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Pak Signaling in the Development and Progression of Cancer

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Abstract

p21-activated kinases (Paks) are positioned at the nexus of several oncogenic signaling pathways. Overexpression or mutational activation of Pak isoforms is frequently seen in various human tumors, and recent data suggests that excessive Pak activity drives many cellular processes that are the hallmarks of cancer. In this review, we discuss the mechanisms of Pak activation in cancer, the key substrates for this family of kinases that mediate their developmental and oncogenic effects, and how small molecule inhibitors of these enzymes might best be developed and deployed in the treatment of cancer.

Several protein kinases have been identified as drivers of the growth, survival, and spread of human cancers. Many oncogenic kinases have been successfully targeted by drugs, but resistance is common and there is a need for additional targets and agents. p21-activated kinases (Paks) are serine/threonine-specific intracellular protein kinases that are positioned at the intersection of a number of signaling pathways required for oncogenesis. When activated by mutation, overexpression, or by upstream elements such as Rac or Cdc42, most Pak isoforms have oncogenic signaling effects in cells, including the acquisition of growth signal autonomy, evasion of apoptosis, and promotion of invasion and metastasis (Fig. 1). For these reasons, it is important to understand the mechanisms of Pak activation in cancer, the key substrates for these kinases that mediate their developmental and oncogenic effects, and their potential value as drug targets for the treatment of cancer.

By sequence and structure, the six mammalian Paks can be categorized into two subgroups: group I (PAK1-3) and group II (PAK 4-6). These two subgroups have both overlapping and distinct functions, and are regulated by different autoinhibitory mechanisms that can be exploited in the design of specific small-molecule inhibitors (Box 1). Gene knockout mouse models vividly demonstrate the distinct roles of Pak family members in normal tissue development, with phenotypes ranging from no apparent effect to early embryonic death (Table 1). The development of such models has also underscored the unique place of each Pak family member in cancer pathophysiology. In addition, these models allow a better understanding of signaling deregulation in Pak-active tumor cells, which may lead to new opportunities for targeted anticancer therapy.

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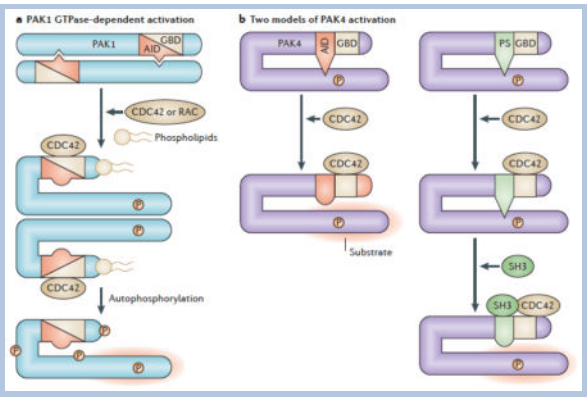
Box 1

Mechanisms of Pak activation

All Paks possess a conserved C-terminal serine/threonine kinase domain with a single phosphorylation site and an N-terminal regulatory domain. The regulatory domain of group I Paks (PAK1-3) is structurally distinct from that of group II Paks (PAK4-6), consistent with the different mechanisms regulating activity of these proteins.

The three Group I Paks are thought to be regulated via a *trans* auto-inhibition mechanism[113]. The N-terminal p21-GTPase binding domain (GBD) overlaps with an autoinhibitory domain (AID) (see the figure). PAK folds into an inactive homodimer, wherein the AID domain binds to the kinase domain of its partner. Binding of active Rho GTPases such as CDC42 and RAC1 to the GBD, and coincident binding of phosphoinositide to an adjacent segment rich in basic amino acids, leads to dissociation of the AID from the kinase domain, re-organization of the dimer, and subsequent autophosphorylation[114–116]. When the phosphorylated kinase domain binds to a substrate, it adopts a monomeric conformation[114]. Subsequent autophosphorylation at multiple sites stabilizes this catalytically active state. Additional mechanisms, including transphorylation by other kinases, and the binding of phospholipids and SRC-homology domain 3 (SH3)-domain-containing proteins such as the adaptor proteins NCK and GRB2, and the exchange factor PIX, can also modify group I Pak activity and function[116–125].

The mechanism(s) of activation of group II Paks is less clear. Unlike group I Paks the kinase domain of the group II Paks is constitutively phosphorylated[126]. Hence, transition to the active form likely depends on conformational changes. Until recently, it was believed that group II Paks, with the possible exception of PAK5[127], lacked an AID and that interactions with CDC42 served mainly to determine subcellular localization[128, 129]. However, a recent study proposes the presence of an AID in the N-terminus of PAK4 that inactivates the kinase domain in *cis*, until binding of GTP-CDC42[126]. An alternative model proposes that PAK4 is inhibited by interaction of the kinase domain to a newly defined pseudosubstrate sequence (PS) within the PAK4 regulatory domain. In this model, the binding of SH3 domain-containing proteins to the PS releases the catalytic domain, thereby promoting kinase activity[130]. It is thought that PAK5 and PAK6 also follow this model, but this has not been experimentally verified.



Upregulation of Pak in cancer

Pak function is increased in many human cancers and is in general positively correlated with advanced grade and decreased survival[1, 2]. The mechanisms underlying increased Pak activity most often entail gene amplification of *PAK1* on chromosome 11q13 or *PAK4* on chromosome 19q13 (Box 2), though in some circumstances Pak mRNA and/or protein may be overexpressed in the absence of gene amplification. In addition, Paks can be hyperactivated by mutations in upstream regulators such as Rac or its exchange factors. For all but the last of these mechanisms, it is assumed that overexpression of wild-type Pak will effectively increase its activity due to increased enzyme concentrations. Recently, activating point mutations in the *PAK4* and *PAK5* gene (the latter, unfortunately, referred to as *PAK7* in genomic databases) have been described in association with colon and lung cancers, but these are not yet validated as drivers of tumor formation[3–6].

Box 2

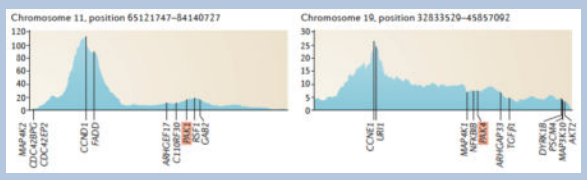
PAK gene amplification

Amplification of *PAK* genes represents the best-described mechanism for increased Pak function in cancer. For example, amplification of chromosomal region 11q13, containing *PAK1*, has been reported in a variety of human cancers, including a large percentage of breast and ovarian cancers[7, 131, 132] (see the figure). In breast cancer, amplification of 11q13 is associated with poor prognosis, and there is much interest in identifying driver genes within this region[132]. *PAK1* amplification is also prevalent in melanoma lacking *BRAF* mutations[96]. This finding, along with reports of activating mutations in the group I Pak activator RAC1 in melanoma[133, 134], suggest that certain *BRAF*-wild-type forms of melanoma might be also driven by PAK1 activation.

The 11q13 amplicon comprises multiple subclusters of amplified genes, many of which have been implicated in breast cancer, including *CCND1* (Cyclin D1)[135–137]. As PAK1 signaling augments Cyclin D1 expression, perhaps via its transcriptional activator, β-catenin[26, 102] and/or Erk, it is possible that co-amplification of *PAK1* and *CCND1* has a cooperative effect. It is also intriguing that several other genes within the amplified cluster encode proteins that activate Erk or act in the DNA repair pathway, indicating potential oncogenic interactions with Pak1.

The *PAK4* gene also lies within a chromosomal region (19q13.2) that is commonly amplified in human malignancies, in particular pancreatic cancer, oral squamous cell carcinoma, basal-like breast cancer, and serous and endometrioid ovarian cancer[138–141]. Amplification of *PAK4* is associated with aggressive disease and poor prognosis[138]. Cells overexpressing PAK4 display sensitivity to PAK4 knockdown by siRNA[140, 142, 143], implying that an oncogene-addicted state exists in such cells. Interestingly, the peak of the 19q13 amplicon includes the *CCNE1* (Cyclin E1) gene, a genomic arrangement that is physically, and perhaps functionally, analogous to the proposed relationship between *CCND1* and *PAK1* on chromosome 11. In addition, like *PAK1*, transgenic over-expression of *PAK4* is sufficient to drive mammary tumorigenesis in 3D cultures and in xenografts[144], consistent with the idea that overexpression of the wild-type allele alone can be sufficient for transformation in the appropriate cellular setting.

Amplification peaks correspond to q-values from the GISTIC analysis presented on www.tumorscape.org. The plots were generated from q values using Microsoft Office Excel.



Interestingly, using an unbiased search for protein kinases that can transform immortalized human mammary epithelial cells, it has been reported that Pak1 exerts a powerful effect on the acquisition of anchorage-independence and other hallmark properties of transformed cells[7]. In this study, the authors showed that overexpression of Pak1 (as occurs in most 11q13 amplified breast cancers) simultaneously augmented activation of Erk and Met (the receptor for hepatocyte growth factor (HGF)) signaling; Met is activated via inhibition of the tumor suppressor protein Merlin (Fig. 2). Importantly, disruption of Erk or Met signaling inhibited PAK1-driven anchorage-independent growth. Also, this and other studies of 11q13-amplified cells are consistent with the idea of PAK1 “addiction”, as such cells exhibited marked sensitivity to PAK1 (but not PAK2) siRNA[7, 8]. Given that most Paks have, in addition to catalytic activity, important scaffolding functions, it will be important to determine if *PAK1*-amplified cells also show enhanced sensitivity to anti-Pak small molecule inhibitors. If so, the presence of *PAK1* amplification might serve as a useful patient selection criterion for designing clinical trials of anti-Pak1 drugs.

Regulation at the transcriptional level has not been described in detail for any of the *PAK* isoforms. *PAK1* mRNA has been reported as a target of miR-7 and also let-7, a miRNA that is thought to play a role as a tumor suppressor in several human malignancies[910]. The expression of *PAK2* and *PAK4* has also been shown to be regulated by miRNAs[11–13]. Finally, *PAK3* transcription was recently reported to be regulated by AP-1[14]. Given reports that expression of certain *PAK* genes is increased by oncogenic signals[15], we can expect that future work will uncover additional regulators of *PAK* transcription or translation that are relevant to cancer.

Promoting growth signal autonomy

In most cell types, Pak isoforms, with the possible exception of PAK2[16–18], promote cell cycle progression when overexpressed, and hinder such progression when removed or inhibited[15, 19, 20]. These observations, coupled with the fact that *PAK* genes are frequently amplified (*PAK1* and *PAK4*) or mutated (*PAK5*) in human cancers, are consistent with a role for these enzymes in promoting oncogenesis by stimulating cell proliferation in the absence of growth signals. The mechanisms underpinning this aspect of Pak signaling are understood in some detail, as Paks have been shown to activate components of the Erk, Akt, and Wnt signaling pathways, all of which are closely tied to cell cycle progression (Fig. 2). In the Erk pathway, various Pak isoforms have been shown to phosphorylate c-RAF at S338 and MEK1 at S298. While it has been proposed that these phosphorylation events are required for efficient Erk activation and subsequent expression of Cyclin D1, a key driver of cell cycle (G1) progression, there are a number of puzzling aspects to this model that remain unresolved. For example, it has recently been reported that over-expression of kinase-dead forms of PAK1 can activate Erk in the absence of phosphorylation of c-RAF on S338 (or the equivalent phosphorylation of b-RAF on S445), perhaps by serving as a scaffold to facilitate Raf/Mek interaction[21]. Also, MEK1 S298 phosphorylation has been shown to be dispensable for ERK activation in some circumstances[22]. Some of these issues may relate to overexpression artifacts, but the weight of data suggests that, in addition to its kinase activity, Pak scaffolding functions contribute to proliferative signal transduction. Such kinase-independent mechanisms have also been invoked to explain the positive effects of Pak on Akt activity and cell survival. In this case, formation of a PAK1/PDPK1 (3-Phosphoinositide-Dependent Protein Kinase 1) complex is thought to promote recruitment of Akt to the plasma membrane and subsequent Akt activation[23].

In a K-ras-driven transgenic mouse model of skin cancer, it has been reported that *Pak1* deletion delayed both cancer initiation and progression, blocked G1 progression, and nearly extinguished activation of Erk and Akt by K-ras (Table 1)[24]. Treatment of these mice with either of two distinct, reasonably specific, small molecule inhibitors of Pak (see below) recapitulated these phenomena, establishing PAK1 as a potential drug target in K-ras driven cancers. Treatment with Erk or Akt pathway selective small molecule inhibitors showed that the major anti-tumor effect in this model was related to loss of the Erk rather than the Akt arm of the K-ras signaling pathway. These data show that, in this genetically engineered mouse cancer model, PAK1 regulates the activation of both Erk and Akt by K-ras, but it is the Erk effects that are more critical to tumorigenesis. Whether these conclusions regarding mechanism will apply in general to K-ras transformation is unclear, as it has been recently reported that depletion of PAK1 or PAK4 in K-ras or b-RAF mutant colon cancer cells resulted in decreased proliferation, but by a mechanism independent of the Erk pathway[25].

Recently, a number of groups have uncovered interactions of Pak with the Wnt/β-catenin pathway[26–28]. PAK1 associates with and phosphorylates β-catenin on at least two sites, S663 and S675, and these phosphorylation events stabilize β-catenin and promote its relocalization to the nucleus and subsequent transcriptional activity, including upregulation of MYC and Cyclin D1 (Fig. 2)[26]. Similar effects have been reported for PAK4 and PAK5[29], but this phenomenon is likely not universal among all the members of the Pak

family, as PAK2 depletion has been shown to have no effect on β-catenin expression levels or phosphorylation in breast epithelial cells[26]. Unlike the aforementioned K-ras skin cancer model, in *ERBB2*- (also known as *HER2*) transformed breast epithelial cells, the role of Pak1 in stabilizing β-catenin appears to be more important than its effects on either Erk or Akt activation, as loss of either PAK1 or PAK2 alone diminishes the activity of these latter two signaling proteins, but only PAK1 loss leads to destabilization of β-catenin and to growth arrest. These data show that different cell types, and/or different oncogenic drivers, are likely to deploy Pak signaling in unique and not-yet predictable patterns, emphasizing the need to assess a variety of tumor models when evaluating the therapeutic potential of Pak isoforms or the Pak family as a whole as drug targets in cancer.

Interestingly, in *Drosophila melanogaster*, the group II Pak mushroom bodies tiny (MBT) (which is most similar to vertebrate PAK4) has been shown to phosphorylate the β-catenin ortholog Armadillo at two sites, one equivalent to mammalian β-catenin S675, destabilizing its interactions with Drosophila E-cadherin and thereby causing decreased cell-cell adhesion[30]. In mammalian cells, it is unclear whether Pak-induced loss of β-catenin from adhesion sites, as opposed to increased transcription of β-catenin target genes in the nucleus, mediates any of the effects of Pak on transformation.

Other cell cycle-related targets of Pak include nuclear hormone receptors, Aurora kinase A (AURKA), and Polo-like kinase 1 (PLK1) [31–34]. PAK1 phosphorylates the estrogen receptor (ER) at S305, promoting its activation and subsequent signaling through Cyclin D1[33]. This event is linked to tamoxifen resistance in ER-positive breast tumors insensitive to hormone-based therapies, suggesting that PAK1 inhibition might be beneficial in the treatment of tamoxifen-resistant breast cancer[19, 35]. On a similar note, PAK6 has been shown to modulate the activity of the androgen receptor[34]. The phosphorylation of PLK1 and AURKA by PAK1 regulates cell-cycle proliferation by affecting cytokinesis and mitotic entry[31, 32].

PAK4 augments G1/S transition by down-regulating the transcription of the cyclin dependent kinase inhibitor, $p21^{Waf1}$ [36], though the details underlying this phenomenon have not been described. In Xenopus oocyte extracts, PAK4 has also been shown to also regulate G2/M transition by phosphorylating the small GTPase RAN, an event that impedes its binding to the guanine nucleotide exchange factor (GEF) RCC1, interfering with nucleotide exchange and the ability of RAN to facilitate the assembly of microtubule asters during mitosis[37, 38]. These mechanisms may explain the mitotic arrest observed in PAK4 depleted cells.

Promoting Cell Survival

Several members of the Pak family have been shown to inhibit apoptosis. Some of these effects are mediated by phosphorylation of BAD, which renders this protein unable to bind BCL2 and participate in apoptotic signaling[39]. The phosphorylation of BAD is regulated by Pak in at least two ways. First, c-RAF is a known substrate of PAK1, PAK2, and PAK5[40, 41]. Pak-mediated phosphorylation of c-RAF at S338 results in its translocation to the mitochondria and subsequent binding to and phosphorylation of BAD[41]. Paks have also

been shown to phosphorylate BAD directly[39]. Reports that PAK1 regulates apoptosis by phosphorylating Dynein Light Chain-1 (DLC1, also known as DYNLL1) at S88 are difficult to reconcile with the observations that the purported phosphorylation site on DLC1 lies within a poor Pak consensus motif, native (non-glutathione-S-transferase (GST) fused) DLC1 is not modified by PAK1 *in vitro*, and phosphorylation of DLC1 on S88 is not represented in mass spectroscopy phosphoproteome databases[42]. Nevertheless, PAK1 clearly binds DLC1[42, 43] and, in this complex, appears to hinder the ability of BIML to bind BCL2, thus impeding apoptotic signaling[43].

It has been also shown that Pak activates the nuclear factor κB (NFκB) pathway. Activation of NFκB signaling by PAK1 has been reported to increase resistance to apoptosis in mammary epithelial cells[44], and to be required for transformation by Kaposi's sarcoma-associated herpes virus[45]. However, the mechanism(s) underlying NFκB pathway activation is still unclear, as a convincing direct PAK1 target in this pathway has yet to be identified. What is known is that, in endothelial cells, PAK1 somehow modulates the ability of reactive oxygen species to activate NFκB in response to disturbances in blood flow[46]. This phenomenon is likely relevant to several aspects of tumorigenesis, including cell survival, angiogenesis, and inflammation.

Based mainly on *in vitro* overexpression studies PAK2 has been reported to have both anti apoptotic and pro apoptotic functions. Inhibition of apoptosis occurs through mechanisms similar to those described for PAK1, but it has been also proposed that PAK2 can phosphorylate Caspase-7 at S30, T173 and S239, decreasing the pro-apoptotic activity of Caspase-7[47]. On the other hand, late in apoptosis PAK2 itself becomes cleaved by Caspase-3 or Caspase-3-like proteases, liberating the kinase domain from the regulatory domain[48]. The resulting proteolytic fragment, PAK2-p34, containing the protein kinase domain, induces nuclear blebbing and reduced protein synthesis, the latter mediated by phosphorylation of MAPK signal-integrating kinase 1 (MNK1)[49]. Interestingly, conditional activation of PAK2 in Hs578T human breast carcinoma cells suppresses activation of caspase-3, generation of PAK2-p34, and apoptosis in response to the anticancer drug cisplatin[50]. These data suggest a feedback process in which PAK2 promotes survival in part by suppressing its own cleavage to a pro-apoptotic fragment.

Much less is known about the mechanisms by which group II Paks augment cell survival. However, there are hints that at least some of these mechanisms differ from those used by group I Paks. For example, PAK4 has been shown to inhibit apoptosis by inhibiting an early apoptotic molecule, Caspase-8, through a kinase-independent mechanism[51].

Activating invasion and metastasis

Tumor cell migration and invasion are key factors in metastatic distribution to distant organs. The initial stages of these processes involve extensive remodeling of the cytoskeleton, disruption of cell adhesions, and release of proteases that digest the extracellular matrix. Paks play an important role in regulating these events, mediated by a number of cytoskeletal effector proteins, including GEFs, GTPase activating proteins

(GAPs), and guanine-nucleotide dissociation inhibitors (GDIs) that control Rho family GTPases, and proteins that act more directly on actin (Figure 1).

As part of a protein complex containing the Rac GEF β-PIX (also known as ARHGEF7), the Arf GAP GIT1, and the adaptor protein Paxillin, PAK1 induces rapid turnover of focal contacts at the leading edge of cells, promoting increased cell motility[525354]. The molecular events underlying this process involve the phosphorylation of paxillin by PAK1, an event that augments the association of Paxillin and GIT1 and targets the GIT1-PIX-PAK signaling module to the leading edge. In line with this model, expression of dominant negative PAK1 in invasive breast carcinoma cell lines is associated with decreased invasion and migration[55], and these cells display stable focal adhesions, increased stress fibers, and enhanced cell attachment.

LIM Kinase (LIMK) represents another important target for Pak in actin remodeling. PAK1 phosphorylates and activates LIMK, which subsequently phosphorylates Cofilin at serine 3, an event that inhibits the ability of Cofilin to sever and depolymerize actin filaments[56]. Such regulation of Cofilin by the PAK1/LIMK pathway is required for RAC1-induced actin reorganization at the cell's leading edge[56]. This activity may be specific to particular Pak isoforms, as it has been reported that, in breast carcinoma cells, PAK1, but not PAK2, mediates the formation of Heregulin-stimulation of lamellipodial protrusions, maturation of focal adhesions, Cofilin phosphorylation, and loss of RHOA activity[57]. A similar dichotomy regarding Pak isoforms and cytoskeletal activity has been observed in mast cells, in which PAK1 and PAK2 appear to play opposing roles with respect to actin organization and degranulation[58, 59].

Tumor cell invasion also requires the reorganization of the extracellular matrix to provide space for cell movement. Destruction of the extracellular matrix is, in part, controlled by the release of matrix metalloproteinases (MMPs). Genetic experiments suggest that Pak mediates certain aspects of extracellular matrix organization downstream of CDC42, as matrix remodeling could not be restored to *Cdc42*$^{-/-}$ MEFs by reintroducing mutants of CDC42 that lacked Pak binding[60]. PAK1, 2, 4, and 5 have been shown to regulate MMP expression in a variety of cancer cell types[61, 626364]. Increased expression of MMPs has been suggested to result from Pak-mediated activation of JUN N-terminal kinase (JNK)[65, 66]. PAK4 has also been reported to interact with MMP-2, and knockdown of PAK4 in glioma cell lines is associated with down-regulation of MMP-2, decreased migration, and loss of invasiveness[64].

Finally, recent work has demonstrated that PAK1 knockdown in prostate cancer cells was associated with reduced motility, reduced MMP9 secretion, and increased expression of TGFβ, which in these cases, is growth inhibitory[67, 68]. Interestingly, in these cells, PAK1 appeared to be the major Pak isoform required for invasiveness, despite the prominent expression of group II PAK4 and PAK6[34, 67, 69].

Pak and Angiogenesis

Pak involvement in endothelial cell biology and angiogenesis has been under scrutiny due to the well-established role of these enzymes in cell proliferation, cytoskeleton rearrangement,

and migration[20, 70, 71]. In mouse models, molecules that signal both upstream and downstream Pak have been shown to be critical for vasculogenesis and angiogenesis[72–74]. For example, endothelial knock out of *Rac1* or *Cdc42* is lethal during mouse development, associated with impaired formation of blood vessels[72, 74]. Furthermore, adult primary endothelial cells that lack *Rac1* or *Cdc42*, show impaired proliferation, attachment, migration and angiogenesis[72, 74]. Whether these effects are mediated through any of the Paks is not known; however, both *Pak2* and *Pak4* knock out mice are embryonic lethal due to multiple organogenesis defects, including severe cardiovascular abnormalities[75, 76]. Recent studies from our group show that endothelial-specific deletion of *Pak2* is associated with embryonic death at E9.5, with grossly impaired blood vessel formation in both the embryo body and the yolk sac (Radu and Chernoff, unpublished observations). In contrast, there is no notable vascular phenotype in *Pak1*-null mice[75]. It should be noted that PAK2 is the main isoform expressed in endothelial cells. For this reason, even though PAK1 and PAK2 may serve different, and perhaps even opposing cellular functions[26, 58], the weight of current evidence favors the view that PAK2 is the more relevant mediator of angiogenic signaling downstream of Rho family GTPases.

Proliferation, survival, migration, and tube formation

In breast cancer cells, it has been shown that PAK1 is required for vascular endothelial growth factor (VEGF) expression downstream of an activator of ERBB signaling, Heregulin, thus promoting angiogenesis[77]. Data from another group demonstrated that the ability of PAK1 to phosphorylate myosin light chain (MLC) is critical for endothelial cell cytoskeletal dynamics that mediate migration[78]. Furthermore, various scaffolding proteins, including NCK and Filamin B, have been shown to form protein complexes that are essential in PAK1- and PAK4-mediated endothelial migration[79, 80]. PAK1 and PAK4 have been found to modulate c-RAF and BAD phosphorylation levels and inhibit apoptosis in endothelial cells[13, 81]. With respect to blood vessel lumen formation, PAK2 and -4 are required for this process *in vitro*, acting in a pathway that involves Rho GTPases, Src, protein kinase C ε (PKCε), and c-RAF[82, 83] (Fig. 3). It has also been suggested that PAK4 plays a role in angiogenesis through phosphorylation of the integrin αvβ5, which affects endothelial cell motility and permeability[84].

Vascular permeability

There is little doubt that Pak (probably PAK2) affects endothelial barrier function[85]; it is whether it promotes or reduces permeability, or both, that is at issue. In a hypoxia-induced hypertension model, activation of Rac/Pak signaling has been shown to protect against hypoxia-induced increase in vascular permeability[86]. In line with these findings, inactivating mutations in zebrafish *Pak2a* (a gene that encodes a protein that is highly homologous to human PAK2) or its binding partner *β-Pix* are associated with brain hemorrhage due to immature vasculature and improper endothelial-mesenchymal contacts[87]. On the other hand, it has also been shown that group I Pak signaling leads to an increase in vascular permeability by modulating cell contraction. It was proposed that a Pak-PIX-GIT1 complex induces phosphoryation of MLC, resulting in a contracted cell with permeable cell junctions[88, 89]. Supporting a positive role for Pak in promoting endothelial permeability, other studies have shown that PAK1 can phosphorylate vascular endothelial (VE)-cadherin.

Upon phosphorylation by PAK1, VE-cadherin dissociates from β-arrestin-2 and translocates from cell:cell junctions to the cytoplasm, weakening endothelial cell:cell contacts and increasing vascular permeability (Fig. 3)[90, 91].

The issue as to whether Paks help or hinder endothelial barrier function could have important clinical implications, as the effects of small molecule Pak inhibitors might be expected to resemble those seen in gene disruption studies. It is possible that Pak serves both functions depending on context and isoform, issues that should be resolved as more endothelial specific *Pak* knock out mice become available for study.

Anti-Pak Therapeutics

Although several broad-range kinase inhibitors demonstrate potent Pak inhibition[92, 93], such non-selective compounds have limited utility. As Paks are increasingly recognized as plausible targets for cancer therapeutics, the search for both pan-Pak inhibitors and group-specific Pak inhibitors has intensified. This task, however, has proven particularly challenging for the Paks due to the large size and high flexibility of the catalytic pocket as well as gaps in our understanding of Pak regulation.

A potent, ATP-competitive pyrrolopyrazole Pak inhibitor, PF-3758309, though originally designed as a PAK4 inhibitor, efficiently targets both group I and II Paks, as well as a number of other, off-target kinases (Fig. 4)[94]. PF-3758309 inhibits growth of many types of tumor cell lines and has also demonstrated potent anti-cancer properties in xenografts and in a K-ras-driven, transgenic mouse model of skin cancer[24, 94–96]. Although the signaling effects of this compound *in vivo* resemble those seen in *Pak1* knockout mice,[24] it remains difficult to ascribe these desirable biological effects to Pak inhibition alone. This will need to be shown using more Pak-specific analogs of PF-3758309, or experiments showing that drug-resistant Pak alleles can overcome PF-3758309-mediated tumor growth inhibition Despite these issues, the suitable potency (IC_{50} ~4.7 nM) of PF-3758309 combined with its oral availability led to its advancement to phase I clinical trials. However, PF-3758309 was withdrawn from clinical use due to undesirable pharmacologic properties, most prominently excessive drug efflux[97].

A group I specific ATP-competitive Pak inhibitor, FRAX-597, was recently shown to reduce the initiation and progression of K-ras-driven tumors in a mouse model of skin cancer, as well as reduce the growth of Merlin-deficient schwannoma xenografts[24, 98]. This compound, however, has substantial off-target activity against receptor tyrosine kinases (Fig. 4). Surprisingly, treatment with FRAX-597 has been shown to result in reduction of total PAK1 and PAK2 levels, and this effect is abolished in cells treated with the proteasome inhibitor MG132 (Chow and Chernoff, unpublished observations), suggesting that FRAX597 acts not only as an ATP-competitive inhibitor but also as a Pak destabilizing agent. Such a combination of inhibitory mechanisms - competition with ATP and destabilization of the kinase - are particularly attractive features of this compound and might be exploited in more specific future analogs.

In an attempt to exploit the capacious ATP binding pocket present in all the Paks, a metallo-pyridocarbazole scaffold has been used to position a rigid, bulky, ruthenium complex within

the ribose binding site[99]. The resulting compound, termed FL172, efficiently fills the large catalytic pocket, thus gaining high PAK1 inhibitory efficacy (IC_{50} ~1 μM) as well as reasonably high selectivity over other related protein kinases. Among 264 kinases tested, only 15 showed an inhibition similar to that of PAK1 (Fig. 4). However, compounds such as this based on organometal conjugates usually suffer from poor solubility and relatively high toxicity and it is therefore unclear whether this strategy will yield clinically useful inhibitors.

Attempts to develop allosoteric Pak inhibitors have also been described[100]. For example, IPA-3 (inhibitor p21-activated kinase-3), a sulfhydryl-containing compound that targets the N-terminal regulatory domain of group I Paks, was isolated in a deliberate attempt to uncover non-competitive PAK1 inhibitors[100]. Reversible covalent binding of IPA-3 to the PAK1 regulatory domain prevents GTPase docking and the subsequent switch to a catalytically active state[101]. This unique mechanism of action likely accounts for the exceptional target specificity of IPA-3, a property that makes it useful as a tool compound for *in vitro* research and as a proof of concept. However, the pharmacokinetic properties of the compound as well as undesirable redox effects in cells, due the continuous reduction of the sulfhydryl moiety, makes IPA-3 unsuitable for further clinical development.

Apart from the small molecule drugs, Pak allosteric peptide inhibitors have been widely used as laboratory tools. Although the isolated PAK1autoinhibitory domain (AID) (Box 1) efficiently regulates PAK1 function, the need to deliver the peptide into cells makes the approach challenging for therapeutic use. Moreover, induction of cell cycle arrest by the PAK1 AID can occur independent of inhibiting PAK1 kinase activity[102], most likely due to AID binding to the fragile-X proteins FMR1 and FRX1, which modulate the stability of the cell-cycle inhibitor $p12^{waf1}$ [103, 104]. However, the AID derived from PAK2 lacks FMR1/FXR1 binding and presumably exerts its biological effects purely through Pak inhibition. Two other peptide inhibitors, comprising the cell permeant TAT peptide fused to the PIX-interacting motif (TAT-Pak18) or the Nck binding motif of Pak1, have also been described. These peptides are thought to prevent proper cellular localization (and activation) of PAK1 through disruption of PAK1-NCK or PAK1-PIX interactions. The Pak-mediated growth suppression effect of TAT-Pak18 has been shown on Pak1-dependent ovarian cancer cell lines[105], while a Pak-Nck inhibitory peptide affects endothelial cell migration and contractility[83, 106].

Conclusions and Future Directions

Paks occupy a central position in oncogenic signaling, driving several processes that are the hallmarks of cancer initiation, growth, and spread. In proliferative signaling, Pak activity is required for efficient activation of ERK, Akt, and β-catenin in many tissues. These effects may render cells particularly sensitive to specific small molecule inhibitors of Pak.

With respect to deciphering the role of Paks in cancer, we have a reasonable signaling framework in hand, but certain basic questions remain. The foremost of these are the identities of the most relevant substrates, and whether these are unique to individual members of the Pak family. What is needed are more comprehensive and unbiased approaches for substrate identification. Efforts in this direction have already begun,

employing diverse technologies such as protein microarray screens [107, 108], substrate capture[109] and phosphoproteome signatures[110], but additional important substrates undoubtedly remain to be discovered. It will also be important to more clearly distinguish scaffolding from catalytic functions, as only the latter are expected to be blocked by conventional small molecule inhibitors.

What sorts of cancers might benefit from Pak inhibitors? We suggest three scenarios. First, given the apparent "addiction" of Pak-amplified cells to Pak activity, we suggest that tumors bearing 11q13 or 19q13 amplifications (Box 2), which are commonly found in breast, ovarian, and pancreatic cancer, should be particularly susceptible to small molecule inhibitors of group I and group II Paks, respectively. In addition, the genomic organization of these amplicons suggests that such tumors might also display synergistic responses to combined inhibition of Pak and signaling proteins that drive cell cycle progression, as genes encoding Cyclins are frequently co-amplified with Paks. Second, because oncogenic signals from ERBB2, K-ras, and Merlin have been shown to depend on Pak1 function in mouse models, tumors driven by mutations in the genes encoding these proteins might also be good candidates for anti-Pak therapeutics. Finally, the stabilization of β-catenin by PAK1 suggests that tumors that depend on overactive Wnt signaling, such as most colon cancers, might also respond well to Pak inhibitors. As with other anti-signaling agents, it is likely that Pak inhibitors will prove most useful in combination with other targeted drugs, as has been suggested in xenograft models[8, 26].

Due to the structural characteristics of their catalytic domains, the Paks, in particular the Group I Paks, are challenging targets with respect to the development of specific competitive inhibitors, but recent progress using a variety of chemical scaffolds suggests that this challenge can be met. In addition, the unusual activation mechanisms for both Pak subgroups (Box 1) provide opportunities for the further development of allosteric inhibitors. Whether competitive or non-competitive, such inhibitors will need to be used with caution, as mouse models indicate that certain Pak functions, in particular, maintenance of normal vascular permeability and hematopoietic stem cell function, may be required even in adult animals (Table 1)[111, 112]. While it is important to acknowledge these provisos, the central position of Paks in key oncogenic signaling and their potential tractability as drug targets make these enzymes worthy of increased study by the community of cancer cell biologists and by the pharmaceutical industry.

Glossary Terms

Cyclin D1	A cyclin that, in partnership with cyclin-dependent kinases, is a key protein in progression through the G1 phase of the cell cycle. The gene encoding this protein (*CCND1*) is frequently coamplified with the *PAK1* gene in human cancers
Kaposi's sarcoma-associated herpes virus	Human herpesvirus that causes Kaposi's sarcoma

Nuclear blebbing	vesicular outpocketing of the nuclear membrane that is a hallmark of apoptosis
Guanine-nucleotide exchange factors (GEFs)	Proteins that promote the exchange of GDP for GTP on a GTPase, thus facilitating its activation
GTPase activating proteins (GAPs)	Accelerate the hydrolysis of GTP to GDP, leading to an increase in the proportion of GDP-bound GTPase molecules and a consequent reduction in their activity
Guanine-nucleotide dissociation inhibitors (GDIs)	enzymes that sequester GDP-bound small GTPases in the cytoplasm
drug efflux	the ability to actively pump out certain small molecule inhibitors from cells
TAT peptide	cell penetrating peptide derived from the HIV Tat protein, which, when fused to a peptide of interest, imparts the ability of the fusion peptide to penetrate through cell membranes into cells
protein microarray screens	recombinant proteins arrayed on a surface such as a glass slide, that can be assessed for phosphorylation following incubation with a protein kinase and ATP

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Key Points

- There are two subgroups of p21-activated kinases (Paks) comprising three members each (group I (PAK1-3) and group II (PAK 4-6)). New genetic models of Pak in mice and fish have illustrated the unique functions of the six Pak isoforms.
- PAK expression and activity, in particular PAK1 and PAK4, are often upregulated in human tumors. Tumor cells with upregulated PAK tend to become dependent on Pak signaling.
- In many cell types, Paks positively regulate at least three key proliferative signaling pathways: Erk, Akt, and Wnt.
- In addition to their roles in proliferation, Paks also play important roles in promoting cell survival, invasion and metastasis, and angiogenesis.
- Several potent and specific small molecule inhibitors of all Paks or of group I or II Paks are in advanced stages of preclinical development. Such agents will need to be used with caution, however, as Pak function may be required for maintaining vascular integrity.
- Anti-Pak drugs may be useful in cancers bearing amplified *PAK* alleles, as well as in cancers that depend on Pak for activation of downstream signaling pathways, such as *HER2*-amplified breast cancer and colon cancers driven by mutations in the Wnt pathway.

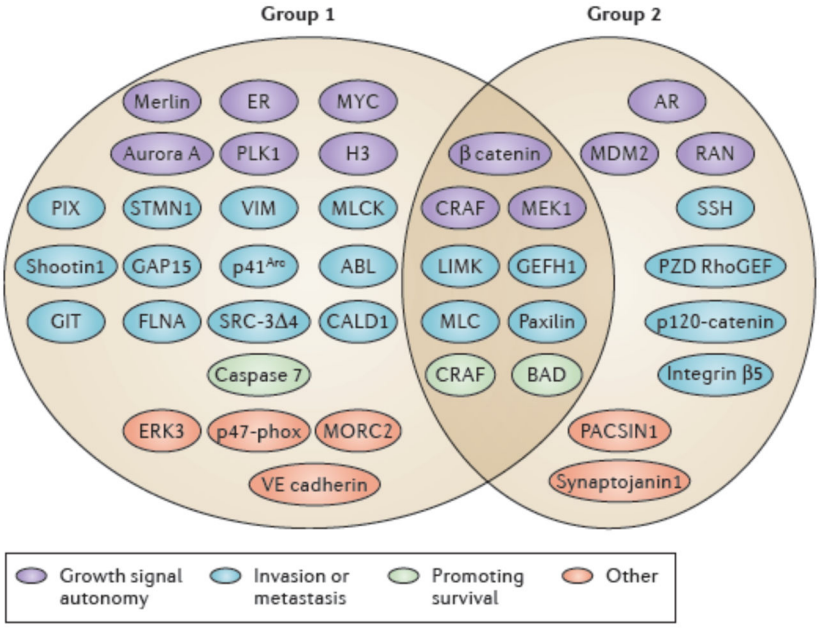

Figure 1. Validated Pak substrates and their roles in the hallmarks of cancer

Substrates of group I and II Paks are listed according to their putative role in oncogenic signaling. In some cases (*e.g.*, c-Raf), given substrates play roles in multiple cellular functions, but may be listed only once to avoid visual clutter. Pak substrates are included only if reported by more than one group or if the reported site of phosphorylation is represented in the PhosphoSitePlus database (http://www.phosphosite.org/homeAction.do). Abbreviations: CALD1, caldesmin 1; ER, estrogen receptor; FlnA, Filamin A; H3, histone 3; LIMK, LIM kinase; MLC, myosin light chain; MLCK, myosin light chain kinase; Plk1, polo-kinase-1; SSH, Slingshot; Stmn1, stathmin-1; Vim, Vimentin.

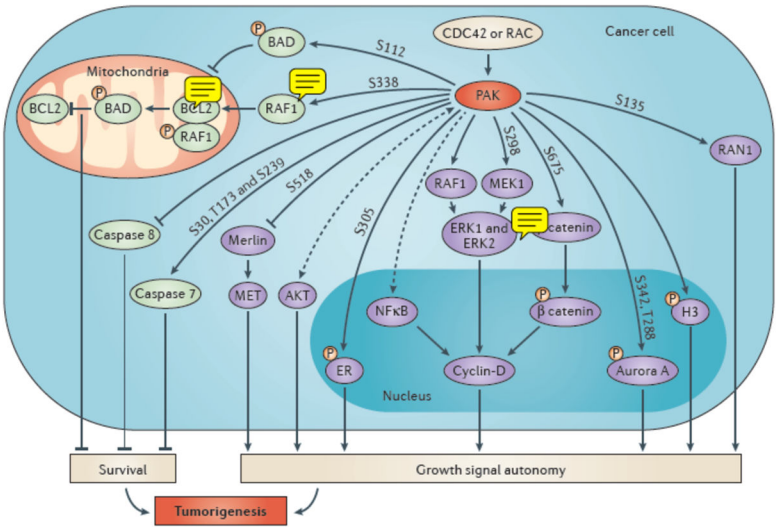

Figure 2. Role of Pak in growth signal autonomy and cell survival

Group I Paks are activated by both Cdc42 and Rac, whereas Group II Paks are activated only by Cdc42. Selected Pak substrates are depicted according to their role in oncogenic signaling. Phosphorylation sites, where known, are listed for each substrate. Dashed lines indicate that Pak has been implicated in activation of the substrate, but that the mechanism is uncertain.

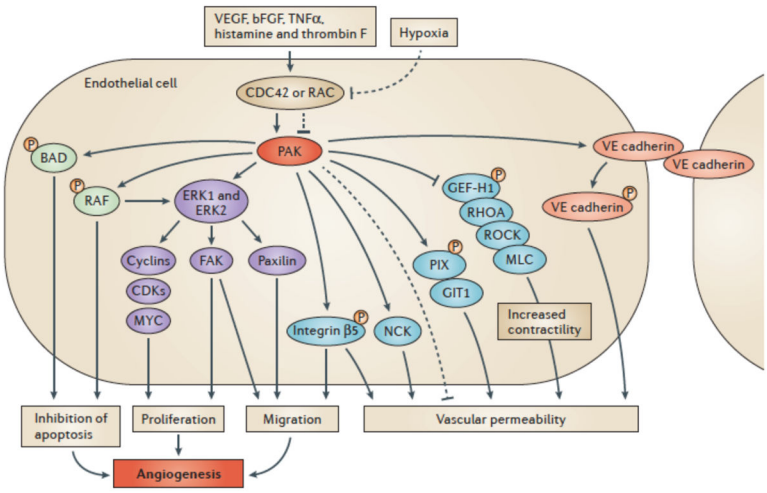

Figure 3. Pak signaling in angiogenesis and modulation of vascular permeability

Paks control critical cellular events required for angiogenesis, including endothelial cell proliferation, survival, attachment and migration. In endothelial cells, the phosphorylation of BAD and RAF1 by Pak protects against apoptotic stimuli by promoting RAF1 translocation to mitochondria and the displacement of BAD/BCL2 complexes. As seen in other cellular contexts, in endothelial cells, the Erk pathway regulates cellular proliferation and migration when initiated by activation of Rac/Pak pathway. The control of vascular permeability by Pak is mediated by modulation of cellular contractility and cell:cell adhesion molecules. In one model, direct phosphorylation of myosin light chain (MLC) by Pak leads to increased contractility and increased endothelial permeability, as has been seen in certain experimental settings. Pak has also been proposed to disrupt endothelial cell:cell junctions by direct phosphorylation and subsequent internalization and degradation of VE cadherin. In another model, activated Pak phosphorylates and inhibits GEF-H1 (also known as ARHGEF2), leading to diminished RHOA/RHO-associated coiled-coil containing protein kinase (ROCK)/MLC activity, decreased contractility and decreased endothelial permeability, This model is consistent with data showing that Pak protects against an increase in permeability in a hypoxia induced pulmonary hypertension model and in a *Pak2a* knockout zebrafish model (dotted lines).

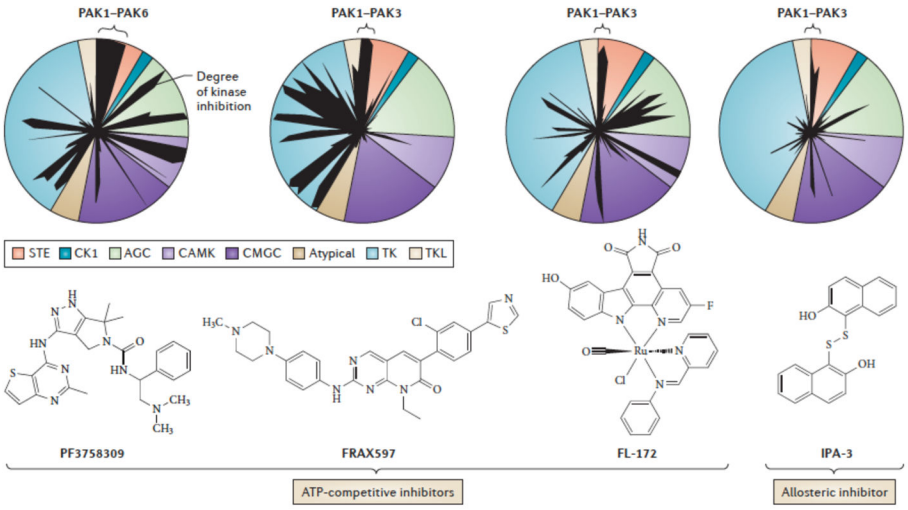

Figure 4. Specificity of Pak inhibitors

The human kinome is represented on a radar plot. The Pak family is oriented to 12 o'clock, emphasized by a red marking. The length of gray areas emanating from the bulls-eye represents the degree of kinase inhibition by each inhibitor shown below. Kinase families are indicated by different colors, as shown in the key to the right of the diagram: TK – Tyrosine kinase; TKL – Tyrosine kinase-like; STE – Homologs of yeast Sterile 20 kinases; CK1 – Casein kinase 1; AGC –Containing PKA, PKG, PKC families; CAMK – Calcium/calmodulin-dependent protein kinase; CMGC –Containing CDK, MAPK, GSK3, CLK families; ATYPICAL – Atypical protein kinase. Primary specificity data are derived from References 24, 94, 99, and 100.

Table 1

Genetic models of Pak function

Group	Genotype	Features	Phenotype	*Signaling Effects*	*Reference*
I	***Pak1***$^{-/-}$		Mast cell and macrophage defects, mild glucose homeostasis defects	↓pMEK	59, 145
	αMHC-cre; Pak1$^{fl/fl}$	Cardiac-specific KO	Cardiac hypertrophy[1]	↓pJNK	146
	MMTV-ErbB2; Pak1$^{-/-}$	ErbB2 breast cancer model	Decreased tumor progression and prolonged survival	↓pERK, ↓pAKT, ↓pβ-catenin	26
	K5-tet-on; tet-K-rasG12D; Pak1$^{-/-}$	Inducible K-ras skin cancer model	Decreased tumor initiation and progression; prolonged survival	↓pERK, ↓pAKT	24
	Nf1$^{+/-}$*; Pak1*$^{-/-}$		Reduction of mast cell dermal accumulation	↓pERK, ↓pp38,	147
	Pak2$^{-/-}$		Lethal E8.5	ND	75
	MSCV-cre; Pak2$^{fl/fl}$	*ex-vivo Pak2* deletion[2]	Mast cell hyperresponsiveness to IgE stimulation	↓pGEF-H1 ↓Rho-GTP	58
	MSCV-cre; Pak2$^{fl/fl}$	*ex-vivo Pak2* deletion[2]	Failure of bone marrow engraftment	↓pERK, ↓pAKT	112
	Pak3$^{-/-}$		Learning and memory defects	ND	148
	Pak1$^{-/-}$*;Pak3*$^{-/-}$		Learning and memory defects, hyperactivity	↓pCofilin	149
	Camk2a-PID[3]	Group I Pak inhibition in forebrain	Impaired memory consolidation	ND	150
	Camk2a-PID[3]*; FMR1*$^{-/-}$	Group I Pak inhibition in forebrain	Rescue of fragile X syndrome phenotypes	ND	151
II	***Pak4***$^{-/-}$		Lethal E10.5. Heart and neural tube defects		76
	Pak5$^{-/-}$		Viable healthy		152
	Pak6$^{-/-}$		Viable healthy		153
	Pak5$^{-/-}$*;Pak6*$^{-/-}$		Impaired learning and locomotion		153

[1] Under conditions of pressure overload

[2] *Ex vivo* deletion in cultured bone marrow cells

[3] Transgenic expression of Pak inhibitor domain (PID), inhibiting all group I Paks

αMHC, (also known as *MYH6*), myosin, heavy chain 6, cardiac muscle, alpha; Camk2a, (also known as *α-CamKII*), calcium/calmodulin-dependent protein kinase II alpha; FMR1, fragile X mental retardation; 1; JNK, JUN N-terminal kinase; KO, knockout, MMTV, mouse mammary tumor virus; MSCV, murine stem cell virus; ND, not determined; NF1, neurofibromin 1; PAK, p21-activated kinase