THE ROLE OF PAK4 IN TUMORIGENESIS *IN VIVO*

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A dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

and

The Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Joint Graduate Program in Molecular and Cellular Pharmacology

Written under the direction of

Professor Audrey Minden

And approved by

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New Brunswick, New Jersey

October, 2010

ABSTRACT OF THE DISSERTATION

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The Pak4 (P21 activated kinase) serine/threonine kinase is an important effector of Rho GTPases and has been implicated in the regulation of cell morphology and motility, as well as cell growth control. Our preliminary data showed that both Pak4 mRNA and protein are highly expressed in primary tumors and almost every tumor cell line, while there is a very low expression level in normal tissues. The goal of this thesis was to study the role of Pak4 in tumorigenesis. First, I investigated whether overexpression of Pak4 is sufficient to induce tumor formation. Fibroblast cell line NIH3T3 and immortalized mouse mammary epithelial cells (iMMECs) transfected with Pak4 were used. We found that overexpression of Pak4 in both cell lines led to formation of tumors in athymic mice. Furthermore, overexpression of Pak4 in iMMECs led to changes in 3D acinar architecture, including decreased central acinar cell death, abrogation of lumen formation, and cell polarity alteration. The results suggest that Pak4 can promote cell survival and proliferation, as well as alter cell shape and polarity, as part of the oncogenic process. Next, I investigated whether Pak4 is necessary for tumorigenesis in response to oncogenes such as Ras and Cdc42. Pak4 conventional knockout 3T3 cell lines and Pak4 conditional knockout 3T3 cell lines transfected with Ras or Cdc42 were used. We found

that in cells transfected with oncogenic Ras, tumors grew more slowly and to a smaller size when Pak4 was deleted. In cells transfected with Cdc42, tumor formation was almost completely abrogated when Pak4 was absent. These results would be consistent with the role for Pak4 as a Cdc42 effector protein involved in signaling pathways leading from Cdc42 and its activators to transformation, and its critical role in cell survival and inhibition of apoptosis. This work shows for the first time that overexpression of Pak4 in epithelial cells leads to tumorigenesis *in vivo*, and that Pak4 is necessary for tumorigenesis in response to certain oncogenes. These findings make Pak4 an attractive target for cancer prevention or therapy.

ACKNOWLEDGEMENTS

My sincere gratitude goes first to my advisor Dr. Audrey Minden. Without her continuous support, encouragement and guidance, I could not complete this dissertation.

I am grateful to the members of my committee: Dr. Chung S. Yang, Dr. Allan Conney, Dr. Suzie Chen, and Dr. Fang Liu, for their valuable time, encouragement and advice.

I am grateful to the former and present members of the Minden lab, Liang Lei, Beatrace Noel, Nadishani Dissanayaka, Yanmei Tian, Tanya Nekrasova, and Lisa Epstein Wong, for their friendship and all the help in my experiments.

I am grateful to my colleagues from other labs: Dr. Hang Xiao, Dr. Xingpei Hao, Dr. Ning Chen, and Dr. Vassiliki Karantza, Dr. Najoo Suh, Dr. Hongjin Lee, Dr. Xi Zheng, Dr. Xiaoxing Cui, Dr. Gary Lu, Dr. Zhihong Yang, Mrs. Yue Liu, and Mrs. Li Deng, for their friendship and/or collaborative work in my thesis.

I am also grateful to Dorothy Wong, Deborah Stalling, Bobbie Busch, Casandra Burrows, Florence Florek, Annette Dionisio and Erica Dipaola, for their patience and help. Without them, the completion of this dissertation is surely much less pleasant.

My utmost gratitude goes to my family. My husband, Hongbin Zhang, my lovely children Sophie and Bill, they are the source of my motivation, and they always stand by me and share all my sadness and happiness. My parents-in-law, Yuehua Zhang and Yongjie Zhang, who continuously encourage and support me. My parents Yan Lu and Jun Liu, and my sister Bin Liu, who give me unconditional love and support.

DEDICATION

I dedicate this dissertation

With love,

To my mother,

Yan Lu,

And

My father,

Jun Liu

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Chapter I

General Introduction

1. The p21 Activated Kinase (Pak) Family of Ser/Thr Kinases

The Pak family of serine /Threoine kinases was first identified as effector proteins for Cdc42 and Rac, members of the Rho GTPase family [1-3]. They have a highly conserved kinase domain homologous to the yeast Ste20 protein, which is identified as a Cdc42 effector essential for the yeast mating pathways in 1992 [4]. Since then, Ste20 and Paks have attracted more attention for their roles in regulating actin cytoskeleton and also many cellular processes such as cell proliferation, apoptosis, cell migration, neuronal development and tumorigenesis [5]. Paks are widely expressed in almost all eukaryotes including yeast, fungi, nematode, fruit fly, frog, mice and human, and all of them express more than one Pak related gene [6]. The six mammalian isoforms have been classified into two groups based on their sequences and functions. Group I comprises Pak1-3 and group II comprises Pak4-6. Extensive information about Paks in lower organisms and in mammals, as well as Pak structures, activation mechanisms, regulators, physiological functions and related pathology conditions will be addressed in detail.

2. General Introduction to Paks in Lower Organisms and in Mammals

2.1 Paks in lower organisms

Lower organisms provide an excellent experimental model system that can be easily manipulated to understand the precise roles of the Paks. Most of our knowledge about Paks function has been derived from genetic approaches in lower organisms and many of these functions are similar to that seen in mammal cells. Paks phenotypes associated

with Paks loss-of-function mutations in lower organisms have been summarized in **Table 1**.

Budding Yeast (Saccharomyces cerevisiae)

The budding yeast encodes three Pak enzymes: Sterile 20 (Ste20), Cla4, and Skm1. Ste20 promotes actin polarization and regulates mating and vegetative growth [7] [8]. Ste20 was identified as a key regulator in the mating pathway and function as an activator of mitogen-activated protein kinase (MAPK) cascades [9]. Cla4 is involved in septin ring assembly, actin polymerization, and mitotic entry and exit [10]. Deletion of Ste20 results in sterility [7], deletion of Cla4 leads to aberrant cytokinesis [10], and deletion of both Ste20 and Cla4 is lethal [10]. Little is known about Skm1.

Fission Yeast (Schizosaccharomyces pombe)

The fission Yeast encodes two Pak kinases: Shk1 (AKA, Pak1, or Orb2) and Shk2 (AKA or Pak2). Shk1 is essential for the viability of Fission Yeast [11], and the loss of functional Shk1 leads to defects in actin and the microtubule cytoskeleton, loss of polarity, and mating [12]. However, Shk2 is not essential for cell viability, morphology or mating.

Amoebae (Dictyostelium discoideum)

The amoebae encodes three Pak kinases: DdPakA, DdPakB (myosin I heavy chain kinase [MIHCK]), and DdPakC. Constitutively active DdPakA leads to the upregulation of myosin II assembly, while DdPakA null cells display defects in myosin II assembly and directional motility, which controls cell polarity and chemotaxis. DdPakA promotes myosin II filament assembly indirectly by regulating the activity of MIHCKs [13]. Activated MIHCK phosphorylates its downstream effector, myosin I, and enhances the

myosin I-dependent process of phagocytosis and pinocytosis, therefore causing severe defects in cytokinesis by entrapping or sequestering actin filaments [14]. Cells lacking MIHCK show no apparent defects in a variety of motile processes [14]. Cells lacking DdPakC exhibit defects in cell polarization and pseudopodia formation but do not develop defects in cell motility [15]. Cells lacking both MIHCK and DdPakC show severe loss of cell movement [15].

Fruit Fly (Drosophila monogus)

Fruit Fly encodes three Paks: DPak1, mushroom bodies tiny (Mbt)/DPak2, and DPak3. DPak1 and Mbt have been found to function in sensory organ development. DPak1 is involved in tissue morphogenesis. It mediates communication between the basement membrane and intracellular proteins during epithelial development, and it regulates photoreceptor axon guidance [16]. Mbt contributes to photoreceptor cell morphogenesis [17]. Mbt null mutants have defects in the central brain structure and have fewer neurons. In addition, Mbt mutant flies have a reduced number of photoreceptor cells in the eye and disorganized adherens junctions [17].

Nematode (Caenorhabditis elegans)

Nematode encodes three Pak kinases; CePak1, CePak2, and MAX-2. CePak1 is expressed in the hypodermal cell boundaries during embryonic body elongation [18]. Both CePak1 and MAX-2 function during axon guidance [19].

Frog (Xenopus laevis)

Xenopus encodes four Pak kinases: xPak1, xPak2, xPak3, and xPak5. Xenopus Paks have been studied extensively, and accumulating evidence indicates that Xenopus Paks have roles in oocyte maturation and embryonic development. xPak1 interupts oocyte

Species	Mutations	Phenotypes	References
Saccharomyces cerevisiae (Budding Yeast)	Ste20	Sterility; cell polarity defects	[4]
	Cla4	Cytokinesis defects; cell polarity defects	[8]
	Ste20 and Cla4	lethal	[10]
	Skm1	N/A	N/A
Schizosaccharomyces pombe (Fission Yeast)	Pak1 (Shk1, AKA, orOrb2)	Lethality; cell polarity defects	[11]
	Pak2 (Shk2 or AKA)	N/A	N/A
Dictyostelium discoideum (Amoebae)	DdPakA	Cell polarity, Myosin II assembly defects, motility defects	[13]
	DdPakB (MIHCK)	No apparent defects	[14]
	DdPakC	Cell polarization defects, pseudopodia formation	[15]
	DdPakC and DdPakB	Loss of cell movement	[15]
Drosophila melanogaster	Dpak1	Lethality; axon guidance defects	[16]
	Mbt (Dpak2)	Mushroom body defects; photoreceptor morphogenesis defects	[17]
Caenorhabditis elegans	Max-2	Darsal guidance defects	[19]
Xenopus	xPak1	Neuronal differentiation defects Embryonic lethality,	[22]
	xPak3	Neurogenin differentiation defects	[23]
	xPak5	Cell motility defects	[25]

Table 1. Phenotypes associated with Pak loss-of-function mutations in lower organisms.

maturation by promoting oocyte arrest in the G2/prophase of the cell cycle. When the catalytic domain released from an N-terminalinhibitory sequence, xPak1 can be activated by caspases, consequently promoting apoptosis [20] [21]. xPak1 also mediates expansion of the neural plate and inhibition of neuronal differentiation through phosphorylating of Tumorhead gene, which are required for cell proliferation and differentiation [22]. xPak3 is expressed in the area of primary neurogenesis in the developing embryo. xPak3 regulates differentiation of neuronally programmed cells downstream of neurogenin [23]. xPak5 is mainly expressed in the regions of the embryo that undergo extensive cell movement during gastrula [24]. xPak5 regulates cellular movement during early embryonic development in xenopus [25].

2.2 Paks in mammals

Individual Pak isoforms show differences in tissue distribution and subcellular localization. In mammals, Pak2 and Pak4 are expressed ubiquitously, whereas Pak1, Pak3, Pak5, and Pak6 have more restricted tissue-specific expression patterns. Pak1, Pak3 [26-27], and Pak5 [28] are all highly expressed in neuronal tissues. Pak5 is also highly expressed in the pancreas [29]. Pak6 is highly expressed in prostate and appears to have a unique role in hormone signaling, as it binds to the androgen receptor and represses androgen receptor-mediated transcription [30-31]. Within individual cells, Pak4 has been localized to a number of different subcellular compartments, including perinuclear region, Golgi when coexpressed with Cdc42 [32], and cell periphery downstream of growth factor [33] and integrin-mediated signaling [34]. Pak5 contains a mitochondria targeting signal [35], a nuclear localization signal (NLS), and a nuclear

export sequence [35]. A substantial portion of Pak5 localizes to the mitochondria, and it has been reported that Pak5 can shuttle between the mitochondrial and the nucleus [35]. In Chinese-hamster ovary cells, Pak6 was found predominantly in the mitochondrial fraction [36], and Pak6 has been reported to be localized in both the cytoplasmic [37] and the nuclear fractions [30]. By stimulation with Platelet-derived growth factor (PDGF), insulin, or wounding of monolayers, Pak1 can be redistributed from the cytosol into cortical actin structures, including lamellae at the leading edge of polarized cells, circular dorsal ruffles, and peripheral membrane ruffles [38].

Targeted deletion of Pak isoforms in mice have further elucidated biological processes controlled by individual Pak isoforms (See **Table 2**). Individual Pak1, Pak3, and Pak5 null mice are viable. Among those viable mice, Pak1 null mice have a defective immune response. Because Pak3 expression is brain specific, it is not surprising that mice lacking Pak3 exhibit abnormalities in synaptic plasticity and deficiencies in learning and memory [27]. In contrast, although Pak5 functions in neurite outgrowth in neuroblastoma cells, mice lacking Pak5 do not appears to have any defects in neuronal systems [29]. Pak6 knockout mice also display no detectable abnormalities. There is a lack of an overt physical phenotype in the Pak5/Pak6-double knockout mice, which are viable and fertile [39]. Pak6 binds to androgen receptor (AR) and is involved in AR signaling during the development of the testes. Testicular development, however, is completely normal in Pak6 knockout mice and Pak5/Pak6 double knockout mice. In fact, all tissues, including neuronal tissue, developed normally in these mice when assessed by histological analysis. Rather than a physical abnormality, the major defect in the Pak5/Pak6-double knockout mice is a deficiency in learning and memory [39]. Results from these studies suggest that

in Pak5/Pak6 single and double knockouts, other members of the Pak family may partially compensate for the functions Pak5 and Pak6.

Pak isoform deletion	Phenotype	References
Pak1	Viable; Immune system defect;	[40]
Pak2	Embryonic lethality	[40]
Pak3	Viable, abnormal long-lasting synaptic plasticity and cognition	[27]
Pak4	Embyronic lethality; cardiac and neuronal defects	[41]
Pak5	Viable, No phenotype	[29]
Pak6	Viable	[39]
Pak5 and pak6	Viable; impaired learning and memory function	[39]

Table 2. Phenotypes associated with Pak isoform deletion in mice.

Pak2 or Pak4 gene deletion result in embryonic lethality. Multiple developmental abnormalities may account for embryonic death in Pak2 knockout mice [40]. Analysis of Pak4-null embryos revealed abnormalities in the heart and nervous system, indicating that Pak4 knockout mice probably died of a heart defect. Analysis of these mice suggest that Pak4 plays an important role in the development of the heart and neural tube, as well as in differentiation and migration of neurons [41]. Our lab also found that in the

placentas of Pak4 knockout embryos, the labyrinth layer was poorly developed, and there was a reduction in size of the spongiotrophoblast and giant cell layers. Throughout the placenta as well as the yolk sac, there was also a decreased number of embryonic blood vessels, suggesting that Pak4 is also a key factor for blood vessels formation [42]. Among the Pak family, only Pak2 and Pak4 are essential for embryonic development.

3. Structural Features of the Paks

The Pak family proteins are architecturally similar and their structure can be divided into three common domains: an N-terminal GTPase-binding domain (GBD), a central region, and a highly conserved C-terminal serine/threonine-kinase domain. The two groups, however, are also structurally distinct from each other (See **Figure 1**).

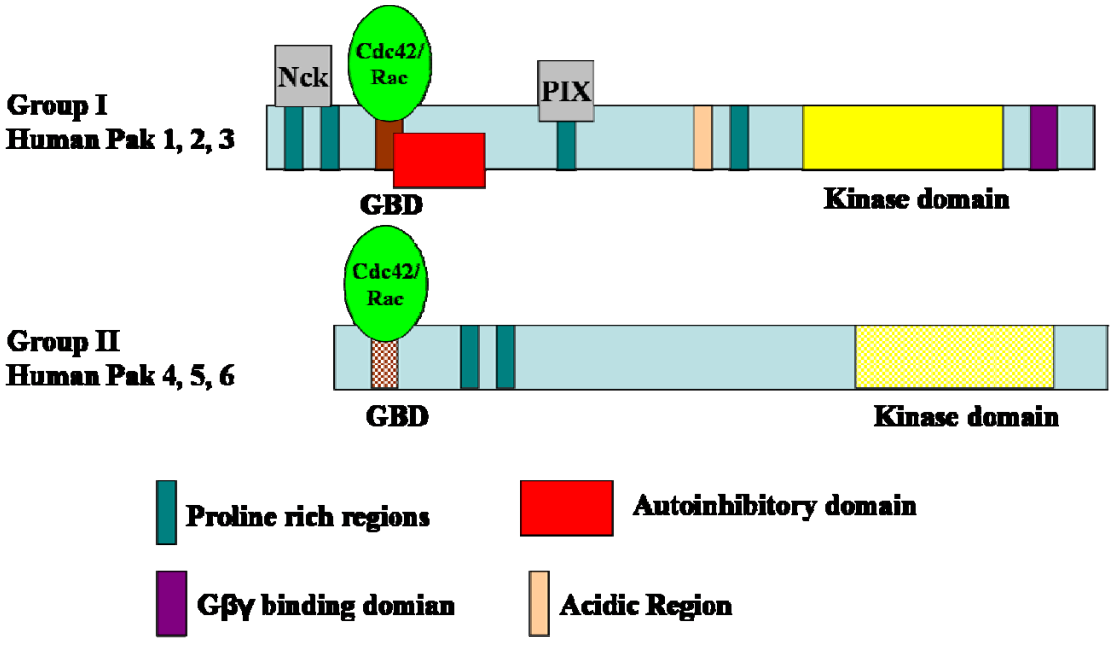

Figure 1. Domain structures of two groups of Pak families.

3.1 Structural features of the group I Paks

This group includes mammalian Pak1, Pak2, and Pak3. They share a number of defining structural characteristics. The members of this family are all quite similar to each other in sequence; exhibiting 73% overall sequence identity and approximately 92% sequence identity within the kinase domain and GBD, which binds to activated Rac and Cdc42, members of the Rho GTPase family. Binding of Cdc42 and Rac stimulates the Paks' kinase activities. GBD contributes to overall binding affinity [43]. Each of these protein kinases has an N-terminal regulatory domain and a highly conserved C-terminal catalytic domain, which are amino acid residues (aa) 225-529, aa 235-509, and aa 254-528 in Paks 1, 2 and 3 respectively. Distinguishing features of the regulatory domain are the presence of five (Pak1), two (Pak2), or four (Pak3) canonical PXXP Src homology 3 (SH3)-binding motifs and one nonclassical (PXP) SH3 binding site [5]. These proline-rich regions in group I Paks are associated with binding to Grb2 [44] and Nck [45], an adaptor protein that is known to be involved in the regulation of actin cytoskeletal dynamics. The noncanonical site is associated with binding to Pak interacting exchange (PIX) family of proteins [46]. The GBD overlaps with an autoinhibitory segment that forms part of an "inhibitory switch" that controls the basal kinase activity of Paks 1-3, and this autoinhibitory domain is specially possessed by group I Paks [47]. The crystal structure of Pak1 in an autoinhibited conformation has been determined to 2.3A resolution [43]. Pak1 exists as a homomdimer in solution and in cells, with the protein in a trans-inhibited conformation where the N-terminal regulatory domain of one Pak1 molecule binds and inhibits the C-terminal catalytic domain of the other [43, 48]. The central part between the N-terminus and the kinase domain is an acidic residue-rich

region of unknown significance. Additionally, a conserved binding site for the Gβγ subunit complex of heterotrimeric G proteins exists at the extreme C terminus [49].

3.2 Structural features of the group II Paks

This group includes mammalian Pak4, Pak5 and Pak6. Group II Paks are structurally distinct from group I Paks. They contain an N-terminal GBD and a C-terminal kinase domain, but lack other motifs found in group I Paks, such as an autoinhibitory switch domain, and they have very little sequence N-terminal to the GBD. The enhancement of the activity of these kinases is not associated with binding of GTPases [28, 32]. In 2007, the structures of the catalytic domains of Pak 4-6 were reported. They share about 75% sequence identity with each other [50]. All enzymes were monophosphorylated at the activation loop positions corresponding to Ser474 in Pak4. The three catalytic domain structures comprise the typical two-domain architecture of protein kinases, with a well-ordered activation segment. Structurally comparison of kinase domains of Pak4, Pak5, Pak6 with Pak1 revealed an unexpected level of domain plasiticity in the active, monophosphorylated group II Paks, suggesting that there are a number of possible movements allowed within the kinase domain during ATP catalysis [50]. The central region of Pak4, Pak5, and Pak6 are more dissimilar and contain various numbers of proline-rich potential SH3-domain-binding sites.

Our lab identified the first member of the group II category of Paks, Pak4 [32]. The GBD and kinase domain of Pak4 have only approximately 50% identity with those of Paks 1, 2, and 3, and the regulatory domain of Pak4 outside of the GBD is completely

different from the group I Paks. Pak4 binds preferentially to Cdc42, though it also binds to Rac.

4. Activation Mechanisms and Regulators of the Paks

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4.1 Mechanisms of activation of the group I Paks

Under basal conditions, group I Paks exist in an autoinhibited inactive dimeric conformation [43]. Uncoupling the intermolecular dimeric complex of autoinhibitory switch and kinase domains is essential for group I Paks activation. Paks are activated in both GTPase-dependent and GTPase-independent manners. Although the multiple events and conformational changes involved in group I Paks activation are complex and remain incompletely elucidated, the major steps involved in the multipart activation cycle of group I Paks have been summarized in the recent published review by Dr. Eswaran et al. (See **Figure 2**) [51], which includes 'resting' antoinhibited dimeric, activation factor bound, regulatory domain phosphorylated, kinase domain transiently phosphorylated (intermediary active) and phosphorylated fully active states.

In 2000, the first crystal structure of the unphosphorylated Pak1 kinase domain in complex with the autoinhibitory domain was solved, revealing the trans autoinhibited, inactive state [43]. The Pak1 AID-GBD domain positions a segment across the typical bi-lobed kinase domain catalytic cleft, mimicking a pseudodubstrate and forcing the kinase domain to adopt an autoinhibited, inactive conformation and thereby preventing activation-loop phosphorylation. When active Cdc42 or Rac interacts with the GBD

domain, it removes the switch away from the kinase domain, thus enabling the activation loop to be phosphorylated and ordered. The GTPase-dependent and GTPase-independent activation precedes the intermediary active phosphorylated state. The comparison of the inactive and intermediary-active Pak1 kinase domain structures indicates that the transition to an active state involves small-lobe rotation, in addition to the concerted movement of the glycine-rich loop towards the activation loop. Structure and analytical studies indicate that phosphorylated group I Paks exist as dimers before substrate binding. When the phosphorylated kinase domain binds to the substrate, it adopts a monomeric conformation.

4.2 Regulators of the group I Paks

Phosphorylation of Paks is important for regulating their activity. There are seven autophosphorylation residues have been mapped for Pak1, including Ser-21, Ser-57, Ser-144, Ser-149, Ser-198, Ser203 and Thr-423. Among these residues, Thr-423 is important for counteracting autoinhibition and maintaining a full catalytic function towards its substrates. Phosphorylation of Ser-21 and Ser-144 also contributes to kinase activation, whereas Ser198 and Ser203 autophosphorylation serves to downregulate PIX-Pak ineraction [52]. There are eight autophosphorylation sites in Pak2, including Ser-19, Ser-20, Ser-55, Ser-192, Ser-197, Ser-141, Ser-165 and Thr-420. The first six sites are phosphorylated by Mg-ATP alone. While activation of group I Paks via Rac and Cdc42 is well characterized, a number of GTPase-independent mechanisms also modify Pak activity and function. Rho GTPase-dependent and Rho GTPase-independent regulatory

mechanism, which include protease digestion, adaptor protein, and other kinase phosphorylation and protein binding, will be addressed in details.

4.2A Rho GTPase-dependent regulatory mechanisms

Pak1 has been found to bind and be activated by Rac1, Rac2, and Rac3, Cdc42 and also by CHP, TC10, and Wrch-1, but not by RhoA-G or by other Ras superfamily members [5]. Reeder et al. mapped residues conferring GTPase selectivity to sites primarily within the overall GTPase domain [53]. Once Paks are activated by the binding of Rac or Cdc42 in vitro, the continued binding of the GTPase is not necessary for Pak kinase activity [1].

4.2B Rho GTPase-independent regulatory mechanisms

4.2B-a Protease digestion

Paks autophosphorylation and activation could be stimulated by limited protease digestion. Pak2 is cleaved by caspase 3 at Asp212, resulting in the formation of 28-kDa N-terminal and 34-kDa C-terminal fragments [54]. A reduction in autoinhibitory constrains normally imposed by the N-terminal inhibitory switch may result in cleaved Pak2 becoming catalytically activated.

4.2B-b Adaptor protein

The regulatory domain of group I Paks contains two canonical PXXP SH3 binding motifs and a nonclassical SH3-binding site PIX factor. The first canonical SH3 site has the capability to bind to the adaptor protein Nck [45, 55], whereas the second SH3 site can bind Grb2 [44]. Membrane recruitment of Pak1 by SH3-containing Nck and Grb2

adaptor proteins results in the stimulation of its kinase activity through either phosphorylation by 3-phosphoinositide-dependent kinase-1 or interaction with lipids such as sphingosine or phosphatidic acid.

4.2B-c Phosphorylation by other kinases and binding to other proteins

A number of kinases have been reported to phosphorylate the Paks and regulate their functions. PDK1 is able to activate Pak1 by direct phosphorylation of Thr423 [56]. AKT phosphorylates Pak1 at Ser21, and its modification both decreases binding Nck to the Pak1 N terminus and stimulates Pak1 activity in a GTPase-independent manner in vivo [57]. Cdk5, a neuron-specific protein kinase, phosphorylates Pak1 at Thr212 [58]. The tyrosine kinase, Abl, was reported to associate with Pak2 in vivo and to decrease Pak2 kinase activity with phosphorylation of Pak2 on multiple sites [59]. The non-receptor tyrosine kinase Etk/Bmx, a tec family member, was reported to directly bind and phosphorylate Pak1 [60]. Src-mediated tyrosine phosphorylation of Pak2 appeared to be dependent upon conformational changes in Pak2 induced by GTPase binding [61]. Through direct binding to other proteins, Paks activity can also be regulated. Paks bind to a family of Rac- and Cdc42-specific guanine nucleotide exchange factors PIX [46, 62]. The interaction of αPIX with Pak1 was shown to induce Pak1 activation. Paks can also be activated by G-protein-coupled receptor kinase-interacting target 1 (GIT1) [63], which associates indirectly with Pak by PIX.

4.2C Group I Pak activation is negatively regulated by protein binding

Many positive regulators of Paks have been found, while less was known about negative regulators of the Paks. Pak-interacting protein (PIP) abolishes kinase activity by

binding to the regulatory domain of Pak1, and inhibits Pak-mediated Jun kinase and NF-κB signaling [64]. Nischarin interacts with the kinase domain of active Pak1 and inhibits the ability of Paks to phosphorylate substrate. Nischarin may be important for local limitation of Pak1 activity in migrating cells [65-66]. Partner of PIX1 and 2 are two phosphatases that dephosphorylate the Pak activation loop [67-68]. Another mechanism is a negative-inhibitory loop generated by Cdc42 homologous protein (Chp) [69-70]. Chp inhibits Pak1 functions through ubiquitination and proteasome-mediated degradation.

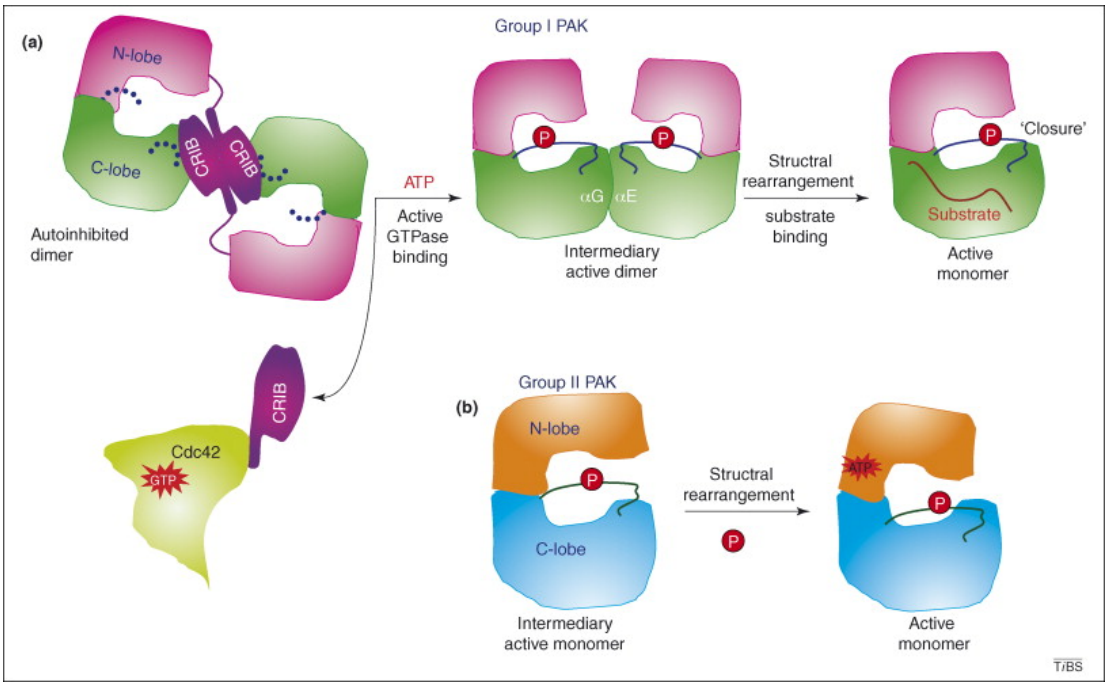


Figure 2. The mechanisms of activation of the group I and group II Pak [51]**.** The group I and group II Paks undergo different modes of activation. **a)** The group I Paks are maintained in an inactive, autoinhibited dimeric complex comprising a kinase domain with a disordered activation loop (blue) and a GTPase-binding CRIB domain (GBD)

(purple).The active GTPase (light green) binds the Pak1 CRIB-AID domain and removes the inhibition, thus enabling autophosphorylation and activation-loop ordering. When the phosphorylated kinase binds the substrate (red), it adopts a monomeric confonmation. **b)** The inactive conformation of group II Paks remains unknown. The intermediary-active, phosphorylated group II Pak kinase domains remain in a monomeric, catalytically non-productive conformation. Further structural rearrangement enables the kinase domain to form a catalytically productive conformation compatible with substrate binding.

4.3 Mechanisms of activation of the group II Paks

The events involved during activation of the group II Paks remain undefined. However, recent investigation of group II Paks provides insight into phosphotransfer and substrate-recognition processes. In contrast to Pak1, the group II Paks are monomeric in solution [50]. The kinase domains of all group II Pak kinase were determined in the active, monophosphorylated conformation. The phosphorylayted, apo structures of Pak4-6 kinase domains adopt an active conformation analogous to the intermediary-active Pak1 conformation (See **Figure 2**). The structures of Pak4 or Pak5 in complex with an ATP-mimicking, small molecules, trisubituted purine inhibitor, exhibit catalytically productive conformations. The inhibitor bound to the ATP-binding site provides an opportunity to capture the dynamic, intricate steps involved in catalysis, thereby demonstrating the plasticity of the kinase domain, which facilitates various conformations during catalysis. The transition from catalytically non-productive, open states to an active, catalytically productive, closed state includes three main characteristic

movements: a ‘clamping’ movement at the interface of the C-terminal and N-terminal lobes, which triggers a closed conformation; glycine-rich loop flipping that stabilizes this closure; and an αC-helix swinging movement that locks the extremely closed conformation. The variants of the first two movements are noticed commonly for many kinases. However, the third swinging motion, acquiring an additional turn of the αC-helix at the N-terminus moving towards glycine-rich loop, independent of the αA-helix, is unique for the group II Pak family.

4.4 Regulators of the group II Paks

The regulatory mechanisms for group II Paks are different with the group I Paks. The group II Paks binds more efficiently to GTP loaded Cdc42 than to activated Rac. Pak4 and Pak6 show no increased kinase activity upon GTPase binding. The Pak5 AID fragments can inhibit kinase activity in the absence of Cdc42 binding [71], so it remains unclear about the functional significance of Pak5 and its interaction with activated cdc42. Indeed, the kinase domains of the group II Paks are reported to be more active in isolation than the full-length protein. It has also been suggested that binding to Cdc42 might be related to the proper localization of the group II Paks rather than kinase activation [32].

Pak4, as the first identified group II Paks member, attracted the most attention. Several Rho GTPase independent activation mechanisms of Pak4 will be addressed below. In MDCK epithelia cells, Pak4 is activated by Hepatocyte Growth Factor (HGF) via a signaling pathway that requires PI3 Kinase [33]. 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 [72]. Pak4 was also shown to interact with the cytoplasmic domain of the keratincyte growth factor (KGF) receptor in a transformed kidney cell line, and may have a role in KGFR-mediated cell survival pathways [73]. Finally Pak4 was shown to interact with integrin αvβ5 [74], and in the breast cancer cell line MCF7, it redistributes from the cytosol to lamellipodial structures when cells are attached to vitronectin [74]. Pak4 has been found as a novel Gab1-interacting protein [75]. In response to HGF, Gab1 and Pak4 associate and colocalize at the cell periphery within lamellipodia. These data suggest that multiple types of signaling proteins may converge upon Pak4 regulation. Although emerging studies on group II Paks illustrates their significant roles in many fundamental cellular functions, we only began to understand the regulation of group II Paks. Many more studies will be necessary to shed light on the regulation of group II Paks *in vitro* and *in vivo*.

5. Biological Functions of the Paks:

Paks function as dynamic control centers that transmit signals from upstream modulators, including G-protein-coupled receptors (GPCRs), integrin cell-adhesion complexes, growth factor receptors and bioactive lipids of various signaling cascades. Then activated Paks phosphorylate their substrates, which in turn activate various biological functions. Although the group I and group II Paks differ from each other in the sequence as well as the mechanisms of activation, they also share functional similarities. Multifunctional regulatory roles of group I and group II Paks will be addressed in detail as follows.

5.1 Cytoskeletal organization and cell motility:

Cell motility, migration and invasion are tightly related with actin cytoskeletal reorganization. Filopodia, lamellipodia, and stress fibers are cell structures that involve actin cytoskeleton reorganization and cell motility, which have been summarized in a review by Dr. Mattila and Dr. Lappalainen (See **Figure 3**) [76]. Filopodia, or microspikes, are long, thin and transient processes that extend out from the cell surface. Bundles of parallel actin filaments, with their plus ends oriented toward the filopodia tip, are cross-linked within filopodia by a small actin-binding protein such as fascin. Lamellipodia are thin but broad projections at the edge of a mobile cell. Lamellipodia are dynamic structures, constantly changing shape. Lamellipodia, at least in some motile cells, have been shown to contain extensively branched arrays of actin filaments, oriented with their plus ends toward the plasma membrane. Stress fibers form when a cell makes stable connections to a substrate. Bundles of actin filaments extend from the cell surface through the cytosol, and myosin mediates sliding of anti-parallel actin filaments during contraction of stress fibers. Among Paks, Pak1 and Pak4 are the major modulators of cytoskeletal reorganization to regulate above cell structures and stimulate cell motility, migration, and invasion. Regulation of actin dynamics by Pak4 has been summarized in **Figure 4**.

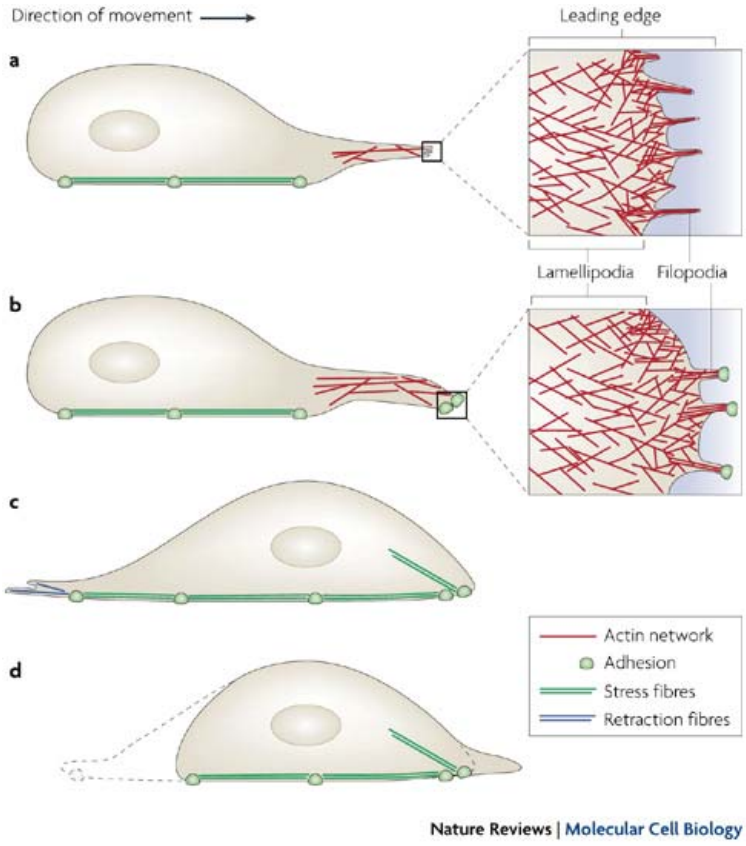


Figure 3. Cell migration is dependent on different actin filament structures [76]. a) Motility is initiated by an actin-dependent protrusion of the leading edge, which is composed of lamellipodia and filopodia. These protrusive structures contain actin filaments, with elongating barbed ends orientated towards the plasma membrane. **b)** During cellular extension, new adhesions with the substratum are formed under the leading edge. **c)** Next, the nucleus and the cell body are translocated forward through actomyosin-based contraction forces that might be mediated by focal adhesion-linked stress fibres, which also mediate the attachment to the substratum. **d)** Then, retraction fibres pull the rear of the cell forward, adhesions at the rear of the cell disassemble and the trailing edge retracts.

5.1A Pak1 in cytoskeletal organization

PDGF [77], insulin, and certain other cell stimuli cause the redistribution of Pak1 from the cytosol into cortical actin structures, such as lamellae at the leading edge, circular dorsal ruffles, and peripheral dorsal ruffles. Pak1 controls cytoskeletal reorganization inducing lamellipodia, filopodia and membrane-ruffle formation through various substrates, including LIMK [78-80], coflin, myosin light chain kinase [81], Op18/stathmin [58], p41-ARC (actin-related protein 2/3 complex 41 kDa subunit), and filamin. Pak1 activates LIMK by phosphorylating it on Thr508 in the kinase activation loop. Active LIMK then phosphorylates the actin binding protein cofilin and inhibits its activity. Inhibition of coflin will promote integrity of the actin filament network in the lamillopodium and cell protrusion efficiency.

Leading edge microtubule dynamics also play a role in cell motility. In the protruding edge of migrating cells, microtubules exhibit decreased catastrophe frequency and increased net growth. Local regulation of Op18/stathmin, a protein that inhibits tubulin polymerization, could account for this [58]. Rac1/Pak1 signaling appears to negatively regulate Op8/stathmin. Pak1 an also phosphorylates stathmin at Ser16 in vitro, a site which downregulates its inhibitory activity on tubulin polymerization [58].

Myosin is another component of the cytoskeleton that is involved in cytoskeletal dynamics. Phosphorylation of the light chains promotes the assembly of myosin into bipolar filaments that generate tension on the actin and bundle actin filaments into stress fibers. Myosin light chain kinase (MLCK), the kinase phosphorylating myosin light chains, is a substrate for Pak1 [81]. Phosphorylation of MLCK by Pak1 decreases its activity, which in turn results in decreased myosin light chain phosphorylation and a

decrease in actin-myosin filament assembly. Studies in inducible, stable NIH3T3 cell lines and in transfected human microvascular endothelial cells showed that expression of active Pak1 enhance overall cell contractility associated with increased phosphorylation of myosin II regulatory light chain [82].

5.1B Pak4 promotes filopodia and lamellipodia formation

We have found that Pak4 promotes filopodia formation in response to activated Cdc42. This effect is dependent on its kinase activity, and on its binding to Cdc42 [32]. Our results suggest that Pak4 is an important link between Cdc42 and filopodia formation. However, there are no reports that knockdown of Pak4 expression prevent filopodium formation. The role of Pak4 in filopodium formation need to be further investigated. Pak4 was shown to translocate to membrane ruffles and lamellipodia following the stimulation of MDCK cells with HGF [33]. A recent finding demonstrated that Pak4 is a specific Gab1 effector downstream target of the Met receptor [75]. The recruitment of Pak4 to Gab1 provides a mechanism to modulate subcellular localization of Pak4. The Gab1 scaffold protein is recruited to membrane ruffles and lamellipodia in response to HGF, and this is dependent on an intact Gab1 PH domain that binds to phosphoinositide 3,4,5 triphosphate (PIP3) phospholipids, in a PI3K-dependent manner.

5.1C Pak4 promotes stress fiber dissociation

Pak4 leads to the dissociation of stress fiber and cell rounding with subsequent loss of focal adhesions. Pak4-induced dissociation of actin stress fibers is not mediated by phosphorylation of myosin light chain or myosin light chain kinase, as has been reported

by Pak1. Pak4-induced stress fiber dissociation is possibly due to its interaction with RhoA GEFs [83]. RhoA induced stress fiber are associated with focal adhesion, and overexpression of GEF-H1 has been shown to induce focal adhesion. As a consequence of its ability to both bind to microtubules and activate RhoA, it has been postulate that GEF-H1 may provide a link between microtubules and actin cytoskeleton. Overexpression of activated Pak4 has been shown to reduce the level of RhoA activity in cells, and Pak4 is reported to phosphorylate GEF-H1 at Ser885, and another study showed that Ser885 phosphorylation of GEF-H1 inhibits RhoA-exchange activity. It might therefore be that Pak4 inhibition of GEF-H1 and a subsequent decrease in the level of active RhoA could lead to the disruption of focal adhesion. However, no reports showed Pak4 directly regulates the level of GTP-RhoA in cells via GEF-H1. Further studies of the relationship between Pak4 and GEF-H1 are required to elucidate how Pak4-mediated phosphorylation affects GEF-H1 localization and activity.

5.1D Pak4 promotes actin filament reorganization and cell migration

Pak4 can regulate the dynamic process of actin depolymerization [84]. Slingshot (SSH) phosphatases and LIMK regulate actin dynamics via a reversible phosphorylation (inactivation) of serine 3 in actin-depolymerizing factor (ADF) and cofilin. Pak4 was demonstrated that being with a multi-protein complex consisting of SSH-1L, LIMK1, actin, and the scaffolding protein, 14-3-3ξ, in the regulation of ADF/cofilin activity. Phosphatase activity of purified SSH-1L is F-actin dependent and is negatively regulated via phosphorylation by Pak4. 14-3-3ξ binds to phosphorylated slingshot, decreases the amount of slingshot that co-sediments with F-actin. At the present time, Pak4 is the only

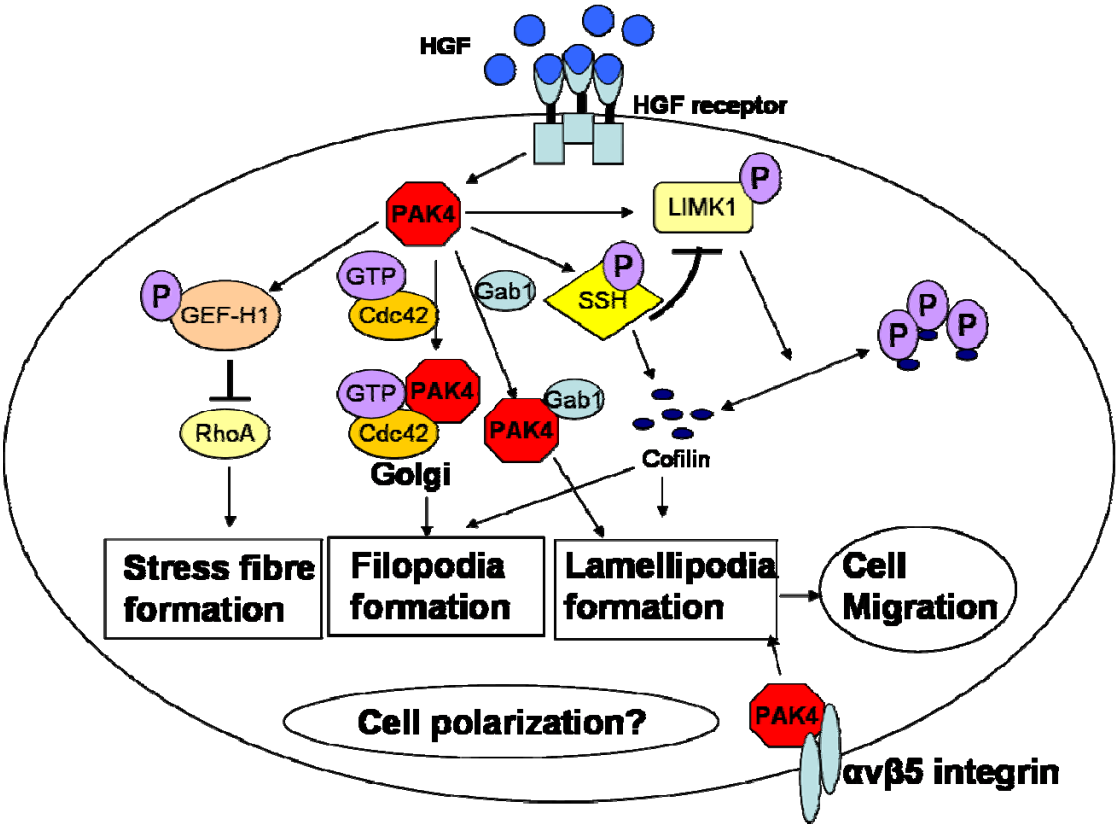


Figure 4. Regulation of actin dynamics by Pak4. Details of the pathways are described in the text.

member of the Pak family involved with this new mechanism for the regulation of ADF/cofilin activity in mediating changes to the actin cytoskeleton. Pak4 and Pak5 also mediate cytoskeletal changes necessary for promoting neurite outgrowth in neuroblastoma cells [28-29], possibly by inhibiting Rho GTPases activation. In the event of cell migration, interaction of Pak4 with the intracellular domain of αvβ5 integrin regulates carcinoma cell motility in a novel integrin-specific manner [74].

5.2 Cell cycle

Paks are likely to be involved in the progression through the different phases of mitosis. In the early phase of mitosis Pak1 may have a role in chromosome condensation. Either directly or indirectly, Pak1 has a regulatory role in chromosome condensation, duplication of the microtubule-organizing centre [58], spindle attachment and maturation and, possibly the coordination of chromosome segregation and cytokinesis. Pak1 phosphoryates Histone H3 on Ser10 [85], an event that is required for the initiation of chromosome condensation. In interphase, a subset of cellular Pak1 is localized in the nucleus. Pak1 directly associates with specific gene promoter and enhancer elements, exerting both positive and negative transcriptional regulatory control.

Pak1 appears to have a critical role during cell cycle progression, and its kinase activity peaks at mitosis entry and remains sustained during mitotic progression. Pak1 localizes to specific structures during mitosis, including chromosomes, centrosomes, mitotic spindles, and the contraction ring during cytokinesis. Overexpresion of activated Pak1 in MCF-7 breast cancer cells leads to abnormal centrosome number and spindle organization, and consequently to aneuploidy. Tubulin cofactor B (TCoB), a cofactor in the assembly of tubulin, was identified as an interacting substrate of Pak1 [86]. Pak1 phosphorylates and colocalizes with TCoB on newly polymerized microtules and centrosomes [87]. Regulation of Cyclin D1 expression may be another mechanism, by which Pak1 promotes cell cycle progression [88-89].

The protein kinases Aurora-A [90] and Polo-like kinase 1 (Plk1) [91] are two other important regulators of mitotic events that are phosphorylated by Pak1. As cell near the M phase, Pak1 is recruited to the centrosomes where it interacts with a GIT1-PIX

complex, interaction with GIT1-PIX activates Pak1 independently of the small GTPases Cdc42 or Rac. Activated Pak1 subsequently phosphorylates Plk1and colocalizes Plk1 on the spindle poles, the central spindle, and the midbody. Pak1-mediated phosphorylation of Plk1 is important in establishing a functional bipolar spindle.

5.3 Cell growth signaling and transformation:

Many studies have implicated Paks in the signaling pathways involved in growth and/or transformation of cells. Anchorage independent growth is an important hallmark of oncogenic transformation. While normal adherent cells stop growing or die when they are not attached to a surface, cancer cells can survive and proliferate when they detached, leading to anchorage independent growth, and often to metastasis.

5.3A Group I Paks promote cell growth and/or transformation

An early study by Field and colleagues demonstrated that the expression of kinase-deficient Pak1 mutant prevented the transformation of Rat-1 fibroblasts by the Ras oncogene [92]. Active Pak1 themselves were not sufficient to induce transformation, although another study observed that Pak1 could synergize with Ras signaling to enhance transformation [93]. The effect of Pak1 to enhance transformation could be correlated with effects of Pak1 on signaling through Erk (mitogen-activated protein kinase) MAPK pathways and dissociable from effects on the JNK or p38 MAPK pathways. Pak1 activation by membrane targeting resulted in stimulation of coexpressed p38, JNK, and Erk1. It also has been reported that the observed synergy between Rho GTPase and Ras in activating Erk required active Pak.

Pak1 has been implicated in the development of anchorage-independent growth by cells that were cultured in soft agar. Pak1 kinase targets such as DLC1 promote breast epithelial cell anchorage-independent growth and tumor formation [94]. When DLC1 is phosphorylated by Pak1 on Ser88, ZR75 breast cancer cells undergo anchorage-independent growth and become highly tumorigenic.

5.3B Pak4 promotes cell growth and anchorage independent growth

Pak4 functions down stream of integrin [74] and HGF receptor [72] mediated signaling pathways that are involved in oncogenic transformation. Pak4 phosphorylates Raf1 and activates the Erk pathway downstream of Raf thus playing a role in cell growth [95]. Our lab has found that Erk and NF-κB pathways lie downstream to Pak4 in TNFα signaling [96]. Since both Erk and NF-κB pathways are important in cell growth and cell survial, the regulation of these two pathways by Pak4 will be addressed in the following sections which address the role for Pak4 in cell survival.

Our lab has found that activated Pak4 promotes anchorage independent growth in immortalized fibroblast, as efficiently as oncogenic Ras, a very strong oncogene [97]. Consistent with this effect, dominant negative Pak4 partially inhibits focus formation in response to oncogenic Dbl in fibroblasts [97], and in some cells it also inhibits transformation by oncogenic Ras [98]. Pak4 null cells have a decreased capacity to be transformed by oncogenic Ras [98] and Dbl [97].

5.4 Cell death and survival signaling:

Apoptosis, or programmed cell death, is a fundamental process in the development of multicellular organisms. It is generally mediated by caspase cascades, and cell survival pathways can be mediated by proteins which inhibit the caspase cascade at various stages. Apoptosis begins with binding to cell surface receptors, and in turn the receptors trimerize. The trimerized receptors recruit a number of proteins through their protein-protein interaction motifs, and these proteins lead to the activation and cleavage of the initiator caspases, such as caspase 8 and caspase 10 [99-100]. The signaling complex II, death-inducing signaling complex (DISC) [101] is the major recruited protein complex that leads to caspase activation. The complex II that forms on the cytoplasmic side of the receptor, and this signaling complex comprised by TRADD, RIP, and FADD [102-103]. The signaling complex I is another recruited protein complex that function to block caspase activation. It assembles as a result of TNFα receptor activation and comprises TRADD, TNFR1, RIP and TRAF2 [104-106]. Following complex I formation at the TNFR1, the NF-κB pathway, which is the major survival pathway, is activated [102, 104, 107-108], and activation of the NF-κB pathway leads to increased expression of several antiapoptotic proteins such as FLIP and c-IAP [106], which can bind to complex II. If the NFκB pathway is fully activated and sufficient amounts of FLIP and C-IAP are presented in complex II, the activation of caspase 8 will be blocked, and the cell will survive [96]. Therefore, either direct activation of the complex I, or disruption of the complex II and in turn full activation of the complex I is a critical initiator of caspase 8 or 10 activation. Once activated, caspase 8 can activate two different apoptotic pathways. First, it can directly cleave and activate effector caspases, such as caspase 3, and 7. Effector caspases then cleave target proteins, which result in cell death [109-110]. Second, caspase 8 can

activate a mitochondria pathway by cleaving its substrate Bid [111-114], and the truncated Bid translocates to the mitochondria, where it interacts with members of the B-cell lymphoma protein 2 (Bcl-2) family to promote cytochrome c release. Release of cytochrome c from mitochondia leads to activation of caspase 9 followed by cleavage and activation of caspase 3, leading to cell death [115].

The mitogen-activated protein kinase (MAPK) cascade is considered to be an importent network involved in the integration of signals for modulating cell survival, as well as other cellular events [116]. MAPK is activated by a MAP kinase kinase (MAP2K, MEK), and MAP2K is activated by a MAP kinase kinase kinase (MAPKKK, MEKK, MAP3K). At present, four MAPK cascades have been described, including extracellular signal-regulated (Erk1/2), c-Jun NH_2-terminal kinase (JNK), p38 and Erk5/BMK. The Erk MAPK cascade is a highly conserved signaling pathway throughout eukaryotic cells, and the Erk cascade can be initiated by numerous growth factors and oncogenes, such as the small G protein Ras. Ras recruits the Raf (MAP3K) from the cytosol to the cell membrane, causing its activation by yet unknown kinases. Raf forwards the signal to MEK (MAP2K), which in turn phosphorylates and activates Erk (MAPK) [117].

5.4A Group I Paks promote cell survival

Multiple mechanisms exist through which Pak1 promotes cell survival. In the presence of a cell survival signal, Pak1 directly phosphorylates proapoptotic protein Bad at Ser112 and Ser136 [118]. This disrupts Bad interactions with Bcl-2 and Bcl-xl on the mitochondria. Bcl-2 or Bcl-xl that are not in complex can suppress cell death response by blocking the release of mitochondrial cytochrome c. In addition, Pak1 interacts with

dynein light-chain 1 (DLC1) [94], which typically sequesters BimL and results in activating the survival functions of Bcl-2. However, in the presence of apoptotic stimuli, Pak1 phosphorylates DLC1and BimL and triggers their degradation, thus blocking the pro-apoptotic signal of BimL. Pak1 can also inhibit apoptosis by phosphorylating and inactivating FKHR [119].

Pak2 has dual functions and regulates both cell survival and cell death pathways. Under cellular stress and serum starvation condition, Pak2 is activated by cleaved caspase3 to generate a proteolytic fragment, Pak2-p34, thereby promoting cell morphology changes characteristic of apoptosis [54]; thus Pak2 performs an antagonistic role in cell survival. On the other hand, activation of full-length Pak2 also promotes cell survival by phosphorylating Bad and reducing the interaction between Bad and Bcl-2, which leads to cell survival as described above for Pak1 [120].

5.4B The role for Pak4 in activating anti-apoptotic signals

Like Pak1, one way Pak4 may protect cells from apoptosis is by phosphorylateing Bad. Unlike Pak1, however, Pak4 phosphorylates Bad specifically at Ser112 and inhibits Bad interaction with Bcl-xl and Bcl-2, which results in turn prevents cytochrome c release, thus promoting cell survival [121]. Moreover, when overexpressed, Pak4 is associated with protection in response to TNF-α treatment, UV irradiation, or serum withdrawal. Pak4 has also been found to inhibit caspase-3 activation [96].

5.4C The role for Pak4 in inhibiting pro-apoptotic signals

Pak4 functions to promote cell survival not only through activating anti-apoptotic signals, but also through inhibiting pro-apoptotic signals. Pak4 operates by a kinase independent mechanism when cells are stimulated by the activate death domain containing receptors, such as TNF or Fas ligand [96]. In this case Pak4 inhibits the formation of the complex II DISC, consequently caspase 8 recruitment and activation are blocked, which will result in inhibiting death signals emanating from the activation of death domain containing receptors such as the TNFR1 and Fas receptors.

5.4D The role for Pak4 in activating the MAPK pathway

Cellular-senescenece stimuli, such as DNA damage, chromatin remodeling and strong mitogenic stimuli, trigger Pak4 upregulation, promoting premature senescence through the Erk MAPK pathway and also through the action of the cell-cycle regulatory proteins, p16INK4 and p19ARF [95]. MAPK activity is strongly stimulated in Pak4-expressing cells. It is noteworthy that expression of RasV12 leads to increase in the level of Pak4 expression in primary cells and that Pak4 was found to phosphorylate and activate Raf at Ser338[95]. Furthermore, the Erk pathway was abrogated in TNFα-treated Pak4 null cells [96]. Pak4 thus activates the Erk pathway downstream of Raf, firmly placing Pak4 in a Ras-Raf-MAPK pathway.

5.4E The role for Pak4 in activating the NF-kB pathway

In addition to the Erk pathway, the NF-κB pathway also plays important roles in cell survival and growth. Our lab has demonstrated that endogenous Pak4 is required for the full activation of prosurvival pathways induced by TNFα [96]. Upon activation of the

TNFα receptor, the cell responds either by undergoing apoptosis or by activating survival pathways. For the cell to survive, full activation of the survival pathways triggered by complex I is critical. The results indicate that Pak4 is required for optimal binding of the scaffold protein TRADD, one of the complex I proteins, to the activated TNFα receptor through both kinase-dependent and kinase-independent mechanisms [122]. Consequently, activation of several prosurvival pathways, including the NF-κB and Erk pathways, is reduced in the absence of Pak4. Interestingly, constitutive activation of the NF-κB and Erk pathways could compensate for the lack of Pak4, indicating that these pathways function downstream to Pak4 [96]. In addition to the NF-κB pathway, other pathways, including the JNK, and p38 pathways, are also activated downstream to complex I. This suggests that Pak4 functions at a level upstream to all of these pathways during cell survival.

5.5 Angiogenesis

Angiogenesis is the development of the smaller branches (capillaries) of the circulatory system. Endothelial cells must respond to external signals and in turn promote cell proliferation and cytoskeleton rearrangements. These change lead to increased endothelial cell motility, alterations of cell morphology, and the formation of tubes that develop into blood vessels [123]. There are several of the proteins which modulate direct endothelial cell motility and morphology in angiogenesis. These include the Rho GTPases (Rho, Rac, and Cdc42) [124]and Paks [125]. Kiosses et al. were the first to provide an in depth study of the role of Pak1 in endothelial cells using microinjection of 14 mutant, truncation, and wild type forms of Pak1 [125]. They found that both activated

and dominant negative forms of Pak inhibited endothelial cell migration compared to wild type, suggesting the essential role of wild type Pak in endothelia cell migration. In a separate study, introduction of a portion of the N-terminal Nck-binding domain of Pak1 inhibited endothelial cell migration and tube formation in vitro [126]. Strikingly, the same peptide blocked angiogenesis in the chick chorioallantoic membrane assay. These results suggest an important role for the Nck adaptor in coupling Pak1 to angiogenic growth factor signaling. Additional supporting evidence for the function of Pak1 in angiogenesis comes from studies in which the recruitment and activation of Pak1 via Nck to the Tek/Tie-2, an endothelial cell specific receptor, potentiated the ability of angiopoietin family of growth factor ligands to stimulate endothelial cell motility [127]. Pak1 activated in breast cancer epithelial cells by heregulin-β1 [128], can also enhance angiogenic response by inducing the up-regulation of VEGF expression.

Pak2 and Pak4 were found to act downstream of protein kinase C (PKC) to stimulate endothelial cell lumen and tube formation [129]. The signaling events that regulate their influence have remained unclear. Our lab recently also found that the Pak4 knockout yolk sac did stain positively for endothelial cells, as indicated by the presence of the vascular plexus, but did not have organized vessels. This data suggests that Pak4 affects angiogenesis and branching, rather than the initial formation of vessels [42].

6. Paks and Pathological Conditions

Paks have been shown to regulate diverse cellular activities, including cytoskeletal organization, cell cycle control, cell growth, cell survival, and angiogenesis. Not

surprisingly, the Paks are also associated with several human pathological conditions, including neurological disorders, infection, and cancer. These three pathological conditions will be addressed in detail below.

6.1 Neurological disorders

Investigations of group I and II Paks reveal their importance in neuronal development and neuro-pathological conditions. Neurofibromatosis patients are predisposed to the development of multiple tumors of the central and peripheral nervous system. Neurofibromatosis types 1 and 2 (NF1 and NF2) are dominantly inherited autosomal diseases caused by the loss-of-function mutation of the tumor suppressor genes NF1 and NF2, respectively [130]. The product of the NF1 gene called Neurofibromin widely expressed across a range of tissues but with high concentration in the nervous system. Neurofibromin is a GTPase activating protein and acts by accelerating the intrinsic GTPase activity of Ras. Consequently, loss of neurofibromin is associated with increased levels of activated GTP-bound Ras, which activated oncogenic pathways, including the MAPK cascade and PI3 kinase. Downstream signals of PI3 kinase activate Pak via Rac and Cdc42. Therefore, loss of gene NF1 or NF2 product can lead to abnormal activation of Pak1. The product of NF2 gene is called Merlin. Merlin is a cytoskeleton-associated tumor suppressor, and structurally related to the moesin/ezrin/radixin proteins. These proteins link the actin cytoskeleton to cell surface glycoproteins that control growth and cellular remodeling. The growth suppressive function of Merlin depends on its phosphorylation status at Ser 518. Both cAMP-dependent protein kinase A and Pak1 are able to phosphorylate Merlin at ser518 and

thereby inhibit its growth suppressive activity [131-132]. There is also an important inhibitory feedback mechanism from Merlin to Pak. Phosphorylation at ser518 induces a conformation change in Merlin and consequently disrupts interaction with Pak1, allowing Pak1 to be activated. Thus, in NF2 patients, loss of Merlin is associated with abnormal Pak1 activity. Taken together, loss of NF1 or NF2 can lead to abnormal Pak1 activity, which lead to elevated levels of Rac as well as pronounced cell ruffling [131]. Dominant negative Pak1 mutants were shown to be potent inhibitors of Ras transformation in rat Schwann cells [133].

In 2007, Pak1 was reported to rescue the adverse effects of fragile X syndrome (FXS) [134], the most commonly inherited form of mental retardation and autism. In Hungtington disease (HD), Pak1 binds and mediates the aggregation of the hungtingtin (htt) protein, thereby increasing its toxicity [135]. In humans, loss-of-function mutations in Pak3 are associated with nonsyndromic X-linked mental retardation [136]. Furthermore, three Pak3 mutations were identified that cause mental retardation and elicit profound defects on spine morphogenesis and density [26]. Similarly, Pak3 knockout mice also exhibit remarkable abnormalities in synaptic plasticity, specifically hippocampal late-phase long-term potentiation and deficiencies in learning and memory [27].

The group II Paks, Pak4 and Pak5, are enriched in the neural crest, which gives rise to multipotential stem cells. These stem cells are precursors of various cell and tissue types and are vital for vertebrate development. In Xenopus laevis embryonic ectoderm, Pak4 is involved in restructuring cytoskeletal organization and directing cell adhesion during craniofacial development [25]. In Parkinson's disease brains, amyloid–β oligomer

protein species, key mediators of memory and cognition deficiency in Alzheimer's disease, were reported to suppress Pak4 autophosphorylation. [137]. The consequence of Pak4 suppression is a decrease in the phosphorylation of its substrate, LIMK1 [79], which regulates cytoskeletal organization and filopodia formation that is essential for neurite outgrowth and neuronal migration. The Pak5 catalytic domain binds and inactivates MAP/microtubule affinity-regulating kinase 1 (MARK1) [138], a crucial component required for the establishment of embryonic polarity. This molecular interaction requires stringent regulation because activated MARK phosphorylates Tau. Tau hyperphosphorylation can initiate tauopathies, a group of diverse dementias and movement disorders caused by the intracellular accumulations of abnormal filaments of Tau protein [138]. Thus, Paks are targets for neurotoxic protein oligomers, potentially contributing to the development of neurodegenerative diseases.

6.2 Infection

Many bacterial virulence "effector" proteins mimic cellular GTPases. Consequently, as downstream effectors of GTPase, the Paks propagate their signal during invasion and survival within the mammal cells. Many studies have implicated the group I Paks and Pak4 in pathways involving viral and bacterial infections. Group I Paks participate in HIV replication and infection. The HIV accessory protein, Nef, is a membrane-associated protein required for the development of AIDS [139-140]. Three major functions of Nef include: enhancing viral particle infectivity, reduction of cell surface expression of the CD4 and MHC I molecules and induction of cell signaling pathways leading to cytoskeletal rearrangements, JNK activation, and enhanced or decreased apoptotic

responses. Pak1 and/or Pak2 was identified as a Nef-binding kinase in various studies [141-142], and these Nef-associated Paks appear to mediate the effects of Rac and Cdc42 to promote viral replication and pathogenesis of HIV [143]. On the other hand, a central role for Pak1 in macropinocytosis, an efficient innate immunity mechanism, has been demonstrated by other studies. In response to cellular stimuli during infection, large plasma-membrane domains are engulfed, subsequently clearing bacterial infection and apoptotic bodies. This endocytic pathway is used by host cells to manage human adenovirus serotype 3 (Ad3) infection, a disease associated with epidemic conjunctivitis as well as fatal respiratory and systemic disease. During Ad3 infection, Pak1 activation triggers phosphorylation of C-terminal-binding protein (CtBP), a nuclear transcription factor that regulates innate immunity, membrane fission and apoptosis. Pak1-mediated CtBP/BARS phosphorylation is essential for fission of the macropinocytic cup, the functional unit involved in the viral degradation process [144]. In addition, activated Pak1 has been defined as a cellular factor essential for intracellular mature virus entry through macropinocytosis and membrane blebbing during vaccinia virus, a proto-poxvirus infection of the host cell [145].

Among the group II Paks, Pak4 is targeted by AKT (v-akt murine thymoma viral oncogene homolog 1) during Mycobacterium tuberculosis and Salmonella typhimurium infections, resulting in intracellular survival by controlling actin dynamics [146]. More studies are required to elucidate the roles of Paks in host-pathogen interaction, which will provide a new way for combating the infectious processes. Pak4 was identified as a key regulator of podosome formation in haemopoietic cells [147]. Podosomes are specialized

adhensive structures found in cells of haemopoietic lineage that are closely related to cancer-cell invadopodia.

6.3 Cancer

Accumulating evidence implicates Paks in oncogenic growth. In cultured cells, ectopic expression of a constitutively active form of Pak1 or Pak4 induces many phenotypic hallmarks of transformation, such as increased cell motility, anchorage-independent growth, and resistance to apoptosis. Pak family members have attracted a lot of attention for their roles in almost all aspects of oncogenesis, including regulation of cell cycle progression, cell survival, motility, angiogenesis and gene regulation. Over the years, the altered expression and activity of Pak family members in different types of cancers are found by many studies (See **Table 3**).

Gene amplification and alteration of upstream regulators may cause aberrant Pak signaling in cancer. Pak1 and Pak4 are localized to genomic regions which are frequently amplified in cancer cells [148-151]. There is little evidence for cancer cells having activating mutations in Pak genes. Among the Paks, Pak1 is the most established isoform being overexpressed in human cancers, and the biological consequences of increased Pak1 signaling have been studied extensively in breast cancer, which will be addressed in detail. The roles of other family members in cancer are beginning to be discovered, such as for Pak4.

Cancer type	PAK isoform	Type of alterations	References
Brain	Pak1	Increased phospho-Pak1 in cytoplasm.	[152]
Esophagus	Pak4	Protein and gene overexpression.	[153]
Breast	Pak1, Pak4,	Protein overexpression and increased nuclear localization; Gene amplification (11q13–>q14 amplicon).	[88, 148, 153-154]
Liver	Pak1	Protein and gene overexpression.	[155]
Kidney	Pak1	Protein overexpression and increased activity.	[156]
Pancreas	Pak4	Gene amplification (19q13 amplicon), protein overexpression.	[157]
Colon	Pak1, Pak4	Protein overexpression. Pak4 gene amplification (19q13 amplicon) and 2 somatic mutations.	[150, 153, 158]
Bladder	Pak1, Pak4	Gene amplification (11q13–>q14 amplicon).	[159] [Chapter III Figure1]
Ovarian	Pak1, Pak4	Protein overexpression and gene amplification (11q13–>q14 amplicon).	[149, 160-161] [Chapter III Figure1]
Prostate	Pak4, Pak6	Protein overexpression.	[31] [Chapter III Figure1]
T-cell lymphoma	Pak1	Gene amplification.	[162]

Table 3. Overexpression and/or activation of Pak family members in different types of cancers. Adapted from Bettina Dummler et al. 2009. Cancer Metastasis Rev 28:51-63.

6.3A Group I Paks and cancer

Overexpression and /or hyperactivation of Pak family members have been detected in various human tumors. The Pak1 isoform is over expressed in almost all types of cancers such as brain, breast, liver, kidney, colon, bladder, ovarian, and T-cell lymphoma. Pak1 expression is increased significantly with malignant progression of human colorectal carcinoma [150, 158]. Pak1 expression level is also found in correlation with the progression stages in a pre-malignant progression series of MCF10A mammary epithelial cell variants [163]. It has been reported that an inverse correlation between Pak1 expression in human cancer cells and the levels of endogenous microRNA miR-7. Moreover, transfection of miR-7 decreased Pak1 expression in breast cancer cells, and suppressed motility and invasiveness of these cells [164]. Abnormal regulation of Pak1 expression, localization and activity may contribute to the tumorigenic properties of certain types of cancers. Studies indicated that a dominant-negative Pak1 mutant inhibits transformation of murine fibroblasts transformed with several oncogenes including Ras, Rac1, Cdc42, or Vav3 [165].

Pak2 was found to act as a tumor repressor by negatively regulating of Myc oncoprotein [166]. Pak2 directly phosphorylates Myc and inhibits the ability of Myc to activate transcription, sustaine cellular proliferation, transform NIH3T3 cells in culture, and elicit apoptosis upon serum withdraw [166]. Pak2 can also be activated in response to transforming growth factor beta (TGF-β) [167-168]. TGF causes growth arrest in epithelial cells and proliferation and morphological transformation in fibroblasts, Pak2 activation is critical for the proliferative/profibrotic action of TGF-β on mesenchymal cells, and yet it is not responsive to TGF-β in epithelial cells. Pak2 also links to NF2 gene

product Merlin, which is mutated in NF2 patients and predisposes affected individuals to intracranial and pinal tumors [169]. Merlin can be phosphorylated by Pak2, leading to merlin relocalization from microvilli to larger membrane protrusions. Merlin inhibited Pak2 activation and recruitment to focal adhesions, while the absence of merlin resulted in inappropriate activation of Pak2. The regulatory protein that prevents inappropriate Pak2 activation in epithelial cultures was the epithelial-enriched protein Erbin. It controls the function of the NF2 tumor suppressor Merlin by determining the output of Merlin's physical interactions with active Pak2. Whereas mesenchymal TGF-β signaling induces Pak2-mediated inhibition of Merlin function in the absence of Erbin, Erbin/Merlin complexes bind and inactivate GTPase-bound Pak2 in epithelia.

6.3B Pak1 in breast cancer

In breast cancer, deregulation of Pak1 is well documented and correlates with increased invasiveness and survival of these cancer cells. More than 50% of human breast cancers display overexpression and/or hyperactivation of Pak1[88]. The molecular mechanisms by which Pak1 promotes mammary epithelial cell transformation have been extensively studied in 3D culture model systems.

Human breast cancer can be classified into two types, steroid hormone dependent or steroid hormone independent. Pak1 can promote cell survival in both types of breast cancer through its interaction with the steroid hormone estrogen receptor (ER). Phosphorylation of the pro-apoptotic proteins Bad [118] and forkhead transcription factor (FKHR) [119], and phosphorylation of dynein light chain 1 (DLC1) [94] are other mechanisms by which Pak1 may promotes breast cancer cell survival. Estrogen binding

to its receptor rapidly activated Pak1 kinase activity, which is independent of PI-3-kinase. Pak1 then directly phosphorylates the FKHR, which relocate FKHR from the nucleus to prenuclear in the cytoplasm and prohibit FKHR to activate its proapoptosis target fas ligand promoter. Pak1 can also promote cell survival through its association with the complex DLC1 and the proapoptotic Bcl2 family member BimL, both of which can be phosphorylated by Pak1 [94]. Phosphorylation of DLC1 and BimL by Pak1 prevents BimL from promoting apoptosis. Overexpression of DLC1 promotes cancerous properties of breast cancer cells and DLC1 protein level is elevated in more than 90% of human breast cancer. Pak1 can also directly phosphorylate ER and induce expression of endogenous ER target genes, which implies that Pak1 might also contribute to the estrogen independent type of breast cancer [119]. Holm et al. showed a mechanistic link between increased nuclear levels of active Pak1 and tamoxifen resistance in breast cancer [154]. Approximately 70% of all breast cancers express ER, and Tamoxifen is a selective antiestrogen medicine, which is widely used for treatment of this group of breast cancers. Pak1 is one of many kinases that phosphorylate ERα [170-171]. Deregulated activation of Pak1 produces multiple or inappropriate phosphorylation of ERα, creating a promiscuous receptor that is resistant to tamoxifen treatment and activates growth mechanisms in absence of estrogen [171]. The link between Pak1 and ERα raises the possibility that tamoxifen resistance might be prevented or reversed by Pak1 inhibition.

Activation of the transcription factor NF-κB appears to be a prominent mechanism by which Pak1 regulates survival of breast cancer cells. Friedland et al. showed a functional link between the resistance of mammary epithelial cells to apoptosis in 3D cultures and Pak1-mediated activation of NF-κB [172]. Notably, NF-κB also promotes

cell proliferation via CyclinD1 transcription in breast cancer cells. A study examined Pak1 activity in a pre-malignant progression series of MCF10A mammary epithelial cell variants. Pak1 expression levels increased in correlation with the progression stages in this series, indicating a role for Pak1 in the early stages of cell transformation [163]. Pak1 substrates that control different aspects of cytoskeletal dynamics, such as LIMK [173], p41-ARC [174], filamin A [175], Op18/stathmin [58], and TCob [86], are likely to promote the invasiveness of breast cancer cells. In addition, multimodular protein Scrib positively regulates activation of Pak1 and participates in lamellipodia formation at the leading edge of migratory breast cancer cells. Pak1 signaling has also been linked with components of the glycolytic pathway. Pak1 binds to, phosphorylates, and enhances the enzymatic activity of phosphoglucomutase 1 (PSG) [176], an important regulatory enzyme in cellular glucose utilization and energy homeostasis. This interaction of PGM with Pak1 is specific since PGM does not bind to Pak2. Pak3, Pak4. The connection of cell survival with glucose utilization by Pak1 provides a model for efficient energy utilization in cell survival. All these data suggest that Pak1 is an important mediator for cell survival function in breast cancer cells.

6.3C. Group II Paks and cancer

Studies now demonstrated that group II Paks, especially Pak4, influences cancer-cell behavior. The expression levels of Pak4 were tested in a panel of 60 tumor cell lines [98]. Remarkably, Pak4 mRNA levels are greatly up regulated in a panel of cell lines derived from leukemia, melanomas, breast cancer, colon cancer, lung cancer, liver cancer, ovarian cancer, and renal cancer, compared with a low level of expression in normal

tissues. We have also found that Pak4 is overexpressed in primary tumors [153]. These studies suggest that there is a link between Pak4 and tumorigenesis.

Constitutively activated Pak4 is highly transforming. Overexpression of activated Pak4 has been shown to induce anchorage independent growth on soft agar to the same level as the notorious oncogenic Ras [97], while expression of dominant-negative Pak4 was found to inhibit Ras-driven transformation in fibroblasts and cancer cells [97-98]. Pak4 is the only Pak-family member that is transforming when overexpressed in cells. Furthermore, Pak4 can also protect cancer cells from apoptosis. The sensitivity of Pak4-knockdown Hela cells to apoptotic stimuli was recovered by activation of the MAPK, but not the JNK, pathway [96]. There is also evidence that Pak4 can promote survival pathways via interaction with the TNFα receptor complex [122]. In addition to the role of Pak4 in cellular transformation, a specific role for Pak4 in prostate cancer-cell migration has been found [72].

Pak5 expression is mainly restricted to adult neuronal tissue and the role of Pak5 in cancer progression has not been fully investigated. One recent report, however, suggests that Pak5 is overexpressed in colorectal cancer and can promote cancer-cell invasion [177]. Pak6 interacts with androgen receptor (AR) and inhibits AR-mediated transcription responses [30]. Effective repression of AR signaling by Pak6 requires its kinase activity, but not GTPase binding to Pak6. It was proposed that elevated Pak6 activity may be responsible for regulation of AR signaling in various forms of prostate cancer. Although Pak6 expression in adult tissue is mainly restricted to the prostate and testis, Pak6 is overexpressed in a number of cancer cell lines, particularly breast and prostate cancer. Moreover, in prostate cancer cell lines that have been hormone-depleted

over long periods, the level of Pak6 expression increases [31]. In contrast, Pak6 was identified as a gene that is hypermethylated in prostate cancer. Hypermethylation is thought to silence gene transcription, thus hypermethylated genes are often associated with inhibition of tumor growth and are otherwise known as tumor suppressors. Pak6 is known to act downstream of the AR, and overexpression of Pak6 may be significant in prostate cancer cells harbouring gain-of-function AR mutations. Pak6 is also activated by MKK6, whose level of expression is increased, and in prostate cancer, where overexpression is correlated with invasiveness in osteosarcoma [178].

7. Concluding Remarks on Pak4

Pak4 was the first identified member of the group II Paks [32]. It is a target for Cdc42, and undergoes autophosphorylation on Ser474. Pak4 is important in cell motility and cell survival. Expression of activated Pak4 in fibroblast leads to a transient induction of filopodia, dissociation of stress fibers, and loss of focal adhesions. One possible mediator of Pak4 induced stress fiber dissociation is GEF-H1, a guanine nucleotide exchange factor (GEF) for Rho. Pak4 specially phosphorylates Serine 810 and Serine 67 on GEF-H1 [83], and this is thought to inhibit its ability to activate Rho, consequently inhibiting stress fiber formation. GEF-H1 is involved in microtubule dynamics and when mutated causes oncogenic transformation. The reorganization of the actin cytoskeleton is dependent on Pak4 kinase activity and on its interaction with Cdc42 [32]. Pak4 also regulates cytoskeletal changes through the modulation of LIMK1 activity [79]. Activated Pak4 phosphorylates LIMK1 and stimulates its ability to phosphorylate cofilin. In

addition to its role in cytoskeletal organization, Pak4 can also promote cell survival through different pathways. It is noteworthy that expression of RasV12 leads to an increase in the level of Pak4 in primary cells, and Pak4 was found to phosphorylate and activate Raf, leading to Erk MAPK activation in primary cells [179]. Pak4 also phosphorylates the pro-apoptotic protein Bad, and delays caspase cleavage [121]. In addition, Pak4 promotes cell survival in response to TNFα treatment or UV irradiation through an exclusive Pak4 mediated pathway that involves inhibition of the caspase 8-dependent apoptotic cascade [122].

In *in vitro* studies, expression of an active Pak4 mutant has transforming potential, leading to anchorage-independent growth of NIH3T3 cells [97]. A kinase-inactive Pak4 efficiently blocks transformation by activated Ras and inhibits anchorage-independent growth of HCT116 colon cancer cells [98]. In humans, Pak4 is expressed at low levels in normal tissue, but its expression is upregulated in a large variety of human tumor cell lines [98] and human tumors [153].

The information above indicates that Pak4 may play an important role in tumorigenesis. While all previous studies of Pak4 were done in cultured cells, in this thesis, we focused on studying the role of Pak4 in tumorigenesis *in vivo*. To investigate whether overexpression of Pak4 is sufficient to induce tumor formation, the fibroblast cell line NIH3T3 and immortalized mouse mammary epithelial cells (iMMECs) were transfected with Pak4. We found that overexpression of Pak4 in both cell lines led to formation of tumors in athymic mice. Furthermore, overexpression of Pak4 in iMMECs led to changes in 3D acinar architecture, including decreased central acinar cell death, abrogation of lumen formation, and cell polarity alteration. The results suggest that Pak4

can promote cell survival and proliferation, as well as deregulate cell shape and polarity, as part of the oncogenic process.

To investigate whether Pak4 is necessary for tumorigenesis in response to oncogenes such as Ras and Cdc42. Pak4 conventional knockout 3T3 cell lines and Pak4 conditional knockout 3T3 cell lines transfected with Ras or Cdc42 were used. We found that in cells transfected with oncogenic Ras, tumors grew more slowly and to a smaller size when Pak4 was deleted. In cells transfected with Cdc42, tumor formation was almost completely abrogated when Pak4 was absent. These results would be consistent with the role for Pak4 as a Cdc42 effector protein involved in signaling pathways leading from Cdc42 and its activators to transformation, and its critical role in cell survival and inhibition of apoptosis. This work shows for the first time that overexpression of Pak4 in epithelial cells leads to tumorigenesis *in vivo*, and that Pak4 is necessary for tumorigenesis in response to certain oncogenes.

Chapter II

Pak4 Protein Kinase Play a Key Role in Cell Survival and Tumorigenesis in Athymic Mice

Abstract

Pak4 is a member of the B group of p21-activated (Pak) kinases, originally identified as an effector protein for Cdc42. Although Pak4 is expressed at low levels in most adult tissues, it is highly overexpressed in tumor cell lines. Here, we show that Pak4 is also overexpressed in primary tumors, including colon, esophageal, and mammary tumors. Overexpression of Pak4 also leads to tumor formation in athymic mice, whereas deletion of Pak4 inhibits tumorigenesis. Although a constitutively active Pak4 mutant was previously shown to promote oncogenic transformation in cultured cells, our results are the first to show that Pak4 also promotes tumorigenesis in experimental animals. Furthermore, these results show for the first time that not only constitutively active Pak4, but also wild-type Pak4, is transforming, when experimental animals are used. These results are highly significant because wild-type Pak4, rather than activated Pak4, is overexpressed in tumor cells. Our results suggest that overexpression or activation of Pak4 is a key step in oncogenic transformation, due to its ability to promote cell survival and subsequent uncontrolled proliferation. The finding that Pak4 is up-regulated in so many types of cancers indicates that Pak4 may play a vital role in a wide range of different types of cancer. This makes it an attractive candidate for drug therapy for different types of cancer.

Introduction

Normal development requires precisely regulated levels of cell survival, apoptosis, proliferation, and differentiation. Increased levels of cell survival, uncontrolled proliferation, or failure to differentiate are often associated with oncogenesis. Understanding the signaling pathways that control these cellular processes is essential for understanding the molecular basis of transformation. Protein kinases play key roles in the intracellular signaling pathways that regulate cell growth control. One group of protein kinases that has important roles in a number of different intracellular signaling pathways is the p21-activated kinase (Pak) family of serine/threonine kinases. The Paks were first identified as effector proteins for Cdc42and Rac, members of the Rho GTPase family. More recently, they have also been found to have Rho GTPase–independent activators. The Paks fall into two categories, group A and group B, based on their sequences and functions. The group A family includes mammalian Pak1, Pak2, and Pak3 [180-182], whereas group B includes Pak4, Pak5, and Pak6 [183]. All of the Paks have an amino-terminal regulatory domain and a carboxyl-terminal kinase domain, with a GTPase-binding domain within the regulatory domain. The group A and B Paks, however, differ significantly from each other in both sequence and function [183].

Among the group B Paks, Pak4 is highly expressed during development and is ubiquitously expressed at low levels in all adult tissues. In contrast, Pak5 and Pak6 are largely expressed in the brain. Pak4 was originally identified as a protein that promotes filopodia formation in response to activated Cdc42and it is an important link between Cdc42and filopodia formation [32]. Pak4 also leads to the dissolution of stress fibers and subsequent loss of focal adhesions, possibly due to inhibition of Rho activity [97].

Although Pak4 is expressed at low levels in most adult tissues, it is highly overexpressed in almost every tumor cell line that has been tested [98]. This is in sharp contrast to Pak6, which is highly expressed in a few adult tissues but is not overexpressed in most tumor cell lines [98]. This suggests an important role for Pak4 in cell growth, survival, and proliferation, all of which are important for tumorigenesis. In fact, we and others have found that like activated Cdc42 [184-186], a constitutively active Pak4 mutant promotes anchorage-independent growth when overexpressed in immortalized fibroblasts [97]. Anchorage-independent growth is an important hallmark of oncogenic transformation [97]. Although normal adherent cells stop growing or die when they are not attached to a surface, cancer cells can survive and proliferate even when detached, leading to anchorage-independent growth and often to metastasis. The transforming ability of activated Pak4 is quite dramatic. In fact, the constitutively active Pak4 mutant is as efficient as oncogenic Ras, a very strong oncogene, in promoting anchorage independent growth in cultured cells [97]. Consistent with this effect, dominant negative Pak4 partially inhibits the formation of anchorage-independent foci in response to oncogenic Dbl in fibroblasts [97], and in some cells it also inhibits transformation by oncogenic Ras [98].

The mechanism by which the activated Pak4 mutant transforms cultured cells is unknown, but one possibility is that its role in cell survival contributes to its role in transformation. When overexpressed, Pak4, as well as several other Paks, is associated with protection from apoptosis [121-122]. Conversely, cells lacking Pak4 have an increased susceptibility toward apoptosis [96]. Pak4 promotes cell survival by different mechanisms, depending on the stimulus. In response to serum withdrawal, Pak4 protects

cells via a kinase-dependent mechanism that is associated with its ability to phosphorylate the proapoptotic protein Bad [122], similar to Pak1, which also protects cells from apoptosis [57, 118]. In response to stimuli that activate death domain–containing receptors, however, such as tumor necrosis factor or Fas ligand, Pak4 functions via a kinase-independent mechanism. In this case, Pak4 inhibits the formation of the DISC complex that forms on the cytoplasmic side of the receptor, thereby inhibiting caspase-8 recruitment and activation [122]. Studies using cells that lack Pak4 have also shown that Pak4 has a role in activating cell survival pathways, which lead to nuclear factor-nB and extracellular signal-regulated kinase (Erk) activation [96]. It is interesting that although the activated Pak4 mutant is transforming in cultured NIH3T3 cells, wild-type Pak4 does not transform cells in culture [97]. Furthermore, although anchorage-independent growth in cultured cells is an important predictor of oncogenesis, there are many other criteria that define a cell as transformed. One very important requirement for defining cells as malignant is the ability to form tumors in experimental animals. Formation of tumors in athymic (nude) mice is often used as a test to determine whether cells are malignant and whether an oncogene is transforming. Here, we show that overexpression of Pak4 leads to tumor formation in athymic mice. We were also able to use our Pak4 knockout cells to show that Pak4 has an essential role in transformation downstream to activated Cdc42 and also plays a role in mediating transformation by oncogenic Ras. Interestingly, when using athymic mice as a model, even wild-type Pak4 is highly transforming. This is the first work to indicate a role for wild-type Pak4 in actually causing tumors to form when it is overexpressed. As part of this study, we also show that Pak4 is overexpressed in primary tumors, including colon, esophageal, and

mammary tumors, rather than only in tumor cell lines. Taken together, our results suggest that Pak4 is a causative agent in tumor formation and not simply a side effect of transformation. Because Pak4 is up-regulated in several types of cancers, our results suggest that it could serve as a general mediator of oncogenic transformation, playing a vital role in a range of different types of cancer.

Materials and Methods

Tumor Samples

Human esophageal squamous cell carcinomas were obtained from patients in Linzhou People's Hospital, Linzhou, Henan, China [187]. Human breast tissues were obtained from the Cooperative Human Tissue Network of University of Pennsylvania medical center. Mouse colon tumor tissue was obtained from azoxymethane-treated CF-1 female mice 44 wk old (33 wk after the last injection of azoxymethane). The dosage of azoxymethane was 10 mg/kg, subcutaneously, and weekly injections were given starting at 5 wk of age. Rat mammary tumor tissue was isolated from N-methylnitrosourea–treated Sprague-Dawley female rats [188].

Plasmids

pLPC-Pak4 WT, pLPC-Pak4 (K350M), and pLPC-Pak4 (S445N) are described before [97, 122]. To construct pLPC-Ras, HindIII/XhoI fragments from pCAN-Myc2-Ras were inserted into the HindIII/XhoI sites of the pLPC vector. Cdc42V12 was inserted into the EcoRI/XhoI sites of the pLPC vector. pLPC is a retroviral vector with a

puromycin resistance marker [97]. The activated Pak4 mutant [Pak4(S445N)] has a single point mutation in which serine 445 is changed to asparagines (S445N). This mutation stabilizes the catalytic loop of the kinase domain and causes Pak4 to have a constitutively high level of kinase activity [97].

Cell Culture, Transfection, and Establishment of Stable Cell Lines

Pak4 null fibroblasts were isolated from Pak4 null embryos at embryonic day (E10.5), as described [41]. Control fibroblasts were isolated from E10.5 wild-type littermates. All fibroblasts were cultured in DMEM (Invitrogen) containing 10% bovine calf serum. Eco Phoenix packaging cells were cultured in DMEM containing 10% fetal bovine serum. The medium was supplemented with 100 units of penicillin/mL, 100 μg of streptomycin/mL, and 1 mmol/L glutamine. Stable cell lines containing wild-type Pak4, activated Pak4, empty vector, or RasV12 were grown as described above in the presence of puromycin (2.0 μg/mL). Stable cell lines were generated by retroviral infection of the vectors, followed by selection with the selectable marker puromycin, as described [97]. Briefly, Phoenix packaging cells were transfected with empty pLPC vector, pLPC-myc-Pak4, pLPC-myc-Pak4 (S445N), or pLPC-myc-RasV12 by the calcium phosphate precipitation method. Supernatants containing the released viruses were collected from the packaging cells 2 d after transfection and filtered through a 0.45-μm pore size filter. The virus was then used to infect either NIH3T3 cells or Pak4 null or Pak4 wild-type fibroblasts. Cells were selected with puromycin (2.0 μg/mL). Expression of Pak4 or Ras was determined by Western blotting.

In vivo Tumorigenesis

Five-week-old Ncr nu/nu male mice were purchased from Taconic Farm. All animals were housed four to a plastic cage with filter top. The animal room was controlled at 20 ± 2°C, 50 ± 10% humidity, and a 12-h light/dark cycle. Fresh AIN-93G diet was replenished twice weekly. For all studies, the mice were allowed to acclimate at least 3 d after receipt of shipment. Before injection, the cells were washed with PBS, harvested by trypsinization, resuspended, and kept on ice until injection. Cells (1×10^6) in a 100 μL mixture containing Matrigel (BD Biosciences) and culture medium at a 1:1 ratio were injected subcutaneously to both flanks of the mice. This is the same concentration that was reported to be used for assessing the tumorigenicity of oncogenic Ras in nude mice [189]. Mice were monitored regularly until termination of the study. Tumor size (length and width) was measured by a caliper and calculated based on tumor volume = $0.5 \times \text{width}^2 \times \text{length}$ [190]. All animals were sacrificed by CO_2 asphyxiation, and tumors were harvested and weighed. Half of the tissue was fixed in 10% formalin for histopathology analysis and the other half was frozen in liquid nitrogen for Western blot analysis.

Histologic Examination

Tumor tissues from athymic mice were fixed in 10% formalin and then dehydrated gradually in alcohol. The tissues were embedded in paraffin and were sectioned at a thickness of 5 μm. The sections were stained with H&E for histologic evaluation.

Immunohistochemistry

A standard avidin-biotin peroxidase complex method was used in this study, as described [191]. In brief, after dewaxing and rehydrating, the slides were heated in a pressure cooker in sodium citrate buffer (0.01 mol/L, pH 6.0) for 3 min after reaching full pressure. Endogenous peroxidase was quenched using 3% hydrogen peroxide in methanol. Sections were then blocked for 1 h at room temperature in PBS containing 3% normal horse or goat serum, depending on the origin of the primary antibody. The sections were then immunostained with cleaved caspase-3 (1:200, Cell Signaling) or Ki-67 (1:50 DakoCytomation) antibodies overnight at room temperature. The antibodies were diluted in 10% goat serum. The sections were rinsed in PBS and incubated with a biotinylated secondary antibody and subsequently incubated in Vectorstain Elite ABC reagent for 30 min, using 3,3-diaminobenzidine (Vector Laboratories) as the chromogen. Sections were then counterstained for 2 to 3 min with hematoxylin (Sigma) and mounted with Permount.

Western Blot Analysis

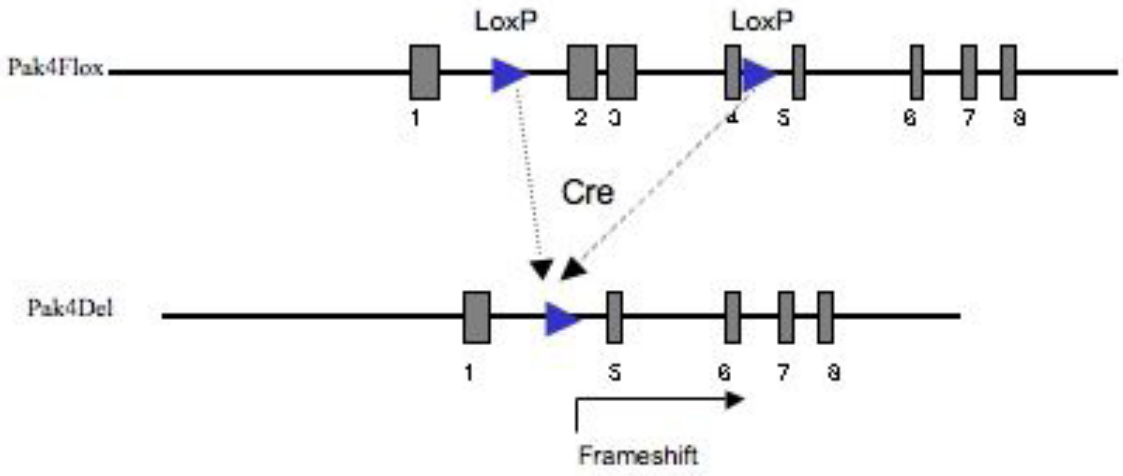
Western blots were carried out as described [32]. Horseradish peroxidase–conjugated secondary antibodies were from Sigma Western blot protein bands were visualized by the enhanced chemiluminescence method (Amersham).

Antibodies

Polyclonal Pak4 antibody, cleaved caspase-3 antibody, phospho-Erk, total Erk, phospho-p38, p38, and myc tag antibodies were from Cell Signaling. Mouse monoclonal Ras antibody was from Calbiochem.

Generation of Conditional Pak4 Knockout Cells

Ad-Cre, an adenovirus that expresses Cre recombinase [192], was prepared in 293 cells as described [193]. Immortalized mouse fibroblasts containing a floxed allele of Pak4 were used for these studies. These cells, referred to as Pak4Flox cells, have two LoxP sites flanking the Pak4 coding sequence (see **Supplementary Figure S1**). The cells were immortalized by serial passaging. Pak4Flox cells were infected with Cre recombinase adenoviruses as described [192]. The Cre recombinase causes the sequences between the LoxP sites to be deleted, resulting in a null allele of Pak4, referred to as Pak4Del. Cells were plated in a six-well plate and used when they were 70% confluent. Cells were incubated with AdCre in DMEM medium overnight at 37°C. After infection, cells were maintained in DMEM medium containing 10% fetal bovine serum, 100 units of penicillin/mL, 100 μg of streptomycin/mL, and 1 mmol/L glutamine. Pak4 protein expression was then evaluated by Western blotting to confirm the deletion of Pak4. Pak4Del is referred to as $Pak4^{-/-}$, and Pak4Flox is referred to as $Pak4^{+/+}$ in **Figure 6**.



Supplementary Figure S1

Supplemental Figure 1. The floxed allele of Pak4 is shown on top. When PakFlox cells are infected with a vector containing Cre recombinase, the result is recombination between the two LoxP sequences. The interventing sequences are deleted and a frameshift is produced so that exons 5-8 are no longer functional. The result is deletion of Pak4. We have included this supplemental material in reference to Figure 6.

Results

Pak4 Is Overexpressed in Tumors

Previous studies showed that Pak4 is overexpressed in cancer cell lines [98]. Cell lines, however, have gone through numerous passages and can be far removed from the actual primary tumor. We therefore cannot rule out the possibility that Pak4 overexpression is somehow a result of the culturing of these tumor cell lines. We were therefore interested in determining whether Pak4 is also overexpressed in primary tumors. To test this, we carried out Western blot analysis of primary tumor tissues from four sources. The first was primary human esophageal squamous cell carcinoma [187]; the second was mouse colon tumor tissue; the third was rat mammary tumor tissue [188]; and the fourth was human breast tumor tissue. For every condition, we also examined the corresponding normal tissue as a control. Although there was variability in the amount of overexpression, we found that in all of these types of cancer, there was a higher level of Pak4 protein in the tumor tissue compared with the normal control tissue (see **Figure 1**). These results indicate that Pak4 is in fact overexpressed in primary tumors in addition to nearly every tumor or cell line that has been tested. Thus, Pak4 seems to be broadly expressed in different types of tumors rather than limited to a specific type of cancer.

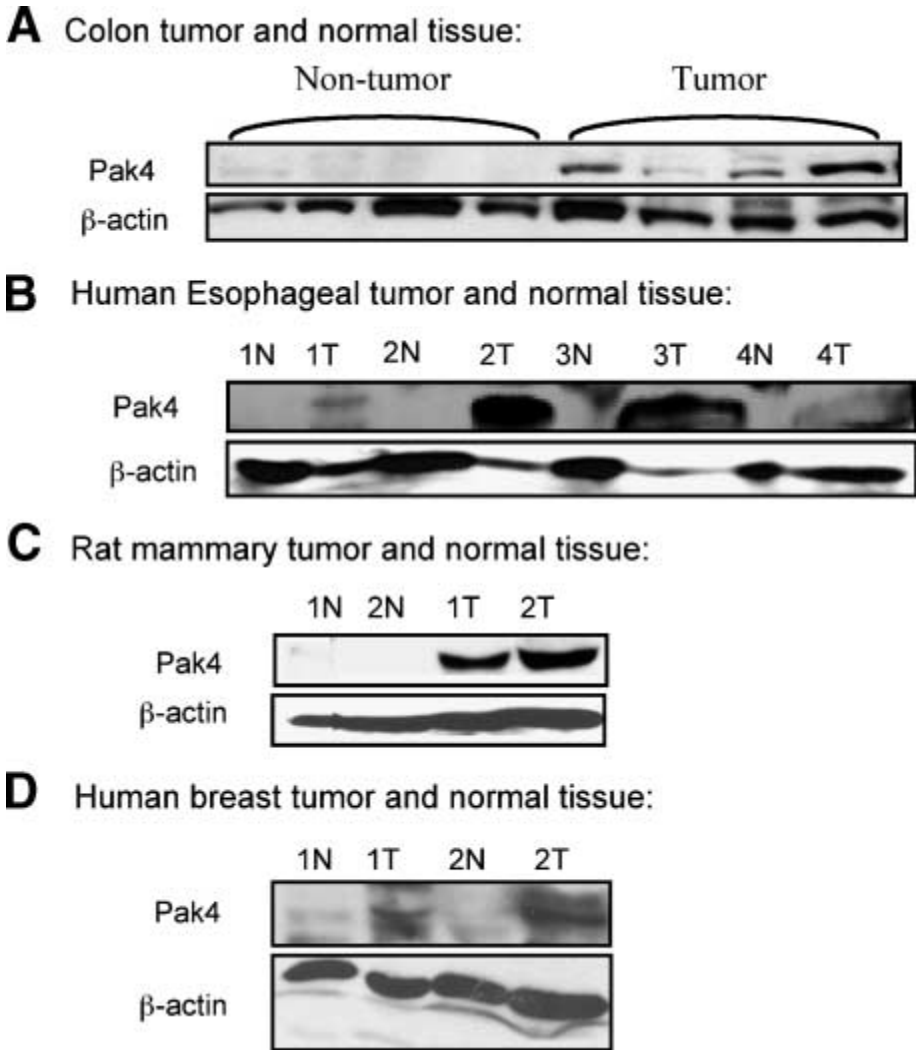


Figure 1

Figure 1. Pak4 is overexpressed in primary tumors. Western blot analysis illustrates Pak4 expression in tumor and normal tissues. The following tumor tissues were analyzed: A. Mouse colon tumor isolated from azoxymethane-treated CF-1 female mice; nontumor tissue was adjacent to normal tissue. B. Human esophageal squamous cell carcinoma (T) and normal tissues (N). Paired normal tissue was pathologically normal and from the

same patient. C. Rat mammary tissue was originally isolated from N-methylnitrosourea treated Sprague-Dawley female rats; normal tissue was isolated from nontreated rats. D. Human breast tumor and normal tissues. Paired normal tissue was pathologically normal and from the same patient. In all cases, 100 μg of protein extract were used; β-actin served as a loading control.

Overexpression of Pak4 Leads to Tumor Formation in Athymic Mice

We next wanted to determine whether Pak4 is sufficient to promote oncogenesis. We previously showed that a constitutively active mutant of Pak4 promotes anchorage-independent growth in cultured immortalized fibroblasts [97]. We did not, however, observe anchorage-independent growth in fibroblasts overexpressing wild-type Pak4. This was somewhat surprising because wild-type Pak4 promotes cell survival [121-122], which is associated with tumorigenesis. We therefore determined whether wild-type Pak4 promotes tumorigenesis in the athymic mouse system, in addition to the activated Pak4 mutant.

NIH3T3 cells that stably overexpress wild-type Pak4, activated Pak4, or empty vector (pLPC) [97] were injected subcutaneously into the flanks of athymic mice. Western blot analysis of a sample of each of these stable cell lines is shown in **Figure 2A**. The different cell lines were injected subcutaneously into the flanks of athymic mice to determine whether they led to tumor formation. After 16 days, we found that cells containing activated Pak4 formed large tumors in the mice, whereas the control cells [containing empty vector (pLPC)] and the cells overexpressing wild-type Pak4 did not

lead to tumor formation (see **Figure 2B**). By 44 days, however, the cells overexpressing wild-type Pak4 also formed large tumors. The control cells still did not form tumors at this time point (see **Figure 2C**) or at any time point tested. Tumor volumes and tumor weights at different time points are illustrated in **Figure 2D** and **E**. From these results, it is clear that both wild-type and activated Pak4 can promote tumor formation in athymic mice.

Tumors Formed by Pak4 Have a Morphology Characteristic of Sarcomas

Next, we observed the morphologies of the tumors formed by wild-type or activated Pak4. Tumors and the corresponding normal tissues were sectioned, stained with H&E, and visualized under a microscope. Histologic analysis indicates that at day 16 postinjection, tumors from the mice injected with cells containing activated Pak4 had an appearance typical of sarcomas, arranged in interlacing fascicles of pleomorphic giant multinucleated cells, which are spindle-shaped cells with a stringy appearance (see **Figure 2F**, top). Tissues taken from the injection sites of the mice injected with wild-type Pak4-expressing cells appeared normal at day 16, but by 44 days postinjection, wild-type Pak4 also promoted tumors with features typical of sarcomas (see **Figure 2F**, bottom). These data support the finding that both wild-type and activated Pak4 promote tumors when overexpressed.

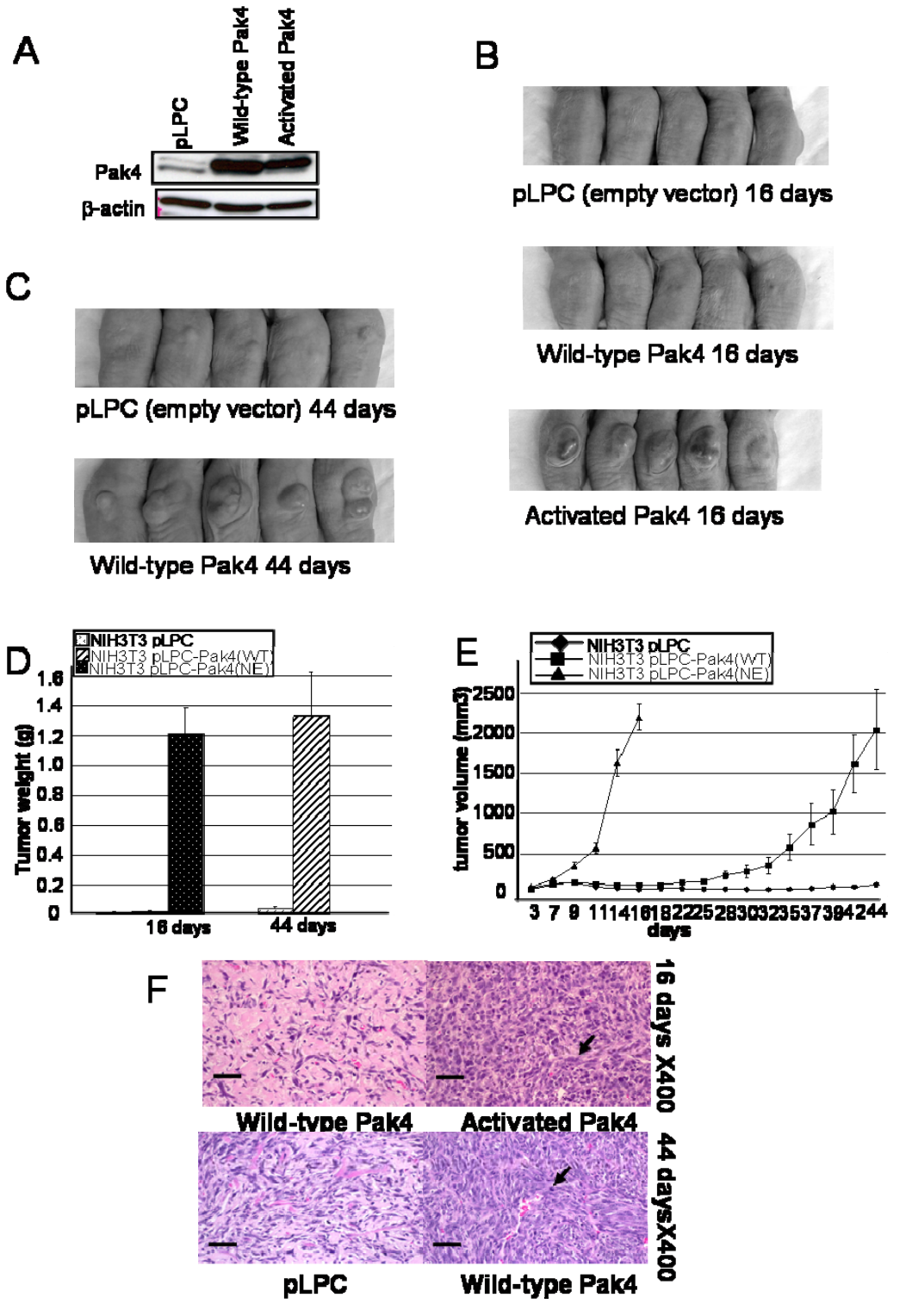

A
pLPC
Wild-type Pak4
Activated Pak4
Pak4
β-actin
B
pLPC (empty vector) 16 days
Wild-type Pak4 16 days
Activated Pak4 16 days
C
pLPC (empty vector) 44 days
Wild-type Pak4 44 days
D
NIH3T3 pLPC
NIH3T3 pLPC-Pak4(WT)
NIH3T3 pLPC-Pak4(NE)
Tumor weight (g)
16 days
44 days
E
NIH3T3 pLPC
NIH3T3 pLPC-Pak4(WT)
NIH3T3 pLPC-Pak4(NE)
tumor volume (mm3)
days
F
Wild-type Pak4
Activated Pak4
16 days X400
pLPC
Wild-type Pak4
44 daysX400

Figure 2

Figure 2. Ectopic overexpression of Pak4 leads to tumor formation. A. NIH3T3 cells were transfected with empty vector (pLPC), wild-type Pak4, or activated Pak4–containing pLPC-puro retroviral vectors. Cell extracts were prepared for Western blotting with the indicated antibodies. In each case, 20 μg of protein extract were used and β-actin served as a loading control. B. Cells containing activated Pak4 lead to tumor formation in athymic mice by 16 d postinjection. C. By 44 d, cells containing wild-type Pak4 also form tumors in athymic mice. D and E. Tumor weights and volumes from mice injected with NIH3T3 cells containing empty vector, wild-type Pak4, or activated Pak4 were assessed (activated Pak4-injected mice were sacrificed after day 16). F. Histology of tumor tissue isolated from mice injected with cells overexpressing wild-type or activated Pak4 (top), or empty vector or wild-type Pak4 (bottom), 16 or 44 d after injection. Arrows, areas with large amounts of spindle-shaped cells typical of sarcoma tissue. Scale bars, 50 μm.

Pak4-Induced Tumors Have a Decreased Level of Apoptosis Compared with Control Tissue

The results described above are the first indication that wild-type Pak4 on its own is sufficient to induce tumors. Next, we were interested in determining the mechanism by which Pak4 promotes tumors. Previously, we have found that Pak4 protects cells from apoptosis [121-122]. One possibility is that the ability to protect cells from apoptosis and thus increase cell survival may be part of the mechanism by which Pak4 triggers tumorigenesis. We therefore examined apoptosis in the tumors formed by Pak4. This was

done by examining the levels of activated caspase-3 as an end point of apoptosis [194-195]. Tumors formed by cells overexpressing wild-type Pak4 were isolated, as well as the corresponding regions from mice that had been injected with empty vector– containing cells. Lysates were prepared from the tissue samples and used to carry out Western blots using an antibody that recognizes cleaved (activated) caspase-3. The level of activated caspase-3 was significantly lower in the tumors compared with control samples (see **Figure 3A**). Immunohistochemistry analysis of tissue sections also revealed a decrease in active caspase-3 and hence a decrease in apoptosis in the tumors overexpressing Pak4 (see **Figure 3B**). In fact, tissue sections taken from mice injected with the wild-type Pak4-overexpressing cells showed a decrease in active caspase-3 levels as early as day 16 postinjection, although tumors had not even begun to form at this time point (see **Figure 3B**). They continued to show a decrease in caspase-3 activity by day 44, at which point tumors were seen. Thus, inhibition of apoptosis seems to begin early, before Pak4 actually causes tumors to form.

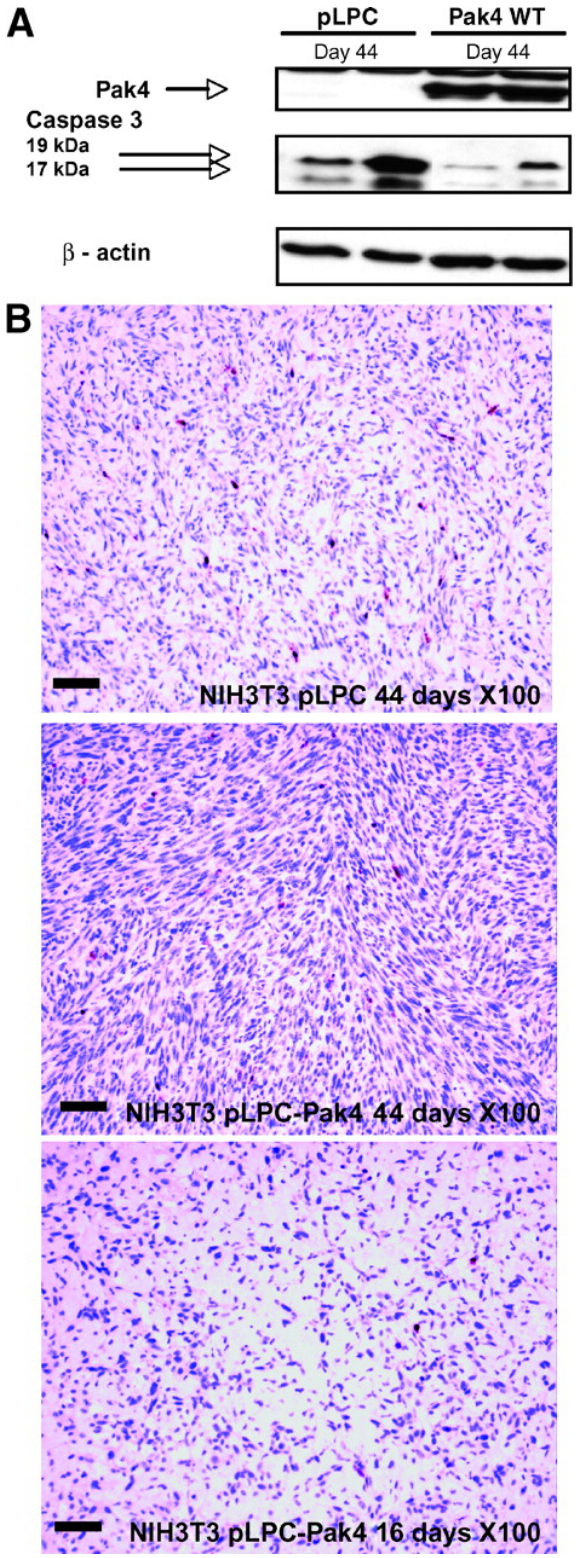

A
pLPC
Pak4 WT
Day 44
Day 44
Pak4
Caspase 3
19 kDa
17 kDa
β - actin
B
NIH3T3 pLPC 44 days X100
NIH3T3 pLPC-Pak4 44 days X100
NIH3T3 pLPC-Pak4 16 days X100

Figure 3

Figure 3. Activated caspase-3 levels are abrogated in tumors derived from Pak4-overexpressing cells. A. Activated caspase-3 (cleaved into 19 and 17 kDa fragments) was assessed by Western blot analysis of tumor or normal tissue that was isolated from mice injected with cells overexpressing empty vector (pLPC) or wild-type Pak4, 44 d after injection. The antibody recognizes only cleaved (activated) caspase-3. Pak4 expression and β-actin expression were also assessed. B. Activated caspase-3 (cleaved into 19 and 17 kDa fragments) was assessed by immunostaining of sections from tumors that were isolated from mice injected with cells overexpressing wild-type Pak4. Tissue sections from mice injected with cells containing empty vector (pLPC) were analyzed as a control. Tumor tissues were examined 16 and 44 d after injection. Areas containing active caspase-3 appear as red spots on the H&E-stained sections. Cells in individual sections were counted and at day 44; an average of 1.32% of the cells in control tissue stained positively for active caspases-3, whereas only 0.25% of the cells in the tumor tissue formed by wild-type Pak4 stained positively. Even at 16 d postinjection, as seen above, only 0.26% of the cells from the injection sites of mice injected with wild-type Pak4 stained positively for caspase-3, although tumors had not begun to form at that time point. In both cases, the difference between Pak4-containing cells and empty vector cells is statistically significant. Scale bars, 100 μm.

Increased Proliferation in Pak4-Induced Tumors

Mice were injected with NIH3T3 cells containing empty vector (pLPC), wild-type Pak4, activated Pak4, or oncogenic RasV12, as a positive control. Tissue sections were taken from the injection sites of all of the mice 16 or 44 days after injection. Tumor sections and control tissues were stained with H&E and with antibody against Ki67, which recognizes proliferating cells. The results, as shown in **Figure 4A**, indicate that wild-type and activated Pak4 lead to an increased number of proliferating cells in the tissue sections. The increased number of proliferating cells was also seen as early as 16 days in the tissue injected with cells containing wild-type Pak4, before the tumors started to form. Increased proliferation is often associated with increased phosphorylation of the Erk mitogen-activated protein kinase. To determine whether Erk phosphorylation is increased in the tumors, we carried out Western blot analysis of tumor and control lysate using anti– phospho-Erk antibody and anti–total Erk antibody. Interestingly, we saw a slight increase in the level of phospho-Erk, relative to the amount of total Erk. This was especially noticeable for Erk2 (p44; see **Figure 4B**). As a negative control, we also examined phosphorylation of p38, another mitogenactivated protein kinase, and did not see an increase in its phosphorylation in the tumors (see **Figure 4B**). Thus, increased proliferation and increased Erk activation may be another contributing factor by which Pak4 leads to transformation, and may occur early, during the lag time before Pak4 actually produces tumors.

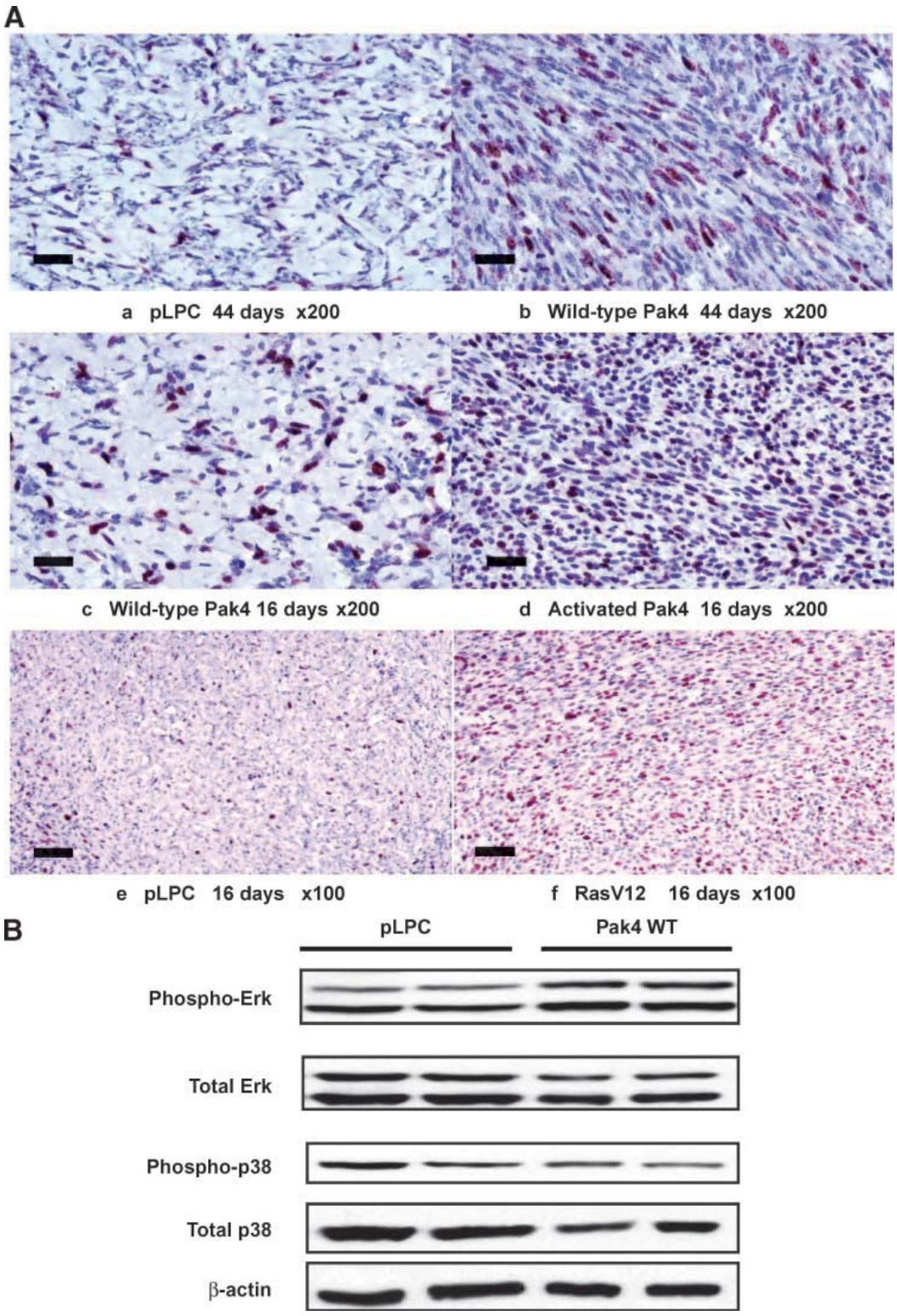

A
a pLPC 44 days x200
b Wild-type Pak4 44 days x200
c Wild-type Pak4 16 days x200
d Activated Pak4 16 days x200
e pLPC 16 days x100
f RasV12 16 days x100
B
pLPC
Pak4 WT
Phospho-Erk
Total Erk
Phospho-p38
Total p38
β-actin

Figure 4

Figure 4. Increased proliferation is in response to Pak4 expression. Mice were injected with NIH3T3 cells containing empty vector (pLPC), wild-type Pak4, activated Pak4, or oncogenic RasV12, as a positive control. **A.** Tissue sections were taken from the injection sites of all of the mice 16 or 44 d after injection. Sections were stained with H&E and with antibody against Ki67, which recognizes proliferating cells (*dark purple*). The results indicate that wild-type and activated Pak4 lead to an increased number of proliferating cells in the tissue sections. Cells in individual sections were counted and at day 44; an average of 20.4% of the cells from control tissue stained positively for Ki67, whereas 36.4% of cells from tumors derived from wild-type Pak4 stained positively. At 16 d, 31.7% of cells from the injection sites of mice injected with wild-type Pak4 stained positively for Ki67. In both cases, the difference between Pak4-containing cells and empty vector cells was statistically significant. This was seen even at 16 d, at which point wild-type Pak4 had not yet caused tumors to form. Scale bars, 50 μm. **B.** Phospho-Erk and total Erk levels were assessed by Western blot analysis of tumor or normal tissue that was isolated from mice injected with cells overexpressing empty vector (pLPC) or wild-type Pak4, 44 d after injection. Top band, Erk2; bottom band, Erk1. As a negative control, phosphorylation of p38 was also examined by Western blot. The results indicated that wild-type Pak4 can lead to a slight increase in phosphorylation of the Erk mitogen-activated protein kinase pathway, which is often associated with increased proliferation.

Tumor Formation in Response to Oncogenic Ras Is Attenuated in Pak4 Null Cells

The above studies indicate that Pak4 is sufficient to form tumors in nude mice. An equally important question is whether Pak4 is also necessary for tumor formation. To address this question, we used fibroblasts that had been isolated from Pak4 null embryos or wild-type controls [41] and immortalized by serial passaging. The Pak4 null and wild-type fibroblasts (which grow at similar rates) were infected with a retrovirus containing oncogenic RasV12, a strong oncogene, or with empty vector (pLPC). Western blot analysis of the wild-type ($Pak^{+/+}$) and Pak4 knockout ($Pak4^{-/-}$) cells infected with either empty vector or RasV12 is shown in **Figure 5A** . When injected into nude mice, RasV12; $Pak4^{+/+}$ cells (wild-type cells infected with oncogenic Ras) formed large tumors by 18 d. At this time point, RasV12; $Pak4^{-/-}$ cells (Pak4 null cells infected with oncogenic Ras) formed significantly smaller tumors (see **Figure 5B**, top). Because of the size of the tumors, mice injected with RasV12; $Pak4^{+/+}$ cells were sacrificed at the 18-d time point. However, mice injected with RasV12; $Pak4^{-/-}$ cells were maintained for 6 more days, by which time larger tumors began to form even in these mice (see **Figure 5B**, bottom). In some cases, these $Pak4^{-/-}$ tumors were especially bloody in appearance (see **Figure 5B**, bottom). **Figure 5C** and **D** shows graphs indicating the weights and volumes of tumors at different time points. The results indicate that although the RasV12; $Pak4^{-/-}$ cells formed tumors, they grew quite a bit more slowly and they never obtained the same weight as the RasV12; $Pak4^{+/+}$ cells.

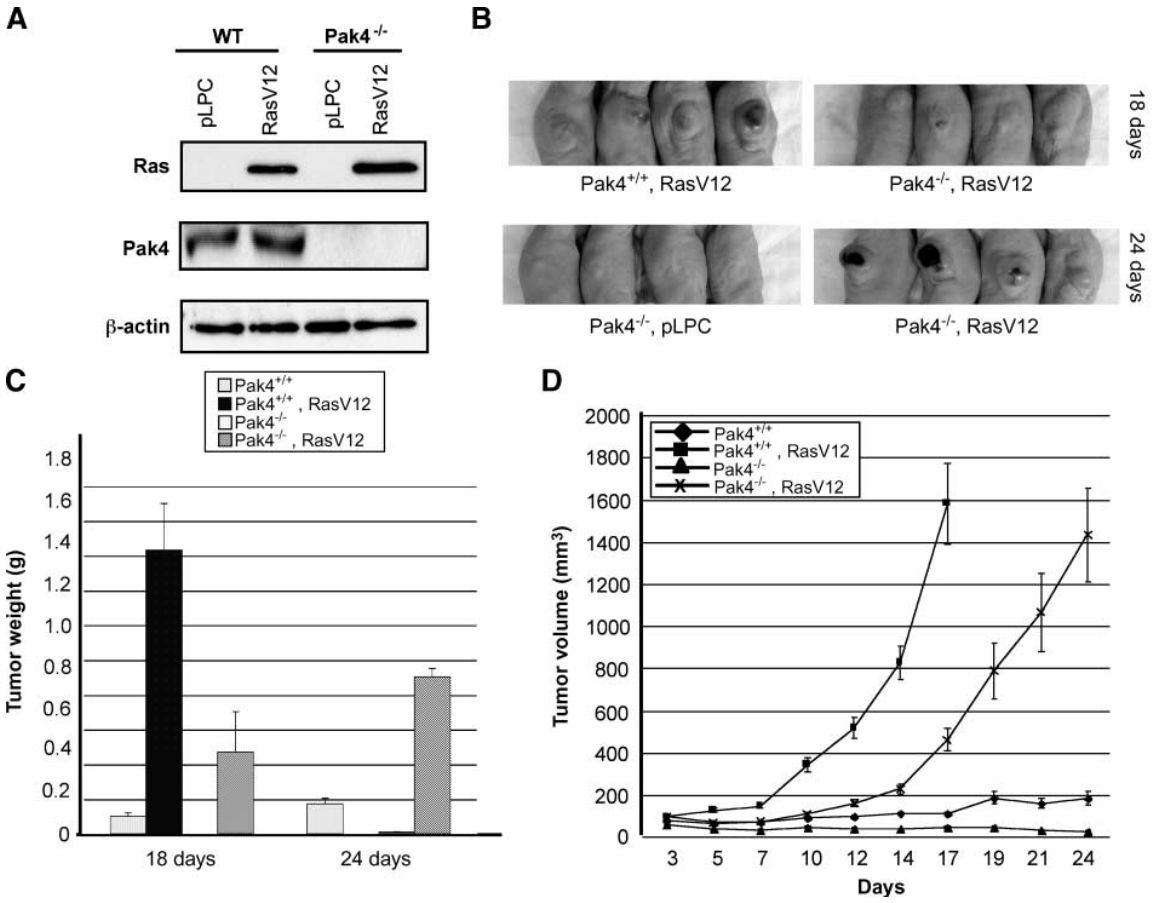


Figure 5

Figure 5. Tumor formation is attenuated in Pak4 null cells when transfected with Ras. **A.** Immortalized wild-type and Pak4$^{-/-}$ fibroblasts were infected with either empty vector (pLPC) or RasV12-containing pLPC retroviral vectors. Two days after infection, cells were selected with puromycin for 2 wk, after which cell extracts were prepared for Western blotting with the indicated antibodies. In each case, 20 μg of protein extract were used. β-Actin served as a loading control. **B.** Top, mice were injected with Pak4$^{+/+}$ cells (wild-type) or Pak4$^{-/-}$ cells that were infected with oncogenic Ras. Tumors were examined 18 d after injection (for both cell types, cells infected with empty vector did not form tumors; not shown). Bottom, mice were injected with Pak4$^{-/-}$ cells containing

empty vector (pLPC; *left*), or with RasV12 (*right*). Tumors were examined 24 d after injection (the $Pak4^{+/+}$, RasV12 condition is not shown at 24 d because the tumors became too large and the mice had to be sacrificed). **C** and **D.** Tumor weight and tumor volume change in mice injected with $Pak4^{+/+}$ and $Pak4^{-/-}$ cells with or without RasV12 were assessed (tumor weight in mice injected RasV12; $Pak4^{+/+}$ cells were only analyzed at day 18 because the mice had to be sacrificed due to the large size of the tumors).

Tumor Formation in Response to Activated Cdc42 Is Almost Completely Abolished in the Absence of Pak4

Pak4 was originally identified as an effector for Cdc42 [32]. We therefore reasoned that Pak4 may function downstream to Cdc42during tumorigenesis. Wild-type or Pak4 knockout immortalized fibroblasts were infected with activated Cdc42 (Cdc42V12) or empty vector. Western blot analysis of the cells is shown in **Figure 6A**. Cdc42V12 transfected cells resulted in large tumors when injected into the athymic mice. Strikingly, however, no tumors formed in mice that were injected with the Pak4 knockout cells, even those infected with Cdc42V12 (see **Figure 6B** and **C**). Our results indicate that Pak4 has a direct role downstream to Cdc42in oncogenic transformation.

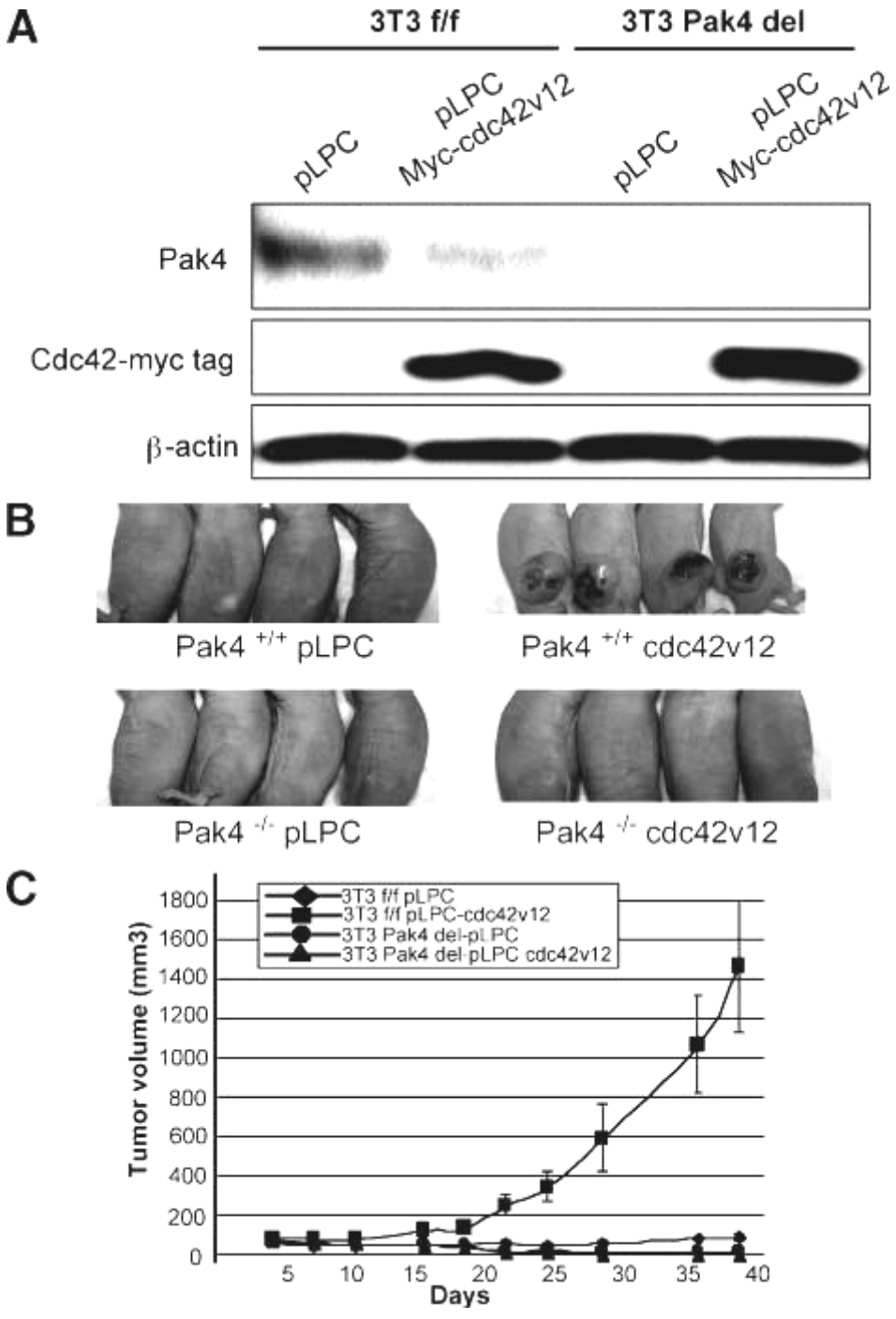

A
3T3 f/f
3T3 Pak4 del
pLPC
pLPC Myc-cdc42v12
pLPC
pLPC Myc-cdc42v12
Pak4
Cdc42-myc tag
β-actin
B
Pak4 +/+ pLPC
Pak4 +/+ cdc42v12
Pak4 -/- pLPC
Pak4 -/- cdc42v12
C
3T3 f/f pLPC
3T3 f/f pLPC-cdc42v12
3T3 Pak4 del-pLPC
3T3 Pak4 del-pLPC cdc42v12
Tumor volume (mm3)
1800
1600
1400
1200
1000
800
600
400
200
0
5
10
15
20
25
30
35
40
Days

Figure 6

Figure 6. Tumor formation is completely abrogated in Pak4 null cells when transfected with Cdc42V12. A. Immortalized wild-type and Pak4 conditional knockout fibroblasts were infected with either empty vector (pLPC) or Cdc42V12-containing pLPC retroviral vectors. Two days after infection, cells were selected with puromycin for 2 wk, after which cell extracts were prepared for Western blotting with the indicated antibodies. In each case, 20 μg of protein extract were used. β-Actin served as a loading control. **B.** Top, mice were injected with $Pak4^{+/+}$ cells (wild-type) that were infected with empty vector (pLPC; *left*) or with Cdc42V12 (*right*). Bottom, mice were injected with $Pak4^{-/-}$ cells containing empty vector (pLPC; *left*) or with Cdc42V12 (*right*). Tumors were examined 38 d after injection. For both cell types, cells infected with empty vector did not form tumors. Wild-type cells infected with Cdc42V12 formed tumors; however, tumor formation were completely abrogated in Pak4 null cells when transfected with Cdc42V12. **C.** Tumor volumes in mice injected with $Pak4^{+/+}$ and Pak4 conditional knockout cells with or without Cdc42V12 were assessed at the indicated time points.

Discussion

Pak4 was originally identified as a protein that plays a role in regulating cytoskeletal organization and cell shape [32]. Later, it was also found to have a role in regulating cell growth and survival [96-97, 122] . Although Pak4 is expressed at high levels in embryos, it is expressed at low levels in most normal adult tissues. It is overexpressed, however, in a large number of different tumor cell lines [98], suggesting an important role for Pak4 in

cancer. Here, we examined Pak4 expression levels in three different types of primary tumors (colon, mammary, and esophagus), and found that, in every case, tumor tissues contained more Pak4 than the corresponding control tissues. This suggests that overexpression of Pak4 is not simply a by-product resulting from the culture of the tumor cells because it is found directly in the primary tumors.

The finding that Pak4 is overexpressed in tumors is compelling, and these results led us to determine whether overexpression of Pak4 is also sufficient to promote tumorigenesis. Interestingly, the expression of a constitutively active mutant of Pak4 promotes anchorage-independent growth in cultured cells, but wild-type Pak4 has no effect [97]. Anchorage-independent growth in cultured cells is an important predictor of oncogenesis because normal adherent cells need to attach to a surface to survive, whereas cancer cells lose this anchorage dependence. However, there are numerous other tests to determine whether a cell is transformed and whether a gene promotes tumorigenesis in the cells that express it, including decreased density dependence, decreased serum requirement, loss of cell cycle control, loss of differentiation, and resistance to apoptosis. One very important requirement for defining cells as malignant is that they form tumors in experimental animals. Formation of tumors in athymic mice is often used as a test to determine whether cells are malignant and whether an oncogene is transforming. To test whether Pak4 promotes tumors in athymic mice, we used NIH3T3 cells that overexpress Pak4. NIH3T3 cells normally only have a low level of Pak4 but the stable cell lines we generated express Pak4 to a level comparable with what is seen in tumor cell lines. Interestingly, we found that overexpression of Pak4 is sufficient to cause tumor formation in athymic mice. These results are quite exciting because they show for the first time that

Pak4 promotes tumorigenesis in animals and not only in cultured cells. The results are especially intriguing because we found that in mice, even wild-type Pak4 is tumorigenic when overexpressed. Our results provide support for the idea that wild-type Pak4 is not only overexpressed in cancer cells but also sufficient to cause tumorigenesis. It should be noted that these experiments were all carried out using NIH3T3 cells. NIH3T3 cells are frequently used as a model to study tumorigenesis. However, because most tumors are of epithelial origin rather than fibroblasts, in future studies it will be important to determine whether Pak4 also plays an important role in transformation using epithelial cells as a model.

An important question is, "What is the mechanism by which Pak4 promotes tumors?" Previously, we found that Pak4 inhibits apoptosis and thus promotes cell survival [121-122]. Interestingly, we have found that there is a decreased level of apoptosis in Pak4-induced tumors compared with control tissues, as assessed by examining caspase-3 levels. Conversely, we see an increase in proliferation in the cells of the Pak4-induced tumors. We propose, therefore, that inhibition of apoptosis and subsequent increased cell survival and cell growth play a key role in Pak4-induced tumorigenesis. Previously, we found that Pak4 inhibits apoptosis by two different mechanisms. The first is a kinase-independent mechanism, mediated by inhibition of initiator caspases [122]; the second is a kinase-dependent mechanism, mediated by phosphorylation of Bad [121]. Interestingly, we have found that Pak4 (K350M), a kinase dead mutant, can promote tumorigenesis as efficiently as wild-type Pak4 (data not shown). This supports the idea that Pak4 can operate via the first mechanism, the kinase-independent mechanism, to promote cell survival and subsequent tumorigenesis.

However, although the Pak4 (K350M) mutant and wild-type Pak4 were both equally transforming, the activated Pak4 mutant, Pak4 (S445N), had an even more dramatic effect on tumor formation. Thus, although kinase activity may not be required for Pak4-induced transformation, it may contribute to cell survival and tumorigenesis. In future experiments, it will be interesting to examine activated caspase-8 levels in the tumors as well as caspase-3, because Pak4 was shown to inhibit initiator caspases (such as caspase-8), as well as initiator caspases (like caspase-3), via a kinase-independent mechanism [122]. It is interesting that there is a lag time before wild-type Pak4-expressing cells form tumors; yet, once tumors begin to form, they do so quite rapidly and grow to a large size. We have found that the decrease in apoptosis and increase in proliferation is seen early, even before tumors form in response to wild-type Pak4. These results suggest that increased proliferation and survival are events that occurs early, during the lag time before tumorigenesis begins in response to wild-type Pak4, and that this eventually primes the cells to proliferate without control and become tumor cells. The sequence of events that must occur before wild-type Pak4 causes tumors to form may require the complex environment of an animal model system. This may explain why transformation in response to wild-type Pak4 is not seen in a simpler cell culture system.

In addition to inhibiting apoptosis, we previously showed that Pak4 specifically activates prosurvival pathways, which lead to activation of pathways such as the nuclear factor-κB pathway and the Erk mitogen-activated protein kinase pathway. Cells lacking Pak4 thus have decreases in nuclear factor-κB and Erk activities [96]. Consistent with this, we see an increase in phosphorylated Erk in the Pak4-induced tumors and this is associated with an increase in proliferation. We propose that cells overexpressing Pak4

have a decrease in apoptosis, an increase in cell survival pathways, and consequently an increase in signaling pathways such as the Erk pathway that promote cell proliferation.

Another issue addressed in this study is whether Pak4 is necessary for tumorigenesis. We found that although RasV12 led to tumor formation, even in the absence of Pak4, the tumors grew more slowly and grew to a smaller size. It is interesting that some of the tumors derived from the RasV12; Pak4$^{-/-}$ cells were especially bloody in appearance (see **Figure 5**). This raises the intriguing possibility that the Pak4$^{-/-}$ tumors are undergoing apoptosis or necrosis and are thus stopped from growing any larger, presumably because of the lack of Pak4. This would be consistent with a critical role for Pak4 in cell survival and inhibition of apoptosis. In contrast to oncogenic Ras, the absence of Pak4 led to nearly complete abrogation of tumors in response to Cdc42V12. This is consistent with the role for Pak4 as a Cdc42 effector protein [32] and suggests the presence of a Pak4-dependent signaling pathway leading to Cdc42 and its activators to transformation.

It is interesting that Pak4 is highly expressed during embryogenesis but expressed at low levels in most adult tissues [41]. This is consistent with a role for this protein in cancer. Although Pak4 most likely plays an important role in the rapid cell growth that occurs during embryogenesis, in adults, proliferation levels decrease and high levels of Pak4 may no longer be needed. Instead, when improperly overexpressed or activated in adult tissues, Pak4 may promote increased cell survival, uncontrolled growth, and tumorigenesis. Because Pak4 is almost undetectable in most normal tissues but up-regulated in several tumors, it may be an attractive candidate for drug therapy for a number of different types of cancer.

Acknowledgements

We thank Dr. Mousumi Bose, for providing mouse colon and normal tissue; the Cooperative Human Tissue Network of University of Pennsylvania Medical Center for providing human breast cancer and normal tissue; and Dr. Lisa Epstein Wong for the Cdc42V12 construct.

Chapter III

The Protein Kinase Pak4 Disrupts Mammary Acinar Architecture and Promotes Mammary Tumorigenesis

Abstract

The Pak4 serine/threonine kinase is highly expressed in many cancer cell lines and human tumors. While several studies have addressed the role for Pak4 in transformation of fibroblasts, most human cancers are epithelial in origin. Epithelial cancers are associated not only with changes in cell growth, but also with changes in the cellular organization within the three dimensional (3D) architecture of the affected tissues. Here we used immortalized mouse mammary epithelial cells (iMMECs) as a model system to study the role for Pak4 in mammary tumorigenesis. iMMECs are an excellent model system for studying breast cancer they can grow in 3D-epithelial cell culture, where they form acinar structures that recapitulate *in vivo* mammary morphogenesis. While Pak4 is expressed at low levels in wild type iMMECs, it is overexpressed in response to oncogenes, such as oncogenic Ras and Her2/neu. Here we found that overexpression of Pak4 in iMMECs leads to changes in 3D acinar architecture that are consistent with oncogenic transformation. These include decreased central acinar cell death, abrogation of lumen formation, cell polarity alterations, and deregulation of acinar size and cell number. Furthermore, iMMECs overexpressing Pak4 form tumors when implanted into the fat pads of athymic mice. Our results suggest that overexpression of Pak4 triggers events that are important for the transformation of mammary epithelial cells. This is likely to be due to the ability of Pak4 to inhibit apoptosis and promote cell survival, and thus subsequent uncontrolled proliferation, and to its ability to deregulate cell shape and polarity.

Introduction

Oncogenic transformation can occur in response to improperly controlled cell proliferation, increased levels of cell survival, failure to differentiate, or failure to maintain proper cell shape and polarity. These changes are often associated with improper regulation of intracellular signaling pathways that control cell growth and survival. Protein kinases play key roles in such pathways. The serine/threonine kinase Pak4 has been implicated in signaling pathways leading to malignant transformation [96-98, 122]. Pak4 was first identified as an effector protein for the Rho GTPase Cdc42, which leads to changes in cytoskeletal organization [32]. Later, Pak4 was also found to be activated by Rho GTPase independent stimuli [75]. Pak4 is expressed at low levels in most adult tissues. Strikingly, however, it is highly overexpressed in many different types of cancer cell lines and tumors [98, 153]. When overexpressed in fibroblasts, activated Pak4 causes cells to grow in an anchorage independent manner, which is an important hallmark of tumorigenesis [97-98]. Pak4 transforms these cells as strongly as activated Ras, a strong and well known oncogene [97], and it causes cells to become resistant to apoptosis [96, 121-122]. When injected into athymic mice, fibroblasts overexpressing Pak4 lead to the formation of large tumors [153].

While a number of studies have shown Pak4 to be highly transforming in fibroblasts, most human cancers are epithelial in origin. Expression studies suggest that Pak4 has a role in epithelial cell tumorigenesis as well. Pak4 is highly overexpressed in many tumor cell lines [98] and primary tumors of epithelial origin [153]. The mechanism by which Pak4 transforms epithelial cells, however, is poorly understood. An extensively studied prototype of epithelial tumorigenesis is breast cancer. Pak4 is highly overexpressed in

human breast tumors and rat mammary tumors [153], and it was found to be overexpressed in 8 out of 8 breast cancer cell lines that were examined [98].

The signaling pathways involved in the progression of breast cancer are complex and incompletely understood. Mammary carcinomas are tumors of the glandular epithelial cells. The normal mammary gland consists of an ordered structure of units in the form of individual spherically shaped acini. These acini contain hollow lumens, surrounded by polarized epithelial cells. Mammary tumorigenesis is associated with disruption of this well ordered structure [196-197], caused in part by increased epithelial cell proliferation and inhibition of apoptosis, resulting in filling of the acinar luminal space with cells [198]. Use of two dimensional (2D) cell cultures can be limiting with respect to studying three dimensional (3D) glandular structures. A more relevant model involves the use of 3D basement membrane cell culture methods that recapitulate glandular epithelium morphogenesis. One such model system involves use of immortalized mouse mammary epithelial cells (iMMECs) generated from wild female mice, which can be grown in 3D cultures such that they recapitulate the structure of the mammary epithelium. iMMECs are poorly transforming on their own, and they are an excellent model for studying genes that promote breast cancer [199]. In regards to 3D morphogenesis, iMMECs behave similarly to the human non-transformed mammary epithelial cell line MCF10A. When grown in 3D culture on reconstituted basement membrane, iMMECs, just like MCF10A cells, follow a temporal sequence of events leading to the formation of spherical acini, mimicking those seen in normal breast epithelium [197]. First, the cells seeded on the basement membrane proliferate and form a cluster of cells, which constitute the beginning of an acinar structure. This is followed by the initial steps of cell polarization. A polarized

outer layer of cells forms and is in direct contact with the basement membrane. An inner group of cells is more poorly polarized and does not come into contact with the matrix. These inner, nonpolarized cells, which do not contact the basement membrane, begin to die by apoptosis, resulting in hollow lumen formation. At the end of this process, there is a suppression of cell proliferation throughout the acinus. The final structure consists of a single layer of polarized epithelial cells around a hollow lumen and surrounded by basement membrane, mimicking the architecture of glandular epithelium. Like normal breast epithelium, the acini formed in this 3D culture system have low levels of proliferation and have a stable cell number [197]. The acinar structures formed by mammary epithelial cells in 3D culture are an excellent model for studying the factors that disrupt the balance between apoptosis and proliferation at a spatial and temporal level.

While changes in cell proliferation and survival are important features of oncogenesis, there are other types of cellular changes that also lead to oncogenic transformation. One important example is the disruption of normal cell polarity. Cells in the glandular epithelium are normally highly polarized. The apical surface of the membrane faces the lumen of the acini, and the basolateral surface contacts the adjacent cells and underlying basement membrane [200]. Disruption of cell polarity is an important hallmark of many epithelial cancers and is sometimes linked to changes in growth and proliferation. Disruption of polarity can contribute to the progression of a premalignant lesion into carcinoma, and it is an important aspect of the epithelial-mesenchymal transition and progression to invasive cancer cells [201]. Acini formed by iMMECs consist of highly polarized cells, and are thus an excellent model for studying factors that can disrupt normal cell polarity.

In this paper we studied the role for Pak4 in controlling growth of mammary epithelial cells. We found that wild type iMMECs have low levels of Pak4. However, when transformed by either activated Ha-ras or Her2/neu, oncogenes which cause iMMECs to become highly tumorigenic [202], Pak4 is highly overexpressed. To study the effects of Pak4 overexpression in iMMECs, we generated stable iMMEC lines which express high levels of Pak4. We found that overexpression of Pak4 in iMMECs leads to changes in 3D acinar morphogenesis that are consistent with oncogenic transformation. These include decreased central acinar cell death, abrogation of lumen formation, and alterations of cell polarity, and deregulation of acinar size and cell number. Furthermore, iMMECs overexpressing Pak4 form mammary tumors when orthotopically implanted in athymic mice. Our results suggest that overexpression of Pak4 triggers events that are important for the transformation of mammary epithelial cells.

Materials and Methods

Real Time PCR

Human normal and tumor quantitative PCR tissue arrays were purchased from OriGene. This array contained 381 tissues covering 22 different cancers. All relevant clinical information can be found on the OriGene web site (http://www.origene.com/geneexpression/disease-panels/products/SCRT102.aspx). *Real time PCR* quantitative measurements of Pak4 mRNA were made on cDNA samples provided by OriGene. Briefly, primers to human Pak4 were designed using ABI Prism Primer Express software (Applied Biosystems, Foster City, CA). Primers were evaluated with National Center for Biotechnology Information Blast (Bethesda, MD) to confirm

product specificity, and products were designed to cross intron/exon borders. The sequences of the forward and reverse primers for Pak4 were 5-GACATCAAGAGCGACTCGATCC -3 and 5-ATCACCATTATCCCCAGCGAC -3 respectively. The sequences of the forward and reverse primers for β-actin were 5-CAGCCATGTACGTTGCTATCCAGG -3 and 5-AGGTCCAGACGCAGGATGGCATG -3, respectively. For all experiments, mRNA expression was measured on an ABI Prism 7900HT Sequence Detection System. Syber Green dye was used for signal detection. Dissociation curves of all analysis were used to ensure specific template amplification. Serial dilutions of a control cDNA (HCT116) were used to determine standard curves, and curves with $R^2 > 0.97$ were then used to determine the mRNA levels in individual samples. Target gene expression level was calculated as a ratio of the mRNA level relative to the mRNA level for β-actin in the same cDNA.

Cell culture, Transfection and Generation of Stable cell lines

iMMECs were cultured in F12 medium (Invitrogen) containing 10% fetal bovine serum, as described [199]. MMECs are isolated from young, virgin female mice of the C57BL/6 genetic background [199] and immortalized by inactivation of the Rb and p53 pathways, as previously described for mouse kidney epithelia cells [203]. Stable cell lines expressing wild-type Pak4 or empty vector, were grown in the medium described above, in the presence of puromycin (3.0 μg per ml). Stable cell lines were generated by retroviral infection, followed by selection with puromycin, as previously described [153].

Growth Curves

Equal numbers of cells from each cell line were seeded in growth medium in six-well plates. Each day after the seeding one set of cells was trypsinized, collected and counted. Each point on the curve is the average of triplicate measurements.

3D Morphogenesis and immunofluorescence (IF)

3D culture of iMMECs on a reconstituted basement membrane was performed according to a previously described protocol [199], which is based on the method used for MCF-10A cells [197]. Mammary acini were grown in F12 medium containing 5 μg/ml insulin, 1 μg/ml hydrocortisone, 5 μg/ml EGF, 3 μg/ml puromycin and 2% Matrigel. Mammary acini were fixed and processed for IF as previously described [199]. Acini were incubated with primary antibodies overnight at 4 ^{o}C, washed, and then incubated with fluorescein- or rhodamine-coupled secondary antibodies (from Jackson ImmunoResearch Laboratory) for 2 hr at room temperature. Finally, acini were stained with DAPI (4', 6'-diamidino-2-phenylindole; Sigma), washed, and mounted with Prolong anti-fade (Molecular Probes). Confocal laser scanning microscope was carried out with a Zeiss LSM510-META confocal microscope system at the W.M. Keck Center for Collaborative Neuroscience, Rutgers University. IF reagents and related antibodies for the analysis of iMMEC-generated acini are shown in **Table 1**.

Western blotting

Western blotting was carried out as previously described [32]. Polyclonal Pak4 antibody was from Cell signaling. Horseradish peroxidase–conjugated secondary antibodies were from Sigma.

Antibody or stain	Purpose	Normal Localization	company	Species
Active (cleaved) caspase3	Apoptosis maker	Apoptotic cells in lumen	Cell Signaling	Rabbit
β-catenin	Cell-cell junction	Basolateral	ZYMED Lab	Mouse
DAPI	Nuclear counterstain	Nuclei	Boehringer Manheim	N/A
GM130	Apical polarity	Golgi apparatus (apically located)	BD	Mouse
Ki67	Proliferation marker	Nuclei of proliferating cells	Dako Cytomation	Rat
Phospho-Erk	Erk kinase activation	Cytosal and nucleus	Cell Signaling	Rabbit
Phalloidin	Cytoskeleton	Actin skeleton	Invitrogen	N/A
Total Erk	Erk kinase activation control	Cytosol and nucleus	Cell Signaling	Rabbit

Table 1. Antibodies and fluorescent reagents used for the analysis of acini formed by iMMEC cells.

Tumorigenicity assays

iMMECs expressing Pak4 or empty vector were harvested by trypsinization and resuspended in PBS (10^8 cells/ml). Orthotopic mammary gland implantation of iMMECs was performed according to an Institutional Animal Care and Use Committee approved protocol NCR nude female mice, 15 weeks old, were anesthetized with 2,2,2-tribromoethanol (avertin, Sigma-Aldrich, St. Louis) at the dose of 175 mg/kg (i.p.). A small incision was made to expose the third pair of mammary fat pads on both sides, and

each mammary gland pad was subjected to implantation of 10^7 cells. The incision was closed with surgical clips that were removed 10 days later. Tumor growth was monitored by weekly measurement of tumor length (L) and width (W). Tumor volume was calculated through $\pi LW^2/6$.

Results

Pak4 Is Overexpressed in Human Tumors and Cancer Cell Lines, and in Oncogene-Expressing iMMECs

To study whether Pak4 is overexpressed in human epithelial cancers, we carried out real-time PCR with human normal and tumor tissue cDNA (see **Figure 1A**). We found that Pak4 is overexpressed in tumors of the breast, endometrium, esophagus, ovary, prostate, and urinary bladder. Thus Pak4 appears to be broadly expressed in different types of human tumors. In previous studies, Pak4 was shown to be overexpressed in primary tumors from human breast and rat mammary gland, and in a panel of breast cancer cell lines [153]. To further study the levels of Pak4 in other epithelial cell lines, we examined the protein levels of Pak4 in human colon and prostate cancer cell lines. We found that Pak4 levels were significantly higher in the tumor cell lines compared with the normal control cell lines (see **Figure 1B**). Pak4 was poorly expressed in iMMECs from wild-type mice (see **Figures 1C and 2A**). Interestingly, however, when the iMMEC cells stably expressed oncogenic Ras or Her2/neu, which are highly transforming in these cells [199, 202], Pak4 levels became highly elevated (see **Figure 1C**). Pak4 is thus overexpressed in a variety of conditions that are associated with malignant transformation.

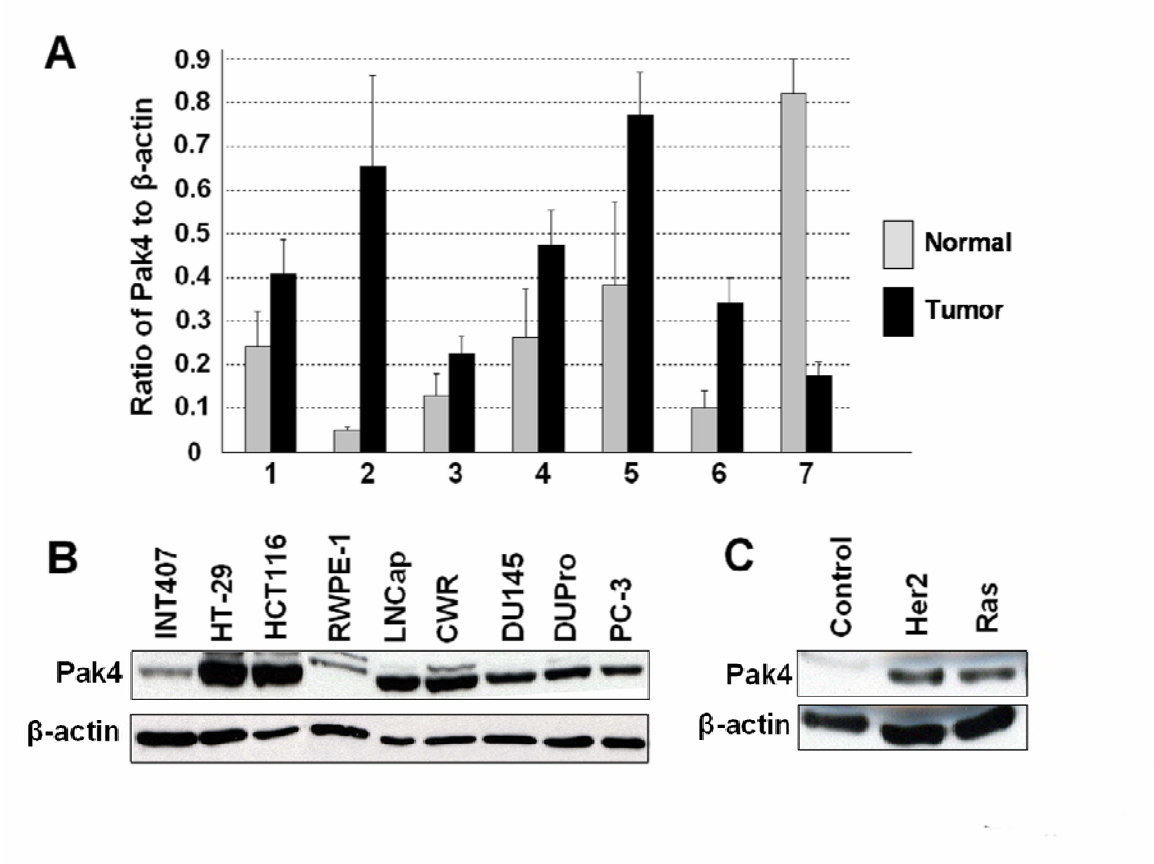


Figure 1

Figure 1. Pak4 is overexpressed in many different types of human tumors and cancer cell lines. A. Quantitative PCR analysis of Pak4 gene expression using cDNA from different types of human tumor tissues and normal tissue. Data are represented as the ratio normalized to β-actin gene expression. 1, Breast. 2, Endometrium. 3, Esophagus. 4, Ovary. 5, Prostate. 6, Urinary bladder. 7, Testis. **B**. Western blot analysis illustrates high levels of Pak4 expression in human cancer cell lines compared to normal cell lines. The following cell lines were analyzed: Colon cancer cell lines (HT29 and HCT116), prostate cancer cell lines (LNCap, CWR, DU145, DUPro, and PC-3), and normal small

intestine and prostate cell lines (INT407 and RWPE-1). **C.** Pak4 is overexpressed in response to oncogene expression in iMMECs. In B and C, 40 μg of protein extract was used; β-actin served as a loading control.

Generation of Stable iMMEC Lines Overexpressing Wild-Type Pak4

Western blot analysis showed that Pak4 is poorly expressed in wild-type iMMEC cells (see **Figure 2A**). This is consistent with the idea that these cells serve as a model for nontransformed mammary epithelial cells. In order to address the role for Pak4 overexpression in mammary cells, retroviral infection was used to generate iMMECs that overexpress Pak4. Western blot analysis of a Pak4-overexpressing iMMEC line is shown in **Figure 2A**.

Overexpression of Pak4 Does Not Affect the Overall Growth Rate of iMMECs

iMMECs expressing Pak4 or empty vector were grown in typical 2D culture conditions. Cells were trypsinized and counted every day, and growth curves were recorded. Growth curves from the first 4 days are shown in **Figure 2B**. Overexpression of Pak4 did not lead to an overall increase in iMMEC growth rate.

Overexpression of Pak4 Abrogates Lumen Formation in 3D Morphogenesis

The Pak4- and empty vector-transfected-iMMECs were grown on a layer of basement membrane in a 3D culture system, as described [199]. Acinar structures were then examined by confocal microscopy at different time points. Similar to the vector

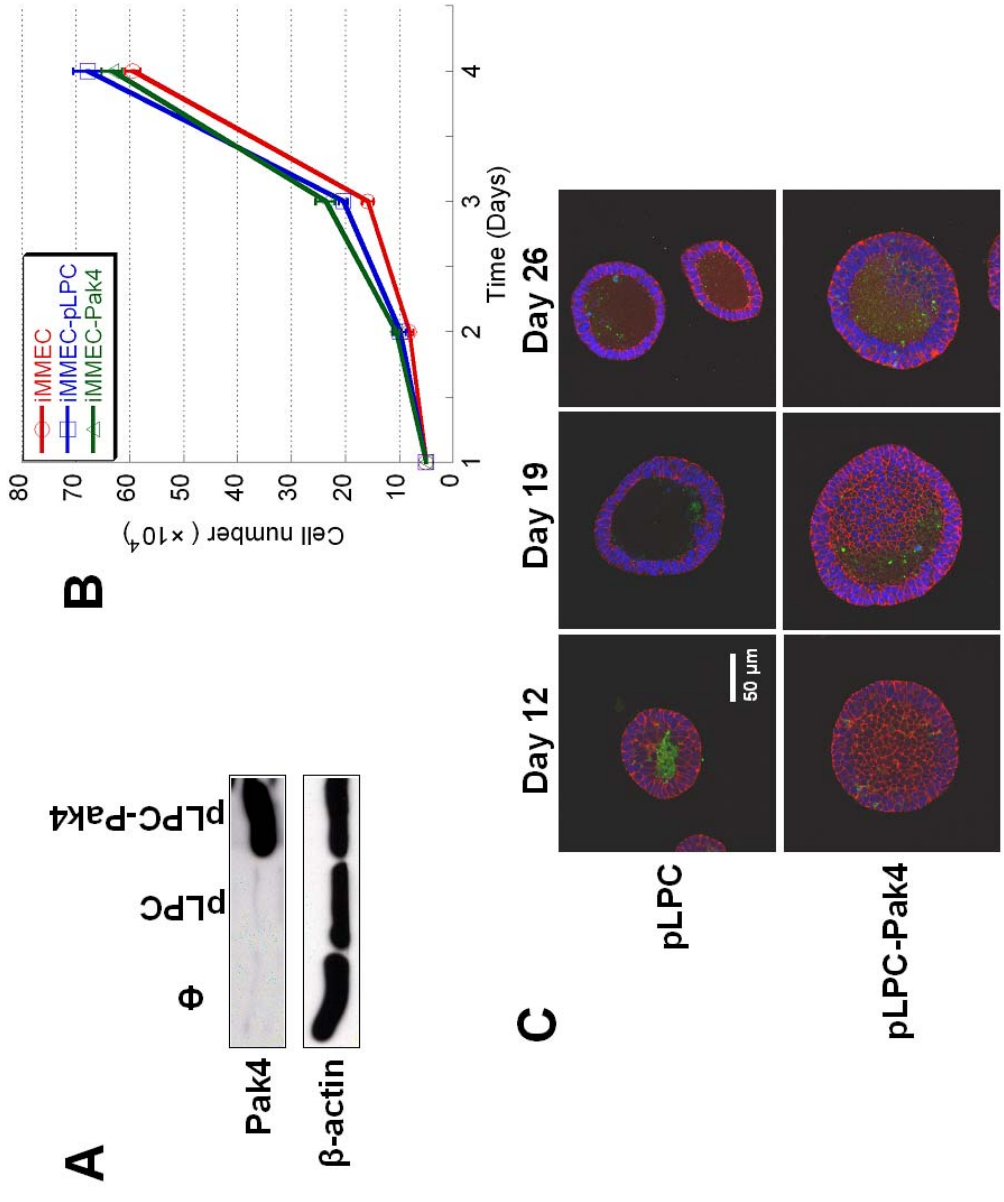

A
Pak4
β-actin
Φ
pLPC
pLPC-Pak4
B
Cell number (×10⁴)
Time (Days)
iMMEC
iMMEC-pLPC
iMMEC-Pak4
C
Day 12
Day 19
Day 26
50 μm
pLPC
pLPC-Pak4

Figure 2

Figure 2. Overexpression of Pak4 inhibits apoptosis in iMMECs in 3D culture. A. Generation of stable cell lines in which iMMECs overexpress wild-type Pak4. Stable iMMEC cell lines were generated by retroviral infection of the empty vector (pLPC), or wild-type Pak4 (pLPC-Pak4) vector. Cell extracts were analyzed by for western blot. In each case, 20 μg of protein extract was used. **B.** Overexpression of Pak4 does not affect the overall growth rate of iMMECs. Control iMMECs (blue), iMMECs infected with empty vector (pLPC, red), and iMMECs infected with Pak4 (yellow) were plated. Cells were counted every day (Y axis is cell number, X axis is days after plating). **C.** Inhibition of apoptosis in iMMECs overexpressing Pak4. iMMECs stably expressing either empty vector (top) or Pak4 (bottom) were grown in 3D cultures. Acini were stained with DAPI (blue), beta-catenin (red; to visualize epithelial cells), and cleaved caspase-3 (green).

control cells, Pak4-overexpressing iMMECs formed spherical acini. There were important differences, however, between the two types of acinar structures, as described below.

The structures formed by iMMECs transfected with empty vector are referred to as wild-type acini, and those formed by Pak4-overexpressing iMMECs are referred to as Pak4 acini. Wild-type acini and Pak4 acini were subjected to immunofluorescence and visualized by confocal microscopy at different time points after plating, as shown in **Figure 2C**. By day 12 after plating, a lumen was clearly beginning to form in the wild-type acini. Apoptotic cells could be seen in the centers of these structures, as assessed by staining with anti active caspase-3 antibody. In contrast, in Pak4 acini, hardly any apoptosis could be seen at this time point, and the lumen was mostly filled with cells. By

day 19 there was a largely hollow lumen in the wild-type acini, while in the Pak4 acini the lumen still contained many cells. Finally, by day 26, typical acini formed from the wild-type cells. These consisted of a single layer of cells surrounding a completely hollow lumen. The Pak4 acini, however, rarely developed a completely hollow lumen, although at 26 days there was an increased volume of empty space, and some apoptosis could be seen in the lumen. Furthermore, rather than a single layer of cells surrounding the lumen, there often appeared to be several layers of cells surrounding the lumens in the Pak4 acini. In addition to these changes in lumenal structure, Pak4 acini generally grew to a larger size than the wild type acicni. The differences between wild-type acini and Pak4 acini with respect to lumen size and overall size of the structures are summarized in **Table 2**.

	pLPC		**pLPC-Pak4**	
	Relative size of the acini (number of acini examined)	**% of empty lumen**	**Relative size of the acini (number of acini examined)**	**% of empty lumen**
10~12 d	1 (85)	25.9%±2.9%	1.65 (84)	17.9%±2.2%
19 d	1.41 (88)	38.8%±2.5%	2.43 (88)	23.3%±2.3%
24~26 d	1.31 (66)	45.8%±2.2%	2.35 (65)	27.1%±2.7%

Table 2. Relative sizes (circumferential area) of the different acini (relative to the size of wild-type acini at 10-12 days) are shown. The amount of empty lumen in the different acini, was shown as a percentage of the total acini. The area of each acini or area of empty lumen was measured using Zeiss LSM software and plotted with the free shape curved drawing mode. These data represents three independent experiments. The mean values with standard error are presented for the empty lumen measurements.

Overexpression of Pak4 in iMMECs Promotes Cell Proliferation

In addition to the changes in acinar structure described above, we also observed that Pak4 expressing acini were often larger than the control acini (see **Figure 3**), although cell size appeared to be similar. To determine whether Pak4 expression led to increased cell proliferation, we stained acinar structures with the proliferation marker Ki67. Acini are shown at day 10 and 19 after plating. Compared with wild-type acini, Pak4 acini showed higher levels of cell proliferation. Proliferating cells can be seen in the lumens of the structures that contain Pak4, while this region is hollow in the wild-type structures (see **Figure 3**).

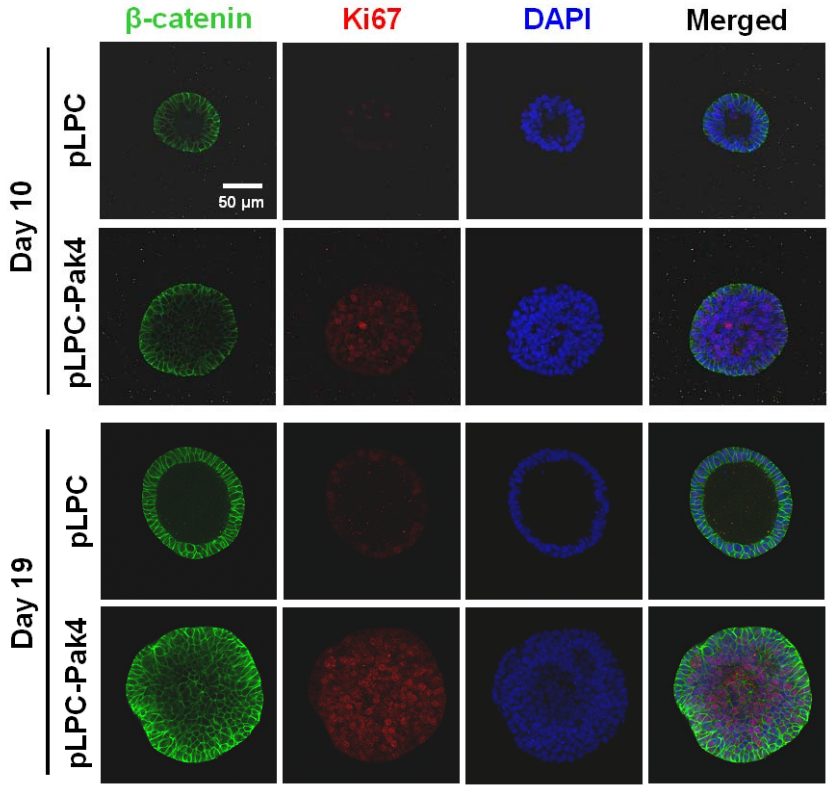


Figure 3

Figure 3. Overexpression of Pak4 promotes proliferation in iMMECs. iMMECs expressing empty vector (pLPC), or Pak4, were plated in 3D cultures. Acini are shown at day 10 and day 19 after plating. Acini were stained with beta-catenin (green) to visualize epithelial cells, DAPI (blue) for nuclear counterstaining, and Ki67 (red/purple) to visualize proliferating cells. Proliferating cells could be seen in the lumens of acini generated by Pak4-expressing iMMECs, while this region was empty in the wild-type acini.

Overexpression of Pak4 in iMMECs Leads to Prolonged Activation of the Erk Pathway

The results described above indicate that Pak4 overexpression promotes cell survival and proliferation. This prompted us to investigate the role for Pak4 in regulating the Erk MAP Kinase pathway, which plays an important role in both cell growth and proliferation [204]. We grew cells in 3D cultures and stained acinar structures with total Erk antibody and phospho-Erk antibody, which recognizes the activated form of Erk. Acini are shown at day 10, 19 and 26 after plating (see **Figure 4**).

At the earliest time point examined, both wild-type acini and Pak4-acini had similar high levels of phospho-Erk. However, after 19 days, wild-type acini had a significant decrease in phospho Erk levels, while Pak4 acini continuously maintained the high level of phospho-Erk activation, both in the lumen and in the surrounding cells. The results indicate that Pak4 has a role in promoting sustained activation of Erk in iMMECs.

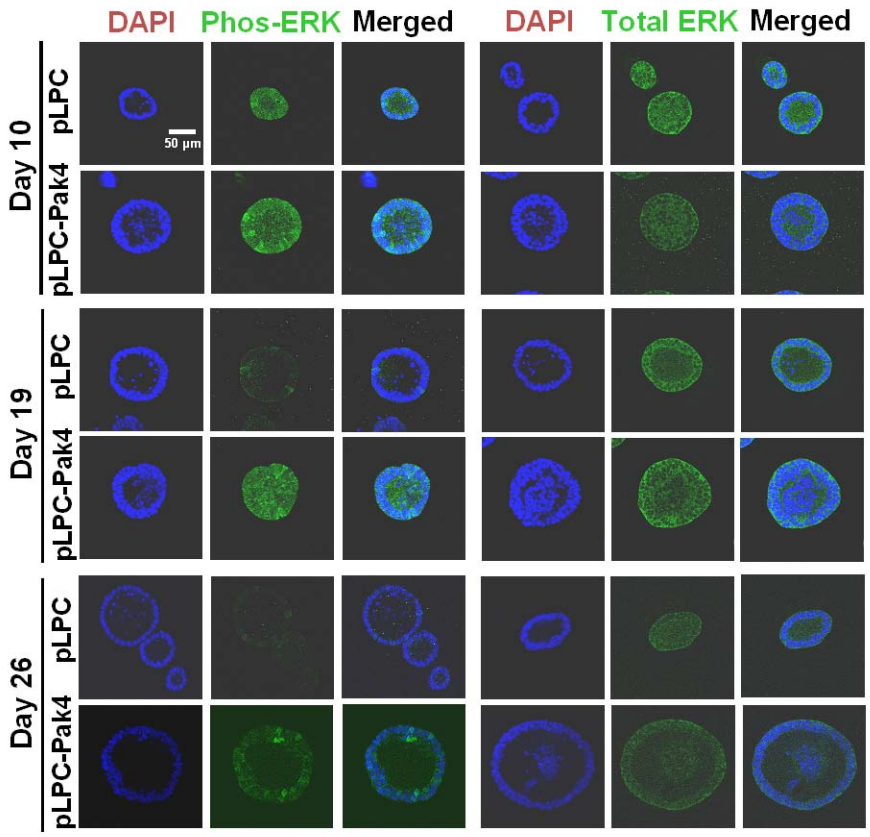


Figure 4

Figure 4. Overexpression of Pak4 promotes Erk activation. iMMECs expressing empty vector (pLPC) or Pak4, were plated in 3D cultures. Acini are shown at day 10, day 19 and day 26 after plating. Acini were stained with phospho-Erk or total Erk (green) to visualize Erk activation, and DAPI (blue) to visualize nuclei of all cells.

Overexpression of Pak4 Leads to Changes in iMMEC Cell Polarity

Disruption of cell polarity is another important hallmark of epithelial cancers and can lead to alterations in the 3D architecture of tissues. To examine cell polarity, cells were stained with anti GM130 antibody, which stains the Golgi and should face the lumen

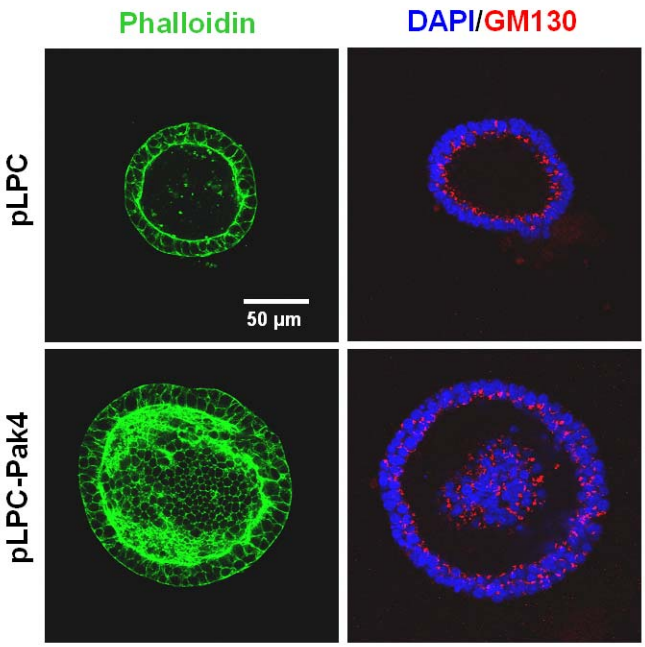


Figure 5

Figure 5. Pak4 overexpression leads to changes in cell polarity. Acini are shown at day 24 after plating. On the right, cells were stained with DAPI (blue), and GM130 (red), which stains the Golgi. In the wild-type structures most of the Golgi faced the lumens of the acini. In the Pak4 structures, the orientation of the Golgi was more random, with many

Golgi facing away from the lumen. On the left, cells were stained with FITC-labeled phalloidin to visualize F-actin.

when cells are properly polarized. Interestingly, while wild-type acini showed a normal pattern of staining (Golgi facing the lumen), in the Pak4 acini the orientation of the Golgi appeared to be somewhat random, with many Golgi facing away from the lumen (see **Figure 5**).

Overexpression of Pak4 Results in Tumor Formation in Athymic Mice

The tumorigenicity of iMMECs stably expressing Pak4 was assessed by orthotopic implantation into the mammary fat pads of athymic mice. Five weeks after implantation, iMMECs expressing wild-type Pak4 resulted in mammary tumor formation in 6 out of 10 mice, whereas control iMMECs (expressing empty vector) did not generate any tumors. By day 58 after implantation, 10 out of 10 mice implanted with wild-type Pak4-expressing iMMECs had mammary tumors (see **Figure 6A**). The tumor growth kinetics for iMMECs expressing Pak4 is shown in **Figure 6B**.

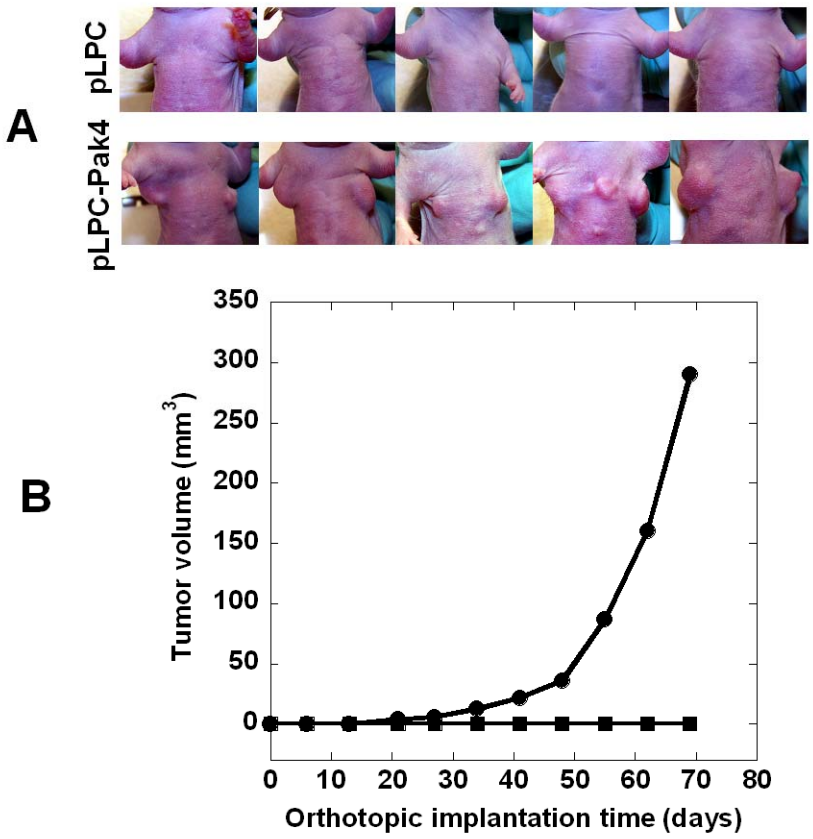


Figure 6

Figure 6. Overexpression of Pak4 results in mammary tumor formation in athymic mice. A. By day 58, iMMECs expressing wild-type Pak4 generated tumors in athymic mice. iMMECs expressing empty vector did not form tumors. **B.** Average tumor volumes from mice implanted with iMMECs expressing empty vector (no tumors, square dots) or wild-type Pak4 (round dots) were assessed.

Discussion

There is increasing evidence that Pak4 is overexpressed in human tumors and cancer cell lines, possibly due to gene amplification [98, 153]. Several studies have been carried out to investigate the role of Pak4 in transformation of fibroblasts. Our lab has found that overexpression of Pak4 in NIH3T3 mouse fibroblasts leads to tumor formation in athymic mice. However, since most tumors are of epithelial rather than mesenchymal origin, it is important to determine whether Pak4 plays an important role in transformation using epithelial cell models. Here we used immortalized mouse mammary cells (iMMECs) from wild-type mice as a model for studying the role for Pak4 in mammary tumorigenesis. These cells are an excellent model system because they have low levels of endogenous Pak4, they represent an early, non-tumorigenic stage in epithelial transformation, and when grown in 3D culture, they form polarized, hollow acini, which recapitulate the in vivo structure of the mammary glandular epithelium. Disruption of the well-ordered acinar architecture and filling of the luminal space is the hallmark of early epithelial tumors [205]. Studying iMMECs allows us to go beyond two dimensional cell culture and to study Pak4 in the context of a more complex and physiologically relevant three dimensional system [199]. In this paper we found that Pak4 overexpressing iMMECs formed mostly non-hollow acinar structures with a lower level of apoptosis and a thicker layer of cells surrounding the lumen. Most strikingly, iMMECs overexpressing Pak4 resulted in mammary tumors when implanted orthotopically in athymic mice, indicating that Pak4 is strongly transforming in these cells. The fact that overall cell growth rates in 2D cultures were unaffected by Pak4, suggests that changes triggered by Pak4 occur in a subset of cells, possibly influenced by cell interactions in a 3D-context.

The development and maintenance of polarized epithelial cells is critical for the normal function of epithelial tissue, and loss of epithelial polarity is frequently carcinomas associated with carcionogenesis [206]. Here we found that in the wild-type acini, the cells were properly polarized, where the apical layer (as assessed by Golgi staining) faces the lumen. In contrast, cell polarity in the Pak4 acini appeared to be somewhat random, with many Golgi facing away from the lumen. Our results for the first time suggest a possible role of Pak4 in disrupting cell polarization. Apicobasal polarization of cells is an important early event in acinar development, and its disruption may lead to uncontrolled cell proliferation and survival, resulting in profound effects on glandular structure.

Lumen formation requires apoptosis of the central acinar cells [196]. Pak4 has been implicated in both cell survival and cell proliferation pathways [96, 153]. We therefore proposed that inhibition of apoptosis and subsequent increased cell survival within the context of the 3D acinar structures play key roles in Pak4 induced tumorigenesis. In the 3D model, we found that a lumen was clearly beginning to form in the wild-type acini by day 12. Apoptotic cells could be seen in the centers of these structures, as assessed by staining with anti active caspase-3 antibody. In contrast, in Pak4 acini, very little apoptosis could be seen at this time point, and the lumen was mostly filled with cells. By day 19 there was a clear hollow lumen in the wild-type acini, while in the Pak4 acini the lumen still contained many cells. Finally, by day 26, typical acini formed from the wild-type cells. These consisted of a single layer of cells surrounding a completely hollow lumen. The Pak4 acini, however, rarely developed a completely hollow lumen, although at 26 days there was an increased volume of empty space, and some apoptosis could be

seen in the lumen. In MCF10A cells, it was previously shown that luminal apoptosis is involved in clearance of cells during lumen formation. Blocking apoptosis during acinar morphogenesis, however, could only delay the clearance of the cells, and the resulting structures eventually exhibited a hollow architecture [196]. Pak4 overexpressing iMMECs rarely formed a completely hollow lumen. One possible explanation is that in addition to inhibiting apoptosis, Pak4 may have other roles, such as promoting cell proliferation.

Cell proliferation occurs during early acini development, followed by suppression of proliferation several days after the onset of luminal apoptosis [196]. This is consistent with our findings that wild-type acini showed low levels of proliferation and a hollow lumen by day 19. In contrast, at this time point more proliferating cells could be seen in the lumens of the acini generated by Pak4-expressing iMMECs (see **Figure 3A**). Interestingly, by day 26, a very late stage in acinar formation, Pak4 expressing acini no longer showed higher levels of proliferating cells (data not shown). One possible explanation is that at earlier stages Pak4 promotes both survival and proliferation pathways, while at later stages of acinar formation, Pak4 may function more in cell survival pathways.

We previously showed that Pak4 specifically activates pro-survival pathways, which lead to activation of pathways such as the NF-κB pathway and the Erk MAP Kinase pathway [96]. The Erk pathway, which also has key roles in cell proliferation, was examined here in the context of 3D morphogenesis. By day 10 after plating, both wild-type and Pak4 acini had similar levels of activated Erk. However, by day 19, the levels of activated Erk dropped off significantly in the control acini, but remained high in the Pak4 acini. At this stage we also observed increased proliferation as well as decreased apoptosis

in the Pak4 acini relative to the control acini (see **Figure 2C**). By day 26 after plating, there was still a high level of activated Erk in the Pak4 acini. Interestingly, at this time point, while we continued to observe a decreased level of apoptosis in the Pak4 acini relative to the controls, there was only a low level of proliferation in the Pak4 acini (data not shown). These data suggest that Pak4 may induce cell proliferation and suppress apoptosis by promoting activation of the Erk pathway, although at least at the later time points, the increase in Erk activity is associated more with protection from apoptosis, rather than increased proliferation.

While we have found that Pak4 overexpression affects acinar structure in a way that is consistent with oncogenesis, resulting in loss of cell polarity, increased proliferation, and decreased apoptosis, the key test for oncogenic transformation is tumorigenicity in animals. To test this, we implanted iMMECs expressing either empty vector or wild-type Pak4 into the mammary fat pads of athymic mice. Remarkably, when Pak4 is overexpressed, iMMECs formed tumors in mice. This is the first report demonstrating that epithelial cells overexpressing Pak4 form tumors in mice. Our studies suggest that the mechanism behind this may involve regulation of cell growth and polarity within the complex 3D structure of the mammary epithelia. Our results, combined with the finding that Pak4 is overexpressed in human breast tumors and breast cancer cell lines, suggest that Pak4 plays an important role in initiation and progression of breast cancer. Our findings may have preventive or therapeutic implications for the rational design of drugs that target proteins that are activated in breast cancer.

Acknowledgements

We thank Dr. Chung S. Yang and Dr. Zhihong Yang for providing human colon cancer cell lines. This work was supported by R01 CA076342 to AM and R00 CA133181 to VK.

Chapter IV

Conclusions and Perspectives

In this thesis, we present for the first time, the important role of Pak4 in tumorigenesis *in vivo*. By using two different cell lines, NIH3T3 and immortalized mouse mammary epithelial cells (iMMECs), we found that overexpression of Pak4 in either cell line is sufficient to induce tumor formation in athymic mice. High rates of cell proliferation, increased cell survival, and decreased cell death are some of the characteristics of tumor cells. Previous *in vitro* studies have shown that Pak4 regulates various cellular activities, including cytoskeletal reorganization [32, 72, 79, 83-84, 207], cell survival [73, 96-97, 121-122] , and cell transformation [34, 95, 98] . In this thesis, we provide further evidence that Pak4 may promote cell growth and cell survival through activating the Erk pathway *in vivo*. We also show that Pak4 can protect cells from apoptosis, thus decreasing cell death. A new interesting finding for Pak4 is its function in changing cell polarity, which is also a hallmark of tumorigenesis, especially in three dimensional (3D) breast tumor formations. By using three cell lines, Pak4 conventional knockout 3T3 fibroblasts, Pak4 conditional knockout 3T3 fibroblasts, and Pak4 knock down HCT116 cells, we found that Pak4 is also necessary for tumorigeneis in response to certain oncogenes such as RasV12 and Cdc42V12.

Pak4 is overexpressed in a variety of types of tumors and cancer cell lines, and its important role in tumorigenesis makes it an attractive cancer prevention and/or therapeutic target. In addition to its implication in cancer, availability of its crystal structures, unique movements of the catalytic motifs [50], and inhibitor profiling further validate Pak4 as an *in vivo* target for the putative Pak inhibitors. The first clinical trial for Pak4 inhibitor is in fact already ongoing.

1. Pak4 Plays an Important Role in Tumorigenesis *in vivo*

1.1 Overexpression of Pak4 promotes tumor formation in athymic mice

Pak4 is overexpressed in tumors and cancer cell lines

Previous studies showed that Pak4 mRNA levels are high in cancer cell lines [98]. Cell lines, however, have gone through numerous passages and can be far removed from the actual primary tumor. We therefore carried out Western blot analysis of primary tumor tissues from four sources: primary human esophageal squamous cell carcinoma, mouse colon tumor tissue, rat mammary tumor tissue, and human breast tumor tissue. To further study the levels of Pak4 in other epithelial cell lines, we also examined the protein levels of Pak4 in human colon and prostate cancer cell lines. Although there was variability in the amount of overexpression, we found that in all of these types of cancer, there was a higher level of Pak4 protein in the tumor tissue compared with the normal control tissue (see Chapter II **Figure 1**). In addition, Pak4 was poorly expressed in iMMECs from wild-type mice (see Chapter III **Figures 1C and 2A**). Interestingly, however, when the iMMEC cells stably expressed oncogenic Ras or Her2/neu, which are highly transforming in these cells, Pak4 levels became highly elevated (see Chapter III **Figure 1C**). We further carried out real-time PCR with human normal and tumor tissue cDNA (see Chapter III **Figure 1A**) to study whether Pak4 mRNA levels are elevated in human epithelial cancers. We found that Pak4 mRNA levels are elevated in tumors of the breast, endometrium, esophagus, ovary, prostate, and urinary bladder.

Taken together, our expression studies indicate that Pak4 mRNA levels and protein level are in fact both elevated in primary tumors or cancer cell lines. These results provide evidence that Pak4 may be broadly expressed in different types of tumors rather than limited to a specific type of cancer. Until now, among the Pak family members, although Pak1 has also been found overexpressed in a variety of types of human cancers [208], Less is known about its direct role in inducing tumorigenesis. In this thesis, we for the first time provide important evidence that Pak4 may play an important role in tumorigenesis, which will be addressed in the following *in vivo* studies.

Overexpression of Pak4 in NIH3T3 cells leads to tumor formation in athymic mice

We used the retroviral infection method to generate NIH3T3 cell lines that stably overexpress wild-type Pak4, activated Pak4, or empty vector (pLPC). The different cell lines were injected subcutaneously into the flanks of athymic mice to determine whether they led to tumor formation. We found that cells overexpressing activated Pak4 and wild-type Pak4 formed large tumors in the mice after 16 days and 44 days of injection, respectively, whereas the control cells (containing empty vector (pLPC)) did not lead to tumor formation (see Chapter II **Figure 2B**). Further data from histologic analysis support the finding that both wild-type and activated Pak4 promote tumor formation when overexpressed. These results show for the first time that Pak4 promotes tumorigenesis in animals, and not only in cultured cells. The results are especially intriguing because we found that in mice, even wild-type Pak4 is tumorigenic when overexpressed. Our results support the idea that wild-type Pak4 is not only overexpressed in cancer cells that it is also sufficient to cause tumorigenesis.

These experiments were all carried out using NIH3T3 cells. Although NIH3T3 cells are frequently used as a model to study tumorigenesis, most tumors are of epithelial origin rather than fibroblasts origin. Therefore, we use another model in which epithelia cells were used, as summarized below. In these studies we investigated whether Pak4 also plays an important role in transformation and tumorigenesis of epithelial cells.

Overexpression of Pak4 in iMMECs abrogates lumen formation in 3D morphogenesis

We used iMMECs from wild-type mice as a model for studying the role for Pak4 in mammary tumorigenesis [199]. These cells have low levels of endogenous Pak4 and represent an early, non-tumorigenic stage in epithelial transformation, and when grown in 3D culture, they form polarized, hollow acini, which recapitulate the *in vivo* structure of the mammary glandular epithelium. The Pak4- and empty vector (pLPC)-transfected-iMMECs were grown on a layer of basement membrane in a 3D culture system, as described before [199]. Acinar structures were then examined by confocal microscopy at different time points. Similar to the vector control cells, Pak4-overexpressing iMMECs formed spherical acini. However, there were important differences between the two types of acinar structures. We found that Pak4 overexpressing iMMECs formed mostly non-hollow acinar structures with a lower level of apoptosis and a thicker layer of cells surrounding the lumen, while a largely hollow lumen was formed in the wild-type acini, which was formed by iMMECs with empty vector. In addition to these changes in lumenal structures, Pak4 acini generally grew to a larger size than the wild type acini.

Studying iMMECs allows us to go beyond two dimensional cell cultures and to study Pak4 in the context of a more complex and physiologically relevant three

dimensional system [199]. Since disruption of the well-ordered acinar architecture and filling of the luminal space is the hallmark of early epithelial tumors [209], overexpressing of Pak4 in iMMECs leading the morphology change of the acinar structure further indicates that Pak4 play a vital role in cell transformation.

Overexpression of Pak4 in iMMECs leads to tumor formation in athymic mice

Strongly transforming ability of Pak4 in iMMECs was further confirmed by the following animal study. iMMECs overexpressing Pak4 resulted in mammary tumors when implanted orthotopically in athymic mice. The tumorigenicity of iMMECs stably expressing Pak4 was assessed by orthotopic implantation into the mammary fat pads of athymic mice. By day 58 after implantation, 10 out of 10 mice implanted with wild-type Pak4-expressing iMMECs had mammary tumors, while control iMMECs did not form tumors. (see Chapter III **Figure 6A)** This is the first report demonstrating that epithelial cells overexpressing Pak4 form tumors in mice.

1.2 Pak4 is necessary for tumorigenesis in response to certain oncogenes

Tumor formation is attenuated in Pak4 null cells in response to oncogenic Ras in athymic mice

The above studies indicate that Pak4 is sufficient to form tumors in athymic mice. An equally important question is whether Pak4 is also necessary for tumor formation. To address this question, we used fibroblasts that had been isolated from Pak4 null embryos or wild-type controls and immortalized by serial passaging [41]. The Pak4 null and wild-

type fibroblasts (which grow at similar rates) were infected with a retrovirus containing oncogenic RasV12, a strong oncogene, or with empty vector (pLPC). We found that although RasV12 led to tumor formation, even in the absence of Pak4, the tumors grew more slowly and grew to a smaller size. It is interesting that some of the tumors derived from the RasV12; Pak4-/- cells were especially bloody in appearance. This result indicates that Pak4 function as one of the downstream signal pathways of Ras, and this is consistent with our finding that Pak4 expression level became highly elevated in iMMEC cells, in which transfected with Ras. These results provide further evidence for the critical roles for Pak4 in cell survival and inhibition of apoptosis [73, 96-97, 121-122] .

Tumor formation is abolished in Pak4 null cells in response to activated Cdc42 in athymic mice

Pak4 was originally identified as an effector for Cdc42 [32]. We therefore reasoned that Pak4 may function downstream to Cdc42 during tumorigenesis. Wild-type or Pak4 knockout immortalized fibroblasts were infected with activated Cdc42 (Cdc42V12) or empty vector (pLPC). We found that the absence of Pak4 led to nearly complete abrogation of tumors in response to Cdc42V12. This is consistent with the role for Pak4 as a Cdc42 effector protein and suggests the presence of a Pak4-dependent signaling pathway leading to Cdc42 and its activators to transformation.

Tumor formation is abrogated in Pak4 knockdown HCT116 cells in athymic mice

Studies described above involve the use of Pak4 knockout fibroblasts to investigate whether Pak4 is necessary for tumor formation. Most cancers, however, are of epithelial

origin. We therefore used small interfering RNA (siRNA) technology to knock down endogenous Pak4 in the human colon cancer epithelial cell line HCT116, which has κ-Ras muation. In order to obtain Pak4 knockdown stable cell lines, we used two siRNA oligonucleotides which were synthesized to target two different regions in the Pak4 cDNA: Pak4-RNAi-1 targeting a linker region between the regulatory domain and the kinase domain AACTTCATCAAGATTGGCGAG and Pak4-RNAi-2 targeting a sequence within the kinase domain AACGAGGTGGTAATCATGAGG. Transient transfection of both siRNAs could disrupt Pak4 expression in HCT116 cells, whereas a scrambled double-stranded RNA did not affect Pak4 expression. pSuper vector, 64-mer oligonucleotide DNA nucleotides targeting the same region as Pak4-RNAi-1 were designed according to the specifications recommended and synthesized by Invitrogen. pSuper-Pak4-RNAi was constructed as decribed before [96]. Stable cell lines were made by co-transfection of pSuper or pSuper-Pak4-RNAi together with the pLPC vector (containing a puromycin-resistant gene) into HCT116 cells using lipofactamine 2000 method. The cells were selected with puromycin (2 μg/ml), and the colonies were picked about two weeks after selection. As the variable expression levels of Pak4 being knocked down in different HCT116 colony, we picked the HCT116 cell line with pSuper-Pak4-RNAi vector showing the lowest Pak4 expression level, and it was injected into the athymic mice. After 24 days of injection, HCT116 cell line with Pak4 knockdown showed a significant reduction in tumor formation. This is the first study to show that Pak4 knockdown in epithelial cells led to a reduction in tumors formation *in vivo*. After examining more HCT116 cell line colonies with different levels of Pak4, we noticed that the level of Pak4 expression may be an issue for us to determine the roles of Pak4 in

tumorigenesis in mice. On the other hand, HCT116, as a cancer cell line, has a *k*-Ras mutation, a strong oncogenic mutation. To generate Pak4 knockdown cell lines, long term selections are required, and HCT116 colon cancer cell lines may undergo unknown cellular changes to compensate for the decreased Pak4 expression during this long period of colony selection. For these reasons experiments with Pak4 knockdown in HCT116 cell lines have some limitations for determining the roles of Pak4 in tumorigenesis in mice.

To circumvent the problems in above Pak4 knockdown experiments, and further determine whether Pak4 is required for the onset of colon cancer in mice, we have already successfully generated "floxed" Pak4 mice in our lab. These mice appear completely normal and breed normally, which will be used to generate colon tissue specific conditional knockout mice using Cre/LoxP system. These mice will be crossed with Tg(Vil-cre)997Gum mice that express Cre in the epithelial cells of the intestine, in turn Pak4 expression will be completely deleted in colon tissue, and other tissues of the body are expected not to be affected. These conditional Pak4 null mice and normal mice will be injected with carcinogen Azoxymethane (AOM), which has been shown to induce colon neoplasms that recapitulate the histological steps of the adenoma-carcinoma sequence [210]. The AOM induced colon carcinogenesis mouse model system has been well established in Dr. Chung S Yang's lab [210]. It is one of the main animal model systems used to study colon cancer, and AOM induced tumors share many characteristics of human tumors. Studying the ability of tumor formation induced by the carcinogene in colon tissue specific Pak4 null mice will provide us more direct evidence for the role of Pak4 in tumorigenesis.

1.3 Perspectives for Pak4 mediated signal pathways in tumorigenesis

Pak4, as the first identified member of group II Paks, shows its important role in cancer development. There is still a great deal to be learned about the mechanism by which Pak4 promotes tumorigenesis. The major features of a cancer cell include uncontrolled cell proliferation, escape from apoptotic signals, prolonged cell survival, altered cytoskeleton dynamics, and deregulated gene expression. Each of the above process for caner cell could be the basic direction for us to study the possible Pak4 mediated signal pathways in tumorigenesis. The findings in this thesis, as well as in some previous studies, focus largely on the role for Pak4 in prosurvival pathways and anti-apoptotic pathways, which are summarized in **Figure 1**. There are, however, other putative functions for Pak4 that may play a role in tumorigenesis including changing cell polarity, angiogenesis, and altering cytoskeleton dynamics. These Pak4 mediated signal events will be addressed in details.

Pak4 leads prolonged activation of Erk MAPK pathway

The Erk MAPK cascade has been addressed in details in the Chapter I, and it is considered to be one of the most important hallmarks in cancer models. There has been much evidence for the involvement of Erk in many human tumors, such as breast carcinoma, glioblastoma, as well as primary tumor cells derived from kidney, colon, and lung tissues.

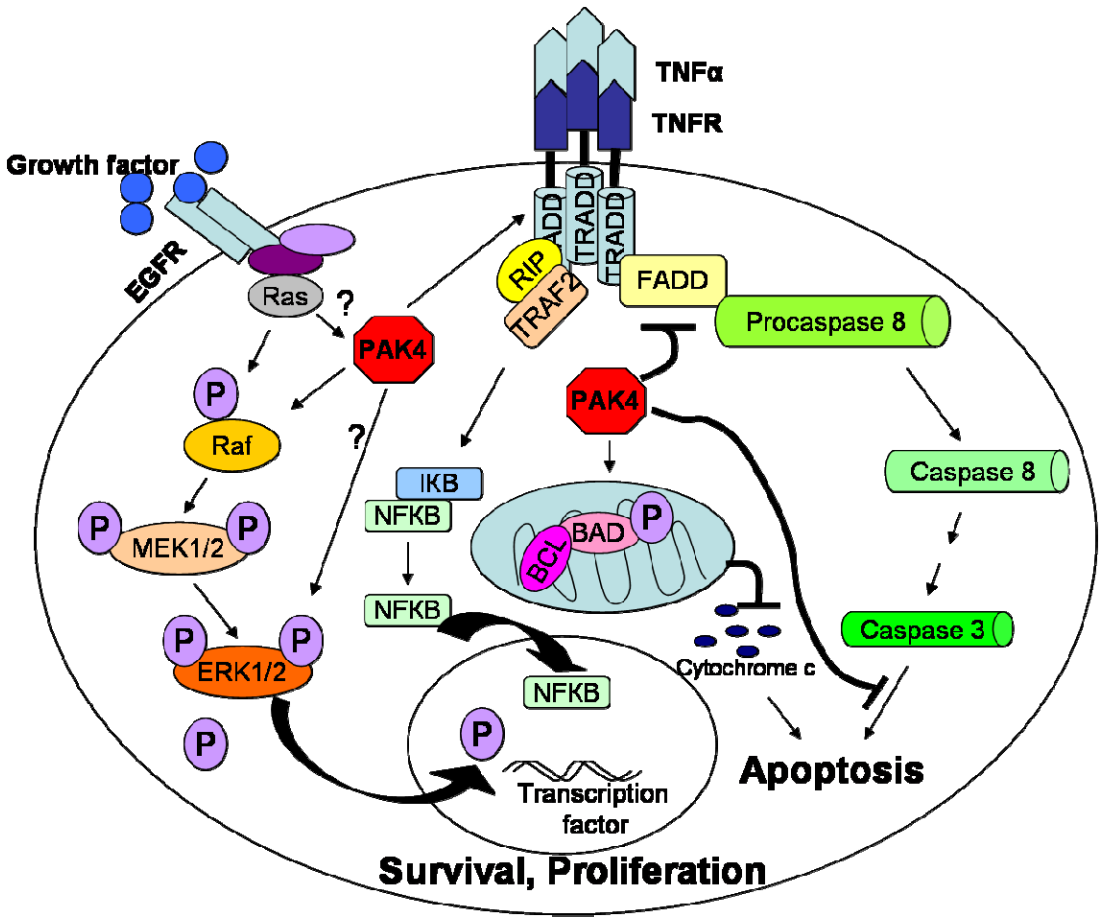


Figure 1. The role of Pak4 in the regulation of prosurvival and apoptotic pathway. The details are described in the text.

We previously showed that Pak4 specifically activates pro-survival pathways, which leads to activation of the Erk MAPK pathway [96]. Cells lacking Pak4 thus have decreased Erk activity [96]. It is noteworthy that expression of RasV12 leads to an increase in the level of Pak4 expression in primary cells, while we found that tumor formation is reduced when Pak4 knockout NIH3T3 cells expressing RasV12 are injected

into athymic mice. Pak4 was also found to phosphorylate and activate Raf. Based on the emerging information above, It is very likely that Pak4 is in part of the Ras-Raf-Erk MAPK pathways, where it functions downstream of Ras, and upstream of Erk.

In the case of the Ras-Raf-Erk pathway, the substrates of Erk1 and Erk2 include transcription factors and other kinases [211]. Genetic evidence from D. melanogaster strongly suggests that transcription factors are the major targets of activated Erk [212]. The diverse fundamental cellular response in which Erk has been implicated depends on the different combination of Erk with its substrates. Spaciotemporal features of Erk dynamics are then critical for establishing a specific cellular signals and mobilization of downstream effectors. In PC12 cells, sustained activation of Erk is associated with translocation of Erks to the nucleus, and nucleus accumulation of activated Erk will result in phosphorylation of transcription factors, consequently leading cellular proliferation and cell survival [213]. Both Ras and oncogenic Raf have been reported to produce prolonged activation of Erks [214]. In the context of 3D morphogenesis, we also found prolonged highly phosphorylation of Erk, which maintained in both cytosol and nucleus through out of all the stages of Pak4 acini formation. These data suggest that Pak4 not only activates Erk in MAPK pathway, but also promotes long-term activation and leads translocation of activated Erk to nucleus, finally inducing cell proliferation and cell survival.

Consistently, we see an increase in phosphorylated Erk in the Pak4-induced tumors, and this is associated with an increase in proliferation. It is interesting that there is a lag time before wild-type Pak4-expressing cells form tumors, once tumors begin to form, they do so quite rapidly and grow to a large size. We have found that the increase in

proliferation and Erk phosphorylation is seen early, even before tumors form in response to wild-type Pak4. These results suggest that increased proliferation and survival are events that occur early, during the lag time before tumorigenesis begins in response to wild-type Pak4. Interestingly, we found similar results from 3D iMMECs system that Pak4 promote proliferation in early stage rather than late stage. More proliferating cells could be seen in the lumens of the acini generated by Pak4-expressing iMMECs at early time points, day 10 and day19. However, by day 26, a very late stage in acinar formation, Pak4 expressing acini no longer showed higher levels of proliferating cells (data not shown). Notebaly, higher phosphorylation of Erk was maintained throughout all of the stages. The above results showed that Pak4 overexpression may induce cell proliferation at an early stage in both an animal model and 3D model. It is expected that at earlier stages, Pak4 promoting activation of Erk pathway is associated more with proliferation pathways, while at later stages of tumor formation, an increase in Erk activity by Pak4 may function more in other cell survival pathways, rather than increasing proliferation.

In summary, Pak4 is associated with prolonged activation of Erk MAPK pathway in certain cells, which may be the major pathway by which Pak4 induces cell proliferation and cell survival in tumorigenesis. Other pathways that Pak4 mediated in promoting cell proliferation need to be studied. Furthermore, it is interesting and meaningful to clearly illustrate the role of Pak4 in promoting cell proliferation at different stages during tumor formation, especially at early stages before tumors actually begin to form.

Pak4 protects cells from apoptosis

Apoptosis, or programmed cell death, is generally mediated by caspase cascades. Cell survival pathways can be mediated by disrupting the caspase cascades at various stages. One of the reasons for tumor formation can be an abnormal balance between the cell death and cell survival. Pak4 has been implicated in promoting cell proliferation pathways [96, 153]. Previously in vitro studies showed that Pak4 inhibits apoptosis and thus promotes cell survival [73, 96-97, 121-122]. We therefore proposed that inhibition of apoptosis and subsequent increased cell survival play a key role in Pak4 induced tumorigenesis.

Our lab also found that Pak4 inhibits apoptosis through two different mechanisms. The first is a kinase-independent mechanism, mediated by inhibition of initiator caspases [96], and the second is a kinase-dependent mechanism, mediated by phosphorylation of Bad [121]. Interestingly, we have found that there is a decreased level of apoptosis in Pak4-induced tumors compared with control tissues, as assessed by examining caspase-3 levels. We propose, therefore, that inhibition of apoptosis and subsequent increased cell survival and cell growth play a key role in Pak4-induced tumorigenesis. Interestingly, we have found that Pak4 (K350M), a kinase dead mutant, can promote tumorigenesis as efficiently as wild-type Pak4. This supports the idea that Pak4 can operate via the first mechanism, the kinase-independent mechanism, to promote cell survival and subsequent tumorigenesis.

In the 3D model, lumen formation requires apoptosis of the central acinar cells. We found that a lumen was clearly beginning to form in the wild-type acini by day 12. Apoptotic cells could be seen in the centers of these structures, as assessed by staining with anti active caspase-3 antibody. In contrast, in Pak4 acini, very little apoptosis could

be seen at this time point, and the lumen was mostly filled with cells. Finally by day 26, the Pak4 acini, rarely developed a completely hollow lumen, although there was an increased volume of empty space, and some apoptosis could be seen in the lumen, while typical acini formed from the wild-type cells. These consisted of a single layer of cells surrounding a completely hollow lumen. Pak4 overexpressing iMMECs rarely formed a completely hollow lumen. One possible explanation is that in addition to inhibiting apoptosis, Pak4 may promote cell proliferation at the same time.

In future experiments, it will be interesting to examine activated caspase-8 levels in the tumors as well as caspase-3, because Pak4 was shown to inhibit initiator caspases (like caspase-8), as well as effector caspases (like caspase-3), via a kinase-independent mechanism [96].

Pak4 leads to changes in cell polarity

Although changes in cell proliferation and survival are important features of transformation, there are other types of cellular changes that can lead to oncogenesis. For example, disruption of cell polarity is an important hallmark of many epithelial cancers [215] [200]. The development and maintenance of polarized epithelial cells is critical for the normal function of epithelial tissue, while disruption of normally organized tissue leads to increased migration, and it can be associated with uncontrolled proliferation. We found in our study that in the wild-type acini, the cells were properly polarized, where the apical layer (as assessed by Golgi staining) faces the lumen. In contrast, cell polarity in the Pak4 acini appeared to be somewhat random, with many Golgi facing away from the lumen [216]. Our results for the first time suggest a possible role of Pak4 in disrupting

cell polarization. Apicobasal polarization of cells is an important early event in acinar development, and its disruption may lead to uncontrolled cell proliferation and survival, resulting in profound effects on glandular structure.

While there are a number of mechanisms could affect cell polarity [217-218], regulating the Par complex by Pak4 may be one of them [219]. The PAR complex is a group of proteins normally located at the apical junction domain. Par6, a scaffold domain containing protein, interacts with Cdc42 through a modified CRIB domain [220]. This in turn leads to activation of atypical PKC (aPKC) by Par6 [220-221]. The activated aPKC then phosphorylates Par3, leading to the formation of an active Par complex at the apical domain, which leads to assembly of the junctional structure. This complex is particularly interesting because Par6, a component of the complex, binds to activated Cdc42. This raises the intriguing possibility that Pak4 competes with Par6 for Cdc42 binding, thus disrupting the formation of the Par complex and establishment normal polarity.

Pak4 in angiogenesis

As described throughout this thesis, Pak4 induced tumorigenesis may be mediated by different mechanisms, including stimulation of proliferation, inhibition of apoptosis, promotion of cell survival, and disruption of cell polarity. Regardless of the mechanism by which the cells are transformed, most tumors have no potential to grow to a large size without a blood supply. We did find that overexpression of Pak4 leads to the formation of large tumors in subcutaneously injected athymic mice. We also found that many capillaries formed around the Pak4 induced tumors, while tumors and capillaries are rarely seen in the sites injected with Pak4 null cells. The studies in this thesis indicate that

Pak4 may be involved in the process of angiogenesis. In fact, the role of Pak4 in angiogenesis is described in more detail in a recent publication from our lab [42]. We have found that Pak4 is absolutely required for embryonic development, and Pak4 null embryos die prior to embryonic day E11.5. When examining the Pak4 null embryos, we made the intriguing observation that there are abnormalities in the blood vessels throughout the Pak4 null embryos as well as 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. Furthermore, in my studies, we also noticed that Pak4 null cells showed no ability to form tumors, and that no blood vessels formed at the injection site. A role for Pak4 in angiogenesis could play an important part in its roles in the oncogenic process.

Pak4 in cytoskeleton reorganization

Pak4 as a regulator of actin cytoskeleton remodeling, promotes filopodia and lamellipodia formation, and also induces stress fiber dissociation, in turn plays an important role in determining cell morphology and motility. Therefore, it is very likely that Pak4 may be the potential factors in enhancing tumorigenesis, invasion, and the metastasis. Pak4 mediates morphological changes through its association with the Rho family GEF-H1 [83]. Pak4 interacts with integrin αvβ5 and selectively promotes integrin αvβ5-mediated migration [74]. Recently, two publications reported possible mechanisms for cancer cell migration induced by Pak4. One is a novel signaling complex downstream from HGF [75], involving a Gab1-Pak4 complex. This complex is critical for the breakdown of cell-cell contacts and for inducing cytoskeletal changes required for

migration and invasion of epithelial cells in response to HGF downstream from the Met receptor. Another novel signaling complex is associated with interaction between DGCR6L and Pak4 [222]. It was reported that DGCR6L specifically interacted with the very C-terminal 126 amino acids of Pak4. Overexpression of DGCR6L promoted Pak4-mediated migration of SGC-7901 gastric cancer cells and enhances the phosphorylation level of LIMK1 and cofilin, and Pak4-induced cell rounding is dependent on cofilin phosphorylation. These data suggest that DGCR6L interacting with Pak4 regulated migration of gastric cancer cells through LIMK1. Taken together, dynamic cytoskeletal changes are required for cell motility and invasion by cancer cells, and increasing evidence indicates that Pak4 as a key integrator of actin cytoskeleton reorganization promotes cancer cell invasion and migration. These findings have particular significance for human cancer.

2. Perspectives for Pak4 as a Drug Target for Cancer

This thesis provides valuable information that Pak4 plays an important role in tumorigenesis. The fact that Pak4 is overexpressed in different types of tumors and cancer cell lines indicates that the role for Pak4 in tumorigenesis may not be limited to the specific tumor type, but could be applied to a variety of types of cancers. Overexpression and loss-of-function studies for Pak4 further elucidate that Pak4 is sufficient and necessary for tumor formation *in vivo*. Among Pak groups, although Pak1 is another member of Pak families to be found overexpressed in many types of tumors [223], no direct evidence has been obtained that Pak1 really induces tumor formation *in vivo*. One paper indicates that Pak1 plays an important role in mammary gland

tumorigenesis in a transgenic mouse model, the catalytically active Pak1, however, was used in this study [224]. This result can not explain the correlation between Pak1 overexpression and a variety of types of cancer. Unlike Pak1, the importance for Pak4 in tummorigenesis has been confirmed in both cell and animal models in this thesis. Our work, therefore, further validate Pak4 to serve as a target for cancer prevention and/or therapy.

Among Pak families, the rationale for targeting group I Paks has been established, owing to their accumulating regulatory function in signal transduction in normal and pathological conditions. Well studied structure information also provides a strong basis for structure-based design. Several approaches for developing group I Paks inhibitor have been explored, and some of them have been successful. These approaches are described below. First, due to the signal-dependent activation of group I Paks, researches have designed specific inhibitors to interfere with upstream regulators of the Paks. A combination of SRC and ETK tyrosine-kinase inhibitors, PPI and AG879 [225], has been used successfully block Pak1 activity. CEP-1347, a synthetic derivative of the ATP antagonist K252a, was reported to directly inhibit Pak1 activity in vitro [226-227]. Second, method involves in manipulating autoinhibitory mechanism of group I Paks or of inhibiting the interations with negatively regulatory proteins. The inactive conformation of Pak1 has been targeted by a screen of 33,000 compounds which led to identification of an ATP noncompetitive, small-molecule allosteric inhibitor, IPA-3. Because of selectively stabilizing the Pak1 autoinhibitory conformation [228], IPA-3 can only prevent Pak1-mediated activation-segment phosphorylation. Emodin is a natural product that inhibits the interaction between Cdc42 and Pak1 [229]. Several peptides that are

derived from Pak1 have been reported to inhibit its activity, one of which is derived from the NCK binding site [128]. Recombinant peptide fragments of the Pak1 autoinhibitory domain efficiently inhibit the activity of the endogenous Pak1 [47]. Third, the accumulating structure information of Pak proteins provides a strong basis for structure-based design. Fine-design ATP competitive inhibitors have been identified in several reviews to lock the movements of active-site components. [40, 51, 223, 230]. Given the diversity and overlapping nature of Paks regulators and effectors, the specificity of some of these inhibitors are still problems in physiological animals. These problems have delayed the use of these inhibitors in clinical studies.

A great deal of information has been obtained for developing inhibitors of group I Paks, especially Pak1, and several major directions have been summarized above. However, we can not simply consider that Pak4 shares similar strategies for developing inhibitors with Pak1. Pak4 differs significantly with Pak1 in many aspects, which are described in details in the Chapter I. First, Pak4 is structurally distinct from Pak1 in its regulatory domain. Pak1 contains defined SH3 domains and an autoinhibitory domain region, which binds and inhibits the catalytic domain activity. However, due to the lack of obvious autoinhibitory region in Pak4, the basal kinase activity level of Pak4 exists in cells, and our results indicated that Pak4 induced tumorigenesis may be through kinase-dependent and/or kinase-independent pathways. Second, the regulatory mechanisms of Pak4 are considerably different from Pak1. Pak1 is activated by direct interaction with GTPase, SH3 domain containing proteins, or proteolytic cleavage. However, interaction of GTPase seems mainly having a targtet function for Pak4, rather than being essential for enzymetic activity. Taken together, structural and regulatory differences between

Pak1 and Pak4 indicate that the strategy for development of Pak4 inhibitors may not be the same as Pak1.

Protein kinases in fact are attractive drug targets, occupying about 30% of drug discovery programs in pharmaceutical companies, as protein kinases are important contributors to the progression of various cancer types [64, 231]. Pfizer's first started Pak4 inhibitor program with high throughput screening of kinase focus library compounds [*ClinicalTrial.gov*]. Multiple series were identified from the screening effort. As a result, PF-03758309 has demonstrated an excellent profile, leading to its selection as a clinical development candidate. PF-03758309 is a potent ATP-competitive inhibitor of Pak4 kinase domain. In engineered cell assays, PF-03758309 inhibited Pak4 dependent phosphorylation of its substrates GEF-H1. It potently inhibited the anchorage independent growth of HCT116 cells. PF-03758309 exhibited broad anti-proliferative activity across a panel of 67 cell lines. In a human xenograft tumor model, PF-03758309 is highly efficacious. Five of seven models tested showed robust tumor growth inhibition by PF-03758309: HCT116, A549, MDAMB231, M24met, and colo205. Pharmacodynamic and antitumor effects of PF-03758309 support its evaluation as an anticancer agent. PF-03758309 is currently being tested in a phase I clinical trial. In September 2009, Pfizer began sponsoring the first study using escalating doses of PF-03758309, an oral compound, in patients with advanced solid tumors. This clinical trial is estimated to be completed in Feburary 2012. In this trial, different doses of PF-03758309 will be administered to different groups of patients. The study will assess the compound's safety, the blood levels of PF-03758309 during the treatment and the effect of the compound on the tumor cells. Broad kinase screening has demonstrated that PF-

03758309 is a selective pan-Pak inhibitor with potential additional activities; therefore it remains to be seen whether PF-03758309 exhibits desired target selectivity [Program and Proceedings, November 15-19, 2009 Boston, MA].

Determining the specificity among closely related members of the same kinase family will be challenge in developing ATP mimetic small molecular inhibitors. However, Dr. Fedorov et al. provide a possible platform to explore this specificity issue. They generates a large number of kinase catalytic domain structures and small molecule high throughput screening profile of many functionally related kinase isoforms that clustered in the same family [232-233]. They found that PIM kinases and CDK like kinases (CLK) but not Casein kinase 1 (CK1) isoform show surprising selectivity between closed related isoforms. The distinct crystal structure comparison among the group II Paks have been found in recent studies, which shed light on a number of possibilities for the generation of Pak4 inhibitors with specificity [50, 234]. The amino acid sequence and structural comparisons of all three isoforms of group II Paks show variability in the region close to the ATP binding site, and this variable region could be applied towards selective small molecule binding of each isoforms of the group II Paks. The plasticity and dynamic nature of ligand bound catalytic domains of Pak4 suggest that the specificity of inhibitor can be achieved not by kinase domain exclusively, but in partnership with other proteins.

The use of the first Pak4 inhibitor in a clinical trial is encouraging. Compared with Pak1, generating Pak4 targets is still challenging, however, especially the problem of inhibitor specificity. Although emerging research on Pak4 illustrates its significant role in many fundamental cellular functions, we are only beginning to understand the regulation

of Pak4. Less information about specific Pak4 regulators limit the development of selective Pak4 inhibitors. Further elucidation of the precise regulatory mechanism by which Pak4 activity is specifically regulated, and identification of novel regulatory Pak4 binding proteins, may provide new avenues for the design of specific Pak4 inhibitors. We hope that specific Pak4 inhibitor can be developed into efficacious drug for cancer treatment.

Chapter V

References

1. Manser, E., et al., *A brain serine/threonine protein kinase activated by Cdc42 and Rac1.* Nature, 1994. **367**: p. 40-46.
2. Martin, G.A., et al., *A novel serine kinase activated by rac1/CDC42Hs-dependent autophosphorylation is related to PAK65 and STE20.* EMBO J, 1995. **14**: p. 1970-1978.
3. Bagrodia, S., et al., *Identification of a mouse p21Cdc42/Rac activated kinase.* J Biol Chem., 1995. **270**(39): p. 22731-22737.
4. Leberer, E., et al., *The protein kinase homologue Ste20p is required to link the yeast pheromone response G-protein beta gamma subunits to downstream signalling components.* EMBO J, 1992. **11**: p. 4815-4824.
5. Bokoch, G.M., *Biology of the p21-Activated Kinases.* Annu Rev Biochem, 2003.
6. Kumar, A., et al., *PAK thread from amoeba to mammals.* J Cell Biochem, 2009. **107**(4): p. 579-85.
7. Ottilie, S., et al., *Fission yeast pak1+ encodes a protein kinase that interacts with Cdc42p and is involved in the control of cell polarity and mating.* EMBO J, 1995. **14**(23): p. 5908-19.
8. Cvrckova, F., et al., *Ste20-like protein kinases are required for normal localization of cell growth and for cytokinesis in budding yeast.* Genes Dev., 1995. **9**: p. 1817-1830.
9. Dan, I., N.M. Watanabe, and A. Kusumi, *The Ste20 group kinases as regulators of MAP kinase cascades.* Trends Cell Biol, 2001. **11**(5): p. 220-230.
10. Benton, G., et al., *Cla4p, a saccharomyces cereisiae Cdc42p-activated kinase involved in cytokinesis, is activated at mitosis.* Mol Cell Biol, 1997. **17**(9): p. 5067-5076.
11. Marcus, S., et al., *Shk1, a homolog of the Saccharomyces cerevisiae Ste20 and mammalian p65PAK protein kinases, is a component of a Ras/Cdc42 signaling module in the fission yeast Schizosaccharomyces pombe.* Proc Natl Acad Sci U S A, 1995. **92**(13): p. 6180-4.
12. Qyang, Y., et al., *The p21-activated kinase, Shk1, is required for proper regulation of microtubule dynamics in the fission yeast, Schizosaccharomyces pombe.* Mol Microbiol, 2002. **44**(2): p. 325-34.
13. Muller-Taubenberger, A., et al., *Differential localization of the Dictyostelium kinase DPAKa during cytokinesis and cell migration.* J Muscle Res Cell Motil, 2002. **23**(7-8): p. 751-63.
14. de la Roche, M., et al., *Cellular distribution and functions of wild-type and constitutively activated Dictyostelium PakB.* Mol Biol Cell, 2005. **16**(1): p. 238-47.
15. Lee, S., et al., *Dictyostelium PAKc is required for proper chemotaxis.* Mol Biol Cell, 2004. **15**(12): p. 5456-69.
16. Rao, Y., *Dissecting Nck/Dock signaling pathways in Drosophila visual system.* Int J Biol Sci, 2005. **1**(2): p. 80-6.
17. Melzig, J., et al., *A protein related to p21-activated kinase (PAK) that is involved in neurogenesis in the Drosophila adult central nervous system.* Curr Biol, 1998. **8**: p. 1223-1226.

18. Chen, W., et al., *The Caenorhabditis elegans p21-activated kinase (CePAK) colocalizes with CeRac1 and CDC42Ce at hypodermal cell boundaries during embryo elongation.* J Biol Chem, 1996. **271**(42): p. 26362-26368.
19. Lucanic, M., et al., *The Caenorhabditis elegans P21-activated kinases are differentially required for UNC-6/netrin-mediated commissural motor axon guidance.* Development, 2006. **133**(22): p. 4549-59.
20. Bisson, N., et al., *The catalytic domain of xPAK1 is sufficient to induce myosin II dependent in vivo cell fragmentation independently of other apoptotic events.* Dev Biol, 2003. **263**(2): p. 264-81.
21. Faure, S., et al., *A member of the Ste20/PAK family of protein kinases is involved in both arrest of Xenopus oocytes at G2/prophase of the first meiotic cell cycle and in prevention of apoptosis.* EMBO J, 1997. **16**(18): p. 5550-5561.
22. Wu, C.F., et al., *Tumorhead distribution to cytoplasmic membrane of neural plate cells is positively regulated by Xenopus p21-activated kinase 1 (X-PAK1).* Dev Biol, 2007. **308**(1): p. 169-86.
23. Souopgui, J., M. Solter, and T. Pieler, *XPak3 promotes cell cycle withdrawal during primary neurogenesis in Xenopus laevis.* EMBO J, 2002. **21**(23): p. 6429-39.
24. Faure, S., et al., *Xenopus p21-activated kinase 5 regulates blastomeres' adhesive properties during convergent extension movements.* Dev Biol, 2005. **277**(2): p. 472-92.
25. Luo, T., et al., *Inca: a novel p21-activated kinase-associated protein required for cranial neural crest development.* Development, 2007. **134**(7): p. 1279-89.
26. Boda, B., et al., *Central Nervous System Functions of PAK Protein Family: From Spine Morphogenesis to Mental Retardation.* Mol Neurobiol, 2006. **34**(1-2): p. 67-80.
27. Meng, J., et al., *Abnormal long-lasting synaptic plasticity and cognition in mice lacking the mental retardation gene Pak3.* J Neurosci, 2005. **25**(28): p. 6641-50.
28. Dan, C., et al., *PAK5, a New Brain-Specific Kinase, Promotes Neurite Outgrowth in N1E- 115 Cells.* Mol. Cell. Biol., 2002. **22**(2): p. 567-577.
29. Li, X. and A. Minden, *Targeted disruption of the gene for the PAK5 kinase in mice.* Mol Cell Biol, 2003. **23**(20): p. 7134-42.
30. Yang, F., et al., *Androgen receptor specifically interacts with a novel p21-activated kinase, PAK6.* J Biol Chem, 2001. **276**(18): p. 15345-15353.
31. Kaur, R., et al., *Increased PAK6 expression in prostate cancer and identification of PAK6 associated proteins.* Prostate, 2008. **68**(14): p. 1510-6.
32. Abo, A., et al., *PAK4, a novel effector for Cdc42Hs, is implicated in the reorganization of the actin cytoskeleton and in the formation of filopodia.* EMBO J, 1998. **17**: p. 6527-6540.
33. Wells, C.M., A. Abo, and A.J. Ridley, *PAK4 is activated via PI3K in HGF-stimulated epithelial cells.* J Cell Sci, 2002. **115**(Pt 20): p. 3947-3956.
34. Zhang, H., Li, Z., Viklund, E.K., Stromblad, S., *P21-activated kinase 4 interacts with integrin alpha v beta 5 and regulates alpha v beta 5-mediated cell migration.* J Cell Biol, 2002. **158**(7): p. 1287-1297.
35. Cotteret, S. and J. Chernoff, *Nucleocytoplasmic shuttling of Pak5 regulates its antiapoptotic properties.* Mol Cell Biol, 2006. **26**(8): p. 3215-30.

36. Cotteret, S., et al., *p21-Activated kinase 5 (Pak5) localizes to mitochondria and inhibits apoptosis by phosphorylating BAD.* Mol Cell Biol, 2003. **23**(16): p. 5526-39.
37. Lee, S.R., et al., *AR and ER Interaction with a p21-Activated Kinase (PAK6).* Mol Endocrinol, 2002. **16**(1): p. 85-99.
38. Dharmawardhane, S., et al., *Localization of p21-activated kinase 1 (PAK1) to pinocytic vesicles and cortical actin structures in stimulated cells.* J Cell Biol, 1997. **138**(6): p. 1265-1278.
39. Nekrasova, T., Jobes, M.L., Tingh, J.H., Wagner, G.C., and Minden A., *Targeted dissruption of the Pak5 and Pak6 genes in mice leads to deficits in learning and locomotion.* Dev Biol, 2008. **322**(1): p. 95-108.
40. Arias-Romero, L.E. and J. Chernoff, *A tale of two Paks.* Biol Cell, 2008. **100**(2): p. 97-108.
41. Qu, J., et al., *PAK4 Kinase Is Essential for Embryonic Viability and for Proper Neuronal Development.* Mol Cell Biol, 2003. **23**(20): p. 7122-7133.
42. Tian, Y., Lei, L., Cammarano, M., Nekrasova, T., Minden, A., *Essential role for the Pak4 protein kinase in extraembryonic tissue development and vessel formation.* Mech Dev, 2009. **126**(8-9): p. 710-720.
43. Lei, M., et al., *Structure of PAK1 in an autoinhibited conformation reveals a multistage activation switch.* Cell, 2000. **102**(3): p. 387-397.
44. Puto, L.A., et al., *p21-activated kinase 1 (PAK1) interacts with the Grb2 adapter protein to couple to growth factor signaling.* J Biol Chem, 2003. **278**(11): p. 9388-93.
45. Bokoch, G.M., et al., *Interaction of the Nck adapter protein with p21-activated kinase (PAK1).* J Biol Chem., 1996. **271**(42): p. 25746-25749.
46. Manser, E., et al., *PAK kinases are directly coupled to the PIX family of nucleotide exchange factors.* Mol Cell, 1998. **1**: p. 183-192.
47. Zhao, Z.-S., et al., *A conserved negative regulatory region in aPAK: inhibition of PAK kinases reveals their morphological roles downstream of Cdc42 and Rac1.* Mol Cell Biol, 1998. **18**(4): p. 2153-2163.
48. Parrini, M.C., et al., *Pak1 Kinase Homodimers Are Autoinhibited in trans and Dissociated upon Activation by Cdc42 and Rac1.* Molecular Cell, 2002. **9**(1): p. 73-83.
49. Leeuw, T., et al., *Interaction of a G-protein beta-subunit with a conserved sequence in Ste20/PAK family protein kinases.* Nature, 1998. **391**: p. 191-195.
50. Eswaran, J., et al., *Crystal Structures of the p21-Activated Kinases PAK4, PAK5, and PAK6 Reveal Catalytic Domain Plasticity of Active Group II PAKs.* Structure, 2007. **15**(2): p. 201-213.
51. Eswaran, J., et al., *UnPAKing the class differences among p21-activated kinases.* Trends Biochem Sci, 2008. **33**(8): p. 394-403.
52. Chong, C., et al., *The mechanism of PAK activation. Autophosphorylation events in both regulatory and kinase domains control activity.* J Biol Chem, 2001. **276**(20): p. 17347-53.
53. Reeder, M.K., et al., *Analysis of small GTPase signaling pathways using p21-activated kinase mutants that selectively couple to Cdc42.* J Biol Chem, 2001. **276**(44): p. 40606-13.

54. Walter, B.N., et al., *Cleavage and activation of p21-activated protein kinase gamma-PAK by CPP32 (caspase 3). Effects of autophosphorylation on activity.* J Biol Chem, 1998. **273**(44): p. 28733-28739.
55. Galisteo, M.L., et al., *The adaptor protein Nck links receptor tyrosine kinases with the serine-threonine kinase Pak1.* J Biol Chem., 1996. **271**(35): p. 20997-21000.
56. King, C.C., et al., *p21-activated kinase (PAK1) is phosphorylated and activated by 3-phosphoinositide-dependent kinase-1 (PDK1).* J Biol Chem, 2000. **275**(52): p. 41201-9.
57. Tang, Y., et al., *The Akt proto-oncogene links Ras to Pak and cell survival signals.* J Biol Chem, 2000. **275**(13): p. 9106-9109.
58. Banerjee, M., et al., *Pak1 phosphorylation on t212 affects microtubules in cells undergoing mitosis.* Curr Biol, 2002. **12**(14): p. 1233-9.
59. Roig, J., P.T. Tuazon, and J.A. Traugh, *Functional interaction between c-Abl and the p21-activated protein kinase gamma-PAK.* Proc Natl Acad Sci, 2000. **97**(26): p. 14346-14351.
60. Bagheri-Yarmand, R., et al., *Etk/Bmx tyrosine kinase activates Pak1 and regulates tumorigenicity of breast cancer cells.* J Biol Chem, 2001. **276**(31): p. 29403-9.
61. Renkema, G.H., K. Pulkkinen, and K. Saksela, *Cdc42/Rac1-mediated activation primes PAK2 for superactivation by tyrosine phosphorylation.* Mol Cell Biol, 2002. **22**(19): p. 6719-25.
62. Bagrodia, S., et al., *A novel regulator of p21-activated kinases.* J Biol Chem, 1998. **273**(37): p. 23633-23636.
63. Turner, C.E., M.C. Brown, and P.S. Leventhal, *Paxillin LD4 motif binds PAK and PIX through a novel 95-kD ankyrin repeat, ARF--GAP protein: A role in cytoskeletal remodeling.* The journal of cell biology, 1999. **145**(4): p. 851-863.
64. Fabian, M.A., et al., *A small molecule-kinase interaction map for clinical kinase inhibitors.* Nat Biotechnol, 2005. **23**(3): p. 329-36.
65. Alahari, S.K., P.J. Reddig, and R.L. Juliano, *The integrin-binding protein Nischarin regulates cell migration by inhibiting PAK.* EMBO J, 2004. **23**(14): p. 2777-88.
66. Ding, Y., T. Milosavljevic, and S.K. Alahari, *Nischarin inhibits LIM kinase to regulate cofilin phosphorylation and cell invasion.* Mol Cell Biol, 2008. **28**(11): p. 3742-56.
67. Koh, C.G., et al., *The p21-activated kinase PAK is negatively regulated by POPX1 and POPX2, a pair of serine/threonine phosphatases of the PP2C family.* Curr Biol, 2002. **12**(4): p. 317-21.
68. Chan, P.M., L. Lim, and E. Manser, *PAK is regulated by PI3K, PIX, CDC42, and PP2Calpha and mediates focal adhesion turnover in the hyperosmotic stress-induced p38 pathway.* J Biol Chem, 2008. **283**(36): p. 24949-61.
69. Aronheim, A., et al., *Chp, a homologue of the GTPase Cdc42Hs, activates the JNK pathway and is implicated in reorganizing the actin cytoskeleton.* Curr Biol, 1998. **8**(20): p. 1125-8.
70. Weisz Hubsman, M., et al., *Autophosphorylation-dependent degradation of Pak1, triggered by the Rho-family GTPase, Chp.* Biochem J, 2007. **404**(3): p. 487-97.

71. Ching, Y.P., et al., *Identification of an autoinhibitory domain of p21-activated protein kinase 5.* J Biol Chem, 2003. **278**(36): p. 33621-4.
72. Ahmed, T., Shea, K., Masters, J.R., Jones, G.E., Wells, C.M., *A Pak4-LIMK1 pathway drives prostate cancer cell migration downstream of HGF.* Cell Signal, 2008. **20**(7): p. 1320-1328.
73. Lu, Y., et al., *p21-activated protein kinase 4 (PAK4) interacts with the keratinocyte growth factor receptor and participates in keratinocyte growth factor-mediated inhibition of oxidant-induced cell death.* J Biol Chem, 2003. **278**(12): p. 10374-80.
74. Zhang, H., et al., *P21-activated kinase 4 interacts with integrin alpha v beta 5 and regulates alpha v beta 5-mediated cell migration.* J Cell Biol, 2002. **158**(7): p. 1287-97.
75. Paliouras, G.N., M.A. Naujokas, and M. Park, *Pak4, a novel Gab1 binding partner, modulates cell migration and invasion by the Met receptor.* Mol Cell Biol, 2009. **29**(11): p. 3018-32.
76. Mattila, P.K. and P. Lappalainen, *Filopodia: molecular architecture and cellular functions.* Nat Rev Mol Cell Biol, 2008. **9**(6): p. 446-54.
77. Dharmawardhane, S., et al., *Localization of p21-activated kinase 1 (PAK1) to pinocytic vesicles and cortical actin structures in stimulated cells.* J Cell Biol, 1997. **138**(6): p. 1265-78.
78. Edwards, D.C., et al., *Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics.* Nat Cell Biol, 1999. **1**(September): p. 253-259.
79. Dan, C., et al., *Cytoskeletal changes regulated by the PAK4 serine/threonine kinase are mediated by LIM kinase 1 and cofilin.* J Biol Chem, 2001. **276**(34): p. 32115-32121.
80. Amano, T., et al., *LIM-kinase 2 induces formation of stress fibres, focal adhesions and membrane blebs, dependent on its activation by Rho-associated kinase-catalysed phosphorylation at threonine-505.* Biochem J, 2001. **354**(Pt 1): p. 149-159.
81. Sanders, L.C., et al., *Inhibition of myosin light chain kinase by p21-activated kinase.* Science, 1999. **283**(5410): p. 2083-2085.
82. Zeng, Q., et al., *Endothelial cell retraction is induced by PAK2 monophosphorylation of myosin II.* J Cell Sci, 2000. **113**(Pt 3): p. 471-482.
83. Callow, M.G., et al., *PAK4 mediates morphological changes through the regulation of GEF-H1.* J Cell Sci, 2005. **118**(Pt 9): p. 1861-72.
84. Soosairajah, J., et al., *Interplay between components of a novel LIM kinase-slingshot phosphatase complex regulates cofilin.* EMBO J, 2005. **24**(3): p. 473-86.
85. Li, F., et al., *p21-activated kinase 1 interacts with and phosphorylates histone H3 in breast cancer cells.* EMBO Rep, 2002. **3**(8): p. 767-73.
86. Vadlamudi, R.K., et al., *p21-activated kinase 1 regulates microtubule dynamics by phosphorylating tubulin cofactor B.* Mol Cell Biol, 2005. **25**(9): p. 3726-36.
87. Rayala, S.K., et al., *Dynamic interplay between nitration and phosphorylation of tubulin cofactor B in the control of microtubule dynamics.* Proc Natl Acad Sci U S A, 2007. **104**(49): p. 19470-5.

88. Balasenthil, S., et al., *p21-activated kinase-1 signaling mediates cyclin D1 expression in mammary epithelial and cancer cells.* J Biol Chem, 2004. **279**(2): p. 1422-8.
89. Parekh, P. and K.V. Rao, *Overexpression of cyclin D1 is associated with elevated levels of MAP kinases, Akt and Pak1 during diethylnitrosamine-induced progressive liver carcinogenesis.* Cell Biol Int, 2007. **31**(1): p. 35-43.
90. Zhao, Z.S., et al., *The GIT-associated kinase PAK targets to the centrosome and regulates Aurora-A.* Mol Cell, 2005. **20**(2): p. 237-49.
91. Maroto, B., et al., *P21-activated kinase is required for mitotic progression and regulates Plk1.* Oncogene, 2008. **27**(36): p. 4900-8.
92. Tang, Y., et al., *Kinase-deficient Pak1 mutants inhibit Ras transformation of Rat-1 fibroblasts.* Mol Cell Biol, 1997. **17**(8): p. 4454-4464.
93. Eblen, S.T., et al., *Rac-PAK signaling stimulates extracellular signal-regulated kinase (ERK) activation by regulating formation of MEK1-ERK complexes.* Mol Cell Biol, 2002. **22**(17): p. 6023-33.
94. Vadlamudi, R.K., et al., *Dynein light chain 1, a p21-activated kinase 1-interacting substrate, promotes cancerous phenotypes.* Cancer Cell, 2004. **5**(6): p. 575-85.
95. Cammarano, M.S., et al., *Pak4 induces premature senescence via a pathway requiring p16INK4/p19ARF and mitogen-activated protein kinase signaling.* Mol Cell Biol, 2005. **25**(21): p. 9532-42.
96. Li, X. and A. Minden, *PAK4 functions in tumor necrosis factor (TNF) alpha-induced survival pathways by facilitating TRADD binding to the TNF receptor.* J Biol Chem, 2005. **280**(50): p. 41192-200.
97. Qu, J., et al., *Activated PAK4 Regulates Cell Adhesion and Anchorage-Independent Growth.* Mol Cell Biol, 2001. **21**(10): p. 3523-3533.
98. Callow, M.G., et al., *Requirement for PAK4 in the anchorage-independent growth of human cancer cell lines.* J Biol Chem, 2002. **277**(1): p. 550-558.
99. Wallach, D., et al., *Tumor necrosis factor receptor and Fas signaling mechanisms.* Annu Rev Immunol, 1999. **17**: p. 331-67.
100. Ashkenazi, A. and V.M. Dixit, *Death receptors: signaling and modulation.* Science, 1998. **281**(5381): p. 1305-8.
101. Kischkel, F.C., et al., *Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor.* Embo J, 1995. **14**(22): p. 5579-88.
102. Hsu, H., J. Xiong, and D.V. Goeddel, *The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation.* Cell, 1995. **81**: p. 495-504.
103. Chinnaiyan, A.M., et al., *FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis.* Cell, 1995. **81**: p. 505-512.
104. Beg, A.A. and D. Baltimore, *An essential role for NF-kappaB in preventing TNF-alpha-induced cell death [see comments].* Science, 1996. **274**(5288): p. 782-784.
105. Liu, Z.G., et al., *Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death.* Cell, 1996. **87**: p. 565-576.

106. Shu, H.B., M. Takeuchi, and D.V. Goeddel, *The tumor necrosis factor receptor 2 signal transducers TRAF2 and c- IAP1 are components of the tumor necrosis factor receptor 1 signaling complex.* Proc Natl Acad Sci U S A, 1996. **93**(24): p. 13973-8.
107. Hsu, H., et al., *TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways.* Cell, 1996. **84**: p. 299-308.
108. Hsu, H., et al., *TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex.* Immunity, 1996. **4**: p. 387-396.
109. Salvesen, G.S. and V.M. Dixit, *Caspases: intracellular signaling by proteolysis.* Cell, 1997. **91**: p. 443-446.
110. Thornberry, N.A. and Y. Lazebnik, *Caspases: enemies within.* Science, 1998. **281**(August 28): p. 1312-1316.
111. Gross, A., et al., *Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death.* J Biol Chem, 1999. **274**(2): p. 1156-63.
112. Li, H., et al., *Cleavage of BID by caspase 8 mediates the mitochondrial damage in the fas pathway of apoptosis.* Cell, 1998. **94**(August 21): p. 491-501.
113. Luo, L., et al., *Bid, a Bcl2 Interacting Protein, Mediates Cytochrome c Release from Mitochondria in Response to Activation of Cell Surface Death Receptors.* Cell, 1998. **94**: p. 481-490.
114. Yin, X.M., et al., *Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis.* Nature, 1999. **400**(6747): p. 886-91.
115. Green, D.R., *Apoptotic pathways: the roads to ruin.* Cell, 1998. **94**(September 18): p. 695-698.
116. Dhillon, A.S., et al., *MAP kinase signalling pathways in cancer.* Oncogene, 2007. **26**(22): p. 3279-90.
117. Marshall, C.J., *Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation.* Cell, 1995. **80**(2): p. 179-85.
118. Schurmann, A., et al., *p21-activated kinase 1 phosphorylates the death agonist bad and protects cells from apoptosis.* Mol Cell Biol, 2000. **20**(2): p. 453-461.
119. Mazumdar, A. and R. Kumar, *Estrogen regulation of Pak1 and FKHR pathways in breast cancer cells.* FEBS Lett, 2003. **535**(1-3): p. 6-10.
120. Jakobi, R., E. Moertl, and M.A. Koeppel, *p21-activated protein kinase gamma-PAK suppresses programmed cell death of BALB3T3 fibroblasts.* J Biol Chem, 2001. **276**(20): p. 16624-34.
121. Gnesutta, N., J. Qu, and A. Minden, *The Serine/Threonine Kinase PAK4 prevents caspase activation and protects cells from apoptosis.* J Biol Chem, 2001. **276**: p. 14414-14419.
122. Gnesutta, N. and A. Minden, *Death Receptor Induced Activation of Initiator Caspase-8 is Antagonized by the Serine/Threonine Kinase PAK4.* Mol. Cell. Biol., 2003. **23**: p. 7838-7848.
123. Bussolino, F., A. Mantovani, and G. Persico, *Molecular mechanisms of blood vessel formation.* Trends biochem Sci, 1997. **22**: p. 251-256.

124. Soga, N., Namba, N., McAllister, S., Cornelius, L., Teitebaum, S.L., Dowdy, S.F., Kawamura, J., Hruska, K.A., *Rho family GTPases regulate VEGF-stimulated endothelial cell motility.* Experimental Cell Research, 2001. **269**: p. 73-87.
125. Kiosses, W.B., et al., *A role for p21-activated kinase in endothelial cell migration.* J Cell Biol, 1999. **147**(4): p. 831-844.
126. Kiosses, W.B., et al., *A dominant-negative p65 PAK peptide inhibits angiogenesis.* Circ Res, 2002. **90**(6): p. 697-702.
127. Master, Z., et al., *Dok-R plays a pivotal role in angiopoietin-1-dependent cell migration through recruitment and activation of Pak.* EMBO J, 2001. **20**(21): p. 5919-28.
128. Bagheri-Yarmand, R., et al., *Vascular endothelial growth factor up-regulation via p21-activated kinase-1 signaling regulates heregulin-beta1-mediated angiogenesis.* J Biol Chem, 2000. **275**(50): p. 39451-7.
129. Koh, W., R.D. Mahan, and G.E. Davis, *Cdc42- and Rac1-mediated endothelial lumen formation requires Pak2, Pak4 and Par3, and PKC-dependent signaling.* J Cell Sci, 2008. **121**(Pt 7): p. 989-1001.
130. Gerber, P.A., et al., *Neurofibromatosis.* Eur J Med Res, 2009. **14**(3): p. 102-5.
131. Yi, C., et al., *Validation of the p21-activated kinases as targets for inhibition in neurofibromatosis type 2.* Cancer Res, 2008. **68**(19): p. 7932-7.
132. Kissil, J.L., et al., *Merlin, the product of the Nf2 tumor suppressor gene, is an inhibitor of the p21-activated kinase, Pak1.* Mol Cell, 2003. **12**(4): p. 841-9.
133. Tang, Y., et al., *A role for Pak protein kinases in Schwann cell transformation.* Proc Natl Acad Sci USA, 1998. **95**(9): p. 5139-5144.
134. Hayashi, M.L., et al., *Inhibition of p21-activated kinase rescues symptoms of fragile X syndrome in mice.* Proc Natl Acad Sci U S A, 2007. **104**(27): p. 11489-94.
135. Luo, S., H. Mizuta, and D.C. Rubinsztein, *p21-activated kinase 1 promotes soluble mutant huntingtin self-interaction and enhances toxicity.* Hum Mol Genet, 2008. **17**(6): p. 895-905.
136. Allen, K.M., et al., *PAK3 mutation in nonsyndromic X-linked mental retardation.* Nat Genet, 1998. **20**(1): p. 25-30.
137. Danzer, K.M., et al., *Functional protein kinase arrays reveal inhibition of p-21-activated kinase 4 by alpha-synuclein oligomers.* J Neurochem, 2007. **103**(6): p. 2401-7.
138. Matenia, D., et al., *PAK5 kinase is an inhibitor of MARK/Par-1, which leads to stable microtubules and dynamic actin.* Mol Biol Cell, 2005. **16**(9): p. 4410-22.
139. Agopian, K., et al., *A hydrophobic binding surface on the human immunodeficiency virus type 1 Nef core is critical for association with p21-activated kinase 2.* J Virol, 2006. **80**(6): p. 3050-61.
140. Nguyen, D.G., et al., *"UnPAKing" human immunodeficiency virus (HIV) replication: using small interfering RNA screening to identify novel cofactors and elucidate the role of group I PAKs in HIV infection.* J Virol, 2006. **80**(1): p. 130-7.
141. Renkema, G.H., et al., *Identification of the nef-associated kinase as p21-activated kinase 2 [In Process Citation].* Curr Biol, 1999. **9**(23): p. 1407-1410.
142. Fackler, O.T., et al., *p21-Activated Kinase 1 Plays a Critical Role in Cellular Activation by Nef.* Mol Cell Biol, 2000. **20**(7): p. 2619-2627.

143. Manninen, A., et al., *SH3-Domain Binding Function of HIV-1 Nef Is Required for Association with a PAK-Related Kinase.* Virology, 1998. **250**(2): p. 273-282.
144. Liberali, P., et al., *The closure of Pak1-dependent macropinosomes requires the phosphorylation of CtBP1/BARS.* EMBO J, 2008. **27**(7): p. 970-81.
145. Mercer, J. and A. Helenius, *Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells.* Science, 2008. **320**(5875): p. 531-5.
146. Kuijl, C., et al., *Intracellular bacterial growth is controlled by a kinase network around PKB/AKT1.* Nature, 2007. **450**(7170): p. 725-30.
147. Gringel, A., Walz, D., Rosenberger, G., Minden, A., Kutsche, K.,Kopp, P., Linder, S., *Pak4 and alphaPIX determine podosome size and number in macrophages througgh localized actin regulation.* J Cell Physiol, 2006(209): p. 2.
148. Bekri, S., et al., *Detailed map of a region commonly amplified at 11q13-->q14 in human breast carcinoma.* Cytogenet Cell Genet, 1997. **79**(1-2): p. 125-31.
149. Brown, L.A., et al., *Amplification of 11q13 in ovarian carcinoma.* Genes Chromosomes Cancer, 2008. **47**(6): p. 481-9.
150. Parsons, D.W., et al., *Colorectal cancer: mutations in a signalling pathway.* Nature, 2005. **436**(7052): p. 792.
151. Chen, S., et al., *Copy number alterations in pancreatic cancer identify recurrent PAK4 amplification.* Cancer Biol Ther, 2008. **7**(11).
152. Aoki, H., et al., *Phosphorylated Pak1 level in the cytoplasm correlates with shorter survival time in patients with glioblastoma.* Clin Cancer Res, 2007. **13**(22 Pt 1): p. 6603-9.
153. Liu, Y., Xiao, H., Tian, Y., Nekrasova, T., Hao, X., Lee, H.J., Suh, N., Yang, C.S., Minden, A., *The Pak4 protein kinase plays a key role in cell survival and tumorigenesis in athymic mice.* Molecular Cancer Research, 2008. **6**(7): p. 1215-1224.
154. Holm, C., et al., *Association between Pak1 expression and subcellular localization and tamoxifen resistance in breast cancer patients.* J Natl Cancer Inst, 2006. **98**(10): p. 671-80.
155. Ching, Y.P., et al., *P21-activated protein kinase is overexpressed in hepatocellular carcinoma and enhances cancer metastasis involving c-Jun NH2-terminal kinase activation and paxillin phosphorylation.* Cancer Res, 2007. **67**(8): p. 3601-8.
156. O'Sullivan, G.C., et al., *Modulation of p21-activated kinase 1 alters the behavior of renal cell carcinoma.* Int J Cancer, 2007. **121**(9): p. 1930-40.
157. Mahlamaki, E.H., et al., *High-resolution genomic and expression profiling reveals 105 putative amplification target genes in pancreatic cancer.* Neoplasia, 2004. **6**(5): p. 432-9.
158. Carter, J.H., et al., *Pak-1 expression increases with progression of colorectal carcinomas to metastasis.* Clin Cancer Res, 2004. **10**(10): p. 3448-56.
159. Ito, M., et al., *P21-activated kinase 1: a new molecular marker for intravesical recurrence after transurethral resection of bladder cancer.* J Urol, 2007. **178**(3 Pt 1): p. 1073-9.
160. Davidson, B., M. Shih Ie, and T.L. Wang, *Different clinical roles for p21-activated kinase-1 in primary and recurrent ovarian carcinoma.* Hum Pathol, 2008. **39**(11): p. 1630-6.

161. Schraml, P., et al., *Combined array comparative genomic hybridization and tissue microarray analysis suggest PAK1 at 11q13.5-q14 as a critical oncogene target in ovarian carcinoma.* Am J Pathol, 2003. **163**(3): p. 985-92.
162. Mao, X., et al., *Genomic alterations in blastic natural killer/extranodal natural killer-like T cell lymphoma with cutaneous involvement.* J Invest Dermatol, 2003. **121**(3): p. 618-27.
163. Li, Q., et al., *p21-Activated kinase 1 coordinates aberrant cell survival and pericellular proteolysis in a three-dimensional culture model for premalignant progression of human breast cancer.* Neoplasia, 2008. **10**(4): p. 314-29.
164. Reddy, S.D., et al., *MicroRNA-7, a homeobox D10 target, inhibits p21-activated kinase 1 and regulates its functions.* Cancer Res, 2008. **68**(20): p. 8195-200.
165. Mira, J.P., et al., *Endogenous, hyperactive rac3 controls proliferation of breast cancer cells by a p21-activated kinase-dependent pathway.* Proc Natl Acad Sci U S A, 2000. **97**(1): p. 185-189.
166. Huang, Z., J.A. Traugh, and J.M. Bishop, *Negative control of the Myc protein by the stress-responsive kinase Pak2.* Mol Cell Biol, 2004. **24**(4): p. 1582-94.
167. Wilkes, M.C., et al., *Cell-type-specific activation of PAK2 by transforming growth factor beta independent of Smad2 and Smad3.* Mol Cell Biol, 2003. **23**(23): p. 8878-89.
168. Suzuki, K., et al., *Transforming growth factor beta signaling via Ras in mesenchymal cells requires p21-activated kinase 2 for extracellular signal-regulated kinase-dependent transcriptional responses.* Cancer Res, 2007. **67**(8): p. 3673-82.
169. Wilkes, M.C., et al., *Erbin and the NF2 tumor suppressor Merlin cooperatively regulate cell-type-specific activation of PAK2 by TGF-beta.* Dev Cell, 2009. **16**(3): p. 433-44.
170. Rayala, S.K., P.R. Molli, and R. Kumar, *Nuclear p21-activated kinase 1 in breast cancer packs off tamoxifen sensitivity.* Cancer Res, 2006. **66**(12): p. 5985-8.
171. Bostner, J., et al., *Estrogen receptor-alpha phosphorylation at serine 305, nuclear p21-activated kinase 1 expression, and response to tamoxifen in postmenopausal breast cancer.* Clin Cancer Res, 2010. **16**(5): p. 1624-33.
172. Friedland, J.C., et al., *alpha6beta4 integrin activates Rac-dependent p21-activated kinase 1 to drive NF-kappaB-dependent resistance to apoptosis in 3D mammary acini.* J Cell Sci, 2007. **120**(Pt 20): p. 3700-12.
173. Edwards, D.C., et al., *Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics.* Nat Cell Biol, 1999. **1**(5): p. 253-9.
174. Vadlamudi, R.K., et al., *p41-Arc subunit of human Arp2/3 complex is a p21-activated kinase-1-interacting substrate.* EMBO Rep, 2004. **5**(2): p. 154-60.
175. Vadlamudi, R.K., et al., *Filamin is essential in actin cytoskeletal assembly mediated by p21-activated kinase 1.* Nat Cell Biol, 2002. **4**(9): p. 681-90.
176. Gururaj, A., et al., *Regulation of phosphoglucomutase 1 phosphorylation and activity by a signaling kinase.* Oncogene, 2004. **23**(49): p. 8118-27.
177. Gong, W., et al., *P21-activated kinase 5 is overexpressed during colorectal cancer progression and regulates colorectal carcinoma cell adhesion and migration.* Int J Cancer, 2009.

178. Kaur, R., et al., *Activation of p21-activated kinase 6 by MAP kinase kinase 6 and p38 MAP kinase.* J Biol Chem, 2005. **280**(5): p. 3323-30.
179. Minden, A., et al., *Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK.* Science, 1994. **266**: p. 1719-1723.
180. Daniels, R.H. and G.M. Bokoch, *p21-activated protein kinase: a crucial component of morphological signaling?* Trends Biochem Sci, 1999. **24**(9): p. 350-355.
181. Knaus, U.G. and G.M. Bokoch, *The p21Rac/Cdc42-activated kinases (PAKs).* Int J Biochem Cell Biol, 1998. **30**(8): p. 857-862.
182. Sells, M.A. and J. Chernoff, *Emerging from the Pak: the p21-activated protein kinase family.* Trends Cell Biol, 1997. **7**: p. 162-167.
183. Jaffer, Z.M. and J. Chernoff, *p21-Activated kinases: three more join the Pak.* Int J Biochem Cell Biol, 2002. **34**(7): p. 713-717.
184. Lin, R., et al., *A novel Cdc42Hs mutant induces cellular transformation.* Curr Biol, 1997. **7**(10): p. 794-797.
185. Lin, R., R.A. Cerione, and D. Manor, *Specific contributions of the small GTPases Rho, Rac, and Cdc42 to Dbl transformation.* J Biol Chem, 1999. **274**(33): p. 23633-41.
186. Qiu, R.G., et al., *Cdc42 regulates anchorage-independent growth and is necessary for ras transformation.* Mol Cell Biol, 1997. **17**(6): p. 3449-3458.
187. Fang, M.Z., et al., *Promoter hypermethylation and inactivation of O(6)-methylguanine-DNA methyltransferase in esophageal squamous cell carcinomas and its reactivation in cell lines.* Int J Oncol, 2005. **26**(3): p. 615-622.
188. Thompson, H.J. and M. Singh, *Rat models of premalignant breast disease.* J Mammary Gland Biol Neoplasia, 2000. **5**(4): p. 409-20.
189. Kroger, A., et al., *IRF-1 reverts the transformed phenotype of oncogenically transformed cells in vitro and in vivo.* Oncogene, 2003. **22**(7): p. 1045-56.
190. Cheung, A.M., et al., *Three-dimensional ultrasound biomicroscopy for xenograft growth*
analysis. . Ultrasound Med Biol, 2005. **31**(6): p. 865-870.
191. Hao, X.P., et al., *Beta catenin expression is altered in human colonoc aberrant crypt foci.* Cancer Res, 2001. **61**(22): p. 8085-8088.
192. Troussard, A.A., Mawji, N.M., Ong, C., Mui, A., St.-Arnaud II, R., Dedhar, S., *Conditional knockout of integrin-linked kinase demonstrates an essential role in protein kinase B Akt activation.* J Biol Chem, 2003. **278**(25): p. 22374-22378.
193. Negrete, A., Chuan, T., Lyddiatt, A., *Production of adenoviral vectors and its recovery.* Process Biochemistry, 2007. **42**(7): p. 1107-1113.
194. Fadeel, B. and S. Orrenius, *Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease.* J Intern Med, 2005. **258**(6): p. 479-517.
195. Budihardjo, I., et al., *Biochemical pathways of caspase activation during apoptosis.* Annu Rev Cell Dev Biol, 1999. **15**: p. 269-290.
196. Debnath, J., Mills, K.R., Collins, N.L., Reginato, M.J., Muthuswamy, S.K., Brugge, J.S., *The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini.* Cell, 2002. **111**: p. 29-40.

197. Debnath, J., Muthuswamy, S.K., Brugge, J.S., *Morphogenesis and oncogeesis of MCF-10A mammary epithelial acini grown in thre-dimensional basement membrane cultures.* Methods, 2003. **30**(2003): p. 256-268.
198. Muthuswamy, S., Li, D., Lelievre, S., Bissell, M., Brugge, J.S. , *ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini.* Nature Cell Biol, 2001. **3**: p. 785-792.
199. Karantza-Wadsworth, V., White, E., *A mouse mammary epithelial cell model to identify molecular mechanisms regulating breast cancer progression.* Meth Enzymol, 2008. **446**: p. 61-76.
200. Moreno-Bueno, G., Portillo, F., Cano, A., *Transcriptional regulation of cell polarity in EMT and cancer.* Oncogene, 2008. **27**: p. 6958-6969.
201. Tanos, B., Rodriguez-Boulan, E., *The epithelial polarity program: machineries involved and their hijacking by cancer.* Oncogene, 2008. **27**: p. 6939-6957.
202. Karantza-Wadsworth, V., Patel, S., Kravchuk, O., Chen, G., Mathew, R., Jin, S., White, E., *Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis.* Genes and Development, 2007. **21**: p. 1621-1635.
203. Degenhardt, K. and E. White, *A mouse model system to genetically dissect the molecular mechanisms regulating tumorigenesis.* Clin Cancer Res, 2006. **12**(18): p. 5298-304.
204. Xia, Z., et al., *Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis.* Science, 1995. **270**: p. 1326-1331.
205. Catteau, A., et al., *Methylation of the BRCA1 promoter region in sporadic breast and ovarian cancer: correlation with disease characteristics.* Oncogene, 1999. **18**(11): p. 1957-65.
206. Zhan, L., et al., *Deregulation of scribble promotes mammary tumorigenesis and reveals a role for cell polarity in carcinoma.* Cell, 2008. **135**(5): p. 865-78.
207. Ahmed, S., et al., *Cryptic Rac-binding and p21Cdc-42Hs/Rac-activated kinase phosphorylation sites of NADPH oxidase component p67phox.* J Biol Chem, 1998. **273**(25): p. 15693-15701.
208. Dummler, B., et al., *Pak protein kinases and their role in cancer.* Cancer Metastasis Rev, 2009. **28**(1-2): p. 51-63.
209. Reginato, M.J. and S.K. Muthuswamy, *Illuminating the center: mechanisms regulating lumen formation and maintenance in mammary morphogenesis.* J Mammary Gland Biol Neoplasia, 2006. **11**(3-4): p. 205-11.
210. Ju, J., et al., *Voluntary exercise inhibits intestinal tumorigenesis in Apc(Min/+) mice and azoxymethane/dextran sulfate sodium-treated mice.* BMC Cancer, 2008. **8**: p. 316.
211. Hill, C.S., J. Wynne, and R. Treisman, *The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF.* Cell, 1995. **81**(7): p. 1159-70.
212. Tsuda, L., et al., *A protein kinase similar to MAP kinase activator acts downstream of the raf kinase in Drosophila.* Cell, 1993. **72**(3): p. 407-14.
213. Traverse, S., et al., *Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor.* Biochem J, 1992. **288 (Pt 2)**: p. 351-5.

214. Leevers, S.J. and C.J. Marshall, *Activation of extracellular signal-regulated kinase, ERK2, by p21ras oncoprotein.* EMBO J, 1992. **11**(2): p. 569-74.
215. Lee, M., Vasioukhin, V., *Cell polarity and cancer - cell and tissue polarity as a non-canonical tumor suppressor.* J Cell Sci, 2008. **121**(8): p. 1141-1150.
216. Bershadsky, A. and A. Futerman, *Disruption of the Golgi apparatus by brefeldin A blocks cell polarization and inhibits directed cell migration.* Proc Natl Acad Sci U.S.A., 1994. **91**: p. 5686-5689.
217. Wallingford, J.B., et al., *Dishevelled controls cell polarity during Xenopus gastrulation.* Nature, 2000. **405**(6782): p. 81-85.
218. Strutt, D.I., U. Weber, and M. Mlodzik, *The role of RhoA in tissue polarity and Frizzled signalling.* Nature, 1997. **387**(6630): p. 292-295.
219. Nolan, M.E., Aranda, V., Lee, S., Lakshmi, B., Basu, S., Allred, D.C., Muthuswamy, S., *The polarity protein Par6 induces cell proliferation and is overexpressed in breast cancer.* Cancer Res, 2008. **68**(20): p. 8201-8209.
220. Noda, Y., Takeya, R., Ohno, S., Naito, S., Ito, ., Sumimoto, H., *Human homologues of the Caenorhabditis elegans cell polarity protein PAR6 as an adaptor that links the small GTPases Rac and Cdc42 to atypical protein kinase C.* Genes To Cells, 2001. **6**: p. 107-119.
221. Georgiou, M., Marinari, E., Burden, J., Baum, B., *Cdc42, Par6, and aPKC regulate Arp2/3-mediataed endocytosis to control local adherens junction stability.* Curr Biol, 2008. **18**: p. 1631-1638.
222. Li, X., et al., *DGCR6L, a novel PAK4 interaction protein, regulates PAK4-mediated migration of human gastric cancer cell via LIMK1.* Int J Biochem Cell Biol, 2010. **42**(1): p. 70-9.
223. Kumar, R., A.E. Gururaj, and C.J. Barnes, *p21-activated kinases in cancer.* Nat Rev Cancer, 2006. **6**(6): p. 459-71.
224. Wang, R.A., et al., *PAK1 hyperactivation is sufficient for mammary gland tumor formation.* Oncogene, 2006. **25**(20): p. 2931-6.
225. He, H., et al., *The Tyr-kinase inhibitor AG879, that blocks the ETK-PAK1 interaction, suppresses the RAS-induced PAK1 activation and malignant transformation.* Cancer Biol Ther, 2004. **3**(1): p. 96-101.
226. Porchia, L.M., et al., *2-amino-N-{4-[5-(2-phenanthrenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]-phe nyl} acetamide (OSU-03012), a celecoxib derivative, directly targets p21-activated kinase.* Mol Pharmacol, 2007. **72**(5): p. 1124-31.
227. Nheu, T.V., et al., *The K252a derivatives, inhibitors for the PAK/MLK kinase family selectively block the growth of RAS transformants.* Cancer J, 2002. **8**(4): p. 328-36.
228. Deacon, S.W., et al., *An isoform-selective, small-molecule inhibitor targets the autoregulatory mechanism of p21-activated kinase.* Chem Biol, 2008. **15**(4): p. 322-31.
229. Huang, Q., H.M. Shen, and C.N. Ong, *Emodin inhibits tumor cell migration through suppression of the phosphatidylinositol 3-kinase-Cdc42/Rac1 pathway.* Cell Mol Life Sci, 2005. **62**(10): p. 1167-75.
230. Bokoch, G.M., *PAK'n it in: identification of a selective PAK inhibitor.* Chem Biol, 2008. **15**(4): p. 305-6.

231. Garber, K., *The second wave in kinase cancer drugs.* Nat Biotechnol, 2006. **24**(2): p. 127-30.
232. Fedorov, O., et al., *A systematic interaction map of validated kinase inhibitors with Ser/Thr kinases.* Proc Natl Acad Sci U S A, 2007. **104**(51): p. 20523-8.
233. Bain, J., et al., *The selectivity of protein kinase inhibitors: a further update.* Biochem J, 2007. **408**(3): p. 297-315.
234. Eswaran, J., Soundararajan, M., Knapp, S., *Targeting group II Paks in cancer and metastasis.* Cancer Metastasis Rev, 2009. **28**: p. 209-217.

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List of Publications

1. **Liu, Y.**, Ju, J., Xiao, H., Simi, B., Hao, X., Reddy, BS., Huang, MT., Newmark, H., and Yang, CS.. Effect of combination of calcium and aspirin on azoxymethane-induced aberrant crypt foci formation in the colons of mice and rats. *Nutr Cancer*. 2008;60(5):660-665.

2. **Liu, Y.**, Xiao, H., Tian, Y., Nekrasova, T., Hao, X., Lee, H., Suh, N., Yang, C., and Minden, A. The PAK4 protein kinase plays a key role in cell survival and tumorigenesis in athymic mice. *Mol Cancer Res*. 2008;6(7):1615-1624.

3. **Liu, Y.**, Ju, J., Huang, MT., Newmark H., and Yang, C.. Effects of a combination of calcium and aspirin on azoxymethane-induced aberrant crypt foci formation in the colons of CF-1 mice. *AACR meeting*, Washington DC. 2006

4. Ju, J., Hong, J., Zhou, J., Pan Z., Bose, M., Liao, J., Yang, G., **Liu, Y.**, Hou, Z., Lin, Y., Ma, J., Shih, W., Carothers, A., and Yang, C.. Inhibition of intestinal tumorigenesis in *Apc*$^{min/+}$ mice by (-)-epigallocatechin-3-gallate, the major catechin in green tea. *Cancer Res.* 2005;65:10623-10631.

5. Zhang, J., **Liu, Y.**, Shi, J., Larson, D., and Watson, R.. Side-stream cigarette smoke induces dose-response in systemic inflammatory cytokine production and oxidative stress. *Exp Biol Med* (Maywood). 2002;227(9):823-829.

6. Yu, Q., Zhang, D., Walston, M., Zhang, J., **Liu, Y.**, and Watson, R.. Chronic methaphetamine exposure alters immune function in normal and retrovirus-infected mice. International Immunopharmacology 2002;2:951-962.

7. **Liu, Y.**, Montes, S., Zhang, D., Sepulvsda, R., Yu, Q., Zhang, J., Larson, D., and Watson, R.. Cocaethylene and heart disease during murine AIDS. International Immunopharmacology 2002;2:139-150.

8. Eggs and health promotion. Edited by Watson, R.. Ames, Iowa: Iowa State University Press,2002. (Chapter 6 in Section 1: **Liu, Y.,** and Watson, R.. Reducing infection in infants with egg phospholipids.)

9. **Liu, Y.**, Zhang, J., and Watson, R.. Enviromental tobacco smoke promotes heart disease. Cardiovascular Reviews and Reports 2000;21:589-594.

10. **Liu, Y.**, Shi, C., Hu, Y., Zheng, Z., and Lu, X.. Studies on the stability of sodium taurocholate in different media and in the course of sterilization. Journal of Chinese Medicine 1999;24:91-93.