Knockdown of PAK4 or PAK1 Inhibits the Proliferation of Mutant KRAS Colon Cancer Cells Independently of RAF/MEK/ERK and PI3K/AKT Signaling

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Abstract

The p21-activated kinase (PAK) serine/threonine kinases are important effectors of the small GTPases Rac and Cdc42, and play significant roles in controlling cell growth, motility, and transformation. Knockdown of PAK4 or PAK1 inhibited the proliferation of mutant KRAS or BRAF colon cancer cells in vitro. Dependence on PAK4 or PAK1 protein for colon cancer cell proliferation was independent of PAK4 or PAK1 protein expression levels. Mutant KRAS HCT116 colorectal cells were the most sensitive to PAK4 or PAK1 knockdown resulting in the potent inhibition of anchorage-dependent and -independent proliferation as well as the formation and proliferation of HCT116 colon cancer spheroids. This inhibition of proliferation did not correlate with inhibition of RAF/MEK/ERK or PI3K/AKT signaling. In HCT116 cells, knockdown of PAK4 or PAK1 caused changes to the actin cytoskeleton resulting in reduced basal spread and cell elongation and increased cell rounding. These cytoskeletal rearrangements seemed to be independent of LIMK/cofilin/paxillin phosphorylation. PAK4 or PAK1 knockdown initially induced growth arrest in HCT116 cells followed by cell death at later time points. Inhibition of the antiapoptotic proteins Bcl-2 and Bcl-XL with the pharmacologic inhibitor ABT-737 increased effector caspase activation and apoptosis, and reduced cell survival with PAK4 or PAK1 knockdown. These results support a role for the PAKs in the proliferation of mutant KRAS-driven colorectal carcinoma cells via pathways not involving RAF/MEK/ERK and PI3K/AKT signaling. Mol Cancer Res; 11(2); 109–21. ©2012 AACR.

Introduction

The p21-activated kinases (PAK) are members of the STE20 family of serine/threonine kinases. In mammalian cells, 6 PAK isoforms have been identified and subclassified into 2 families: group I (PAK1-3) and group II (PAK4-6) based on their sequence homology but also on the presence of an inhibitory region present in group I but not group II PAKs (1, 2). The PAKs function as major downstream effectors of the Rho family of small GTPases such as Rac and Cdc42 (3, 4), and therefore play a major role in modulating changes to the actin cytoskeleton organization and dynamics, thereby controlling cell shape and motility in response to extracellular stimuli. PAK1 and PAK4 have been implicated in a number of cellular processes critical for oncogenic transformation including protection from apoptosis and programmed cell death (5, 6), inhibition of cell adhesion (7), and promotion of cell migration (8–10) and anchorage-independent growth (11, 12). Changes to PAK1 mRNA, protein and activity have been reported in variety of human malignancies including bladder (13), breast (14), colorectal (15), and ovarian carcinoma (16). Overtexpression of a kinase-activated mutant form of PAK1 induced the anchorage-independent growth of breast cell lines in vitro and breast carcinomas when transgenically expressed in the mouse mammary gland albeit with a long latency (17). PAK1 genomic amplification has been identified as prevalent in luminal breast cancer, whereas strong nuclear and cytoplasmic PAK1 expression was prevalent in squamous non–small cell lung carcinomas (NSCLCs; ref. 14). Selective targeting of PAK1 with short hairpin RNA (shRNA) inhibited breast and NSCLC cell proliferation in vitro and in vivo. Pharmacologic inhibition of X chromosome–linked inhibitor of apoptosis (XIAP) increased effector caspase activation and apoptosis in NSCLC cells following PAK1 knockdown (14).

PAK4 upregulation has been identified in a variety of human cancer cell lines and amplification of the chromosome region containing PAK4 has been frequently observed in pancreatic (18), colorectal (19), and ovarian cancer (20). Overexpression of a kinase-activated mutant form of PAK4 transforms NIH-3T3 cells facilitating anchorage-independent growth and tumor formation in nude mice (7, 11). PAK4 knockdown has an opposing effect on cell transformation. Oncogenic Ras-dependent transformation was
severely attenuated in PAK4−/− mouse embryonic fibroblast cells, which were also resistant to tumor formation by an oncogenic variant of the upstream activator Cdc42 (Cdc42V12; refs. 19, 21). Nuclear and cytoplasmic PAK4 and pPAK4 overexpression were observed in ovarian cancer cell lines both in vitro and in vivo. Targeted shRNA-mediated knockdown of PAK4 inhibited the migration, invasion, and proliferation of these ovarian cell lines and their tumorigenesis in nude mice (20).

Mutation of KRAS is a common, early event in the adenoma to carcinoma transition in colorectal cancer. KRAS mutations have been identified in around 40% of human primary cancers (22) and 53% of colorectal cancer cell lines. KRAS mutation, and the resulting activation, activates both mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways. In colorectal carcinoma, overexpression of both PAK1 and PAK4 has been identified in primary human cancers and cancer cell lines (15, 19). PAK1 overexpression was found to correlate with lower survival rates in patients with colorectal cancer (23). PAK1 and PAK4 have been shown to phosphorylate Raf-1 and mitogen-activated protein/extracellular signal-regulated kinase (MEK), and facilitate signaling through the Ras/Raf/MAPK signaling pathways (3, 24, 25) and the downstream potentiation of cell death following PAK inhibition. In this study, we show the importance of PAK4 and PAK1 in colon cancer proliferation through pathways that are apparently independent of RAF/MEK/ERK and PI3K/AKT signaling.

**Materials and Methods**

**Cell lines and cell culture**

All cell lines were purchased from the American Type Culture Collection and passaged in our laboratory for less than 6 months after resuscitation. HCT116 cells were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS) and penicillin/streptomycin at 37°C in a 5% CO2-humidified atmosphere.

**Compounds**

ABT-737 was purchased from Selleck Chem and dissolved in dimethyl sulfoxide (DMSO) as a 20-mmol/L stock.

**Immunoblotting**

Anti-PAK1, PAK2, PAK4, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pMEK1 (S298), pMEK1 (S217/221), pERK (T202/Y204), cyclin D, pRAF-1 (S338), pCo-filin (S3), pLIMK (T508), pPaxillin (T118), Actin, PARP, pAKT (S473), pGSK3β (S9), and caspase-3 antibodies were purchased from Cell Signaling Technologies.

Cells were washed once with PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitor cocktails (Roche). Protein concentration was determined using BCA Kit (Pierce). Equal amounts of lysate were separated by SDS-PAGE and Western blot analysis conducted using the antibodies indicated earlier.

**RNAi and transfection**

RNA interference (RNAi) specific to PAK4 (Hs_PAK4_5 UACAUUCUCCACCAAUUCUG, Hs_PAK4_6 UUC-CUUCUUCCAACAUGGTG, Hs_PAK4_11 UUCUUG-GACGGCCACGCTGUG and Hs_PAK4_12 UUGUG-CAGGGUCUUCAGUGG) and PAK1 (Hs_PAK1_6 G-UAUAUCAACAUUGUUUUGTT, Hs_PAK1_7 UCUC-UGGGCGCUCUUUCUCTT, Hs_PAK1_8 UUAGGU-GCAGCAUCAGUGGA and Hs_PAK1_9 UUAC-GUGCGAGUCUCUCCAA) were purchased from Qia-gen as were AllStars negative control RNAi and AllStars Hs cell death control RNAi. A total of 1.5 × 10^5 HCT116 cells per well of a 6-well plate were transfected using Lipofectamine RNAiMAX (Invitrogen) in OptiMEM (Invitrogen) according to the manufacturer’s protocol.

**Cell proliferation and survival assays**

HCT116 cells were seeded in 96-well plates at a density of 1 × 10^5 cells per well in DMEM containing 10% FCS for 72 or 96 hours posttransfection. Cell viability was determined using a CellTiter-Glo Luminescent Cell Viability Assay (Promega). For colony formation assays, HCT116 cells were trypsinized 24 hours after transfection and plated in 6-well plates at 200 to 2,000 cells per well. Colonies were allowed to form for 10 days at 37°C in a 5% CO2 humidified atmosphere, fixed, and stained with crystal violet in formaldehyde and counted. Viable colonies were determined as those containing 50 cells or more. For anchorage-independent growth assays, HCT116 cells were trypsinized 24 hours after transfection and plated in 24-well plates at 200 to 2,000 cells per well in 0.4% low melting point agarose on a layer of 0.6% agarose in media containing 10% FCS. The agarose layers were subsequently overlaid with 1 mL of DMEM containing 10% FCS and incubated at 37°C in a 5%
Co2-humidified atmosphere for 14 days. Colonies were stained using MTT reagent (Sigma) and counted.

Spheroid growth assays

Multicellular tumor spheroid assays were conducted essentially as described previously (29). A total of 1 x 10^3 HCT116 cells per well in DMEM containing 10% FCS were seeded in 96-well round-bottomed ultra-low attachment microlites (Corning Costar), centrifuged at 1,000 x g for 3 minutes and allowed to form into spheroids at 37°C in a 5% CO2 incubator for 72 hours. Spheroid cell viability was determined using a CellTiter-Glo Luminescent Cell Viability Assay (Promega) and imaged using a Zeiss Axiovert 200 microscope and x10 or x20 objective. Colonospheres were cultured essentially as described earlier (30). HCT116 cells were cultured in DMEM containing B27 supplement (Invitrogen) plus EGF and fibroblast growth factor (FGF; Invitrogen).

Immunofluorescence microscopy

A total of 1 x 10^4 HCT116 cells/chamber were seeded in 8-chamber glass slides. Following fixation with formaldehyde and permeabilization, F-actin was selectively labeled with an Alexa Fluor 488–labeled phalloidin (Invitrogen). Cells were subsequently imaged using a Zeiss Axiovert 200 microscope and x40 objective.

7-Aminoactinomycin D (7-AAD) cell viability assay

RNAi-transfected cells were harvested by trypsinization, washed once with PBS, and stained with 7-AAD for 10 minutes at room temperature in the dark. Viable (7-AAD-negative) and dead (7-AAD-positive) cell populations were quantitated by flow cytometry using a FACSAarray flow cytometer.

Flow cytometry

RNAi-transfected HCT116 cells were harvested by trypsinization, washed, and fixed in ice-cold 70% ethanol before being stained with propidium iodide (PI) containing RNAse A. Cell-cycle profiles were collected on a FACSAarray flow cytometer and analyzed with FACSDiva software (Becton Dickinson).

Growth in 3-dimensional culture conditions

HCT116, HT29, and DLD1 cells were grown in DMEM containing 10% FCS in 12-well Alvetex plates (Reinnervate) or 6-well AlgiMatrix plates (Invitrogen) according to the manufacturer’s recommended protocol.

Statistical analysis

Results were analyzed using a Student t test tool within the data analysis package provided by Microsoft Excel.

Results

PAK4 is required for the proliferation of colon carcinoma cells in vitro in anchorage-dependant culture

The role of PAK4 and PAK1 in the proliferation of colon carcinoma cells in vitro was evaluated in a panel of KRAS mutant (HCT116, SW620, DLD1, and SW480) or BRAF mutant (HT29, Colo205, and RKO) colon carcinoma cell lines. Knockdown of PAK4 resulted in a 43% to 89% reduction (P < 0.001) in cell proliferation in 6 of 7 cell lines (Fig. 1A). Conversely, knockdown of PAK1 reduced cell proliferation by 45% to 50% (P < 0.001) in 2 of 7 cell lines. Simultaneous knockdown of PAK4 and PAK1 did not decrease the cell proliferation beyond that seen with knockdown of PAK4 alone in all 7 colon cancer cell lines (data not shown). Mutant KRAS HCT116 cells exhibited the greatest sensitivity to either PAK4 or PAK1 knockdown, and so the effect of PAK4 or PAK1 knockdown using specific RNAi oligonucleotides on the proliferation of this cell line was investigated in more detail under anchorage-dependant growth conditions. Knockdown of PAK4 inhibited the proliferation of HCT116 cells up to 95% (P < 0.001), whereas knockdown of PAK1 inhibited proliferation up to 80% (P < 0.001) after 96 hours transfection (Fig. 1B). Transient knockdown of PAK4 but not PAK1 for 72 hours produced a small but significant (P < 0.05 compared with nontargeting RNAi) reduction in cell proliferation (Fig. 1C). Increasing the transient knockdown to 96 hours significantly increased the inhibition of cell proliferation by both PAK4- and PAK1-targeting RNAi (P < 0.001). RNAi-mediated knockdown of PAK4 with Hs_PAK4_6 reduced cell proliferation by 46% at 72 hours to 97% after 96 hours and PAK1 knockdown with Hs_PAK1_7 by 14% at 72 hours to 85% at 96 hours.

Assessment of the ability of PAK4 or PAK1 RNAi to inhibit the colony forming ability of HCT116 cells was also assessed. Knockdown of PAK4 reduced HCT116 colony forming ability by 97%, whereas PAK1 knockdown reduced it by 74% (P < 0.001, Fig. 1D). The reduction of PAK4 or PAK1 protein resulted in the initial inhibition of cell proliferation as evidenced by the small increase in 7-AAD-positive cells after 72-hour incubation with specific RNAi (Fig. 1E). Increasing the incubation time from 72 to 96 hours resulted in increased cell death. For example, with the PAK4-specific RNAi PAK4_6, the fraction of 7-AAD-positive cells increased from 23.5% at 72 hours to 52.6% at 96 hours. Simultaneous knockdown of PAK4 and PAK1 did not dramatically decrease the proliferation of HCT116 cells beyond that induced by PAK4 knockdown alone (Fig. 1C, D, and F). To determine the effect of PAK4 or PAK1 protein depletion on the cell cycle, the cell-cycle profile of HCT116 cells was determined 72 or 96 hours after transfection with specific RNAi. Knockdown of PAK4 or PAK1 reduced the fraction of cells in G1 and increased the fraction of cells with sub-G1 DNA content (Fig. 1G and Supplementary Fig. S1). The effect of PAK protein knockdown on the fraction of sub-G1 cells was time dependent. The percentage of sub-G1 cells increased following a 96-hour incubation with specific RNAi as compared with 72-hour incubation. Despite knocking PAK4 or PAK1 protein levels down to a similar extent as the other 3 targeted RNAis, 1 RNAi targeting PAK4 (PAK4_5) and 1 targeting PAK1 (PAK1_6) exhibited a reduced effect on HCT116 cell...
Figure 1. Knockdown of PAK4 reduces the proliferation of mutant KRAS or BRAF colon carcinoma cells in vitro. A, proliferation of a panel of colon cancer cell lines following transfection with specific RNAi oligonucleotides to PAK4 (PAK4_6) or PAK1 (PAK1_7) was determined using CellTiter-Glo 96 hours after transfection. Values are normalized against the scrambled RNAi-negative control. **, P < 0.01. B, proliferation of HCT116 colon carcinoma cells transfected with specific RNAi oligonucleotides to PAK4 or PAK1 was determined by CellTiter-Glo 96 hours after transfection. C, the time dependence of PAK inhibition on
proliferation in the Cell Titer Glo assay compared with the other RNAis (Fig. 1B and C). Hs_PAK4_5 RNAi, however, was able to reduce the colony forming ability of HCT116 cells by 84% (Fig. 1D). The reason for the reduced activity of these 2 RNAis was not apparent and was not investigated further.

Expression levels of PAK1 or PAK4 do not correlate with growth inhibition sensitivity to knockdown

PAK1 and PAK4 amplification has been identified in a range of human cancer types namely breast, colon, ovarian, and NSCLC for PAK1 and pancreatic, colon, and ovarian cancer for PAK4. The protein expression levels of PAK1, 2 or PAK4 in whole-cell lysates from a range of solid cancer cell lines was assessed by immunoblotting (Fig. 2). The expression levels of the 3 PAK isoforms varied across the panel of 14 solid cancer cell lines studied. In the 5 colon cancer cell lines in the panel (Colo205, DLD1, HCT116, HT29, and SW620), no consistent expression profile was evident. All of the colon cancer cell lines had detectable levels of PAK1 and PAK4 though the levels of PAK4 were low in the Colo205 and DLD1 cell lines. With the exception of the A549 and MDA-MB-453 tumor lysates, the expression levels of PAK2 seemed relatively consistent across the panel of cell lines. MDA-MB-453 had noticeably higher expression levels of PAK2 but had virtually no detectable levels of PAK1 or PAK4. No correlation between inhibition of proliferation by PAK4- or PAK1-specific RNAi and expression levels of PAK4 or PAK1 protein was evident.

Inhibition of proliferation of HCT116 colon carcinoma cells by PAK4 or PAK1 RNAi is independent of RAF/MEK/ERK signaling

Previous studies have shown an association between the PAKs and the RAF/MEK/ERK and PI3K/AKT signaling pathways. To assess the mechanism by which PAK4 or PAK1 contribute to HCT116 colon carcinoma cell proliferation, we examined the effect of transient RNAi-mediated knockdown of PAK4 or PAK1 on RAF/MEK/ERK and PI3K/AKT signaling. The 4 RNAi-targeting PAK4 efficiently reduced PAK4 protein levels with minimal effect on PAK1 protein levels. Likewise, the 4 RNAi-targeting PAK1 efficiently reduced PAK1 protein levels with minimal effect on PAK4 protein levels (Fig. 3A and Supplementary Fig. S2). The effects of PAK1 or PAK4 knockdown on RAF, MEK, ERK, and AKT phosphorylation were not consistent across the 4 RNAi oligonucleotides used and did not result in consistent changes to cyclin D levels. Reduction in PAK4 protein levels resulted in a reduction in cyclin D protein levels with 1 RNAi, an increase with another RNAi and no change with 2 additional RNAis (Fig. 3A). Following knockdown of PAK1, cyclin D levels were reduced with 3 oligonucleotides and induced by a fourth. These changes in cyclin D protein levels did not correlate with suppression of cell proliferation. In HCT116 cells, the cellular phenotype associated with knockdown of PAK4 seemed to predominate over PAK1 (Fig. 3B). Suppression of PAK4 protein expression following transfection of HCT116 cells with the specific RNAi PAK4_6 was observed to at least 120 hours post-transfection. Likewise, transfection of HCT116 cells with the PAK1-specific RNAi PAK1_7 suppressed PAK1 protein expression in the Cell Titer Glo assay compared with the other RNAis (Fig. 1B and C). Hs_PAK4_5 RNAi, however, was able to reduce the colony forming ability of HCT116 cells by 84% (Fig. 1D). The reason for the reduced activity of these 2 RNAis was not apparent and was not investigated further.

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expression for at least 120 hours posttransfection (Fig. 3C). In summary, the effect of PAK4 and PAK1 on HCT116 cell proliferation seems to not be mediated via the RAF-1/MEK dependent control of cyclin D protein levels. PAK4 and PAK1 seem to drive cell proliferation via additional, as yet unidentified, mechanisms that are independent of the RAF/MEK/ERK and PI3K/AKT signaling pathways.

**PAK4 or PAK1 knockdown modulates actin cytoskeletal rearrangements but not via the LIMK and paxillin signaling pathways.**

The PAKs have been associated with the regulation of the actin cytoskeleton in normal and cancer cells (21). PAKs are the major effectors of the small Rho GTPases Rac1 and Cdc42, which play critical roles in cell morphology, and subsequent cell migration, proliferation, and invasion. Knockdown of either PAK4 or PAK1 with specific RNAi oligonucleotides induced a reduction in the basal spread area of cells and a concomitant increase in cell rounding (Fig. 4A). Furthermore, PAK4 or PAK1 reduced the amount of HCT116 cell elongation compared with those treated with a control RNAi. The LIMK/cofilin/paxillin pathway plays a key role in controlling actin dynamics and subsequent cytoskeletal rearrangements. Modulation of actin dynamics through PAK4-dependent cofilin phosphorylation has been shown to affect cell migration and polarization and thereby cell proliferation in a variety of human cancer cell types (8, 20). In HCT116 cells, the observed F-actin rearrangements (as determined by phalloidin staining) induced by knockdown of PAK4 or PAK1 did not correlate with

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**Figure 3.** Inhibition of proliferation of HCT116 colon carcinoma cells by PAK4 or PAK1 RNAi is independent of RAF/MEK/ERK signaling. HCT116 cells were transfected with specific RNAi oligonucleotides to PAK4 or PAK1 (A), or combinations of PAK4 plus PAK1 (B), and changes to signaling pathways determined by immunoblot analysis 48 hours after transfection. C, the duration of PAK4 or PAK1 knockdown was evaluated in HCT116 cells transfected with the specific RNAi oligonucleotides PAK4_6 or PAK1_7 for up to 5 days.
Figure 4. PAK4 or PAK1 knockdown alters actin cytoskeletal arrangements in HCT116 colon cancer cells. A, HCT116 cells were transfected with specific RNAi oligonucleotides to PAK4 (PAK4_6) or PAK1 (PAK1_7) for 48 hours. Fixed cells were stained with fluorescent phalloidin to detect F-actin and imaged using a ×40 objective. Enlarged sections of the image (as defined by the white box) are shown later. Changes to the phosphorylation levels of components of the LIMK signaling pathway were determined by immunoblotting 48 hours after transfection with oligonucleotides specific to PAK4 or PAK1 (B) or in combination (C).
decreased coflin phosphorylation on serine 9 or paxillin on tyrosine 118 (Fig. 4B). Combinatorial knockdown of PAK4 and PAK1 resulted in the same changes to coflin and paxillin phosphorylation as the single gene knockdowns (Fig. 4C). In HCT116 colon carcinoma cells, control of the actin cytoskeleton by PAK4 and PAK1 does not seem to proceed via the LIM kinase (LIMK) and paxillin signaling pathways.

PAK4 and PAK1 are essential for HCT116 colon cancer cell proliferation in anchorage-independent culture

The PAKs have been shown to have a clear role in cytoskeletal remodeling, cell motility and invasion, and the promotion of anchorage-independent growth. Given the increased cell–cell interactions in 3-dimensional culture (anchorage-independent culture), we postulated that cancer cells may be more PAK dependent under these growth conditions. To examine the role of PAK4 and PAK1 on anchorage-independent survival, mutant KRAS HCT116 cells were transiently transfected with RNAi oligonucleotides specific to either PAK4 or PAK1 before being plated in low melting point agarose. Knockdown of PAK4 or PAK1 inhibited the anchorage-independent proliferation and survival of HCT116 colon cancer cells growing in low melting point agarose (Fig. 5A). PAK4 knockdown reduced HCT116 cell survival by 88.3%, whereas knockdown of PAK1 was less effective reducing cell survival by 65.4% (P < 0.001%) compared with the negative control, scrambled RNAi. Dual knockdown of PAK4 plus PAK1 did not dramatically reduce cell survival compared with PAK4 knockdown alone.

Multicellular tumor spheroids more closely resemble the avascular regions of human tumors with respect to their morphologic and histopathologic features than the same cell line grown anchorage dependently. Tumor cells from such spheroids assemble an appropriate extracellular matrix along with complex cell–cell and cell–matrix interactions as well as oxygen and pH gradients in larger spheroids (29). Knockdown of PAK4 or PAK1 did not inhibit the formation of HCT116 colon cancer spheroids. However, spheroids formed from HCT116 cells lacking either PAK4 or PAK1, and especially those lacking both PAK4 and PAK1, appeared less compacted and the extracellular matrix supporting the spheroid less well organized (Fig. 5B). Knockdown of PAK4 reduced spheroid cell growth, as evidenced by the reduction in total ATP content of the spheroid, by 79% and PAK1 by 52% (Fig. 5C; P < 0.001 compared with control RNAi). The effect on spheroid cell proliferation following PAK knockdown seemed independent of FCS concentration.

We evaluated further the effect of growing colon cancer cells in a variety of 3-dimensional culture systems on signaling pathways postulated to be PAK dependent. A comparison of MEK/ERK and coflin signaling was made between (i) normal anchorage-dependent growth on cell culture plastic plates; (ii) anchorage-independent growth as multicellular colon tumor spheroids (MCTS in media postulated to increase the stem cell such as properties of the cells (colonospheres)), on a highly porous polystyrene scaffold (Alvetex); and (iii) embedded in a porous alginate gel (AlgiMatrix). Anchorage-independent growth-modulated MEK/ERK and LIMK signaling compared with anchorage-dependent growth that was cell line and culture system dependent (Fig. 6). For HCT116 cells, growth as spheroids, colonospheres, or in AlgiMatrix resulted in a reduction in MEK1 S298 phosphorylation, but only under the AlgiMatrix and spheroid growth conditions did this result in a decrease in cyclin D levels (Fig. 6). Conversely, growth in AlgiMatrix but none of the other anchorage-independent systems dramatically induced phospho-cofilin in HCT116 cells. Growth on the Alvetex scaffold reduced protein expression levels of both PAK4 and PAK1 in HCT116 and DLD1 cells. This reduction in PAK protein levels, however, had little effect on MEK1 S298 and ERK T202/Y204 phosphorylation and cyclin D levels. Despite differences in MEK and cofilin phosphorylation, and cyclin D levels, HCT116 cells remained sensitive under 3-dimensional culture conditions to growth inhibition following PAK4 or PAK1 knockdown. This provides additional evidence to suggest that PAK4 and PAK1 drive colon cancer cell proliferation through additional pathways independently of RAF/MEK/ERK.

Bcl-2/Bcl-X<sub>L</sub> inhibition potentiates cell killing following PAK inhibition

ABT-737 is a small-molecular antagonist of the prosurvival Bcl-2, Bcl-X<sub>L</sub>, and Bcl-w but not Mcl-1 proteins. It mimics the prosapoptotic BH3 proteins through binding to the prosapoptotic Bcl-2 homology domain (BH3) groove on the Bcl-2 family proteins thereby inhibiting their prosurvival activity. As a single agent, ABT-737 induces apoptosis in a limited number of tumor types (31, 32). However, ABT-737 (and the closely related analog ABT-263) has been shown to enhance the apoptotic potential of a range of novel-targeted therapeutics and cytotoxic chemotherapeutics.

Knockdown of PAK4 or PAK1 with RNAi induced a small increase in apoptotic HCT116 cells as measured by an increase in cleaved caspase-3 and -7 (Fig. 7A and C), cleaved PARP (Fig. 7C), and PI staining tumor spheroid cells (Fig. 7E) compared with cells transfected with scrambled RNAi. Addition of ABT-737 to HCT116 cells transfected with PAK4 or PAK1 RNAi increased (P < 0.001) the levels of activated caspase-3 and -7 (Fig. 7A). This increased activation of caspase-dependent apoptosis by ABT-737 resulted in a decrease in HCT116 survival. Following the knockdown of PAK4, HCT116 cell survival was reduced from 54% to 20% (P < 0.001) in the presence of ABT-737 (Fig. 7B). Likewise, ABT-737 in combination with the knockdown of PAK1 resulted in a reduction in HCT116 cell survival from 90% to 42% (P < 0.001). The decreased cell viability induced by the combination of either PAK4 or PAK1 RNAi with ABT-737 resulted in an induction of apoptosis as measured by an increase in PARP and caspase-3 cleavage by immunoblotting (Fig. 7C).

In multicellular tumor spheroids deficient in either PAK4 or PAK1, treatment with ABT-737 resulted in a small but significant decrease (P < 0.001) in the fraction of viable cells within the tumor spheroid (Fig. 7D). ABT-737 treatment
Figure 5. PAK4 and PAK1 are essential for HCT116 colon cancer cell proliferation in anchorage-independent culture. A, HCT116 cells were transfected with RNAi oligonucleotides specific to PAK4 (PAK4_6) or PAK1 (PAK1_7) for 24 hours before being replated in low melting point agarose. Colonies were allowed to form for 14 days before being stained with MTT and counted. Values are the mean of 3 determinations ± SD. ***, P < 0.001 compared with -ve control RNAi. B, HCT116 colon cancer cells were transfected with specific RNAi oligonucleotides to PAK4 or PAK1 for 24 hours before being transferred to round-bottomed, low-adherence 96-well plates containing 1% or 10% FCS. Spheroids were allowed to form for a further 72 hours before being imaged under a Zeiss Axiovert microscope. C, cell viability was determined using CellTiter-Glo and normalized to the untreated (UT) control 72 hours after transfection. The values are the mean of 8 determinations ± SD. ***, P < 0.001. Scale bar, 100 μm.
reduced the fraction of viable cells from 26% to 17% following PAK4 knockdown and from 75% to 40% following PAK1 knockdown. Cell death following combinatorial knockdown of PAK4 with PAK1 was not increased beyond that of the single gene knockdowns. This increased cell death following ABT-737 of PAK4 or PAK1 RNAi-treated spheroids was confirmed by PI staining of the treated spheroids. The fraction of the cells staining positive for PI within PAK4 or PAK1 RNAi knockdown spheroids was increased following treatment with ABT-737 (Fig. 7E).

Discussion

The PAK family of kinases have been shown to modulate cell signaling pathways critical for cancer cell proliferation, transformation, migration, and invasion (3, 25), and therefore suggest themselves as potentially important targets for therapeutic intervention. In this study, we evaluated the role of PAK4 and PAK1 in the proliferation and survival of a range of colon carcinoma cell lines harboring mutations in either KRAS or BRAF. Knockdown of PAK4 inhibited the proliferation of 3 of 4 of the KRAS-mutant and 3 of 3 of the BRAF-mutant colon cancer cell lines. Combinatorial knockdown of PAK4 and PAK1 was not significantly more antiproliferative than knockdown of either PAK4 or PAK1 alone. The mutant KRAS HCT116 cell line showed the greatest sensitivity to PAK4 or PAK1 knockdown. Knockdown of PAK4 or PAK1 inhibited the anchorage-dependent growth on plastic and the anchorage-independent growth in low melting point agarose of HCT116 colon cancer cells as well as the growth and formation of multicellular colon cancer spheroids. Under all growth conditions studied, knockdown of PAK4 had a greater inhibitory effect on cell proliferation than knockdown of PAK1. Inhibition of proliferation occurred at an earlier time point following PAK4 RNAi treatment compared with PAK1 RNAi despite similar kinetics of protein downregulation. As was seen with the other cell lines, combinatorial knockdown of PAK4 and PAK1 was not significantly more antiproliferative than knockdown of either PAK4 or PAK1 alone under any of the different growth conditions tested. Despite reducing protein levels of PAK4 or PAK1 to a similar extent as the other RNAi, PAK4_5 and PAK1_6 did not inhibit cellular proliferation to the same extent. The reasons for this are unclear but potential explanations include differences in either the kinetics or duration of mRNA and subsequent protein reduction, or knockdown of an off target protein that renders the cells insensitive to PAK knockdown. These observations are in keeping with previous studies showing a crucial role for PAK4 or PAK1 in cancer cell proliferation and survival. Knockdown of PAK4 reduced the proliferation and survival of ovarian cancer cells (20), pancreatic cancer 8988T cells (18), JEG3, and JAR choriocarcinoma cells (33) and increased the levels of apoptotic proteins in gastric cancer cell lines (34). In comparison, knockdown of PAK1 inhibited the anchorage-dependent (14) and -independent (35) growth of breast cancer cells, the
proliferation of NSCLC cancers in vitro and in vivo (14), colon cancer in vitro (27) and in vivo (28), and the growth of gastric cancer cells anchorage-dependently and -independently as well as tumor xenografts (36). In previous studies, gene amplification or increased protein expression of PAK4 or PAK1 has been suggested to correlate with dependence on this pathway for tumor progression and tumor cell proliferation. In this study, the dependence of colon cancer cells on PAK4 or PAK1 for proliferation did not correlate with increased expression of either PAK4 or PAK1 protein. Compensation for PAK1 loss by PAK2 did not seem to be responsible for the variable effect of PAK1 RNAi on cell proliferation in the colon cancer cell lines studied. A recent study by Baskaran and colleagues (37) suggests that PAK4 (unlike PAK1) is constitutively phosphorylated on serine 474 and held inactive by an N-terminal auto-inhibitory domain. Binding of Cdc42 relieves this auto-inhibition resulting in the full activation of PAK4. Reliance on gene
amplification or protein overexpression to select patient populations that might be sensitive to PAK-directed small-molecule therapeutics could result in potentially sensitive patient populations being overlooked. Further work is needed to identify additional cellular biomarkers of PAK activation status to aid this patient selection.

Downregulation of PAK4, PAK1, or PAK4 plus PAK1 in HCT116 colon cancer cells induced a predominantly cytostatic effect on cell proliferation that converted to cell death on prolonged protein knockdown. Inhibition of Bcl-2/Bcl-XL with ABT-737 increased caspase-dependent apoptosis and cell death in HCT116 cells following RNAi knockdown of PAK4 or PAK1. Previous studies in NSCLC cells have shown that antagonism of the anti-apoptotic inhibitor of apoptosis (IAP) proteins with a pharmacologic inhibitor in combination with PAK1 ablation increased the number of cells undergoing apoptosis (14). This differed substantially from the antiproliferative effect observed after single-agent inhibition of PAK1 in these cells. PAK4 and PAK1 have both been shown to protect against apoptotic cell death by direct phosphorylation of Bad on serine 136 (6, 38). Targeting PAK4 or PAK1 may be a mechanism to prime tumor populations resistant to small-molecule inhibitors of apoptosis proteins, such as Bcl-2, Bcl-XL, or XIAP to apoptosis induction by these small molecules. Inhibitors of both Bcl-2/Bcl-XL and XIAP are currently in early-stage clinical trials and the combination of PAK inhibitors with inhibitors of apoptosis may be a useful therapeutic regimen for a variety of human cancers.

To further understand the mechanism by which PAK4 and PAK1 regulate cell proliferation in mutant KRAS HCT116 colon cancer cells, we examined the effect of PAK4 and PAK1 knockdown on pathways previously shown to be modulated by the PAKs. PAK4 and PAK1 have been found to phosphorylate common substrates in cells and modulate the same signaling pathways namely the RAF/MEK, LIMK/Cofilin, HGF/MET, Rho GTPase exchange factors, PI3K/AKT, and apoptotic regulatory (BAD) pathways (2–4, 24, 25, 39). In the case of the RAF/MEK pathway, PAK4 and PAK1 have both been shown to phosphorylate MEK on serine 298 (40, 41) and RAF on serine 338 (42, 43). In HCT116 colon cancer cells, inhibition of cell proliferation induced by knockdown of PAK4 or PAK1 did not correlate with consistent reduction in phosphorylation of RAF-1, MEK, or ERK and subsequent decrease in cyclin D protein levels. The relationship between PAK4 and MEK/cyclin D activation was independent of cell culture conditions. In anchorage-independent culture, the phosphorylation of MEK1 on serine 298 (40, 41) and RAF on MEK pathway, PAK4 and PAK1 have both been shown to protect against apoptotic cell death by direct phosphorylation of Bad on serine 136 (6, 38). Targeting PAK4 or PAK1 may be a mechanism to prime tumor populations resistant to small-molecule inhibitors of apoptosis proteins, such as Bcl-2, Bcl-XL, or XIAP to apoptosis induction by these small molecules. Inhibitors of both Bcl-2/Bcl-XL and XIAP are currently in early-stage clinical trials and the combination of PAK inhibitors with inhibitors of apoptosis may be a useful therapeutic regimen for a variety of human cancers.

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Despite the ability of PAK4 and PAK1 to phosphorylate identical protein substrates albeit often in different cancer cell lines, and are activated by the same small G-proteins, PAK4 and PAK1 do not show functional redundancy. In all the colon cell lines studied, including HCT116s, PAK1 did not compensate for the loss of PAK4 and vice versa. It is also unclear as to whether PAK5 and PAK6 can functionally substitute for PAK4. This was further reinforced by the observation that PAK1 RNAi does not increase the anti-proliferative activity of PAK4 RNAi and vice versa. Knockdown of PAK4 or PAK1 did not result in consistent changes to MEK1, RAF-1, paxillin, or cofilin phosphorylation in HCT116 cells and, following knockdown of PAK4 and PAK1, the cellular phenotype associated with knockdown of PAK4 seemed to predominate over PAK1.

Overall our studies suggest that PAK4 and PAK1 are crucial for the anchorage-dependent and-independent proliferation but not the survival of colon cancer cells in vitro via pathways independent of RAF/MEK/ERK and that protein overexpression was not a determinant of PAK signaling dependence. Further work is needed to identify biomarkers of the activation status of PAK1 and PAK4 in human cancer cells, and further elucidate the mechanisms by which group I and group II PAK substrate specificity is determined in a cancer cell context, and the signaling pathways modulated by the PAKs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.J. Massey
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