Signaling Intermediates (PI3K/PTEN/AKT/mTOR and RAF/MEK/ERK Pathways) as Therapeutic Targets for Anti-Cancer and Anti-Angiogenesis Treatments

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Abstract: Protein phosphorylation catalyzed by protein kinases play critical roles in the regulation of signal-transduction pathways. Deregulated kinase activity is observed in a variety of human diseases, such as cancer, making them targets for the development of molecular therapies. The PI3K/PTEN/AKT/mTOR and RAF/MEK/ERK signaling pathways play fundamental roles in transmitting signals from membrane receptors to downstream targets that regulate apoptosis, cell growth and angiogenesis. Accumulating evidence suggests that both pathways are constitutively activated through multiple genetic and epigenetic mechanisms in a wide variety of human malignancies and play several key functions in cancer development and progression; in that respect, both the PI3K and MAPK pathways function at the bottleneck of signal transduction through protein kinase cascades, thereby constituting attractive therapeutic targets for anti-cancer treatments. These pathways, however, are part of complicated and interwoven regulatory networks and recent evidence suggests that combining inhibitors targeting both the PI3K/PTEN/AKT/mTOR and the RAF/MEK/ERK pathways may avoid tumor escape from single-pathway blockade and ultimately suppress both malignant growth and survival more efficiently. Moreover, both pathways may converge on the regulation of crucial functions, such as neo-angiogenesis, involving not only the cancer cell but also the tumor stroma and the surrounding "normal" compartment. In this review, we describe recent advances in understanding the PI3K and MAPK pathways, in particular the mechanisms by which they regulate tumor growth and angiogenesis, and highlight the potential therapeutic opportunities for targeting these pathways for cancer treatment.

INTRODUCTION

Deregulation of cell proliferation/survival pathways is widely accepted to be a fundamental aspect of tumorigenesis [1-3]. Several key cellular signaling pathways that work independently, in parallel, and/or through interconnections to promote cancer development have been identified in a number of tumor models [3-7].

Physiologically, components of signal transduction cascades modulate signals from cell surface receptors to transcription factors, which regulate gene expression. Most extracellular signals are amplified and transduced inside the cell by protein kinase cascades. Deregulated kinase activity is observed in a variety of human diseases, including cancer, and development of selective molecules that modulate kinase activity is widely considered a promising approach for drug development [2, 3, 6, 8].

Two of the most important signaling cascades frequently deregulated in cancer are the phosphatidylinositol 3-kinase (PI3K)/phosphatase and tensin homolog (PTEN)/AKT/ mammalian target of rapamycin (mTOR) and RAF/mitogen-activated protein kinase-extracellular signal-regulated kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathways. These signaling cascades play critical roles in the transmission of signals from growth factor receptors to regulate diverse biological process such as cell growth, metabolism, differentiation, apoptosis and angiogenesis. Components of these pathways are mutated or aberrantly expressed in human cancer (e.g., RAS, B-RAF, PI3K, PTEN, AKT) [2, 3]. In addition, some components of the PI3K/ PTEN/AKT/mTOR and RAF/MEK/ERK signaling pathways, such as RAF-1, MEK-1/2, AKT or mTOR, represent excellent targets for the development anticancer drugs; because of the converging function of these signaling molecules, their specific inhibition is expected to quite effectively intercept a wide variety of mitogenic and/or pro-survival signals [3, 9, 10].

In rare cases, such as in chronic myelogenous leukemia (CML), a single "apical" genetic lesion (the t(9;22) chromosomal translocation that gives rise to the BCR-ABL fusion protein) can be identified that drives the activation of an array of diverse signaling pathways, including Nuclear Factor κB (NF-κB), AKT and Signal Transducer and Activator of Transcription 5 (STAT5) cascades among others [11]. In such cases, pharmacological interference with the "causative" genetic alteration severely impairs the ability of transformed cells to proliferate and survive and dramatically alters the natural history of the disease, leading to arguably the most impressive "success story" in the field of cancer therapy over the past 20 years [12]. At the other end of the spectrum lies perhaps the deadliest of human cancers, pancreatic cancer, in which an average of 63 genetic alterations per case, the majority of which are point mutations, were recently elucidated by comprehensive genetic analysis. These alterations defined a core set of 12 different cellular signaling pathways and processes that were each genetically altered in 67 to 100% of the tumors [13]. Although most of

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human cancers lie between these two extremes, a single genetic alteration necessary and sufficient to drive the diverse array of phenotypic hallmarks of malignancy (as it is the case for the BCR-ABL fusion protein in CML) is the exception rather than the rule and malignant behavior is usually driven by the accumulation of several genetic and epigenetic aberrations [14]. For this very reason, in many solid tumor models highly selective or specific blocking of only one of the kinases involved in these signaling pathways has been associated with limited clinical responses [15]. Improved understanding of the complexity of signal transduction processes and their roles in cancer has suggested that simultaneous inhibition of several key kinases may help optimize the overall therapeutic benefit associated with molecularly targeted anticancer agents. Using targeted agents to inhibit multiple signaling pathways has thus emerged as a new paradigm for anticancer treatment [2].

In this review, we describe the current status of inhibitors of the MEK/ERK and PI3K pathway, and the possibility that combination of ERK inhibitors and PI3K inhibitor may provide an effective basis for development of new chemotherapeutic strategies against cancer.

THE RAF/MEK/ERK PATHWAY

The ubiquitous Mitogen-Activated Protein Kinase (MAPK) pathway regulates diverse cellular functions, including cell differentiation, cell division, cell movement and apoptosis [16-19]. Five distinct groups of MAPK have been characterized in mammals: ERK-1/2, c-Jun N-terminal kinase 1/2/3 (JNK-1/2/3), p38 isoforms α, β, γ and δ, ERK-3/4 and ERK-5 [16, 17]. MAPK are regulated by modular phosphorylation cascades. The main kinase module consists of three protein kinases that are sequentially activated by a phosphorylation cascade: a MAPK kinase kinase (MAP3K), a MAPK kinase (MAP2K), and a MAPK [17, 20]. Accumulating evidence has demonstrated that the magnitude and duration of MAPK activities, as well as their re-localization to specific cellular compartments [1, 18, 21-23], regulate signaling specificity. Thus, inactivation of MAPK plays an important role in determining signaling outcomes and is carried out by specific MAPK phosphatases (MKPs) that catalyze the dephosphorylation of activated MAPK [24-26]. An interesting feature of MAPK signaling is that the activation of a single MAPK pathway is able to transduce multiple extracellular stimuli to their specific cellular responses. Among the different MAPK modules, the RAF/MEK/ERK cascade is the most extensively studied and perhaps the most relevant to cancer pathogenesis and therapy [3, 8, 27, 28]. Activation of this pathway is triggered by a diverse range of stimuli acting through cell-surface receptors and is under the control of the small G-protein RAS [17, 20, 29]. Initially, activated RAS recruits RAF to the plasma membrane, where it is activated, in a key step of a complex activation process. RAF then acts as a MAP3K and phosphorylates the dual-specificity protein kinases MEK-1 and -2, which catalyze the phosphorylation of ERK on both serine/threonine and tyrosine residues, allowing their activation. ERK, in turn, modulates the function of numerous substrates involved in a multitude of cellular processes such as cell growth, survival and angiogenesis (c-Jun, c-Myc, CREB, HIF-1α, IKK, among

many others). MAPK signaling is not a simple linear pathway and the complexity of the MEK/ERK cascade is enhanced by the presence of more than one of each of the kinases, namely three RAF (A-RAF, B-RAF and C-RAF) and two distinct MEK and ERK (MEK-1 and -2, and ERK-1 and -2) [1, 17, 20, 27, 30].

Targeting the MAPK Pathway

Proliferation, differentiation, and apoptosis are coordinated processes that help maintain homeostasis among the diverse cell types in higher organisms. Accumulating evidence has shown that some component of these processes may become unregulated and contribute to transformation and tumorigenesis.

The pivotal role played by RAF/MEK/ERK in signaling networks regulating cell growth and survival, provides the conceptual framework to understand the oncogenic potential of deranged signaling through this MAPK module [1, 17, 20, 27, 31]. Indeed, the MAPK pathway has been shown to be deregulated in various human malignancies and its constitutive activation has been associated with an aggressive neoplastic phenotype [31]. Accordingly, over-expression or activating mutations of Epidermal Growth Factor Receptor (EGFR) and other membrane RTK, activating mutations of RAS or activating mutations of RAF have been detected. Mutations in RAS and BRAF typically demonstrate mutual exclusivity in tumors; therefore, either mutation might exert its oncogenic activity through common downstream proteins such as MEK-1/2 and ERK-1/2, and these enzymes may be better exploited as drug targets [8, 28]. Germline MEK mutations have been demonstrated in patients with cardio-facio-cutaneous (CFC) syndrome, a complex developmental disorder involving the heart, face, and skin [32], with currently unknown potential for predisposition to cancer; more recently, somatic activating mutations in exon 2 of the MEK1 gene have been reported in an ovarian cancer cell line [33] and in two patients with lung adenocarcinoma [34]. Although the oncogenic nature of such mutations remains to be demonstrated, both MEK and ERK can efficiently transform mammalian cells to a neoplastic phenotype when expressed in constitutively active forms [35-37]; similarly, disruption of their activation by pharmacological inhibitors severely impairs the transforming ability of many upstream-acting cellular oncogenes [17, 38, 39].

A wide spectrum of inhibitors against the components of this network have been developed and investigated both *in vitro* and *in vivo*. These include farnesyltransferase inhibitors (FTI) that interfere with the translocation of RAS to the cell membrane, and direct inhibitors of RAF, such as Sorafenib (BAY 43-9006), or MEK, such as ARRY142886/AZD6244 or PD0325901.

- **RAS inhibitors:** FTI are a family of inhibitors of RAS and the antineoplastic properties of these agents have been extensively evaluated. By inhibiting the post-translational addition of a farnesyl group to RAS, it was thought that FTI would be able to target a broad range of human tumors in which RAS was constitutively activated. R115777 (Zarnestra) has undergone the most extensive clinical evaluation and has been studied in pa-

tients diagnosed with a range of cancers, encompassing acute and chronic leukemias, multiple myeloma, non-small-cell lung, breast, pancreatic and prostate cancers. The most notable effects were observed when this drug was administered to patients with hematopoietic malignancies [40, 41]. However, clinical testing of FTI has been largely disappointing, as it is the case for tipifarnib in pancreatic cancer and lonafarnib in non small cell lung cancer [42-44]. The root of the problem lies in the fact that, although H-RAS is exclusively modified by farnesyltransferase, K-RAS and, to a lesser extent, N-RAS can also be modified by gernylgeranyltransferase (GGT), that is still able to support the biological activity of RAS. Geranylgeranylation of K-RAS and N-RAS becomes important only when farnesylation is blocked. As the vast majority of RAS mutations in human are in K-RAS followed by N-RAS, with very few in H-RAS, it is likely that inhibition of mutant RAS farnesylation is not responsible for any antitumour effects of FTI. Attempting to inhibit the function of K-RAS and N-RAS by using FTI and GGTI together has failed because of the very high toxicity that is associated with this combination [43, 45].

- **RAF inhibitors:** as reviewed by Bollag *et al.*, several small-molecule RAF inhibitors have now been reported [46]. According to published reports, only one of these, BAY 43-9006 (Sorafenib), has reached the clinical testing stage. BAY 43-9006 is a potent inhibitor of both wild type and mutant RAF [47, 48], inhibits the MAPK signaling pathway both *in vitro* and *in vivo* [49], and is generally well tolerated, with the most common toxicities involving the gastrointestinal tract (diarrhea) and the skin. BAY 43-9006 has undergone extensive clinical evaluation and is now an approved standard of care in renal cell and hepatocellular carcinoma [50, 51]. However, sorafenib has been shown to hit multiple additional targets (it binds to ~10% of the kinases tested with a binding affinity within 10-fold of that for the primary target), with a strong preference for tyrosine, rather than serine/threonine, kinases [47]. Indeed, at least some of the antitumor effects of this compound are likely due to inhibition of tyrosine kinases [52, 53]. Recently, another selective inhibitor of oncogenic B-RAF kinase with potent antimelanoma activity (PLX4720) has been described [54].

- **MEK inhibitors:** the first MEK inhibitor to be disclosed was PD098059 [2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one] [55, 56]. Because of its pharmaceutical limitations, this inhibitor was almost exclusively used in cell systems to study MEK inhibition in order to further delineate the role of the MAPK pathway in carcinogenesis. Similarly, U0126, a second MEK inhibitor with more potency than PD098059, has been mostly used as an *in vitro* laboratory reagent [57]. Based on such preclinical activity, PD184352 (subsequently named CI-1040) became the first MEK inhibitor to move into clinical trials [58]. Because of the poor metabolic stability and bioavailability of this agent observed in Phase I trials, higher doses had to be administered to patients in Phase II trials, that clearly resulted in significant MEK inhibition and were clinically well tolerated. A second-generation oral MEK inhibitor, compound PD0325901, was subsequently developed [8, 28, 59, 60]. PD0325901 is structurally highly similar to CI-1040 but is significantly more potent than CI-1040 *in vivo*. Anticancer activity of PD0325901 has been demonstrated for a broad spectrum of human tumor xenografts [61-63]. The benzimidazole ARRY-142886 (also known as AZD6244) which is 10-fold less potent than PD0325901, was found to be tumor-specific and ranged from antiproliferative effects to the induction of apoptosis and differentiation [64, 65]. ARRY-142886 was advanced into full development and recently entered clinical trials [62, 66, 67]. MEK inhibitors differ from most of the other currently available kinase inhibitors, since they do not compete with ATP binding and therefore are endowed with an unusually high specificity towards their target [68]; indeed, none of these compounds significantly inhibit the activity of a large panel of protein kinases that include ERK-1, JNK-1 and p38 MAPK in an *in vitro* assay [69]. Recently, crystal structures of MEK-1 and -2 have been determined as ternary complexes with Mg-ATP and PD184352-like inhibitors, showing that both enzymes have a unique inhibitor-binding site located in an interior hydrophobic pocket adjacent to, but not overlapping with, the Mg-ATP-binding site [70]. Binding of MEK inhibitors to this hydrophobic pocket induces several conformational changes in unphosphorylated MEK, locking them into a closed and catalytically inactive conformation. Notably, the MEK inhibitor binding-site is located in a region where the sequence homology to other protein kinases is quite low. With the exception of MEK-2 (100% identical) and MKK-5 (81% identical), all other protein kinases share low sequence identity (60%–70%) with MEK-1 in the inhibitor-binding site, thereby explaining why PD184352-like MEK inhibitors are exceptionally specific for MEK-1, MEK-2, and MKK-5 (although to a much lesser extent), but do not inhibit many other protein kinases [8, 70].

THE PI3K/PTEN/AKT/MTOR PATHWAY

The PI3K/PTEN/AKT/mTOR constitutes an important signaling pathway regulating multiple biological processes such as apoptosis, metabolism, cell growth and proliferation in response to extracellular signals. The PI3K pathway has been implicated in cancer since its discovery 20 years ago, and in recent years it has become apparent that it is one of the most frequently targeted pathways in sporadic human tumors, with estimates suggesting that mutation in one or another PI3K pathway component accounts for 30% of all human cancers [71, 72].

A large number of the plasma membrane receptors, in particular those with tyrosine kinase activity (RTK), can activate PI3K. For instance, binding of Insulin-like Growth Factor-1 (IGF-1) to its receptor IGF-1R leads to receptor activation and autophosphorylation on tyrosine [73, 74], resulting in activation of the PI3K network. Other RTK whose activity prominently relies on the activation of the PI3K pathway include the EGFR family of receptors, most notably EGFR and HER-2 [75, 76].

PTEN is by now one of, if not the most, frequently mutated, deleted and silenced tumor suppressor in the history of

cancer genetics. Although there is no doubt that PTEN function in disease is predominantly impacted by genetic events compromising the PTEN locus, numerous factors regulating PTEN transcription as well as post-transcriptional modifiers (such as PTEN-targeting miRNA) and post-translational regulators have been recently identified. As expected for an important tumor suppressor, PTEN is dynamically modified at multiple levels, for instance PTEN is acetylated, phosphorylated and ubiquitinylated [77, 78].

In response to extracellular stimuli, PI3K phosphorylates the 3'-hydroxyl phosphatidylinositol-4, 5 biphosphate (PIP$_2$) to generate phosphatidylinositol-3, 4, 5 triphosphate (PIP$_3$). PIP3 serves as a second messenger that helps to activate the serine-threonine kinase AKT. In fact, these specialized lipids serve to recruit pleckstrin homology (PH) domain-containing proteins such as the serine-threonine kinase AKT and Phosphoinositide-Dependent Kinase 1 (PDK1) to the plasma membrane. After recruitment to the membrane PDK1 phosphorylates and consequently activates AKT. The tumor suppressor phosphatase PTEN, instead, dephosphorylates PIP$_3$ reversing the action of PI3K [79, 80].

Several direct substrates of AKT phosphorylation have crucial roles in cell-cycle regulation. These substrates include the forkhead box transcription factors (FOXO) and Tuberous Sclerosis Complex 2 (TSC2) [5, 81, 82]. Forkhead transcription factors of the FOXO family are important downstream targets of AKT that mediate apoptosis and cell cycle arrest. Direct phosphorylation by PKB/AKT inhibits transcriptional activation by FOXO factors, resulting in cell proliferation and survival [81].

Activated AKT phosphorylates and inhibits the TSC complex thereby removing its inhibitory effects on mTOR. mTOR is a serine-threonine kinase that regulates both cell growth and cell cycle progression through its ability to integrate signals from nutrient availability and growth factor stimulation. mTOR activation by growth factor receptors proceeds both through and in parallel to PI3K and AKT [82-86]. Mammalian TOR was discovered in the early 1990s in studies into the mechanism of action of rapamycin (also known as sirolimus), which is a macrolide that was originally found as an antifungal agent and was later recognized as having immunosuppressive and anticancer properties. Even today, exactly how rapamycin perturbs mTOR function is not completely understood. The complex of rapamycin with its intracellular receptor FKBP12 binds directly to the mTOR complex 1 (mTORC1), containing two associated proteins (raptor, regulatory- associated protein of mTOR, and mLST8, also known as GβL), and suppresses mTORC1-mediated phosphorylation of the substrates S6K1 and 4EBP1. Rapamycin also weakens the interaction between mTOR and raptor [87], thereby inhibiting recruitment substrates to the mTOR kinase domain [88]. It is not known if mTORC1 has functions that depend on its kinase activity but are not sensitive to rapamycin, so it is still unclear if a molecule that directly inhibited the mTORC1 kinase domain would have different biological effects to those of rapamycin. Mammalian TOR complex 2 (mTORC2) also contains mTOR and mLST8 but, instead of raptor, it contains two proteins, rictor (rapamycin-insensitive companion of mTOR) and mSin1

(also known as mitogen-activated-protein-kinase-associated protein 1), that are not part of mTORC1. This second mTOR-containing complex is less understood than mTORC1 but recent work indicates that it should be considered part of the PI3K–AKT pathway as it directly phosphorylates AKT [84, 89] on one of the two sites that are necessary for AKT activation in response to growth-factor signalling. This finding makes mTORC2 a key part of the pathway that activates AKT and, like PDK1 and PI3K, a potential drug target for cancers in which there is AKT deregulation. The AKT-activating function of mTORC2 sets up the intriguing situation in which mTOR, as part of two distinct complexes, is potentially both 'upstream' and 'downstream' of itself. Mammalian TORC2 has other functions besides activating AKT, such as regulating the cytoskeleton [90, 91], but the implications for cancer of these roles are still unknown. A potentially important wrinkle in this seemingly closed story has recently emerged [92]. It turns out that prolonged treatment with rapamycin — clearly a situation that is relevant to its use in patients — perturbs mTORC2 assembly and, in about 20% of cancer cell lines, the drop in intact mTORC2 levels is sufficient to strongly inhibit AKT signalling. The binding of FKBP12–rapamycin to mTOR seems to block the subsequent binding of the mTORC2-specific components rictor and mSin1 [92, 93] but it is unknown why in certain cell types rapamycin only partially inhibits mTORC2 assembly. No absolute correlation exists between the tissue of origin of a cell line and the sensitivity of mTORC2 formation to rapamycin, although many cell lines with this property are derived from the haematological system. Recent work provides the first evidence that mTORC2 function can be rapamycin-sensitive in patients. In more than 50% of patients with AML, rapamycin and its analogues inhibited AKT phosphorylation in primary leukaemic cells and the inhibition correlated with the loss of intact mTORC2 [94]. So, rapamycin and its analogues are universal inhibitors of mTORC1 and S6K1, and cell-type specific inhibitors of mTORC2 and AKT. As the inhibition of mTORC2 by rapamycin is time and dose dependent, AKT activity in tumours will vary with the length of rapamycin treatment and the dosing regimen.

The PI3K pathway is highly conserved among different species including *Drosophila melanogaster*, *Caenorhabditis elegans* and mammals [95-97]. This pathway is abnormally activated in many tumors and many mechanisms for pathway activation have been described, including loss of tumor suppressor PTEN function, amplification or mutation of PI3K, amplification or mutation of AKT, activation of growth factor receptors, and exposure to carcinogens [98-102]. The tumor suppressor PTEN is frequently mutated or epigenetically lost in primary glioblastomas, breast cancer, lung cancer, and melanoma; in addition, decreased levels of PTEN expression are correlated with an aggressive neoplastic phenotype. AKT gene amplification has been observed in many human cancers, including glioblastoma, gliosarcoma and gastric carcinoma. Several observations make AKT an attractive target for anticancer drug discovery, and it has been postulated that inhibition of AKT alone or in combination with standard cancer chemotherapeutics will reduce the apoptotic threshold and preferentially kill cancer cells. In fact, AKT sits at the crossroads of multiple oncogenic and

tumor suppressor signaling networks; frequent deregulation of many components of the AKT signaling pathway has been observed in human cancer [103]; ectopic expression of constitutively active AKT results in oncogenic transformation *in vitro* and *in vivo* [104-108]; and finally knockdown of AKT by antisense or siRNA significantly reduces tumor growth and invasiveness in tumor cells overexpressing AKT [109, 110]. Indeed, AKT can be activated by growth factors and cytokines and stimulation of its kinase activity correlates with resistance to chemotherapy [111].

The introduction of modulators of the PI3K pathway as potential targeted anticancer agents is still in an early stage but several interesting compounds have entered clinical trials.

Targeting the PI3K Pathway

The basic players in the PI3K/AKT pathway have now been defined and the importance of the cascade in various human cancers is firmly established. These facts should put the issue of developing targeted drugs for the treatment of cancers that have PI3K/AKT pathway deregulation at the forefront of the translational cancer-research field. Considering the broad experience gained in the past 20 years with small molecule kinase inhibitors, therapeutic targeting of this pathway including AKT itself as well as its upstream regulators (PI3K) and downstream effectors (mTOR) should now be close to becoming a clinical reality. The majority of small molecule inhibitors are classic ATP-competitive inhibitors, which provide little specificity. However, rapamycin-like drugs that inhibit mTOR have a unique mechanism of action that ensures extremely high specificity [112, 113].

Proteins that have received the most attention as targets for pharmacological intervention the PI3K pathway are: PDK1, PI3K, AKT and mTOR.

- PDK1 is an upstream regulator of AKT [114]; therefore, a PDK1 inhibitor should significantly block activation of AKT. One of the most potent, but nonselective PDK1 kinase inhibitor reported to date is UCN-01 (7-hydroxy-staurosporine). This drug is currently being evaluated as an antineoplastic agent in clinical trials both alone and in combination with chemotherapeutic agents and ionizing radiation. UCN-01 exerts antiproliferative activity both in vitro and in vivo [115-117]. A class of aminopyrimidine derivates has also been reported to inhibit PDK1 kinase activity. A representative example of this class of compounds are BX-795, BX-912 and BX-320, recently identified by screening of compound libraries. Inhibitory activity against PDK1 has also been reported for compounds originally designed to antagonize a different therapeutic target. For example, it has been found that celecoxib, which is a cyclooxygenase-2 inhibitor, can block the activation of AKT by inhibiting PDK1 enzymatic activity in a variety of cancer cells [118].

- Two pharmacological PI3K inhibitors are the fungal metabolite Wortmannin and LY294002. These non selective compounds, block the enzymatic activity of PI3K by different mechanisms: wortmannin is an irreversible inhibitor that forms a covalent bond with a conserved lysine residue involved in the phosphate-binding reaction, whereas LY294002 is a classical reversible, ATP-competitive PI3K modulator [69, 119, 120]. Wortmannin or LY294002 alone may inhibit cell proliferation, promote apoptosis and/or inhibit tumor growth, but have important pharmaceutical limitations. To overcome these shortcomings, broad-spectrum PI3K inhibitors using wortmannin and LY294002 as a structural reference are being developed [121, 122]. A new generation of PI3K inhibitors is thus emerging, overcoming earlier problems of poor selectivity, unfavorable pharmacokinetic profiles, and unacceptable toxicity [123]. These new inhibitors (such as PWT-458, PX-866, SF-1126) are more potent and have less toxic effects that Wortmannin or LY294002. PWT-458 and PX-866 have not entered clinical trial at this time. SF-1126 is currently being tested in phase I trials and a number of other agents are approaching early-phase clinical testing. Among these, dual PI3K/mTOR inhibitors that simultaneously target two crucial points along the same pathway appear very promising [124]. NVP-BEZ235 (Novartis Pharma) is a synthetic low molecular mass compound belonging to the class of imidazoquinolines that potently and reversibly inhibits class 1 PI3K catalytic activity by competing at its ATP-binding site. NVP-BEZ235 also inhibits mTOR catalytic activity but does not target other protein kinases and is currently in phase I clinical testing [124-127].

- Lipid-based inhibitors of AKT were the first group of to be developed. These AKT non-selective inhibitors include perifosine, phosphatidylinositol ether lipid analogues, and D-3-deoxy-phosphatidylmyoinositol-1-[(R)-2-methoxy-3- octadecyloxyropyl hydrogen phosphate] (PX-316). Perifosine is the best-characterized AKT inhibitor, which inhibits the translocation of AKT to the cell membrane, and inhibits the growth of several different solid tumors [128]. Perifosine treatment overcomes tumor resistance to chemotherapeutic drugs and radiation. Several other AKT inhibitors being developed include peptide-based inhibitors of AKT, pseudopeptide substrates of AKT, a single-chain antibody (scFv) against AKT, an inhibitory form of AKT expressed by adenoviral vectors, and siRNA against AKT [129]. From a drug discovery perspective, other pockets besides the ATP-binding cleft can be exploited for development of AKT kinase modulators, forming the basis for the identification of allosteric inhibitors of AKT [124, 130]. Although the mechanism of inhibition by these allosteric kinase inhibitors has not been fully elucidated and there are no structural data yet, it seems that these molecules may bind outside the ATP-binding pocket, interacting with the PH domain and/or hinge region likely promoting the formation of an inactive conformation. This appears to be the case for the allosteric AKT inhibitor VIII, as recently reported [131].

- Among the possible targets for cancer therapy, mTOR is one of the most promising. Currently, the mTOR inhibitor rapamycin and its analogues (CCI-779, RAD001, AP23573), which induce cell-cycle arrest in the G1 phase, are being evaluated in cancer clinical trials. Preclinical studies with these compounds indicate that they have cytostatic activity as single agents in animal models

and exert synergistic growth-inhibitory effects when are used in combination with the conventional hormonal agent tamoxifen, or with radiation treatment. In clinical studies, these compounds have been shown to be effective against many types of solid cancers [129]. CCI-779 (Temsirolimus) is a more water-soluble ester of rapamycin for which there are both i.v. and oral formulations. In preclinical studies, the drug resulted in significant antitumor activity in a variety of human cancer models, such as gliomas, rhabdomyosarcomas, prostate cancer, breast cancer, small cell lung cancer (SCLC), melanoma, and leukemia. After extensive clinical testing, Temsirolimus has now been approved for the treatment of poor-prognosis renal cell carcinoma patients [132] and is being tested in phase II-III trials in hematological and gynecological malignancies. Another rapamycin-like mTOR inhibitor (RAD-001, Everolimus) has also recently proven effective in advanced renal cell cancer patients [133] and a third one (AP23573, Deforolimus) is currently undergoing phase III clinical testing. As rapamycin-like mTOR inhibitors enter the stage of clinical testing in combination with other agents [134], an eagerly awaited progress in this field is the development of TOR inhibitors that directly target the kinase domain, thereby inhibiting the activity of both the mTORC1 and mTORC2 complexes (see above).

INVOLVEMENT OF PI3K/PTEN/AKT/MTOR AND RAF/MEK/ERK PATHWAYS IN ANGIOGENESIS

Angiogenesis is a dynamic process in which new blood vessels grow from a preexisting primitive network. In adults, this vascular network is relatively stable and the formation of a new or modified microvasculature is generally associated with pathological conditions including cancer. Angiogenesis is an essential process for tumor growth and progression. In fact, tumor growth and metastasis require angiogenesis when the tumor reaches 1–2 mm in diameter [135]. The angiogenesis process includes dissolution of the basement membrane of the vessel, migration and proliferation of endothelial cells, formation of a new vessel lumen and vessel branches. Angiogenesis may be regulated by hypoxia, a condition where the level of oxygen is diminished [136]. Oxygen limitation is central in controlling neovascularization and tumor spread. This pleiotropic action is orchestrated by Hypoxia-Inducible Factor (HIF-1), which is a master transcriptional factor in nutrient and stress signaling. HIF-1 can induce a vast array of gene products including: Vascular Endothelial Growth Factor (VEGF), Angiotensin II, TGF-β-1, PDGF and endothelin-1 [137-139]. This protein is a heterodimeric transcription factor comprised of two subunits: HIF-1α and HIF1-β [140]. HIF-1α can be induced by hypoxia, growth factors and oncogenes; whereas HIF1-β protein is constitutively expressed in human cells. Indeed, HIF-1α is the major regulator of VEGF transcriptional activation through the binding to the Hypoxia Response Element (HRE) of VEGF promoter. VEGF is a potent and critical vascular regulator required to initiate the formation of immature vessels by angiogenic sprouting. Furthermore, VEGF acts as survival factor for vascular endothelial cells, thereby protecting tumor cells from apoptosis and necrosis [141-143]. The MAPK and PI3K pathways are crucially involved in the regulation of angiogenesis. Indeed, it is remarkable that, in addition to cancer cell proliferation and survival, these two pathways also control the expression of many key factors in vascularization/angiogenesis such as VEGF [138, 144, 145].

PI3K Pathways in Angiogenesis

As discussed above, the PI3K signaling pathway plays a central role in the regulation of cell survival, proliferation, and tumor growth. Recently it was also found that this pathway plays an important role in regulating normal vascularization and pathological angiogenesis [138]. Recent evidence strongly support the use of PI3K inhibitors in antiangiogenic therapy.

PI3K is activated downstream of key receptors expressed by endothelial cells: VEGFR1-3, TIE-1/2, FGFR1-2, PDGFR- and ERBB1-4 RTK [146]. Each of these receptors acts as a master regulator of angiogenic signaling in the endothelium. Studies in mice using conditional or germline knockouts of PI3K effector genes illustrate the importance of the PI3K pathway in angiogenesis. In fact, complete loss of PTEN in the endothelium results in abnormal vascular remodeling, bleeding and embryonic lethality [147-149]; and constitutive AKT activity in the endothelium results in abnormal vessel patterning, vessel congestion and breaching [150]. Direct evidence of PI3K and AKT involvement in regulating angiogenesis *in vivo* was provided by the enforced expression of PI3K and AKT in chicken chorioallantoic membrane (CAM) by the RCAS retroviral vector [151]. Fujikava *et al*. reported that the PI3K pathway is also involved in endothelial cell survival and migration induced by Angiopoietin I [152].

In addition to its role in endothelial cell pathophysiology, PI3K transmits the upstream signals from growth factors, cytokines, and oncogenes to regulate VEGF and HIF-1 expression in human cancer cells [137]. In these studies, it was also observed that RAS/PI3K/AKT signaling is involved in hypoxia-dependent induction of VEGF [138]. In addition, it has been found that PI3K/AKT regulates VEGF and HIF-1 expression through HDM2 and p70S6K1 activation [153]. Blocking the PI3K pathway by downstream-acting mTOR inhibitors, also suppresses angiogenesis in many tumor models through direct (i.e. inhibition of endothelial cell functions) and indirect (i.e. inhibition of pro-angiogenic cytokine production by cancer cells) mechanisms [9, 154, 155]. Thus signal transduction through PI3K is implicated in multiple key angiogenic pathway at both afferent and efferent levels, and may therefore represent excellent pivotal points for therapeutic intervention. This is exemplified by the finding that TOR inhibitors can reduce both induction of VEGF, endothelial cell proliferation and tube formation resulting in significant inhibition of angiogenesis, tumor growth and metastasis [9, 155-157].

MAPK Pathways in Angiogenesis

MAPK have been implicated in the regulation of angiogenic process; in fact, over the past few years, activation of the MAPK pathway has been shown to be involved in the modulation of cell migration, protease induction, dissolution of the basement membrane and VEGF induction [19, 144, 158-161]. The basement membrane forms a cellular support for tumors, and is made up of a complex mix of Extracellular

Matrix (ECM) proteins, including laminins, collagens and proteoglycans. MAPK have been shown to be involved in regulating the proteolytic enzymes that degrade the basement membrane [159, 162, 163]. In response to extracellular stimuli, phosphorylated ERK activates a host of transcriptional factors including Activating Protein-1 (AP-1), that modulate the expression of many proteolytic enzymes implicated in progression and invasion, such as Matrix Metalloproteinases (MMP) and urokinase-type Plasminogen Activator (uPA). MMP can degrade ECM, and therefore have been implicated in progression and invasion of cancer in addition to their involvement in normal tissue remodeling, wound healing and angiogenesis. Indeed, persistent activation of ERK in malignant cells can lead to enhanced induction of MMP and this could lead to ECM and basement-membrane degradation allowing the cancer cells to invade into surrounding tissues and metastasize [164, 165]. Many growth factors have been reported to stimulate cell migration, through activation of receptor tyrosine kinase involving RAS/MAPK signaling pathways [166].

The RAF/MEK/ERK pathway also plays a key role in the control of VEGF expression [144]; in fact, under normoxic conditions VEGF was rapidly induced by activation of the RAF/MEK/ERK kinase cascade and this effect was observed to be suppressed by MEK inhibitors. Moreover, activation of the RAF/MEK/ERK pathway and hypoxia exerted additive effects on VEGF mRNA induction. It was indeed shown that VEGF mRNA is upregulated by the ERK pathway through the phosphorylation of the transcription factor Sp1. This regulation is independent of hypoxic stress and reflects the intensity of growth-factor stimulation or oncogenic signals [144]. MAPK not only activate the VEGF promoter through the Sp1/AP-2 transcriptional factor complex but also phosphorylate HIF-1α leading in turn to the enhancement of HIF-1 dependent transcriptional activation of VEGF [143].

Several evidence indicate that RAF/MEK/ERK signaling regulates NF-κB activity [167-169]. NF-κB, is an inducible transcription factor that regulates the expression of many inflammatory mediators, including chemokine (e. g. CXCL-8). Recently, it was demonstrated that MAPK signaling regulates NF-κB through activation of p90^{RSK}. In fact, p90^{RSK} phosphorylates the regulatory N terminus of IκBα on serine 32 and triggers effective IκBα degradation *in vitro* [168]. IκBα is a cytoplasmic inhibitor that binds NF-κB complex sequestering it in the cytoplasm. After degradation of IκBα, the NF-κB complex is freed and rapidly translocates into the nucleus [170-172]. However, other reports demonstrated that constitutive activation of the RAF/MEK/ ERK pathway negatively regulates NF-κB transcriptional activity [167, 169].

Moreover, ERK activation has been shown to directly promote endothelial cell survival and vessel sprouting during angiogenesis by downregulating Rho kinase activity. The activity of the prototypical member of the family, Rho, has been shown to be necessary for VEGF-driven angiogenesis in the chorioallantoic membrane and VEGF-mediated organization of endothelial cells into vessels in a skin angiogenesis model *in vivo* [173], suggesting that the regulation of Rho signaling plays an important role during angiogenesis.

Mavria *et al.*, have recently demonstrated that ERK activation opposes Rho-kinase-dependent actomyosin contractility to promote endothelial cell survival and vessel sprouting [174].

SIGNALING CROSS-TALK

Emerging evidence indicates that the RAF/MEK/ERK pathway is intimately linked with the PI3K/PTEN/AKT pathway. These signaling cascades are frequently deregulated in cancer and there is accumulating data supporting the hypothesis that these pathways may cooperate to promote the survival of transformed cells [3]. In fact, RAS activation regulates activation of both pathways [175]. Both pathways may result in the phosphorylation of many downstream targets and impose a role in the regulation of cell survival and proliferation.

The PI3K pathway may impact on MAPK signaling at multiple levels. In some cell types, the PI3K pathway can directly modulate the RAF kinase bypassing the GTPase RAS. RAF activity is negatively regulated by AKT indicating a cross-talk between the two pathways. AKT phosphorylates c-RAF and B-RAF on Ser259, thereby inhibiting RAF activity and downstream MAPK signaling [176, 177]. In addition, the GTPase Rheb has also been shown to negatively regulate RAF [178, 179]. A novel mTOR-MAPK/ERK feedback loop has recently been demonstrated [180]. In this study, the authors reported the involvement of S6 kinase in the negative regulation of ERK activation, while treatment with mTOR inhibitors resulted in a hyperactive PI3K pathway, increasing the signal toward the RAS/RAF/MEK/ERK pathway.

The PI3K pathway also receives regulatory signals from the MAPK pathway. PTEN transcription is regulated by RAS in cancer cells leading to tumor progression [181, 182]. The TSC complex is also regulated by MAPK at two levels: p90RSK1 phosphorylates TSC2 at Ser1798, inhibiting the tumor suppressor function of the tuberin/hamartin complex and resulting in increased mTOR signaling to S6K1 [183]; and ERK can subsequently phosphorylate TSC2 at Ser664 leading to TSC1-TSC2 inhibiting mTOR activity [184]. In addition, a novel link between the RAS/MAPK pathway and mTOR signaling was recently described. In this study, the authors demonstrate that Raptor is phosphorylated by p90RSK1 and p90RSK2 protein kinases *in vitro* and *in vivo* and that RSK-mediated phosphorylation of Raptor positively regulates mTOR kinase activity [185].

Treatment of human disease with drug combinations might be exploited therapeutically. It has recently been demonstrated that aggressive melanoma cell lines are resistant to both MEK and PI3K inhibitors, whereas the combination of MEK- with PI3K-inhibitors suppresses the growth and invasion of metastatic melanoma cells [186, 187]. These data support the hypothesis that in the treatment of melanoma it is not sufficient to inhibit only a single constitutively activated signaling pathway and that an effective treatment strategy must take into account more than one deregulated signaling pathway. In a subsequent study, the authors reported the effects of simultaneous treatment with an inhibitor of MEK-1/2 (PD0325901) and mTOR (rapamycin) using PTEN defi-

cient transgenic prostate cancer [2, 188]. They reported that these agents effectively inhibited their targets and, when combined, interacted synergistically to prevent prostate cancer cell growth both *in vitro* and *in vivo*. In patient specimens, activation of ERK and the PI3K/PTEN/AKT/mTOR pathway was associated with prostate cancer progression; moreover, the authors found that combined MEK/ERK and mTOR inhibition was effective in the adjuvant setting. The authors concluded that a strategy combining MEK/ERK and mTOR inhibition may be effective in the treatment of advanced cancer [2, 188].

CONCLUSIONS AND FUTURE PERSPECTIVES

Our knowledge of signal transduction pathways has evolved, over the past 20 years, from the classical notion of "linear" signaling pathways, whereby a single receptor would transduce signals through specific "intermediates" to a

limited number of final "effectors", to the much more complex vision of "signaling networks", in which every single component is closely intertwined with an array of different players, thereby creating an extremely complex scheme of vertical and parallel signaling pathways regulated by positive and negative feedback loops (Fig. **1**). In this context, even the most specific interference with a single signaling component may actually lead to unexpected, and sometimes "undesired" from a therapeutic perspective, functional outputs. Such new level of complexity obviously requires completely novel strategies to both pathway investigation (for example the use of high throughput technologies and "omics" approaches) and interpretation of the results (the thriving science of "systems biology" applied to cancer biology and anti-cancer drug discovery) [189-191]. This may help explain why, in addition to a handful of success stories (such as the development of imatinib for the treatment of CML and
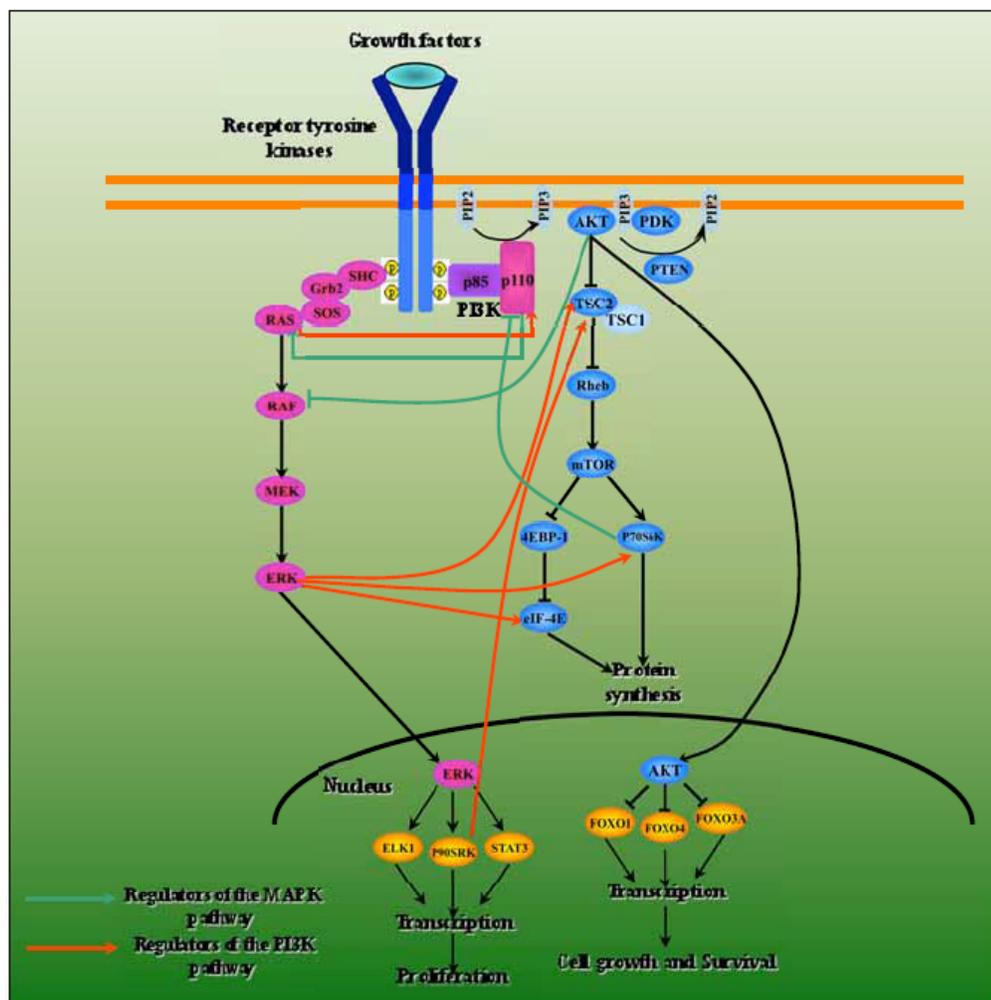
Fig. (1). PI3K/PTEN/AKT/mTOR and RAF/MEK/ERK pathways and their potential interactions in transformed cells. The RAS/RAF/MEK/ERK and PI3K/PTEN/AKT/mTOR signaling cascades transduce many signals from growth factor receptors to regulate gene expression. These pathways interact with each other to regulate growth and in some cases tumorigenesis. The RAS signalling pathway can be triggered by a set of RTK that are activated by growth factors. RAS can then activate PI3K or RAF, as described. Several members of the PI3K (PI3K, AKT, p70S6K) control the activation status of the RAS-MAPK pathway (green arrows). On the other hand, the PI3K signaling pathway is also regulated by other pathways, such as signaling through the MEK/ERK module. The RAS-MAPK pathway modulates the PI3K pathway at multiple levels (red arrows): RAS can regulate the activity of PI3K, ERK can regulate the activity of TSC2, p70S6K, and eIF-4E, and p90SRK can regulate TSC2 activity.

GIST or that of trastuzumab for breast cancer), the clinical development of other compounds that specifically target protein kinases has been more troublesome [15].

As depicted in Fig. (**2**), such complex functional cross-stalk between, for example, the PI3K and MAPK pathways is operational in the regulation of angiogenesis as well. Such effects on angiogenesis may often be overlooked when combinations of different pathway disrupting agents are carried out in experimental models *in vitro*. With particular regard to combined MAPK and PI3K pathway inhibition, an intriguing observation in the recently published study by Carracedo *et al.* [180] is that there was no clear increase in apoptosis in cells simultaneously exposed to MAPK and mTOR inhibitors *in vitro*, whereas apoptosis was pronounced in tumor cells exposed to this agents *in vivo*. One possible explanation for these findings is that concurrent MAPK and mTOR inhibition may cooperate to disrupt tumor angiogenesis, which could promote cancer cell death *in vivo*

could promote cancer cell death *in vivo* through an indirect mechanism.

A deeper understanding of such intricate signaling networks and extensive preclinical and early clinical modeling, also taking into account indirect mechanisms of action that counteract paraphysiological processes, such as neo-angiogenesis, turning normal tissue surrounding the tumor into a powerful cancer ally, will likely be required to translate such exciting preclinical findings into effective new therapeutic strategies for our patients suffering from cancer.

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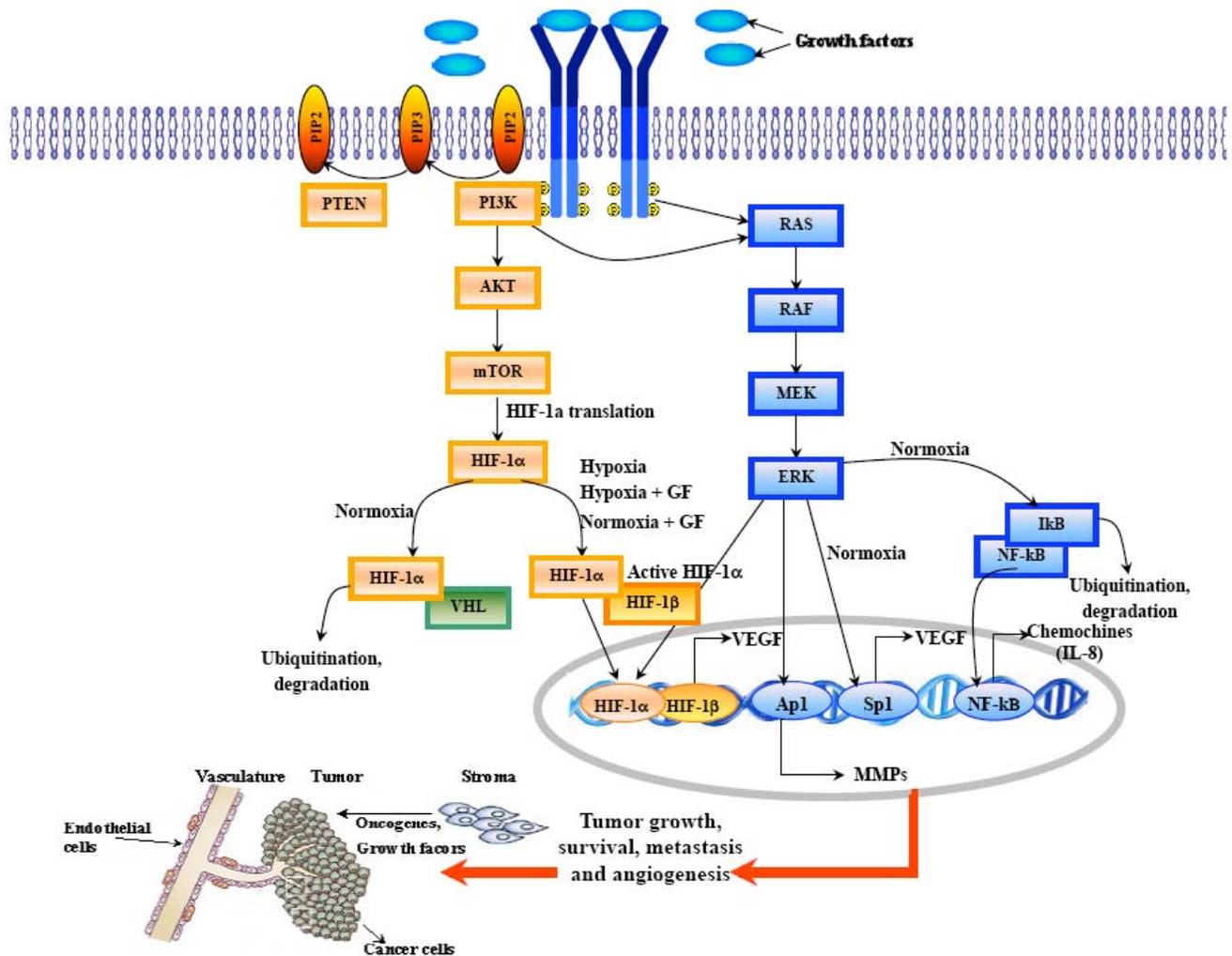
Fig. (2). PI3K/PTEN/AKT/mTOR and RAF/MEK/ERK pathways are crucially involved in the regulation of angiogenesis. MAPK and PI3K pathways control the activity of many transcription factors that may operate to promote angiogenesis, such as HIF-1α and NF-κB. HIF-1 is a master transcription factor in the regulation of oxygen homeostasis. Under normoxic conditions, the Von Hippel-Lindau tumor suppressor protein (VHL) targets HIF-1α for rapid ubiqutination. Under hypoxic condition, the HIF-1 heterodimer binds the promoter of genes that mediate angiogenesis such as VEGF. NF-κB is normally under strict regulation by its sequestration in the cytoplasm in assotiation with the inhibitor of κB (IκB). Activation of MAPK pathway activates NF-κB that rapidly translocates into the nucleus. NF-κB, in turn, activates genes that mediate angiogenesis such as CXCL-8.

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