PAK4–6 in cancer and neuronal development

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PAKs 4, 5 and 6 are members of the group B family of p21-activated kinases. Among this group, PAK4 has been most extensively studied. While it has essential roles in embryonic development, in adults high levels of PAK4 are frequently associated with cancer. PAK4 is overexpressed in a variety of cancers, and the *Pak4* gene is amplified in some cancers. PAK4 overexpression is sufficient to cause oncogenic transformation in cells and in mouse models. The tight connection between PAK4 and cancer make it a promising diagnostic tool as well as a potential drug target. The group B PAKs also have important developmental functions. PAK4 is important for many early developmental processes, while PAK5 and PAK6 play roles in learning and memory in mice. This chapter provides an overview of the roles of the group B PAKs in cancer as well as development, and includes a discussion of PAK mediated signaling pathways and cellular functions.

Introduction

The p21 activated kinase (PAK) family of Ser/Thr kinases were first identified as effector proteins for Cdc42 and Rac, members of the Rho GTPase family, but they can also respond to Rho GTPase independent signals. The PAKs fall into two groups (A and B), based on their sequences and functions (see **Fig. 1**).

Group A includes mammalian PAK1, PAK2 and PAK3.[1-3] Each of these kinases has an N-terminal regulatory domain and a C-terminal kinase domain. Within the regulatory domain is a GTPase binding domain (GBD), which binds to activated Rac or Cdc42. PAK1–3 also have several other conserved motifs, as illustrated in **Figure 1**. Cdc42 or Rac stimulates their kinase activities, by relieving an intramolecular interaction between the kinase domain and an autoinhibitory domain (AID). In addition to Rho GTPases, PAK1–3 need several other signal transducers for their full activation in cells, such as the SH3 adaptor proteins (PIX and NCK), Tyr-kinases (Fyn and ETK) and sphingolipids.[2,4,5] Furrthermore, PAK1–3 are negatively regulated by a few proteins such as merlin, nischarin and the kinase LKB1.

PAK4–6, like PAK1–3, also have N-terminal GBD and a C-terminal kinase domains, but they lack other conserved domains found in PAK1–3 [although PAK5 does contain an AID (auto-inhibitory domain)] (see **Fig. 1**). Furthermore, the GBD and kinase domains of PAK4–6 have only approximately 50% identity with those of PAK1–3, and the regulatory domains outside of the GBD are completely different from PAK1–3. PAK4 is the first among the group B PAKs to be cloned.[6] PAK4 binds preferentially Cdc42, but it also binds Rac. It differs from PAK1–3 in its substrate specificity, although there is also some overlap.[7,8] PAK4 expression is high throughout development, but in many adult tissues PAK4 protein levels are low. In contrast, PAK5 and PAK6 are expressed in a limited number of tissues, and are expressed at an especially high level in the adult brain.[9-12] Interestingly, PAK6, which is also expressed in testes and prostate, was shown to have an important role in androgen receptor signaling, in a pathway that is not known to be linked to Rho GTPases.[11,13,14] Thus, the group B PAKs may have both Rho GTPase dependent and Rho GTPase independent functions. This chapter will focus on the group B PAKs, their cellular functions, and their roles in oncogenesis and development.

Cellular Activities and Signal Transduction Pathways Mediated by PAK4–6

Cytoskeletal organization. PAK4 promotes filopodia formation in response to activated Cdc42,[6] and it appears to be an important link between Cdc42 and filopodia formation. PAK4 also leads to the dissolution of stress fibers and subsequent loss of focal adhesions, partly by inactivating a Rho activator called GEFH1.[8] Unlike PAK1 (T423E), the "constitutively activated" PAK5 (S573N) leads to cytoskeletal changes, which include filopodia formation and the formation of neurite-like extensions in neuroblastoma cells.[9]

Substrates. Like PAK1,[15] PAK4 phosphorylates LIM kinase 1 (LIMK1),[7] which in turn phosphorylates the actin depolymerization protein cofilin, thereby inhibiting actin depolymerization.[16,17] The phosphorylation of LIMK1 by PAK4 may help explain how PAK4 leads to polymerized actin structures such as filopodia. In addition to direct phosphorylation of LIMK1, PAK4 also activates LIMK1 indirectly, via inhibition of SlingShot phosphatase (SSH1), a LIM kinase phosphatase.[18] LIMK1 phosphorylation by PAK4 may also be regulated by another protein, DGCRL
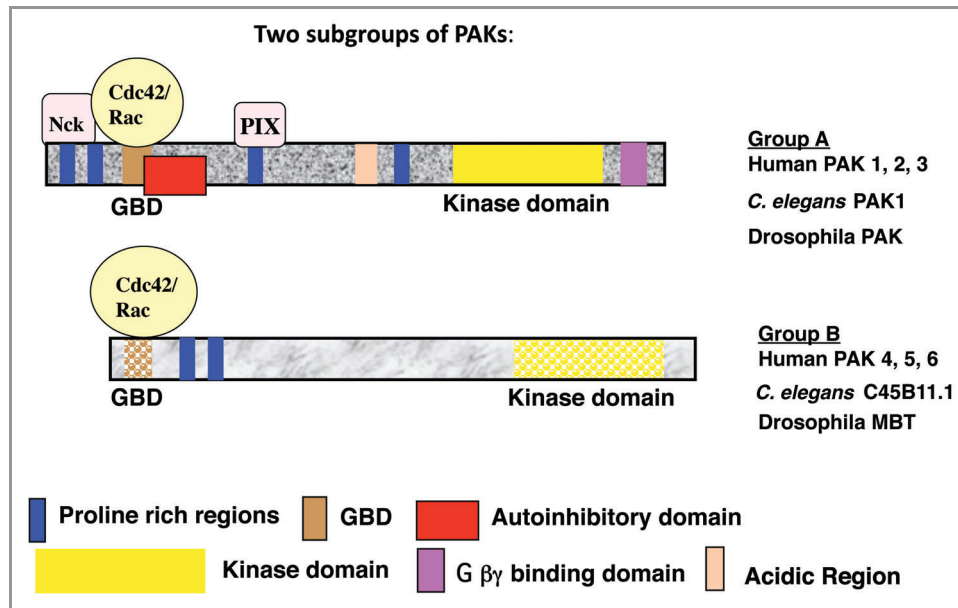
Figure 1. Structural features of the group A and group B PAK family members.

(DiGeorge critical region 6). DGCRL was identified as a PAK4 binding protein, which has links to cancer and metastasis.[19] Binding of DGCR6L to PAK4 enhances LIMK phosphorylation in response to PAK4, and this may promote the migration of gastric cells. In addition to the formation of filopodia, PAK4 and other PAK proteins lead to the dissolution of stress fibers. In the case of PAK1, this has been shown to be mediated by myosin light chain kinase (MLCK). PAK1 directly phosphorylates MLCK, and this in turn leads to stress fiber dissolution.[20] In contrast, PAK4 does not phosphorylate MLCK, but it still causes stress fiber dissolution, suggesting that it operates by a different mechanism.[8] One possible mediator in PAK4-induced stress fiber dissolution is GefH1, a guanine nucleotide exchange factor (Gef) for Rho. PAK4 specifically phosphorylates GefH1, and this is thought to inhibit its ability to activate Rho, consequently inhibiting stress fiber formation.[21]

Other group B PAK substrates are linked to cell adhesion. PAK5 interacts with p120- catenin in vitro and leads to its phosphorylation. A constitutively active PAK4 also leads to phosphorylation of p120.[22] p120 catenin has important roles in controlling cell shape and adhesion and also has a role in anchorage-independent cell growth.[23] The exact role for p120 downstream to group B PAKs, however, is still under investigation. Another substrate that may be important for cell adhesion is a member of the integrin family. Specifically, PAK4 has been shown to phosphorylate the cytoplasmic tail of β-5 integrin. Cytoplasmic tails of integrins have many functions, including key roles in adhesion and migration. Phosphorylation of β-5 integrin may have important implications in cell adhesion and migration in response to PAK4.[24]

PAK4 phosphorylates a number of substrates that are involved in the regulation of cell growth and survival. For example, PAK4, as well as PAK5 and PAK1–3,[25-28] has been shown to phosphorylate cRaf-1. In the case of PAK4, this leads to ERK MAP Kinase activation in primary cells.[29] PAK4 and PAK1–3[5,30] also phosphorylate the pro-apoptotic protein Bad, which contributes to PAK mediated cell survival.[5,30-32] Xenopus and mammalian PAK4 were shown to phosphorylate the GTPase Ran, and Ran phosphorylation increases during mitosis. PAK4-mediated phosphorylation of Ran may regulate the assembly of Ran-dependent complexes on the mitotic spindle, suggesting a role for this complex in mitosis.[33]

Mechanisms of activation of the group B PAKs. The PAKs bind to Cdc42 and Rac via their GBDs, but the contribution of these Rho GTPases to activation of the PAKs can vary. In the case of the group A PAKs, PAK kinase activity is activated in response to binding to Cdc42 and Rac. This appears to be regulated by an autoinhibitory domain (AID) located within the N-terminal regulatory domain, and overlapping the GBD. The AID may operate by interacting with the kinase domain of a dimerizing PAK. Activated Rho GTPases can bind the AID and relieve the autoinhibition, leading to PAK activation.[34] In contrast to the group A PAKs, the group B PAKs are not necessarily activated by Rho GTPases, and it is not clear whether the group A PAKs dimerize or operate via an autoinhibitory mechanism. Instead, Cdc42 or Rac may lead to changes in cellular localization of the group B PAKs, possibly bringing them into proximity to their substrates. An autoinhibitory site has been identified in PAK5, however, and in one study PAK5 has been shown to be activated by activated Cdc42.[35] Activation of the PAKs is therefore complex, and may vary among the different PAK family members.

CDC42/RAC-independent activation of PAK4–6. Although PAKs are bind to Cdc42 and Rac, a number of stimuli which could operate independently of these GTPases may also lie upstream of the PAKs. In MDCK epithelial cells, for example, PAK4 is activated by hepatocyte growth factor (HGF) via a

signaling pathway that requires PI3 kinase. HGF also causes PAK4 to localize to the cell periphery, and PAK4 is in turn thought to contribute to HGF induced changes in cytoskeletal organization and cell adhesion.[36] PAK4 was also shown to interact with the cytoplasmic domain of the keratinocyte growth factor (KGF) receptor in a transformed kidney cell line, and may have a role in KGFR mediated cell survival pathways.[37] PAK6 has been linked to androgen receptor signaling,[11,13,14] by a mechanism not known to be linked to CDC42/RAC. PAK6 was also shown to be activated by MAP kinase kinase 6 (MKK6) and by the p38 MAP Kinase.[38] These data suggest that multiple types of signaling pathways may converge upon PAK4–6 to modulate their activity or cellular localization.

PAK4 and Oncogenesis

Numerous studies point to a role for the PAK kinases in oncogenic transformation and in cellular processes associated with transformation. These include regulation of cell survival, proliferation, cytoskeletal organization and migration.[30,39-45] Among PAK4–6, PAK4 is most closely linked to cancer, and it is overexpressed in many cancer cells.[39,46-49] Some of the key studies linking PAK4–6 with oncogenesis are described below.

PAK4 promotes anchorage independent growth in cultured cells. Like activated Cdc42,[50-52] activated PAK4 promotes anchorage-independent growth in immortalized fibroblasts.[8,21] Anchorage independent growth is an important in vitro hallmark of oncogenic transformation, which can be assessed by focus formation in soft agar. Interestingly, constitutively activated PAK4 (S445N) is as efficient as oncogenic RAS mutants in this assay system.[8] Dominant negative PAK4 mutants can partially inhibit focus formation of fibroblasts induced by oncogenic Dbl which activates both Rho and CDC42.[8]

PAK4 and the cell cycle. Precise control of the cell cycle is important for normal cell function. Improper regulation of the cell cycle in contrast, can be associated with uncontrolled growth and cancer. PAK4 protein levels fluctuate throughout the cell cycle. In immortalized fibroblasts they increase early in G1 phase and then decline within several hours, remaining low during the rest of the cycle. Deletion of PAK4 significantly extends the life time of p21, a CDK (cyclin-dependent kinase) inhibitor,[22] suggesting that PAK4 is important for p21 degradation. This could have important implications in cell cycle control and cancer. In contrast, in primary cells PAK4 was shown to promote cell cycle arrest and increased levels of cell cycle inhibitory proteins.[29] The role for PAK4 in cell cycle regulation is therefore complex, and may be different in primary cells as opposed to established cell lines.

PAK4 promotes cell survival. Increased survival in the face of apoptotic stimuli is an important aspect of tumorigenesis. When overexpressed, PAK4 is associated with survival and protection from apoptosis.[30,40] Conversely, cells lacking PAK4 have an increased susceptibility toward apoptosis.[41] PAK4 promotes cell survival by different mechanisms, depending on the stimulus (see **Fig. 2**). In response to serum withdrawal, PAK4 protects cells via a

kinase dependent mechanism that is associated with its ability to phosphorylate the pro-apoptotic protein Bad.[30] In response to stimuli that activate death domain containing receptors, however, such as TNF or Fas ligand, PAK4 functions via a kinase independent mechanism.[40] In this case PAK4 inhibits recruitment of caspase-8 to the DISC complex that forms on the cytoplasmic side of the receptor. This leads to inhibition of caspase-8 activity.[40] PAK4 also has a role in activating cell survival pathways, which lead to NFκB and ERK activation.[41] Like PAK4, PAK5 also protects cells from apoptosis, and this may be due to direct phosphorylation of BAD.[53,54] PAK5 localizes to the mitochondria, and this is required for its protective function.[53] PAK5 over-expression also inhibits camptothecin-induced apoptosis in colorectal cancer cells, and this is mediated by inhibition of caspase-8.[54]

PAK4 plays roles in invasion and migration. Invasion and migration are important parts of the oncogenic process, and they are tightly linked with metastasis. The role for PAK4 in actin cytoskeletal organization suggests an important role for in migration. In fact, when a constitutively active PAK4 mutant was overexpressed in pancreatic ductal cells the result was an increase in migratory capacity and increased invasion in in vitro assays. In contrast, PAK4 siRNA reduces invasion in an in vitro assay in a pancreatic tumor cell line.[55] Overexpression of PAK4 also promotes invasion, migration and proliferation of chorio-carcinoma cells, and its inhibition has the opposite effect.[56] PAK4 was also shown to have a role in migration and adhesion of prostate cancer cells. As described above, PAK4 is regulated by HGF.[36] PAK4, along with LIMK1, functions downstream to HGF in prostate cancer cells and in turn mediates cell migration.[43] The level of auto-phosphorylated PAK4 is elevated in prostate cancer cells,[57] but when PAK4 levels are reduced using siRNA, the cells become deficient in the ability to migrate in response to HGF. The PAK4 deficient cells also have alterations in cytoskeletal organization and adhesion, and they display reduced cell-adhesion turnover rates. These data point to an important link between PAK4 and invasion and migration in several different types of cancer cells. This role may be closely linked to its role in regulating cytoskeletal organization, cell adhesion, and integrin phosphorylation.

Overexpression of PAK4 leads to tumor formation in mice. NIH3T3 cells that stably overexpress PAK4 cause tumors to form when injected into athymic mice.[58] Interestingly, while a constitutively active mutant of PAK4 causes tumors to form rapidly, even wild-type PAK4 leads to a high level of tumorigenesis. In the case of wild-type PAK4 tumors takes longer to appear, but ultimately grow to a large size.[58] Thus there appears to be a lag time before tumors begin to form in response to wild-type PAK4.[58] These results are important because there is little evidence for activating mutants of PAK4 in cancer. Rather, it may be overexpression of wild-type PAK4 that is associated with many types of cancer. It is therefore crucial to understand how wild-type PAK4 can lead to tumor formation and growth. Interestingly, PAK4 causes an increase in cell proliferation, and a corresponding decrease in apoptosis in the mouse tissue well before tumors can be detected.[58] These changes in growth
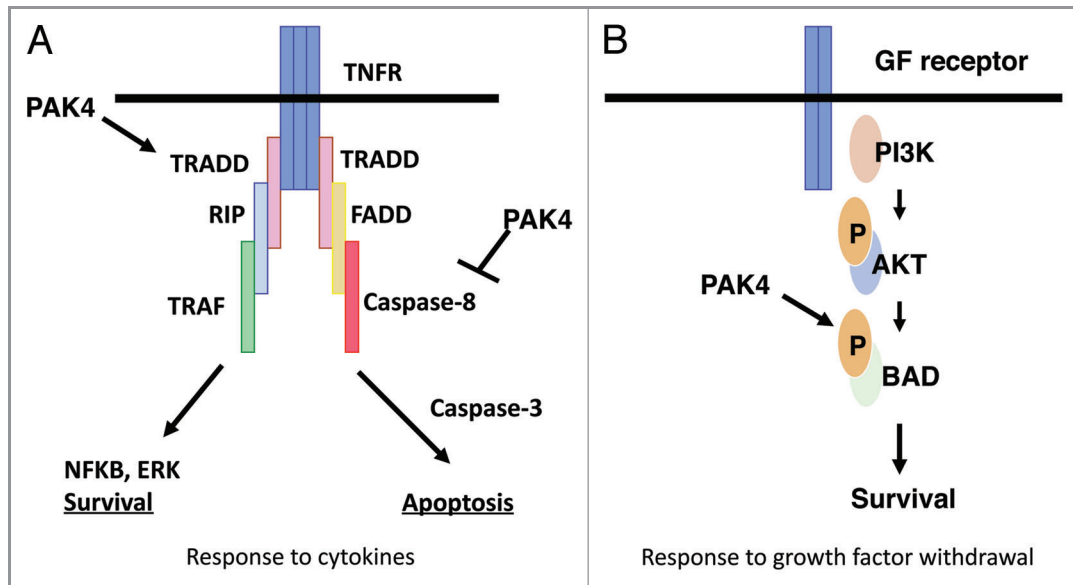
Figure 2. Different mechanisms by which PAK4 inhibits apoptosis. In response to cytokines PAK4 blocks caspase-8 binding to the DISC, thereby inhibiting apoptosis. It also stimulates NFκB and ERK pathways leading to survival. These pathways occur by kinase independent mechanisms. In response to serum withdrawal PAK4 acts by phosphorylating Bad, thereby promoting cell survival. This occurs by a kinase dependent mechanism.

characteristics could be part of what primes the cells to become tumor cells after the lag period.

The studies described above indicate that PAK4 is sufficient to form tumors in athymic mice. Another important question is whether PAK4 is also necessary for tumor formation. This seems to be the case at least in part, as the oncogenic Ha-RasV12 expressed in PAK4 knockout (PAK4$^{-/-}$) fibroblasts forms tumors significantly less efficiently than that in the wild-type control cells.[58] Tumors from PAK4$^{-/-}$ cells form more slowly and grow to a smaller size. Furthermore, PAK4$^{-/-}$ tumors that do form are especially bloody in appearance (angiogenic). One possible explanation for this is that the PAK4 null cells begin to undergo apoptosis after a certain point, due to the lack of PAK4 and its corresponding cell survival functions. This in turn would prevent the tumors from growing beyond a certain size. An even more dramatic result was obtained when cells were transfected with Cdc42V12. Although wild-type cells transfected with Cdc42V12 are highly transforming in athymic mice, virtually no tumors form when PAK4$^{-/-}$ cells transfected with Cdc42V12 are used. In the latter study the cells were conditionally PAK4 knockout by the Cre/LoxP system,[58] which may eliminate some of the compensatory effects that could be relevant in conventional knockout cells.

PAK4 is overexpressed in cancer. When PAK4 levels were tested in a panel of 60 tumor cell lines, it was found, remarkably, to be overexpressed in almost all of them.[12] By comparison, PAK4 is expressed at low levels in most normal tissues. This is in contrast to PAK6, which is highly expressed in a few adult tissues but is not overexpressed in most tumor cell lines.[12] In addition to cell lines, PAK4 has been reported to be elevated in many primary tumors. PAK4 was shown to be overexpressed in a subset of gastric tumors, and overexpression of PAK4 tends toward more poor survival rates of gastric cancer patients.[59] Liver cancer is

frequently linked to PAK4 overexpression. PAK4 is overexpressed in human hepatocellular cancer carcinoma (HCC) samples.[59] In these HCC samples, PAK4 overexpression and activation correlates with overexpression of the kinase CDK5-Associated Protein CDK5RAP3. CDK5RAP3 has a role in controlling apoptosis and genotoxic stress.[60] It has been implicated both in enhancing and inhibiting cell growth[61,62] and is amplified In HCC.[63] In HCC CDK5RAP3 is associated with more aggressive tumor behavior and increased invasiveness.[64] CDK5RAP3 activates PAK4, and PAK4 is in turn hypothesized to trigger cell invasiveness in HCC.[64] Further evidence for the role for PAK4 in liver cancer comes from studying microRNAs.[65] The microRNA miR-199a/b-3p is highly expressed in liver, but consistently decreased in HCC. Interestingly, this microRNA, which has an antitumor effect in cells, downregulates PAK4 and blocks the downstream ERK activation,[65] strongly linking PAK4 to the etiology of the disease.

Ovarian cancer cell lines have also been reported to have high levels of PAK4. High PAK4 and phospho-PAK4 levels are strongly associated with metastasis of ovarian cancers, and with poor survival and reduced chemosensitivity.[66] Knockdown of PAK4 in ovarian cancer cell lines abrogates cell migration, invasion, and proliferation, and it reduces a number of signaling pathways associated with cell growth. It also blocks tumor growth in mice. Overexpression of PAK4 in ovarian cancer cell lines has the opposite effect, and leads to increased cell migration and invasion.[66] Breast tumors also frequently express PAK4. PAK4 mRNA levels are elevated in breast cancer cell lines,[12] and PAK4 protein level is elevated in human breast tumors and rat mammary tumor samples.[58]

PAK4 is clearly overexpressed in a variety of cancers and tumor cell lines as described above.[12,58,59,66,67] In some cases point

mutations were also found in PAK4, such as in some colorectal cancers,[68] but in other cases overexpression of wild-type PAK4 may be sufficient for oncogenesis. The mechanism of PAK4 overexpression in cancer is thus of considerable interest. In at least some cases, PAK4 overexpression may be due to gene amplification. The *Pak4* gene is located on a chromosomal region that is frequently amplified in cancer. The wild-type *Pak4* gene was shown to be amplified in a panel of pancreatic cancer samples, including pancreas ductal adenocarcinomas (PDACs),[55,69,70] and squamous cell carcinomas.[71] The chromosomal region containing *Pak4*, 19q13.2, is also frequently amplified at a high rate in aggressive breast cancers with basal-like features.[72] The frequent amplification of the *Pak4* gene in cancer could make PAK4 an excellent diagnostic tool for early detection of cancer.

PAK4 and breast cancer. In addition to serving as a marker for cancer, PAK4 may also be a driving force in cancer and thus holds potential as a drug target. In breast cancer, for example, PAK4 is highly overexpressed, and mouse and cell culture models show that it is sufficient to lead to disease. The role for PAK4 in disrupting the normal structure of the mammary gland has been examined in vitro by studying the mouse mammary epithelial cell line iMMEC.[73] When grown in 3D culture, iMMECs form spherical acini, mimicking those seen in normal non-cancerous breast epithelia[74] (see **Fig. 3**). Normal iMMECs have nearly undetectable levels of PAK4, similar to the normal mammary epithelium. When they are stably transfected with an expression vector containing wild-type PAK4, the resulting acinar structures exhibit several changes that are typically associated with cancer. These include increased cell proliferation and survival, filling of the luminal space with cells,[73] increased acinar size, loss of cell polarity, and a thicker layer of epithelial cells surrounding the lumen (see **Fig. 3**). Importantly, these changes are reminiscent of changes seen in the glandular epithelium during pre-cancerous conditions and early tumorigenesis. Some of the changes, such as filling of the luminal space, are particularly reminiscent of atypical hyperplasia and DCIS.[75] Importantly, the PAK4 expressing cells form tumors at a high frequency when implanted into the mammary fat pads of mice,[73] indicating that PAK4 can be a driving force in oncogenic transformation of these cells. Oncogenes such as *Her2Neu (ErbB2)* and oncogenic *Ras* also cause iMMECs to become tumorigenic.[74,76] Interestingly, the levels of PAK4 are also strongly increased in response to *ErbB2* and *Ras* in iMMECs,[73] suggesting a role for PAK4 downstream to these oncogenes in breast cancer.

One interesting result from the above study was the finding that PAK4 overexpression disrupts cell polarity.[73] Alterations in cell polarity can be important in cancer, and the role for PAK4 in polarity is intriguing. It is not yet clear how PAK4 can alter cell polarity when it is overexpressed. It is interesting that PAK4 along with Par6b, have recently been shown to be required for regulation of apical junction formation by Cdc42 in human bronchial epithelial cells.[77] Par6 is a cdc42 binding protein, known to play a role in cell polarity.[78,79] Its binding to Cdc42 is important for establishing cell polarity, and it will be interesting to determine whether PAK4 may interfere with this interaction

when it is overexpressed, thereby disrupting cell polarity in cancer. The exact role for pak4 in cell polarity, and possible downstream mediators, is an area that needs to be explored further.

In addition to PAK4, PAK1 is also associated with breast cancer, and there are both similarities and differences between PAK1 and PAK4 in cell culture and animal models of the disease. Transgenic mice that express the constitutively active PAK1 mutant (T423E) in the mammary gland develop mammary tumors, but at low penetrance and with a long latency period, suggesting that other genetic events are required in the transformation process.[80] PAK1 was also shown to be activated in breast cancer cells, specifically in estrogen receptor (ER) negative tumors that overexpress the oncogene Erb-B2.[81] Blocking PAK1 activity, in contrast, inhibits transformation of MCF10A cells by ErbB2, and blocks tumor formation in mice in response to ErbB2 positive breast cancer cell lines. The T423E mutant of PAK1 can bypass the requirement for ErbB2 activity in transformation. These results suggest an important role for PAK1 in ErbB2 positive ER negative breast cancer.[81] One important difference between the PAK1 and PAK4 studies is that in contrast to PAK1, even overexpression of wild-type PAK4 in mammary epithelial cells and fibroblasts causes them to be tumorigenic in mice, and it does so at a high frequency.[58,73] It can be difficult to compare these results because different types of conditions were used for the different studies, but it is intriguing that wild-type PAK4 is sufficient to cause tumorigenesis. This is important because wild-type PAKs are often overexpressed in tumors, and both in vitro studies and mouse models are likely to be increasingly valuable for studying the mechanisms behind PAK4 protein-mediated tumorigenesis.

An interesting cell culture model for breast cancer is the MCF10A progression series. MCF10A, neoT, ATI and DCIS cells are all derived from MCF10A cells. MCF10A represent normal human breast epithelium,[82] similar in many respects to the mouse iMMECs. The other cells in the progression series are models for increasing levels of oncogenic transformation.[83-85] When grown in 3D culture, there is increasing derangement of normal acinar structure in the more malignant cells. PAK4 levels increase in the more malignant versions of the cells.[86] Likewise, PAK1 expression and auto-phosphorylation levels were also shown to increase in the more malignant versions of the cells,[87] and the abnormal morphologies can be partially reversed by the expression of dominant negative PAK1. Overexpression of exogenous wild-type or activated PAK1, however, has no effect on cell proliferation, invasion or acinar growth.[87] In contrast, as described above, overexpression of wild-type PAK4 has striking effects on mouse iMMECs and causes them to become tumorigenic.[73] In other words, PAK1 is essential, but not sufficient for malignant growth, whereas PAK4 is both essential and sufficient for malignant growth of human cells.

PAK4 as a drug target. There is currently a significant amount of interest by pharmaceutical companies in developing drugs that target the different PAK family members in cancer. An inhibitor that blocks PAK4 kinase activity has been generated by Pfizer Oncology. This potent pan-PAK inhibitor, PF-3758309 has growth inhibitory activity toward a large number of tumor cell
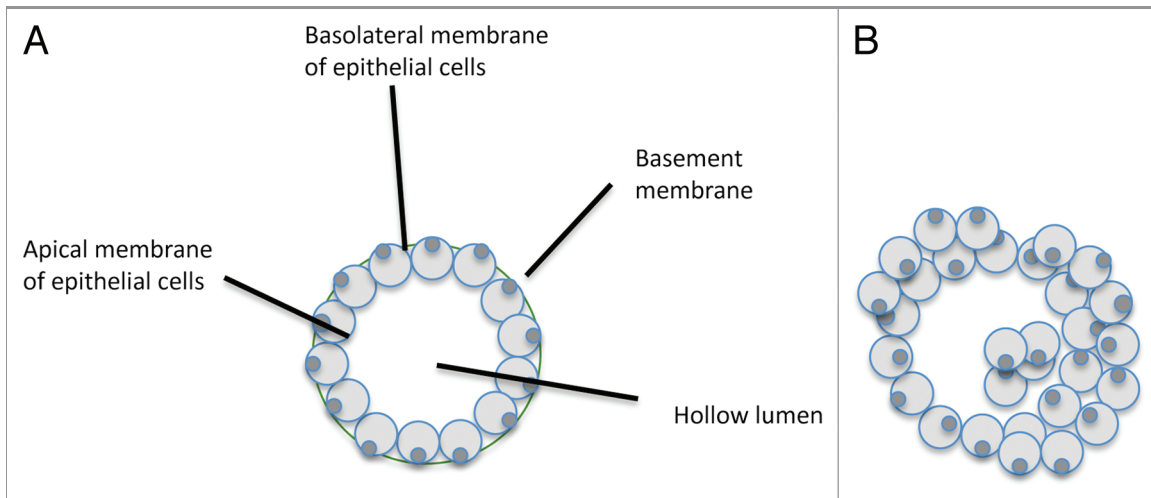
Figure 3. Diagram of typical acini formed by iMMECs. (A) When grown in 3D culture conditions, wild-type iMMECs form spherical acini with a completely hollow lumen, surrounded by a single layer of polarized epithelial cells. (B) Overexpression of PAK4 leads to formation of acini, which lack a completely hollow, with a thicker outer layer of epithelial cells, and leads to disruption in cell polarity.

lines with the IC_{50} raging 5–15 nM.[88,89] The Pfizer inhibitor is currently being tested clinically for effectiveness against various solid tumors[88] (clinicaltrials.gov). A newer PAK4 inhibitor, LCH-7749944 has also been reported recently.[90] This PAK4 inhibitor affects several cell signaling pathways. Specifically, it down-regulates a pathway mediated by PAK4/c-Src/EGFR and cyclin D1, and it inhibits EGFR activity. LCH-7749944 also affects cell morphology, by blocking filopodia formation and leading to cell elongation. This may be due to the fact that it blocks the cofilin pathway, which is tightly linked to cytoskeletal organization, as well as the ERK/MMP2 (matrix metalloproteinase) pathways. Importantly, this inhibitor suppresses proliferation of human gastric cancer cells and blocks their migration and invasion capacity. These results suggest that a far more (1,000 times) potent derivative (s) of LCH-7749944 could have a cancer therapeutic potential in the future.

PAK4 is a promising drug target for cancer, but in order for PAK4 to be a fully effective drug target, a better understanding of the mechanisms by which it causes oncogenesis may be needed. One area of particular importance is the role for PAK4's catalytic activity. Most drugs that target protein kinases are designed to block kinase activity, but PAK4 and other PAKs may have some kinase independent functions.[30,40,91,92] For example, PAK4 has an important role in suppressing apoptosis, which could be directly related to its role in cancer, but under some conditions this occurs completely independently of PAK4's kinase activity.[30,40,41] PAK4 may thus serve not only as a protein kinase, but it may also have other roles, which could include scaffolding roles, and roles in sequestering other regulatory proteins in the cell. The possibility that PAK4 could promote tumorigenesis by a kinase-independent mechanism, or by a combination of kinase-dependent and kinase-independent mechanisms needs to be considered. In this case, drugs designed specifically to block PAK4 kinase activity alone may be incompletely effective in some types of cancer and new strategies for blocking PAK4 should be investigated. Another

consideration is that different PAK family members may have different functions in cancer, and may be overexpressed in some of the same types of cancers, as discussed above for PAK1 and PAK4. The possibility should be considered, therefore, that multiple different PAK family members may need to be blocked, in order to combat some types of cancer.

PAK5 and PAK6 in cancer? Although PAK4 is most strongly linked to cancer among PAK4–6, PAK5 or PAK6 may also been linked to the disease. For example, both PAK4 and PAK5 can protect pancreatic cells from apoptosis.[93] There has also been evidence for PAK5 overexpression in some colorectal cancers, and PAK5 plays a role in invasiveness of colorectal cancer cells.[94] PAK6 mRNA overexpression can be detected in certain cancer cells,[12] and PAK6 protein levels are elevated in some prostate and breast cancer cell lines.[95] PAK6 levels increase in prostate tumors that relapsed after androgen deprivation therapy, and it may play a role in motility and stress responses of tumor cells.[95] PAK6 may have a role in radiosensitivity in prostate cancer cells, because inhibition of PAK6 combined with irradiation, significantly decreases survival of prostate cancer cells.[96] The role for PAK6 in prostate cancer is not entirely clear though, because it was also identified as a gene that is sometimes hypermethylated in prostate cancer, which is more often associated with genes that suppress tumorigenesis.[97] Overall, there is no direct or concrete evidence indicating that either PAK5 or PAK6 is essential for the malignant growth as yet.

The Roles of PAK4–6 in Development

Knockout mice have been developed for all of the group B PAKs, in order to study their roles in development. PAK4 null mice are embryonic lethal, which is consistent with high PAK4 expression during embryogenesis, but conditional PAK4 knockout mice have been generated to study its role in specific tissues. PAK5 and PAK6 knockout mice are viable, but the double knockouts have

deficits in learning and memory. Some of the key studies regarding the group B Pak knockout mice are described below.

PAK4 and angiogenesis. PAK4 is highly expressed in embryos but PAK4 protein levels are typically low in adult tissues. PAK4 is absolutely required for embryonic development, as PAK4 null embryos die prior to embryonic day E11.5.[98] There are multiple possible causes for embryonic death at this early stage. One interesting phenotype exhibited by the PAK4 null embryos is an abnormality in the blood vessels throughout the embryo and extraembryonic tissue. Although some early vessels form, there is almost a complete lack of branching, suggesting a defect in angiogenesis. This may help explain the early death of the PAK4 null embryos,[98] and a role for PAK4 in angiogenesis could also play an important part in the oncogenic process.

The role for PAK4 in the heart. PAK4 null embryos have severe abnormalities in the heart. $Pak4^{-/-}$ embryos have a thinning of the myocardial walls of the bulbus cordis and the ventricle, and the sinus venosus region of the heart is dilated and distorted.[98] Conditional deletion of PAK4 specifically in the secondary heart field in mice led to a different result. These mice are viable but they exhibit abnormalities in the outflow track of the heart. Deletion of PAK4 in vitro in cardiomyocytes gives some important clues about its role in heart development. PAK4 null cardiomyocytes exhibit severe disruption of the sarcomeric structure.[99] Specifically, the sarcomeres have a drastic reduction in F-actin (actin thin filaments), and the normal striated structure of the sarcomere is disrupted. This is consistent with the role for PAK4 in cytoskeletal organization, although it is also somewhat surprising because the cytoskeleton in the sarcomere is much less dynamic than in other cells such as fibroblasts. Other PAKs have also been shown to have functions in the heart. Ablation of PAK1, for example, does not lead to embryonic lethality, but in adult mice it leads to an increase in stress induced cardiac hypertrophy.[100] The roles for PAKs in the heart are important, and need to be taken into consideration if they are used as therapeutic targets for drug development. An important consideration for PAK4 is that PAK4 levels were found to be high in the embryonic heart, but nearly undetectable in adult heart.[99] This expression pattern suggests that the role for PAK4 in the heart is likely to be a developmental one, and that its role in the adult heart may be negligible. This raises the promising possibility that inhibition of PAK4 may not lead to adverse side effects in the adult heart.

Group B PAKs in neuronal development. PAK4 knockout mice are embryonic lethal, most likely due to defects in the heart, extraembryonic tissue and blood vessels, but abnormalities are also seen in the developing nervous system. In particular, the neuroepithelium around the brain and spinal cord in PAK4 null embryos are especially thin, and motor neurons fail to develop normally.[98] To examine the role for PAK4 in neuronal development in more depth, conditional PAK4 knockout mice were generated in which PAK4 was deleted specifically in the nervous system. PAK4 conditional knockout mice are viable but they display growth retardation and die prematurely. The brains show a dramatic decrease in proliferation of cortical and striatal neuronal progenitor cells. In vitro analysis revealed a reduced proliferation and self-renewing capacity of neural progenitor cells

isolated from PAK4 knockout brains. The mice also display cortical thinning, impaired neurogenesis, and loss of neuroepithelial adherens junctions. By the time the mice die, by four weeks after birth, they exhibit severe hydrocephalus.[101] These results suggest that PAK4 plays a critical role in neural progenitor cell proliferation and establishing the foundation for development of the brain.

PAK5 and PAK6 are highly expressed in the brain.[9,102] PAK5 has been shown to be important for filopodia formation in neuroblastoma cells,[9] and PAK6 may be important in the response to traumatic brain injury (TBI).[103] The guanine nucleotide exchange factor GEFT is highly expressed in the brain, and was shown to lead to increased dendritic spine formation and neurite outgrowth. Interestingly, studies with dominant negative mutants indicate that both PAK1 and PAK5 have essential roles in these processes.[104] Surprisingly though, even though PAK5 and PAK6 are highly expressed in the brain, the phenotypes of PAK5 and PAK6 knockout mice are quite subtle, and much less dramatic than the PAK4 knockouts. Knockout of either PAK5 or PAK6 alone does not result in any noticeable phenotype. Double deletion of PAK5/PAK6 results in mice that are viable and fertile. The double knockout mice do not have noticeable gross abnormalities, but when behavioral tests were performed, it became evident that PAK5 and PAK6 are important for certain neuronal functions.[105] Specifically, the double knockout mice display a lower activity level than the wild-type mice, and they have a decreased level of aggression. Importantly, behavioral tests revealed specific deficits in learning and memory. This is consistent with the finding that there are high levels of both PAK5 and PAK6 in the cortex, hippocampus and striatum, which are structures that are critically involved in cognitive functions. No histological abnormalities were observed in the PAK5/PAK6 knockout brains, but in vitro, cultured neurons from the knockouts showed abnormally small growth cones and less neurite outgrowth, which could be related to the role for the group B PAKs in cytoskeletal organization. It is interesting that a Drosophila homolog of the group B PAKs, mushroom body tiny (mbt) is involved in neuronal development, specifically of the cells of the mushroom body, a structure also involved in learning and memory.[106] These results strongly support an evolutionarily conserved role for group B PAKs in the development of neurons which are necessary for learning and memory. Recently two new PAK5 substrates were identified which may provide some clues as to the mechanism by which group B PAKs affect learning and memory. These two substrates, Pacsin1 and Synaptojanin1, are phosphorylated by PAK5, and PAK5 stimulates their interactions with each other.[107] These two proteins regulate synaptic vesicle endocytosis and recycling, and this study raises the intriguing possibility that the cognitive problems exhibited by the PAK5/PAK6 knockout mice could be due to altered endocytosis and vesicle trafficking at the synapse.

The differences between the phenotypes of the PAK4 null mice and the PAK5/PAK6 knockout mice are interesting, but may have more to do with the expression patterns of the different genes as much as differences in their functions. High levels of PAK5 and PAK6 are generally seen later in development in the brain,

compared with PAK4, which is higher during embryogenesis. This would support the idea that PAK4 has a role in early neuronal differentiation, whereas PAK5 and PAK6 have roles later in neuronal development or maintenance. It is also intriguing that the PAK5/PAK6 double knockouts have a more dramatic phenotype than either knockout alone, suggesting functional redundancy (compensation) between these two PAKs.

Conclusions

PAK4–6 have important roles in cellular processes including cytoskeletal organization, cell cycle regulation, and cell survival. PAK4 is frequently overexpressed in cancer,[12,58,59,66,67] and in some types of cancer the PAK4 gene is amplified.[55,69-72] Furthermore, PAK4 is sufficient to lead to cancer in animal models, particularly in a mouse breast cancer model.[73] These studies make PAK4 an attractive candidate both as a diagnostic tool for early detection of cancer, and as a drug target for cancer treatment, In addition to its role in cancer, PAK4–6 were shown to have roles in the nervous system.[98,103,105,107] While the role for

PAK4 in the nervous system is most likely developmental, PAK5 and PAK6 have roles in learning and memory in mouse models. Other PAK family members have also been linked with learning. Blocking the activity of PAKs1–3 in mice impairs spatial memory function.[108] Moreover, mutations in PAK3 are linked with nonsyndromatic mental retardation[109] and the Drosophila group B PAK, mbt, is involved in neurogenesis in regions that are important for learning.[110] A link between PAK5 and PAK6 in human learning and memory disorders has not yet been established, but it will be interesting to determine whether PAK4–6 could serve a diagnostic role in learning disorders. PAK4–6 also have been shown to be involved in neurite outgrowth in cells and mouse models,[9,98,101,105] which raises the question of whether activation or overexpression of these PAKs could be valuable for treating neuronal abnormalities or injury. Because of the link between PAK4 in cancer, however, and such treatments would have to be used with caution.

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