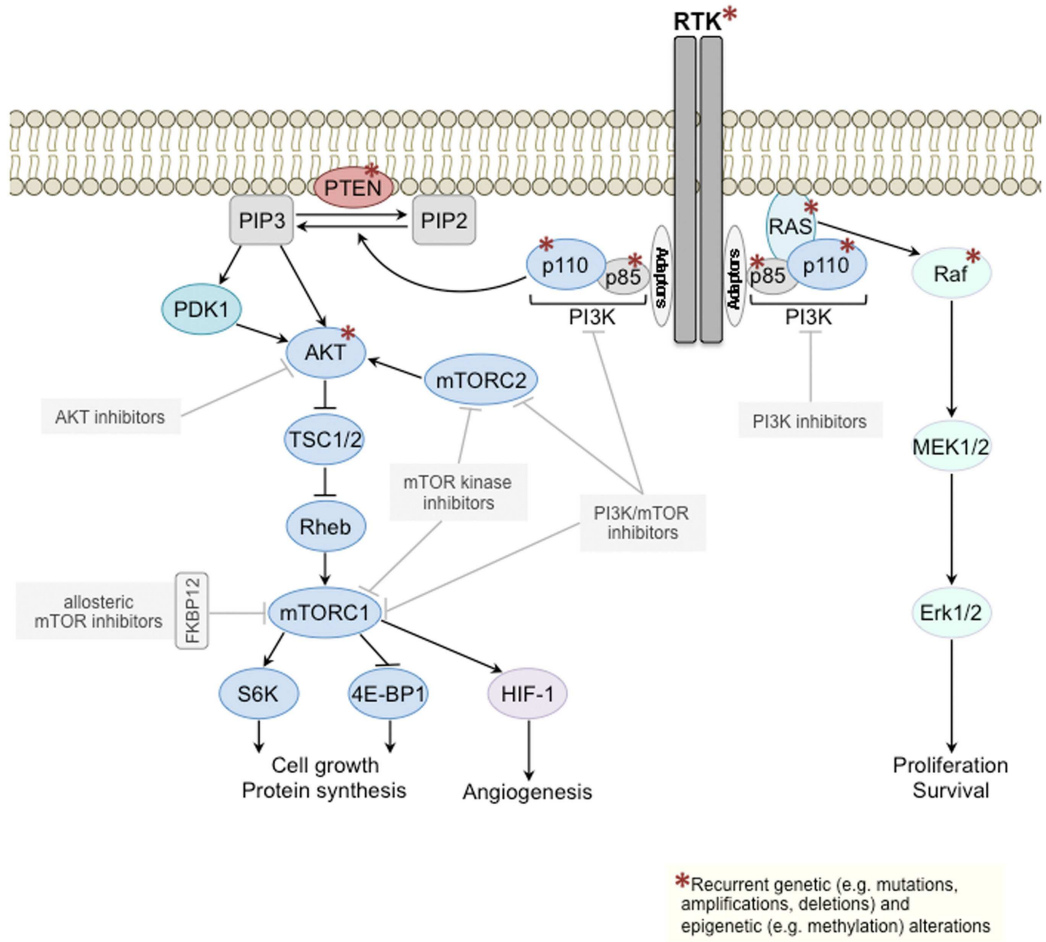

FIGURE 1 | Class I PI3K signal transduction pathway. Components of the class I PI3K signaling pathway (left) and of the mitogen-activated protein kinase (MAPK) pathway (right) recurrently targeted by genetic/epigenetic alterations in cancer are depicted with a red asterisk. Several PI3K pathway inhibitors downstream of RTKs are currently being tested in clinical trials (gray boxes). mTOR, mechanistic target of rapamycin; mTORC, mTOR complex; PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-triphosphate; PTEN, phosphatase and tensin homolog; RTK, receptor tyrosine kinase; TSC, tuberous sclerosis protein.

the genes mentioned above seem not to be sensitive to the same agents.

As PI3K pathway inhibitors progress into trials focusing on their clinical efficacy (**Table 1**), it is critical to identify their genomic determinants of response and to select the patient population most likely to benefit from treatment. In fact, it has been suggested to incorporate predictive biomarkers throughout the clinical drug development process from phase I studies onward in order to enrich trials with patients more likely to respond to a given targeted therapy and to increase the chances of drug registration (Carden et al., 2010). For the guidance and prioritization of predictive biomarker candidates in early clinical trials, results derived from the study of preclinical models are of importance.

In this review, we focus on the genomic determinants of response to PI3K pathway inhibitors in cancer identified in preclinical models and clinical trials to date, and discuss the challenges for the development of molecular tools for the stratification of cancer patients.

GENOMIC DETERMINANTS OF PI3K PATHWAY INHIBITOR RESPONSE IN PRECLINICAL MODELS

The ease of therapeutic intervention using *in vitro* cell culture and the wealth of data available on the mutational landscape of known cancer genes in the most common cell lines obtainable from commercial repositories have made cancer cell line panels the model of choice for the preclinical study of drug response. Furthermore, with the advent of methods for massively parallel sequencing, it is now possible to identify the genomic determinants of therapy response in *in vitro* models in a genome-wide fashion (Barretina et al., 2012; Garnett et al., 2012). In general, sensitivity or resistance of cancer cell lines to a given targeted agent are determined by short-term treatment ranging from 48 to 120 h of cells grown on tissue culture plastic using several dilutions of the inhibitor. At the endpoint, cell number or cell viability is assessed and drug response reported as half-maximal inhibitory concentration (IC_{50}), or the concentration needed to reduce the growth of treated cells to half that of untreated or vehicle treated

Table 1 | Open clinical trials testing PI3K pathway inhibitors in cancer*.

Inhibitor name	Company	Target	Clinical trial phase	Cancer type
PAN-CLASS I PI3K INHIBITORS				
BAY80-6946	Bayer	Class I PI3K	I	Advanced solid cancers
ZSTK474	Zenyaku Kogyo	Class I PI3K	I	Advanced solid cancers
GSK1059615	GlaxoSmithKline	Class I PI3K	I	Terminated
BKM120	Novartis	Class I PI3K	I and II	(Advanced) solid cancers; NSCLC, endometrial, prostate, breast, colorectal, pancreatic, renal cell, GIST, melanoma, glioblastoma, leukemia, SCCHN, TCC
GDC-0941	Roche/Genentech	Class I PI3K	I and II	Solid cancers; breast, NSCLC, non-Hodgkin's lymphoma
PX866	Oncothyreon	Class I PI3K	I and II	Prostate, NSCLC, SCCHN, colorectal, glioblastoma
XL147 (SAR245408)	Exelixis/Sanofi-Aventis	Class I PI3K	I and II	Solid cancers; endometrial, ovarian, breast, NSCLC
ISOFORM SPECIFIC PI3K INHIBITORS				
BYL719	Novartis	p110α	I	Advanced solid cancers; SCCHN
GDC-0032	Roche/Genentech	p110α	I	Solid cancers
INK-1117	Intellikine	p110α	I	Advanced solid cancers
GSK2636771	GlaxoSmithKline	p110β	I/IIa	Advanced solid cancers (PTEN deficient)
IPI-145	Infinity	p110γ, p110δ	I	Advanced hematological malignancies
AMG319	Amgen	p110δ	I	Relapsed or refractory lymphoid malignancies
CAL-101 (GS-1101)	Gilead sciences	p110δ	I, II, and III	Chronic lymphocytic leukemia, Hodgkin lymphoma, non-Hodgkin's lymphoma; mantle cell lymphoma, acute myeloid leukemia, multiple myeloma
DUAL PI3K/mTOR INHIBITORS				
DS-7423	Daiichi Sankyo	PI3K/mTOR	I	Advanced solid cancers; colorectal, endometrial
GDC-0980	Roche/Genentech	PI3K/mTOR	I	(Advanced) solid cancers; non-Hodgkin's lymphoma, breast, prostate, endometrial, renal cell
GSK2126458	GlaxoSmithKline	PI3K/mTOR	I	Advanced solid cancers
PWT33597	Pathway Therapeutics	PI3K/mTOR	I	Advanced solid cancers or malignant lymphoma
SF1126	Semafore	PI3K/mTOR	I	Advanced solid cancers
BEZ235	Novartis	PI3K/mTOR	I and II	Advanced solid cancers; renal cell, breast
BGT226	Novartis	PI3K/mTOR	I and II	Completed (advanced solid cancers; breast)
PF-04691502	Pfizer	PI3K/mTOR	I and II	Advanced solid cancers; breast, endometrial
PF-05212384 (PKI-587)	Pfizer	PI3K/mTOR	I and II	Advanced solid cancers; endometrial
XL765 (SAR245409)	Exelixis/Sanofi-Aventis	PI3K/mTOR	I and II	Advanced breast, gliomas, glioblastoma multiforme
mTOR KINASE INHIBITORS				
AZD2014	AstraZeneca	mTOR	I	Advanced solid cancers; breast
AZD8055	AstraZeneca	mTOR	I	Recurrent glioma
INK-128	Intellikine	mTOR	I	Advanced solid cancers; multiple myeloma, Waldenstrom macroglobulinemia
OSI-027	Astellas Pharma	mTOR	I	Advanced solid cancers; lymphoma
CC-223	Celgene Corporation	mTOR	I and II	Advanced solid cancers; non-Hodgkin's lymphoma, multiple myeloma, NSCLC
ALLOSTERIC mTOR INHIBITORS (RAPAMYCIN ANALOGS)				
Sirolimus (Rapamycin)	Wyeth/Pfizer	mTOR	I, II, and III	Advanced solid cancers; breast, liver, rectum, NSCLC, leukemias, lymphomas, head and neck, pancreatic, ovarian, fallopian tube, glioblastoma, fibromatosis
Everolimus** (RAD001)	Novartis	mTOR	I, II, and III	Solid cancers; leukemias, lymphomas, breast, bladder, head and neck, kidney/renal cell, liver, gastric, thyroid, neuroendocrine tumors, ovarian, fallopian tube, cervical, colorectal, brain and central nervous system, prostate, endometrial, esophageal, melanoma, NSCLC, SCLC, germ cell, soft tissue sarcoma, osteosarcoma, nasopharyngeal, glioma, Waldenstrom's macroglobulinemia

(Continued)

Table 1 | Continued

Inhibitor name	Company	Target	Clinical trial phase	Cancer type
Temsirolimus** (CCI-779)	Wyeth/Pfizer	mTOR	I and II	Advanced solid cancers; breast, endometrial, ovarian, prostate, liver, kidney/renal cell, SCCHN, NSCLC, melanoma, sarcoma, lymphomas, leukemia, brain and central nervous system, bladder, urethral
Ridaforolimus (MK-8669)	Merck/Ariad	mTOR	I	Advanced solid cancers; endometrial, ovarian, breast, NSCLC, renal cell, soft tissue sarcoma
AKT INHIBITORS (ATP-Competitive)				
ARQ 092	ArQule/Daiichi Sankyo	AKT	I	Advanced solid cancers
AZD5363	AstraZeneca	AKT	I	Advanced solid cancers
GSK2141795	GlaxoSmithKline	AKT	I	Completed/not recruiting (advanced solid cancers; lymphoma)
GDC-0068	Roche/Genentech	AKT	I and II	Advanced solid cancers; prostate cancer
GSK2110183	GlaxoSmithKline	AKT	I and II	Solid cancers, hematological malignancies, multiple myeloma, Langerhans cell histiocytosis, chronic lymphocytic leukemia
ALLOSTERIC AKT INHIBITORS				
MK-2206	Merck	AKT	I and II	Advances solid cancers; breast, endometrial, ovarian, fallopian tube, peritoneal, gastric, gastroesophageal junction, colorectal, prostate, NSCLC, SCLC, melanoma, kidney, leukemias, lymphomas, biliary, head and neck, liver, thymic, nasopharyngeal

**Data retrieved from http://clinicaltrials.gov and http://www.fda.gov/ (May 2012).*

***Temsirolimus: approved for the treatment of advanced renal cell carcinoma; Everolimus: approved for the treatment of progressive neuroendocrine tumors of pancreatic origin, for advanced renal cell carcinoma after failure of treatment with sunitinib or sorafenib, for renal angiomyolipoma and tuberous sclerosis complex, and for subependymal giant cell astrocytoma associated with tuberous sclerosis.*

GIST, gastrointestinal stromal tumor; NSCLC, non-small cell lung cancer; SCCHN, squamous cell carcinoma of the head and neck; SCLC, small cell lung cancer; TCC, transitional cell carcinoma of the urothelium.

cells (GI_{50}). In addition, xenograft studies in immunodeficient mice injected with human cancer cell lines or human tumor tissues, as well as transgenic mouse models have been employed to assess anti-tumor activity of PI3K pathway inhibitors *in vivo* using tumor growth, proliferation, apoptosis, and/or levels of pathway activation state as read-out of treatment response.

Using these preclinical approaches, several groups attempted to define genomic determinants of response to PI3K pathway inhibitors. It should perhaps not come as a surprise that genetic alterations leading to PI3K pathway activation, including *PIK3CA* gain-of-function mutations and/or *PTEN* mutations/PTEN loss of function and/or amplification of *HER2*, have been repeatedly identified as predictors of response to these agents (**Table 2**). However, tumor type-specific differences have been observed. For example, in ovarian cancer cells both *PIK3CA* mutations and PTEN deficiency have been reported to predict PI3K pathway inhibitor response (Ihle et al., 2009; Di Nicolantonio et al., 2010; Meuillet et al., 2010; Santiskulvong et al., 2011; Tanaka et al., 2011; Meric-Bernstam et al., 2012), whereas in breast cancer the associations between PTEN loss of function and response are less clear (She et al., 2008; Brachmann et al., 2009; Lehmann et al., 2011; Sanchez et al., 2011; Tanaka et al., 2011; Weigelt et al., 2011), which will be discussed in greater detail below.

Several studies provided evidence to suggest that cancer cells harboring *PIK3CA* gain-of-function mutations are selectively sensitive to inhibitors of different components of the PI3K pathway. In breast cancer, cell culture, and/or xenograft models identified *PIK3CA* mutations as determinant of response to PI3K inhibition (O'Brien et al., 2010; Sanchez et al., 2011), dual PI3K/mTOR inhibition (Serra et al., 2008; Brachmann et al., 2009; Lehmann et al., 2011; Sanchez et al., 2011), mTOR kinase inhibition (Weigelt et al., 2011), allosteric mTOR inhibition (Sanchez et al., 2011; Weigelt et al., 2011), and AKT inhibition (She et al., 2008; Meuillet et al., 2010; **Table 2**). In one report, however, which assessed seven estrogen receptor (ER)-positive breast cancer cell lines and their response to the allosteric mTOR inhibitor Rapamycin (Sirolimus), no correlation with *PIK3CA* mutation status but to some extent with a *PIK3CA* mutation associated gene signature was found (Loi et al., 2010). *In vitro* and xenograft models of breast cancer have also demonstrated that cells harboring amplification of the RTK *HER2* are dependent on PI3K pathway activation and sensitive to its inhibition through targeting of PI3K (O'Brien et al., 2010; Tanaka et al., 2011), dual PI3K/mTOR (Brachmann et al., 2009), AKT (She et al., 2008), and mTOR kinase (Weigelt et al., 2011). In fact, mTOR kinase inhibitors seem to lead to a more effective decrease of PI3K pathway signaling than allosteric mTOR inhibitors given that *HER2* amplified breast cancer cells *in vitro* have been found to be unresponsive to the rapamycin analog ("rapalog") Everolimus (RAD001; glossary box; Weigelt et al., 2011). Whereas *PIK3CA* mutations and *HER2* amplification have been identified in the majority of preclinical breast cancer studies as determinant of sensitivity to PI3K pathway inhibition downstream of RTKs, the correlation between PTEN deficiency and response is less clear. In some studies, results were inconclusive as

Table 2 | Genomic determinants of response to PI3K pathway inhibitors identified in preclinical cancer models.

Inhibitor (target)	Cancer type	Preclinical model	Genomic determinant of response	Reference
GDC-0941 (Class I PI3K)	Breast	Cell lines Cell line xenografts	*PIK3CA* mutation *HER2* amplification	O'Brien et al. (2010)
BEZ235 (PI3K/mTOR)	Breast	Cell lines Cell line xenografts	*PIK3CA* mutation	Serra et al. (2008)
BEZ235 (PI3K/mTOR)	Breast	Cell lines Cell line xenografts	*PIK3CA* mutation *HER2* amplification	Brachmann et al. (2009)
BEZ235 (PI3K/mTOR)	Breast	Cell lines	*PIK3CA* mutation (PTEN deficiency)	Lehmann et al. (2011)
BKM120 (Class I PI3K), BGT226 (PI3K/mTOR), Everolimus (mTOR)	Breast	Cell lines	*PIK3CA* mutation	Sanchez et al. (2011)
PP242 (mTOR kinase) Everolimus (mTOR)	Breast	Cell lines	*PIK3CA* mutation *HER2* amplification (only for PP242)	Weigelt et al. (2011)
Rapamycin (mTOR)	Breast	Cell lines	None (not *PIK3CA* mutations)	Loi et al. (2010)
AKTi-1/2 (AKT)	Breast	Cell lines Cell line xenografts	*PIK3CA* mutation *HER2* amplification	She et al. (2008)
Everolimus (mTOR)	Non-malignant breast	Cell lines (isogenic)	*PIK3CA* mutation (knock-in)	Di Nicolantonio et al. (2010)
Temsirolimus (mTOR)	Multiple myeloma	Cell lines	PTEN deficiency	Shi et al. (2002)
Everolimus (mTOR)	Glioblastoma multiforme	Cell lines Human tumor xenografts	None (not PTEN deficiency)	Yang et al. (2008)
BEZ235 (PI3K/mTOR)	Ovarian	Cell lines	*PIK3CA* mutation PTEN deficiency	Santiskulvong et al. (2011)
WAY-175, WAY-176 (Class I PI3K)	Various (breast, prostate, melanoma, lung, colon)	Cell lines	*PIK3CA* mutation	Yu et al. (2008)
PX866 (PI3K)	Various (non-small cell lung cancer, colon, breast, pancreatic, prostate, ovarian, multiple myeloma)	Cell line xenografts	*PIK3CA* mutation PTEN deficiency	Ihle et al. (2009)
CH5132799 (PI3K)	Various (breast, ovarian, prostate, endometrial)	Cell lines Cell line xenografts	*PIK3CA* mutation	Tanaka et al. (2011)
Temsirolimus (mTOR)	Various (glioblastoma, prostate)	Cell lines	PTEN deficiency	Neshat et al. (2001)
Everolimus (mTOR)	Various (prostate, glioblastoma, breast, ovarian, cervical)	Cell lines	*PIK3CA* mutation PTEN deficiency	Di Nicolantonio et al. (2010)
Rapamycin (mTOR)	Various (neuroendocrine, cervical, hepatocellular, melanoma, ovarian, colon, breast, renal cell, glioblastoma, breast)	Cell lines	*PIK3CA* mutation PTEN deficiency	Meric-Bernstam et al. (2012)
PHT-427 (AKT/PDPK1)	Various (pancreatic, prostate, ovarian, breast, lung)	Cell line xenografts	*PIK3CA* mutation	Meuillet et al. (2010)
25 PI3K pathway inhibitors (PI3K, PI3K/mTOR, AKT)	Various (lung, colorectal, gastric, breast, ovarian, brain, renal, melanoma, prostate)	Cell lines	None (p-AKT levels)	Dan et al. (2010)
A-443654 (AKT)	Various (bladder, blood, bone, breast, CNS, GI tract, kidney, lung, ovary, pancreas, skin, soft tissue, thyroid, upper aerodigestive, uterus)	Cell lines	*SMAD4* mutation	Garnett et al. (2012); (http://www.cancerrxgene.org/; Release 2, July 2012)
AKT inhibitor VIII (AKT)	Various (bladder, blood, bone, breast, CNS, GI tract, kidney, liver, lung, ovary, pancreas, prostate, skin, soft tissue, thyroid, upper aerodigestive, uterus)	Cell lines	*PIK3CA* mutation *ERBB2* mutation	Garnett et al. (2012); (http://www.cancerrxgene.org/; Release 2, July 2012)

(Continued)

Table 2 | Continued

Inhibitor (target)	Cancer type	Preclinical model	Genomic determinant of response	Reference
MK-2206 (AKT)	Various (bladder, blood, bone, breast, CNS, GI tract, kidney, liver, lung, ovary, pancreas, prostate, skin, soft tissue, thyroid, upper aerodigestive, uterus)	Cell lines	*PTEN* mutation	Garnett et al. (2012); (http://www.cancerrxgene.org/; Release 2, July 2012)
AZD6482 (p110β)	Various (bladder, blood, bone, breast, CNS, GI tract, kidney, liver, lung, ovary, pancreas, prostate, skin, soft tissue, thyroid, upper aerodigestive, uterus)	Cell lines	*PTEN* mutation *PIK3CA* mutation	Garnett et al. (2012); (http://www.cancerrxgene.org/; Release 2, July 2012)
BEZ235 (PI3K/mTOR)	Various (bladder, blood, bone, breast, CNS, GI tract, kidney, liver, lung, ovary, pancreas, prostate, skin, soft tissue, thyroid, upper aerodigestive, uterus)	Cell lines	*CDKN2A* mutation *NRAS* mutation	Garnett et al. (2012); (http://www.cancerrxgene.org/; Release 2, July 2012)
Temsirolimus (mTOR)	Various (bladder, blood, bone, breast, CNS, GI tract, kidney, liver, lung, ovary, pancreas, prostate, skin, soft tissue, thyroid, upper aerodigestive, uterus)	Cell lines	*PTEN* mutation	Garnett et al. (2012); (http://www.cancerrxgene.org/; Release 2, July 2012)
GDC-0941 (Class I PI3K), AZD8055 (mTOR kinase), Rapamycin (mTOR), JW-7-52-1 (mTOR)	Various (bladder, bone, breast, CNS, GI tract, kidney, liver, lung, ovary, pancreas, prostate, skin, soft tissue, thyroid, upper aerodigestive, uterus)	Cell lines	None (*TET2* mutations associated with AZD8055 response, however only 3/554 cell lines were *TET2* mutant)	Garnett et al. (2012); (http://www.cancerrxgene.org/; Release 2, July 2012)
CONFIRMATORY STUDIES USING ANIMAL MODELS				
BEZ235 (PI3K/mTOR)	Prostate and glioblastoma	Cell line xenografts	PTEN deficiency	Maira et al. (2008)
Rapamycin (mTOR)	Breast and pancreatic	Cell line xenografts	*PIK3CA* mutation	Meric-Bernstam et al. (2012)
WYE-354 (mTOR kinase)	Prostate and glioblastoma	Cell line xenografts	PTEN deficiency	Yu et al. (2009)
BEZ235 (PI3K/mTOR)	Lung	*PIK3CA* H1047R mouse model	*PIK3CA* H1047R mutation	Engelman et al. (2008)
Rapamycin (mTOR), API-2 (AKT)	Ovarian endometrioid adenocarcinoma	$Apc^{flox/flox}$; $Pten^{flox/flox}$ mouse model	PTEN deficiency	Wu et al. (2011)

CNS, central nervous system; GI, gastrointestinal.

only a subset of PTEN null breast cancer cell lines were sensitive to PI3K pathway inhibition (She et al., 2008; Lehmann et al., 2011; Sanchez et al., 2011), whilst others found PTEN deficient breast cancer cells to be preferentially resistant to treatment with PI3K (Tanaka et al., 2011), dual PI3K/mTOR (Brachmann et al., 2009), mTOR kinase, and allosteric mTOR inhibitors (Weigelt et al., 2011). These data are consistent with the notion that aberrations in the different components of the PI3K pathway are not necessarily equivalent in their biological impact and their potential to activate the signaling pathway (Stemke-Hale et al., 2008; Vasudevan et al., 2009; Dan et al., 2010). Moreover, these observations also suggest that sensitivity of PTEN deficient breast cancer cells to PI3K pathway inhibitors may be dependent on epistatic interactions between PI3K pathway genes and genes from other signaling pathways such as the MAPK pathway, as well as the release of negative feedback loops and the node targeted by pharmacologic inhibition (Efeyan and Sabatini, 2010; Zhang and Yu, 2010). Recent work in preclinical models has suggested that PTEN deficient cancers may depend on p110β rather than p110α signaling (Jia et al., 2008; Wee et al., 2008; Edgar et al., 2010; Ni et al., 2012), and a p110β isoform specific inhibitor (GSK2636771) is currently being tested in a clinical trial of PTEN deficient malignancies (NCT01458067). In fact, as in different disease contexts selective targeting of specific p110 isoforms may be more beneficial and less toxic than pan-PI3K inhibition (Jia et al., 2009; Vanhaesebroeck et al., 2010; Jamieson et al., 2011; Tzenaki et al., 2012), also p110α, p110γ, and p110δ specific inhibitors are being assessed in clinical trials (**Table 1**). The contribution of the p85 isoforms (glossary box) to PI3K inhibitor response is however not yet fully understood. There is evidence to suggest that different cancer types express different levels of p110 and p85 isoforms (Cortes et al., 2012; Tzenaki et al., 2012), which may lead to tumor type-specific combinations of catalytic and regulatory PI3K subunits. It remains to be determined whether certain PI3K inhibitors show preferential activity against specific

p110/p85 isoform combinations and whether distinct mutations in the regulatory subunits *PIK3R1* or *PIK3R2* have an impact on PI3K inhibitor response.

The general effect of *PIK3CA* gain-of-function mutations in the sensitization to PI3K pathway inhibitors has been confirmed in a mouse model with inducible expression of human oncogenic p110α (i.e., p110α H1047R), where treatment of the p110α H1047R driven lung adenocarcinomas with the dual PI3K/mTOR inhibitor BEZ235 led to marked tumor regression (Engelman et al., 2008). In xenografts derived from the breast cancer cell line MCF7 and the pancreatic carcinoid cell line BON, both harboring an activating *PIK3CA* mutation, treatment with the allosteric mTOR inhibitor Rapamycin (Sirolimus) was associated with a significant decrease in tumor volume (Meric-Bernstam et al., 2012). Moreover, using *PIK3CA* wild-type human breast immortalized epithelial cells (hTERT-HME1) or non-malignant MCF10A breast cells, knock-in of the E454K or H1047R *PIK3CA* mutant alleles sensitized non-transformed human breast cells to the rapalog Everolimus (Di Nicolantonio et al., 2010).

Also when focusing on an array of tumor types rather than on a single disease entity, *PIK3CA* mutant cell lines, or cell line derived xenografts were found to be selectively sensitive to PI3K pathway inhibition (**Table 2**). For example, analysis of xenografts derived from pancreatic, prostate, ovarian, NSCLC, and ovarian cancer cells revealed that those harboring *PIK3CA* mutations were among the most sensitive to the AKT inhibitor PHT-427 (Meuillet et al., 2010). This observation has been validated in a large panel of breast, ovarian, prostate, and endometrial cancer cells, given that those with *PIK3CA* mutations were found to be significantly more sensitive to the PI3K inhibitor CH5132799 *in vitro* than those without (Tanaka et al., 2011). Other studies assessing mixed tumor type cell line panels, however, have identified both activating *PIK3CA* mutations and PTEN loss of function as determinant of PI3K pathway inhibitor response. This was observed in a panel of breast, melanoma, lung, colon, prostate cancer cells treated *in vitro* with the PI3K inhibitors WAY-175 and WAY-176 (Yu et al., 2008), in a panel of human lung, colon, breast, pancreatic, ovarian, and multiple myeloma cell line derived xenografts treated with the PI3K inhibitor PX866 (Ihle et al., 2009), and in cell line panels of various tumor types treated *in vitro* with the allosteric mTOR inhibitors Everolimus (Di Nicolantonio et al., 2010) or Rapamycin (Meric-Bernstam et al., 2012). In one study, the evaluation of the *in vitro* efficacy of 25 PI3K pathway inhibitors in a panel of 39 human cancer cell lines did not identify any genetic determinant of sensitivity (Dan et al., 2010).

It is interesting to note that whilst loss of PTEN function has been shown to be a strong activator of the PI3K pathway as determined by levels of AKT phosphorylation (Stemke-Hale et al., 2008), only a few studies identified *PTEN* mutations/PTEN deficiency as a single genomic determinant of response to PI3K pathway inhibitors. Murine PTEN deficient ovarian endometrioid adenocarcinomas arising in $Apc^{flox/flox}$; $Pten^{flox/flox}$ mice have been shown to be sensitive to Rapamycin and the AKT inhibitor API-2 (Wu et al., 2011). Also in a panel of multiple myeloma (Shi et al., 2002), glioblastoma, and prostate cancer cell lines (Neshat et al., 2001), PTEN deficiency was reported to be associated with enhanced sensitivity to the allosteric mTOR inhibitor Temsirolimus (CCI-779). Consistent with this result, the PTEN null glioblastoma cell line U-87MG and the prostate cancer cell line PC3 were found to be sensitive to Rapamycin *in vitro* (Di Nicolantonio et al., 2010), and when grown as xenografts to the dual PI3K/mTOR inhibitor BEZ235 (Maira et al., 2008) and the ATP-competitive mTOR inhibitor WYE-354 (Yu et al., 2009). At variance with these findings, PTEN loss of function was shown to be a poor predictor of Everolimus response in a panel of 17 glioblastoma multiforme cell lines, and in human glioblastoma xenograft models (Yang et al., 2008).

The data discussed above on the genomic determinants of PI3K pathway inhibitor response identified *in vitro* are based on the analysis of up to 60 cancer cell lines, which were selected based on different criteria by independent investigators. Recently, two large-scale studies subjected hundreds of cancer cell lines derived from tumors stemming from different anatomical sites and tissue types to transcriptomic profiling, copy number profiling, and massively parallel sequencing. Owing to their unprecedented scale and approach employed, these studies unraveled several associations between genetic aberrations and response to specific targeted therapies (Barretina et al., 2012; Garnett et al., 2012). Garnett et al. (2012) tested up to 714 cell lines for their response to 138 anticancer agents including ten PI3K pathway inhibitors downstream of RTKs (http://www.cancerrxgene.org/; Release 2, July 2012), and observed that, in line with previous findings, cancer cells harboring mutations in *PIK3CA* and *PTEN* were sensitive to treatment with the AKT inhibitor VIII and MK-2206, respectively. Of note, also *ERBB2* mutations were associated with AKT inhibitor VIII response. Sensitivity to the AKT inhibitor A-443654 and the dual PI3K/mTOR inhibitor BEZ235, however, was not determined by PI3K pathway aberrations but by the presence of *SMAD4* and *CDKN2A* mutations, respectively (**Table 2**). In Garnett et al. (2012) mutations in *PTEN* were associated with response to the mTOR inhibitor Temsirolimus, and not only *PTEN* but also *PIK3CA* mutations predicted response to the PI3K isoform specific p110β inhibitor AZD6482 (http://www.cancerrxgene.org/; Release 2). On the other hand, contrary to previous reports, no mutations predictive of response to the PI3K inhibitor GDC-0941, the mTOR kinase inhibitor AZD8055, and the mTOR inhibitors Rapamycin and JW-7-52-1 were identified (http://www.cancerrxgene.org/; Release 2; Garnett et al., 2012; **Table 2**).

Mutation analysis has already become part of the diagnostic armamentarium for lung and colon cancers (Allegra et al., 2009; Keedy et al., 2011), and is also likely to be implemented in the management of other tumor types. In fact, the potential determinants of PI3K pathway inhibitor response identified in preclinical studies may provide a rationale for the guidance of predictive biomarkers to be assessed in early clinical trials. It should be noted here that in addition to genomic response predictors also non-genetic predictors of PI3K inhibitor response have been put forward, yet none of them has been fully validated. In breast cancer, a gene expression signature predictive of *in vitro* sensitivity to the PI3K inhibitor GDC-0941 (O'Brien et al., 2010), and a *PIK3CA* mutation associated gene signature (*PIK3CA*-GS) derived from exon 20 *PIK3CA* mutations able to predict *PIK3CA* mutation status in primary breast cancers and predictive of Rapamycin response *in vitro* have recently been described (Loi et al., 2010). In addition, several

groups found increased phosphorylated (p)-AKT baseline levels as a read-out for PI3K pathway activation to be associated with its therapeutic intervention (Noh et al., 2004; Yu et al., 2008; Dan et al., 2010; Meric-Bernstam et al., 2012). Despite the potential utility of these approaches, it should be mentioned that gene expression signatures and immunohistochemical assessment of phosphorylated proteins have proven challenging to implement in routine clinical practice (Pinhel et al., 2010; Weigelt et al., 2012).

Although predictive markers of sensitivity to PI3K pathway inhibitors, such as *PIK3CA* mutations, are of importance for treatment tailoring, markers predictive of resistance may be useful. In fact, tumors harboring a given therapeutic target not uncommonly display primary (i.e., *de novo*) resistance or develop resistance over time (van der Heijden and Bernards, 2010; Turner and Reis-Filho, 2012). In several studies discussed here assessing determinants of single agent PI3K pathway inhibitor response, *KRAS* mutations were found to be associated with resistance to these targeted agents (Engelman et al., 2008; Brachmann et al., 2009; Ihle et al., 2009; Dan et al., 2010; Meuillet et al., 2010; Garnett et al., 2012), as were mutations in *APC*, *BRAF*, or *MYCN* (http://www.cancerrxgene.org/; Garnett et al., 2012).

Finally, in addition to genetic alterations of components of the PI3K pathway germline polymorphisms may affect response of patients treated with targeted therapies. Ng et al. (2012) have recently identified a common intronic deletion polymorphism of the *BIM* gene that leads to the generation of an alternative spliced BIM isoform lacking the BH3 domain, which is required for tyrosine kinase inhibitor induced apoptosis. This polymorphism was shown to confer intrinsic resistance to RTK inhibitors in chronic myeloid leukemia and *EGFR* mutated NSCLC cell lines (Ng et al., 2012). It is plausible that this and other germline polymorphisms may results in resistance to agents targeting the PI3K pathway.

Taken together, preclinical studies focusing on breast cancer only have repeatedly identified *PIK3CA* mutations and *HER2* amplifications as predictors of sensitivity to PI3K pathway inhibitors. In other tumor types, however, the genotype-drug response associations are less defined and *PIK3CA* mutations, PTEN loss of function or both, or *CDKN2A* mutations have been reported as determinants of response. Furthermore, the *in vitro* and animal model studies revealed that in cancer cells other than breast cancer, where MAPK pathway mutations are rare (COSMIC), *KRAS* mutations may confer resistance to single agent PI3K pathway inhibitor treatment, as do mutations in *BRAF*, *APC* and *MYCN*.

GENOMIC DETERMINANTS OF PI3K PATHWAY INHIBITOR RESPONSE IN CLINICAL TRIALS

Rapamycin analogs ("rapalogs"; glossary box) were the first PI3K pathway inhibitors to be tested in clinical trials for the treatment of cancer, and Everolimus and Temsirolimus have been approved by the US Food and Drug Administration (FDA) for the treatment of advanced renal cell carcinoma (ARCC), and Everolimus has also been approved for the treatment of progressive neuroendocrine tumors of pancreatic origin and non-malignant kidney and brain tumors (**Table 1**).

The determinants of mTOR inhibition in renal cell carcinomas may differ from those of other solid malignancies. In fact, clear cell renal cell carcinomas rarely harbor mutations in PI3K pathway components (COSMIC), however commonly show loss of function of the tumor suppressor genes PTEN (Brenner et al., 2002; Velickovic et al., 2002) or von Hippel Lindau (VHL), a critical regulator of the hypoxic response (Kim and Kaelin, 2004; Linehan et al., 2010). Clear cell renal cell cancer has been suggested to be a cell metabolism, angiogenesis-dependent and hypoxia-driven disease, and its response to mTOR inhibition thought to stem from its impact on proliferation and cell survival but also from the fact that the hypoxia-inducible-factor 1-α (HIF1-α) is under translational control of the mTOR complex 1 (mTORC1; glossary box; Thomas et al., 2006; Linehan et al., 2010). Exploratory subgroup analysis of the 209 patients from the Temsirolimus single agent arm of the phase III global ARCC trial (Hudes et al., 2007) investigated PTEN and HIF1-α protein expression levels by immunohistochemistry (IHC) on formalin fixed paraffin embedded nephrectomy or core biopsy derived tissues. Importantly, baseline PTEN or HIF1-α levels were shown not to be associated with single agent Temsirolimus response (Figlin et al., 2009; **Table 3**). Furthermore, in a retrospective subgroup analysis from a phase II clinical trial of ARCC (Atkins et al., 2004) including 20 patients (Cho et al., 2007), carbonic anhydrase IX (CA9), p-AKT, and PTEN protein expression levels using IHC or *VHL* mutation status were shown not to be significantly associated with single agent Temsirolimus response. There was, however, a significant positive association between higher p-rpS6 expression, a downstream effector of mTORC1 (**Figure 1**), and clinical Temsirolimus response (Cho et al., 2007). It should be noted that the analysis above was performed in a limited number of patients and their statistical power to reveal the associations should be taken into account.

The vast majority of completed to date trials testing PI3K pathway inhibitors in tumor types other than renal cell carcinoma also focused on rapalogs, but only few studies assessed potential genomic predictors (**Table 3**). Based on the rationale that *PIK3CA* mutations may predict response to PI3K pathway inhibitors, breast, cervical, endometrial, and ovarian cancers were sequenced for the presence of activating *PIK3CA* mutations and treated with different allosteric mTOR inhibitors (i.e., rapalogs) or the PI3K inhibitor PX866 either as single agent or combination in a prospective phase I clinical trial. A partial response was observed in 30% of the 23 patients with tumors harboring a *PIK3CA* mutation in contrast to 10% of 70 patients whose tumors were *PIK3CA* wild-type (Janku et al., 2012), consistent with the preclinical observations. Interestingly, whilst in preclinical models mutations in *KRAS* have been found to confer resistance to PI3K pathway inhibition, as discussed above, in this trial 2/7 ovarian cancer patients with coexisting *PIK3CA* and *KRAS* or *BRAF* mutations responded to the anti-PI3K pathway treatment (Janku et al., 2012). This finding may be tumor type-specific, given that the same group had previously described that colorectal cancers harboring simultaneous *PIK3CA* and *KRAS* mutations were resistant to PI3K pathway inhibitor treatment (Janku et al., 2011). A similar, but not statistically significant, trend was observed in a retrospective subgroup analysis of 43 patients with different tumor types but most frequently colorectal cancer from phase I/II clinical study of single agent Everolimus (Tabernero et al., 2008; Di Nicolantonio et al., 2010). Patients whose tumors harbored *PIK3CA* mutations or PTEN loss

Table 3 | Genomic determinants of response to PI3K pathway inhibitors identified in clinical trials.

Inhibitor name (target)	Cancer type	Clinical trial	Patients (n)	Genomic determinant of sensitivity	Non-genomic determinant of sensitivity	Genomic determinant of resistance	Reference
Temsirolimus (mTOR), single agent	Renal cell carcinoma	NCT00065468 (retrospective subgroup analysis; phase III)	209 (Temsirolimus arm; ∼60% assessed)	NA	None (PTEN or HIF1-α protein expression assessed)	NA	Figlin et al. (2009)
Temsirolimus (mTOR), single agent	Renal cell carcinoma	ND (retrospective subgroup analysis; phase II)	20	None (VHL mutation assessed)	p-rpS6 (Ser235)	NA	Cho et al. (2007)
Temsirolimus (mTOR), single agent, or combined; Rapamycin (mTOR), combined; PX866 (PI3K), single agent	Breast, cervical, endometrial, ovarian cancer (colorectal, head and neck)	NCT00761644, NCT00877773, NCT01054313, NCT00610493, NCT00726583 (phases I/II)	140 (217)	*PIK3CA* mutation	NA	Coexisting *KRAS* mutation (tissue-specific)	Janku et al. (2011, 2012)
Everolimus (mTOR), single agent	Colorectal, breast, melanoma, pancreas, HNSCC	ND (retrospective subgroup analysis; phase I/II)	43	*PIK3CA* mutation PTEN loss of function	NA	Coexisting *KRAS* mutation	Di Nicolantonio et al. (2010)
Temsirolimus (mTOR), combined	Ovarian, uterine, cervix, breast cancer	NCT00761644 (phase I)	74	*PIK3CA* mutation PTEN loss of function	NA	NA	Moroney et al. (2011)
Everolimus (mTOR), combined	Neuroendocrine carcinoma	NCT00113360 (retrospective subgroup analysis; phase II)	60 (17 assessed)	NA	p-AKT (Thr308; PSF)	NA	Meric-Bernstam et al. (2012)
Everolimus (mTOR), single agent	NSCLC	NCT00124280 (phase I)	58 (40 assessed)	NA	p-AKT (Ser473, Thr308; PFS)	NA	Soria et al. (2009)
Everolimus (mTOR), single agent	SCLC	NCT00374140 (phase II)	40 (22 assessed)	NA	S6K	NA	Tarhini et al. (2010)
Temsirolimus (mTOR), single agent	Glioblastoma multiforme	NCT00016328 (phase II)	56 (43 assessed)	NA	p-S6K (Thr421/Ser424)	NA	Galanis et al. (2005)
Deforolimus (Ridaforolimus; mTOR), single agent, and combination	Sarcoma	NCT00288431 NCT00093080 (phase I/II; retrospective subgroup analysis)	20	NA	p-rpS6 (Ser235/236)	NA	Iwenofu et al. (2008)
Temsirolimus (mTOR), single agent	Neuroendocrine carcinoma	NCT00093782 (phase II)	37 (35 assessed)	NA	p-mTOR (Ser2448)	NA	Duran et al. (2006)

(Continued)

Table 3 | Continued

Inhibitor name (target)	Cancer type	Clinical trial	Patients (n)	Genomic determinant of sensitivity	Non-genomic determinant of sensitivity	Genomic determinant of resistance	Reference
Everolimus (mTOR), single agent	Breast cancer	NCT00255788 (phase II)	49 (47 assessed)	NA	None (PTEN, p-AKT, CA9, ER, PR, HER2 assessed)	NA	Ellard et al. (2009)
Ridaforolimus (mTOR), single agent	Bone and soft tissue sarcoma	NCT00093080 (phase II)	212 (~80 assessed)	NA	None (p27Kip1, IGF-1R, PTEN, p-S6K, 4E-PB1, eIF4E, p-AKT, FKBP12)	NA	Chawla et al. (2012)
Temsirolimus (mTOR), single agent	Epithelial ovarian and peritoneal malignancies	NCT00429793 (phase II)	54 (51 assessed)	NA	None (p-AKT, p-mTOR, p-S6K, p-4E-BP1, cyclin D1)	NA	Behbakht et al. (2011)

CA9, carbonic anhydrase IX; HNSCC, head and neck squamous cell carcinoma; NA, not assessed; ND, not defined; NSCLC, non-small cell lung cancer; PFS, progression-free survival; SCLC, small cell lung cancer.

of function were more likely to benefit from Everolimus, except in presence of coexistent *KRAS*/*BRAF* mutations (Di Nicolantonio et al., 2010). A high percentage of responders with PI3K pathway aberrations as determined by *PIK3CA* mutations or PTEN loss of function was also reported in a phase I trial of liposomal Doxorubicin, Bevacizumab, and Temsirolimus (Moroney et al., 2011). Taken together, these results suggest *PI3KCA* gain-of-function mutations may predict sensitivity to PI3K pathway inhibitors, whereas *KRAS* and *BRAF* mutations may lead to resistance in some tumor types such as colorectal cancer. Importantly, however, the data available demonstrate that *PIK3CA* activating mutations are neither required nor sufficient for a tumor to be sensitive to PI3K pathway inhibitors, and that a substantial proportion of cases with *PIK3CA* activating mutations may be *de novo* resistant to these agents.

Not only genomic predictors but also PI3K pathway activation state as determined by expression levels of markers upstream of mTORC1, such as p-AKT, or downstream of mTORC1, such as p-S6K (**Figure 1**), have been shown to correlate with sensitivity to allosteric mTOR inhibitors in breast cancer cell lines *in vitro* (Noh et al., 2004; Meric-Bernstam et al., 2012). In fact, several clinical trials testing rapalogs evaluated PI3K signaling biomarkers on baseline tumor tissue by IHC rather than performing sequencing analysis. In a phase II study, high p-AKT levels on baseline and on-treatment fine needle aspirations of tumors from patients with neuroendocrine carcinoma ($n = 17$) assessed by reverse phase protein arrays correlated with longer progression-free survival (PFS; Meric-Bernstam et al., 2012). Moreover, in NSCLC patients treated with Everolimus ($n = 40$), p-AKT levels at baseline determined by IHC were reported to be independent predictors of PFS (Soria et al., 2009; **Table 3**). It should be noted, however, that the authors emphasized that tissue fixation had large effects on immunoreactivity when assessing phosphorylated proteins, which may compound the implementation of this IHC predictive test in clinical practice.

In a phase II trial, p-S6K levels assessed by IHC in baseline glioblastoma multiforme samples were associated with single agent Temsirolimus response ($n = 44$; Galanis et al., 2005). Also in a small retrospective subgroup analysis of two phase I/II clinical trials p-rpS6, downstream of p-S6K, was correlated with early response of sarcomas to the rapalog Deforolimus (i.e., Ridaforolimus) alone or in combination with doxorubicin ($n = 20$; Iwenofu et al., 2008). Not only expression levels of activated (i.e., phosphorylated) S6K/rpS6 have been found to correlate with response to allosteric mTOR inhibitors, but in phase II study, total S6K expression in baseline SCLC tumor tissue defined by IHC was reported as a potential predictive biomarker for the therapeutic benefit of Everolimus ($n = 22$; Tarhini et al., 2010). In addition, higher baseline levels of p-mTOR itself assessed by IHC predicted for a better response to Temsirolimus in patients with neuroendocrine carcinoma in a phase II study ($n = 35$; Duran et al., 2006).

Other, similarly powered phase II trials however did not identify any correlates between potential biomarkers assessed in archival tumor material using IHC and treatment response (**Table 3**). In metastatic breast cancers, no association between p-AKT, PTEN, CA9, ER, progesterone receptor or HER2 expression, and response

to Everolimus was found (Ellard et al., 2009). In bone and soft tissue sarcomas, an extended subgroup analysis ($n \approx 80$; Chawla et al., 2012) did not confirm the previously published analysis on 20 patients, which identified p-rpS6 levels as Ridaforolimus response predictors (Iwenofu et al., 2008). In fact, neither the potential biomarkers upstream of mTORC1, including PTEN, p-AKT, FKBP21, or IGF-1R, nor downstream of mTORC1, p-S6K, 4E-BP1, eIF4E, or p27kip1, were predictive of clinical benefit response to Ridaforolimus (Chawla et al., 2012). Furthermore, p-AKT, p-mTOR, p-4E-BP1, and cyclin D1 expression levels in epithelial ovarian and peritoneal tumors were shown not to be associated with partial/complete tumor responses to Temsirolimus, however cyclin D1 expression seemed to correlate with PFS $\geq$6 months (Behbakht et al., 2011).

The completed phase I/II clinical trials to date are to some extent consistent with the preclinical observations in that tumors harboring *PIK3CA* mutations may be more likely to respond to PI3K pathway inhibitors. It is important to note, however, that not all patients with tumors harboring *PIK3CA* mutations are sensitive to PI3K pathway inhibitor treatment, and, on the other hand, that also subsets of patients with wild-type *PIK3CA*/*PTEN* cancers are responsive. The results from studies analyzing the activation state of PI3K pathway components by IHC are variable and no consistent determinant of response has been identified to date.

DEVELOPMENT OF MOLECULAR TOOLS FOR THE STRATIFICATION OF CANCER PATIENTS

For the development of molecular markers for patient stratification in clinical trials testing the efficacy of PI3K pathway inhibitors, it is crucial to take into account the observations that in some tumor types, either *PIK3CA* activating mutations or PTEN loss of function are predictors of sensitivity, whereas in other tumor types, both predict sensitivity to these agents (**Table 2**). These data imply that the mutational repertoire and the epistatic interactions between different components of the PI3K pathway may be distinct in different tumor types, that genetic lesions in different components of the pathway may not have the same functional effects in different tumor types, and that a genetic determinant identified in one cancer type may not necessarily be applicable to another. This is perhaps best exemplified by $BRAF^{V600E}$ mutations, which are predictive of response to Vemurafenib in melanoma, however colorectal cancer patients harboring oncogenic $BRAF^{V600E}$ mutations derive limited if any benefit from this drug due to increased EGFR expression (Prahallad et al., 2012). Likewise, the clinical trials discussed above provide evidence to suggest that ovarian cancers with coexisting *PIK3CA* and MAPK pathway mutations may be sensitive to PI3K pathway inhibition, whereas colorectal cancers harboring the same repertoire of mutations affecting these genes may be resistant (Di Nicolantonio et al., 2010; Janku et al., 2011, 2012).

Results from preclinical studies performed have further suggested that cancer cells harboring *PIK3CA* mutations might be among the most sensitive to single agent PI3K pathway inhibitors. These data were in part confirmed in the three clinical trials assessing tumor *PIK3CA* mutational status (Di Nicolantonio et al., 2010; Janku et al., 2011, 2012; Moroney et al., 2011). The predictive value of the PTEN status is however less clear, as in some clinical trials an association between PTEN deficiency and PI3K pathway inhibitor response was found (Di Nicolantonio et al., 2010; Moroney et al., 2011) but not in others (Ellard et al., 2009; Figlin et al., 2009; Chawla et al., 2012). A similar picture is seen when expression levels of phosphorylated proteins of the PI3K pathway are used as a read-out of its activation state and determinant of response (**Table 3**). IHC of phosphorylated proteins has proven challenging (Soria et al., 2009; Pinhel et al., 2010) and also PTEN staining is not routinely performed. Recent reports focused on the reproducibility of PTEN staining protocols and scoring (Sakr et al., 2010; Garg et al., 2012), however guidelines for accurate PTEN testing and its utility as predictive marker have yet to be established.

Early clinical trials often analyze archival tissue of the primary tumor for the presence of specific mutations and the response of the metastatic lesions are correlated with the mutational status. Recent analyses of paired primary tumors and metastases have revealed that there is a high level of discordance in PTEN expression level and *PIK3CA* mutation status, which may influence patient selection and response to PI3K targeted therapies (Dupont Jensen et al., 2011; Gonzalez-Angulo et al., 2011).

Despite the interest in the development of biomarkers for patient selection in clinical trials testing PI3K pathway inhibitors, none of the biomarkers tested so far is supported by level I evidence. Importantly, however, one of the most exciting results of allosteric mTOR inhibitors in the context of a clinical study was the BOLERO-2 trial, where patients with ER-positive advanced breast cancers resistant to aromatase inhibitors were randomized to receive Exemestane (a non-steroidal aromatase inhibitor) plus Everolimus or Exemestane plus Placebo (Baselga et al., 2012). The rationale for this stemmed from preclinical observations that resistance to endocrine therapy in breast cancer is associated with activation of the PI3K pathway (Miller et al., 2011). Despite the lack of a patient stratification biomarker, this trial demonstrated that addition of Everolimus to Exemestane increased the median PFS from 4.1 to 10.6 months (Baselga et al., 2012). Although a substantial proportion of patients included in this trial may harbor *PIK3CA* activating mutations, given that they are more likely to occur in ER-positive postmenopausal patients (Kalinsky et al., 2009), other mechanisms resulting in PI3K pathway activation are likely to play a role in resistance to endocrine therapy. The material from this trial will constitute a unique resource to determine the genomic and epigenomic determinants of sensitivity to concurrent mTOR inhibition and endocrine treatment in breast cancer.

FUTURE PERSPECTIVES AND CHALLENGES

Despite the critical role of the PI3K pathway in cancer, the introduction of single-agent PI3K pathway inhibitors into the clinic may be challenging. In fact, of all PI3K pathway inhibitors discussed here, one of the most exciting targeted agents is the p110δ inhibitor CAL-101, which has shown remarkable clinical activity in certain hematological diseases including chronic lymphocytic leukemia. Inhibition of p110δ is though to target both the malignant B cells and the tumor microenvironment of chronic lymphoid leukemia (Fruman and Rommel, 2011). The clinical trials performed thus far using allosteric mTOR inhibitors as single agents have seen some stable diseases and partial responses, however by no means are these responses as dramatic as for example for Vemurafenib in *BRAF* mutant melanoma (Flaherty et al., 2010).

Based in the preclinical data, kinase inhibitors seem to target the PI3K pathway more robustly and, in contrast to allosteric mTOR inhibitors, also promote apoptotic effects *in vitro* and *in vivo* (Brachmann et al., 2009; O'Brien et al., 2010; Weigelt et al., 2011). Several feedback loops upon PI3K/AKT/mTOR inhibition have been described, which amongst others lead to activation of the MAPK signaling pathway or re-activation of the PI3K pathway (reviewed in Carracedo and Pandolfi, 2008; Efeyan and Sabatini, 2010; Chandarlapaty, 2012; Laplante and Sabatini, 2012). These feedbacks may play a role in the modest responses observed thus far using single agent rapalogs, and for the optimal activity of PI3K pathway inhibitors, co-administration with other agents may be required.

Numerous clinical trials are currently testing the safety and efficacy of combination PI3K pathway and MEK inhibitors in advanced solid tumors to target both the driver and potential "escape" pathways. As discussed above, such combinatorial approach has been successfully performed in breast cancer, where the combination of an aromatase inhibitor with Everolimus led to substantial improvement of PFS (Baselga et al., 2012). For the identification of optimal combinations of PI3K pathway inhibitors with other agents, *Drosophila* models may provide an effective tool as these have been successfully employed for the identification of agents with optimized pharmacological profiles (Das and Cagan, 2010; Dar et al., 2012). For combinatorial treatments it will be crucial to understand the activation of negative feedback loops in the PI3K pathway and the cross-talk with other pathway upon inhibition of its different components, and whether the feedback activation is dependent on specific epistatic interactions and distinct in tumors from different anatomical sites.

Drug sensitivity and resistance are likely to constitute convergent phenotypes, meaning that they may be driven by distinct genetic aberrations in the same tumor type (Gerlinger and Swanton, 2010; Turner and Reis-Filho, 2012; Weigelt et al., 2012; Yap et al., 2012). It has become apparent from *in vitro* studies that there are significant correlations between specific mutations and treatment response, however the negative predictive value of these mutations is often poor and not all sensitive cancers are identified by single mutations/single gene panels. For example, O'Brien et al. (2010) showed that in their cell line panel tested, *PIK3CA* mutations and *HER2* amplification showed excellent specificity (100 and 95%, respectively) and a high positive predictive value, but relatively low sensitivity (∼30%) and a poor negative predictive value as single markers in predicting drug responsiveness in the cell line panel analyzed. Additional biomarkers will therefore be required to identify all patients likely to respond to PI3K pathway inhibitors. To date, the majority of studies have focused on the analysis of *PIK3CA* mutations or PTEN deficiency as potential determinants of PI3K pathway inhibitor response. However, also activating mutations in other components of the pathway, such as *PIK3R1* (Jaiswal et al., 2009; Urick et al., 2011) or *mTOR* (Sato et al., 2010; Hardt et al., 2011), or loss of function of TSC1/2 (COSMIC; El-Hashemite et al., 2003; Sjodahl et al., 2011) or INPP4B (Gewinner et al., 2009; Fedele et al., 2010) may play a role in PI3K pathway inhibitor response. Furthermore, and as mentioned above, the remarkable single agent activity of the PI3K isoform specific p110δ inhibitor CAL-101 in chronic lymphocytic leukemia, a disease in which PI3K pathway aberrations are relatively rare, emphasizes that some targeted agents may be effective *in vivo* due to targeting of tumor microenvironment interactions (Fruman and Rommel, 2011), which are unlikely to be uncovered using conventional *in vitro* cell culture models or by the genomic characterization of tumor cells only.

With the advent of massively parallel sequencing technologies, several studies have documented intra-tumor genetic heterogeneity in solid cancers (reviewed in Turner and Reis-Filho, 2012; Yap et al., 2012), and revealed that certain mutations, including *PIK3CA* or *PTEN* mutations, may be only prevalent in a subset of tumor cells in a given cancer (Gerlinger et al., 2012; Shah et al., 2012). This has not only consequences for cancer drug resistance and the clinical utility of single agent targeted therapy, but also questions whether potential biomarkers assessed in a single biopsy will be representative of the entire tumor.

Given the crucial role of the PI3K pathway in cancer, inhibitors of its components are expected to be effective in subsets of many different cancer types. Preclinical models have proven useful in the identification of potential predictive biomarkers, however tissue collection and assessment of biomarkers even in early clinical trials are crucial, as is the development of robust and accurate companion diagnostics. With the number of ongoing clinical trials currently testing a wide gamut of PI3K pathway inhibitors, our community should expect a wealth of data, which will help improve therapeutic strategies for cancer patients.

GLOSSARY

PHOSPHOINOSITIDE 3-KINASE CLASSES

According to their structures and substrate specificities, PI3Ks are divided into three classes, and class I PI3Ks are directly activated by cell surface receptors (Liu et al., 2009). Class IA PI3Ks are heterodimeric lipid kinases composed of a p110 catalytic subunit (isoforms p110α, p110β, and p110δ, encoded by *PIK3CA*, *PIK3CB*, and *PIK3CD*, respectively), and a regulatory subunit (p85α and its splice variants p55α and p50α), p85β, and p55γ, encoded by *PIK3R1*, *PIK3R2*, and *PIK3R3*, respectively); the class IB PI3K is composed of the p110γ catalytic subunit, encoded by *PIK3CG*, and the regulatory subunit p101, p84/p87 (Liu et al., 2009; Vanhaesebroeck et al., 2010).

RAPAMYCIN AND RAPAMYCIN ANALOGS ("RAPALOGS")

Mechanistic target of rapamycin is a serine/threonine kinase that interacts with several proteins to form two distinct signaling complexes called mTORC1 and mTORC2 (Laplante and Sabatini, 2012). Rapamycin and rapamycin analogs ("rapalogs") bind the FK506-binding protein (FKBP12) and together target preferentially the mTORC1 by an allosteric mechanism, however prolonged treatment may also inhibit mTORC2 and disrupt its main substrate AKT, possibly in a tissue-specific manner (Sarbassov et al., 2006; Lamming et al., 2012).

ACKNOWLEDGMENTS

We apologize to those authors whose work we could not cite directly due to space constraints. We would like to thank J. S. Reis-Filho (The Breakthrough Breast Cancer Centre, London) for advice and critical reading of the manuscript. Britta Weigelt is funded by a Cancer Research UK postdoctoral fellowship.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 21 June 2012; accepted: 14 August 2012; published online: 31 August 2012.

*Citation: Weigelt B and Downward J (2012) Genomic determinants of PI3K pathway inhibitor response in cancer. Front. Oncol. **2**:109. doi: 10.3389/fonc.2012.00109*

This article was submitted to Frontiers in Molecular and Cellular Oncology, a specialty of Frontiers in Oncology.

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frontiers in ONCOLOGY

REVIEW ARTICLE
published: 16 October 2012
doi: 10.3389/fonc.2012.00145

Abrogating endocrine resistance by targeting ERα and PI3K in breast cancer

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Antiestrogen therapies targeting estrogen receptor α (ER) signaling are a mainstay for patients with ER+ breast cancer. While many cancers exhibit resistance to antiestrogen therapies, a large body of clinical and experimental evidence indicates that hyperactivation of the phosphatidylinositol 3-kinase (PI3K) pathway promotes antiestrogen resistance. In addition, continued ligand-independent ER signaling in the setting of estrogen deprivation may contribute to resistance to endocrine therapy. PI3K activates several proteins which promote cell cycle progression and survival. In ER+ breast cancer cells, PI3K promotes ligand-dependent and -independent ER transcriptional activity. Models of antiestrogen-resistant breast cancer often remain sensitive to estrogen stimulation and PI3K inhibition, suggesting that clinical trials with combinations of drugs targeting both the PI3K and ER pathways are warranted. Herein, we review recent findings on the roles of PI3K and ER in antiestrogen resistance, and clinical trials testing drug combinations which target both pathways. We also discuss the need for clinical investigation of ER downregulators in combination with PI3K inhibitors.

Keywords: PI3K, breast cancer, antiestrogen, aromatase, fulvestrant, tamoxifen, estrogen receptor

INTRODUCTION

At least 75% of breast cancers express estrogen receptor α (ER) and/or progesterone receptor (PR), which are tumor biomarkers of estrogen dependence. Antiestrogen treatments for patients with ER+ or PR+ breast cancer inhibit ER by antagonizing estrogen ligand binding to ER (tamoxifen and other selective estrogen receptor modulators, SERMs), inhibiting dimerization and downregulating ER (fulvestrant and other selective estrogen receptor downregulators, SERDs), or blocking estrogen production (aromatase inhibitors, AIs; letrozole, anastrozole, exemestane). While such endocrine therapies have changed the natural history of ER+ breast cancer, many tumors exhibit *de novo* or acquired drug resistance (**Table 1**). The only clinically validated mechanism of resistance to endocrine therapy is overexpression or amplification of the *ERBB2* (HER2) protooncogene (Arpino et al., 2004; De Laurentiis et al., 2005; Ellis et al., 2006). However, only 10% of ER+ breast cancers exhibit HER2 overexpression, prompting the need for discovery of other mechanisms of antiestrogen resistance.

A large body of experimental and clinical evidence suggests that hyperactivation of the phosphatidylinositol 3-kinase (PI3K) pathway, the most frequently mutated pathway in breast cancer, promotes antiestrogen resistance. PI3K is commonly activated by growth factor receptor tyrosine kinases and G-protein-coupled receptors in breast cancer cells. The signaling cascades triggered by PI3K, including PDK1, AKT, and SGK among others, promote cell growth and survival. For detailed information, we refer the reader to a recently published, comprehensive review of this material (Miller et al., 2011a). Herein, we focus on updated findings, clinical testing of drug combinations targeting the ER and PI3K pathways, and the need to clinically address the potential for continued ER signaling in patients treated with endocrine therapies.

RATIONALE FOR COMBINED TARGETING OF THE ER AND PI3K PATHWAYS

We and others identified a requirement for PI3K in the estrogen-independent growth of long-term estrogen-deprived (LTED) ER+ breast cancer cells, which mirror clinical resistance to AIs (Sabnis et al., 2007; Crowder et al., 2009; Miller et al., 2010). Proteomic profiling revealed amplification of PI3K signaling via the mTOR substrates p70S6 kinase and p85S6 kinase, and the PI3K effector AKT in ER+ human breast cancer cells adapted to hormone deprivation. Treatment with the ATP-competitive PI3K/mTOR dual inhibitor BEZ235 (Maira et al., 2008) completely suppressed the emergence of hormone-independent ER+ cells and induced apoptosis in cell lines harboring activating mutations in *PIK3CA* (gene that encodes the p110α subunit of PI3K) or PTEN loss (PTEN antagonizes PI3K signaling). In contrast, the TORC1 inhibitor everolimus (Schuler et al., 1997) had only a partial effect (Miller et al., 2010; Sanchez et al., 2011). This partial effect may be attributable to feedback activation of PI3K/AKT upon inhibition of TORC1 (O'Reilly et al., 2006; Carracedo et al., 2008; Miller et al., 2009), suggesting that direct inhibitors of PI3K may be more effective than rapalogs in this setting.

In a siRNA screen against 779 kinases, we implicated insulin receptor (InsR) in the hormone-independent growth of MCF-7/LTED cells. InsR and its homolog IGF-1R dimerize and, upon ligand binding, potently activate PI3K. IGF-1R has also been

Table 1 | Frequencies of breast cancer recurrence and resistance to anti-estrogen therapies in patients with ER+ breast cancer.

Population	Treatment	Effect	Trial/reference
		Follow-up: 5 years 10 years	
Early-stage	Adjuvant anastrozole × 5 years	Distant recurrence: 9.8% 19.7%	ATAC (Cuzick et al., 2010)
Post-menopausal	Adjuvant tamoxifen × 5 years	Distant recurrence: 12.5% 24%	
		Follow-up: 5 years 8 years	
Early-stage	Adjuvant letrozole × 5 years	Recurrence: 14.5% 23.6%	BIG 1-98 (Regan et al., 2011)
Post-menopausal	Adjuvant tamoxifen × 5 years	Recurrence: 18% 28%	
Advanced			
Post-menopausal	Anastrozole	No clinical benefit: 33%	FIRST (Robertson et al., 2009a)
No prior Tx	Fulvestrant (high-dose regimen)	No clinical benefit: 27.5%	
Advanced			
Post-menopausal	Exemestane	No clinical benefit: 68.5%	EFECT (Chia et al., 2008)
Progressed on AI	Fulvestrant (loading-dose regimen)	No clinical benefit: 67.8%	
		Median follow-up: 5.3 years	
Disease-free following	Letrozole	With disease: 2%	MA.17
5 years of adjuvant tamoxifen	Placebo	With disease: 4.9%	(Goss et al., 2008)
		Median follow-up: 2.5 years	
Disease-free following	Exemestane	With disease: 9%	NSABP B-33
5 years of adjuvant tamoxifen	Placebo	With disease: 11%	(Mamounas et al., 2008)

shown to confer antiestrogen resistance in MCF-7 cells (Zhang et al., 2011). Treatment with the ATP-competitive IGF-1R/InsR inhibitor OSI-906 suppressed PI3K activation and hormone-independent ER+ cell growth (Fox et al., 2011). Network mapping of the 42 kinases individually implicated in MCF-7/LTED cell growth in this screen revealed that PI3K is a central hub in these signaling pathways (**Figure 1**). Interestingly, a recent study showed that in ER+ breast cancer cells treated with BEZ235 or with PI3K siRNA, exogenous 17β-estradiol rescued the cells from drug- and siRNA-induced apoptosis (Crowder et al., 2009; Sanchez et al., 2011). This suggests that in ER+ cancers treated with PI3K inhibitors, estrogen suppression should be maintained and, therefore, combined inhibition of both PI3K and ER may be more effective than single-agent therapies.

Clinical evidence further indicates that PI3K pathway activation is associated with antiestrogen resistance. Patients bearing primary ER+ breast tumors which exhibit a protein expression/phosphorylation signature of PI3K activation, as determined using reverse-phase protein arrays (RPPA), have a shorter recurrence-free survival (Miller et al., 2010). RPPA analysis of ER+ primary breast tumors obtained from patients following 2–3 weeks of treatment with the AI letrozole showed that a protein signature of insulin signaling was associated with high post-AI tumor cell proliferation (Fox et al., 2011). Overexpression of HER2 or FGFR1, or loss of INPP4B, molecular lesions which activate the PI3K pathway, also confer antiestrogen resistance in patients with ER+ breast cancer (Arpino et al., 2004; De Laurentiis et al., 2005; Ellis et al., 2006; Gewinner et al., 2009; Turner et al., 2010). Also noteworthy is the inverse correlation between levels of PI3K activation and ER protein in human tumors. This ER/PI3K balance can be shifted using PI3K and ER inhibitors in preclinical models (**Figure 2**; Creighton et al., 2010; Miller et al., 2010), suggesting that cells may defer to the other pathway when one is inhibited.

Crosstalk between the PI3K and ER pathways has also been suggested as a mechanism of endocrine resistance (Musgrove and Sutherland, 2009). PI3K activation was shown to induce ER phosphorylation at the putative AKT/p70S6K site Ser_{167} and estrogen-independent transcriptional activity (Campbell et al., 2001; Yamnik et al., 2009). However, treatment of such cells in hormone-depleted conditions with everolimus or the pan-PI3K inhibitor BKM120 (Maira et al., 2012) did not decrease ER phosphorylation at Ser_{167}, ER-DNA binding, or ER transcriptional reporter activity (Miller et al., 2011b). These data collectively suggest that PI3K effectors do not modulate ER in the absence of estrogens. Analysis of the effects of BKM120 and fulvestrant on hormone-independent cell growth showed synergy in 6/8 ER+ lines. In mice bearing ER+ breast cancer xenografts, single-agent treatment with BKM120 or fulvestrant slowed tumor growth, while the combination induced tumor regression. Similarly, treatment with the ATP-competitive IGF-1R/InsR dual inhibitor OSI-906, which blocks downstream activation of PI3K in MCF-7 cells, slowed tumor growth and induced regression when combined with fulvestrant (Fox et al., 2011). These data further imply that combined targeting of the ER and PI3K pathways is more effective than single-agent therapies.

CLINICAL TRIALS TESTING DRUG COMBINATIONS TARGETING THE ER AND PI3K PATHWAYS

Herein, we will review three recent clinical studies that evaluated the benefit of adding the TORC1 inhibitor everolimus to

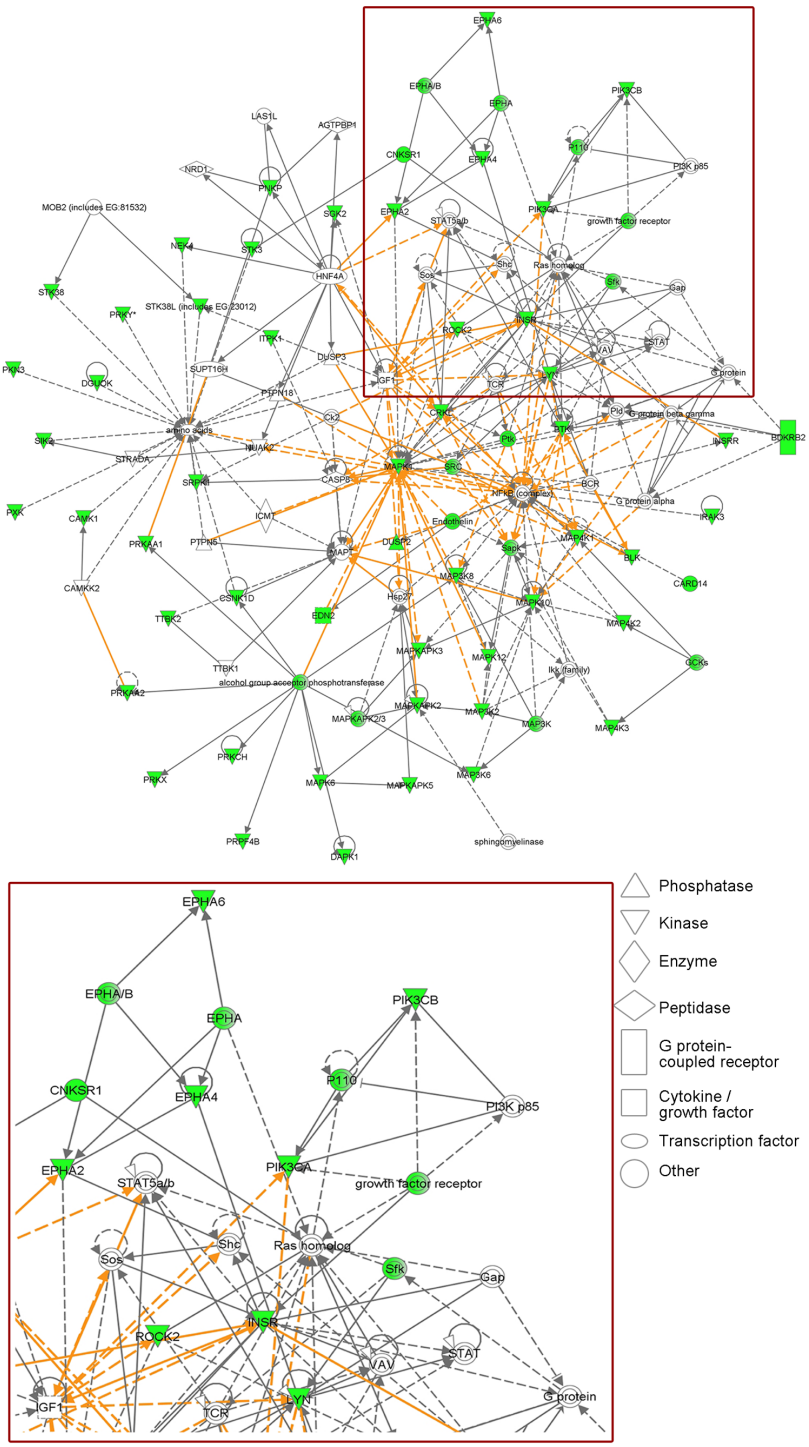

FIGURE 1 | Phosphatidylinositol 3-kinase is a central hub in signaling pathways required for estrogen-independent ER+ breast cancer cell growth. MCF-7/LTED cells transiently transfected with a siRNA library targeting 779 kinases were reseeded in hormone-depleted medium. Cell viability was measured 4–5 days later by Alamar blue assay. Median cell growth in four independent experiments was calculated for each kinase siRNA relative to non-silencing controls. Individual knockdown of 42 kinases inhibited MCF-7/LTED cell growth ≥33% ($p \leq 0.05$) in at least three of four experiments (detailed in Fox et al., 2011). Ingenuity Pathways Analysis revealed that these 42 kinases map to several protein networks that overlap with PI3K signaling (red box, enlarged in bottom panel). Proteins involved in these networks are displayed as nodes. Solid lines indicate direct relationships between proteins, and dotted lines indicate indirect interactions. Green nodes represent the kinases identified in the screen, as well as others whose knockdown was predicted by the Ingenuity software to negatively affect cell growth. The various nodal shapes represent the functional class of the gene product.

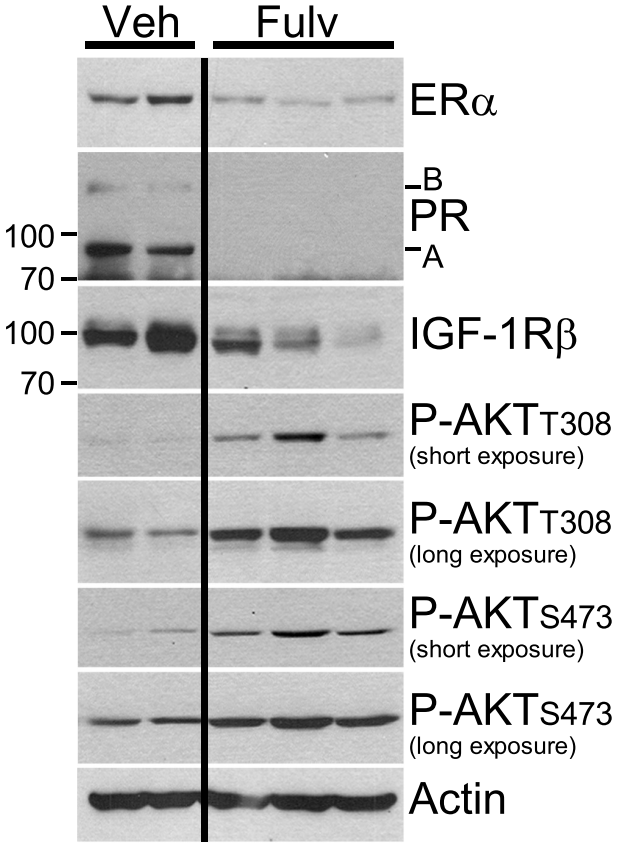

FIGURE 2 | Estrogen receptor inhibition with fulvestrant induces upregulation of PI3K signaling. Ovariectomized athymic mice were s.c. implanted with MCF-7 cells and a 10-day-release E2 pellet (0.12 mg). Twelve days later, mice were randomized to treatment with vehicle or fulvestrant (5 mg/week, s.c., clinical formulation). Tumors were harvested after 3–4 weeks of treatment. Tumor lysates were analyzed by immunoblotting using the indicated antibodies; each lane contains equal amount of protein from two to three tumors. Fulvestrant treatment decreased the levels of ER and ER-regulated genes products (PR, IGF-1R), but increased levels of P-AKT-T308 and P-AKT-S473, suggesting increased activation of PI3K. All lanes were from the same membrane.

endocrine therapy. (1) In the first study, post-menopausal women with early-stage ER+ breast cancer were randomized to neoadjuvant therapy with the AI letrozole ± everolimus for 4 months. The addition of everolimus increased clinical response and suppression of tumor cell proliferation at 2 weeks (measured by Ki67 IHC) compared to letrozole alone (Baselga et al., 2009). (2) In the TAMRAD study, post-menopausal patients with metastatic, ER+, AI-resistant breast cancer were randomized to treatment with tamoxifen ± everolimus. The addition of everolimus improved clinical benefit rate, time-to-progression, and disease-free survival compared to tamoxifen alone (Bachelot et al., 2010). (3) The phase III BOLERO-2 study included 724 post-menopausal women with metastatic, ER+, HER2-negative breast cancer. While 84% of patients exhibited sensitivity to prior endocrine therapy, all were resistant to non-steroidal AIs (letrozole, anastrozole) at the time of randomization to treatment with the steroidal AI exemestane ± everolimus. The addition of everolimus increased progression-free survival (PFS) from 4.1 months (exemestane alone) to 10.6 months (Baselga et al., 2012).

While the addition of a TORC1 inhibitor prevents disease progression in patients with antiestrogen-resistant breast cancer, inhibition of TORC1 relieves negative feedback on activators of PI3K (e.g., IGF-1R, IRS-1, HER3; O'Reilly et al., 2006; Carracedo et al., 2008; Miller et al., 2009). These data suggest that direct inhibitors of PI3K may be more effective. Early clinical testing of PI3K inhibitors in combination with antiestrogens suggests that this strategy is feasible. In a phase Ib trial, post-menopausal patients with advanced ER+ disease are being treated with letrozole plus the PI3K inhibitor BKM120. This drug combination is safe and exhibits promising anti-tumor activity (Mayer et al., 2012).

RATIONALE FOR AN ER DOWNREGULATOR IN COMBINATION WITH A PI3K INHIBITOR IN AI-RESISTANT BREAST CANCER

A recent comparison of high-dose fulvestrant (an ER downregulator) to the AI anastrozole as first-line treatment for advanced breast cancer revealed that fulvestrant provided a longer time-to-progression (Robertson et al., 2009a). In other studies, ~35% of patients who progressed on an AI responded to second-line

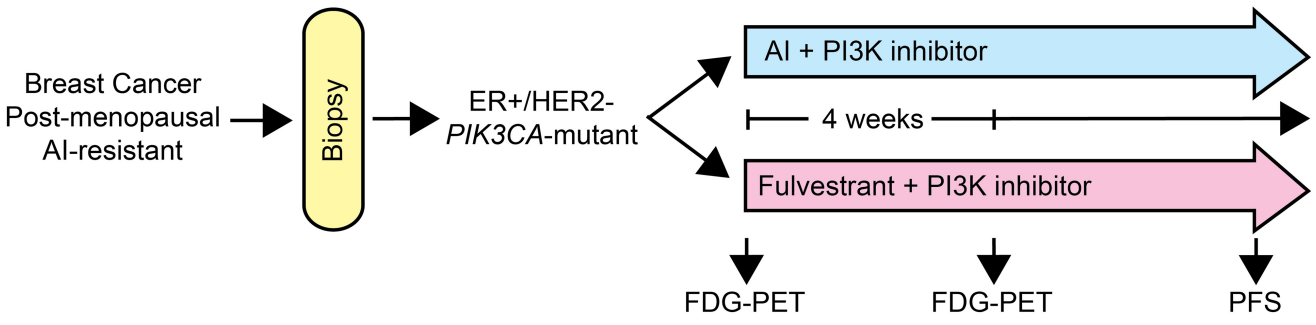

FIGURE 3 | Diagram of a clinical trial with a PI3K pathway inhibitor in AI-resistant breast cancer. Patients with breast cancer that progressed on AI therapy will be subjected to a biopsy to confirm ER+, HER2-negative, *PIK3CA*-mutant status. Eligible patients would then be randomized to another AI plus a PI3K inhibitor, or fulvestrant plus the PI3K inhibitor. FDG-PET scans would be performed before and after 4 weeks of therapy to identify early metabolic changes. Patients will be treated until progression.

fulvestrant (Ingle et al., 2006; Perey et al., 2007). This suggests that in some clinical situations, downregulation of ER may be superior to estrogen deprivation (AI) therapy (Robertson et al., 2009a). We recently reported that ER retains transcriptional activity in estrogen-independent LTED cells and primary human breast tumors (i.e., following AI therapy), and drives the estrogen-independent growth of LTED cells (Miller et al., 2011b). These data suggest that estrogen (ligand)-independent ER activity may promote resistance to AI therapy. While their side effect profiles are generally similar, AI therapy increases the risk of bone fractures and joint disorders more so than fulvestrant (Howell et al., 2002, 2005; Osborne et al., 2002; Goss et al., 2003; Robertson et al., 2003; Howell and Sapunar, 2011). Fulvestrant, which is administered intramuscularly, is associated with injection site pain, and only induces partial ER downregulation in tumors (Robertson et al., 2009b). Hence, the development of a more potent, orally available ER downregulator/inhibitor may provide a convenient and effective treatment option for patients with ER+ breast cancer.

Cancer cells harboring activating mutations in *PIK3CA* exhibit increased sensitivity to PI3K inhibition (Miller et al., 2010; Sanchez et al., 2011; Maira et al., 2012), suggesting that this class of drugs may be most effective against tumors with mutations in the PI3K pathway. In mice bearing ER+, HER2-negative, *PIK3CA*-mutant MCF-7 breast cancer xenografts, treatment with the combination of fulvestrant and BKM120 induced tumor regression (Miller et al., 2011b). Using [^{18}F]FDG-PET imaging as an early biomarker of metabolic inhibition, treatment with BKM120 but not fulvestrant decreased tumor FDG uptake. BKM120 increased tumor cell apoptosis, while fulvestrant decreased tumor cell proliferation. These findings may be validated clinically in a phase II clinical trial where post-menopausal patients with AI-resistant, ER+, HER2-negative, *PIK3CA*-mutant breast cancer are randomized to treatment with another AI plus a PI3K inhibitor vs. fulvestrant plus a PI3K inhibitor (**Figure 3**). The novel agent in such a trial would be the PI3K inhibitor, but the comparison would be an AI vs. fulvestrant. The primary endpoint would be PFS. Incorporation of non-invasive imaging with [^{18}F]FDG-PET at baseline and after several weeks of treatment could identify metabolic changes indicative of a pharmacodynamic effect. This comparison would inform us whether (1) the addition of a PI3K inhibitor to an AI is beneficial, (2) downregulation of ER is superior to estrogen deprivation (AI) therapy in the context of PI3K inhibition, and (3) metabolic inhibition at an early time point as reflected by FDG-PET is predictive of PFS.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health K99CA142899, R00CA142899 (Todd W. Miller), Breast Cancer Specialized Program of Research Excellence (SPORE) P50CA98131, Vanderbilt-Ingram Cancer Center Support Grant P30CA68485; a grant from the Breast Cancer Research Foundation (Carlos L. Arteaga); American Cancer Society Clinical Research Professorship Grant CRP-07-234 (Carlos L. Arteaga) and Postdoctoral Fellowship Grant PF-10-184-01-TBE (Emily M. Fox); the Lee Jeans Translational Breast Cancer Research Program (Carlos L. Arteaga); and Stand Up to Cancer/American Association for Cancer Research Dream Team Translational Cancer Research Grant SU2C-AACR-DT0209 (Carlos L. Arteaga).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 21 August 2012; paper pending published: 18 September 2012; accepted: 30 September 2012; published online: 16 October 2012.

*Citation: Fox EM, Arteaga CL and Miller TW (2012) Abrogating endocrine resistance by targeting ERα and PI3K in breast cancer. Front. Oncol. **2**:145. doi: 10.3389/fonc.2012.00145*

This article was submitted to Frontiers in Molecular and Cellular Oncology, a specialty of Frontiers in Oncology.

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frontiers in ONCOLOGY

REVIEW ARTICLE
published: 08 May 2013
doi: 10.3389/fonc.2013.00108

Targeting PI3K in cancer: any good news?

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The phosphatidylinositol 3-kinase (PI3K) signaling pathway regulates several cellular processes and it's one of the most frequently deregulated pathway in human tumors. Given its prominent role in cancer, there is great interest in the development of inhibitors able to target several members of PI3K signaling pathway in clinical trials. These drug candidates include PI3K inhibitors, both pan- and isoform-specific inhibitors, AKT, mTOR, and dual PI3K/mTOR inhibitors. As novel compounds progress into clinical trials, it's becoming urgent to identify and select patient population that most likely benefit from PI3K inhibition. In this review we will discuss individual *PIK3CA* mutations as predictors of sensitivity and resistance to targeted therapies, leading to use of novel PI3K/mTOR/AKT inhibitors to a more "personalized" treatment.

Keywords: PI3K, cancer, therapeutics, genetic determinants, class II phosphatidylinositol 3-kinase

INTRODUCTION

Over the past years, it has become widely accepted that cancer is a multistep genetic disease that arises by the activation of specific oncogenes, inactivation of tumor suppressor genes, and stochastic accumulation of genetic alterations driving tumor progression (Vogelstein and Kinzler, 2004). Despite the genetic and epigenetic complexity observed in cancer, tumor growth and survival can be impaired by the inactivation of a single oncogene. This phenomenon is called "oncogene addiction" (term coined by Weinstein, 2000), and reveals a possible "Achilles heel" within the cancer cell that can be therapeutically exploited (Weinstein, 2000, 2002). The hypothesis of oncogene addiction refers to the observation that a tumor cell, despite several genetic alterations, is dependent on a single oncogenic pathway responsible for sustaining the malignant phenotype. An important implication is that switching off this crucial pathway upon which cancer cells are dependent should have negative effects on cancer while sparing normal cells. Therefore pharmacological inhibition of this crucial pathway cause an "addiction shock," resulting in the blockade of cell growth or in cell death (Sharma and Settleman, 2007; Janne et al., 2009).

The "addiction paradigm" has been pharmacologically exploited and drugs designed to specifically inhibit mutated proteins have led to what is commonly known as "personalized cancer medicine." At present only a small subset of anticancer therapies are administered based upon the genetic alterations present in individual tumors (Martini et al., 2012). For example, breast cancer patients with amplification/overexpression of the human epidermal growth factor receptor (EGFR) 2 (HER-2) are selectively sensitive to Trastuzumab and Lapatinib (Stern, 2012), melanomas harboring *BRAF* V600E mutations to Vemurafenib (Flaherty et al., 2010), non-small cells lung cancers (NSCLC) with mutated EGFR to Erlotinib and Gefitinib (Pallis et al., 2011), and KIT and PDGFRA mutant gastrointestinal stromal tumors (GIST) to Imatinib (Antonescu, 2011).

The phosphatidylinositol 3-kinase (PI3K) signaling pathway regulates several processes in normal cell such as survival, metabolism, and motility and it's one of the most frequently deregulated pathway in human cancer (Cantley, 2002; Samuels et al., 2004; Liu et al., 2009a). Mutations and/or amplifications of the PI3K catalytic subunits p110α (*PIK3CA*) and p110β (*PIK3CB*), the PI3K regulatory subunits p85α (*PIK3R1*) and p85β (*PIK3R2*), the PI3K effector AKT (*AKT1*) are often observed in cancer[1]. Moreover, mutations, deletions, or epigenetic changes of negative regulators of PI3K axis (Gewinner et al., 2009; Hollander et al., 2011), such as phosphatase and tensin homolog (PTEN) and inositol polyphosphate-4-phosphatase, type II (INPP4B), may alter sensitivity to chemo- and targeted-therapies (Steelman et al., 2008; Kim et al., 2012).

While class I is the most well characterized of the PI3K to date, the family comprises other two groups; class II and class III. Each class of PI3-kinase has unique preferences for phosphoinositide substrates and produces specific lipid second messengers, responding to a wide variety of signaling molecules.

Class II PI3Ks consists of three members named PI3K-C2α, PI3K-C2β, and PI3K-C2γ. Unlike class I, they lack a regulative subunit and appear to be monomers of high molecular weight, predominantly associated with intracellular membranes (Falasca et al., 2007). PI3K-C2α and PI3K-C2β have a broad tissue distribution and are almost ubiquitously expressed, while PI3K-C2γ displayed a very restricted expression pattern, limited to liver, pancreas, and prostate (Kok et al., 2009). The precise nature of class II substrates and lipid products is still debated. Class II members are

[1] www.sanger.ac.uk/cosmic

thought to act similarly to class III mainly generating PtdIns(3)P *in vitro* and *in vivo* (Falasca and Maffucci, 2007), nonetheless, they can also produce PtdIns(3,4)P_2 *in vitro* (Vanhaesebroeck et al., 2010) and PI3K-C2α has been also reported to produce PtdIns(3,4,5)P_3 *in vitro* (Gaidarov et al., 2001). Class II PI3Ks are activated downstream of different receptor types including RTKs (EGFR and PDGFR) (Brown et al., 1999; Arcaro et al., 2000, 2002; Falasca and Maffucci, 2007) and GPCRs (Maffucci et al., 2005). Several stimuli promote PI3K-C2α activation such as hormones (insulin) (Brown et al., 1999), chemokines (Turner et al., 1998), and cytokines (TNFα and leptin) (Ktori et al., 2003). Similarly PI3K-C2β is activated by growth factor (EGF) (Arcaro et al., 2002) and phospholipids (LPA) (Maffucci et al., 2005) while at the present there are no study investigating PI3K-C2γ upstream activators. A recent study reported that PI3K-C2α has an essential role in angiogenesis resulting in embryo lethality, impaired endothelial cell signaling, and RhoA activation (Yoshioka et al., 2012).

The increasing understanding of the mechanisms underlying the role of the PI3K pathway in tumorigenesis has encouraged many pharmaceutical companies and academic laboratories to focus their efforts on the development of inhibitors targeting the PI3K signaling pathway at different levels.

In this review, we will discuss the challenges for the development of novel inhibitors to target the PI3K signaling pathway and the binary relationship between PI3K mutations in cancer genotype and personalized medicine.

TARGETING PI3K SIGNALING PATHWAY IN CANCER

Since PI3K/AKT/mTOR axis has been classified among the most frequently activated pathway in cancer, members of the cascade represent an attractive target for cancer therapeutics (Miled et al., 2007). The activation of the PI3K signaling pathway contributes to several aspects of tumorigenesis as tumor development, progression, invasiveness, and metastasis formation. A number of molecules targeting members of the PI3K axis have been developed and evaluated in preclinical studies as well as in clinical trials (**Figure 1**). Based on pharmacokinetics properties and isoform selectivity for the ATP binding site, PI3K inhibitors have been classified into different groups (**Table 1**).

The first group encompasses inhibitors able to bind all class I PI3Ks (pan inhibitors), and in particular PI3Kα, PI3Kβ, PI3Kγ, and PI3Kδ. Wortmannin and LY294002, the first two prototype PI3K inhibitors, represented for a long time a useful tool in the study of PI3K function in cellular processes, given their effectiveness at low concentration (nM). Nevertheless, given their poor pharmacokinetic properties and lack of selectivity, these compounds have limited their therapeutic potential. The availability of the crystal structure of the p110 isoform-specific catalytic subunits gave a boost to the development of new PI3K inhibitors (Vadas et al., 2011). Therefore, several novel compounds have been further developed in order to improve pharmacokinetic profiles, to increase target specificity and to minimize toxicity. At the present, several promising pan-PI3K inhibitors are under development and evaluation in clinical trials for cancer therapy. These molecules predominantly display cytostatic effects with consequent G1 phase arrest *in vitro* and favorable anticancer effects *in vivo*.

Lately, a second group of PI3K inhibitors has been developed to overcome the toxicity displayed by the treatment with pan-PI3K inhibitors. They are characterized by greater selective activity (isoform-specific) and several molecules are currently under evaluation in preclinical and clinical studies.

On the other hand, since PI3K and mTOR share several structural similarities, many chemical compounds, under evaluation in clinical trials, are able to inhibit both catalytic subunits. This third group of inhibitors is termed "dual PI3K/mTOR" and they have the advantage of inhibiting not only all class I isoforms but also mTORC1 and mTORC2 thus having a strongest effectiveness in switching off the PI3K signaling pathway.

Given the growing interest in inhibiting PI3K signaling pathway, the drug development landscape is becoming increasingly crowded and highly competitive, so that several pharmaceutical companies, such as Novartis, Sanofi-Aventis, Roche/Genentech, Bayer, and GlaxoSmithKline, are currently in competition to

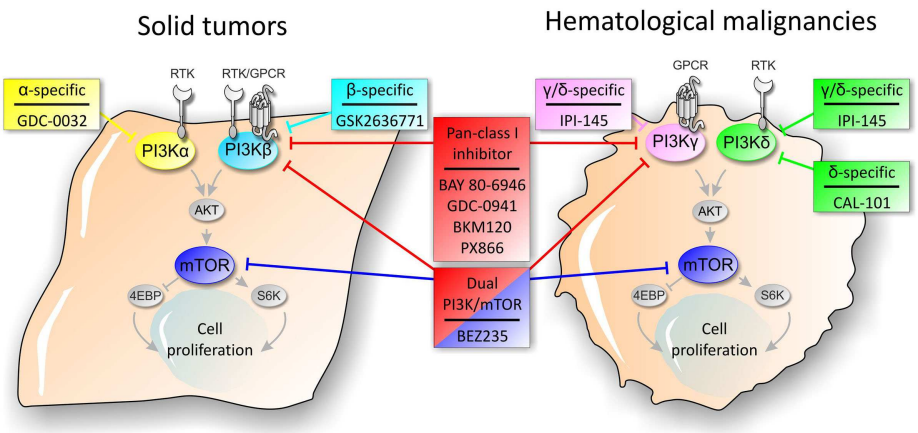

FIGURE 1 | Schematic representation of PI3K pathway and sites of action of PI3K signaling pathway inhibitors in solid tumors and hematological malignancies.

Table 1 | PI3K inhibitors tested in preclinical and clinical models, genetic determinants of response, and open clinical trials described for each compound.

Group	Selectivity	Compound	Cancer type	Genetic determinant of response	Clinical trial status
I Pan-class I	Class I PI3K	GDC-0941 (Roche/Genentech)	Breast	HER-2 amplification PIK3CA mutations	I-II in breast, non-Hodgkin's lymphoma, NSCLC
			Melanomas, MM, non-Hodgkin's lymphoma, NSCLC, ovarian	–	
		BKM120 (Novartis)	Breast	PIK3CA mutations	I-II in breast CRC, endometrial, GIST, GBM, leukemia, melanoma, NSCLC, pancreatic, renal cell, SCCHN, TCC
			CRC, endometrial, GIST, GBM, leukemia, melanoma, NSCLC, pancreatic prostate	–	
		PX866 (Oncothyreon)	Breast, CRC, MM, NSCLC, pancreatic prostate, ovarian	PIK3CA mutation, PTEN loss	I-II in CRC, GBM, NSCLC, SCCHN
		BAY 80-6946 (Bayer)	Advanced solid cancers	PIK3CA mutations	I-II in advanced solid cancers
II Isoform specific	PI3Kα	GDC-0032 (Roche/Genentech)	Solid cancers	–	I-II in solid cancers
	PI3Kβ	GSK2636771 (GlaxoSmithKline)	Advanced solid cancers	–	I-IIa in advanced solid cancers with PTEN deficiency
	PI3Kγ and PI3Kδ	IPI-145 (Infinity)	Hematological malignancies	–	I-IIa in advanced hematological malignancies
	PI3Kδ	CAL-101 (Gilead Sciences)	AML, CLL, Hodgkin's and non-Hodgkin's lymphoma, MCL	–	I-II-III in AML, CLL, Hodgkin's and non-Hodgkin's lymphoma, MCL, MM
III Dual PI3K/mTOR	PI3K and mTOR	BEZ235 (Novartis)	Breast	PIK3C2a mutation, HER-2 amplification, PTEN loss	I-II in breast, renal cell
			Ovarian	PIK3C2a mutation, PTEN loss	

bring their own inhibitors in the market. On the basis of these considerations, we will describe and discuss results for the most important PI3K inhibitors currently in clinical trial.

PI3K SIGNALING INHIBITION IN SOLID TUMORS

Several PI3K inhibitors have progressed through early clinical safety and dose-escalation studies to phase I clinical trials on

solid tumors. This allowed to define the treatment efficacy and to identify patient population that will benefit from PI3K inhibitor administration. Furthermore, a few number of compounds are now progressing to phase Ib expansion cohort and phase II single agent efficacy studies.

Several early reports were presented in the last 3 years revealing safety and some preliminary activity data about the use of class I PI3K and dual mTOR/PI3K inhibitors in patients.

GDC-0941 (GENENTECH-ROCHE)

GDC-0941 is a potent and selective oral inhibitor of class I PI3K with activity against DNA-PK and also mTOR but at high concentrations (Folkes et al., 2008). GDC-0941 is currently under evaluation in several phase I clinical trials on patients with advanced solid tumors, such as HER-2 positive metastatic breast cancer and advanced NSCLC[2]. Several studies have documented the effects of GDC-0941 on cell viability inhibition at submicromolar concentration in several tumor types including glioblastoma, breast, and prostate cell lines carrying specific alteration in the PI3K signaling pathway. GDC-0941 exhibited excellent inhibition on MCF7, T-47D, and SK-BR-3 breast derived cancer cell lines as a single agent while in combination with rapamycin promotes apoptosis and down regulates cell cycle machinery components, such as cyclin D1 (Zheng et al., 2012). In addition, the combinatorial treatment of GDC-0941 and the Docetaxel in a panel of 25 breast tumor cell lines (HER-2+, luminal, and basal subtypes) increases the rate of apoptosis and enhances sensitivity to Docetaxel (Wallin et al., 2012). The efficacy of combined GDC-0941 and chemo- and targeted-therapies has also been demonstrated for other agents such as Trastuzumab, Pertuzumab, and Docetaxel (Yao et al., 2009). In NSCLC GDC-0941 synergizes also with the MEK inhibitor (U0126) by promoting G0-G1 arrest and cell apoptosis (Zou et al., 2012). Several clinical studies are currently evaluating the relative bioavailability, absorption, metabolism, excretion, and effects of GDC-0941 in patient with advanced or metastatic tumors, alone or in combination with Paclitaxel, Erlotinib, Carboplatin, or Bevacizumab (see text footnote 2) GDC-0973 (MEK inhibitor). Safety data about GDC-0941 monotherapy regimen have been recently released. Phase I studies with a $3+3$ dose-escalation design showed that GDC-0941 is generally well tolerated at doses below 450 mg every day (QD) and twice a day (BID) in patient with advanced solid tumors. After treatment, signs of clinical activity have been reported, including a partial response (PR) by the response evaluation criteria in solid tumors (RECIST) in a patients with melanoma, ovarian, endocervical, and ER+/HER-breast cancer (Wagner et al., 2009; Moreno Garcia et al., 2011; Von Hoff et al., 2011). Several side effects have been described after administration of GDC-0941 in about 10% of patients, in particular nausea, diarrhea, fatigue, dysgeusia, and decreased appetite. In two different studies, the maximum tolerated dose (MTD) was enriched at 450 mg with a dose limiting toxicities (DLT) of grade 3 (Gr) macular rash and asymptomatic *T*-wave inversion in ECG, Gr3 thrombocytopenia, and Gr4 hyperglycemia (Moreno Garcia et al., 2011; Von Hoff et al., 2011).

[2]www.clinicaltrials.gov

BKM120 (NOVARTIS)

BKM120 is an oral pyrimidine-derived pan-PI3K inhibitor with potent activity at nanomolar concentrations against all class I PI3K isoform while didn't show any activity against other classes of PI3K as well as mTOR. *In vitro* preclinical models showed that BKM120 has a strong anti-proliferative activity in more than 400 cancer cell lines. The antitumor effects of BKM120 was also described in several xenograft models of lung cancer (Fruman and Rommel, 2011) and metastatic HER-2+ breast cancer (Nanni et al., 2012). Clinical data indicate that it is unlikely that BKM120 will achieve exposures sufficient to significantly engage the off-target activity at tolerated doses and schedules, however careful dose range selection is required to ensure specific targeting of PI3K signaling pathway (Brachmann et al., 2012). Given its ability to penetrate the blood-brain barrier, BKM120 may represent an attractive option for the treatment of glioblastoma multiforme (GBM), the most common and aggressive malignant primary brain tumor (Koul et al., 2012). The first-in-human phase I dose-escalation study investigated the MTD, safety, preliminary activity, and pharmacodynamics of BKM120 (Bendell et al., 2012). The study reports that BKM120 was well tolerated with a dose-dependent safety profile and it describes related side effects, such as hyperglycemia, rash, nausea, fatigue, and mood alterations. In particular hyperglycemia is consistent with inhibition of PI3K signaling and has been observed with other PI3K/mTOR/Akt pathway inhibitors. Disturbance of glucose homeostasis, as evidenced by hyperglycemia, was more common at higher doses and may be attributed to BKM120 inhibition of p110. Pharmacodynamics data demonstrate a dose-related inhibition of the PI3K signaling with significant decrease in pS6 phosphorylation and decreased [18F]fluorodeoxyglucose uptake. In another study, 77 patients with CRC, breast, lung, and endometrial cancers received oral BKM120 in monotherapy once daily (Call et al., 2010). PR were observed in two patients, a triple negative breast cancer with *KRAS* and *p53* mutation and a ER+/HER− metastatic breast cancer carrying *PIK3CA* mutations. At the same time, 58% of patients showed stable disease (SD) response.

BAY 80-6946 (BAYER HEALTHCARE)

BAY 80-6946 is a potent pan-class I PI3K inhibitor with IC50 at sub-nanomolar concentration against PI3Kα (0.5 nM), PI3Kβ (3.7 nM), PI3Kδ (0.7 nM), and PI3Kγ (6.4 nM) while it is inactive against around 240 protein/lipid kinases and RTKs. BAY 80-6946 has shown antitumor activity against a panel of 140 tumor cell lines with an IC50 of 1–100 nM in about 60 tumor cell lines. BAY 80-6946 displayed a strong activity of the PI3K signaling pathway inhibiting AKT (Thr308 and Ser473) as well as PRAS40, 4EBP1, and FOXOs phosphorylation in tumor cells carrying *PIK3CA* activating mutations. BAY 80-6946 has been demonstrated to induce apoptosis in a subset of *PIK3CA* mutant tumors at concentrations lower that 100 nM in preclinical studies. The pharmacokinetics, pharmacodynamics, and MTD of BAY 80-6946 have been determined in a phase I escalation multicenter study in patients with advanced solid tumors. Unlike other PI3K inhibitors, BAY 80-6946 is administered intravenously as 1-h infusion once weekly for 3 weeks every month. Data deriving from phase I study revealed a MTD at 0.8 mg/kg and several

side effects including hyperglycemia, fatigue, nausea, alopecia, diarrhea, mucositis, dysgeusia, and grade 2/3 anemia. Moreover a phase I study assessed safety, pharmacokinetics, and clinical benefit in patients with advanced solid tumors, including breast, endometrial, gastric cancer.

NVP-BEZ235 (NOVARTIS)

NVP-BEZ235 is a reversible, orally available, and selective inhibitor of PI3K and TORC1/2. Several preclinical studies have already demonstrate its efficacy in a variety of solid tumors such as melanomas (Roper et al., 2011), breast (Brunner-Kubath et al., 2011), CRCs (Manara et al., 2010; Roper et al., 2011), and sarcomas (Manara et al., 2010). This compound suppresses cell proliferation, induces G1 cell cycle arrest and promotes autophagy by inhibiting the activity of AKT, S6K, S6, and 4EBP1 target proteins (Serra et al., 2008; Cerniglia et al., 2012). In addition, data on glioma xenograft demonstrate a reduction of the expression of the vascular endothelial growth factor (VEGF) on tumor vasculature, thus suggesting an anti-angiogenetic effect for NVP-BEZ235 (Liu et al., 2009b). Recently, NVP-BEZ235 also emerged as inhibitor of ATM and DNA-PK at low concentration (100 nM). In this context, treatment with NVP-BEZ235 may have significant radio sensitizing effects with important implications in the rational design of clinical trials (Mukherjee et al., 2012). At present, this molecule is under evaluation in phase I/II clinical trials in patients with advanced solid malignancies, including GBM (Salkeni et al., 2012), breast, renal cell carcinoma (RCC), castration-resistant prostate cancer (CRPC), endometrial carcinoma, and pancreatic neuroendocrine tumors (see text footnote 2), alone or in combination with other drugs such as Paclitaxel, Trastuzumab, Everolimus, and MEK162. A 3 + 3 dose-escalation schedule with NVP-BEZ235 revealed tolerability at 600 mg BID dose and preliminary signs of clinical and pharmacodynamic activity (Arkenau et al., 2012). In a phase IB dose-escalation study, NVP-BEZ235 was administered in combination with Trastuzumab in HER-2+ metastatic breast cancer (15 pts) with altered PI3K/PTEN status, showing an acceptable safety profile and PR or SD in one and four patients respectively (Krop et al., 2012). NVP-BEZ235 was also tested as a single agent or Trastuzumab-combined with a novel formulation based on a solid dispersion system (SDS) sachet (Peyton et al., 2011). The MTD for the new formulated NVP-BEZ235 was determined as 1600 mg/day, dose chosen for the ongoing phase II clinical trials. After treatment, in 28 patients with advanced solid tumors, a stable response has been evidence in a 40% of cases (Wen et al., 2012). Overall, these studies display that NVP-BEZ235 is generally well tolerated and the most common side effects include nausea, diarrhea, AST/ALT elevation, and headache. DLT include fatigue, asthenia, Gr3 thrombocytopenia, and Gr3 mucositis (Peyton et al., 2011; Arkenau et al., 2012; Wen et al., 2012).

PI3K SIGNALING INHIBITION IN HEMATOLOGICAL MALIGNANCIES

Although the majority of PI3K inhibitors are under development for the treatment of solid tumors, hematological malignancies also represent a therapeutic area of interest, especially for isoform-selective PI3K inhibitors. Constitutive activation of class I PI3K isoform has been identified in high percentage of acute and chronic leukemia. In addition, in some cases leukemias cell display overexpression of PI3Kα, PI3Kβ, and PI3Kγ. Conversely, the expression of PI3Kδ has been found up-regulated in acute myelogenous leukemia (AML) and in a subset of promyelocytic leukemia (APL). AML comprises a heterogeneous group of tumors characterized by uncontrolled proliferation of hematopoietic precursors thus leading to accumulation of blast cells in the bone marrow. These blasts are blocked in their differentiation program at different stage of maturation. This blast accumulation causes a progressive failure in the hematopoiesis process, which in turn leads to anemia, neutropenia, and thrombocytopenia (Smith et al., 2004). The standard therapeutic approach for the treatment of AML is based on high-dose chemotherapy, nonetheless the prognosis of AML remains poor with a 5-year survival rate in a 15–30% of patients. A recent evidence demonstrated that up-regulation of the PI3K/AKT/mTOR axis is a common feature in AML. Consequently, several pan-PI3K and dual PI3K/mTOR inhibitors, in particular BKM120 and BEZ235, are undergoing phase I clinical development to assess safety, dose, and preliminary efficacy in patients with advanced leukemias, relapsed or refractory acute lymphoblastic and myelocytic leukemia (see text footnote 2).

Several studies demonstrated that treatment with the PI3Kδ selective inhibitor, EC87114, inhibits AML cell proliferation without affecting the proliferation of normal hematopoietic progenitor cells (Sujobert et al., 2005; Billottet et al., 2006). In this context, PI3Kδ inhibitors represent a promising therapeutic treatment for AML without producing undesirable side effects that are conversely expected for other pan-PI3K inhibitors (Di Nicolantonio et al., 2010). Additionally, since PI3Kδ is expressed in leukocytes and plays a key role in B-cell signaling (Jou et al., 2002; Bilancio et al., 2006), it represents an interesting target in B-cells malignancies. Mice with deleted or kinase dead PI3Kδ exhibit B-cell defects, such as lack of B1 lymphocytes, decreased number of mature B-cell and impaired antibody production. The absence of PI3Kδ in B-cell leads also to a reduction in AKT phosphorylation and decreased PtdIns(3,4,5)P_3 levels (Okkenhaug and Vanhaesebroeck, 2003). All these data support the use of PI3Kδ inhibitors in B-cell malignancies including the chronic lymphocytic leukemia (CLL).

Given the increasing interest in inhibiting PI3Kδ for the treatment of hematopoietic malignancies, several inhibitors are currently under evaluation in preclinical and in clinical trials. Presently, the most promising PI3Kδ inhibitor is represent by CAL-101 (Calistoga Pharmaceuticals/Gilead Sciences), an orally available selective inhibitor with an IC50 of 2.5 nM for PI3Kδ and 820, 565, 89 nM for PI3Kα, PI3Kβ, PI3Kγ respectively (Lannutti et al., 2011). At the present, CAL-101 is undergoing in preclinical and clinical development in a variety of lymphoid malignancies thanks to its high selectivity. Phase I studies, on patients with relapsed or refractory hematologic malignancies, including non-Hodgkin lymphoma (NHL), CLL, and AML, revealed a very favorable toxicity profile and pharmacokinetics during a week of CAL-101 oral administration (Fruman and Rommel, 2011). The predominant toxicity, caused by high serum transaminases, was observed in 21% of patients at doses of 200 and 350 mg. However, this side effect resulted to be reversible disappearing with a temporary discontinuation of the drug (Fruman and Rommel, 2011). In

this study, 57 patients were treated with different dosages, corresponding to 50, 100, 200, and 350 mg. Clinical responses were seen at all dose levels resulting in 50% of reduction in lymphadenopathy and around 6 months of stable disease. In a successive phase I clinical trial CAL-101 was administered orally one or two times per day for a cycle of 28 day in 54 patients with CLL. After treatment with CAL-101, 26% of patients achieved a PR with 80% of patients showing reduced lymphadenopathy by ≥50% (Di Nicolantonio et al., 2010; Fruman and Rommel, 2011).

RELEVANCE FOR SELECTIVE INHIBITION OF CLASS II PI3Ks

Pharmacological inhibitors selectively targeting class II PI3Ks have not been described yet. In particular, *in vitro* PI3K-C2α is refractory to inhibition by LY294002 and Wortmannin (Virbasius et al., 1996; Domin et al., 1997), two very well-known PI3Ks inhibitors. However, emerging evidence suggests that class II PI3Ks may have crucial role in different types of tumor independently from class I PI3K activity.

PI3K-C2α

RNAi-based silencing of PI3K-C2α in a large set of cancer cell lines showed that this enzyme is crucially required for cancer cell survival *in vitro* (Elis et al., 2008). A small but significantly difference in the DNA copy number and mRNA levels, was then reported *in vivo* in 19 hepatitis B-positive hepatocellular carcinoma compared with non-tumor tissues counterparts (Ng et al., 2009). The PIK3C2A gene was also found to be up-regulated in breast cancer stem-like cells characterized by increased tumorigenicity compared with the normal counterpart (Zhou et al., 2007). Moreover, it was reported that PI3K-C2α is directly down-modulated at a translational level by *miR-30e-3p* in DLD1 CRC cells (Schepeler et al., 2012). In particular *miR-30e-3p* levels are significantly decreased during early events of CRC carcinogenesis thus raising the possibility that PIK3C2A gene may be up-regulated in the initial steps of CRC onset. On the other hand, Yoshioka et al. (2012) showed that PI3K-C2α has an essential role during angiogenesis process and in vascular barrier function. After subcutaneous injection of Lewis lung carcinoma (LLC) or B16-BL6 melanoma tumors, Pik3c2a endothelial-restricted knock-out mice had reduced tumor volumes/weights compared to control, suggesting that the *in vivo* pro-angiogenetic function of PI3K-C2α is required for tumor growth and maintenance. Further studies are needed to better understand the role PI3K-C2α in tumor angiogenesis, however designing of specific inhibitors targeting PI3K-C2α could represent a promising new anti-angiogenetic approach to arrest tumor growth.

PI3K-C2β

An increasing number of studies described the involvement of the gene PIK3C2B, encoding for PI3K-C2β, in cancer. Amplification of *PIK3C2B* gene at 1q32 was reported in 6 out of 103 GBM tumors and in 4 of these cases amplification correlates with *PI3KC2B* mRNA over-expression (Knobbe and Reifenberger, 2003). Amplification of chromosomal region 1q32.1 (*PIK3C2B**MDM4*) was also reported in around 8% of GBM tumor samples (Rao et al., 2010) and in whole genome amplification analysis (Nobusawa et al., 2010). On the other hand increased PI3K-C2β protein levels significantly correlates with resistance to Erlotinib in GBM thus suggesting that targeting of PI3K-C2β in resistant GBM may represent a new therapeutic approach (Low et al., 2008).

To further support the hypothesis the role of PI3K-C2β in drug resistance, a siRNA-based study reported that in a panel of kinases, down regulation PIK3C2B is one of the Tamoxifen sensitizing target in breast cancer cells (Iorns et al., 2009). Furthermore, overexpression of PI3K-C2β significantly inhibited cisplatin-induced apoptosis and cleavage of caspase-3 in esophageal squamous cell carcinoma (Liu et al., 2011). Altogether these data suggest that PI3K-C2β may have a role in promoting resistance to chemotherapeutic drugs and that interference with PI3K-C2β activity might be a rational possibility for treatment of cisplatin-resistant esophageal cancer patients. On the other hand, it has been recently showed that down regulation of PI3K-C2β may specifically confer resistance to leukemia cells to chemotherapy (thioguanine and mercaptopurine) (Diouf et al., 2011). Reduced levels of PI3K-C2β results in increased degradation of MSH2, a DNA mismatch repair enzyme involved in genomic integrity maintenance and drug resistance. Although it's becoming increasingly clear the involvement of PI3K-C2β in chemotherapy resistance, further studies are needed to clarify when the targeting of this enzyme could promote drug sensitivity.

A recent study also reported that PIK3C2B gene single-nucleotide polymorphisms (SNP) is associated to cancer risk susceptibility (Koutros et al., 2010). The authors examined the association between several SNPs in PI3Ks genes (PIK3CD, PIK3C2A, PIK3R3, PIK3AP1, and PIK3C2B) and prostate cancer risk: among the five genes, only PIK3C2B showed a cluster of SNPs related to prostate cancer risk. PIK3C2B was also found to be significantly mutated in a recent whole-exome sequencing screening in NSCLC (Liu et al., 2012).

PI3K-C2γ

At the present very little is known about the role of PI3K-C2γ in cancer. The chromosomal region 12p12, containing the human PIK3C2G gene, is found significantly amplified in a subset of ovarian cancer (Lambros et al., 2005) and in about 60% of pancreatic ductal adenocarcinoma (PDAC) (Harada et al., 2008). Recently bioinformatics analysis reported significative association between PIK3C2G mutations and GBM related signaling pathways (Dong et al., 2010). Another study also suggests a new mechanism through which Bcr-Abl induces abnormal homing of leukemia cells by reducing PI3K-C2γ expression (Yu et al., 2010). In this way, Bcr-Abl is not only responsible for class I PI3K activation in cell migration (Martelli et al., 2006) but also for PI3K-C2γ reduced expression (Yu et al., 2010).

Altogether these data suggest that class II PI3Ks may exert additional or complementary role to class I PI3Ks in promoting cancer, being involved in specific kind of tumors. The development of selective inhibitors targeting class II PI3Ks may thus represent a promising way of action, once the precise role of these enzymes in cancer will be elucidated.

FUTURE PERSPECTIVES

As PI3K inhibitors progress into trials focusing on their clinical efficacy, it is critical to identify genomic determinants of response

and to stratify the patient population that will most likely benefit from the treatment (Weigelt and Downward, 2012).

Historically rapalogs were the first PI3K pathway inhibitors tested in clinical trials for cancer therapy and currently preclinical models and early clinical data suggested that *PIK3CA* mutations may predict sensitivity to treatment with PI3K/AKT/mTOR inhibitors (Engelman et al., 2008; Ihle et al., 2009). In particular, it has been shown that patients with advanced malignancies carrying *PIK3CA* pathway aberrations, showed high percentage of response to rapalogs and/or PI3K pan-inhibitor (Janku et al., 2011b; Moroney et al., 2011). Moreover, PI3K pathway aberrations due to *PIK3CA* mutations or PTEN loss correlate with increase response to Doxorubicin, Bevacizumab, and Temsirolimus in patients with advanced gynecologic and breast malignancies (Moroney et al., 2011). Likewise, ovarian cancers with coexisting *PIK3CA* and *MAPK* pathway mutations are sensitive to PI3K inhibition, whereas CRC with the same repertoire of mutations are resistant (Di Nicolantonio et al., 2010; Janku et al., 2011a, 2012). The identification of feedback loops leading to MAPK activation, upon PI3K inhibition, underscores the potential of a combined therapeutic approach with PI3K and MAPK inhibitors (Carracedo et al., 2008; Laplante and Sabatini, 2012). According to these findings, it has been proposed to incorporate predictive biomarkers during the clinical drug development process from phase I studies onward in order to enrich trials with patients more likely to respond to a given targeted therapy and to increase the chances of drug registration (Carden et al., 2010). For the guidance and prioritization of predictive biomarker candidates in early clinical trials, results derived from the study of preclinical models are of primary importance to develop accurate companion diagnostic tools.

CONCLUSION

In summary, although available dataset showed that a high percentage of tumors harboring *PIK3CA* will likely benefit from inhibition of the PI3K pathway, a substantial proportion of patients with *PIK3CA* activating mutations may be *de novo* resistant to these agents. On the other hand, not all the patients with *PIK3CA* mutations are sensitive to PI3K inhibitors and not all the patients with wt *PIK3CA*/*PTEN* tumors are responsive. Moreover, while the majority of the studies have restricted the analysis on the *PIK3CA* and PTEN status, also occurring in other members of the pathway, such as mTOR activating mutations, or INPP4B loss of function, may play a role in the response to inhibitors. Therefore, the possibility to target PI3K signaling pathway in cancer requires deeper investigation, in order to identify additional biomarkers and to improve therapeutic strategies in the clinic.

ACKNOWLEDGMENTS

This work was supported by a grant from Fondazione Cariplo.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 February 2013; paper pending published: 04 March 2013; accepted: 19 April 2013; published online: 08 May 2013.

*Citation: Martini M, Ciraolo E, Gulluni F and Hirsch E (2013) Targeting PI3K in cancer: any good news? Front. Oncol. **3**:108. doi: 10.3389/fonc.2013.00108*

This article was submitted to Frontiers in Molecular and Cellular Oncology, a specialty of Frontiers in Oncology.

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frontiers in ONCOLOGY

REVIEW ARTICLE
published: 24 July 2013
doi: 10.3389/fonc.2013.00191

S6K2: the neglected S6 kinase family member

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S6 kinase 2 (S6K2) is a member of the AGC kinases super-family. Its closest homolog, S6K1, has been extensively studied along the years. However, due to the belief in the community that the high degree of identity between these two isoforms would translate in essentially identical biological functions, S6K2 has been largely neglected. Nevertheless, recent research has clearly highlighted that these two proteins significantly differ in their roles *in vitro* as well as *in vivo*. These findings are significant to our understanding of S6 kinase signaling and the development of therapeutic strategies for several diseases including cancer. Here, we will focus on S6K2 and review the protein–protein interactions and specific substrates that determine the selective functions of this kinase.

Keywords: S6K2, S6 kinase, selectivity, specificity, function, RPS6KB2, cancer

INTRODUCTION

The ribosomal protein S6 kinases constitute a super-family of proteins initially discovered based on their ability to phosphorylate a 40S ribosomal subunit component, the ribosomal S6 protein. The p90 ribosomal S6 kinases (RSKs), comprising RSK1–4 (1), were first identified followed by the p70 ribosomal S6 kinase, S6K1 (2, 3). It took an additional 10 years for the p70 ribosomal S6 kinase homolog, S6K2, to be discovered (4–6). The high degree of homology between S6K1 and S6K2 has for many years led researchers to assume that these were redundant kinases with essentially overlapping functions. This introduced a bias toward S6K1-oriented research, as this isoform came to be considered the prototypical S6K. However, more recent research clearly indicates that these two isoforms also have distinct biological functions the understanding of which may have implications for therapeutic intervention. Therefore, while other publications exist that review S6Ks and their upstream pathways (7, 8), here we will focus specifically on S6K2 and highlight the distinct biological function of this isoform.

STRUCTURE OF S6K2

Human S6K2 is encoded by the 15 exons of the *RPS6KB2* gene on chromosome 11 (11q13). The S6K2 mRNA (ID ENST00000312629) gives rise to two protein products through the use of alternative translational start sites: a long form (p56 S6K2) and a short form (p54 S6K2) that differ by the presence or absence of an N-terminal 13 amino acid segment. The overall structure of S6K2 is very close to that of S6K1 (**Figure 1A**). The kinase domain of S6K2 shares 83% amino acid identity with that of S6K1, a fact that has long justified the lack of interest in finding isoform-specific substrates for these proteins. The kinase domain is followed toward the C-terminus by a kinase extension domain and a pseudo-substrate inhibitory region. The greatest degree of divergence between S6K1 and S6K2 lies in the C-and N-terminus, a fact that has enabled the development of S6K2-specific antibodies (9). The presence in the C-terminus of S6K2 of a nuclear localization sequence (NLS) means that this isoform is predominantly localized to the nuclei of quiescent cells (10). In addition, the long form of S6K2 contains in its 13 amino acid extension an additional putative NLS. This results in the different cellular distribution of these two isoforms as the two NLS motifs in p56 S6K2 confers constitutive nuclear localization to this variant, while p54 S6K2 shuttles between the nucleus and the cytoplasm in response to growth factor signaling. The C-terminus of S6K2 also contains a proline-rich region which has been proposed to promote interaction with SH3 and WW domains putatively present in its binding partners (4). While shorter isoforms of S6K1 have been shown to be generated by alternate mRNA splicing (11), no such variants have yet been reported for S6K2. However, the high degree of conservation between the two proteins raises the possibility that similar regulation may take place for the *RPS6KB2* gene. Indeed, eight transcripts have been reported for S6K2 with a corresponding protein found for only one (ID ENST00000312629) of the four protein coding transcripts (ID ENST00000539188, ENST00000524934, ENST00000524814, ENST00000312629). This may have important functional consequences as, unlike its full length counterpart, an S6K1 splice variant, p31S6K1, was shown to have oncogenic potential (12).

S6K2 ACTIVATION AND POST-TRANSLATIONAL MODIFICATION

S6K2 ACTIVATION

Many of the residues that are required for kinase activation are common between S6K1 and S6K2 as seven of the eight serine/threonine phosphorylation sites present on S6K1 are conserved in S6K2 (Thr-228, Ser-370, Thr-388, Ser-403, Ser-410, Ser-417, and Ser-423 on p54 S6K2) (4, 6, 10) (**Figure 1A**). The activation of S6K2 occurs in a step-wise manner (**Figure 1B**). An initial barrier to overcome is the repression exerted by the

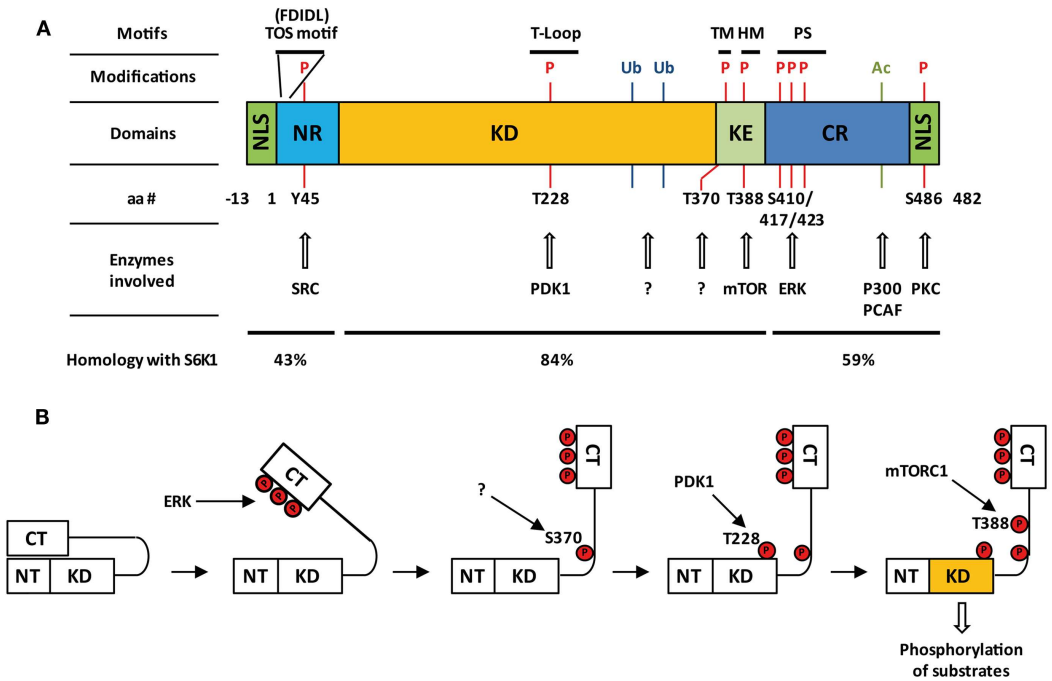

FIGURE 1 | Structure and activation of S6K2. **(A)** Domain organization of S6K2, post-translational modifications together with involved enzymes, and percentage homology with S6K1. Nuclear localizations sequences (NLS); N-terminal regulatory region (NR); kinase domain (KD); kinase extension region (KE); C-terminal regulatory region (CR); pseudo-substrate domain (PS); turn motif (TM); hydrophobic motif (HM); pseudo-substrate region (PS); phosphorylation (P), ubiquitination (Ub); acetylation (Ac). **(B)** Step-wise model of activation of S6K2.

C-terminal autoinhibitory pseudo-substrate domain. This is dealt with by phosphorylation of the three proline-directed serines in the autoinhibitory domain, Ser-410, Ser-417, and Ser-423 downstream of MEK/ERK signaling. We and others have found this first step to be crucial for S6K2 activation in various cell types (13, 14), as this domain exerts a far more repressive role on S6K2 activity than it's equivalent for S6K1 (15, 16). This event is presumed to open the kinase conformation, exposing additional phosphorylation sites to activating kinases. In agreement with this, deletion of the autoinhibitory region increases basal activity of S6K2 and sensitizes the kinase to activation by various agonists (15). Subsequent phosphorylation of Ser-370 then enables phosphorylation of Thr-388 by the mTORC1 complex followed by that of Thr-228 by PDK1 (17). The T388 site lies within a conserved sequence of the kinase extension domain (F-X-X-F/Y-S/T-F/Y) known as the hydrophobic motif, a region found in many AGC kinases. Phosphorylation of this site by mTOR is achieved following the binding of the mTORC1 complex component Raptor to the TOR signaling (TOS) motif present in both S6K1 and 2 (18, 19). Interestingly, despite the conservation of the hydrophobic motif, substitution of Thr-388 by a glutamic acid (T388E) renders S6K2, but not S6K1, constitutively active. However, phosphorylation of both the Ser-370 and Thr-228 is crucial for S6K2 activity. Indeed, substitution of the latter site for alanine renders the T388E mutant inactive while that of the first prevents Thr-388 phosphorylation. As S6K2 is mainly a nuclear protein and mTOR shuttles between the cytoplasm and the nucleus, it was shown that S6K2 activity was increased by targeting mTOR expression to the nucleus (20).

Despite S6K1 and S6K2 both lying downstream of mTOR (**Figure 2**), there is evidence to indicate that they may be regulated through different pools of this upstream kinase. Indeed, both S6K isoforms react differently to nutrient deprivation, a known modulator of mTOR activity. For instance inhibition of protein synthesis by leucine deprivation in myotubes, results in dephosphorylation of S6K1, without affecting S6K2 activity (21). The existence of two separate pools of mTOR regulating the two S6K isoforms is further suggested by the differential sensitivity of S6K1 and 2 kinase activity to the mTOR inhibitor, rapamycin. Indeed, the involvement of mTORC1 in the activation of S6K2, led several researchers to report the sensitivity of S6K2 to this inhibitor (14, 17, 22). However, the majority of reports suggesting equivalent sensitivity of S6K1 and 2 to rapamycin used concentrations of this drug that non-selectively inhibit the MEK/ERK pathway, therefore indirectly targeting S6K2 independently of its effect on mTOR (13). Hence, when used at the minimal concentrations that fully inhibit S6K1 activity, rapamycin often fails to significantly alter S6K2 activity in several cell systems [(13) and unpublished data from our lab]. These findings are consistent with the reported existence of a rapamycin-resistant mTORC1 activity pool (23, 24) that can efficiently be targeted by mTOR ATP-competitive inhibitors (23, 25).

ADDITIONAL PHOSPHORYLATION EVENTS

While expression of an mTOR variant targeted to the nucleus increases activation of S6K2, nuclear localization of S6K2 is not indispensable for activation of this kinase. Indeed, S6K2, but not S6K1, is phosphorylated *in vitro* as well as *in vivo* by protein

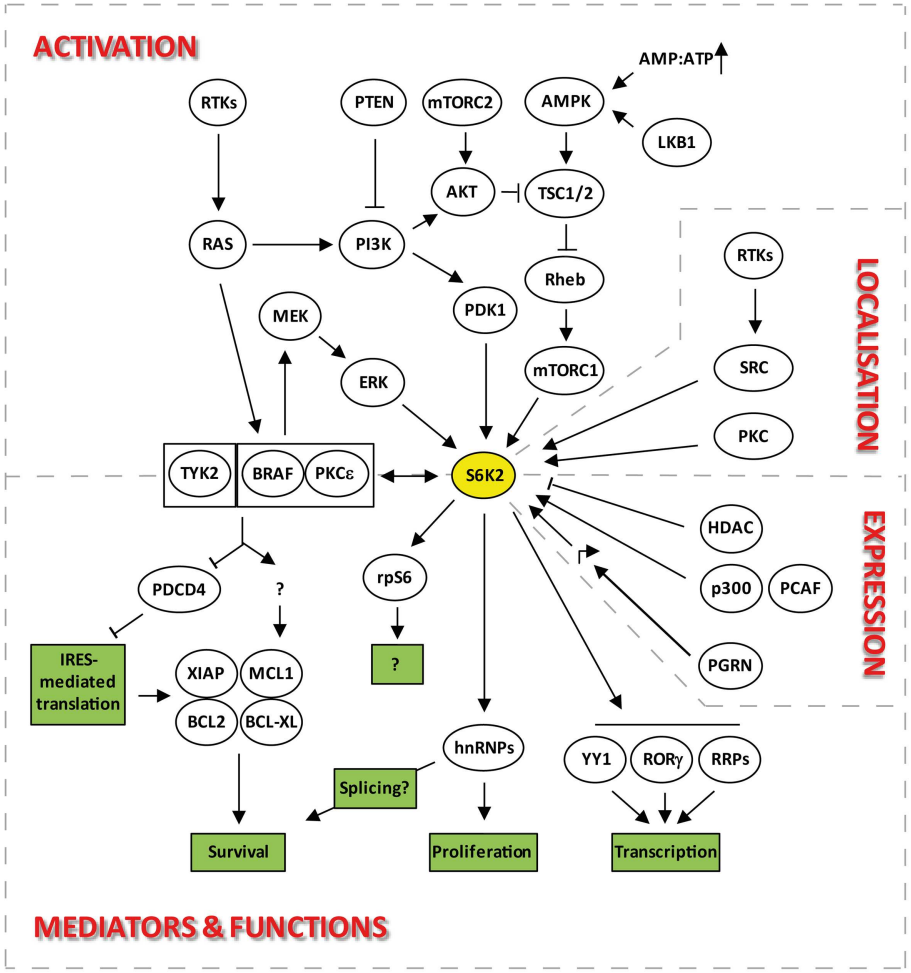

FIGURE 2 | Signaling pathways upstream and downstream of S6K2 that regulate its activation, localization, expression, and functions.

kinase C (PKC) (26). The site of phosphorylation was identified as S486 in p54 S6K2 (S486 in p56 S6K2), located within the C-terminal NLS. While phosphorylation of this site did not affect the activity of S6K2, it impaired the function of the NLS leading to cytoplasmic accumulation of the kinase upon cell stimulation with PKC agonists such as PMA. In contrast, S6K1 sub-cellular localization was not modulated by this treatment, highlighting a specific mechanism regulating S6K2 nucleo-cytoplasmic shuttling. All PKC isoforms were capable of phosphorylating S6K2, with PKCδ appearing to be the most efficient *in vitro*. However, this specificity seemed to disappear *in vivo* with all PKCs being equally potent.

In addition to being serine/threonine phosphorylated, S6K2, as well as S6K1, can be tyrosine phosphorylated downstream of receptor tyrosine kinase activation (27). Both S6Ks were found to associate with the PDGFR, HGFR, and CSFR. Upon stimulation of these receptors, N-terminal tyrosine phosphorylation of S6Ks occurred (Y39 on S6K1 and Y45 on S6K2) in a SRC-dependent manner. This event did not result in modulation of S6Ks activity or the gross redistribution of these enzymes, although a fraction of S6K1 was found to relocates to membrane ruffles where the activated RTKs are expected to reside. Although this pathway seems shared between S6K1 and 2, it is worth noting that while SRC family members were equally able to phosphorylate both isoforms *in vitro*, S6K2, but not S6K1, was tyrosine phosphorylated in response to FYN transgene expression *in vivo*. This may reflect differential wiring of these isoforms to the SRC family members through alternate cellular multi-protein complexes.

In addition to phosphorylation events, S6K2 is also the target of ubiquitination (28, 29) and of acetylation on a lysine residue close to the C-terminal PDZ binding motif (30). The latter modification does not impact on S6K2 kinase activity or sub-cellular localization but increases the stability of this kinase (see Control of S6K2 Steady-State Levels).

CELLULAR EXPRESSION AND LOCALIZATION OF S6K2

CONTROL OF S6K2 STEADY-STATE LEVELS

There is currently little to no information on the transcriptional or translational regulation of S6K2 expression. However, much more is known about the regulation of S6K2 stability. Steady-state

levels of S6K2 and 1 are regulated through the opposing effects of ubiquitination and acetylation. Indeed, degradation of S6K1 and S6K2 is mediated by ubiquitination followed by proteosomal degradation (28, 29). This is promoted by growth factor signaling in cell lines although independently of phosphorylation/activation of these kinases. Conversely, cell stress, such as that induced by UV exposure, stabilizes both proteins. However, unlike for S6K1, the molecular pathway regulating S6K2 ubiquitination is currently unknown. Indeed, while the ROC1 ubiquitin ligase was shown to specifically interact and ubiquitinate S6K1 (31), the corresponding partner for S6K2 has not yet been identified. S6K1/2 degradation is counteracted by the stabilization of this protein through a C-terminal lysine acetylation event. This occurs through interaction with the acetyltransferases p300 and p300/CBP-associated factor (PCAF) (**Figure 2**). Hence, overexpression of p300 or inhibition of deacetylases leads to an increase in the levels of both kinases (30). Interestingly, overexpression of p300 has been linked to decreased overall survival in patients suffering from a wide variety of malignancies (32–36) while that of PCAF has been associated with drug resistance (37–39). However, the role of S6K2 in these backgrounds still remains to be established. S6K1 seems to be targeted by both HDAC and sirtuins for de-acetylation, but S6K2 seems entirely dependent on HDAC showing differential regulation of these isoforms. The involvement of HDACs in this process may provide a further link between these kinases and the transcriptional machinery (see Regulation of Transcription). Moreover, the stabilization of S6K2 by acetylation together with the pro-survival and drug resistance phenotypes associated with overexpression of this kinase (see Control of Cell Survival) may hinder the efficiency of HDAC inhibitors in the clinic.

SUB-CELLULAR LOCALIZATION OF S6K2

As mentioned above, S6K2 mainly resides in the nucleus of resting cells. Closer examination reveals that it is the long form of S6K2 that is predominantly nuclear by virtue of its two NLSs. In contrast, the short form of S6K2 shuttles between the nucleus and the cytoplasm in response to growth factor signaling. In addition to its diffuse nuclear localization, a proportion of S6K2, but not S6K1, has been shown to co-localize with CTR453 and γ-tubulin at the level of the centrosome. This localization, demonstrated in multiple cell lines using both immunofluorescence and immunoblotting of purified centrosome (40), was stable throughout the cell cycle. Finally, cytoplasmic S6K2 has been shown to have a speckled distribution (40), although the structural components of these speckles remains undetermined.

TISSUE EXPRESSION OF S6K2 IN HEALTH AND DISEASE

PHYSIOLOGICAL EXPRESSION OF S6K2

S6K2 is expressed at various levels in different mouse and human tissues, and its expression levels often inversely correlate with those of S6K1 (41). In Humans, S6K2 expression was found in all tissues with the exception of the neuropil, the peripheral nervous system, and adipocytes. However, expression levels between organs vary considerably, with highest levels found in the gastrointestinal tract, the central nervous system and the lung. In contrast, most mesenchymal cells stain weakly for S6K2. Although S6K2 is detected in normal tissues, its expression levels are often very low compared to those found in corresponding tumor samples [(42) and see S6K2 Protein Levels in Cancer and Normal Corresponding Tissues].

EXPRESSION OF S6K2 IN CANCER AND ITS SIGNIFICANCE

S6K2 protein levels in cancer and normal corresponding tissues

S6K2 has been shown to be expressed in the overwhelming majority (88%) of cancer samples investigated and the level for this kinase compared between several cancer types and corresponding normal tissue. These studies demonstrated that normal tissue usually express low levels of this kinase as compared to those found in tumor samples (42–45) and that overexpression of S6K2 was more common than that of S6K1 (e.g., 80 versus 25% in breast and 18 versus 8% for endometrial cancer). In addition to changes in the levels of expression of S6K2, investigators also identified changes in the sub-cellular localization of this kinase between normal and malignant tissues. For instance, Filonenko et al. demonstrated that nuclear accumulation of S6K2 was a distinguishing feature of breast cancer cell *in situ*, whereas this kinase was only found in the cytoplasm of normal breast cells (43). Moreover, presence of S6K2 in the nucleus positively correlated with proliferating cell nuclear antigen (PCNA) and Ki-67 staining in breast cancer tissues, demonstrating a link between the presence of S6K2 in this compartment and cell proliferation (46). It is noteworthy that no such correlation existed in the case of S6K1. Interestingly, nuclear localization of S6K2 was further increased in cells localized at the periphery of the tumor where tumor cells are in contact with healthy tissue. Hence, it is tempting to speculate that the nuclear activity of S6K2 is somehow promoting tumorigenesis. The role of S6K2 in tumorigenesis is further suggested by the fact that expression levels of S6K2 in various tissues seem to influence the role of S6K1 in mediating PTEN haplo-insufficiency-driven tumorigenicity. Indeed, S6K1 downregulation impaired tumor development downstream of mTORC1 hyperactivation in *Pten*$^{\pm}$ mice only in tissues where S6K2 expression levels were low (41). Furthermore, in endometrial cancer, increased nuclear localization of S6K2 correlated with tumor grade (44), while in lung cancer, increased expression of S6K2 correlated with drug resistance (42). This would further suggest that S6K2 expression is linked to cancer progression.

Interestingly, phosphorylation of the ribosomal S6 protein was shown not to correlate with expression levels of either S6K1 or S6K2 in endometrial and breast cancer (43, 44). This lack of correlation is in contradiction with the results obtained from animal models showing that S6K2 knockout mice, unlike their S6K1 counterpart, showed a dramatic reduction in the cellular levels of S6 phosphorylation (47). Hence, the lack of correlation found in tissue samples may be an artifact generated by the modalities of tissue processing or the saturation of these phosphorylation events beyond a certain threshold of S6K expression. However, conclusions on this issue are further complicated by results obtained in knock-in mice where the five phosphorylation sites in S6 are replaced by alanine residues. Indeed, these animals show a phenotypic overlap with that of S6K1$^{-/-}$ mice including a cell growth defect associated with reduction in cell size (48). This is somehow surprising considering the lack of impact on S6 phosphorylation of knocking out S6K1 and the previously mentioned privileged

link between S6K2 and S6 phosphorylation in mice (47), a finding that our lab corroborated in human cell lines using an siRNA approach (42). However, some reports suggest that reliance of S6 phosphorylation on one or the other S6K isoform may depend on the nature of the mitogen stimulation (49). Also, these discrepancies may be explained by date failure to take into account the sub-cellular localizations of S6K1, S6K2, and S6, variables that may impact considerably on the overall phosphorylation of the latter protein. The change in sub-cellular distribution of S6K2 observed between tumor and healthy tissues would support this hypothesis (43, 44). Finally, it may be postulated that increased expression of S6K1 and S6K2 does not correlate with increased activity of these kinases.

Amplification of S6K2 in cancer

Amplification of the chromosomal region 11q13 where the *S6K2* resides is found in 15–20% of breast cancers samples studied, an event implicated in resistance to endocrine therapy (50). Amplification of *S6K2* among these samples correlated with increased mRNA levels for this kinase, ER positive status and worse prognosis (45). However, *S6K2* copy number gain and nuclear localization of the protein was related to an improved benefit from tamoxifen among patients with ER+/PgR+ tumors, while in the ER+/PgR− subgroup, nuclear S6K2 rather indicated decreased tamoxifen responsiveness. The presence of the *S6K2* amplicon correlated significantly with amplification of the 8p12 region, containing the *FGFR1*, *PPAPDC1B*, and *4EBP1* genes (50). This latter amplification was associated with increased mRNA levels for all three genes. Of the genes present in the proximal 11q13 region, *S6K2* expression alone correlated with that of *FGFR1*, *PPAPDC1B*, and *4EBP1*. Using univariate analysis, it was found that 8p12 gain/amplification was significantly associated with increased risk of distant recurrence among patients with 11q13 positive tumors. This analysis could be further refined to demonstrate that high level of *FGFR1* and *4EBP1* mRNA expression alone predicted a worse outcome in this patient group. Although co-expression of FGFR1 and S6K2 would be logical in view of the link between FGF2-mediated survival and downstream S6K2 activity (42), an associated increased expression of 4EBP1, a reported tumor suppressor, may seem surprising. However, phosphorylated 4EBP1 has been suggested to stimulate mTORC1 activity (51), a process that would in turn increase S6K2 activation. In addition to the case of breast cancer, *S6K2*, but not *S6K1*, was found amplified in about 5% of gastric carcinoma patient samples (52). This amplification was associated with a significance decrease in the overall survival of patients with advanced disease.

S6K2 variants and disease

One of the neuropathological hallmarks in Alzheimer's disease (AD) is the neurofibrillary tangles formed by hyperphosphorylated microtubule-associated Tau protein. Vazquez-Higuera et al. investigated genetic variations in a set of 20 candidates kinases involved in tau phosphorylation at sites correlating with AD (53). They reported that the distribution of the minor allele frequencies for these kinases did not differ significantly between sufferers and control groups, except for *S6K2* where variations in intron 2 was increased in patients (50%) versus controls (39%). In addition to correlating with increased risks of developing the disease, this minor allele was also associated with late onset of AD. Genetic variations in the *S6K2* gene have also been linked to the risk of developing colon cancer where they are found in tumors with SNPs in *PIK3CA*, CIMP positivity, and mutated KRAS2 (54). While CIMP positivity in colon cancer generally correlates with poor tumor differentiation and patient prognosis (55, 56), it was also shown to independently predict the survival benefit from 5-FU chemotherapy (57, 58). Hence, the polymorphism in *S6K2*, and its pathway associations, may have prognostic value and therapeutic significance. However, the true impact of these SNPs to S6K2 biological activity is yet to be experimentally confirmed.

BIOLOGICAL FUNCTIONS OF S6K2

MOUSE MODELS OF S6K2 REVEAL LITTLE OF ITS BIOLOGICAL FUNCTIONS

Knockout mice for S6K1 and S6K2, in isolation and combination, have been bred (47). Single-knockout animals were viable and born at the expected Mendelian ratio. S6K1$^{-/-}$ mice were significantly smaller than their wild-type counterpart while S6K2$^{-/-}$ animals tended to be slightly larger. The latter change is thought to result from compensatory mechanisms whereby S6K2 knockout leads to increased S6K1 activity. This possibility is independently supported by cell line-based experiments in which RNAi-mediated silencing or inhibition of S6K2 leads to increased baseline S6K1 activity (13, 42). Taken together, these data provide confirmation for the proposed role of S6K1 in the control of cell size in mammalian cell lines (59). Conversely, S6K1 knockout mice presented with increased S6K2 mRNA levels in all organs tested, a fact that may explain the observed physiological inverse correlation between the tissue expression for S6K1 and S6K2 (see Physiological Expression of S6K2). In contrast to single-knockout animals, mice lacking both S6K1 and S6K2 suffered from perinatal lethality. This was not due to defects in cell cycle progression or 5′-TOP mRNAs translation in these animals. Also, analysis of embryos at 18.5 days of gestation indicated normal Mendelian distribution. Instead, one third of the double-knockout animals were born dead and the majority of those born alive developed signs of cyanosis leading to death shortly after birth. The minority of mice surviving past the first few days following delivery succeeded in reaching adulthood with similar growth rates as the S6K1$^{-/-}$ animals and were fertile. Histopathological analysis performed to understand the reason for perinatal lethality amongst the double-knockout litters revealed no gross anatomical abnormalities. However non-viable animals showed hyperemic internal organs, occasional dilated heart chambers as well as several hemorrhagic sites. In short, none of the phenotypes observed in these animal models could be attributed to some distinct role of S6K2 and further biochemical analysis was required before biological functions of this kinase started to transpire.

REGULATION OF TRANSCRIPTION

Genome-wide microarray experiments revealed that S6K1 and 2 regulate the general transcriptional program. Indeed, 456 mRNAs were downregulated in the whole-cell extracts from starved S6K1/S6K2 double-knockout mice livers as compared to that of wild-type controls following re-feeding (60). Specifically, S6K2,

but not S6K1, has been shown to directly interact in a mitogen-inducible fashion with the general transcription factor Yin Yang 1(YY1), an association that required the C-terminal region of S6K2 (61). YY1 has been involved in a wide range of biological processes through the recruitment of general components of the transcriptional machinery such as RNA polymerase II, ATF and SP1 as well as various transcriptional co-activators and co-repressors including DNA methyltransferases (DNMTs), histone acetyl transferases (p300, CBP, PCAF), histone deacetylases (HDAC 4), protein arginine methyltransferases (PRMTs), histone-lysine *N*-methyltransferase (Ezh1/2), Sumo-conjugating enzyme (Ubc9), and ubiquitin ligases (Mdm2). The function of YY1 is regulated by post-translational modifications including phosphorylation, sumoylation, acetylation, and ubiquitination so it is possible that interaction with S6K2 results in the phosphorylation and regulation of transcriptional activity of this protein. S6K2, along with S6K1, has also been involved in the transcription of ribosomal proteins (60). Indeed, analysis of the ribosome biogenesis transcriptional program after feeding in liver cells from S6K1$^{-/-}$/S6K2$^{-/-}$ mice showed that over 75 factors involved in ribosome biogenesis were controlled by S6Ks. Importantly, these changes were also observed in knock-in mice for a non-phosphorylatable mutant of rpS6. However, this was not associated with changes in the recruitment of RNAs into polysomes, revealing a role for S6Ks in the regulation of transcription that is dissociated from the translational program. Interestingly, S6K1 and 2 were functionally redundant in this biological process as overexpression of either isoform into the double-knockout cells rescued the phenotype.

The regulation of transcription by S6K2 also plays an important role in immune cells differentiation. Indeed, the import into the nucleus of RORγ, a critical transcription factor for the differentiation of a sub-class of IL-17-secreting CD4^{+} T-helper lymphocytes, is dependent on the binding of this protein to S6K2 (**Figure 2**) (62). This occurs because RORγ, a protein that lacks an NLS, uses S6K2 to piggyback its ways into the nucleus. The interaction of S6K2 with RORγ was resistant to rapamycin treatment. However, the import of RORγ into the nucleus was rapamycin-sensitive, suggesting a role for mTORC1 in S6K2-mediated nuclear import of RORγ.

REGULATION OF PROTEIN TRANSLATION

S6K2, like S6K1, appear not to be involved in the general protein translation program. Indeed, despite the impairment in S6 phosphorylation found in cells from S6K1/2 double-knockout mice, translation of 5′-TOP mRNAs were still promoted by mitogen stimulation (47). This and other data (63, 64) indicated that S6 phosphorylation is dispensable for this process., Furthermore, these results demonstrated that other kinases lying downstream of the MAPK pathway substituted for S6K1 and S6K2 in phosphorylating two serine sites (235 and 236) on S6 in response to mitogen stimuli. This role has since been attributed to members of the RSK family. However, unlike RSKs, S6Ks are capable of catalyzing the ordered phosphorylation of the five sites present in the C-terminus of the S6 protein (S236, S235, S240, S244, S247) (65), although the physiological significance of these events still remains unclear (66). While it is clear that S6K2 does not play a major role in the cap-dependent translation of housekeeping proteins, this kinase is involved in the cap-independent translation of selective mRNAs (**Figure 2**). These, including the Bcl-XL, MCL1, and XIAP mRNAs, may be selected for regulation by S6K2 through the presence in their 5′UTR of an internal ribosome entry site (IRES) (67, 68), the activity of which S6K2 controls through the phosphorylation of IRES transactivating factors (ITAFs) such as PDCD4 (69) (see Control of Cell Survival).

CELL CYCLE REGULATION

S6K2 has been suggested to play a role in mitosis, as a pool of this kinase localizes at the centrosome and S6K2 activity peaks at the G2 and M phases of the cell cycle (70). However, it is worth noting that neither MEFs from S6K1/S6K2 double-knockout mice nor their corresponding embryonic stem cells show significant defects in cell proliferation or cell cycle distribution (47). Hence, the role of S6K2 in the cell cycle may be context dependent and limited to situations where mitogenic stimulus is applied or compensatory mechanisms are not available. Indeed, in IL3-dependent immortalized murine bone marrow-derived pro-B-cells and primary mast cells, IL3 stimulation activated S6K2, which contributed to the mitogenic effect of this growth factor (71). In these cells, S6K2 activity was able to shorten the G1 phase of the cell cycle, enabling cells to enter the S-phase at an increased rate. However, S6K2 activity alone was not able to substitute the need for IL3, as cells expressing a kinase-active S6K2 in the absence of IL3 failed to proliferate. This suggests that S6K2 is only playing a facilitating role in this process, a finding that may help explain the non-essential role of this kinase for cell cycle progression in animal models. It is worth noting that our recent collaborative research has highlighted specific S6K2 partners that mediate the effect of this kinase on the cell cycle. We showed that S6K2 binds several heterogenous ribonuclear proteins (hnRNPs) in a mitogen-inducible manner. One such RNP, hnRNPF was required for cell proliferation driven by S6K2 (72). Indeed, silencing of hnRNPF thwarted the proliferative effects of S6K2 while overexpression of this RNP increased cell proliferation in a rapamycin-sensitive manner. Consistent with the latter result, mitogen stimulation led to the recruitment of preformed S6K2-hnRNPF complexes to mTORC1.

The contradictory findings published on the role of S6K2 in the regulation of the cell cycle may also be explained by the extensive rewiring of signaling pathways occurring during tumorigenesis together with accompanying overexpression of S6K2. These may significantly change the contribution of this kinase to the promotion of mitosis, a possibility supported by the correlation between Ki-67 staining, a marker for cell proliferation, and S6K2 overexpression found in tumor samples (44). In addition, the increased nuclear localization of S6K2 in cancer (43, 44) may also impact on its pro-mitotic function. A possible increased involvement of S6K2 in the regulation of cell cycle in malignant tumors as compared to normal tissue could provide a therapeutic window for the targeting of this kinase in the treatment of cancer patients.

CONTROL OF CELL SURVIVAL

The role of S6K2 in the regulation of apoptotic cell death was first demonstrated by research from our lab (42). The serum levels for basic fibroblast growth factor (FGF2) are often elevated in patients

with a variety of malignancies and are a poor prognostic factor on uni- and multi-variate analysis (73–78). We found that treatment of lung cancer cells with FGF2, used at concentrations commonly found in the serum of carcinoma patients, promoted cell survival, and drug resistance through translational up-regulation of anti-apoptotic proteins such as Bcl-XL and XIAP (79, 80). The mRNAs for these proteins are characterized by the presence in their 5′UTR of an IRES, a three-dimensional structure that represses the efficiency of their translation. Hence, increased expression of these proteins requires the unwinding of these structures and the recruitment of ITAFs that de-represses their translation. Investigation into the signaling involved in this response revealed that S6K2, but not S6K1, was required for the increased translation of these anti-apoptotic proteins in the absence of *de novo* mRNA synthesis (42, 80). In support of this, silencing of S6K2 using siRNAs prevented FGF2-induced drug resistance as well as up-regulation of Bcl-XL and XIAP. Moreover, in un-stimulated cells, S6K2 down-regulation was accompanied by a decrease in the steady-state levels of both anti-apoptotic proteins, suggesting that this kinase is not only involved in promoting their production downstream of pro-survival signaling but also participates to their baseline translation. In contrast, silencing S6K1 had no impact on either protein whether in the presence or absence of mitogen stimulation. Conversely, overexpression of a kinase-active mutant for S6K2, but not S6K1, increased the translation of Bcl-XL and XIAP, promoted baseline cell survival and induced drug resistance in the absence of FGF2 stimulation (42). The anti-apoptotic function of S6K2 was dependent on the FGF2-inducible formation of a multi-protein complex comprising S6K2, BRAF, and PKCε (**Figure 2**). Indeed, disruption of this complex through the silencing of BRAF or PKCε prevented the pro-survival activity of S6K2. The composition of this multi-protein interaction was selective as it did not include other PKC or RAF isoforms. Similarly, S6K1 was unable to form a complex with these S6K2 partners. Tandem affinity purification using S6K2 as bait in the presence and absence of FGF2 stimulation in HEK93 cells enabled the identification of downstream mediators that regulate the translation of S6K2's anti-apoptotic targets. One such interactor, the tumor suppressor programed cell death 4 (PDCD4) that normally binds to the IRES of Bcl-XL and XIAP to repress their translation, is phosphorylated by S6K2 (69). This post-translational modification leads to the degradation of PDCD4 and subsequent derepression of Bcl-XL and XIAP translation. It is also noteworthy that hnRNPF and hnRNPH, two ribonucleoproteins previously found to regulate the differential splicing of BCL-X into the anti-apoptotic protein BCL-XL or the pro-apoptotic proteins Bcl-XS (81) were found to associate with S6K2 in a mitogen-inducible manner (72). Hence, in addition to being able to regulate the translation of this protein, S6K2 may also be able to promote the preferential splicing of BCL-X toward BcL-XL.

Further work in U2OS osteosarcoma cells demonstrated that the Janus kinase TYK2 participated to the initially identified S6K2/BRAF/PKCε complex downstream of FGF2 signaling (82). In this cell system, TYK2 was required for the induction of the anti-apoptotic proteins BCL2 and MCL1 and for the promotion of cell survival in response to this growth factor. Whether this finding extends to lung, or other, cancer cells in which FGF2 signaling is relevant to the development of chemoresistance is at this point unknown. However, it suggests that inhibition of TYK2 may represent a new therapeutic strategy to target drug resistance downstream of FGF2 signaling.

The role of S6K2 in the control of cell survival may extend to neurodegenerative disorders. Frontotemporal dementia (FTD) is a form of pre-senile dementia associated with focal atrophy of the frontal or temporal lobes accompanied by deficits in cognition, behavior, and language. Mutations in progranulin (PGRN), a protein involved in cell growth and survival (83–85), are a common cause of FTD (86). Human neurons obtained from FTD patients with mutant PGRN were shown to have reduced cell viability that correlated with a downregulation of S6K2 transcription (87). All these changes were rescued by expression of wild-type PGRN, directly linking this factor with expression of S6K2. This link may have far wider relevance as serum levels of PGRN are a clinically significant predictive marker for recurrence in patients with HR-positive breast cancer during adjuvant tamoxifen therapy (88). Also, high PGRN expression levels correlate with an aggressive phenotype in cancer cell lines and clinical specimens from gliomas, breast, ovarian, and renal cancers (83). However, the link between PGRN and S6K2 in these settings is yet to be established.

S6K2 AND COGNITION

Behavioral analysis of S6K knockout mice highlighted non-overlapping cognitive functions for S6K1 and 2. S6K1 and S6K2-deficient mice were tested for contextual fear memory, conditioned taste aversion, Morris water maze acquisition, exploratory behavior, and long-term potentiation (89). Deficit in individual S6K isoforms resulted in distinct patterns of behavioral modifications with S6K1 being associated with the most pronounced phenotype. While both isoforms participated to contextual fear memory, conditioned taste aversion, and early-phase long-term potentiation, S6K2 deficit impacted particularly on long-term contextual fear memory and reduced latent inhibition of conditioned taste aversion. However, S6K2$^{-/-}$ mice displayed normal spatial learning in the Morris water maze.

S6K2 AS A THERAPEUTIC TARGET FOR CANCER

IMPORTANT CONSIDERATIONS IN THE DESIGNS OF S6K2 TARGETING THERAPY

Since knockout mice for S6K2 have shown that this kinase was dispensable for normal development and homeostasis, this kinase may represent an excellent therapeutic target for cancer. However, several lines of evidence suggest that S6K2 targeting should be selective and not impinge on S6K1 activity. Indeed, as indicated above, unlike S6K2 single-knockout animals, S6K1$^{-/-}$/S6K2$^{-/-}$ mice displayed perinatal lethality (47), suggesting that acute inhibition of both isoforms simultaneously in the absence of compensatory mechanisms may be deleterious to normal homeostasis. This is confirmed by work done in *Drosophila* where disruption of the unique S6K gene, *dS6K*, results in the death of the majority of flies at the larval stage or early pupation (90). Moreover, the S6Ks have been involved in negative feedback loops that regulate the PI3K and mTOR pathways. First, it was found that S6K1 and S6K2 phosphorylate IRS-1 on serine 302, a site adjacent to its PTB domain. This phosphorylation event inhibits the binding of IRS-1 to the insulin receptor, preventing further stimulation

of the PI3K pathway by insulin (91, 92). Conversely, silencing of S6K1 using siRNAs was shown to decrease IRS-1 phosphorylation on several serine residues leading to an increase in PI3K/AKT signaling (93). In addition to targeting IRS-1, it has recently been revealed that S6K1, but not S6K2, modulates the activation of AKT by phosphorylating the mTORC2 complex component RICTOR on T1135 (49, 94, 95). This phosphorylation event does not seem to directly modulate the kinase activity of the mTORC2 complex. However, expression of a phospho-deficient mutant version of RICTOR promoted phosphorylation of AKT on S473, a site associated with activation of this kinase (49). Hence, considering the well-documented function of AKT in tumorigenesis (96) and the lethality of S6K double-knockout animals, a therapeutic strategy targeting both S6K isoforms in cancer patient may not be advisable. This hypothesis is further supported by results obtained using the pan-S6K inhibitor, LYS6K2, which increases basal and mitogen-induced AKT phosphorylation in treated cell lines (97). Conversely, the use of rapamycin analogs to inhibit the mTOR pathway, an approach that has been the subject of numerous cancer clinical trials (98), would not be appropriate in targeting the biological effects of S6K2 as some of the functions of this kinase are resistant to this inhibitor (13, 42, 79). Hence, it would be highly desirable to develop S6K2-specific compounds. However, at present, no drug discovery project has attempted to specifically target S6K2.

SMALL-MOLECULE ATP COMPETITORS

While S6K kinase inhibitors do exist, these are either pan-S6K compounds that also target other AGC kinases [e.g., Ref. (99, 100)] or compounds that show relative selectivity for S6K1 (101, 102). It was initially thought that the high degree of identity between the kinase domains of S6K1 and S6K2 would prevent the development of S6K2-selective kinase inhibitors. However, the existence of "selective" S6K1 inhibitors (101, 102) together with data acquired through 3-D modeling suggest that the development of such compounds would be possible. Indeed, comparison of the crystal structure of the kinase domain of S6K1 bound to Staurosporine (103) with the 3-D model of the same region in S6K2 reveals that the two kinases may differ in their contact with this inhibitor. This divergence occurs at the level of cysteine 150, a residue within the hinge region of S6K2 and may be exploitable to tweak selectivity of pan-S6K compounds toward this isoform. In support for this idea, the modulation of interaction of chemical compounds with the hinge region has previously been exploited to introduce selectivity among kinase inhibitors (104). Nevertheless, the high level of homology between the kinase domains of AGC kinases family members will always render the broader selectivity of these compounds hard to secure, especially *in vivo* where their intracellular concentration cannot be easily controlled. Hence, other approaches for the selective inhibition of S6K2 should also be considered.

PROTEIN–PROTEIN INTERACTION INHIBITORS

We propose that the development of protein–protein interaction inhibitors would be a more appropriate strategy to specifically target S6K2. Indeed, drug discovery efforts into protein–protein interaction inhibitors have multiplied in the last few years [e.g., Ref. (105–109) to only cite a few] and some have already yielded compounds that entered the clinic (105). The heightened interest in this approach has been fueled by the relative lack of success of small-molecule kinase inhibitors in the clinic, together with the development of novel bioinformatics tools to predict disruption of protein–protein interactions (110, 111). We and others have performed co-purification of S6K2 with its interacting partners downstream of mitogen stimulation in various cell lines [(69, 72) and unpublished], an effort that resulted in the identification of S6K2-specific interactors that regulate the biological functions of this kinase (42, 69, 72). For instance, interaction of S6K2 with BRAF and PKCε was shown to regulate the anti-apoptotic function of this kinase while that of S6K2 and hnRNPF modulated its effect on the cell cycle. Hence, one could envision drug discovery efforts aimed at targeting these identified interactions.

ALLOSTERIC S6K2 INHIBITORS

Two more characteristics of S6K2 may be potentially exploitable for the development of selective compounds. The first is its C-terminal domain which differs significantly from the same region in S6K1 and is predicted through 3-D modeling to be fairly unstructured and exposed. This region could be used to fish out interacting compounds that may, through binding, alter the 3-D conformation of S6K2. As this region also contains the NLS for S6K2, interacting compounds may also interfere with its subcellular localization. The second S6K2 characteristic is its higher reliance for activation on the MEK/ERK-mediated derepression from the pseudo-substrate domain. Compounds that would bind to the kinase extension domain or hinder access to the proline-directed phosphorylation sites may prevent S6K2 from exposing the serine/threonine sites responsible for its activation to upstream kinases.

CONCLUSION

It has now been 15 years since the cloning of S6K2. However, many aspects of the regulation and biological functions of this enzyme are still a mystery. Indeed, the high degree of homology between S6K1 and S6K2 led us to bias most of our research toward the first of these two isoforms, as these were thought to have identical roles. Instead, it is now becoming clear that these enzymes have distinct biological functions mediated by their distinct repertoires of substrates and interactors. Our limited knowledge of S6K2 suggests that this isoform may play a particularly important role in the pathobiology of cancer and targeting this isoform could provide a therapeutic benefit in patients. However, this will most probably require the development of S6K2 isoform-selective compounds that exploit its known specific protein–protein interactions and downstream substrates that mediate its functions. The time has come to embrace S6K2, recognize the biological diversity that this enzyme introduces into the S6 kinase pathway and exploit this information for new therapies.

ACKNOWLEDGMENTS

Michael J. Seckl is supported by the Department of Health and Cancer Research UK funded Imperial College NIHR/Biomedical Research Center and Imperial Experimental Cancer Medicine Center. Olivier E. Pardo and Michael J. Seckl are funded by the European Union FP7 as part of the LungTarget consortium.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 22 May 2013; paper pending published: 11 June 2013; accepted: 08 July 2013; published online: 24 July 2013.
Citation: Pardo OE and Seckl MJ (2013) S6K2: the neglected S6 kinase family member. Front. Oncol. 3:191. doi: 10.3389/fonc.2013.00191
This article was submitted to Frontiers in Molecular and Cellular Oncology, a specialty of Frontiers in Oncology.
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frontiers in ONCOLOGY

REVIEW ARTICLE
published: 14 April 2014
doi: 10.3389/fonc.2014.00064

Targeting PI3K/Akt/mTOR signaling in cancer

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The phosphatidylinositol-3-kinase (PI3K)/Akt and the mammalian target of rapamycin (mTOR) signaling pathways are two pathways crucial to many aspects of cell growth and survival, in physiological as well as in pathological conditions (e.g., cancer). Indeed, they are so interconnected that, in a certain sense, they could be regarded as a single, unique pathway. In this paper, after a general overview of the biological significance and the main components of these pathways, we address the present status of the development of specific PI3K, Akt, and mTOR inhibitors, from already registered medicines to novel compounds that are just leaving the laboratory bench.

Keywords: PI3K, Akt, mTOR, inhibitors, temsirolimus, everolimus, ridaforolimus, novel agents

INTRODUCTION

The phosphatidylinositol-3-kinase (PI3K)/Akt and the mammalian target of rapamycin (mTOR) signaling pathways are both crucial to many aspects of cell growth and survival, in physiological as well as in pathological conditions. They are so interconnected that, in a certain sense, they could be regarded as a single, unique pathway (**Figure 1**) that, in turn, heavily interacts also with many other pathways, including that of the hypoxia inducible factors (HIFs).

The PI3K/Akt pathway is a key regulator of survival during cellular stress (1). Since tumors exist in an intrinsically stressful environment (characterized by limited nutrient and oxygen supply, as well as by low pH), the role of this pathway in cancer appears to be crucial.

Mammalian target of rapamycin is a serine/threonine kinase ubiquitously expressed in mammalian cells (2). It picks up and integrates signals initiated by nutrient intake, growth factors, and other cellular stimuli to regulate downstream signaling and protein synthesis. Through its downstream effectors, 4EBP1 and P70S6 kinase (S6K), it is involved in the initiation of ribosomal translation of mRNA into proteins necessary for cell growth, cell cycle progression, and cell metabolism.

Somatic mutations and/or gains and losses of key genes are among a number of genetic alterations affecting these pathways in a number of different solid and hematological tumors [including big killers such as breast and colon cancer, as well as relatively less frequent neoplasms such as neuroendocrine tumors (NETs), kidney cancer, and some lymphomas]. The activation of the PI3K/Akt/mTOR pathway results in a profound disturbance of control of cell growth and survival, which ultimately leads to a competitive growth advantage, metastatic competence, angiogenesis, and therapy resistance.

Thus, this complex pathway has been taken into consideration as one of the most attractive targets for the development of anticancer agents (3, 4).

PI3K STRUCTURE AND FUNCTIONS

Phosphatidyl-inositol-3-kinases (PI3Ks) constitute a lipid kinase family characterized by the capability to phosphorylate inositol ring 3′-OH group in inositol phospholipids (5). Class I PI3Ks are heterodimers composed of a catalytic (CAT) subunit (i.e., p110) and an adaptor/regulatory subunit (i.e., p85).

This class is further divided into two subclasses: subclass IA (PI3Kα, β, and δ), which is activated by receptors with protein tyrosine kinase activity, and subclass IB (PI3Kγ), which is activated by receptors coupled with G proteins (5).

Activation of growth factor receptor protein tyrosine kinases results in autophosphorylation on tyrosine residues. PI3K is then recruited to the membrane by directly binding to phosphotyrosine consensus residues of growth factor receptors or adaptors through one of the two SH2 domains in the adaptor subunit. This leads to allosteric activation of the CAT subunit. In a few seconds, PI3K activation leads to the production of the second messenger phosphatidylinositol-3,4,5-triphosphate (PI3,4,5-P_3) from the substrate phosphatidylinositol-4,4-bisphosphate (PI-4,5-P_2). PI3,4,5-P_3 then recruits a subset of signaling proteins with pleckstrin homology (PH) domains to the membrane, including protein serine/threonine kinase-3′-phosphoinositide-dependent kinase 1 (PDK1) and Akt/protein kinase B (PKB) (5, 6). Akt/PKB, on its own, regulates several cell processes involved in cell survival and cell cycle progression.

As far as cell survival is concerned, Akt/PKB can inactivate pro-apoptotic factors such as Bad and Procaspase-9, as well as the Forkhead family of transcription factors that induce the expression

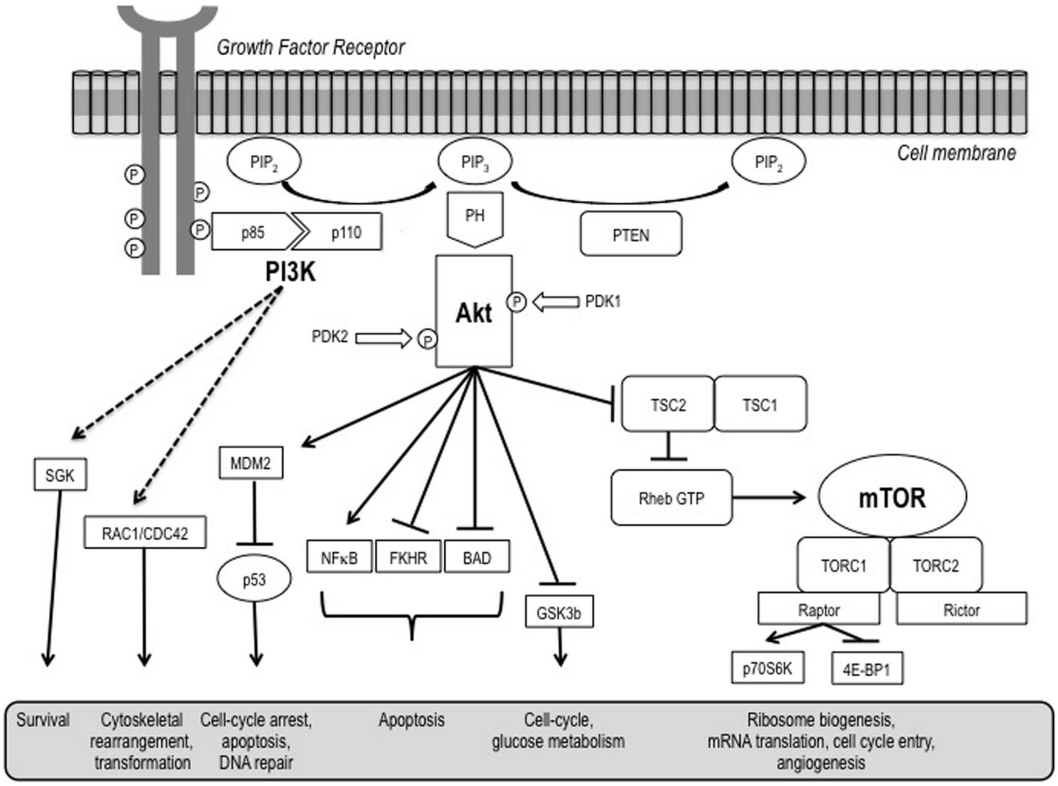

FIGURE 1 | A schematic representation of the PI3K/Akt/mTOR pathway.

of other pro-apoptotic factors, such as Fas-ligand (FasL) (7, 8). Akt/PKB activation has been related to an increased resistance of prostate cancer cells to apoptosis mediated by tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL)/APO-2L (9). Finally, Akt/PKB also activates the IκB kinase (IKK), a positive regulator of the survival factor NFκB. Notably, a strong biological link between the NFκB and the PI3K/Akt pathways in the modulation of anti-apoptotic effects in lymphoma cells exposed to the irreversible inhibitor of the activation of NFκB and the phosphorylation of IκBα BAY11-7085 has been also shown (10).

As for cell cycle progression and cell growth, several targets of Akt are involved in protein synthesis, glycogen metabolism, and cell cycle regulation (6), including the same mTOR, glycogen synthase kinase-3 (GSK3), insulin receptor substrate-1 (IRS-1), the cyclin-dependent kinase inhibitors $p21^{CIP1/WAF1}$ and $p27^{KIP1}$, and possibly also Raf-1, a member of the MAPK pathway. With regard to GSK3, Akt/PKB triggers a network that positively regulates G1/S cell cycle progression through inactivation of GSK3-β, leading to increased cyclin D1, and inhibition of the Forkhead family transcription factors and the tumor suppressor tuberin (TSC2), ultimately resulting in the reduction in p27Kip1 (11).

Akt STRUCTURE AND FUNCTIONS

Akt kinases belong to the AGC kinase family, related to AMP/GMP kinases and protein kinase C. They consist of three conserved domains, an N-terminal PH domain, a central kinase CAT domain, and a C-terminal extension (EXT) containing a regulatory hydrophobic motif (HM) (12). Among the Akt isoforms, the PH domains are ~80% identical and ~30% identical to PH domains in pleckstrin and other proteins. The linker (LINK) region connecting the PH domain to the CAT domain is poorly conserved among the Akt isoforms (17–46% identical) and has no significant homology to any other human protein (12). The consensus CAT domain is ~90% identical among the Akt isoforms and is closely related the PKC, PKA, SGK, and S6 subfamilies of the AGC kinase family (12). The C-terminal EXT is ~70% identical among the Akt isoforms and is most closely related to the PKC family (12).

mTOR STRUCTURE AND FUNCTIONS

Mammalian target of rapamycin is a key protein evolutionarily conserved from yeast to man and is essential for life. Indeed, embryonic mutations in mTOR proved to be lethal.

In normal cells, mTOR activity is controlled by positive and negative upstream regulators (13). Positive regulators include growth factors and their receptors, such as insulin-like growth factor-1 (IGF-1) and its cognate receptor IFGR-1, members of the human epidermal growth factor receptor (HER) family and associated ligands, and vascular endothelial growth factor receptors (VEGFRs) and their ligands, which transmit signals to mTOR through the PI3K-Akt. Negative regulators of mTOR activity include phosphatase and tensin homolog (PTEN) that inhibits signaling through the PI3K-Akt pathway, and tuberous sclerosis complex (TSC) 1 (hamartin) and TSC2 (tuberin). Phosphorylation of TSC2 by Akt releases its inhibitory effect on mTOR and

up-regulates mTOR activity. Another negative regulator, LKB1, is in an energy-sensing pathway upstream of TSC (14).

Mammalian target of rapamycin activity is carried out by two distinct complexes: mTORC1 and mTORC2.

The mTORC1 complex is made up of mTOR, Raptor, mLST8, and PRAS40. It is extremely sensitive to rapamycin and thus represents the target of first-generation mTOR inhibitors. It also activates S6K and inactivates 4EBP1, leading to protein translation and cell growth (13).

The mTORC2 complex is composed of mTOR, Rictor, Sin1, and mLST8. It is less sensitive to rapamycin and its role in normal cell function and oncogenesis has not been well clarified. However, it is known to activate Akt, thereby promoting cell proliferation and survival. The canonical pathway of mTOR activation depends on mitogen-driven signaling through PI3K/Akt, although alternative non-Akt dependent activation through the Ras/MEK/ERK pathway is now recognized (15).

Altogether, mTOR activation leads to increased synthesis of multiple proteins. These include several that have been implicated in the pathogenesis of multiple tumors, e.g., cyclin D1, which allows progression of cells through the cell cycle (16), and HIF, which drive the expression of pro-angiogenic growth factors such as VEGF (17).

PTEN AS A REGULATOR OF THE PI3K/Akt/mTOR PATHWAY

The PTEN deleted on chromosome 10 (PTEN) is a key molecule downstream of the PI3K/Akt pathway. This phosphatase, endowed with dual activity on both lipids and proteins, acts as a tumor suppressor by inhibiting cell growth and enhancing cellular sensitivity to apoptosis and anoikis, i.e., an epithelial cell-peculiar type of apoptosis triggered by alterations in integrin–extracellular matrix interactions (18).

Phosphatase and tensin homolog is frequently mutated in several advanced human cancers. In addition, PTEN mutations in germ cell lines result in the rare hereditary syndrome known as Cowden's disease, which is associated with a higher risk of different cancers, including breast, thyroid, and endometrial cancers (19).

The main lipid substrate of PTEN is PI3,4,5-P_3, and indeed PTEN acts as a negative regulator of PI3K/Akt signaling. Thus, loss of PTEN activity leads to permanent PI3K/Akt pathway activation.

DEVELOPMENT OF mTOR INHIBITORS AS ANTICANCER AGENTS

Rapamycin (sirolimus), an antifungal agent with immunosuppressive properties, was first isolated in 1975 from the soil of the island of Rapa Nui or Easter Island (20). Already back in the 1980s, when tested against a panel of human cancer cell lines, rapamycin showed a broad anticancer activity (21). However, clinical development of rapamycin as an anticancer agent was hampered by unfavorable pharmacokinetic properties (22).

The relatively recent development of rapamycin analogs endowed with a more favorable pharmacokinetic profile, i.e., temsirolimus, everolimus, and ridaforolimus (a.k.a. deforolimus), opened up the present era of mTOR inhibitors as anticancer agents.

All these agents have similar structure (**Figure 2**) and mechanism of action, but different pharmacokinetic properties. Indeed, all these drugs are small molecule inhibitors that function intracellularly, forming a complex with the FK506 binding protein-12 (FKBP-12) that is then recognized by mTOR. The resulting complex prevents mTOR activity, leading to inhibition of cell cycle progression, survival, and angiogenesis. Notably, all these inhibitors are similar to the parental compound rapamycin in that they affect only mTORC1, and not mTORC2 (22).

TEMSIROLIMUS: PHASE III TRIALS

Temsirolimus is a pro-drug whose primary active metabolite is rapamycin. Temsirolimus is administered intravenously on a once-weekly schedule (23). It has been approved for the treatment of patients with advanced renal cell carcinoma (RCC) with poor prognostic features, and of mantle cell lymphoma (MCL) patients.

RENAL CELL CARCINOMA

Temsirolimus registration in RCC was obtained on the basis of the positive results of a randomized, controlled, phase III trial of temsirolimus, interferon-α, or a combination of the two (24). In this study, 626 patients with previously untreated, poor-prognosis, metastatic RCC were randomized to receive 25 mg of intravenous (i.v.) temsirolimus weekly, 3 MU of interferon-α (with an increase to 18 MU) subcutaneous (s.c.) three times weekly, or a combination-therapy with 15 mg of temsirolimus weekly plus 6 MU of interferon-α three times weekly. Overall survival (OS) was the primary endpoint of the trial. Patients who received temsirolimus alone had longer OS and progression-free survival (PFS) than patients who received interferon-α alone, while there was no significant difference in OS between the combination-therapy group and the interferon group. Indeed, median OS in the temsirolimus group, the interferon-α group, and

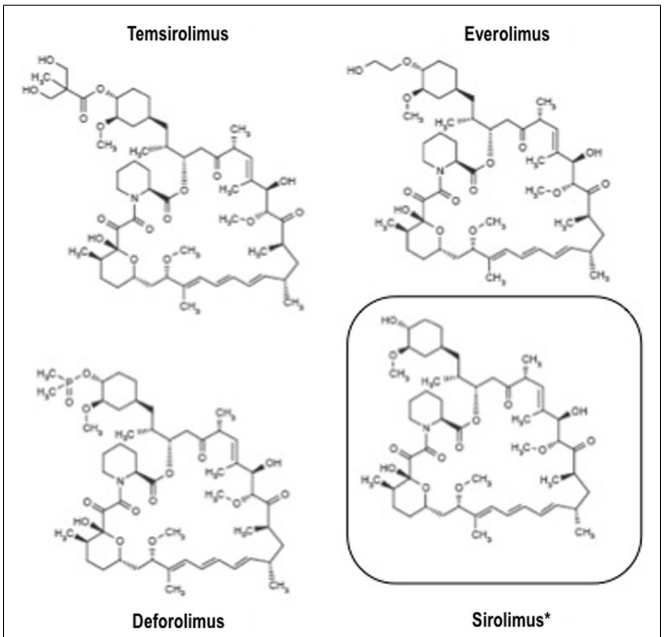

FIGURE 2 | Structure of clinically available mTOR inhibitors.

the combination-therapy group were 10.9, 7.3, and 8.4 months, respectively (24).

MANTLE CELL LYMPHOMA

As far as MCL is concerned, the pivotal registration trial was a phase III trial evaluating two dose regimens of temsirolimus in comparison with single-agent therapy in relapsed or refractory disease (investigator's choice) (25). Sixty-two patients with relapsed or refractory MCL were randomly assigned to receive one of two temsirolimus regimens: 175 mg weekly for 3 weeks followed by either 75 mg (175/75 mg) or 25 mg (175/25 mg) weekly, or investigator's choice therapy from approved options. PFS was the primary endpoint of the study. Median PFS was 4.8, 3.4, and 1.9 months for the temsirolimus 175/75, 175/25 mg, and investigator's choice groups, respectively (25). Patients treated with temsirolimus 175/75 mg had significantly longer PFS than those treated with investigator's choice therapy [$p = 0.0009$; hazard ratio (HR) = 0.44]. Furthermore, objective response rate (ORR) was significantly higher in the 175/75-mg group (22%) compared with the investigator's choice group (2%; $p = 0.0019$), while there was no statistical difference in OS (25).

RIDAFOROLIMUS: PHASE III TRIAL

Ridaforolimus is not a pro-drug, but like temsirolimus, it was originally administered intravenously on an intermittent schedule, while an oral formulation has also been subsequently developed (26, 27).

MAINTENANCE TREATMENT FOR ADULT SOFT TISSUE AND BONE SARCOMAS

Recently, a large randomized, placebo-controlled, phase III trial was carried out aiming to evaluate ridaforolimus activity as a maintenance treatment in advanced sarcomas (28). In this study, 711 patients with metastatic soft tissue or bone sarcomas who achieved an objective response or at least a stable disease after standard chemotherapy were randomly assigned to receive ridaforolimus 40 mg or placebo once per day, per oral administration (o.s.) for 5 days every week. The primary endpoint was PFS. Overall, ridaforolimus treatment led to a modest, although statistically significant, improvement in PFS compared with placebo (17.7 vs. 14.6 weeks; HR: 0.72; 95% CI: 0.61–0.85; $p = 0.001$) (28).

EVEROLIMUS: PHASE III TRIALS

Everolimus is another orally available mTOR inhibitor that is usually administered on a continuous daily schedule (even though a weekly schedule has been also tested, especially for combination regimens) (29).

RENAL CELL CARCINOMA

Everolimus has recently been approved by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) for the treatment of advanced RCC after failure of treatment with Sunitinib and/or Sorafenib, following the presentation of the results of the RECORD-1 trial.

RECORD-1 was a phase III double-blind, randomized, placebo-controlled trial aimed at evaluating the activity of everolimus in patients whose disease had progressed under treatment with one or two VEGFR tyrosine kinase inhibitors (TKIs) (30). A total of 416 patients were enrolled and stratified according to the number of previous treatments [Sorafenib or Sunitinib (1 TKI) vs. Sorafenib as well as Sunitinib (2 TKIs)] and prognostic risk group. Patients were then randomized in the ratio of 2 to 1 to receive everolimus (given at the standard dose of 10 mg daily, per o.s.) plus best supportive care (BSC), or to placebo plus BSC. After the second interim analysis, the study was terminated since the pre-specified efficacy endpoint had been met (30). Indeed, at the final trial analysis, everolimus proved able to significantly improve PFS when compared to placebo: 4.9 vs. 1.9 months, respectively (HR: 0.33; 95% CI: 0.25–0.43; $p < 0.001$) (31). Furthermore, everolimus significantly increased median PFS in each risk group regardless of whether patients had received one or two prior TKIs (32), had stopped prior therapy for intolerance (33), or of patient age (34).

NEUROENDOCRINE TUMORS

As most NETs are hypervascular (35) and synthesize and secrete high levels of VEGF-A (36, 37), targeted (such as everolimus and sunitinib) and untargeted (such as somatostatin analogs, interferon-α, and thalidomide) therapies, with certain or possible anti-angiogenic properties, have been tested in metastatic NET.

Everolimus, in association with octreotide LAR, first demonstrated a promising antitumor activity in a phase II trial with 30 low- to intermediate-grade NET (carcinoids) patients, showing 17% of partial remission and 80% of stable disease, added to a median PFS of 15.7 months (38).

The open-label, phase II trial RADIANT-1 enrolled 160 advanced, low- or intermediate-grade pancreatic NET (pNET) patients, with progressive (according to RECIST criteria) disease during or after cytotoxic chemotherapy (39). One hundred and fifteen patients were assigned to everolimus 10 mg/day o.s., and 45 patients were submitted to everolimus 10 mg/day o.s. + octreotide LAR 30 mg/28 days intramuscular (i.m). The response rates were 9.6% in the everolimus arm and 4.4% in the everolimus + octreotide LAR group. Median PFS by central radiology review were 9.7 months for patients receiving everolimus and 16.7 months for those receiving the combination (39). Furthermore, high baseline levels of chromogranin A and neuron-specific enolase circulating neuroendocrine markers were associated with shorter median PFS and OS (40).

The favorable results of these previous phase II trials were then confirmed in two international, multicenter, randomized, placebo-controlled, phase III studies (RADIANT-2 and RADIANT-3).

In the RADIANT-2 study (41), 429 patients with advanced progressive midgut NETs were randomized to receive everolimus 10 mg/day plus octreotide LAR 30 mg/month or octreotide LAR plus placebo. A clinically significant improvement in PFS was recorded in the everolimus arm compared with octreotide LAR/placebo arm (16.4 vs. 11.3 months, respectively), even though the pre-defined threshold for statistical significance was not reached, according to central radiological reading (41). A subsequent multivariate analysis and the local radiological reading sustained the efficacy of everolimus. Furthermore, a subgroup analysis underlined some potential primary tumor sites in particular that could benefit, such as bronchial/lung NETs or colonic NETs (42). Nevertheless, the precise therapeutic activity

of everolimus in advanced progressive midgut NETs remained to be defined (43).

In RADIANT-3 (44), the largest clinical trial conducted in pNET patients, 410 patients with advanced pNET and progressive disease were randomly assigned to treatment with oral everolimus 10 mg/day or placebo. Octreotide LAR was administered at the discretion of the investigator. Everolimus was associated with an improvement in median PFS compared with placebo (11.0 vs. 4.6 months, respectively; $p < 0.0001$), and with an overall tumor response rate of 5% (44). The most common drug-related toxicities were G1–2 stomatitis or aphthous ulceration (44). Furthermore, everolimus therapy correlated with a reduction in VEGF pathway markers, such as soluble VEGF receptor 2 and placental growth factor, suggesting an anti-angiogenic activity of everolimus in pNET patients (45).

Even though everolimus evidently inhibited tumor growth and delayed time-to-progression, the percentages of progression events (i.e., appearance of new metastasis as the only cause of progression, appearance of new metastasis concurrent with progression of preexisting metastases, lesion growth at baseline without new metastases appearing) in the two arms (everolimus, placebo) were similar, suggesting that everolimus delayed tumor progression without affecting the pattern of progression in advanced pNET patients (46).

Following the RADIANT-3, in 2011, everolimus was approved for the treatment of progressive pNETs, but its efficacy in other NETs remains uncertain. Given that RADIANT-2, including 51% of small intestinal carcinoids, failed to achieve its primary endpoint, a placebo-controlled trial with everolimus as monotherapy in progressive gastro-intestinal and lung carcinoids (RADIANT-4) is now ongoing. In the meantime, NCCN guidelines recommend everolimus among several therapeutic options in clinically significant progressive NETs, and by ENETS guidelines in non-pNETs with progressive disease after all other medical treatments (43).

BREAST CANCER

Recently, the combination of everolimus with the aromatase inhibitor Exemestane has been evaluated in a randomized, phase III trial, since a large amount of evidence supported the hypothesis that aberrant signaling through the mTOR pathway is associated with resistance to endocrine therapies (47).

In the BOLERO-2 phase III trial (48), 724 patients with hormone-receptor-positive advanced breast cancer who recurred or progressed while receiving treatment with a non-steroidal aromatase inhibitor in the adjuvant or metastatic setting, were randomized two to one to receive everolimus and exemestane or exemestane and placebo. PFS was the primary endpoint of the study. A pre-planned interim analysis was performed by an independent data and safety monitoring committee after 359 PFS events were observed. At the time of this analysis, median PFS assessed by the Investigators PFS was 6.9 months with everolimus plus Exemestane and 2.8 months with Placebo plus Exemestane (HR: 0.43; 95% CI: 0.35–0.54; $p < 0.001$), while centrally assessed PFS was 10.6 and 4.1 months, respectively, again in favor of the everolimus-containing combination (HR: 0.36; 95% CI: 0.27–0.47; $p < 0.001$) (48).

OTHER INDICATIONS

Everolimus also has an established role in the treatment of two rare conditions: renal angiomyolipomas associated with TSC or lymphangioleiomyomatosis, as well as TSC-related subependymal giant cell astrocytoma (SEGA), both characterized by constitutive activation of the mTOR pathway (49).

In the EXIST-2 double-blind, placebo-controlled, phase III trial (50), 118 patients with at least one angiomyolipoma 3 cm or larger in its longest diameter and a definite diagnosis of TSC or sporadic lymphangioleiomyomatosis were randomly assigned 2:1 to either everolimus 10 mg/day or Placebo. The primary efficacy endpoint of the study was the proportion of patients with confirmed angiomyolipoma response of an at least 50% reduction in total volume of target angiomyolipomas relative to baseline. Response rate (as defined above) was 42% [33 of 79 (95% CI: 31–53%)] for everolimus and 0% [0 of 39 (95% CI: 0–9%)] for placebo ($p < 0.0001$) (50), thus suggesting the usefulness of everolimus in this setting.

Similarly, in the EXIST-1 double-blind, placebo-controlled, phase III trial (51), 117 patients were randomized in a 2:1 ratio to everolimus 4.5 mg/m^2/day (titrated to achieve blood trough concentrations of 5–15 ng/mL) or Placebo. Eligible patients had a definite diagnosis of TSC and at least one lesion with a diameter of 1 cm or greater, and either serial growth of an SEGA, a new lesion of 1 cm or greater, or new or worsening hydrocephalus. The primary endpoint of this study was the proportion of patients with confirmed response, i.e., a reduction in target volume of 50% or greater relative to baseline in SEGA. Twenty-seven (35%) patients in the everolimus group had an at least 50% reduction in SEGA volume as compared to none in the Placebo group (95% CI: 15–52; $p < 0.0001$) (51).

Taken together, these two studies suggest the possibility that everolimus might represent a disease-modifying treatment also for other aspects of tuberous sclerosis.

THE SAFETY PROFILE OF mTOR INHIBITORS

Adverse events observed in patients treated with mTOR inhibitors are fairly constant, irrespective of each specific indication. They include cutaneous and mucosal events (i.e., stomatitis and skin rash), pulmonary dysfunction (non-infectious pneumonitis), metabolic abnormalities (elevated blood levels of glucose, cholesterol, and triglycerides), as well immune-related events (i.e., increased incidence of infections) (52). As far as the risk of infections is concerned, we should not forget that mTOR inhibitors were first developed as immune suppressive agents and are still widely used as such in the transplantation setting.

Metabolic and immune-related adverse events are clearly on-target effects of mTOR inhibition, while cutaneous and mucosal effects may have a less direct association with mTOR inhibition, although inhibition of mTOR-mediated growth and tissue repair and/or immune dysregulation have been proposed to be a factor in mucosal epithelia with high turnover (53, 54).

In general, the incidences of key class-effect adverse events in the three largest phase III trials of everolimus (i.e., RECORD-1 in RCC, RADIANT-3 in pNET, and BOLERO-2 in hormone receptor-positive, HER2-negative, advanced breast cancer) were comparable (30, 44, 48), as summarized in **Table 1**.

Table 1 | Incidence of the main adverse events (all grades and grade 3/4) reported in the three largest phase III studies of everolimus in advanced solid tumors (RCC, pNET, and breast cancer).

	RCC (27)		pNET (41)		Breast cancer (46)	
	Everolimus + BSC[a] ($n = 274$)		Everolimus monotherapy ($n = 204$)		Everolimus + exemestane ($n = 482$)	
	All grades (%)	Grade 3/4 (%)	All grades (%)	Grade 3/4 (%)	All grades (%)	Grade 3/4 (%)
Stomatitis	44	4	64	7	59	8
Rash	29	1	49	<1	39	1
Non-infectious pneumonitis	14	4	17	2	16	3
Hyperglycemia	57	15	13	5	14	5
Infections	37	10	23	2	50	5

[a] *BSC, best supportive care.*

THE DEVELOPMENT OF PI3K AND Akt INHIBITORS AS ANTICANCER AGENTS

In contrast to the three mTOR inhibitors discussed above, PI3K and Akt inhibitors are still at an early development phase, and so far no compound has reached the bedside. Despite this, three generations of compounds targeting PI3K have already been developed over time.

PI3K AND DUAL PI3K/mTOR INHIBITORS

The first-generation of PI3K inhibitors included compounds like Wortmannin and LY294002, which were able to bind all class I PI3Ks, thus being called "pan-inhibitors." These compounds have been widely used in pre-clinical models to better characterize this complex pathway. However, due to very poor pharmacokinetic properties, they were never fully developed as anticancer drugs for clinical use (55).

More recently, compounds with better pharmacokinetic properties have been developed and are currently being evaluated within clinical trials in several malignancies (55), including genitourinary cancers (56) and others. These second-generation inhibitors are characterized by greater and isoform-specific selective activity (55).

The third generation of compounds comprises the so-called "dual PI3K/mTOR inhibitors." These were developed after consideration that the CAT sites of PI3K and mTOR share a high degree of sequence homology. The potential advantage of these novel compounds (an advantage which still has to be confirmed *in vivo*) is that they inhibit not only all PI3K class I isoforms, but also mTORC1 and (more notably) mTORC2. In theory, this combined activity would lead to the strongest inhibition of the whole PI3K/Akt/mTOR pathway (57).

A list of PI3K inhibitors in pre-clinical and clinical development is reported in **Table 2**.

Akt INHIBITORS

So far, compounds that target Akt ATP binding site, its PH domain, LINK, and the protein substrate sites have been developed (12, 58). Compared to PI3K, and especially mTOR inhibitors, fewer Akt-targeting agents have entered clinical development (58), even though one of them, Miltefosine, has already completed a phase III trial (59). A list of Akt inhibitors under pre-clinical and clinical development is given in **Table 3**.

MECHANISMS OF RESISTANCE TO PI3K/Akt/mTOR INHIBITORS

Despite all the successes (achieved with mTOR inhibitors) and expectations (related to novel anti-PI3K and Akt drugs), none of the above drugs is currently able to cure a single patient with cancer.

As with all antineoplastic agents, this is mainly due to the development of resistance. The underlying molecular basis of resistance, either intrinsic or acquired, remains largely unknown and has not been well characterized. So far, multiple mechanisms of resistance to targeted agents have been proposed, including secondary target mutations, activation of alternative, parallel, signaling pathways, and amplification of downstream alterations within the same pathway (60).

Resistance to mTOR inhibitors has been at least partially clarified. Indeed, it is often linked to different negative feedback loops. In one loop, mTORC1 inhibition leads to upregulation of receptor tyrosine kinases (RTKs or substrates) such as platelet-derived growth factor receptors (PDGFRs) and insulin receptor substrate-1 (IRS-1), resulting in increased PI3K-dependent Akt phosphorylation at Ser473. In another loop, mTORC1 inhibition leads to PI3K-Ras activation, which leads to an increase in MAPK signaling (61–63).

Furthermore, aberrant activation of MYC may contribute to acquired resistance to PI3K/Akt/mTOR-targeted therapy. Indeed, targeting this pathway may cause MYC activation through PDK1-dependent MYC phosphorylation and MYC amplification, which is parallel to PIK3CA-dependent Akt and MAPK activation, thus attenuating the therapeutic effect of PI3K/Akt/mTOR inhibitors (64–66).

Finally, a recent report analyzed SGK (serum- and glucocorticoid-regulated kinase) levels and the relative sensitivity of a panel of breast cancer cells toward two distinct Akt inhibitors (67). This study showed a number of Akt-inhibitor-resistant lines displaying markedly elevated SGK1 that also exhibited significant phosphorylation of the SGK1 substrate NDRG1 [neuroblastoma-derived Myc (N-Myc) downstream-regulated gene 1]. In contrast,

Table 2 | Phosphatidylinositol-3-kinase and "dual PI3K and mTOR" inhibitors in development [modified from ref. (53)].

Group	Selectivity	Compound/company/route of administration	Main feature(s)	Ongoing trials in
Pan-class I	Class I PI3K	GDC-0941 (Roche/Genentech) oral	Proved able to synergize with different agents (e.g., rapamycin, docetaxel, HER-targeting agents)	Breast, NHL, NSCLC
		BKM120 (Novartis) oral	Peculiar ability to penetrate the blood-brain barrier	Breast, colo-rectal, endometrial, GIST, leukemia, melanoma, NSCLC, pancreatic, RCC, transitional cell carcinoma, squamous cell carcinoma of the head and neck
		PX866 (Oncothyreon) oral	Proved able to synergize with chemotherapy, radiation, and targeted agents (e.g., EGFR inhibitors)	Colo-rectal, glioblastoma, NSCLC, squamous cell carcinoma of the head and neck
Isoform-specific	PI3Kα	GDC-0032 (Roche/Genentech) oral	Sparing the β-isoform of PI3K, it may reduce some undesired adverse events, e.g., metabolic abnormalities	Different solid cancers
	PI3Kβ	GSK2636771 (GSK) oral	Studied especially in patients whose tumors lack PTEN expression	Different solid cancers
	PI3K-γ and -δ	IPI-145 (Infinity) oral	Since PI3K-γ and -δ isoforms are preferentially expressed in leukocytes, where they have distinct and non-overlapping roles in key cellular functions (e.g., cell proliferation, differentiation, migration, and immunity) it may be particularly active in hematological malignancies (as well as in inflammatory diseases)	Different hematological malignancies
	PI3Kδ	CAL-101 (Gilead sciences) oral	Since PI3K-δ is preferentially expressed in leukocytes, may be particularly active in hematological malignancies; furthermore, the targeted inhibition of PI3K-δ is designed to preserve PI3K signaling in normal cells	AML, CLL, HL, NHL, multiple myeloma
Dual PI3K/mTOR	PI3K and mTOR	NVP-BEZ235 (Novartis) oral	This drug also potently inhibits ATM and DNA-PKcs, the two major kinases responding to ionizing radiation-induced DNA double-strand breaks, resulting in significant attenuation of double-strand breaks repair. May thus be developed as a radiosensitizer. Also the first PI3K inhibitor to enter clinical trials, in 2006; issues in its bioavailability are presently hampering its development	Breast, RCC

NHL, non-Hodgkin's lymphoma; NSCLC, non-small cell lung cancer; GIST, gastro-intestinal stromal tumor; RCC, renal cell carcinoma; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; HL, Hodgkin's lymphoma.

most Akt-inhibitor-sensitive cell lines displayed low or undetectable levels of SGK1. Intriguingly, despite low SGK1 levels, several Akt-inhibitor-sensitive cell lines showed marked NDRG1 phosphorylation that, unlike resistant cells, were suppressed by Akt inhibitors. Furthermore, SGK1 knockdown markedly reduced proliferation of Akt-inhibitor-resistant cells, but not Akt-sensitive cells (67). Taken together, these results clearly suggest that SGK1 levels, as well as responses of NDRG1 phosphorylation to Akt inhibitor administration, could help us predict the sensitivity or resistance of tumor cells to Akt-targeting drugs.

Autophagy may represent another mechanism of resistance from Akt/mTOR targeting. Indeed, autophagy induction proved able to protect MCL cells from Akt/mTOR inhibition. Furthermore, selective triple knockdown of the autophagy genes ATG7, ATG5 and ATG3, and pre-treatment with the autophagy inhibitor hydroxychloroquine, efficiently overcame the resistance

Table 3 | Akt inhibitors in development.

Compound/drug (company)	Characteristics	Clinical development
Miltefosine (Zentaris GmbH)	• As 6% topical solution, proved able to increase time to treatment failure (in a double-blind, placebo-controlled, phase III trial) in cutaneous metastases from breast cancer patients • Registered and used in India, Colombia, and Germany for the treatment of visceral and cutaneous leishmaniasis • Targets HIV-infected macrophages. The HIV protein Tat activates pro-survival PI3K/Akt pathway in primary human macrophages. Miltefosine acts by inhibiting the PI3K/Akt pathway, thus removing the infected macrophages from circulation, without affecting healthy cells	
Perifosine (Keryx/Aeterna Zentaris)	• Orally active alkyl-phosphocholine compound • Modulates membrane permeability, membrane lipid composition, phospholipid metabolism, and mitogenic signal transduction, resulting in cell differentiation and inhibition of cell growth • Inhibits the anti-apoptotic mitogen-activated protein kinase (MAPK) pathway and modulates the balance between the MAPK and pro-apoptotic stress-activated protein kinase (SAPK/JNK) pathways, thereby inducing apoptosis	Stopped after several phase II studies
MK2206 (Merck/Astra Zeneca)	• Orally bioavailable allosteric inhibitor of the serine/threonine protein kinase Akt (protein kinase B) • Binds to and inhibits the activity of Akt in a non-ATP competitive manner, which may result in the inhibition of the PI3K/Akt signaling pathway and tumor cell proliferation and the induction of tumor cell apoptosis	Phase I and II trials ongoing as single-agent or in combination with other drugs – e.g., chemotherapeutic, hormonal, and other targeted agents
RX-0201 (Rexahn pharmaceuticals)	• A 20-mer antisense oligodeoxynucleotide directed against Akt • Binds to Akt-1 mRNA, inhibiting translation of the transcript; suppression of Akt-1 expression may result in the inhibition of cellular proliferation, and the induction of apoptosis in tumor cells that overexpress Akt-1	Phase II study in combination with gemcitabine in pancreatic cancer closed
Erucylphosphocholine (a.k.a. ErPC or AEZS-127) Aeterna Zentaris	• Structurally related to Perifosine, it inhibits Akt, but also impacts other signaling pathways (most prominently, Raf-MEK-ERK) • Intravenous use	Currently under pre-clinical development
PBI-05204 (a.k.a. Oleandrin) (Phoenix biotechnology)	• A lipid soluble cardiac glycoside derived from *Nerium oleander* • Specifically binds to and inhibits the α3 subunit of the Na/K-ATPase pump in human cancer cells. This may inhibit the phosphorylation of Akt, upregulate MAPK, inhibit NF-κb activation, and inhibit FGF-2 export and may downregulate mTOR thereby inhibiting p70S6K and S6 protein expression, ultimately resulting in the induction of apoptosis • As cancer cells with relatively higher expression of the α3 subunit and with limited expression of the α1 subunit are more sensitive to oleandrin, one may predict the tumor response to oleandrin based on the tumors Na/K-ATPase pump protein subunit expression	Early clinical development
GSK690693 (GSK)	• An aminofurazan-derived inhibitor of Akt kinases 1, 2, and 3 • May also inhibit other protein kinases including protein kinase C (PKC) and protein kinase A (PKA)	Early clinical development
XL-418 (Exelixis)	• A dual inhibitor of Akt and p70S6K	• Enhance apoptosis in combination with XL647, an inhibitor of multiple receptor tyrosine kinases including EGFR, HER2, and VEGFR, in pre-clinical tumor models • In a phase I study, low drug exposure was achieved and the trial was thus stopped

to Akt/mTOR inhibitors in this model, leading to the activation of the mitochondrial apoptotic pathway (68, 69). Taken together, these results suggest that counteracting autophagy may represent an attractive strategy for sensitizing lymphoma cells to everolimus-based therapy. Furthermore, autophagy facilitates cancer cell resistance also to cytotoxic chemotherapy and radiation treatment (70).

CONCLUSION

The PI3K/Akt/mTOR pathway represents a good example of the concept of redundancy in biological systems, particularly in cancer cells. Indeed, cancer responds to chronic treatment with drugs targeting a single pathway by adapting its signaling circuitry, taking advantage of pathway redundancy and routes of feedback and crosstalk to maintain their function and thus escape from drug-induced growth inhibition and death (71–73).

That is why, despite recent successes (achieved in completely different diseases such as kidney and breast cancer, pNETs, and other malignancies), tumors ultimately evade inhibition of this pathway.

Novel agents targeting PI3K/Akt/mTOR promise further improvement of the results achieved so far through higher selectivity and potency, as well as to combinability with other therapeutic strategies. However, only translational research, addressing this variegated and complex network of highly integrated signaling pathways and mechanisms of resistance to their inhibition, will be able to help us take another step forward.

AUTHOR CONTRIBUTION

All the authors equally contributed to the preparation of this manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 September 2013; accepted: 17 March 2014; published online: 14 April 2014.

*Citation: Porta C, Paglino C and Mosca A (2014) Targeting PI3K/Akt/mTOR signaling in cancer. Front. Oncol. **4**:64. doi: 10.3389/fonc.2014.00064*
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology.
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frontiers in ONCOLOGY

REVIEW ARTICLE
published: 01 March 2013
doi: 10.3389/fonc.2013.00040

p110δ PI3 kinase pathway: emerging roles in cancer

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Class IA PI3Ks consists of three isoforms of the p110 catalytic subunit designated p110α, p110β, and p110δ which are encoded by three separate genes. Gain-of-function mutations on *PIK3CA* gene encoding for p110α isoform have been detected in a wide variety of human cancers whereas no somatic mutations of genes encoding for p110β or p110δ have been reported. Unlike p110α and p110β which are ubiquitously expressed, p110δ is highly enriched in leukocytes and thus the p110δ PI3K pathway has attracted more attention for its involvement in immune disorders. However, findings have been accumulated showing that the p110δ PI3K plays a seminal role in the development and progression of some hematologic malignancies. A wealth of knowledge has come from studies showing the central role of p110δ PI3K in B-cell functions and B-cell malignancies. Further data have documented that wild-type p110δ becomes oncogenic when overexpressed in cell culture models and that p110δ is the predominant isoform expressed in some human solid tumor cells playing a prominent role in these cells. Genetic inactivation of p110δ in mice models and highly-selective inhibitors of p110δ have demonstrated an important role of this isoform in differentiation, growth, survival, motility, and morphology with the inositol phosphatase PTEN to play a critical role in p110δ signaling. In this review, we summarize our understanding of the p110δ PI3K signaling pathway in hematopoietic cells and malignancies, we highlight the evidence showing the oncogenic potential of p110δ in cells of non-hematopoietic origin and we discuss perspectives for potential novel roles of p110δ PI3K in cancer.

Keywords: p110δ PI3K, cancer, hematologic malignancies, PTEN, solid tumors

GENERAL ASPECTS OF THE CLASS IA PI3Ks SIGNALING PATHWAY

Class I phosphoinositide-3 kinases (PI3Ks) consist of a group of enzymes that transmit signals inside cells by the production of intracellular second messenger lipid signals. PI3Ks phosphorylate inositol lipids at the 3-position of the inositol ring, generating phosphatidylinositol (PI)-3-phosphate (PI3P), phosphatidylinositol-3,4-bisphosphate [$PI(3,4)P_2$] and phosphatidyl-inositol-3,4,5-trisphosphate [$PI(3,4,5)P_3$]. These lipids trigger signal transduction cascades that control cell division, survival, metabolism, intracellular trafficking, differentiation, re-organization of the actin cytoskeleton, and cell migration under the control of PI3Ks (Vanhaesebroeck et al., 2001; Hawkins et al., 2006; Low et al., 2010; Zwaenepoel et al., 2012). The PI3K isoforms that are activated by tyrosine kinases and G-protein coupled receptors (GPCRs) are known as class IA and IB PI3Ks, respectively (**Figure 1**). Class IA PI3Ks are constitutive heterodimers of a 110 kDa catalytic subunit (p110) with one of the five regulatory adaptor proteins (p85α, p55α, p50α, p85β, or p55γ, collectively called "p85s") that recruits the p110 to intracellular locations of tyrosine kinase activation (Vanhaesebroeck et al., 1997a, 2010) (**Figure 1**). Mammals have genes for 3 class IA catalytic subunits designated p110α, p110β, and p110δ (Vanhaesebroeck et al., 2010) (**Figure 1**). p110γ is the only class IB PI3K. This kinase occurs in complex with the p101 (Stephens et al., 1997; Krugmann et al., 1999) or p84 (Suire et al., 2005; Voigt et al., 2006) adaptor protein and is activated by the Gβγ subunits of heterotrimeric G-proteins (**Figure 1**). However, several studies have linked the p110β and p110δ isoforms of class IA to GPCRs and the class IB p110γ to tyrosine kinases, the mechanisms though are not yet clear (Sadhu et al., 2003; Reif et al., 2004; Condliffe et al., 2005; Guillermet-Guibert et al., 2008; Durand et al., 2009; Hoellenriegel et al., 2011; Schmid et al., 2011) (**Figure 1**). All catalytic subunits of the class I PI3Ks contain binding domains for Ras GTPases (**Figure 1**) and their binding to certain Ras proteins contributes to activation (Rodriguez-Viciana et al., 1994; Jimenez et al., 2002).

Activation of tyrosine kinase receptors by growth factors recruits class IA PI3 kinases to the cell membrane. Activated growth factor receptors possess phosphorylated Tyr-X-X-Met motifs in which bind with high affinity the regulatory subunits of class IA PI3Ks by their SH2 domains (**Figure 1**). In cells, activated class IA PI3Ks phosphorylate primarily the phosphatidylinositol (PI)-4,5-bisphosphate [PI(4,5)P2] yielding the product $PI(3,4,5)P_3$ (Vanhaesebroeck et al., 2001) (**Figure 2**). The generation of $PI(3,4,5)P_3$ leads to the recruitment of adaptor and effector proteins containing pleckstrin-homology (PH)-domains, including regulators of small GTPases [such as guanosine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs)] and Ser/Thr kinases (such as PDK1 and Akt/PKB), which thus become located at the plasma membrane (Klarlund et al., 1997; Krugmann et al., 2002; Welch et al., 2002;

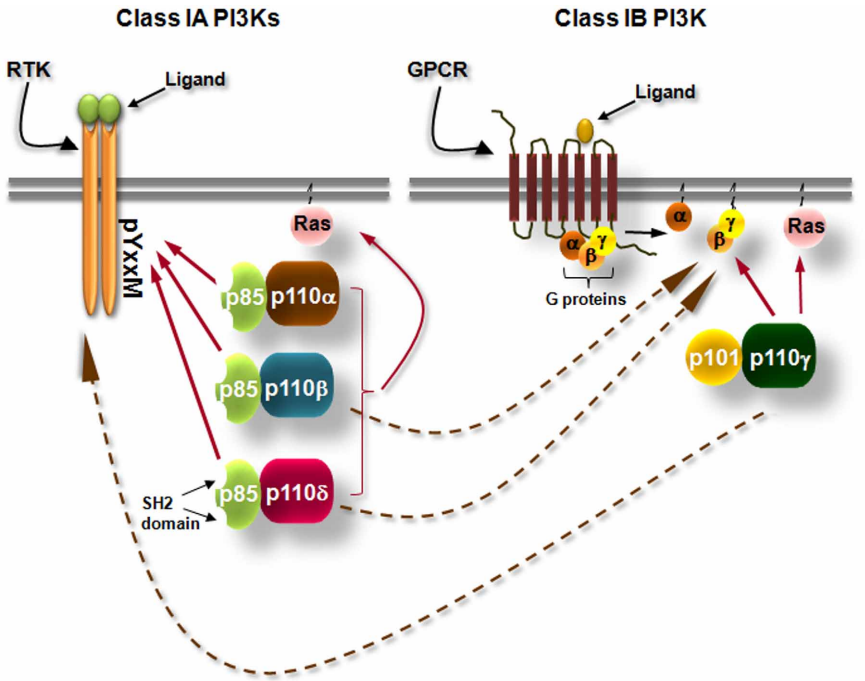

FIGURE 1 | Simplified scheme showing the differential activation of class IA and class IB PI3K isoforms. Class IA PI3Ks are heterodimers consisting of a 110 kDa catalytic subunit (p110α, p110β, and p110δ) in complex with a p85 regulatory subunit, of which five isoforms exist. Class IA PI3Ks are activated by growth factor and cytokine receptors or adaptor proteins (e.g., CD19/BCAP in B cells). Binding of the ligand to its receptor leads to receptor dimerization and auto-phosphorylation of tyrosines (Y) which are located in pYxxM motifs. The p85 regulatory subunits have Src-homology 2 (SH2) domains which bind to phosphorylated tyrosines in YxxM motifs recruiting thus the class IA PI3Ks to the plasma membrane where their lipid substrates are located. Class IB PI3K consists of the p110γ isoform which binds to p101 or p84 regulatory subunits. Class IB PI3K is activated by G protein-coupled receptors (GPCRs). Binding of the ligand (e.g., a chemokine) to its cognate GPCR induces the dissociation of heterotrimeric G-proteins and the Gβγ subunits interact with the class IB PI3K. Arrows with dashed lines represent activation of p110β and p110δ downstream of GPCRs and activation of p110γ downstream of tyrosine kinases by currently unknown mechanisms.

Marone et al., 2008). Small GTPases are activated (become GTP-bound) by GEFs whereas the return from their active state to an inactive state (GDP-bound) is catalyzed by GAPs (**Figure 2**). Cyclic activation-inactivation of the small GTPases is required for cell body to move properly (Ridley et al., 2003). PDK1, which is in an active state under basal conditions, becomes additionally activated on cell stimulation (Alessi et al., 1997a; Pullen et al., 1998; Currie et al., 1999) and phosphorylates Akt on Thr308 (Alessi et al., 1997a,b; Stokoe et al., 1997; Stephens et al., 1998). Akt is also phosphorylated on Ser473 (Alessi et al., 1996) by mTORC2 (mTOR complexed with the Rictor protein) (Sarbassov et al., 2005) (**Figure 2**). Full activation of Akt kinase activity requires the phosphorylation of both kinase domains of Akt (Bellacosa et al., 1998).

Akt activates or inhibits a broad range of proteins including mTORC1 (mTOR in complex with Raptor), BAD, FOXO, GSK-3β, and p27, which are involved in the control of cell growth, metabolism, survival, cell cycle, and migration (Manning and Cantley, 2007) (**Figure 2**). Akt phosphorylates and inactivates the tuberous sclerosis complex 1/2 (TSC1/2) which acts as a GAP protein on Ras homologue enriched in brain (RHEB), a guanosine triphosphate (GTP)-binding protein (Garami et al., 2003; Li et al., 2004). The role of GTP-bound RHEB is to activate mTORC1 and consequently its downstream effector proteins (Inoki et al., 2003). Thus, increased Akt activity promotes the activation of mTORC1 because Akt inactivates TSC1/2 (**Figure 2**). The multiple roles of mTORC1 and especially those correlated with mRNA translation and cell cycle has made the PI3K/Akt/mTORC1 axis an attractive target for the development of dual PI3K/mTOR inhibitors, mTOR-selective inhibitors and Akt inhibitors as anti-cancer drugs (Marinov et al., 2007; Sabbah et al., 2011; Castillo et al., 2012; Sheppard et al., 2012; Weigelt and Downward, 2012; Willems et al., 2012).

Akt also phosphorylates the death promoter BAD leading to the release of the anti-apoptotic proteins Bcl-2 and Bcl-XL (Datta et al., 1997; Peso et al., 1997). GSK-3 which regulates glucose metabolism and apoptosis is also controlled by Akt (Jope and Johnson, 2004). Phosphorylation of GSK-3β by Akt prevents its activity leading to the accumulation of cyclin D1 and the consequent transition of cells from G1 to the S phase of the cell cycle (Liang and Slingerland, 2003). Other substrates of Akt are the class O of transcription factors (FOXOs) that are known regulators of the cell cycle. Phosphorylated FOXOs bind to the 14-3-3 proteins resulting in the exclusion of FOXOs of the nucleus which leads to the increased transcription of cyclin D1 and to the reduced transcription of the p27 CDK inhibitor (CKI) (Alvarez

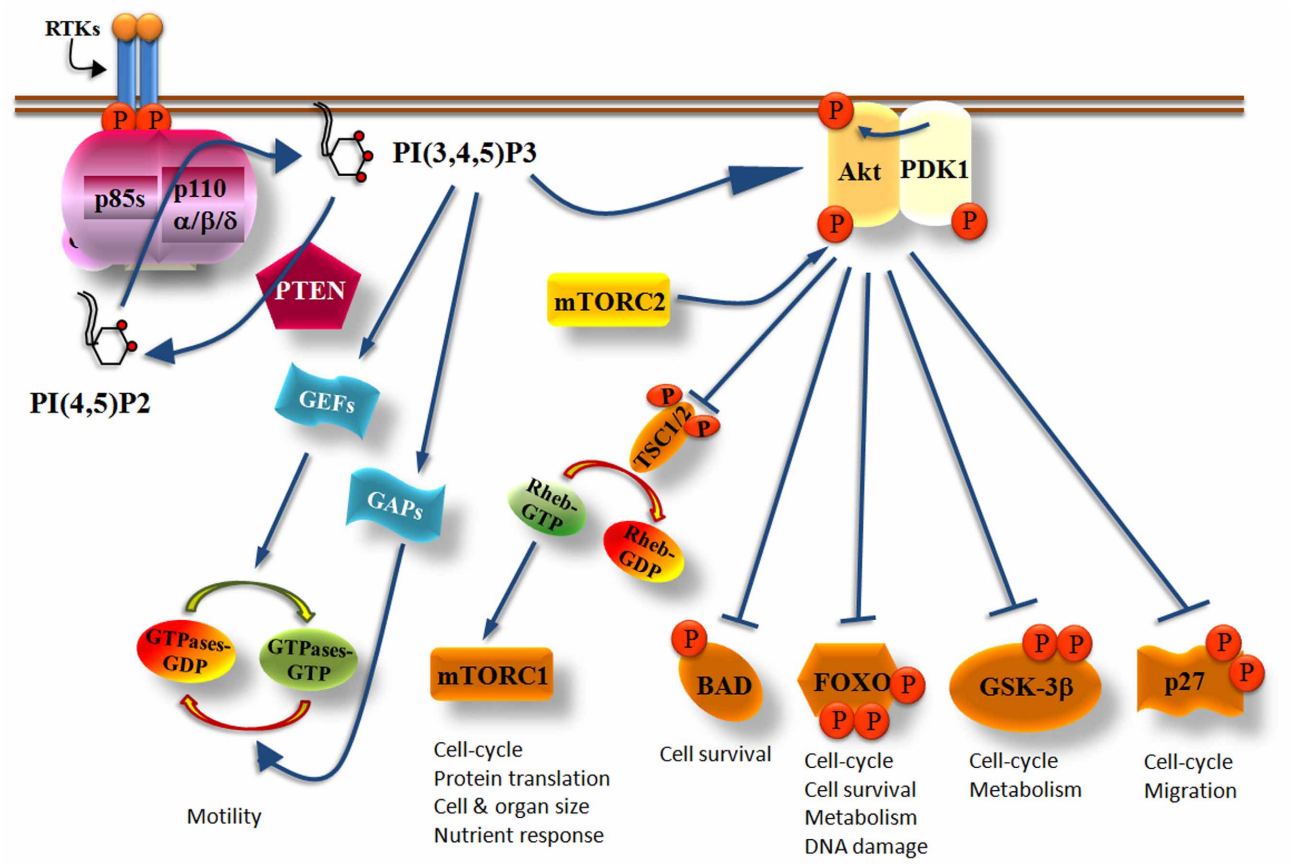

FIGURE 2 | Simplified scheme showing the critical role of Class IA PI3Ks in multiple cellular functions. Upon PI3Ks activation, PI(3,4,5)P3 is produced and recruits Akt at the cell membrane where it becomes phosphorylated at The308 by PDK1 and at Ser473 by mTORC2. Fully activated Akt phosphorylates a variety of effector molecules including the TSC1/2, BAD, FOXO, GSK-3β, and p27 which then control cell growth, metabolism, survival, cell cycle, or migration. PI(3,4,5)P3 also activates GEFs or GAPs which then regulate the activity of small GTPases controlling cell motility. The PI(3,4,5)P3 levels produced by PI3Ks are regulated by the PTEN phosphatase which counteracts the PI3K reaction by dephosphorylating the 3-position of the inositol ring of PI(3,4,5)P_3 yielding back PI(4,5)P_2.

et al., 2001; Burgering and Medema, 2003). Upon cytosolic localization of FOXOs also the transcription of FasL is prevented leading to the blockage of apoptosis. Akt also regulates post-translationally the p21 and p27 CKIs by phosphorylating them resulting in their exclusion of the nucleus (Zhou et al., 2001; Fujita et al., 2002; Liang et al., 2002) which consequently leads to increased cell proliferation due to decreased inhibition of cyclins. p27 acts as an oncoprotein in the cytoplasm where it binds to and inhibits RhoA thus promoting cell migration (Besson et al., 2004). The cytoplasmic localization of p21 and p27 is associated with high tumor grade, tumor cell invasiveness and metastasis (Sáez et al., 1999; Slingerland, 2000; Philipp-Staheli et al., 2001).

The PI3K/Akt signaling pathway is regulated by phosphatases with the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) lipid phosphatase being the most extensively investigated. The PTEN tumor suppressor protein antagonizes the PI3K activity by dephosphorylating the 3-position of the inositol ring of PI(3,4,5)P_3 (Maehama and Dixon, 1998) (**Figure 2**) thus controlling cell survival (Stambolic et al., 1998; Leslie and Downes, 2002; Sulis and Parsons, 2003). Reduced or lost activity of PTEN creates a state in which PI(3,4,5)P_3 production is mis-regulated contributing to the constitutive activation of the PI3K pathway (Leslie and Downes, 2004; Parsons, 2004; Sansal and Sellers, 2004; Cully et al., 2006) and to abnormal cell growth (Ali et al., 1999; Vivanco and Sawyers, 2002; Luo et al., 2003).

The tissue distribution and the regulation of class IA PI3Ks expression have been determined using various approaches (Kok et al., 2009a,b). Reporter mice with a β-Gal-LacZ reporter gene inserted into endogenous p110 loci by homologous recombination were proven very useful in determining the distribution of p110α (Foukas et al., 2006) and p110δ (Okkenhaug et al., 2002; Eickholt et al., 2007). Whereas p110α and p110β were found to be globally expressed (Hu et al., 1993; Bi et al., 1999, 2002; Geering et al., 2007), p110δ is predominantly expressed in white blood cells (Chantry et al., 1997; Vanhaesebroeck et al., 1997b). p110δ is also expressed at high levels in some cancer cell lines and human tissues of non-leukocyte origin such as breast cancer cells (Sawyer et al., 2003; Tzenaki et al., 2012) and at moderate levels in neurons (Eickholt et al., 2007). The mechanism by which the expression of p110δ PI3K is regulated has recently been explored (Edwards

et al., 2009; Kok et al., 2009b; Calvanese et al., 2012; Whitehead et al., 2012). A highly conserved transcription factor binding cluster in the *PI3KD* gene was identified and found to display higher promoter activity in leukocyte compared to non-leukocyte cells providing an explanation for the highly enriched p110δ levels in leukocytes (Kok et al., 2009b; Whitehead et al., 2012). Transcriptional regulation of *PIK3CD* by RUNX1 (Edwards et al., 2009) and leukocyte-dependent promoter DNA hypomethylation (Calvanese et al., 2012) were also proposed to be involved in high p110δ expression. It is possible that p110δ expression is transcriptionally regulated also in non-leukocyte cells that express high levels of p110δ, such as breast cancer cells, by leukocyte-related transcription factors which have been found to be activated in breast cancers (Teschendorff et al., 2007).

The three isoforms of class IA PI3K have identical enzymatic activities but they have non-redundant functions in cell signaling, metabolism, and tumorigenesis (Roche et al., 1994, 1998; Vanhaesebroeck and Waterfield, 1999; Hill et al., 2000; Hooshmand-Rad et al., 2000; Leverrier et al., 2003; Vanhaesebroeck et al., 2005; Foukas et al., 2006; Ali et al., 2008; Graupera et al., 2008; Papakonstanti et al., 2008). Since cancer-specific gain-of-function mutations were reported in *PIK3CA* gene (Campbell et al., 2004; Samuels and Velculescu, 2004), which encodes the p110α PI3K, this isoform has been placed in the center of cancer research. In contrast, no somatic mutations of genes encoding p110β or p110δ have been reported (Samuels and Velculescu, 2004; Thomas et al., 2007; Wood et al., 2007; Parsons et al., 2008; TGCA, 2008). Gene targeting and pharmacological studies have revealed a key role of p110β in platelet biology and thrombosis (Jackson et al., 2005) whereas recent studies have also shown a role of p110β in certain cancers and especially in tumor cells lacking PTEN (Ciraolo et al., 2008; Jia et al., 2008; Torbett et al., 2008; Wee et al., 2008; Zhu et al., 2008). Given that p110δ is preferentially expressed in leukocytes, the functional role of p110δ has been studied in immune system (Clayton et al., 2002; Jou et al., 2002; Okkenhaug et al., 2002; Ali et al., 2004; Aksoy et al., 2012) and this isoform has been more considered as target in immunity and inflammation (Rommel et al., 2007; Rommel, 2010; Soond et al., 2010). However, findings have been accumulated showing a seminal role of p110δ PI3K in lymphoid and myeloid malignancies. Furthermore, p110δ-selective inhibitors have entered clinical studies showing effective clinical outcomes in some hematologic malignancies (Fruman and Rommel, 2011; Castillo et al., 2012). Further data have also suggested a promising role of p110δ PI3K in oncogenesis and cancers of non-hematopoietic origin (Knobbe and Reifenberger, 2003; Mizoguchi et al., 2004; Boller et al., 2008; Zhao and Vogt, 2008a; Jia et al., 2009; Vogt et al., 2009; Jiang et al., 2010; Tzenaki et al., 2012). The malignancies with aberrant p110δ signaling that will be discussed below are summarized in **Table 1**.

In this review, we go over the main points of the evidence showing the critical role of p110δ PI3K in hematopoietic cells and malignancies, we highlight findings suggesting an emerging role of p110δ in non-hematologic cancers and discuss how a better understanding of p110δ regulation and function might reveal cancer contexts in which p110δ-selective inhibitors alone or in combination with inhibitors of other components of PI3K pathway could be beneficial.

ROLE OF p110δ PI3K IN B CELLS AND B-CELL MALIGNANCIES

B cells express all isoforms of the class I PI3K catalytic subunit (Bilancio et al., 2006), however, the p110δ PI3K was found to play a predominant role in most of the functions of B cells. The role of p110δ in B-cell development has been demonstrated by studies in p110δ knock-out (KO) and p110δ knock-in (KI) mice (Clayton et al., 2002; Jou et al., 2002; Okkenhaug et al., 2002; Beer-Hammer et al., 2010; Ramadani et al., 2010). These mice comprise significantly reduced numbers of mature circulating B cells because of delayed B cell maturation at the pro-B cell stage within the bone marrow whereas in B cells that eventually become mature the chemokine-induced migration, B-cell receptor (BCR) signaling, and BCR-induced proliferation were found to be impaired (Clayton et al., 2002; Jou et al., 2002; Okkenhaug et al., 2002; Reif et al., 2004). Although, the class IB p110γ PI3K is not essential for B-cell development (Sasaki et al., 2000), combined inactivation of both p110γ and p110δ led to greater reduction of peripheral B-cell numbers than p110δ inactivation alone, suggesting that p110γ and p110δ may have overlapping functions in B-cell development (Beer-Hammer et al., 2010). Studies using mice with p110α deficiency in lymphocytes showed that p110α is not essential for B cell development and BCR signaling, however, deletion of both

Table 1 | Malignancies with aberrant p110δ signaling highlighted in this review.

	Malignancy	p110δPI3K aberration
Hematological malignancies	Chronic lymphocytic leukemia (CLL) Multiple myeloma (MM) Diffuse large B-cell lymphoma (DLBCL) Hodgkin's lymphoma (HL) Acute myeloid leukemia (AML) Acute promyelocytic leukemia (APL)	Over-activation of p110δ signaling
Solid non-hematologic tumors	Glioblastoma	Overexpression of p110δ mRNA, increased copy number of *PIK3CD*
	Prostate carcinoma	Overexpression of p110δ mRNA
	Neuroblastoma Breast carcinoma	Overexpression of p110δ protein

p110α and p110δ isoforms blocked B cell development suggesting that only p110δ is required for antigen-dependent B-cell activation triggered by the BCR whereas p110α contributes to antigen independent tonic pre-BCR and BCR signaling (Ramadani et al., 2010). Lymphocyte-specific inactivation of p110β or combined inactivation of p110β and p110δ did not affect B cell development and activation (Ramadani et al., 2010).

In resting B cells, the TC21 GTPase was found to recruit the p85α/p110δ PI3K to non-phosphorylated BCR immunoreceptor tyrosine-based activation motifs (ITAMs) (Delgado et al., 2009). Although this finding is not supported by the data suggesting a role of p110α in antigen-independent pre-BCR and BCR survival signals (Ramadani et al., 2010), it is possible that both p110α and p110δ are recruited to the BCR by TC21, however, this remains to be determined. Upon antigen binding to BCR, the ITAMs in the cytoplasmic tails of Ig-α and Ig-β (Reth, 1992) are tyrosine phosphorylated by Lyn leading to recruitment and activation of Syk initiating thus the downstream signaling cascade (Kurosaki et al., 1994; Beitz et al., 1999) (**Figure 3**). The tyrosine phosphorylation of the scaffolding proteins CD19 and B-cell adaptor protein (BCAP) creates Src-homology 2 (SH2)-binding domains which allow the binding of the SH2 domains of the p85 subunit and the recruitment of the p85/p110 complex to the cell membrane (Tuveson et al., 1993; Fujimoto et al., 2000; Okada et al., 2000; Yamazaki et al., 2002; Aiba et al., 2008) (**Figure 3**). Downstream of PI3K, the BCR signaling pathways include the activation of Akt which then regulates the GSK-3, mTOR, and NF-kB pathway as well as the activation of Bruton's tyrosine kinase (Btk), which then induces the activation of phospholypase C-γ (Spaargaren et al., 2003; Fruman, 2004) (**Figure 3**). PLCγ is an enzyme that catalyzes the hydrolysis of PI(4,5)P2 to generate the second messengers inositol 1,4,5-trisphosphate [I(1,4,5)P3] and diacylglycerol (DAG) that regulate the reorganization of cytoskeleton and cell adhesion by inducing an increase

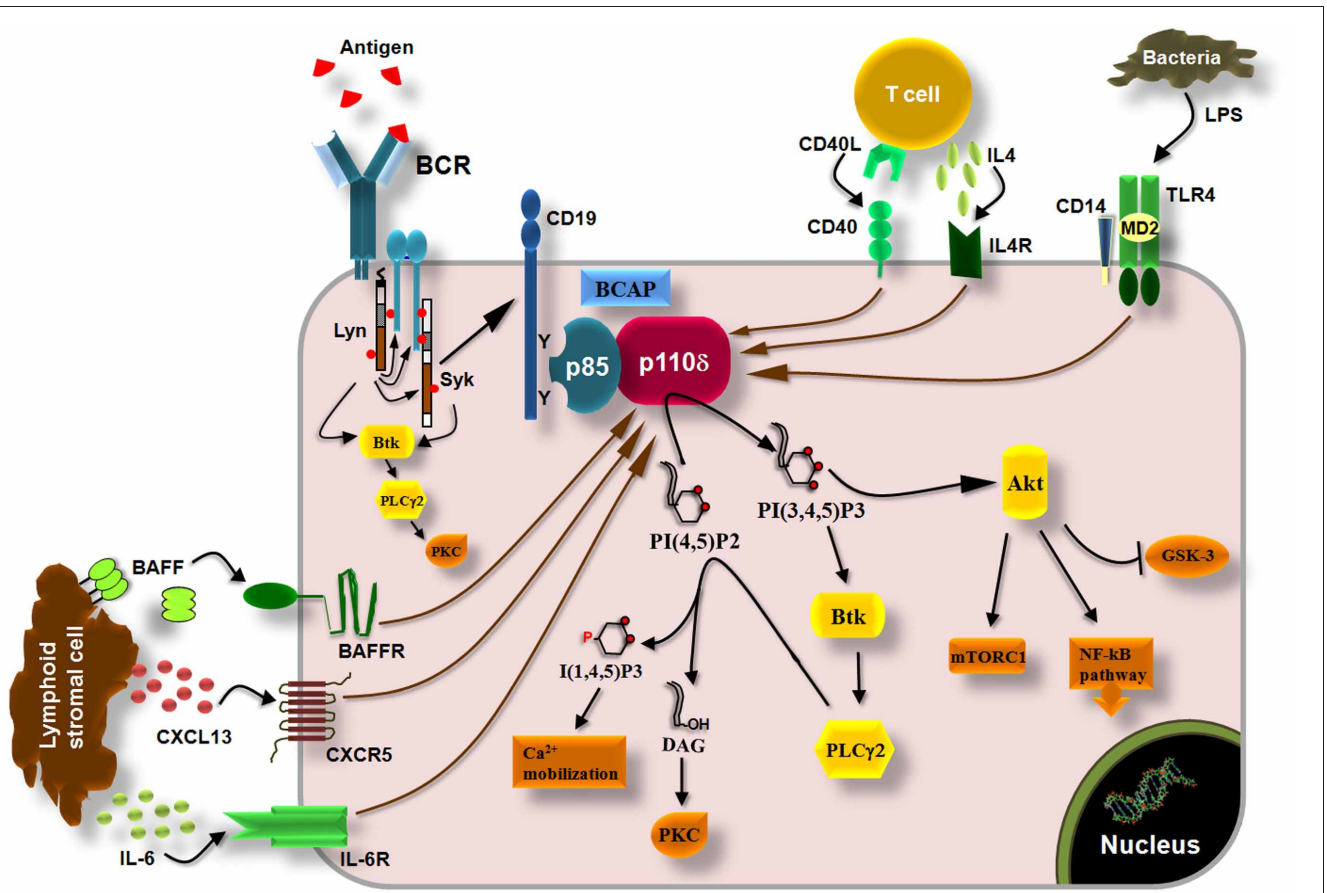

FIGURE 3 | p110δ PI3K pathway in B cells. Engagement of BCRs by antigen induces the phosphorylation of ITAMs in the cytoplasmic tails of Ig-α and Ig-β by Lyn leading to recruitment and activation of Syk via ITAMs and to initiation of downstream signaling cascade. Tyrosine phosphorylation of the co-receptor CD19 and BCAP recruits p110δ PI3K through SH2 interactions leading to production of PI(3,4,5)P3 which recruits PH-domain containing proteins such as Akt, Btk, and PLCγ2. Akt controls the activity of multiple signaling molecules and pathways such as the mTORC1, GSK-3, and the NF-kB pathway. Btk phosphorylates and activates PLCγ2 which then catalyzes the hydrolysis of PI(4,5)P2 yielding I(3,4,5)P3 and diacylglycerol (DAG). I(3,4,5)P3 initiates Ca2+ mobilization and DAG induces the activation of protein kinase C (PKC) isoforms. The p110δ PI3K also functions downstream of the cytokine receptors BAFFR and IL6R, which are activated by BAFF and IL-6, respectively, derived from lymphoid stromal cells, and downstream of the IL4R which is activated by IL-4 derived from T cells. Chemokine receptors (CXCR5) and co-stimulatory receptors (CD40, TLRs) also induce the activation of p110δ PI3K in B cells.

in intracellular free-calcium levels and the activation of multiple protein kinase C (PKC) isoforms (**Figure 3**) and downstream signaling molecules (Papakonstanti et al., 2000; Fruman, 2004). The p110δ PI3K signaling pathway is also activated by cytokines like BAFF and IL6 (Patke et al., 2006; Henley et al., 2008) and chemokines like CXCL13 (Reif et al., 2004) derived from lymphoid stromal cells, by cytokines like IL-4 derived from T cells (Bilancio et al., 2006) and by co-stimulatory receptors such as CD40 and Toll-like receptors (TLRs) (Arron et al., 2001; Ni et al., 2012; So and Fruman, 2012; Troutman et al., 2012) (**Figure 3**). The mechanism that links the activation of p110δ to G protein-coupled chemokine receptors (such as the CXCR5) is not currently known. That p110δ PI3K mediates the effects of multiple receptors on B cells is consistent with substantial evidence documenting a central role of this enzyme in B cell development and activation (Okkenhaug and Fruman, 2010).

The critical role of p110δ in homeostasis and function of B cells combined with the fact that *PIC3CA* and *PTEN* gene mutations are rare in B cell malignancies (Leupin et al., 2003; Georgakis et al., 2006; Ismail et al., 2010) made the p110δ PI3K pathway to attract the interest in understanding its potential involvement in malignant B cells. Indeed, there is mounting evidence showing that the p110δ PI3K pathway is over-activated in B cell malignancies because of alterations in BCR signaling and other signals provided by factors from tumor microenvironment (Pauls et al., 2012). Significantly higher levels of p110δ PI3K activity have been determined in cells from patients with chronic lymphocytic leukemia (CLL) compared with normal hematopoietic cells (Herman et al., 2010) whereas overactivation of p110δ has also been found in multiple myeloma (MM) cell lines and cells from patients with MM (Ikeda et al., 2010) as well as in cell lines and cells from patients with Hodgkin's lymphoma (HL) (Meadows et al., 2012).

The critical role of p110δ in B cells led to the development of highly p110δ-specific inhibitors for treatment of B-cell malignancies (Norman, 2011) including the initially developed CAL-101 (GS-1101) (Fruman and Rommel, 2011; Lannutti et al., 2011). The activity of CAL-101 and other p110δ-selective inhibitors have been studied in cell lines and patient cells from different B-cell malignancies including CLL, MM, diffuse large B-cell lymphoma (DLBCL), and HL (Herman et al., 2010; Ikeda et al., 2010; Hoellenriegel et al., 2011; Lannutti et al., 2011; Meadows et al., 2012) (**Table 2**). Inhibition of p110δ PI3K by CAL-101 in cells from patients with CLL led to inhibition of Akt and ERK and consequently to reduced B-CLL survival (Herman et al., 2010; Hoellenriegel et al., 2011). Furthermore, inhibition of p110δ leads to blockade of protective microenvironmental signals on B-CLL. Indeed, survival signals induced by *in vitro* stimulation of B-CLL with TNFα, BAFF and CD40L were attenuated by CAL-101 treatment (Herman et al., 2010). The protective effect provided in B-CLL by their culture on fibronectin or stomal cells or their co-culture with nurse-like cells (NLC) was also blocked by inhibition of p110δ activity (Herman et al., 2010; Hoellenriegel et al., 2011). In these co-culture systems, elimination of p110δ activity additionally led to decreased secretion of the chemokines CCL2, CCL3 from CLL cells, CXCL13 from stromal cells, various survival factors from NLC and inhibited the chemotaxis of B-CLL to CXCL12, CXCL13 and stromal cell lines (Hoellenriegel et al., 2011). The later result is in line with previous findings showing that B cells derived from p110δ KO mice poorly respond to CXCL13 and exhibit reduced homing to lymphatic tissues (Reif et al., 2004). Similarly, the p110δ selective inhibitor IC87114 (Sadhu et al., 2003) inhibited the B cell chemotaxis to CXCL13 and S1P *in vitro* and led to depletion of MZ B cells from the spleen *in vivo* (Durand et al., 2009). A potential involvement of p110α, p110β, and p110γ in CLL has been indicated by a study showing that pharmacological inhibition of each of these isoforms inhibited the proliferation of CLL cells (Shehata et al., 2010).

The above summarized data suggested that inhibition of p110δ by CAL-101 reduces the B-CLL survival driven by B-cell molecules and furthermore acts by blocking cells to access protective niches inhibiting the environmental protective interactions that otherwise would promote B-cell survival and proliferation. These were promising results for potential efficacy of p110δ inhibition in CLL patients since CLL is characterized by the accumulation of B lymphocytes in the peripheral blood, lymph nodes, and bone marrow (Cheson et al., 1996; Hallek et al., 2008). Indeed, the phosphorylation of Akt in B-CLL and the plasma levels of CXCL13, CCL3, CCL4, and TNFα were found to be significantly reduced in patients treated with CAL-101 (Hoellenriegel et al., 2011; Sharman et al., 2011) suggesting that inhibition of p110δ disrupts the interactions of B-CLL from their protective microenvironment. These results are consistent with the increased numbers of B-CLL found in the peripheral blood of the CAL-101-treated patients (Sharman et al., 2011) indicating a release of lymphocytes from lymphoid tissues or a failure to home into lymph nodes which consequently leads to reduced lymph node size (Hoellenriegel et al., 2011; Castillo et al., 2012). It seems therefore that inhibition of p110δ in B-CLL *in vivo* is more efficient to release B-CLL from their microenvironment than to kill them which is consistent with recent data showing that even extremely reduced levels of class I PI3K activity are sufficient to sustain cell survival (Foukas et al., 2010). Inhibitors of Syk or Btk that also involved in BCR signaling (**Figure 3**) led to similar clinical responses (Herman et al., 2011; Ma and Rosen, 2011; Burger, 2012; De Rooij et al., 2012; Ponader et al., 2012; Puri and Gold, 2012).

Similar effects of CAL-101 and other p110δ inhibitors have been observed on other B-cell malignancies such as MM, DLBCL, and HL (Ikeda et al., 2010; Lannutti et al., 2011; Meadows et al., 2012) (**Table 2**). The important role of p110δ in MM pathogenesis was indicated by its high expression in patient MM cells (Ikeda et al., 2010). Suppression of p110δ expression or inhibition of p110δ activity by CAL-101 in MM cell lines and patient MM cells decreased the phosphorylation of Akt and P70S6K and inhibited cells growth (Ikeda et al., 2010). The implication of p110δ in protective signals derived from bone marrow microenvironment seems to be the case also in MM since CAL-101 inhibited MM cells growth and Akt and P70S6K phosphorylation in cells treated with IL-6 and insulin growth factor-1 (IGF-1) or co-cultured with bone marrow stromal cells (Ikeda et al., 2010). The effects of p110δ inhibition was also confirmed in two xenograft mouse models of human MM, where p110δ-inhibors prevented the growth of transplanted human MM cells

Table 2 | Summarized findings showing the effects of p110 isoform-selective inhibitors in cell lines and patient cells from different hematological malignancies and in animal models described in the text.

Malignancy	Targeted isoform			
	p110δ	**p110α**	**p110β**	**p110γ**
B-CLL	• Inhibition of p-Akt and p-ERK • Inhibition of B-CLL survival • Inhibition of TNFα-, BAFF- and CD40L-induced survival • Inhibition of protective effects provided by fibronectin, stomal cells or NLC • Decreased secretion of CCL2 and CCL3 from CLL cells, CXCL13 from stromal cells, various survival factors from NLC • Inhibition of chemotaxis of B-CLL to CXCL12, CXCL13, S1P and stromal cell lines • Depletion of MZ B cells from the spleen	• Decreased proliferation of CLL cells	• Decreased proliferation of CLL cells	• Decreased proliferation of CLL cells
References	Durand et al. (2009), Herman et al. (2010), Hoellenriegel et al. (2011)	Shehata et al. (2010)	Shehata et al. (2010)	Shehata et al. (2010)
MM	• Inhibition of phosphorylation of Akt and P70S6K • Inhibition of MM cells growth • Inhibition of IL6-, IGF-1- or stromal cells-induced phosphorylation of Akt, P70S6K and cell growth • Inhibition of growth of transplanted human MM cells in xenograft mouse models	Not determined	Not determined	Not determined
Reference	Ikeda et al. (2010)			
DLBCL	• Inhibition of phosphorylation of Akt and S6 • Increased cleavage of the apoptotic markers caspase 3 and poly (ADP-ribose) polymerase	Not determined	Not determined	Not determined
Reference	Lannutti et al. (2011)			
HL	• Inhibition of phosphorylation of Akt • Induction in apoptosis • Inhibition of stroma cells-induced Akt activation • Disruption of survival signals mediated by CCL5, CCL17, and CCL22 in co-cultures of HL cells with stromal cells	Not determined	Not determined	Not determined
Reference	Meadows et al. (2012)			
AML	• Inhibition of Akt phosphorylation • Reduction in viable cells number • Reduction in NF-kB activity • Enhancement of cytotoxic effects of VP16	• Reduction in AML blast colony forming cells	• Modest effect in AML blast colony forming cells	• Modest effect in AML blast colony forming cells
References	Sujobert et al. (2005), Billottet et al. (2006)	Xing et al. (2012)	Xing et al. (2012)	Xing et al. (2012)
APL	• Suppression of the ATRA-induced phosphorylation of Akt and S6 • Induction in apoptosis	• No effect	• Suppression of the ATRA-induced phosphorylation of Akt and S6 • Induction in apoptosis	• No effect
References	Billottet et al. (2009)	Billottet et al. (2009)	Billottet et al. (2009)	Billottet et al. (2009)

and prolonged the host survival (Ikeda et al., 2010). Inhibition of p110δ activity by CAL-101 in DLBCL cell lines reduced the phosphorylation of Akt and S6 and increased the cleavage of the apoptotic markers caspase 3 and poly(ADP-ribose) polymerase (Lannutti et al., 2011). CAL-101 also decreased the phosphorylation of Akt and induced apoptosis in HL cell lines and moreover blocked the stroma cells-induced Akt activation in HL cells and disrupted the survival signals mediated by CCL5, CCL17, and CCL22 in co-cultures of HL cells with stromal cells (Meadows et al., 2012).

Various mechanisms that alter the activity of protein tyrosine kinases and phosphoinositide phosphatases that are involved in BCR signaling have been proposed to account for the over-activation of the PI3K pathway in malignant B-cells. Lyn kinase was found to be over-expressed in CLL and its inhibition led to induced apoptosis (Contri et al., 2005; Trentin et al., 2008). Syk is also over-expressed and constitutively phosphorylated and activated in CLL (Buchner et al., 2009; Efremov and Laurenti, 2011). The zeta-associated protein of 70-kD (ZAP-70) kinase, which is a Syk family kinase, is expressed in a subset of B-CLL patients (Rosenwald et al., 2001) and has been implicated in the elevated PI3K activity since its introduction in B cells that do not express ZAP-70 led to increased Akt phosphorylation (Gobessi et al., 2007). Other experiments have revealed that ZAP-70 functions as an adaptor protein in BCR signaling (Chen et al., 2008) and that the phosphorylation of Syk, PLCγ, and BLNK is enhanced in B-cell ZAP-70 positive compared to B-cell ZAP-70 negative CLL (Chen et al., 2005) which could indirectly alter the PI3K activity. B cells from ZAP-70 positive CLL patients were also found to express decreased levels of the SHIP phosphatase which affects PI3K signaling by dephosphorylating the product of PI3Ks PI(3,4,5)P3 producing PI(3,4)P2 (Brauweiler et al., 2000). The lipid phosphatase PTEN which directly antagonizes the PI3K pathway (Maehama and Dixon, 1998) has been found to be rarely mutated in B cell malignancies (Grønbæk et al., 1998; Sakai et al., 1998), however, its expression was found to be reduced or lost in CLL (Leupin et al., 2003). This has been attributed to be a result of miR-17-92 overexpression which negatively regulates PTEN expression in various leukemias (Lenz et al., 2008; Rao et al., 2012). Reduced PTEN activity has also been found in CLL (Shehata et al., 2010) which might be a result of overexpression and overactivation of CK2 that were also detected in CLL and blockade of CK2 decreased PTEN phosphorylation leading to PTEN activation (Shehata et al., 2010; Martins et al., 2011). Other than the control of PTEN activity by CK2 (Torres and Pulido, 2001), a variety of mechanisms regulate the activity of the PTEN tumor suppressor in B cells (Pauls et al., 2012) and might also be involved in B-cell malignancies, a possibility that remains to be determined.

ROLE OF p110δ PI3K IN MYELOID MALIGNANCIES

Besides the B-cell malignancies, the role of class IA PI3Ks has been also studied in some myeloid malignancies (**Table 2**). Acute myeloid leukemia (AML) is characterized by the uncontrolled survival and proliferation of immature myeloid cells and their abnormal accumulation in the bone marrow. PI3K/Akt pathway was found to be constitutively activated in leukemic cells of AML patients, contributing to unrestricted cell survival and proliferation (Min et al., 2003; Xu et al., 2003; Zhao et al., 2003; Doepfner et al., 2007). Further studies have demonstrated that p110δ was the main PI3K isoform that was involved, as it was indicated from the higher p110δ expression levels, compared to other isoforms, in blast cells of AML patients (Sujobert et al., 2005). Treatment of these cells with the p110δ-specific inhibitor IC87114, suppressed the constitutive Akt activation (Sujobert et al., 2005; Billottet et al., 2006) to equal levels as those observed upon the pan-PI3K inhibitor LY294002 treatment (Sujobert et al., 2005) confirming that the p110δ is the main isoform contributor of PI3K activity in AML cells. It is of note that IC87114 did not affect the proliferation of normal hematopoietic progenitor cells (Sujobert et al., 2005). The combination of IC87114 with other antineoplastic agents such a topoisomerase II inhibitor VP16, which is used in AML patients treatment, further reduced AML cell numbers and NF-kB activity and most profoundly induced apoptosis (Billottet et al., 2006). Thus, the use of p110δ-specific inhibitors in combination with other cytotoxic drugs, may offer the maximum therapeutic efficiency in AML pathology accompanied with minimum overall toxicity. A recent study has shown that inhibition of p110α is also effective in killing AML blast colony forming cells (Xing et al., 2012), however, the concentration of p110α inhibitor used in this study was much higher (more than 1000 fold higher) than the IC50 value of this compound (Hayakawa et al., 2007) making thus unclear whether this inhibitor retains its isoform selectivity. Inhibitors for p110β or p110γ had a very modest effect (Xing et al., 2012).

Acute promyelocytic leukemia (APL) is a relative to AML malignancy, characterized by the increased accumulation of abnormal promyelocytes to the bone marrow due to their inability to differentiate normally and to their increased resistance in apoptotic signals. Similar to AML, p110δ seems to contribute in the constitutive PI3K signaling observed in APL promyelocytes (Billottet et al., 2009). However, p110β is also involved in APL and in line with this, both isoforms are consistently expressed in APL cells (Billottet et al., 2009). All-trans retinoic acid (ATRA) is an agent used in APL treatment because of its potential to promote the differentiation of promyelocytic leukemic cells by regulating the PI3K/Akt/mTOR pathway (Lal et al., 2005). Inhibition of p110δ or p110β suppressed the ATRA-induced Akt and S6 phosphorylation without affecting the ATRA-induced differentiation (Billottet et al., 2009). Dual inhibition of both p110δ and p110β activity promoted the apoptosis of primary APL cells in the presence and in the absence of ATRA treatment (Billottet et al., 2009). These results suggested that inhibition of p110δ and p110β combined with differentiation induced treatments may represent potential therapeutic targets in APL.

The mechanism leading to the dominant activity of p110δ in myeloid malignancies is not yet completely clear. The most profound reason seems to be its high expression in leukocytes since no mutations on *PIK3CD* gene have been found in samples from AML patients (Cornillet-Lefebvre et al., 2005). The PTEN expression levels were also readily detected in AML samples and its phosphorylation on S380/T382/T383 was marginally affected (Billottet et al., 2006), indicating that other mechanisms are involved in the constitutive Akt activation in AML.

The constitutive p110δ activity in AML could be triggered by upstream factors and autocrine mechanism. Previous studies had shown that insulin growth factor-1 receptor (IGF-1R) signaling is constitutively activated in AML cells through IGF-1 autocrine production (Doepfner et al., 2007; Tazzari et al., 2007). The IGF-1R was found to be constitutively phosphorylated in all leukemic cells tested, whereas its inhibition with neutralizing anti-IGF-1R strongly inhibited the phosphorylation of Akt and cell proliferation in AML cells (Chapuis et al., 2010). Inhibition of both p110β and p110δ impaired the IGF-1 stimulated Akt activation, cell growth and survival, suggesting that both isoforms are activated downstream of IGF-1 signaling in AML cells (Doepfner et al., 2007).

A PROMISING ROLE OF p110δ PI3K EMERGES IN NON-HEMATOLOGIC CANCERS

Since the p110δ PI3K was cloned and characterized (Chantry et al., 1997; Vanhaesebroeck et al., 1997b) an increasing catalog of evidence showing p110δ-isoform specific functions in hematopoietic cells have suggested p110δ as a potential therapeutic target in immunity, inflammation, and hematological malignancies (Rommel et al., 2007; Okkenhaug and Fruman, 2010; Rommel, 2010; Soond et al., 2010; Fruman and Rommel, 2011). The p110δ-isoform specific functions were demonstrated by mice with inactivated p110δ (Clayton et al., 2002; Jou et al., 2002; Okkenhaug et al., 2002; Ali et al., 2004; Aksoy et al., 2012) and by p110δ-selective inhibitors such as the IC87114 compound, which was the first isoform-selective inhibitor published (Sadhu et al., 2003), and the CAL101 which has recently entered clinical studies for hematologic malignancies (Fruman and Rommel, 2011; Castillo et al., 2012). It was the preferential expression of p110δ in leukocytes (Chantry et al., 1997; Vanhaesebroeck et al., 1997b) together with the absence of somatic mutations in *PIK3CD* gene that placed p110δ PI3K in the realm of immune system and hematologic cancers.

A non-expecting role of p110δ in oncogenesis of non-hematopoietic cells was first observed in avian fibroblasts in which overexpression of wild-type p110δ induced oncogenic transformation (Kang et al., 2006). The p110δ-overexpressing cells were found to express elevated levels of phosphorylated Akt, comparable to those detected in cells expressing the oncogenic H1047R p110α mutant (Kang et al., 2006). The p110δ oncogenic activity was not required binding of p110δ to RAS and was resistant to inhibitors of the MAPK pathway (Zhao and Vogt, 2008a; Vogt et al., 2009). Further data have also suggested a role of p110δ in non-hematologic human cancers (**Table 1**). Overexpression of p110δ mRNA and increased copy number of the *PIK3CD* gene were found in some cases of glioblastoma (Knobbe and Reifenberger, 2003; Mizoguchi et al., 2004). p110δ mRNA was also found to be increased in prostate carcinoma compared with normal prostate (Jiang et al., 2010). Abnormally high p110δ expression levels were found in primary neuroblastoma tissue compared with the normal adrenal gland tissue (Boller et al., 2008) and suppression of p110δ expression in neuroblastoma cells led to impaired cell growth and survival (Boller et al., 2008). All this evidence suggested that the expression levels of wild-type p110δ might correlate with its oncogenic potential.

Recent data documented that p110δ PI3K inhibits the activity of the PTEN tumor suppressor via a negative signaling pathway that involves inhibition of RhoA/ROCK (Papakonstanti et al., 2007) (**Figure 4**). The activation of p110δ PI3K was found to positively regulate the p190RhoGAP activity and to result in the accumulation of p27 in the cytoplasm (Papakonstanti et al., 2007). Given that p190RhoGAP catalyzes the return of RhoA-GTP (active state) to RhoA-GDP (inactive state) (Bernards and Settleman, 2005) and p27 prevents the return of RhoA-GDP to RhoA-GTP (Besson et al., 2004) the activation of p110δ leads to decreased RhoA activity and consequently to decreased PTEN activity. Upon genetic or pharmacological inactivation of p110δ

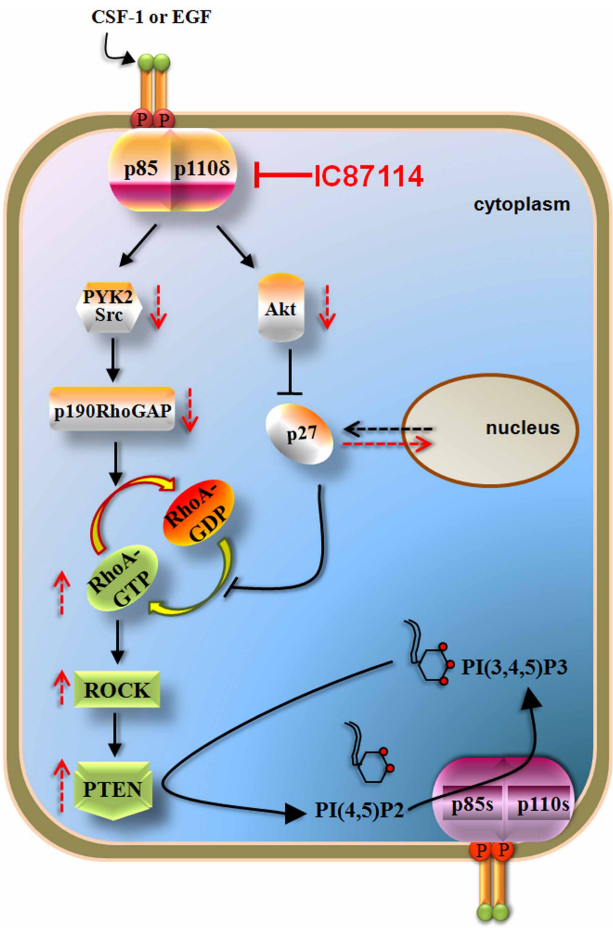

FIGURE 4 | p110δ PI3K mediates the effects of CSF-1 or EGF via RhoA and PTEN in macrophages and some solid tumor cells. Activation of p110δ leads to increased activity of p190RhoGAP (through PYK2/Src activation) and to cytoplasmic accumulation of p27 (which is mediated by the increased Akt activity). p190RhoGAP induces the inactivation of RhoA whereas p27 prevents the activation of RhoA both thus leading to reduced RhoA activity and consequently to decreased PTEN activity. Inactivation of p110δ by IC87114 reverses these pathways leading to PTEN activation which then opposes the PI3K reaction of the remaining active p110 isoforms. The p190RhoGAP-driven mechanism almost solely keeps RhoA activity low under basal whereas that of p27 contributes upon stimulated conditions. Arrows with dashed lines represent alterations in activity or location in the presence (red) or in the absence (black) of IC87114.

by IC87114, PTEN becomes activated and dampens the PI3K pathway (**Figure 4**). The isoform-selective role of p110δ in the negative regulation of RhoA and the mechanism by which RhoA regulates PTEN activity under the control of p110δ are not currently understood but it seems that these are related with activation of p110δ at certain cellular compartments (Papakonstanti, unpublished data). This feedback mechanism was originally found to be the case in primary (Papakonstanti et al., 2007) and transformed macrophages (Papakonstanti et al., 2008) and in mouse brain tissue (Eickholt et al., 2007). More recently we showed that the negative regulation of PTEN by p110δ is also the case in those cancer contexts where p110δ is expressed at high levels (Tzenaki et al., 2012). The p110δ protein was found to be expressed at different levels in different cancer types e.g., the p110δ PI3K is the predominant isoform expressed in human primary breast carcinoma, whereas ovarian and cervical human carcinomas mainly express p110α and p110β (Tzenaki et al., 2012). The activity of wild-type PTEN was found to be suppressed in breast and prostate cancer cells that express high levels of p110δ suggesting that the elevated expression of p110δ might provide these cells with a competitive advantage to keep their wild-type PTEN inactive (Tzenaki et al., 2012). Breast and prostate cancer cells expressing functional PTEN were also sensitive to anti-proliferative effect of p110δ inhibitors through PTEN activation. In contrast, inhibition of p110δ in ovarian and cervical cancer cells which express very low levels of p110δ had no effect neither in PTEN activity nor in cell proliferation (Tzenaki et al., 2012).

The p110δ expression levels might therefore represent one of the parameters that correlate with the cancer type-specific response to PI3K pathway inhibitors, a possibility that will be important to be explored in future studies. This hypothesis is also corroborated by other published data. For example, breast cancer cells were found to be sensitive to growth inhibition by PI3K inhibitors without having mutations in *PTEN* or *PIK3CA* genes (O'Brien et al., 2010). On the other hand, breast cancer cells with PTEN deficiency were found to be resistant to PI3K inhibitors (Tanaka et al., 2011), PI3K/mTOR inhibitors (Brachmann et al., 2009) or mTOR inhibitors (Weigelt et al., 2011) whereas some of the PTEN-deficient breast cancer cell lines were sensitive to inhibitors of the PI3K pathway (She et al., 2008; Lehmann et al., 2011; Sanchez et al., 2011; Tanaka et al., 2011). In ovarian cancer cells, however, *PIK3CA* gain-of-function mutations and PTEN deficiency were correlated with their response to PI3K pathway inhibitors (Ihle et al., 2009; Di Nicolantonio et al., 2010; Meuillet et al., 2010; Santiskulvong et al., 2011; Tanaka et al., 2011; Meric-Bernstam et al., 2012). There are also evidence showing that in human breast tumor cells and cancer cell lines there is no good correlation between the presence of *PIK3CA* gain-of-function mutations and the basal or growth factor stimulated PI3K and Akt activity (Stemke-Hale et al., 2008) suggesting that other regulatory mechanism may affect the status of PI3K activity. It will be important to determine whether in breast cancers that *PIK3CA* gene is mutated and *PTEN* gene is wild-type, induction of PTEN activity by inhibition of p110δ PI3K dampens the production of $PI(3,4,5)P_3$ and cell growth. An open question is also whether in cells with heterozygously mutated PTEN, the remaining wild-type PTEN allele is under the influence of high levels of p110δ. Given that *PTEN* gene is often wild-type in human breast cancers (Stemke-Hale et al., 2008; Chalhoub and Baker, 2009), further experiments may reveal that p110δ-selective inhibitors alone or combined with inhibitors of other components of PI3K pathway could be beneficial in this cancer type.

CONCLUSIONS

The PI3K signaling pathway was brought at the center of attention in the field of cancer research by the discovery of cancer-specific gain-of-functions mutations in *PIK3CA* gene (Campbell et al., 2004; Samuels and Velculescu, 2004). Deregulated PI3K signaling in cancer has also been attributed to gain of function in receptor tyrosine kinases, activated Akt or to loss-of-function mutations in *PTEN* gene (Vivanco and Sawyers, 2002; Engelman et al., 2006; Yuan and Cantley, 2008). Although the p110α PI3K pathway and the loss of function of PTEN have received a great attention for their involvement in human cancers there are still some unexplained observations. Indeed, recent studies have shown that there is poor correlation between the *PIK3CA* or *PTEN* mutational status in cancer cell lines and the response of these cells to anti-proliferative effect of PI3K inhibitors (Edgar et al., 2010; O'Brien et al., 2010; Tanaka et al., 2011) indicating that unidentified mechanisms or PI3K isoform(s) other than p110α are also involved in the control of cancer cells survival. There are also evidence documenting no correlation between the oncogenic activity of p110α PI3K and signaling through Akt (Gymnopoulos et al., 2007; Zhao and Vogt, 2008b; Vasudevan et al., 2009) suggesting that Akt can be a non-obligatory partner in PI3K signaling and that Akt-independent PI3K pathways may be important in cancer cells.

The relationship of p110δ PI3K with cancer had received much less attention but recently p110δ has entered the realm of hematologic cancers and p110δ-selective inhibitors have provided promising results in some hematological malignancies (Fruman and Rommel, 2011; Castillo et al., 2012). p110δ is exceptional in that it regulates not only homeostasis and function of B-cells but also it is involved in the transduction of microenvironmental signals including chemokines and cytokines derived from lymphoid tissues or T-cells (Okkenhaug and Fruman, 2010; Puri and Gold, 2012). p110δ-selective inhibitors have been studied in multiple hematologic malignancies and the most promising results are currently available for B-CCL. It was remarkable that inhibition of p110δ in B-CLL cells or treatment of patients with the p110δ-selective inhibitor CAL-101 prevented B-CLL survival and moreover disrupted the signals from supporting cells of B-CLL microenvironment thus providing an anti-tumor activity (Herman et al., 2010; Hoellenriegel et al., 2011; Castillo et al., 2012). The fact, however, that CAL-101 inhibited cytokine production by human T cells (Herman et al., 2010; Hoellenriegel et al., 2011) together with data showing that p110δ plays an important role in functions of NK cells (Kim et al., 2007; Saudemont et al., 2007), in anti-tumor response of cytotoxic T lymphocytes (Putz et al., 2012) and in the development of regulatory T cells (Patton et al., 2006), raise the question if the efficacy of p110δ inhibition might be counterbalanced by a potential suppression of anti-tumor immunity. Nevertheless, although a single agent

treatment was unexpected to have clinical activity, the outcome of the patients treated with CAL-101 went far beyond the expectations.

A critical role of p110δ in solid tumor cells has just emerged by published data showing that an oncogenic potential of p110δ might correlate with its expression levels (Knobbe and Reifenberger, 2003; Mizoguchi et al., 2004; Boller et al., 2008; Zhao and Vogt, 2008a; Vogt et al., 2009; Jiang et al., 2010) and by evidence documented that in those solid tumor cells expressing leukocyte-levels of p110δ, this isoform suppresses the activity of wild-type PTEN rendering these cells sensitive to growth-inhibitory effects of p110δ-selective inhibitors (Tzenaki et al., 2012). The mechanism that accounts for the high expression levels of p110δ PI3K in some cancer types whilst in others the expression of p110δ is very low (Tzenaki et al., 2012) is unclear at the moment. The differential expression of p110δ in human cancers might be a result of transcriptional regulation by differentially activated transcription factors in each cancer type or a consequence of epigenetic aberrations. It is also of note that the expression levels of the PI3K regulatory subunit p85β were found to be elevated in breast carcinomas and that altered *PIK3R2* expression affected tumor progression (Cortés et al., 2012). It will be important to determine whether a specific combination of p110δ with p85β exists in breast carcinomas and whether it affects the activity of p110δ.

There is also evidence to suggest that GEFs and GAPs, which regulate the activity of small GTPases, could play important roles in cancer biology as components of PI3K signaling. Indeed, P-REX2a (phosphatidylinositol 3,4,5-trisphosphate Rac exchanger 2a), which activates the small GTPase Rac, was found to interact with PTEN and directly inhibit PTEN function (Fine et al., 2009). It is also of note that the p190RhoGAP-driven mechanism (the p110δ/p190RhoGAP/RhoA/PTEN branch in **Figure 4**), contributes almost solely to inhibition of RhoA and PTEN under non-stimulated conditions (Papakonstanti et al., 2007) suggesting that p110δ can signal to a large extend independently of Akt through p190RhoGAP and RhoA.

The p110δ PI3K has just become eminent in the field of hematologic malignancies and it seems to have the potential to come into the spotlight of non-hematologic cancers. More work is needed to delineate the role of p110δ in cancer and to determine important aspects of its regulation and function such as the possible activation of p110δ at certain cellular locations, the mechanisms by which p110δ regulates its cellular locations, the role of p110δ in cancers cells expressing mutated p110α and/or heterozygously mutated PTEN, the p110δ triggered signaling through effectors other than Akt and last but not least the mechanism that regulates the differential expression of p110δ in different cancer types. Answering these questions will reveal a ground of discoveries that might illuminate some currently unexplained observations potentially improving therapeutic strategies against cancer.

ACKNOWLEDGMENTS

Authors' work is supported by grants from EU and the Research Committee of the University of Crete. The article publishing fee was covered by the Research Committee of the University of Crete (KA 3774). Niki Tzenaki is graduate student of the Graduate Program in the Molecular Basis of Human Disease of the Medical School, University of Crete.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 December 2012; paper pending published: 23 December 2012; accepted: 12 February 2013; published online: 01 March 2013.

*Citation: Tzenaki N and Papakonstanti EA (2013) p110δ PI3 kinase pathway: emerging roles in cancer. Front. Oncol. **3**:40. doi: 10.3389/fonc.2013.00040*

This article was submitted to Frontiers in Molecular and Cellular Oncology, a specialty of Frontiers in Oncology.

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frontiers in ONCOLOGY

OPINION ARTICLE
published: 15 July 2013
doi: 10.3389/fonc.2013.00186

Did we get Pasteur, Warburg, and Crabtree on a right note?

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Antoine Lavoisier (eighteenth century) demonstrated that living organisms consume oxygen to slowly burn the fuels in their bodies to release energy (1). Louis Pasteur (2) in an epoch making discovery, recognized as "*the Pasteur effect*," declared "fermentation is an alternate form of life and that fermentation is suppressed by respiration" [reviewed in (3–5)]. Yet another observation of Pasteur, that yeast cells consume oxygen to multiply, is debated less among cancer biologists. This nevertheless, forms the first scientific observation that proliferating cells need oxygen to divide. Pasteur was involved in a path breaking debate with Liebig on whether fermentation is a chemical or a biological process? By 1880s Pasteur could establish that fermentation is a physiological process, and oxygen, considered to be a putrefying factor by Liebig and others, was a growth promoting factor [reviewed in (3)]. Six decades later, Warburg (6) proposed that damaged respiration and enhanced fermentation of glucose is the prime cause of cancer formation. It became popular as "*aerobic glycolysis*" and a counter to "*the Pasteur effect*." Warburg was uncompromising and was intolerant to any alternate theory on cancer formation and declared to the German Central Committee for Cancer control in 1955 at Stuttgart "… there is today no other explanation for the origin of cancer cells, either special or general. From this point of view, mutation and carcinogenic agent are not alternatives, but empty words, unless metabolically specified…" (7). Crabtree (8), a contemporary of Warburg, suggested that pathological over growths use aerobic glycolysis as a source of energy and glucose uptake and glycolytic activity has a depressive effect on oxygen consumption. His conclusion gained the popularity as the "inverted Pasteur effect" or the "Crabtree effect" (9). Several authors in the middle of twentieth century reported that glucose is a negative regulator of respiration. These reports indicate that there is an initial stimulation of oxygen consumption for about 20–120 s following glucose consumption followed by an inhibitory period, which after equilibration stabilizes to a constant of about 30% of the endogenous rate until all the glucose was consumed [reviewed by (9)].

PASTEUR, WARBURG, AND CRABTREE – THE THREE EDGES OF A TRIANGLE COIN?

A critical analysis of the statements suggests that Pasteur, Warburg, and Crabtree represent the three edges of a *Triangle Coin*, while glucose and oxygen form the two sides of the coin. Semantics apart, the three authors conclude that glucose utilization and the presence of oxygen are required for proliferating cells. Pasteur observed that when sufficient oxygen is available, yeast "seizes to be ferment and increases in mass," but renews its capacity to ferment under depleted oxygen. His observations that ammonia transformed into a complex "*albuminoid*" (protein) compound during fermentation and growth is a recognition of the fact that the two processes can coexist when nitrogen source and oxygen are available [see the reviews (3, 4)]. Warburg's hypothesis that "*damaged respiration promotes fermentation even in the presence of oxygen*" is a reiteration of Pasteur's statement on growth in principle. Warburg considered fermentation but not oxygen is a deciding factor in proliferation. He was aware that glucose consumption and the oxygen levels in different regions of the tumors can vary and fermentation decreases in the direction of capillary blood flow (10). Tumors grafted in low oxygen tension were shown to grow slowly (11); Crabtree demonstrated 50% higher respiration in subcutaneous tumors than those in abdomen which has 50% higher oxygen tension (8). It is noteworthy that Harvey (12), around Warburg's times, demonstrated that oxygen is needed for the proper division of Sea Urchin eggs, and lack of oxygen arrests the development. Recent studies indicate that proliferative cells exhibit altered metabolism (13–15) and tumor is a heterogeneous tissue; a metabolic symbiosis exists between cells in sharing glucose and lactate between well oxygenated and low oxygenated population of cells (16).

In short, with regard to proliferative cells, there are two unifying principles in the three apparently diverging hypotheses of Pasteur, Warburg, and Crabtree, i.e., an inverse relation exists between glucose uptake and oxygen utilization (respiration); while fermenting cells require more glucose, the proliferative cells require both glucose and oxygen. Pasteur, in addition, points to the requirement of nitrogen as an additional source (albuminoid) for growth of yeast

ADENYLATE PHOSPHATE ESTERS (ATP, ADP, AND AMPS) REGULATE METABOLISM

The debate on Warburg and Pasteur, gave a lead to deciphering the details of glycolysis, citric acid cycle, and the role of adenylate phosphate esters (ATP, ADP, and AMPs) in metabolic regulation [see (17–21)]. The discovery of ATP by Fiske and Subbarow and Lohmann paved the way for understanding the role of oxygen in ATP generation through mitochondrial respiration and oxidative phosphorylation (OXPHOS) [reviewed by (5)]. The debate on the mitochondrial function, reactive oxygen species (ROS), and regulation of OXPHOS, however, is still alive (22, 23). Parallel to the research

on cancer cells were the studies on energetics of muscle contraction, mainly by Lundsgard, Meyerhof, A. V. Hill, and others, where lactate production, heat generation, and oxidative recovery of the energy remained the main focus [reviewed in (4)]. Some of these early researchers came out with the theories of competitive limitation of inorganic phosphates (Pi), ADP, and hexokinase as regulating agents of OXPHOS and glycolysis. Two key inhibitors, the indoleacetic acid (IAA) and nitrophenols which block glycolysis, and OXPHOS respectively, were developed to examine the relative influence of glycolysis and OXPHOS on metabolism of cells and exercising muscles which produced enormous amounts of lactate [reviewed in (4) and (24)]. Most of these researchers were influenced by either Warburg or Pasteur hypotheses. For example, Nigam (25), demonstrated that in Novikoff Ascites-Hepatoma cells, when OXPHOS is blocked in the presence of oxygen by nitrophenols, glucose consumption is rapid but reaches a plateau within 10–15 min, and glycolysis (lactate production) is up regulated at the expense of the glycogen synthesis. In the absence of nitrophenols glucose up take was proportional to the glycogen synthesis and increased slowly but exponentially when compared to the cells with blocked OXPHOS. The author tried to explain the results as a different type of Pasteur effect, but a critical analysis of the results indicates that glucose uptake in the presence of oxygen is proportional to its utility in macromolecular biosynthesis.

THE RATIO OF AMP AND ATP (ENERGY CHARGE) REGULATES GLYCOLYSIS AND OXPHOS

Phosphofructokinase (PFK) was identified as a key regulator of glycolysis (4, 26). Ramaiah et al. (27) demonstrated that the ratio of AMP and ATP in cells decides whether PFK is inhibited or activated. Atkinson (28) suggested that feed back cycles regulate the metabolic fate of cells and this regulation depends on the energy charge of cells, i.e., [(ATP) + 0.5(ADP)]/[(ATP) + (ADP) + (AMP)]. The ratio of ATP production between glycolysis and mitochondrial OXPHOS is 1:15 per one molecule of glucose and it takes only few molecules of glucose for healthy cells to reach the saturating levels of ADP:ATP ratio of 1:10 (29). Mitochondria have several other functions, in addition to ATP producing function. During biosynthetic processes they act as regulators of the metabolic homeostasis in cells and regulate the carbon and nitrogen fluxes between proteins, lipids by activating anaplerotic and cataplerotic reactions for reconstruction of membranes, and supra molecular structures [see (30–33)].

ENERGY STATUS OF CELLS ALSO MODULATES CELL SIGNALING PATHWAYS

The explosion of genomic research that followed the discovery of DNA double helix in the last half of twentieth Century has shifted focus of cancer research from metabolism to gene mutations (14, 34–36). One of the key contributions of the genomic era is the discovery of the oncogenes and tumor suppressors (37), which work in a regulated series of networks of signaling pathways that respond to environmental cues in modulating the energy metabolism and cell cycle progression. There was a renewed search for pathways responsible for metabolic reprograming in cancer cells [see (38–40)] and Akt was named as "Warburg enzyme" (41). Akt and its downstream target, the mechanistic target of rapamycin (mTOR; earlier known as mammalian target of rapamycin), were recognized to play crucial role in several metabolic disorders including cancer [Reviewed by (42)]. The mechanistic target of rapamycin, especially the complex1 (mTORC1) plays a crucial role in regulation of metabolism and promotes the biosynthetic activity of cells (an energy consuming process) and activates cell survival pathways by inhibiting GSK3β and autophagy. It has come to be recognized that ATP/ADP ratio plays critical role in modulating the functions of Akt (43) as well as those of mechanistic target of rapamycin (44).

MITOCHONDRIAL DYSFUNCTION IS CENTRAL TO PATHOGENESIS

Warburg considered dysfunctional mitochondria (respiration) as the cause of cancer. Generation of superoxide radical (O_2^-) leading to ROS has become the symbol of mitochondrial dysfunction. The key factors that promote the ROS generation are high proton motive force (Δp), a reduced coenzyme Q (CoQ), a high NADH/NAD ratio, and the presence of intra mitochondrial O_2 (22). We have earlier presented a view that inhibition of autophagy has a crucial role in promoting cell cycle progression and metabolic reprograming (45). One of the consequences of inhibition of autophagy is also the inhibition of mitophagy (46). In addition, inhibition of FoxO3a by activated Akt down regulates the anti oxidant enzymes MnSOD and catalases (47); mutational deregulation of mitochondrial genome by ROS disables mitochondrial ability to produce ATP. ROS in fact, are suggested to act as rheostat in deciding the cell fate and metabolism in hematopoietic stem cells by regulating the Bcl-2 proteins (48). As already indicated, high energy (ATP/ADP) conditions promote activation of Akt (43) and the mTOR (44). The Akt-mTORC1 signaling also modulates the mitochondrial survival by regulating the activity of GSK3β, which has crucial regulatory role in activation of proapoptotic mitochondrial proteins (49, 50). Mitochondria are required for amino acid metabolism, citrate production, urea production, heme synthesis, and FeS assembly etc (51, 52). In cancer cells, GSK3β remains inhibited; inhibition of mitophagy during proliferation of cells may also lead to activation of aspartate, malate, and citrate shuttles, which are very much essential for carbon and nitrogen recycling during biosynthetic process. The life style healthy practices like aerobic exercise, yoga, which promote expenditure of energy, the dietary restriction, which limits the energy (calorie) intake and therapeutic drugs like metformin, PPARγagonists, which promote activation of AMPK and autophagy, therefore, play crucial roles in amelioration of metabolic pathologies (53, 54).

CONCLUSION

In summary, it should be recognized that there is a unifying concept in the hypotheses of Pasteur, Warburg, and Crabtree that proliferative cells require oxygen, nitrogen source, and glucose (albeit lesser than that required for fermentation). Mitochondria, which are central to Warburg's theory of damaged respiration, have diverse functions. Taking cues from signal pathways, it is suggested that high ATP/ADP ratio activate both Akt and mTORC1, inhibit autophagy/mitophagy, up regulate ROS, and promote biosynthetic activity (see **Figure 1**). Mitochondrial metabolism shifts from ATP production to anaplerotic reactions that replenish fatty acids and non-essential amino acids (55) for biosynthesis of macromolecules and membrane structures of proliferating cells. During the discussion of

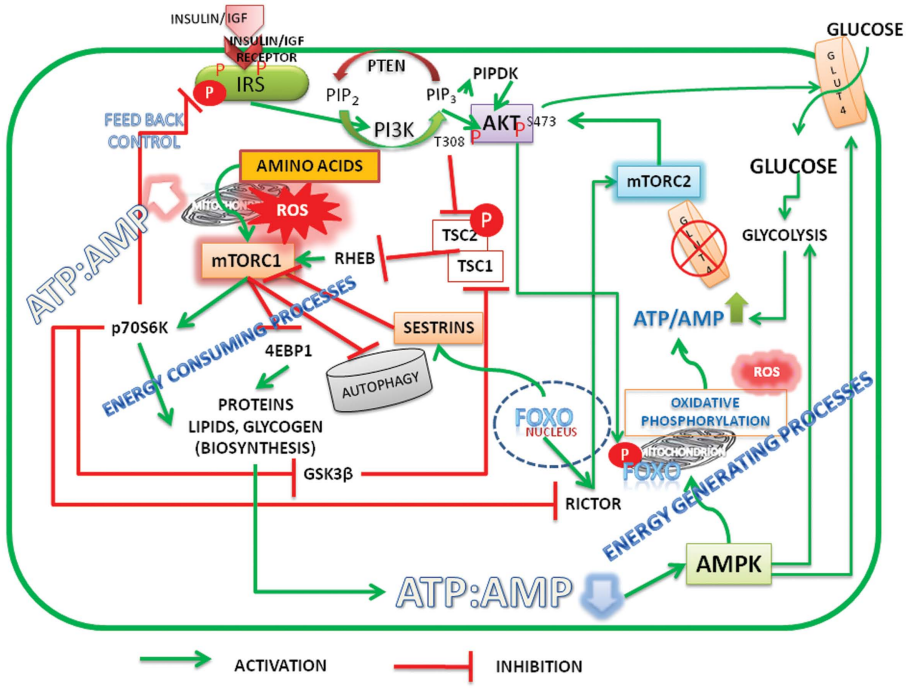

FIGURE 1 | Schematic representation of energy status of cells modulating cell signaling and mitochondrial function. Under high energy (ATP/AMP ratio)/nutrient levels the insulin/insulin growth factor signaling (IIS) activates PI3K-Akt pathway. Initiation of PI3K-Akt signaling takes place when mTORC2 is active and phosphorylates Akt on Serine473. FoxO is the transcription factor of rictor, a critical component of mTORC2. Akt is further phosphorylated at Threonine 308 by IIS mediated PIPDK (originally PDK1). High ATP/AMP ratio stabilizes the phosphorylations and activated Akt phosphorylates FoxO, which leads to its exclusion from the nucleus. Akt inactivates tuberous sclerosis complex (TSC) 1/2 resulting in activation of mTORC1. mTORC1 promotes biosynthetic activity, inhibits autophagy. When ATP/AMP ratio is high, mitochondria stop synthesizing ATP and generate reactive oxygen species (ROS) and activate metabolite shuttles to replenish the amino acids and citrate, the precursors of protein and membrane lipids. Reduction in the ATP/ADP ratio, on the other hand, results in activation of AMPK, which activates autophagy. FoxO translocates into nucleus transcribes sestrins which inhibit mTORC1 and activates mTORC2 by transcribing rictor. The activation of glycolysis and glucose transport by Akt S473 and AMPK increases the ATP/ADP ratio there by reactivating the cycle. Under normal and healthy dietary conditions a perfect balance between ATP production, ROS generation, and biosynthetic processes is cyclically maintained by activation–inactivation cycles of autophagy modulated by alternate activation and inactivation cycle of AMPK and mTORC1. Under surplus nutrients/inflammatory conditions, a deregulated hyper activated mTORC1 leads to either carcinogenesis or insulin resistance. A growing tumor, with increased population of cells is heterogeneous with mixed population of cells, either deprived of oxygen or having access to it. It maintains a metabolic symbiosis with hypoxic cells surviving on glucose uptake and anaerobic glycolysis, while those having access to oxygen thrive on lactate accumulating in the neighborhood (microenvironment). Akt, protein kinase B (T308, S473 – Phosphorylated sites Threonine 308 and Serine 473); AMPK, AMP activated protein kinase; FoxO, fork head transcription factors of O group; GSK3β, glycogen synthase kinase3β; GLUT, glucose transporter; IGF, insulin growth factor; IRS, insulin receptor substrate; mTORC1, 2, mechanistic target of rapamycin Complex 1 and 2 (mTOR: formerly known as mammalian target of rapamycin); PIP2, phosphatidylinositol 4,5 bisphosphate; PIP3, phosphatidylinositol 3,4,5 trisphosphate; PIPDK, phosphoinositide dependent kinase 1 (the abbreviation PIPDK is preferred over the original PDK1 in the article to avoid confusion with the pyruvate dehydrogenase kinase, which is also abbreviated as PDK1 in the literature); PI3K, Phosphatidylinositol 3-kinases; Rictor, a Component of mTORC2; p70S6K, The p70 Ribosomal S6K; ROS, reaction oxygen species; Sestrins, stress response proteins.

the article, two new publications (56, 57), highlighted the role of nutrients/energy, Akt, and mTORC1 in modulating biosynthetic activity of cells.

ACKNOWLEDGMENTS

Dr. Lakshmipathi Vadlakonda is thankful to the Director of CRRAO AIMSCS and the DST, New Delhi for placement as the honorary adjunct faculty. Dr. Mukesh Pasupuleti thanks the management of SRM University for providing facilities at SRM Research Institute. Abhinandita Dash is thankful to the Director, CRRAO (AIMSCS) and DST for the JRF fellowship under the grants DST: SR/S4/MS:516/07 dated 21-04-2008. Dr. Reddanna Pallu thanks the Department of Biotechnology for placement in NIAB.

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Received: 29 May 2013; accepted: 02 July 2013; published online: 15 July 2013.
*Citation: Vadlakonda L, Dash A, Pasupuleti M, Anil Kumar K and Reddanna P (2013) Did we get Pasteur, Warburg, and Crabtree on a right note? Front. Oncol. **3**:186. doi: 10.3389/fonc.2013.00186*
This article was submitted to Frontiers in Molecular and Cellular Oncology, a specialty of Frontiers in Oncology.
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frontiers in ONCOLOGY

HYPOTHESIS AND THEORY ARTICLE
published: 20 June 2013
doi: 10.3389/fonc.2013.00165

The paradox of Akt-mTOR interactions

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The serine threonine protein kinase, Akt, is at the central hub of signaling pathways that regulates cell growth, differentiation, and survival. The reciprocal relation that exists between the two activating phosphorylation sites of Akt, T308 and S473, and the two mTOR complexes, C1 and C2, forms the central controlling hub that regulates these cellular functions. In our previous review "PI3Kinase (PI3K)-AKT-mTOR and Wnt signaling pathways in cell cycle" we discussed the reciprocal relation between mTORC1 and C2 complexes in regulating cell metabolism and cell cycle progression in cancer cells. We present in this article, a hypothesis that activation of Akt-T308 phosphorylation in the presence of high ATP:AMP ratio promotes the stability of its phosphorylations and activates mTORC1 and the energy consuming biosynthetic processes. Depletion of energy leads to inactivation of mTORC1, activation of AMPK, FoxO, and promotes constitution of mTORC2 that leads to phosphorylation of Akt S473. Akt can also be activated independent of PI3K; this appears to have an advantage under situations like dietary restrictions, where insulin/insulin growth factor signaling could be a casualty.

Keywords: FoxO, rictor, Akt, insulin/IGF signaling, glucose transport

INTRODUCTION

Protein kinases have been implicated in affecting many aspects of metabolism and cell fate and play key roles in the pathogenesis of human diseases, including metabolic disorders, degenerative diseases, and cancer. Akt or Protein kinase B was discovered in the year 1991, as a novel serine threonine (ser/thr) protein kinase, by three independent groups. It was variously named as rac protein kinase (rac, for related to the A and C protein kinases; RAC-PK) (Jones et al., 1991); Protein kinase B (between protein kinase A and C) (Coffer and Woodgett, 1991); or Akt (designated after the oncogenic provirus, AKT-8) (Bellacosa et al., 1991). Downward (1995) proposed the use of the kinase name as Akt/PKB to avoid confusion with Ras related GTPase RAC. The use of Akt has become popular in literature since then. Structurally, the protein Akt/PKB can be divided into three regions; the N-terminal pleckstrin homology containing (AH/PH) domain, the centrally located kinase domain, and a C-terminal regulatory domain containing the hydrophobic motif (HM) phosphorylation site (Nicholson and Anderson, 2002). Akt is activated in response to the insulin or insulin growth factor signaling (IIS). Akt exists in three isoforms Akt1, 2, 3 with a varying tissue distribution but with similar domain structure (Jones et al., 1991; Konishi et al., 2007).

Abbreviations: AH/PH domain, a unique N-terminal domain of the AKT protein which contains pleckstrin homology domain for binding of PIP3; Akt, protein kinase B (T308, S473 – phosphorylated sites threonine 308 and serine 473); FoxO, fork head transcription factors of O group; GLUT, glucose transporter; GSK3β, glycogen synthase kinase3β; IGF, insulin growth factor; IRS, insulin receptor substrate; mTORC1, 2, mechanistic target of rapamycin complex 1 and 2 (mTOR, formerly known as mammalian target of rapamycin); PDK2, phosphoinositide dependent kinase2 (a putative kinase thought to phosphorylate Akt on S473); PI3K, phosphatidylinositol 3-kinases; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PIPDK, phosphoinositide dependent kinase 1 (the abbreviation PIPDK is preferred over the original PDK1 in the article to avoid confusion with the pyruvate dehydrogenase kinase, which is also abbreviated as PDK1 in the literature); rictor, a component of mTORC2; ROS, reaction oxygen species; S6K, the p70 ribosomal S6K; Sestrins, stress response proteins.

AKT IS THE CENTRAL HUB OF INSULIN/INSULIN GROWTH FACTOR SIGNALING

Insulin or insulin like growth factor signaling is the major signaling pathway that responds to the nutrient signals. Insulin is the major

hormone promoting the anabolic activity in the body. Deregulation of this pathway had been shown to be the principal cause of the metabolic syndromes like type 2 diabetes, obesity, and cancer (Laplante and Sabatini, 2012; Boosani and Agrawal, 2013; Zhu et al., 2013). White (2003), in his brief review, presented the summary of events that led to the description of the role of Akt in insulin signaling pathway (see also the pathway map http://stke.sciencemag.org/cgi/cm/CMP_12069?cookietest=yes) (White, 2003). In brief, the IIS pathway is activated by the insulin or insulin like growth factor (IGF) binding to the insulin/IGF receptor, a hetero-tetramer protein, comprising of two α and two β subunits spanning across the membrane. Upon stimulation by IGF, the tyrosine residues of the cytosolic domains are phosphorylated. This leads to the recruitment of the key scaffold protein, the insulin receptor substrate 1 or 2 (IRS 1/2), to the receptor site; IRS in turn activates the PI3Kinase (PI3K)-Akt pathway.

PHOSPHORYLATION OF AKT IS THE KEY TO ITS ACTIVATION

Akt is phosphorylated at several sites (Mahajan and Mahajan, 2012), although the significance of these phosphorylations remains to be fully understood. Its activity depends on two phosphorylated sites; one located in the catalytic domain [also known as the T- or activation loop, the threonine 308 (Akt T308)] and the second in the HM, the serine 473 (Akt S473). Insulin was shown (Kohn et al., 1995) to activate PI3K mediated activation of Akt. PI3K is a heterodimer consisting of a regulatory p85 subunit and a catalytic p110 subunit, which mediates the phosphorylation of phosphoinositides (PIs) at the three-position of the inositol ring. The enzyme phosphatidylinositol (PI) 3-kinase converts PI 4,5-bisphosphate (PIP2) to the putative second messenger PI 3,4,5-trisphosphate (PIP3) (Fruman et al., 1998). The AH/PH domain was shown to be required for the binding of PIs to PH domain and recruit Akt to the membrane (Franke et al., 1995). There are also reports that Akt can be translocated to the membrane independent of PI3K (Brugge et al., 2007; Mahajan and Mahajan, 2012). Alessi et al. (1996) identified T308 and S473 phosphorylations on Akt as the activating sites, and that these phosphorylations do not depend on one another. A 67 kDa protein was shown to be responsible for phosphorylation of Akt exclusively on T308 and it was purified in 1997 from skeletal muscle by Alessi et al. (1997) and from rat brain by Stokoe et al. (1997), Alessi et al. (1997), and Stokoe et al. (1997). The kinase was named as 3-phosphoinositide-dependent protein kinase 1 [PIPDK; the abbreviation PIPDK is preferred in this article over the original PDK1 (Alessi et al., 1997) to avoid confusion with the pyruvate dehydrogenase kinase, which is also abbreviated as PDK1 in the literature]. An unknown kinase, PDK2, was thought to mediate the S473 phosphorylation. The identity of this unknown kinase was complicated with reports that more than ten heterologous kinases were able to phosphorylate Akt on Ser 473 (reviewed in Dong and Liu, 2005). Among the kinases that are reported to phosphorylate Akt S473, there is a broad consensus, that mTORC2 (mechanistic target of rapamycin complex 2; mTOR was formerly known as the mammalian target of rapamycin) could be the kinase that phosphorylates Akt S473 (Sarbassov et al., 2005). It is reported to phosphorylate Akt under a variety of physiological conditions (Frias et al., 2006). ILK was also shown to phosphorylate Akt S473 in association with rictor and siRNAs against rictor/ILK resulted in inhibition of Akt S473 (McDonald et al., 2008). But, there is no consistency in the reports on ILK's role in this phosphorylation. Chan and Tsichlis (2001), suggested that ILK could be acting as a scaffold protein. The double-stranded DNA-dependent protein kinase (DNA-PK) has gained some acceptance (Feng et al., 2004; Bozulic et al., 2008) as the possible kinase especially under the conditions of DNA damage (Stronach et al., 2011). An atypical IκB kinase ε and TANK-binding kinase 1 (IKKε/TBK1) was also suggested to phosphorylate S473 in rictor$^{-/-}$ cells; it also needed PI3K signaling for activation of Akt (Xie et al., 2011). Tumor necrosis factor α (TNFα) was earlier reported (O'Toole et al., 2001) to promote phosphorylation of Akt exclusively on S473. It is reported that inflammation is associated with hyper active mTORC1 and inhibition of mTORC1 was recently shown to control inflammation (Thiem et al., 2013). Inhibition of mTORC1 and the presence of rictor is required for phosphorylation of AktS473 was earlier reported (Breuleux et al., 2009). It is possible that under the conditions of inhibited state of rictor by mTORC1, activation of IKKε/TBK1 promotes the phosphorylation of Akt S473 for cell survival. TBK1 was recently shown to be an activator of autophagy in the clearance of *Salmonella enterica* (Weidberg and Elazar, 2011).

ROLE OF AKT S473 PHOSPHORYLATION IN CELLULAR FUNCTION

There are conflicting views on the role of Akt S473 phosphorylation in cellular function. It has been suggested that phosphorylation of the Ser473 may be independent of its activity (Hill et al., 2001) or it may not be necessary for the full activation of Akt (Moore et al., 2011) and that phosphorylation of Thr308 is a more reliable biomarker than that of Ser473 for Akt activity especially in tumor samples (Guertin et al., 2006; Vincent et al., 2011). There is an increasing evidence that selective mTORC1 inhibition can elicit increased AKT S473 phosphorylation and attenuates the signal effects on tumor cell proliferation (Guertin et al., 2006; Ikenoue et al., 2008; Breuleux et al., 2009). It is clear from the foregone discussion that there is a reciprocal relation between Akt and the two mTOR complexes. IIS mediated phosphorylation of Akt-T308 activates mTORC1, and mTORC2 phosphorylates Akt on S473.

THE ACTIVATING PHOSPHORYLATIONS OF AKT T308 AND AKT S473 HAVE DIVERSE DOWNSTREAM EFFECTORS

In most of the phosphoproteomic data on Akt, the two phosphorylation sites are shown to be activated concurrently on IIS signaling and this has led to the suggestion that the phosphorylation at two sites is required for its maximal activity, and IIS stimulates these functions. However, there is a division in the functional role of Akt in cells and the two phosphorylations play critical role in this functional division (Chandrasekher and Sailaja, 2004; Vadlakonda et al., 2013). It has been demonstrated that, Akt-T308 phosphorylated form is essential and S473 is not needed for activation of mTORC1 (Guertin et al., 2006; Rodrik-Outmezguine et al., 2012). mTORC1 activates protein synthesis, S6K and inhibits autophagy; S6K is a feedback inhibitor of IIS and represses the rictor/mTORC2 functions. Actively proliferating cells therefore, require an active mTORC1 to initiate the process of cell cycle but inhibition of mTORC1 and the activation of

mTORC2 is required for progression of cell cycle beyond S phase (Vadlakonda et al., 2013). The phosphorylation status of Akt in three different types of leukemia presents an interesting case. Tazzari et al. (2004) reported high levels of S473 phosphorylation in *acute myeloid leukemia blasts* (AML blasts), similarly Nyakern et al. (2006) reported high levels of Akt S473 phosphorylation in mononuclear cells from bone marrow of the patients with high-risk *myelodysplastic syndrome* (MDS) when compared to normal or low risk MDS patients; Gallay et al. (2009) on the other hand, reported higher T308 phosphorylation in patients with AML, which was shown to be associated with high-risk cytogenetics and poor overall survival. Although apparently contradictory, the results reflect the status of proliferation of the cells examined; actively proliferating AML cases have high T308, while the AML blasts and MDS, which are poorly dividing cells have high levels of S473. In our earlier review (Vadlakonda et al., 2013) we suggested that activation of mTORC2, which is the upstream regulator of Akt S473, requires inhibition of mTORC1. mTORC2 has two key functions, phosphorylation of Akt (Sarbassov et al., 2005) and at the plasma membrane mTORC2 was shown to promote reorganization of cytoskeleton by activating RhoA GTPases and protect cell survival by up regulating anti-apoptotic proteins, the BCL2 (Goncharova et al., 2011). It is not clear, how these two functions of mTORC2 are partitioned in actively proliferating cells and quiescent cells. It was shown that mTORC1 activity is needed for translation of mRNA of RhoA GTPases and for mTORC2 mediated cytoskeleton reorganization (Lee et al., 2012). Akt phosphorylated at S473 inhibits FoxO in non-proliferative senescent cells and there appears to be waves of activation and inactivation cycles between Akt, mTOR complexes, autophagy, and FoxO in such cells to keep the survival of the cells intact (Young et al., 2009). Besides, Akt S473 phosphorylation is needed for uptake of glucose (Kumar et al., 2010) and the quiescent or senescent cells require glucose more as they rely on glycolysis for energy needs. It is coming to be realized that in tumor tissue, there is a metabolic symbiosis between non-proliferative cells depending on glucose, while actively proliferating cells on lactate (Semenza, 2008).

THE PRESENCE OF TWO PHOSPHORYLATIONS IN THE PHOSPHOPROTEOMIC DATA IS A PARADOX!

There is evidence that prior phosphorylation of Akt S473 was required for enabling PIPDK (PDK1) to phosphorylate at T308 (Scheid et al., 2002; Yang et al., 2002). Sabatini group, who demonstrated that mTORC2 is the phosphorylating kinase of Akt S473 also confirmed this (Sarbassov et al., 2005). In our previous review (Vadlakonda et al., 2013), we have shown that Akt S473 phosphorylation follows the inhibition of mTORC1, activation (or reactivation) of FoxO, AMPK, mTORC2, and the progression of cell cycle. This dichotomy in Akt's relation with mTOR complexes is also reflected in other physiological functions of the cells; for example, anti-apoptotic and proliferative signals from IGF-1 were shown to bifurcate downstream of PI3K in lens epithelial cells (Chandrasekher and Sailaja, 2004). IGF-1-mediated stimulation of the PI3K/p70 S6K cascade was shown to promote cell proliferation, but inactivation of proapoptotic Bad protein and suppression of caspase activation was shown to be independent of PI3K/p70 S6K signaling (Chandrasekher and Sailaja, 2004). A similar situation was also noticed in human non-small cell lung cancer (Vincent et al., 2011). Akt phosphorylated at T308, primarily targets TSC2 and PRAS40 leading to the activation of mTORC1, S6K, and protein synthesis (Kwiatkowski and Manning, 2005), while Akt phosphorylated at S473 was shown to target mainly the FoxO proteins (Guo et al., 2006) and promote anti apoptotic and cell survival pathways (Guan et al., 2011). Akt S473 is a key player in promoting GLUT4 translocation by phosphorylation of As 160 (Zong et al., 2009; Kumar et al., 2010).

HOW DO THE TWO PHOSPHORYLATED SITES OF AKT TRANSLATE THEIR MESSAGES TO THE DIVERSE TARGETS?

In our previous review, we suggested that IIS activated PI3K-Akt-mTORC1 is in fact a negative regulator of mTORC2 and FoxO; the two complexes, mTORC1 and mTORC2 regulate each other by a feedback control (Vadlakonda et al., 2013). Such antagonistic relation between mTORC1 and mTORC2 was also recognized earlier (Breuleux et al., 2009). This raises a question whether Akt T308 and Akt S473 also regulate each other? In a perspective article "PDK2: a complex tail in one Akt," Chan and Tsichlis (2001) presented evidence that in embryonic stem cells which carry inactive PDK1 (PIPDK), phosphorylation of T308 has an inhibitory effect on AktS473 phosphorylation. In a study on pancreatic islet β-cell specific Rictor and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) knockout mice (Gu et al., 2011) demonstrated that βPtenKO mice exhibit a 12-fold increase in AKT-T308 phosphorylation and increase in cell proliferation; rictor null mice on the other hand, were shown to exhibit reduction in β-cell mass, mild hyperglycemia, and glucose intolerance Gu et al. (2011). The authors suggested a critical role for Akt S473 in maintaining the normal β-cell mass and negatively regulates the T308 phosphorylated functions. The two phosphorylations of Akt thus have an antagonistic effects on each other, but in their review Chan and Tsichlis (2001) suggested that the interaction of some unknown molecules in the Akt activation complex might alter its conformation to protect and stabilize the phosphorylations of T308 and S473 on Akt.

INTER DOMAIN INTERACTIONS ARE KEY IN REGULATION OF AKT FUNCTIONS

As already indicated, Akt contains three main domains; an N-terminal pleckstrin homology containing (AH/PH) domain, the centrally located kinase domain, and a C-terminal regulatory domain containing the HM phosphorylation site. The presence of two linker peptides, one between the PH and catalytic domain, and the other between the catalytic and the c-terminal HM, which could not be crystallized is a major hindrance in obtaining the crystal structure of full length Akt protein (Calleja et al., 2009a), and in studying its inter domain interactions. However, using combined techniques like protein mass spectrometry, Förster resonance energy transfer (FRET) by fluorescence life time imaging microscopy, molecular dynamics, and classical biochemical approaches (Calleja et al., 2009a,b) proposed that in an inactive state the PH domain assumes a PH-in conformation and its interaction with the catalytic domain creates a cavity, which leads to the dephosphorylation of the two activating phosphorylations T308

and S473. Two amino acid residues, tryptophan 80 (W80) in the PH domain, and phenylalanine 469 (F469) in the hydrophobic domain interact to keep this inactive state. Upon stimulation by growth factors, the PH domain assumes the PH-out conformation and translocation of Akt to plasma membrane induces conformational changes in the protein facilitating its phosphorylation of the two activating sites T308 and S473 (Macreadie et al., 1991). Upon phosphorylation, Akt is detached from the membrane and translocates to the target sites in the cytoplasm and nucleus. In an earlier study on activation and inactivation dynamics of Akt signaling, Kunkel et al. (2005) employed biosensors and the real time imaging with FRET, and reported a turnover of the activated Akt with inactive forms at membrane site and that phosphatases promote the inactivation of Akt signaling. The inactivation was shown to be rapid in cytosol when compared to that of the membrane site or in nucleus.

PHOSPHATASES REGULATE THE AMPLITUDE AND DURATION OF KINASE ACTIVITY OF AKT

The PTEN, a lipid phosphatase and a tumor suppressor (Maehama and Dixon, 1998), is one of the key regulators of the PI3K-Akt pathway. It dephosphorylates the D3 position of phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Mutations resulting in the functional loss of this phosphatase leading to up regulation of Akt activity are reported in several cancers (Hollander et al., 2011; Mester and Eng, 2013). Apart from this, Akt activity itself is shown to be controlled directly by three phosphatases, the protein phosphatase 2A (PP2A) (Resjo et al., 2002) and protein phosphatase 1 (PP1) (Xu et al., 2003; Thayyullathil et al., 2011), and PH domain leucine-rich repeat protein phosphatase (PHLPP), which is insensitive to okadaic acid and specifically dephosphorylates the S473 (Bayascas and Alessi, 2005; Gao et al., 2005). An increased phosphorylation of T308 was shown to be associated with reduced PP2A in AML patients (Gallay et al., 2009). These phosphatases also play crucial role in conferring resistance to radiation and chemo therapies; Eke et al. (2010) recently demonstrated that an adapter protein, downstream of focal adhesion, PINCH1 [5 Lin-1, Isl-1, Mec-3 (LIM) domain – containing particularly interesting new cysteine-histidine-rich 1], inhibits protein phosphatase 1α and confers resistance to cancer cells against ionizing radiation and chemo therapies by increasing the Akt phosphorylation.

THE ADENYLATES ATP/ADP MODULATE THE STABILITY OF AKT PHOSPHORYLATIONS AND DENY ACCESS OF ACTIVATED AKT TO PHOSPHATASE ACTION

Chan and Tsichlis (2001), in their review, predicted that some unknown molecules in the Akt activation complex might stabilize its phosphorylations. In a recent study, two different groups (Chan et al., 2011; Lin et al., 2012) demonstrated that ATP bound to the phosphorylated Akt protects the phosphorylated sites by preventing their access to the phosphatases, which in turn results in increased phosphorylations of these sites. Adenylate nucleotides, ATP and ADP, were shown to act as "on – off switch" to maintain the stability of the phosphorylations at T308 and S473. Both ATP and the ATP competitive inhibitors were shown to stabilize the two phosphorylation sites, while hydrolysis of ATP to ADP was shown to destabilize the kinase and expose the two phosphorylated sites to phosphatases. Reviewing these two works, Humphrey and James (2012) suggested that Akt creates a cage in the ATP bound form between the two phosphorylations in preventing their access to phosphatases and the stabilized form was shown to detach from the membrane to be transported to the locations of its targets. The phosphorylation at T308 was suggested to increase the affinity between the PH domain and the phosphorylated kinase domain leading to the detachment of Akt from the membrane (Ananthanarayanan et al., 2007). Although the question as to how this stabilization of phosphorylation sites results in the diversified functions of the Akt remains unanswered, it is not difficult to speculate that the energy charge and the localization site of its targets appears to play a critical role in its effects on the downstream targets.

ENERGY CHARGE (ATP/AMP RATIO) OF CELLS BECOMES CRUCIAL IN MODULATING AKT'S FUNCTION

The stabilized phosphorylations under ATP/energy rich environment by activating Akt (**Figure 1**) primarily target TSC2 and PRAS 40 in cytosol and activate mTORC1 functions. With the reduction in ATP levels under energy consuming processes activated by mTORC1, ADP accumulates, and destabilizes the phosphorylations of Akt; this exposes the phosphorylated sites to phosphatases. In our previous article (Vadlakonda et al., 2013), we proposed that the constitution of active mTORC2 takes place only when FoxO, AMPK, and autophagy are activated and mTORC1 is inhibited. It is well recognized that activation of AMPK and autophagy and inhibition of mTORC1take place only when the energy levels in cells drop (Hardie and Hawley, 2001; Hardie, 2011). The ATP-ADP switch controlling the stability of Akt therefore, depends on the energy charge, a concept defined by Atkinson in 1960s (Ramaiah et al., 1964; Atkinson, 1968). The ratio of concentration of AMP:ATP which varies at equilibrium as the square of the ADP:ATP ratio is maintained by the enzyme adenylate kinase, which is highly expressed in all eukaryotic cells. The role of AMPK in responding to the equilibrium of adenylate pool and the equations related to these interactions were discussed in detail by Hardie and Hawley (2001) and reviewed recently by Oakhill et al. (2011, 2012). An energy charge of healthy cells is maintained around 0.9 (10:1) (Hardie and Hawley, 2001); this was shown to adjust the partitioning of substrates among competing metabolic functions of energy producing or energy consuming processes by balancing the feedback regulation cycles (Hardie and Hawley, 2001). Drugs activating AMPK were shown to dephosphorylate and inactivate Akt but activate the Akt target GSK3 β (King et al., 2006). This confirms that under high ATP:AMP ratio, the targets of Akt will be TSC2 and PRAS 40, whose inhibition activates mTORC1. But under low ATP:AMP ratio, the targets will be the factors that mobilize resources (glucose) for energy production. It is therefore not illogical to speculate that the energy dynamics play critical role in activation inactivation cycles of Akt and modulate glucose up take.

We present a model below on the interacting dynamics of the various signal molecules, viz., the Akt, FoxO, AMPK, mTOR complexes (**Figure 1**) and how the pathways behave under energy/nutrient poor, and energy/nutrient surplus environments (**Figure 2**).

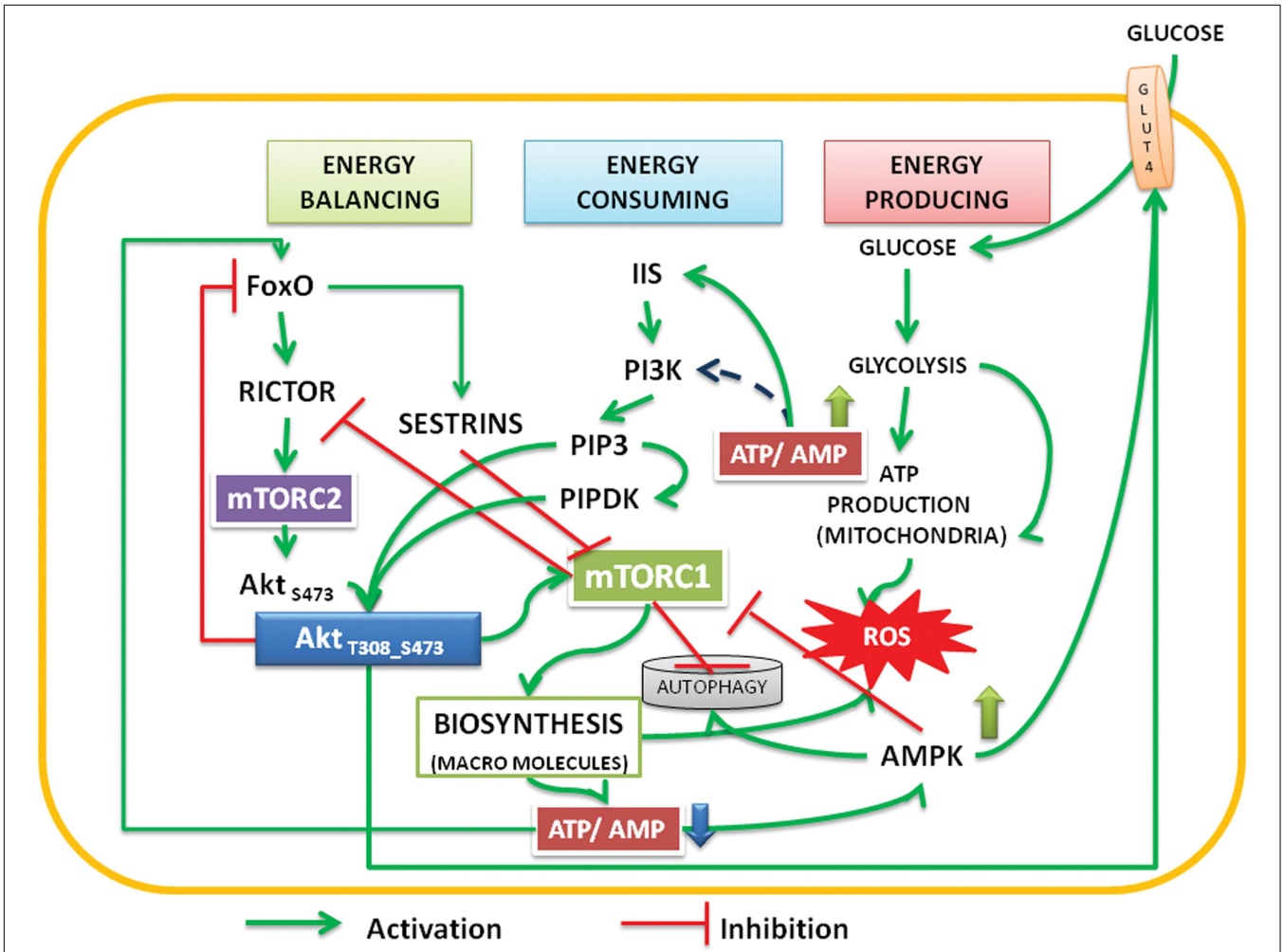

FIGURE 1 | The interactions and feedback loops between Akt and mTOR complexes and energy consuming and energy producing processes. Insulin/insulin growth factor signaling (IIS) activates PI3Kinase, which phosphorylates PIP2 to PIP3. Binding of PIP3 to AH/PH domain translocates Akt to the membrane. FoxO transcription factors transcribe rictor leading to assembly of mTORC2 complex, which phosphorylates Akt S473. Activated by PIP3, the PIPDK (originally the PDK1) phosphorylates Akt T308. PI3K-Akt pathway can also be activated independent of IIS (see Mahajan and Mahajan, 2012). The phosphorylations are stabilized by high ATP:AMP ratio, Akt migrates into cytoplasm and nucleus. In the nucleus it phosphorylates FoxO, translocates it into the cytoplasm, and under low energy levels AMPK promotes its translocation into mitochondria. In cooperation with Sirtuin3, FoxO promotes mitochondrial OXPHOS (Peserico et al., 2013). Sestrins, transcribed by FoxO inhibit mTORC1 and the energy consuming processes come down. This up regulates ATP:AMP ratio. At basal level of activation, Akt inhibits AS 160 and activates translocation of glucose transporter (GLUT4) to the membrane there by facilitating the glucose entry into the cells. Activated AMPK also promotes glucose transport under low energy conditions (Schwenk et al., 2010; Wu et al., 2013). The cycle continues under healthy environments. Under inflammatory environment, either due to the Dietary surplus environment or under stress activated conditions, the IIS is destabilized and causes insulin resistance (see **Figure 2**).

THE MODEL

A simplified sequence of events of phosphorylation/dephosphorylation or activation/inactivation cycles of Akt *vis-a-vis* the mTOR complexes can be described as hereunder.

Under energy/nutrient depleted conditions, Akt is in an inhibited state; FoxO and AMPK transcription factors are activated. AMPK activates glycolysis and krebs cycle and generates ATP, but inhibits mTORC1 (Hardie and Hawley, 2001; Liang and Mills, 2013). AMPK also activates autophagy, which recycles the cellular cargo and degrades defective/damaged mitochondria in cells starved of energy (Egan et al., 2011; Hardie, 2011). Within the nucleus, the activation of FoxO leads to transcription of rictor (Chen et al., 2010), IRS (Tsunekawa et al., 2011; Tsuchiya et al., 2012) and enzymes of gluconeogenesis (Zhang et al., 2006; Liu et al., 2011; Shin et al., 2012). Amino acids released from recycled cargo by autophagy release the inhibition on mTORC1 by AMPK and provide the substrates for protein synthesis needed for the reprogramming of metabolism. Activation of mTORC1 also facilitates the translation of transcription products of FoxO like rictor and IRS. The protein rictor promotes the constitution of mTORC2 which then translocates to membrane to activate the Akt S473 phosphorylation (see the text). Since insulin or IGFs are absent under nutrient deprived conditions, it is probable that Akt is activated independent of PI3K/insulin signaling (Brugge et al.,

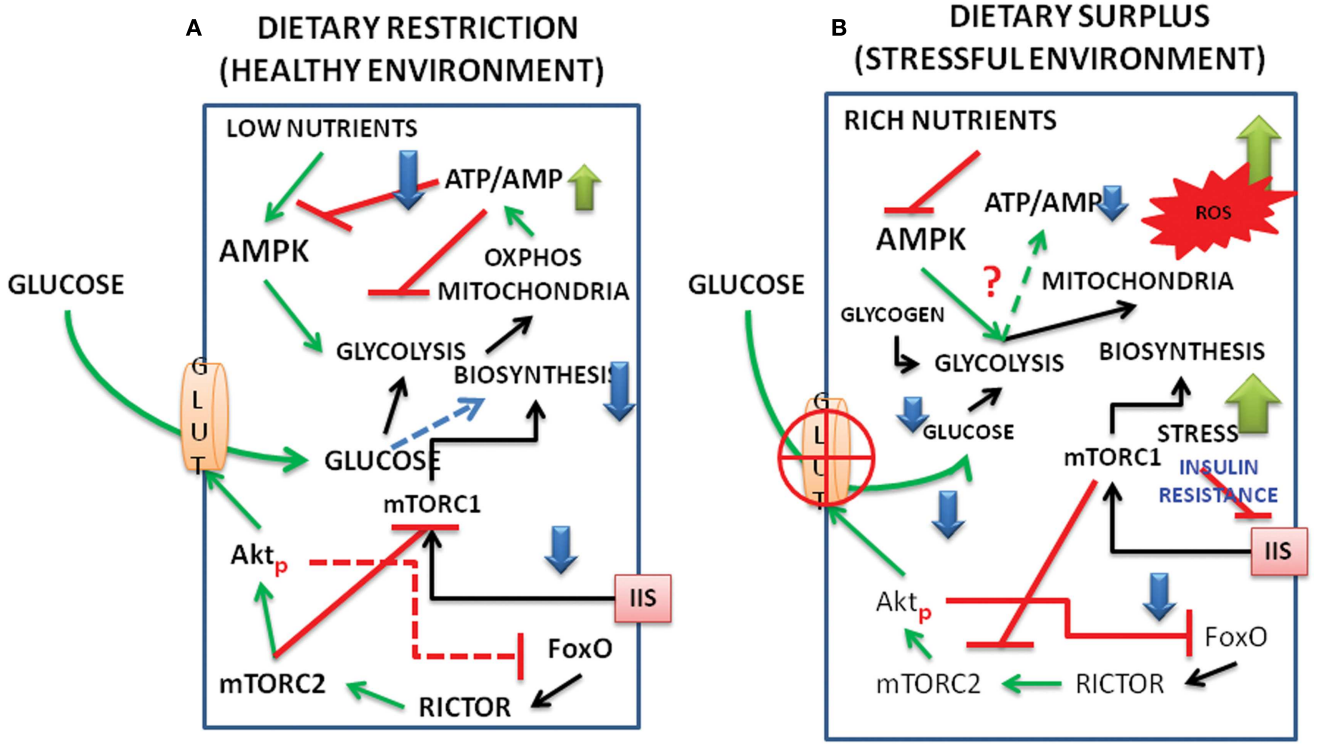

FIGURE 2 | Schematic representation of interacting signal pathways under (A) dietary restriction and healthy environment and (B) under dietary surplus and stress environment. The interactions between FoxO, Akt, mTORC1, and C2, as well as those of AMPK and metabolism are shown under dietary restriction and dietary surplus conditions. (For abbreviations see the text.)

2007; Mahajan and Mahajan, 2012). Activated Akt is liberated from the membrane and migrates into cytoplasm and nucleus. In the cytoplasm it phosphorylates As 160, which leads to translocation of GLUT4 to the membrane. It has been shown that both Akt S473 and mTORC2 are key factors in GLUT4 translocation (Zong et al., 2009; Kumar et al., 2010; Fukuda, 2011; Kim et al., 2011). Within the nucleus, Akt phosphorylates FoxO, which then translocates into the cytoplasm (Guertin et al., 2006). In the cytoplasm, active AMPK drives FoxO into the mitochondria and in cooperation with the SIRTUIN3 activates mitochondrial respiration (Peserico et al., 2013).

This increases production of ATP, raising the levels of ATP/AMP ratio. Enhanced ATP/AMP ratio stabilizes Akt, which targets TSC2, PRAS40; this results in further activation of mTORC1. Coupled with the exclusion of FoxO from the nucleus, activation of mTORC1 leads to the inhibition of rictor and disassembly mTORC2, which reduces the mTORC2 mediated S473 phosphorylation of Akt and release of inhibition on AS 160 and making GLUT4 dysfunctional.

THE NUTRIENT STATUS OF ORGANISMS APPEARS TO PLAY A CRITICAL ROLE IN THIS ENERGY AND SIGNALING DYNAMICS

Under dietary restriction, limited entry of glucose keeps the rise in ATP:AMP ratio at the bare minimum levels to maintain living processes, mTORC1 remains largely inhibited and IIS is not in the picture (Mercken et al., 2013). When the nutrients are maintained in a balanced state, especially during the growth phase of the organisms, the energy expenditure due to active mTORC1 reduces the ATP/AMP ratio destabilizing Akt and the alternate activation, reactivation cycle of Akt, and glucose entry regulate IIS. Higher activation of mTORC1 due to enriched nutrients/amino acids inside the cells might lead to inhibition of rictor (mTORC2) and a feedback inhibition of IIS (see the text). Inhibition of autophagy/mitophagy by mTORC1 will also produce reaction oxygen species (ROS) and create conditions of inflammation (Thiem et al., 2013). It is possible that when ROS generated during active biosynthetic processes are under control, the protective phase of inflammation might lead to activation of cytokines and TNFα, and promote phosphorylation of AktS473 exclusively even in the absence of mTORC2 (O'Toole et al., 2001). This retains the GLUT4 active at the membrane and promotes the replenishment of glucose in the cell for ATP production. But under malnutrition and surplus nutrients, especially in aging organisms, the deregulation of Akt activation, deactivation cycles promote the stress activated state. Increased levels of cytokines and stress activated kinases like JNK (Salminen and Kaarniranta, 2013) under such situations hampers both IIS and the glucose flux resulting in the state of insulin resistance (**Figure 2**).

CONCLUSION

In summary, we suggest that the nutrient inputs, energy cycles regulate the functions of several signal molecules. Akt is positioned

at the central hub of regulating these interactions. The reciprocal relation that exists between the two activating phosphorylation sites, T308 and S473, of Akt and the two mTOR complexes, C1 and C2, forms the central controlling hub that regulates cellular function. The energy charge, the ratio of concentration of ATP to that of AMP, decides the active and inactive state of Akt. In general, the stability of Akt phosphorylations is coupled to high ATP:AMP ratio in cells. It activates mTORC1 and the energy consuming biosynthetic processes. Under nutrient deprived conditions, active FoxO transcribes rictor promoting constitution of mTORC2; phosphorylation of T308 and activation of Akt may be independent of IIS and Akt maybe stable for short time, which is required for maintenance of basal metabolism.

ACKNOWLEDGMENTS

Dr. Lakshmipathi Vadlakonda is thankful to the Director, Prof. Allam Apparao and the DST, New Delhi for providing a placement as the honorary adjunct faculty, at CRRAO AIMSCS. Dr. Mukesh Pasupuleti thanks the management of SRM University for providing facilities for carrying out the research work at SRM Research Institute. Abhinandita Dash is thankful to the Director, CRRAO AIMSCS and DST for the JRF fellowship under the grants DST: SR/S4/MS: 516/07 dated 21-04-2008. The financial support by the Department of Biotechnology, Council of Scientific and Industrial Research and Indian Council of Medical Research to Dr. Pallu Reddanna is gratefully acknowledged.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 May 2013; accepted: 07 June 2013; published online: 20 June 2013.
*Citation: Vadlakonda L, Dash A, Pasupuleti M, Anil Kumar K and Reddanna P (2013) The paradox of Akt-mTOR interactions. Front. Oncol. **3**:165. doi: 10.3389/fonc.2013.00165*
This article was submitted to Frontiers in Molecular and Cellular Oncology, a specialty of Frontiers in Oncology.
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frontiers in ONCOLOGY

PERSPECTIVE ARTICLE
published: 12 April 2013
doi: 10.3389/fonc.2013.00085

Role of PI3K-AKT-mTOR and Wnt signaling pathways in transition of G1-S phase of cell cycle in cancer cells

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The PI3K-Akt pathway together with one of its downstream targets, the mechanistic target of rapamycin (mTOR; also known as the mammalian target of rapamycin) is a highly deregulated pathway in cancers. mTOR exists in two complexes, mTORC1 and mTORC2. Akt phosphorylated at T308 inhibits TSC1/2 complex to activate mTORC1; mTORC2 is recognized as the kinase phosphorylating Akt at S473. Inhibition of autophagy by mTORC1 was shown to rescue disheveled (Dvl) leading to activation of Wnt pathway. Cyclin D1 and the c-Myc are activated by the Wnt signaling. Cyclin D1 is a key player in initiation of cell cycle. c-Myc triggers metabolic reprograming in G1 phase of cell cycle, which also activates the transcription factors like FoxO and p53 that play key roles in promoting the progression of cell cycle. While the role of p53 in cancer cell metabolism in arresting glycolysis and inhibition of pentose phosphate pathway has come to be recognized, there are confusions in the literature on the role of FoxO and that of rictor. FoxO was shown to be the transcription factor of rictor, in addition to the cell cycle inhibitors like p21. Rictor has dual roles; inhibition of c-Myc and constitution of mTORC2, both of which are key factors in the exit of G1-S phase and entry into G2 phase of cell cycle. A model is presented in this article, which suggests that the PI3K-Akt-mTOR and Wnt pathways converge and regulate the progression of cell cycle through G0-G1-S-phases and reprogram the metabolism in cancer cells. This model is different from the conventional method of looking at individual pathways triggering the cell cycle.

Keywords: mTORC1, autophagy, Wnt, G1-S, cell cycle

INTRODUCTION

The protein kinase B (*between protein kinase A and C*; Coffer and Woodgett, 1991), or Akt designated after the viral acute transforming retrovirus, Akt8 (Staal et al., 1977; Bellacosa et al., 1991; Downward, 1995) is recognized as the regulator of cell survival. Aberrant activation of the kinase is associated with many diseases, including cancer and diabetes (Pearce et al., 2010). Phosphorylation of Akt at threonine 308 (T308; in activation loop) and the serine 473 (S473; in hydrophobic motif) are considered important for its activity (Nicholson and Anderson, 2002). Akt is activated by insulin/insulin like growth factor (IGF) signaling (IIS). The autophosphorylation of the internal domains of IIS receptors leads to the recruitment of insulin receptor substrate (IRS) and activation of phosphatidylinositol 3-kinases (PI3K). PI3K phosphorylates phosphatidylinositol 4, 5 bisphosphate (PIP2) to PIP3 (Engelman et al., 2006; Manning and Cantley, 2007). PIP3 activates the phosphatidylinositol dependent protein kinase 1 (PDPK1) and recruits Akt to the plasma membrane. PDPK1 phosphorylates Akt T308 in the activation loop (Alessi et al., 1997). Several kinases, integrin-linked kinase (ILK), protein kinase Cα

Abbreviations: Akt, protein kinase B (T308, S473 – phosphorylated sites Threonine 308 and Serine 473); APC, adenomatous polyposis coli; AXIN, axis inhibition protein; CDK2, cyclin-dependent kinase 2; CK, casein kinase; CKI, cyclin-dependent kinase inhibitors; c-Myc, the oncoprotein activated by Wnt signaling; COX-2, cyclooxygenase-2; Dvl, Dsh homolog in mammals; 4E-BP, eukaryotic translation initiation factor (eIF4E) binding protein1; FoxO, fork head transcription factors of O group; G0, G1, and S are phases of cell cycle; GLUT, glucose transporter; GSK3β, glycogen synthase kinase3β; HIF, hypoxia inducible factor; IGF, insulin like growth factor; IIS, insulin/insulin like growth factor signaling; IRS, insulin receptor substrate; LEF, lymphoid enhancer-binding factor; LRP, LDL receptor protein; mTORC1, 2, mechanistic target of rapamycin complex 1 and 2 (mTOR, formerly known as mammalian target of rapamycin); p16INK 4a ARF, cyclin-dependent kinase inhibitor 2A family of cell cycle inhibitors; p16INK 4a^{ARF}, cyclin-dependent kinase inhibitor 2A family of cell cycle inhibitors; PDPK1, phosphoinositide dependent kinase 1 (the abbreviation PDPK1 is preferred over the original PDK1 in the article to avoid confusion with the pyruvate dehydrogenase kinase, which is also abbreviated as PDK1 in the literature); PDK2, phosphoinositide dependent kinase2 (a putative kinase thought to phosphorylate Akt on S473); PI3K, phosphatidylinositol 3-kinases; PIP2, phosphatidylinositol 4,5 bisphosphate; PIP3, phosphatidylinositol 3,4,5 trisphosphate; PPP, pentose phosphate pathway; *PTEN*, phosphatase and tensin homolog deleted from chromosome ten; rictor, a component of mTORC2; ROS, reaction oxygen species; S6K, the p70 ribosomal S6K; sestrins, stress response proteins; snail/slug, transcriptional inhibitors of E-Cadherin; TCF, T-cell factor; TIGAR, *TP53*-induced glycolysis and apoptosis regulator.

(PKCα), double-stranded DNA-dependent protein kinase (DNA-PK) ataxia telangiectasia mutated (ATM) gene product, and the mammalian target of rapamycin (mTOR) were proposed to phosphorylate Akt on Ser-473 (Dong and Liu, 2005). The mTORC2 (Sarbassov et al., 2005) is widely recognized as the key kinase that phosphorylates the Akt at S473. Ambiguity on the phosphorylation of this site however, remains; an atypical IκB kinase ε and TANK-binding kinase 1 (IKKε/TBK1) was reported to induce this phosphorylation in rictor (−/−) cells (Xie et al., 2011). There are reports that phosphorylation of Akt S473 could be cell specific (Riaz et al., 2012) or may not be required for full activation (Moore et al., 2011). T308 phosphorylation is considered a reliable biomarker of Akt activity especially for mTORC1 function (Jacinto et al., 2006; Breuleux et al., 2009; Vincent et al., 2011). Several tyrosine kinases are reported to phosphorylate Akt at different sites (Mahajan and Mahajan, 2012). Two phosphatases, the phosphatase and tensin homolog deleted from chromosome ten (PTEN) and the SH2 domain containing inositol-5-phosphatase 2 (SHIP2) regulate Akt function through dephosphorylation of 3-OH position of PIP3 (Leslie et al., 2003) and the 5-OH position (Aman et al., 1998) respectively.

The mechanistic target of rapamycin (mTOR: formerly known as mTOR; also known as FK506 binding protein 12-rapamycin associated protein 1 (FRAP1; Moore et al., 1996), in mammals exists in two multi protein complexes, mTORC1 and mTORC2, distinguished by their sensitivity to rapamycin. The catalytic cores of the two complexes have the kinase mTOR domain. While raptor (regulatory associated protein of mTOR) regulates the function of mTORC1, rictor (Rapamycin insensitive companion of mTOR) was shown to control the activity of mTORC2 (reviewed by Loewith et al., 2002; Laplante and Sabatini, 2009). DEPTOR is a negative regulator of the two complexes (Wang et al., 2012).

The complex mTORC1 responds to the nutrients and conditions that promote cellular growth. It is activated by AktT308 downstream of IIS (Wullschleger et al., 2006; Gamper and Powell, 2012). mTORC1 is activated both by the oncogenic PI3K-Akt as well as the Ras-Erk pathways, which inhibit the tuberous sclerosis complex (TSC1 and TSC2) (TSC complex) through the phosphorylation of the TSC2 (Manning and Cantley, 2007). The inhibition of TSC complex releases the inhibitory effect of TSC on the GTP-bound Rheb (Ras homolog enhanced in brain), which controls the activity of mTORC1. TSC is also inhibited by the Wnt pathway (Inoki et al., 2006). Activation of mTORC1 by amino acids is mediated by Rag GTPases. (Sancak et al., 2010), which is independent of IIS. AMP activated protein kinase (AMPK) inhibits mTORC1 by activating the TSC2 (Corradetti et al., 2004; Kwiatkowski and Manning, 2005; Inoki et al., 2006) and drugs that activate AMPK reverse the activation of mTORC1 (Guppy et al., 2011; He et al., 2011).

mTORC1 IS A FEEDBACK REGULATOR OF IIS PATHWAY AND IT ALSO REGULATES mTORC2

One of the key downstream targets of mTORC1, the p70 ribosomal S6 Kinase (S6K) phosphorylates IRS and inhibits the IIS in a feedback regulatory step (Zhang et al., 2008; Veilleux et al., 2010; Kang et al., 2011). An inverse relation is reported both in relative abundance and activation of mTORC1 and mTORC2 in cells (Sarbassov et al., 2004). S6K also phosphorylates rictor and inhibits mTORC2 assembly (Dibble et al., 2009; Julien et al., 2010; Treins et al., 2010).

S6K is also shown to inhibit glycogen synthase kinase3β (GSK3β) (Zhang et al., 2006). Recognized as one of the key targets of Akt, GSK3β was also shown to phosphorylate rictor (Chen et al., 2011). GSK3β has multiple roles ranging from glucose homeostasis (Kim and Kimmel, 2000) to inflammation (Wang et al., 2011), and it plays a key role in Wnt signaling (Wu and Pan, 2010). GSK3β phosphorylates the voltage-dependent anion channel (VDAC) and regulates the mitochondrial metabolite exchange and apoptosis (Shoshan-Barmatz et al., 2010); its depletion was shown to increase the beta cell proliferation (Stein et al., 2011). GSK3β cooperates with AMPK in activation of TSC complex that leads to inactivation of mTORC1 (Kwiatkowski and Manning, 2005).

Regulation of protein synthesis is recognized as one of the conserved role of mTORC1; it phosphorylates and inhibits, the eukaryotic initiation factor 4E (eIF4E)-binding proteins (4E-BP1/2), which are the inhibitors of translation (Castellvi et al., 2006; Ma and Blenis, 2009). The two functions of mTORC1, phosphorylation of S6kinase and inhibition of 4E-BP, have come to be accepted as routine markers for its activity and activation of protein synthesis in cells (Miron et al., 2003).

RAS-Erk MAP KINASE SIGNALING ALSO ACTIVATES mTORC1

Over expression of epidermal growth factor receptors belonging to the proto-oncogene erbB (Thompson and Gill, 1985) and abberrant activation of RAS-Erk MAP kinase signaling was recognized as the cause of several cancers and antibodies targeting the receptors were developed during early 1980s (Sato et al., 1983; Schlessinger, 2000; Mendelsohn and Baselga, 2003; Lemmon and Schlessinger, 2010). The MAP kinase Erk was shown to phosphorylate and inactivate TSC2 (Ma et al., 2005) leading to activation of mTORC1. Several drugs that target the nutrient and growth factor (PI3K-Akt and Ras-Erk MAP kinase) pathways claim that the targeted drugs arrest the progression of cell cycle. The convergence of the two pathways at mTORC1 led to a surge in the activity in targeting of mTORC1 for the control of carcinogenesis. Rapamycin, which was initially recognized as an immunosuppressant for its ability to reduce organ rejection (Abraham and Wiederrecht, 1996) was subsequently found useful in treatment of cancers (Mita et al., 2003). But, the realization that rapamycin is inadequate in completely inhibiting mTORC1 functions (Shor et al., 2009) led to a search for the ATP competitive inhibitors (Bhagwat and Crew, 2010; Schenone et al., 2011). These inhibitors are claimed to arrest the cells in quiescent or gap1 (G0/G1) phase of the cell cycle (Evangelisti et al., 2011). But the exact link between the growth factor, mTOR pathways and cell cycle remains unexplained.

Wnt PATHWAY IS THE KEY PATHWAY IN ACTIVATION OF CELL CYCLE

Wnt pathway is the key pathway in activation of cell cycle. Wnt signaling in general activates the Cyclin D, the *c-Myc*; matrix metalloproteinases, COX-2, peroxisome proliferator-activated receptors (PPARs), and the growth factors, and their receptors, and down regulates E-Cadherin, the cell cycle inhibitor P16ink4A

(ARF) and p53 (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/human_genetic_diseases; 2010). Wnt pathway thus, regulates the cancer cells entry into the cell cycle through the production of cyclin D. Cyclin D complexes with cyclin-dependent kinase 4/6 (Cdk4/6), inactivates the tumor suppressor protein retinoblastoma (Rb), and promotes the entry of the cell from G0 to G1 phase of cell cycle. E2F uncoupled from the phosphorylated Rb transcribes the cyclin E, which binds to Cdk2 and promotes the progression of the cell cycle. The up regulation of cyclin E/CDK2 is reported to correlate with the G1/S transition (Stott et al., 1998; Arima et al., 2004; Soto Martinez et al., 2005; Sun et al., 2007). Aberrant activation of Wnt pathway was shown to lead cells to malignant transformation (Polakis, 2012).

Activation of Wnt pathway is usually based on destabilizing the commonly known "destruction complex" comprising of the APC, the Axin, and the casein kinase I (CKI) and GSK3β. The disheveled (*Dvl*; Dsh gene homolog in mammals) protein is recognized as the key component in the signaling (Nusse, 2005; Gao and Chen, 2010).

AUTOPHAGY PROMOTES DEGRADATION OF Dvl AND NEGATIVELY REGULATES THE Wnt PATHWAY

The process of autophagy involves the fusion of phagophores with lysosomes (Yang and Klionsky, 2010); it plays a key role in human diseases like immune disorders (Deretic, 2011), neurodegenerative disorders (Weihl, 2011), and also in cancers (Brech et al., 2009; Stipanuk, 2009; White and Lowe, 2009; Chen and Klionsky, 2011; Nyfeler et al., 2011). mTORC1 was shown to inhibit autophagy by phosphorylation of ULK1 at Ser 757 (Kim et al., 2011).

Gao et al. (2010) demonstrated that autophagy is a negative regulator of the Wnt pathway by promoting the degradation of Dvl, a component of Wnt pathway. All the three isoforms of Dvl (Dvl1, Dvl2, and Dvl3) were shown to be degraded. Inhibition of autophagy by mTORC1 therefore, releases the Dvl. Autophagy mediated down regulation of Wnt signaling was confirmed by rapamycin treatment, which resulted in down regulation of the Wnt target genes *axin2*, *c-Myc*, and *cyclin D1*. Dvl appears to be the link for the cooperative interaction between the MAP kinase and PI3K-Akt-mTOR pathways converging at autophagy to activate cell proliferation. In addition, *c-Myc*, a downstream target of the Wnt signaling, was shown to be involved in carcinogenesis along with erbB2 as early as in 1980s (Dotto et al., 1986; Land et al., 1986). Pacheco-Pinedo et al. (2011) recently demonstrated that cooperation between K-Ras mutant and the Wnt/β-catenin signaling is the cause of aggressive lung tumor phenotype. Heallen et al. (2011) demonstrated that Hippo pathway inhibits the Wnt signaling to prevent cardiomyocyte proliferation; Dvl was shown to be the link between the two pathways. Apart from reusing Dvl from autophagic degradation, mTORC1 inactivation of GSK3β by S6K (Zhang et al., 2006) blocks the β-catenin degradation. Dvl was also shown to translocate into nucleus and in conjugation with the transcription factor of AP1 complex, c-jun is reported to stabilize the β-catenin-TCF/LEF signaling (Gan et al., 2008).

ONCOGENES AND TUMOR SUPPRESSORS ARE BOTH INVOLVED IN METABOLIC REPROGRAMING

Progression of cell cycle also requires the activation of metabolic pathways in G1 phase. Glycolysis, Krebs cycle, and the pentose phosphate pathways are the key pathways involved in metabolic reprograming of cells. Warburg, in the early twentieth century, was the first to suggest that cancer cells utilize the aerobic glycolysis for promotion of tumorigenesis (Warburg, 1956). A re-examination of Warburg hypothesis in the last part of twentieth century led to a search for the role of oncogenes and tumor suppressors in metabolism (**Figure 1A**). Akt was named as the "Warburg enzyme" (Robey and Hay, 2009), p53 was recognized to suppress both glycolysis through TIGAR (*TP53*-induced glycolysis and apoptosis regulator; Bensaad et al., 2006) and Pentose phosphate pathway by inhibiting the G6PD (Jiang et al., 2011). It was shown to activate glutamine metabolism and control the ROS production (Gottlieb, 2011; Maddocks and Vousden, 2011). HIF and *c-Myc* were shown to up regulate the enzymes of glycolysis (Kim et al., 2007). The signature of cancer cells is recognized by loss of function of the tumor suppressors p53, PTEN and by activation of Akt, Myc, HIF-1α, and NFkB (Markert et al., 2012). The oncogene *c-Myc* is recognized to play an important role in activation of genes of enzymes of glycolysis and Krebs cycle as well as those involved in chromatin structure, and its transcriptional networks that are involved predominantly in cell cycle regulation and cellular metabolism and protein synthesis specific to the G0-G1-S transition in cancer cells, lymphocytes, and in embryonic stem cell (Kim et al., 2010; Swami, 2010; Lin et al., 2012).

THE ROLE OF FoxO TRANSCRIPTION FACTORS

One of the consequences of metabolic reprograming in cancer cells is activation of FoxO transcription factors (Ronnebaum and Patterson, 2012). The FoxO transcription factors are reported to sequester β-catenin away from the TCF/LEF transcription factors (Hoogeboom et al., 2008; Hoogeboom and Burgering, 2009). Although the exact mechanism is still enigmatic, deregulation of adherens junction (AJ) was reported to result in translocation of the FoxO transcription factors into nucleus (Fournier et al., 2009). One of the critical steps in the progression of cell cycle is the crossover of restriction point in G1, which is regulated by the E2F-pRb. The role of FoxO in up regulating cell cycle inhibitors p15 (INK4b) and p19 (INK4d) is viewed as an arrest of cell cycle (Katayama et al., 2008). Rictor transcribed by FoxO is a key component of the mTORC2 (Chen et al., 2010). Guo et al. (2012) reported that rictor promotes ubiquitination and degradation of c-Myc and cyclin E and suggested that it leads to the arrest of cell cycle in G1 phase. Rictor is also reported to be involved in regulation of Rho GTPases (Jacinto et al., 2004) and RhoA activation is crucial for G1-S progression of cell cycle (Zhang et al., 2009). The up regulation of cell cycle inhibitors (p21WAF1/CIP1 and p27KIP1) and transcription of rictor by FoxOs and inhibition of c-Myc and cyclin E reported by Guo et al. (2012) should therefore, be viewed as a regulation of the exit of G1/S phase of cell cycle rather than inhibition of cell cycle in G1 phase. FoxO is also reported to transcribe the antioxidant genes like sestrins (Chen et al., 2010; Lee et al., 2010) which inhibit mTORC1 and inhibit the mitochondrial metabolism (Ferber et al., 2012). Young et al. (2009) demonstrated a feedback link between mTORC1 and C2 signaling and the timing of inhibition of mTORC1 correlated with activation of autophagy and cyclin A. These results suggest the role of FoxO in progression of cell cycle and assembly of mTORC2. mTORC2 was shown to be required for proliferation

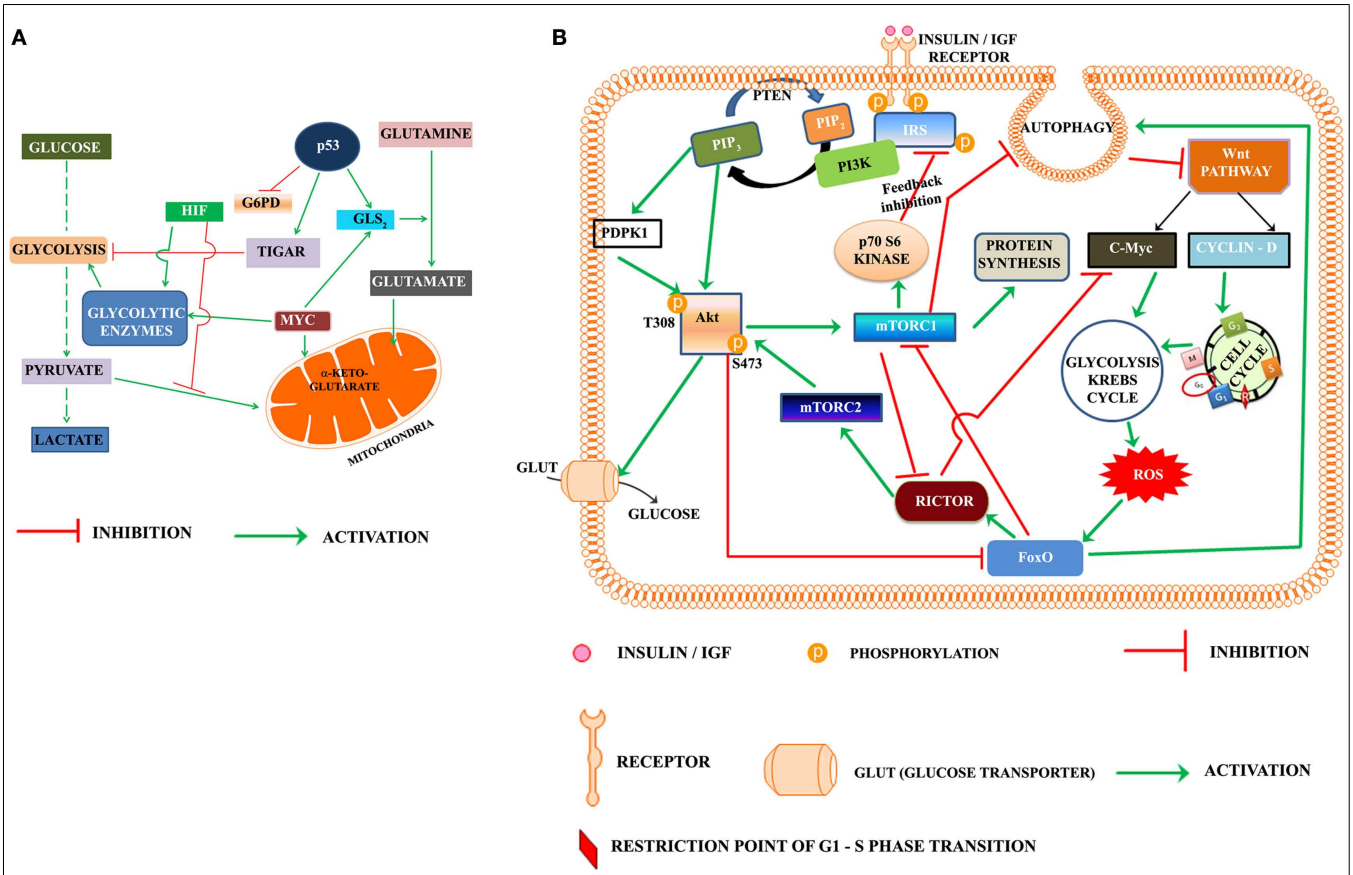

FIGURE 1 | (A) Oncogenes and tumor suppressor modulate metabolic reprograming in cancer cells. HIF, hypoxia inducible factor; TIGAR, TP53-induced glycolysis and apoptosis regulator; MYC, Proto oncogene *c-Myc*; G6PD, glucose-6-phosphate dehydrogenase; GLS_2, glutaminase 2. **(B)** Proposed model highlighting the role of PI3K-Akt-mTOR and Wnt pathways in regulation of the cell cycle progression in cancer cell: according to the presented model, activation of insulin/IGF receptor by nutrients/growth factors activates PI3K-Akt pathway. Akt phosphorylated on T308 activates mTORC1. One of the downstream target of mTORC1, the p70S6Kinase, phosphorylates the serine residues on IRS and inhibits the insulin/IGF signaling in a regulatory feedback control. The activated mTORC1 activates the protein synthesis, but inhibits autophagy. Inhibition of autophagy rescues Dvl and this lead to activation of Wnt pathway. The activated Wnt pathway up regulates cyclin D and *C-Myc*, which trigger the activation of cell cycle and metabolic reprograming. The metabolic activation in cancer cells leads to the production of reaction oxygen species (ROS), which activate the FoxO. FoxO is the transcription factor of rictor, one of the key components of mTORC2 recognized as the kinase phosphorylating Akt on S473. Rictor also inhibits c-Myc, which according to the present model is required for exit of G1/S restriction point. Akt, protein kinase B (T308, S473 are the phosphorylated sites Threonine 308 and Serine 473 of Akt), c-Myc is the oncoprotein activated by Wnt signaling, FoxO, fork head transcription factors of O group; GLUT, glucose transporter; IGF, insulin growth factor; IRS, insulin receptor substrate; mTORC1, 2, mammalian target of rapamycin (mTOR) Complex 1 and 2, PIP2, phosphoinositide 4,5 bisphosphate; PIP3, phosphoinositide 3,4,5 trisphosphate; rictor, a component of mTORC2; P70S6K, the p70 ribosomal S6K; PTEN, phosphatase and tensin homolog deleted from chromosome ten; ROS, reaction oxygen species; PDPK1 (also abbreviated as PDK1), phosphoinositide dependent protein kinase.

and survival of TSC2-Null cells (Goncharova et al., 2011). The hypothesis that FoxOs are involved in the progression of cell cycle is further strengthened by the fact that FoxOs also regulate the expression of mitotic genes such as cyclin B, polo-like kinase (Plk) (Alvarez et al., 2001). In addition, recent reports indicate the role of FoxO1 in dedifferentiation of pancreatic β-cells (Talchai et al., 2012) and in osteoblast proliferation (Kode et al., 2012).

THE PROPOSED MODEL

Based on the foregone discussion, we propose that activation of PI3K-Akt-mTORC1 leads to inhibition of autophagy and rescues Dvl, which activates the Wnt pathway (**Figure 1B**). The transcriptional activation of *Cyclin D* by Wnt pathway triggers the entry of cells from G0 to G1 phase. *c-Myc* promotes reprograming of cancer cell metabolism in the G1 phase, which apart from generating ROS, activates the transcription factors like p53 and FoxO and autophagy. The transcription of rictor by FoxO leads to the inhibition of *c-Myc* and promotes exit of the restriction point of G1-S phase of cell cycle. Rictor also constitutes mTORC2 in G2 phase. Phosphorylation of Akt at S473 by mTORC2 leads to feedback inhibition of FoxO.

SOME UNANSWERED QUESTIONS

Cancer cells consume lots of glucose, but it is reported that glucose transporters are activated only following phosphorylation of Akt

at S473 (Kumar et al., 2010) and it coincides with the inactivation of FoxO proteins. Under hypoxic conditions, loss of p53 promotes the expression of mono carboxylate transporters (MCT1) and lactate export which is reported to promote cell proliferation by fueling mitochondrial respiration (Boidot et al., 2012). Do cancer cells exiting the divisional phase depend on excess glucose and glycolytic flux fuels respiration, while actively proliferating cells depend on lactate as a fuel resulting in a Warburg effect?

ACKNOWLEDGMENTS

Dr. Lakshmipathi Vadlakonda is thankful to the Director of CR RAO AIMSCS, Prof. Allam Apparao, and the DST, New Delhi for providing a placement as the adjunct faculty. Dr. Mukesh Pasupuleti thanks the management of SRM University for providing funds and facilities for carrying out the research work at SRM Research Institute. The financial support by the Department of Biotechnology and Indian Council of Medical Research to Dr. Pallu Reddanna is gratefully acknowledged.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 07 February 2013; accepted: 31 March 2013; published online: 12 April 2013.

Citation: Vadlakonda L, Pasupuleti M and Pallu R (2013) Role of PI3K-AKT-mTOR and Wnt signaling pathways in transition of G1-S phase of cell cycle in cancer cells. Front. Oncol. ***3****:85. doi: 10.3389/fonc.2013.00085*

This article was submitted to Frontiers in Molecular and Cellular Oncology, a specialty of Frontiers in Oncology.

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