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# POTENTIAL ANTITUMOR TARGET: ROLE OF PAK-4 SIGNALING PATHWAY IN CANCER

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## SUMMARY

*Cancer metastasis is a multistage process involving invasion into surrounding tissue, intravasation, transit in the blood or lymph, extravasation and growth at a new site. Many of these steps require cell motility, which is driven by cycles of actin polymerization, cell adhesion and actomyosin contraction. The p21-activated kinases (serine/threonine-protein kinase PAK) have functions as downstream effectors of Ras-related C3 botulinum toxin substrate (Rac) in the regulation of the actin cytoskeleton and stimulate cell motility and invasion. PAK-4 is an important regulator of growth factor signaling, cytoskeletal reorganization and growth factor-mediated cell migration. Based on its role in cell transformation, PAK-4 is acknowledged as a viable antitumor target. In this review, we focus on the present research on the role of PAK-4 in tumorigenesis and metastasis.*

**Key words:** Serine/threonine-protein kinase PAK-4 – Cancer – Metastasis – Cell transformation – Tumorigenesis

## INTRODUCTION

Tumor invasion and metastasis consist of a series of sequential steps resulting in the formation of metastatic foci distant from the primary tumor (1). The process of cancer metastasis requires, among other steps, changes in cytoskeletal signaling pathways, increased directional motility and enhanced cell survival (2). During malignant transformation, the signaling pathways controlling these cytoskeletal dynamics are altered, and an increase in cell motility allows cancer cells to invade surrounding tissues and metastasize (3). Cell adhesions can be highly dynamic and cell migration depends on the continuous formation and disassembly of adhesions at both the front and rear of a migrating cell (4, 5). It is well established that the Rho family of GTPases, including RhoA, Rac and CDC42, orchestrate cell migration and adhesion turnover (4, 6), and that p21-activated kinases (PAKs) function as downstream effectors of Rac in the regulation of the actin cytoskeleton and stimulate cell motility and invasion (3). PAKs are major regulators of growth factor signaling, cytoskeletal reorganization and growth factor-mediated cell migration, and are overexpressed in many cancers, with effects on migration potential, anchorage-independent growth and metastasis (7).

Six PAK isoforms are expressed in humans: group I (PAK-1, PAK-2 and PAK-3) and group II (PAK-4, PAK-5 and PAK-6) (7). Among the group II PAK isoforms, PAK-4 was first identified as an effector of CDC42 essential for regulating cytoskeleton reorganization (8). PAK-4 is ubiquitously expressed, with highest levels in the prostate, testis and colon (9). Although PAK-4 is expressed at low levels in most adult tissues, it is highly overexpressed in tumor cell lines, including prostate (5), colon (10), esophageal and mammary tumors (10). This suggests an important role for PAK-4 in cell growth, survival and proliferation, all of which are important for tumorigenesis. Therefore, its role in cell transformation makes it an attractive therapeutic target.

Meanwhile, a drug discovery effort focused on this unique drug target could yield potentially unique and useful therapeutics. Several compounds have been found to exert antitumor effects via down-regulation of the enzyme (11, 12). In this review, we summarize the signaling pathway of PAK-4 regulation and review compounds that may be used as chemopreventive agents via modulation of the PAK-4 signaling pathway.

## THE STRUCTURE OF PAK-4

All six mammalian PAK isoforms share a highly conserved *C*-terminal kinase domain and an *N*-terminal CDC42/Rac-interactive binding (CRIB) domain (13), also known as the GTPase-binding domain (P21-binding domain, GBD, PBD) (3, 14). Unlike the group I PAKs, PAK-4 lacks an autoinhibitory domain (15, 16) (Fig. 1). However, PAK-4 still interacts with GTPases; these interactions target the kinase to certain cellular locations, but they have no influence on enzymatic activity (9).

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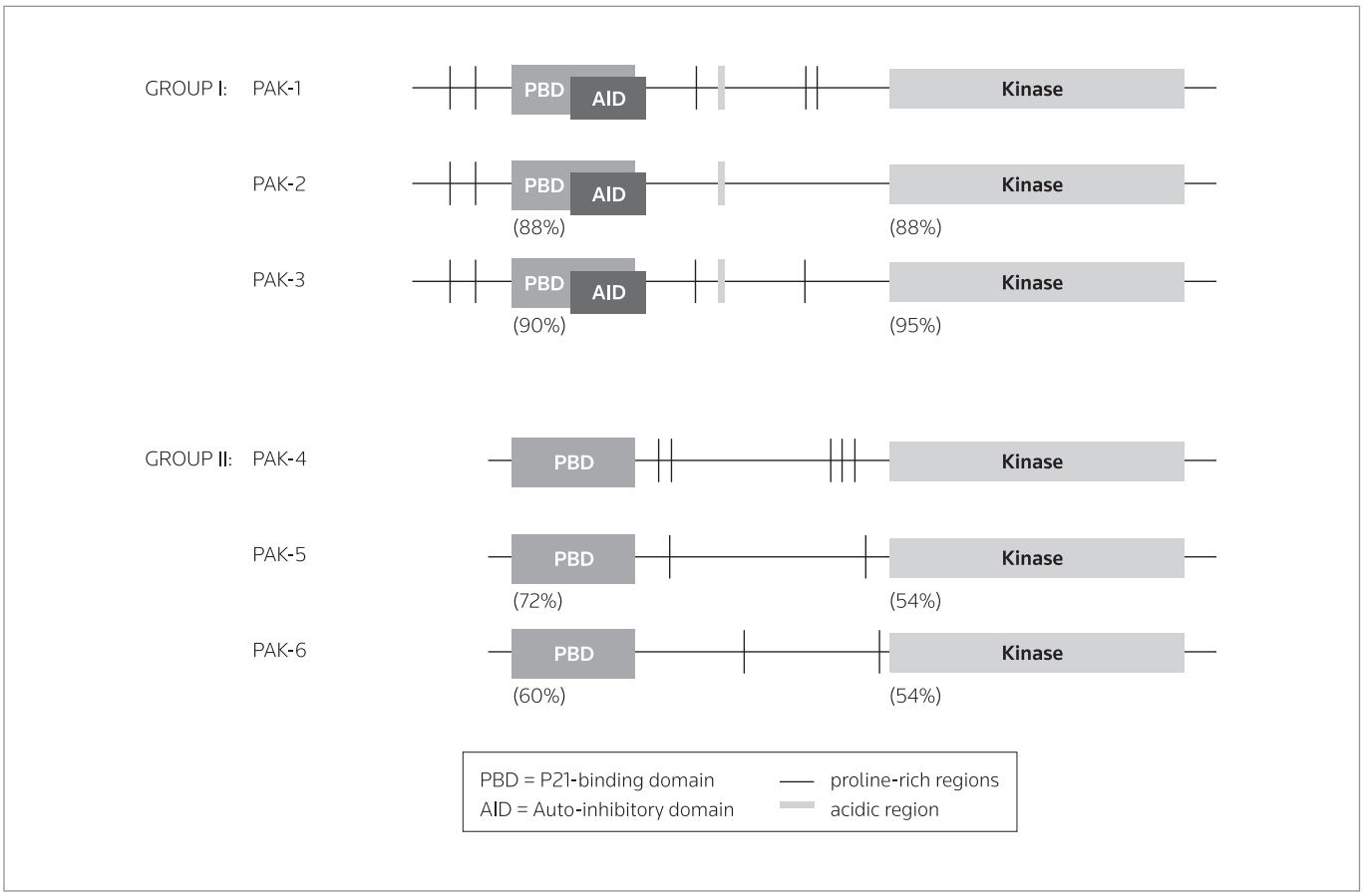


**Figure 1.** Structural comparison of the group I and II PAKs. Reproduced with permission from Jaffer, Z.M., Chernoff, J. *Potential antitumor target: Role of PAK4 signaling pathway in cancer.* Int J Biochem Cell Biol 2002, 34(7): 713-7.

Most serine/threonine-protein kinases have a conserved Asn, which stabilizes the catalytic loop by hydrogen bonding with a conserved Asp (17). The group II PAKs have a Ser at this site in place of the conserved Asn (S445 in PAK-4). The substitution of this Ser with Asn results in constitutive activation of PAK-4 (18). The group I PAKs contain an SH3 binding site *N*-terminal to the GBD domain, which is responsible for binding to the NCK adaptor protein (19). In contrast, PAK-4 has no SH3 binding sites *N*-terminal to the GBD and does not bind NCK or alpha-Pix, but has seven putative SH3 binding sites between the GBD and kinase domains (20). The enzyme is monophosphorylated at the activation loop positions corresponding to Ser474 in PAK-4 (11).

## THE FUNCTIONS OF PAK-4

The PAKs play an essential role in cell signaling and control a variety of cellular functions, including cell motility, survival, angiogenesis and mitosis. To date, several proteins have been identified as substrates for PAK-4, reflecting the significant roles played by PAK-4 in a range of biological activities. The most prominent functions are discussed below, including stimulation of cytoskeletal regulation (21), cell survival (22) and cell motility (23, 24). Deregulation of these cellular processes promotes carcinogenesis and PAK-4 signaling can thus play a mechanistic role in cell transformation. Of note, PAK-4 has also been implicated in other cellular processes that are relevant in tumorigenesis, including angiogenesis (7, 25) and anchorage-independent growth (26-28).

### Cytoskeletal regulation

Reorganization of the actin cytoskeleton is a fundamental component of several cellular processes (29), including determination of cell polarity, shape and motility, all of which are controlled by spatial and temporal polymerization and depolymerization of actin (2). PAK-4 regulates actin polymerization and filopodia formation by direct interaction with active CDC42 (30). Active PAK-4 is required for Ras-driven transformation to facilitate anchorage-independent growth (18, 28), and affects profound cell morphological and cytoskeletal effects (18, 31).

The cytoskeletal function of group II PAKs is therefore primarily an outcome of their direct interaction with Rho GTPases (32); however, PAKs also interact directly with Rho guanine nucleotide exchange factors (RhoGEFs) (33), such as the Rho family guanine nucleotide

exchange factor H1 (GEF-H1), proto-oncogene DBL (18), PDZ-RhoGEF (33, 34) and alpha-Pix (35).

### Metastasis

Cancer cell metastasis involves a series of changes in cell behavior and the Rho family GTPases Rho, Rac and CDC42 orchestrate many of the required processes (36). PAK-4 directly interacts with an integrin intracellular domain and regulates carcinoma cell motility in an integrin-specific manner (2). PAK-4 closely correlated with metastasis is expressed mainly in the cytoplasm of cancer cells, occasionally in the cell nuclei, and virtually not expressed in the matrix surrounding the cells (37). Meanwhile, PAK-4 significantly increases cell migration and invasion of SK-OV-3 cells, an ovarian cancer cell line stably transfected with PAK-4. There is a link between PAK-4 and proto-oncogene c-Src, MEK-1/ERK-1/2 and MMP-2 in the regulation of cell migration and invasion, which is kinase-dependent (8).

### Tumorigenesis

Normal cell development requires precisely regulated levels of cell survival, apoptosis, proliferation and differentiation (38). Increased levels of cell survival, uncontrolled proliferation or failure to differentiate are often associated with tumorigenesis. It is known that the *PAK4* gene is mutated in a number of tumor cell lines, including breast tumors (39), and primary tumors, including colon, esophageal and ovarian tumors (8, 10). There is increasing evidence that *PAK4* is overexpressed in human tumors and cancer cell lines, possibly due to gene amplification (40). The *PAK4* gene is frequently overexpressed without the presence of gene-activating mutations in many tumor types (28). It is suggested that this gene is an important target during carcinogenesis. When mutated/activated, *PAK4* can promote anchorage-independent growth. It has been demonstrated that the PAK-4 protein can be active in pancreatic cancer cells without the need for mutation (41).

Through its interaction with CDC42, PAK-4 is involved in altering the cytoskeleton by promoting the formation of filopodia (42). It is proposed that activation of PAK-4 is critical to neoplastic pancreatic cells as they transition to invasiveness (41). In addition, the kinase activity of PAK-4 is increased in some breast cancer samples with genomic amplification (37, 40). PAK-4 plays a role in integrin-mediated breast cancer cell motility (43). PAK-4 promotes tumorigenesis in animals, as well as in cultured cells. Overexpression of *PAK4* leads to tumor formation in athymic mice (10). The development and maintenance of polarized epithelial cells is critical for the normal function of epithelial tissue (32), and loss of epithelial polarity is frequently associated with carcinogenesis. Overexpression of *PAK4* is associated with disruption of cell polarization, especially apical polarization. The disruption of apicobasal polarity is thought to lead to uncontrolled cell proliferation and survival, resulting in profound effects on glandular structure (40). It is proposed that inhibition of apoptosis and subsequent increased cell survival and cell growth play a key role in PAK-4-induced tumorigenesis.

### Apoptosis

Oncogenic transformation can occur in response to the cytological changes mentioned above. These changes are often associated with improper regulation of intracellular signaling pathways that control cell growth and survival. Further investigation of the role of group II PAKs in cell survival pathways shows that PAKs mediate survival through interaction with Bcl family members (25). The anti-apoptotic Bcl family members Bcl-2 and Bcl-XL, located in the mitochondrial outer membrane, control the release of cytochrome c from mitochondria (44). PAK-4 phosphorylates BAD at Ser112 and inhibits the BAD interaction with Bcl-XL and Bcl-2, which results in prevention of cytochrome c release, promoting cell survival (25, 45). Meanwhile, it was found that there is a reduced level of apoptosis in PAK-4-induced tumors compared with control tissues, as assessed by examining caspase-3 levels (10). Moreover, PAK-4 activation provides resistance to apoptosis in response to TNF-$\alpha$ treatment. Importantly, PAK-4 has been shown to promote cell survival by inhibiting proapoptotic signals, as well as promoting TNF-$\alpha$-stimulated survival pathways (27). It was also found that stable overexpression of *PAK4* in immortalized mouse mammary epithelial cells (iMMECs) led to the formation of a mostly non-hollow acinar structures with a lower level of apoptosis and a thicker layer of cells surrounding the lumen (38).

## THE PAK-4 SIGNALING PATHWAY

As mentioned above, PAK-4 plays a role in a range of biological activities which are activated by hepatocyte growth factor (HGF) (46, 47). HGF is associated with tumor progression and increases the invasiveness of tumor cells (5, 46). Migration and invasion require coordinated reorganization of the actin cytoskeleton and regulation of cell adhesion dynamics. Proto-oncogene c-Met is the tyrosine kinase receptor for HGF that undergoes tyrosine autophosphorylation and triggers the activation of several pathways controlling epithelial–mesenchymal morphogenesis, angiogenesis and cell–cell adhesion (48). The HGF/c-Met signaling pathway is generally associated with the promotion of cellular growth, as well as with tumor progression (49).

HGF-stimulated migration requires reorganization of the actin cytoskeleton; these activities are mediated by Rho family GTPases (46). The GTPases Rac and CDC42 control diverse cellular functions. In addition to being mediators of intracellular signaling cascades, they have important roles in cell morphogenesis and mitogenesis (50). These include roles in cell proliferation, progression through the cell cycle and oncogenic transformation. A great deal of effort has been made to identify the downstream molecular targets for Rac and CDC42. PAK-4 was originally identified as a protein that promotes filopodia formation in response to activated CDC42, and is an important link between CDC42 and filopodia formation. PAK-4 interacts tightly with the GTP-bound form of CDC42 and subsequently induces actin polymerization at the Golgi apparatus. The interaction between CDC42 and PAK-4 is essential for targeting PAK-4 to the Golgi compartment and subsequently for reorganization of the actin cytoskeleton and filopodia formation. In contrast to group I PAKs, the kinase activity of PAK-4 is not regulated by CDC42. Rather, PAK-4 appears to have constitutive kinase activity in the absence or presence of CDC42. It appears to be recruitment of PAK-4 by CDC42 rather than stimulation of its kinase activity that is important for actin polymerization and cytoskeletal changes. PAK-4 interacts specifically with the GTP-bound form of CDC42 via the GTPase-binding domain (GBD) motif (30). Moreover, PAK-4 is

required for efficient HGF-dependent cell scattering and migration (46, 47). Activated PAK-4 induces a decrease in stress fibers and in HGF-stimulated cells a loss of focal complexes and cell rounding (5, 47), a response dependent on PAK-4 kinase activity, which does not require CDC42 interaction. PAK-4 kinase activity is also stimulated by HGF, and the activated PAK-4 induces a loss of cell adhesion in an HGF-dependent manner. Furthermore, both activity and subcellular localization of PAK-4 during HGF signaling are regulated by phosphoinositide 3-kinase (PI3K) (47). A number of PAK-4-interacting proteins have been identified, including LIM domain kinase 1 (LIMK-1), GEF-H1, Slingshot (SSH) and GRB2-associated binder 1 (GAB1) (Fig. 2).

LIMK-1 activity is associated with cancer cell motility, and LIMK-1 operates by phosphorylating and inactivating another cytoskeletal regulatory protein, cofilin, thereby inhibiting cofilin's actin depolymerization activity (51). Cells overexpressing LIMK-1 can show reduced migration (52, 53). Conversely, LIMK-1 is essential for directed protrusion towards HGF (54). It has also been reported that PAK-4 can enhance the activity of exogenous LIMK-1 towards its target cofilin in vitro (51). SSH is a phosphatase that downregulates the activity of LIMK-1 towards cofilin. PAK-4 is a pivotal player in this pathway by acting as a dual regulator that activates LIMK-1 and inactivates SSH (55).

GAB1 and PAK-4 have been reported to associate and co-localize at the cell periphery within lamellipodia in response to HGF, and enhance epithelial cell dispersal, migration and invasion. The GAB1–PAK-4 interaction is essential for HGF-induced cell migration, invasion and tubulogenesis (56). The PAK-4 domain required for binding to GAB1 was previously identified to bind to GEF-H1, a RhoGEF involved in cell motility, polarization, dendritic spine morphology, cell cycle regulation and cancer. It has been shown that PAK-4 influences cytoskeletal changes by phosphorylating GEF-H1. The Rho-dependent activity of GEF-H1 is also attenuated by PAK-4-dependent phosphorylation on Ser810 (GEF-H1), which leads to the formation of actin-rich lamellipodia in NIH/3T3 cells (12). It was demonstrated that GEF-H1 serine phosphorylation correlates with HGF stimulation in cells and that PAK-4 can negatively regulate RhoA exchange activity via GEF-H1 (5). PAK-4 is reported to phosphorylate GEF-H1 at Ser885 in vitro (31), and phosphorylation of this residue by Aurora inhibits RhoA exchange activity during mitosis (57).

There are also several other proteins upstream from PAK-4, including integrin alpha v beta 5. Integrin alpha v beta 5 mediates cell adhesion to the extracellular matrix (ECM) and triggers intracellular signaling pathways that regulate cell spreading and migration. Integrin alpha v beta 5 binding to vitronectin leads to PAK-4 activation, and the activated PAK-4 functions to limit integrin-mediated vitronectin adhesion, thereby reducing total cell–ECM adhesion and

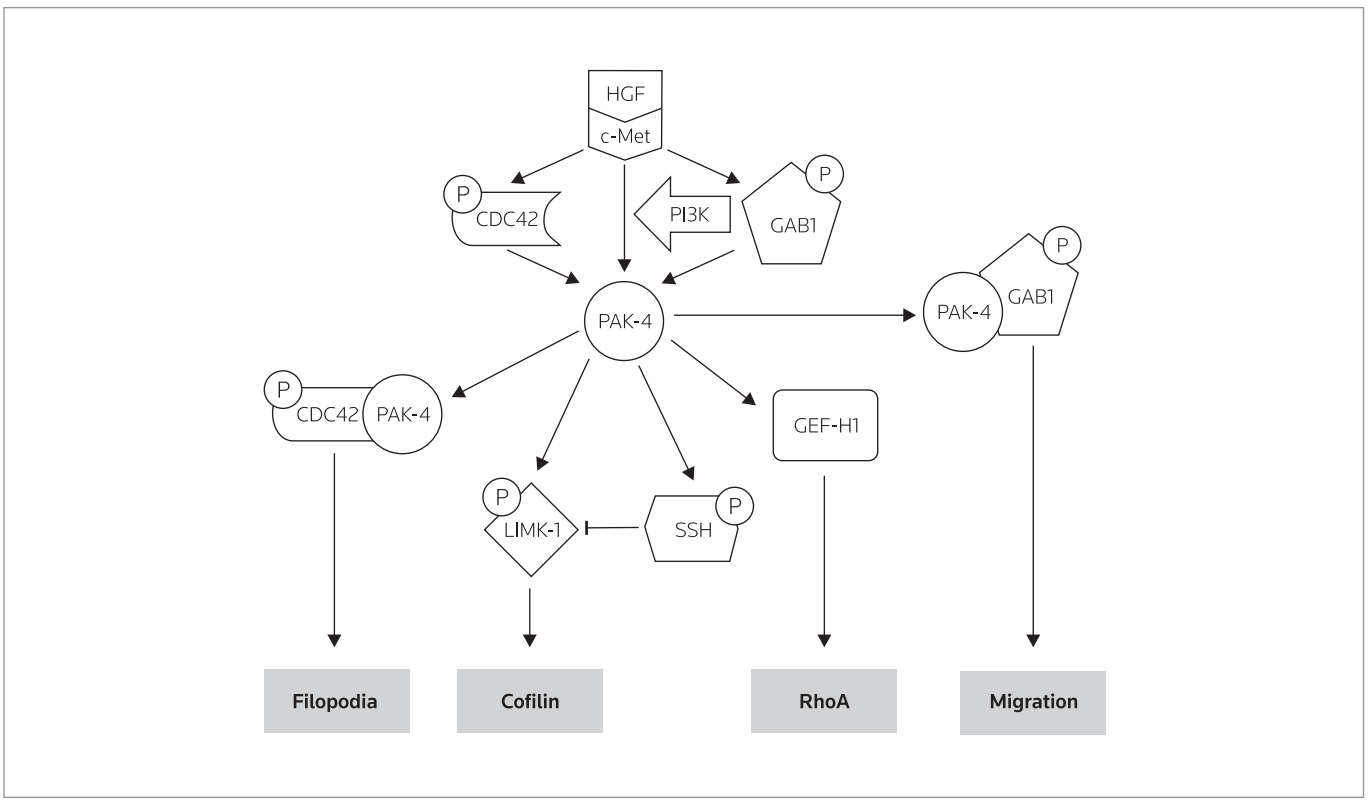


**Figure 3.** Regulation of actin dynamics by PAK-4. Activating interactions are indicated by an arrow and inhibitory interactions are depicted using blocked lines. Details of this pathway are described in the text.

facilitating enhanced cell migration. This pathway represents a novel autoinhibitory negative feedback loop that is initiated within the core machinery of cell adhesion (43).

## INHIBITORS OF PAK-4

Taken together, it appears that a drug discovery effort focused on this target could yield a potentially unique and useful therapeutic. Several inhibitor scaffolds were identified by using a fluorescence-based temperature-shift screening assay. The most potent was the nonspecific kinase inhibitor staurosporine, and the related compound K-252a also inhibited group II PAKs (11). In preclinical models the potent, ATP-competitive, pyrrolopyrazole PAK-4 inhibitor PF-3758309 demonstrated antitumor activity mediated through several mechanisms, i.e., cytoskeletal, apoptosis and cell cycle signaling (12). LCH-7749944, a novel and potent PAK-4 inhibitor, suppressed the proliferation of human gastric cancer cells by downregulating the PAK-4/c-Src/EGFR/cyclin D1 pathway, as well as inhibiting the migration and invasion of human gastric cancer cells via blockade of the PAK-4/LIMK-1/cofilin and PAK-4/MEK-1/ERK-1/2/MMP-2 pathways (58).

Moreover, several endogenous inhibitors have been identified. MicroRNA miR-199a/b-3p is one of the most highly expressed miRNAs in human liver and hepatocellular carcinoma, and it was found that PAK-4 protein expression could be inhibited by miR-199a/b-3p, mainly through translational inhibition (59).

However, most details regarding PAK-4 biology, including the nature of pathways controlling tumor cell growth and survival, are largely unknown, and only a few potent inhibitors of PAK family kinases have been reported. Based on its role in cell transformation, inhibition of PAK-4 is acknowledged as a viable antitumor strategy.

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