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Overexpression of PAK1 Promotes Cell Survival in Inflammatory Bowel Diseases and Colitis-associated Cancer

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Background: Chronic gut inflammation predisposes to the development of colorectal cancer and increased mortality. Use of mesalamine (5-ASA) in the treatment of ulcerative colitis modulates the risk of neoplastic progression. p21 activated kinase 1 (PAK1) mediates 5-ASA activity by orchestrating MAPK signaling, Wnt- β catenin pathway, and cell adhesion; all implicated in the colon carcinogenesis. We evaluated the role of PAK1 in IBD and in colitis-associated cancer (CAC).

Methods and Results: PAK1 expression was scored by immunohistochemistry in human samples from IBD, CAC, and in normal mucosa. Compared with controls, a higher PAK1 expression was detected in IBD which further increased in CAC. The consequence of PAK1 overexpression was investigated using normal diploid colon epithelial cells (HCEC-1CT), which showed higher proliferation and decreased apoptosis on overexpression of PAK1. Analysis of IBD and CAC samples showed activation of AKT (p-AKT). However, mTOR pathway was activated in IBD but not in CAC. Treatment of cells with specific inhibitors (PD98059/LY294002/rapamycin) of growth signaling pathways (MEK/PI3K/mTOR) demonstrated that in HCEC-1CT, PAK1 expression is regulated by MEK, PI3K, and mTOR. In colorectal cancer cell lines, PAK1, and beta-catenin expression correlated and inhibition of PAK1 and addition of 5-ASA elicited similar molecular affects by reducing ERK and AKT activation. Moreover, 5-ASA disrupted PAK1 interaction and colocalization with β -catenin.

Conclusions: Our data indicate that (1) PAK1 is upregulated in IBD and CAC (2) PAK1 overexpression is associated with activation of PI3K-AKT/mTOR prosurvival pathways in IBD.

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Key Words: PAK1, ulcerative colitis, colitis-associated cancer, chemoprevention, mesalamine

Ulcerative colitis (UC) and Crohn's disease (CD) are characterized by chronic gut inflammation associated with architectural distortion of intestinal epithelium and impaired mucosal barrier functions. Chronic gut inflammation also disposes to an increased risk of colitis-associated cancer (CAC). Mesalamine

(5-ASA; 5-aminosalicylic acid) is an anti-inflammatory drug used in the treatment of UC with therapeutic benefits in mucosal healing and consequently impeding colon carcinogenesis. It has been demonstrated that in addition to reduction of oxidative stress,^{1,2} 5-ASA acts through induction of cell cycle arrest,³ increases PPAR- γ ,⁴ reduces PI3K/AKT and β -catenin signaling,⁵ and facilitates membranous expression of E-cadherin through modulation of N-glycosylation.^{6,7} This confers 5-ASA with chemopreventive potential, which remains controversial in the meta-analyses.^{2,8} We performed a microarray analysis on colorectal cancer (CRC) cells to investigate targets of 5-ASA and PAK1 (p21-activated kinase 1) emerged as its consensus target that orchestrates Wnt/ β -catenin, MAP kinase pathways, and cell adhesion interfered by 5-ASA.

PAK1 is a serine-threonine protein kinase implicated in multiple pathways through its kinase activity and scaffolding functions.⁹ Besides its role in antiapoptotic signaling, PAK1 is critical for cytoskeletal dynamics, cell motility and EMT (epithelial to mesenchymal transition).¹⁰ Moreover, PAK1 signaling contributes in combating gastrointestinal viral/microbial infections.^{11,12} In CRC, PAK1 expression is found to be amplified with the disease progression,¹³ and accumulating evidence establish its role in the promotion of colon carcinogenesis.^{14,15} However, the cause of PAK1 overexpression throughout disease initiation and progression is currently unclear.

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In contrast to sporadic CRC, cellular pathways contributing to progression from chronic inflammation to CAC are primarily driven by activation of proinflammatory signaling such as NF- κ B, JNK, and p38MAPK.^{16,17} Although, PAK1 is implicated in the activation of NF- κ B,¹⁸ knowledge is lacking about role of PAK1 in intestinal inflammation and homeostasis.¹⁹ Interestingly, the small GTPase RAC1 is associated with the susceptibility and development of colitis,²⁰ and PAK1 is one of its critical downstream effector molecules. In CAC, p53 and K-Ras mutations are early events in the progression from colitis to cancer. K-Ras activation propagates growth factor signaling through Raf/MEK/ERK cascade or through PI3K/AKT, and PAK1 contributes to both ERK and AKT pathways to promote tumor growth.²¹

Here, we examined the expression of PAK1 in IBD and CAC. Using normal diploid human colon epithelial cells (HCEC-1CT), we investigated the regulation of PAK1 expression in intestinal epithelium through overexpression and inhibition.

MATERIALS AND METHODS

Immunohistochemistry

The human samples were obtained from the Department of Pathology at Medical University of Vienna. Control specimens representing normal mucosa were taken from normal colon tissue as judged by pathologists. Immunohistochemistry was done from paraffin-embedded tissue sections. Briefly, slides were dried, dewaxed in xylol, and rehydrated using a decreasing alcohol series. After blocking of endogenous peroxidase with 15% H₂O₂ in methanol, antigen retrieval was performed in 10 mM citrate buffer, pH 6. Subsequently, slides were blocked in 2% horse serum, 3% BSA in TRIS buffer. Staining was performed using the avidin–biotin complex method. Antibodies against PAK1 (#2602, cell signaling; n = 12 controls; n = 17 UC; n = 13 CD; n = 9 CAC) or p-AKT1 (Thr308; sc-135650, Santa Cruz; n = 12 controls; n = 17 UC; n = 13 CD; n = 6 CAC); p-mTOR (Ser 2448; cell signaling; n = 5 controls; n = 5 UC; n = 5 CD; n = 8 CAC) were incubated at 4°C overnight, followed by incubation with biotinylated anti-rabbit antibody and avidin–biotin–HRP complex. Staining was visualized using 3,3'-Diaminobenzidine, and nuclear counterstaining was performed using hematoxylin. Slides were dehydrated and embedded in Histofluid (Marienfeld superior, Lauda-Königshofen, Germany). A 4-grade immunoreactivity scoring system (IRS) was used (percentage of cells with no staining = 0; 1 = weak; 2 = moderate; 3 = strong). The mean intensity and percentage of positively stained epithelial cells was multiplied to generate the IRS. Averaged IRS score of 2 independent investigators (M.J. and K.D.) was used for the analysis. For p-AKT and p-mTOR, combined score of cytoplasmic and nuclear staining was used. Staining with secondary antibody alone was performed as control.

Cell Lines and Reagents

Primary human colon epithelial cells, HCEC-1CT cells (obtained from Jerry W. Shay and Andres I. Roig, University of

Texas, Dallas), were cultured in basal X media (DMEM: M199, 4:1; GIBCO, Eggenstein, Germany), supplemented with epidermal growth factor (20 ng/mL; BD Biosciences, Heidelberg, Germany), hydrocortisone (1 μ g/mL; Sigma, Deisenhofen, Germany), insulin (10 μ g/mL), transferrin (2 μ g/mL), sodiumselenite (5 nM; all from Gibco, Life Technologies GmbH, Karlsruhe, Germany), 2% cosmic calf serum (HyClone, Bonn, Germany), and gentamicin sulfate (50 μ g/mL; Sigma). Cells were cultured in Primaria flasks (Becton Dickinson, Heidelberg, Germany) at 37°C, 5% CO₂ and 100% humidity. Human colorectal carcinoma cell lines including HCT116 and HT-29 (obtained from ATCC) were grown in Iscove's Modified Dulbecco's Medium (Gibco/Invitrogen, Lofer, Austria) containing 10% fetal bovine serum (Biochrom, Berlin, Germany). Mesalamine (>99.9% pure; a generous gift from Shire Inc., Eysins, Switzerland) was dissolved in the culture medium at 20 mM final concentration (pH adjusted to 7.2 with NaOH) as described earlier.⁷ This concentration of 5-ASA is within the range of physiological relevance in humans.⁴ IPA3 (Sigma–Aldrich) is an allosteric inhibitor of PAK1 and targets its autoinhibitory domain was dissolved in dimethyl sulfoxide and used at 5 to 20 μ M concentrations. Other inhibitors (Cell signaling) were solubilized in dimethyl sulfoxide and used at following concentrations: MEK inhibitors PD98059, U0126 (20 μ M), PI3 Kinase inhibitor LY294002 (20 μ M), mTOR inhibitor rapamycin (40 nM).

PAK1 Overexpression

Normal diploid colon epithelial cells (HCEC-1CT) were transiently transfected through electroporation with 5 μ g of pCMV empty vector (Con) or wild-type pCMV6M-PAK1 (WT-PAK1) plasmid DNA, a kind gift from Jonathan Chernoff, Fox Chase Cancer Center, Philadelphia, PA. HCEC-1CT cells were electroporated using the Amaxa nucleofactor 2b device with program number T023 and basic Nucleofactor Kit for primary mammalian epithelial cells (Lonza) according to manufacturer's instructions.

Cell Proliferation Assay

One million HCEC-1CT cells were transiently transfected with 5 μ g pCMV empty vector (Con) or wild-type pCMV6M-PAK1 (WT-PAK1) plasmid DNA and seeded into 10 cm plates. Twelve hours after transfection, the cells were counted, and 1 \times 10⁴ cells per well were seeded into 96-well plates. The remaining cells were lysed in RIPA buffer, and transfection efficiency was determined by Western blot. Cell proliferation was evaluated after 72 hours with a standard MTT assay. Briefly, MTT (Thiazolyl Blue Tetrazolium Bromide; Sigma, M5655) reagent was freshly diluted. Twenty microliter of the 5 mg/mL reagent was added to each well and incubated for 3 hours at 37°C in the dark. The media was removed and 150 μ L of dimethyl sulfoxide/ethanol solvent was added per well. The plate was covered in tinfoil and placed on a shaker for 15 minutes at 25°C. The absorbance was measured on a microplate reader (Anthos 2010) at 570 nm with a reference filter set at 620 nm. Each measurement was performed in biological triplicates.

Annexin V Staining

The apoptosis assay was performed using the Annexin V detection kit (eBioscience) in accordance with the manufacturer's instructions. Briefly, 1×10^6 HCEC-1CT cells were transiently transfected with 5 μ g of Con or WT-PAK1 plasmid DNA as previously described. Seventy-two hours after transfection, the cells were counted, and 1×10^5 cells were resuspended in binding buffer. Five microliter of the fluorochrome-conjugated Annexin V solution was added to the cell suspension and incubated for 15 minutes at 25°C. The cells were washed in binding buffer and incubated with propidium iodide solution for 3 hours in the dark at 4°C. Flow cytometry was performed on a Cell Lab Quanta SC flow cytometer (Beckman Coulter, Brea, CA) and analyzed with Quanta Analysis software.

Western Blotting

Whole cell lysates were prepared in RIPA buffer (50 mM Tris-cl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 1x Roche complete mini protease inhibitor cocktail). Protein concentrations were measured by Bradford assay (Bio-Rad, Hercules, CA). Twenty microgram of protein sample was incubated with Laemmli sample buffer containing 10% β -mercaptoethanol at 95°C for 10 minutes. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted onto a polyvinyl difluoride membrane. The protein bands were visualized with IRDye coupled anti-rabbit or anti-mouse antibodies (either or both mouse/rabbit; LI-COR) and scanned on Odyssey imager (LI-COR Biotechnology, Homburg, Germany). Primary antibodies used were as follows: PAK1 #2602, Phospho-p44/42 MAPK #9106, p44/42 MAPK #9102, Phospho-mTOR (Ser2448) #2971, Raptor, Ric-tor, AKT#2920, p-AKT (Ser473) #4060 (Cell Signaling), β -catenin clone 14 (BD Transduction Laboratories, San Jose, CA), alpha-tubulin, (Abcam), p-AKT (Thr308), and β -actin sc-47778 (Santa Cruz Biotechnology, Dallas, TX).

Immunofluorescence Microscopy

Cells were fixed in methanol, and immunostaining was performed using antibodies against β -catenin (clone 14/BD Transduction Laboratories) and E-cadherin (clone 36/BD Transduction Laboratories). For protein visualization Alexa-Fluor 488 and 568 antibodies (Invitrogen) were used. Nuclear staining was performed using Vectashield with DAPI (Vector Laboratories, Peterborough, United Kingdom) for mounting. Images were scanned at $\times 40$ magnification on an LSM 510 (Zeiss, Munich, Germany) or acquired on Olympus BX51 microscope. Digital images were processed with Zeiss LSM Browser.

RNA Interference

For silencing RNA (siRNA) experiments, cells were plated at a density of 1×10^5 cells per well in a 6-well plate and transfected using 50 and 100 nM of PAK1 siRNA duplex (dose was selected after titrating 10–100 nM duplex RNA) with siRNA transfection

reagent (Santa Cruz Biotechnology). Fresh medium was added 24 hours after transfection. siRNA oligonucleotides were purchased from Dharmacon (catalog number D-003521-03, Accession Numbers: NM_002576, target sequence CAUCAAAUAUCACUAAGUC). Control siRNA-A: sc-37007 (Santa Cruz Biotechnology) was prepared based on the manufacture's instructions.

Statistical Analysis

Statistical analysis was performed using SPSS (version 21.0). Metric outcome variables were compared using univariate analysis of variance and Tukey's honest significant difference post hoc tests. IRS were analyzed using a 2-tailed *t* test. *P*-values less than 0.05 were considered significant. All data are expressed as mean \pm SD. Pearson's correlation analysis was performed on Excel (Microsoft office).

Ethical Considerations

The study was approved under the ethics by the local ethics committee. Samples were selected from endoscopic biopsies or surgical specimens of patients with IBD and CAC.

RESULTS

PAK1 Is Overexpressed in IBD and CAC and Contributes to Cell Proliferation and Survival

Patient samples were analyzed for PAK1 expression by immunohistochemistry in CD, UC, and CAC (as described in Methods) and compared with normal mucosa. In normal colonic tissue, epithelial PAK1 expression was low, whereas PAK1 expression was comparatively higher in the samples from patients with CD and UC (Fig. 1A, B) and was mostly cytoplasmic. PAK1 immunoreactivity increased further in CAC. These observations suggest that PAK1 overexpression is an early event in the disease progression from colitis to CAC.

To investigate the functional effect of PAK1 overexpression in intestinal epithelial cells, HCEC-1CT was transfected with control (Con) and wild-type (PAK1-WT) expression vectors, and cell proliferation was analyzed. HCEC-1CT showed higher proliferation ($46\% \pm 3.1\%$) on overexpression of PAK1-WT compared with control (Fig. 1C). Apoptosis (Annexin V positive cells) was reduced in HCEC-1CT overexpressing PAK1-WT ($0.96\% \pm 2.8\%$) compared with control ($16.1\% \pm 6.2\%$) (Fig. 1D).

AKT1 and mTOR Pathways Are Activated in IBD

To investigate the activation of cell proliferation and survival pathways associated with PAK1 overexpression in IBD and CAC, MEK/ERK, PI3K/AKT, and mTOR pathways were examined. Immunohistochemistry was performed on these samples with p-ERK1/2, p-AKT (Thr 308), and p-mTOR (Ser 2448) for activation of respective pathways. Both p-mTOR (Fig. 2A, B) and p-AKT (Fig. 2C, D) levels were increased in the epithelium from IBD samples and exhibited nuclear and cytoplasmic

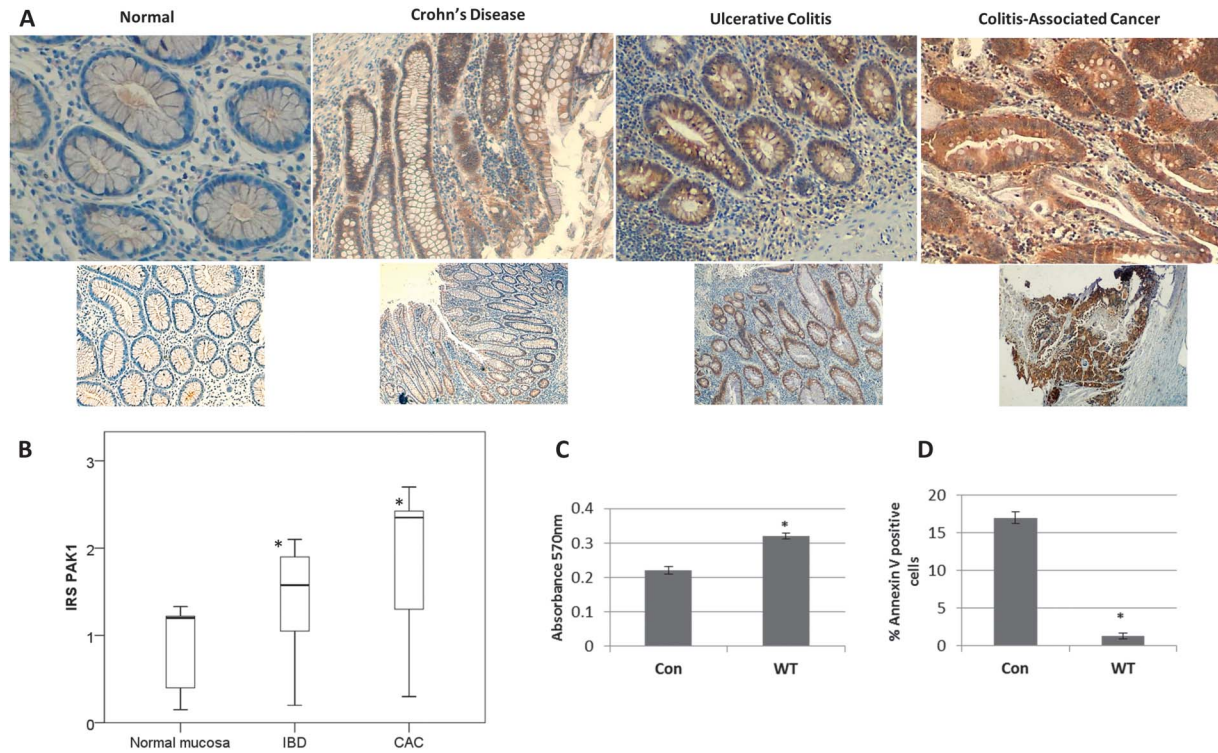


FIGURE 1. PAK1 is overexpressed in IBD and CAC. Immunohistochemical analysis was performed to examine PAK1 expression in patient samples from IBD and CAC. A, PAK1 staining was increased in the epithelial cells in CD, UC, and colitis-CAC compared with normal mucosa showing low basal expression of PAK1. Although PAK1 expression was higher in IBD, not all samples of CD stained positive. However, in UC, PAK1 expression was consistently higher and further increased with disease progression to CAC, indicating PAK1 overexpression is an early event in CAC. Magnifications are $\times 20$ and $\times 4$. B, PAK1 IRS was significantly higher in IBD ($*P < 0.05$) and increased further in CAC ($*P < 0.005$). C, HCEC-1CT transfected with control (con) and PAK1-WT (wild type) vectors, and MTT assay was performed for cell proliferation. PAK1-WT cells exhibited enhanced proliferation. D, Apoptosis was measured by FACS analysis for Annexin-V staining. Total percentage of Annexin-V positive cells in PAK1 overexpressing HCEC-1CT was significantly less compared with control ($*P < 0.005$).

staining. However, only p-AKT1 was increased further in CAC (Fig. 2). Noticeably, p-mTOR staining was predominantly nuclear in both IBD and CAC. Expression of p-ERK1/2 was also examined; however, expression was not altered either in IBD or CAC compared with controls (see Fig., Supplemental Digital Content 1, <http://links.lww.com/IBD/A679>).

PAK1 Contributes to PI3K/AKT, MAPK/ERK, and mTOR Pathways in Colon Epithelial Cells

It was clear that PAK1 overexpression in HCEC-1CT contributes to cell proliferation and survival. Western blot analysis was performed on PAK1 overexpressing HCEC-1CT cells to examine activation of cell proliferation/survival pathways (Fig. 3A). 5-ASA was effective in reducing PAK1 expression. However, PAK1 overexpression did not induce any change in p-ERK1/2, p-AKT, or p-mTOR (see Fig., Supplemental Digital Content 2, <http://links.lww.com/IBD/A680>); indicating that neither of these pathways was affected by PAK1 overexpression per se. This suggested that PAK1 might be contributing downstream of these molecules.

To assess if PAK1 is downstream of these pathways, HCEC-1CT cells were treated with specific inhibitors (U0126/

LY294002/rapamycin) of these respective pathways (MEK/PI3K/mTOR) in the presence or absence of 5-ASA. Interestingly, PAK1 expression was reduced by all inhibitors tested (Fig. 3B) indicating these pathways regulate PAK1 in HCEC-1CT. There was no additional effect of 5-ASA on PAK1 inhibition in the presence of LY294002 and U0126. However, in rapamycin-treated cells, PAK1 inhibition was more pronounced on combination with 5-ASA, indicating inhibition of additional pathway regulating PAK1. Western blot for effector molecules of these pathways (p-AKT (308), p-mTOR, and p-ERK1/2) showed that 5-ASA inhibits p-AKT1 and p-mTOR. This explained that the additional effect of 5-ASA on PAK1 inhibition on rapamycin treatment was through inhibition of p-AKT. Overall, these data indicated that in HCEC-1CT, 5-ASA uses either of these pathways resulting in PAK1 inhibition.

To assess the effect of these inhibitors on the functional consequence of PAK1 overexpression, cell proliferation analysis was performed on PAK1 overexpressing cells. As was expected, all inhibitors reduced cell proliferation in the control where cells were transfected with empty vector (Fig. 3C). However, in PAK1 overexpressing HCEC-1CT, compared with MEK inhibitor U0126, PI3K inhibitor LY294002, and mTOR inhibitor, rapamycin were

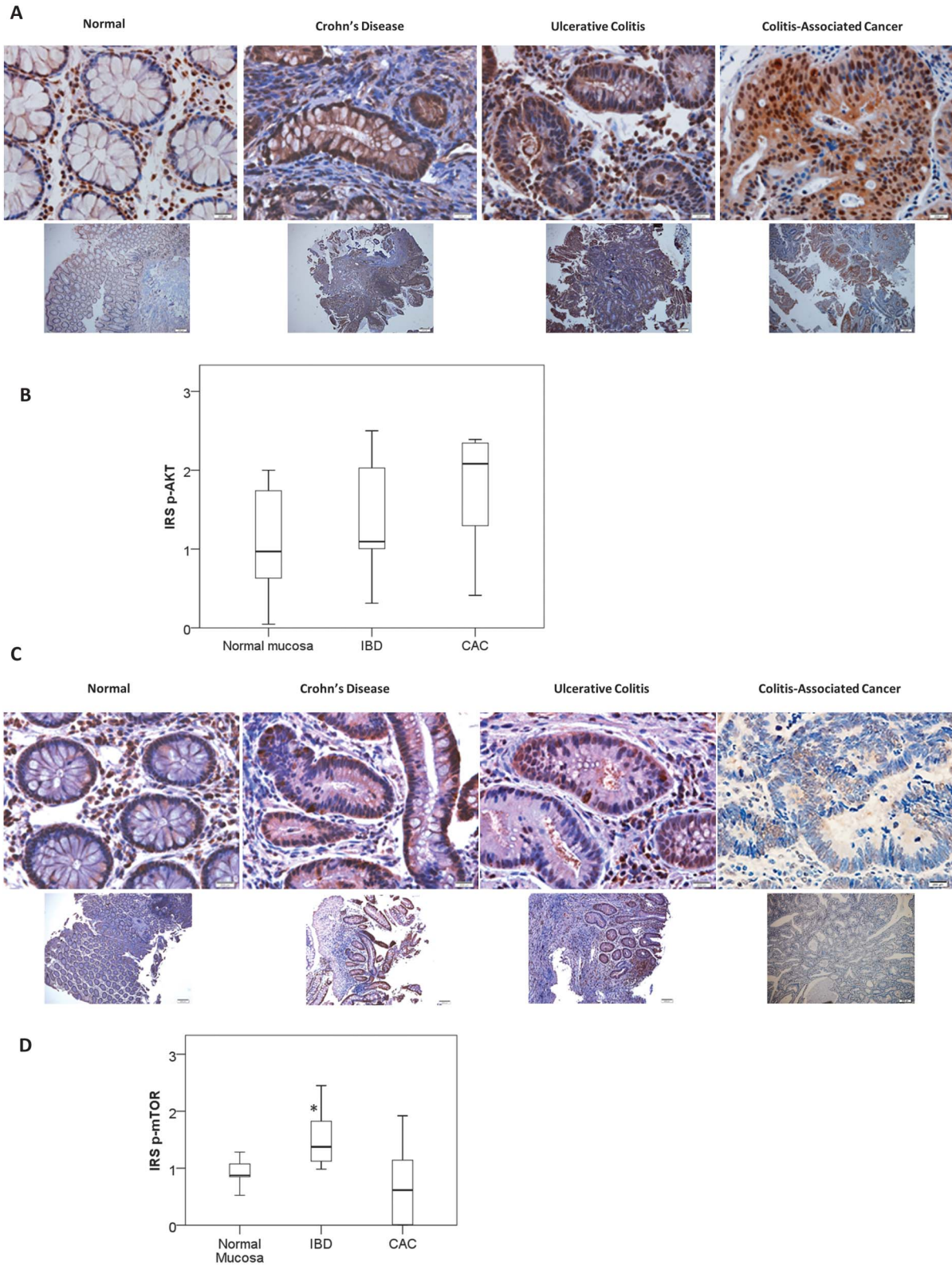


FIGURE 2. Activation of AKT and mTOR signaling in IBD and CAC. A, Immunostaining of IBD and CAC samples with phospho-AKT (Thr 308). Compared with controls, epithelial p-AKT1 showed higher cytoplasmic and nuclear staining in IBD that further increased in CAC. B, IRS showed a trend toward an increase in AKT activity with the progression of disease to CAC ($P = 0.09$). C, Activation of mTOR was increased in IBD samples but not in CAC as compared with controls. D, IRS scores demonstrated that mTOR activation was significantly increased in IBD ($*P \leq 0.05$).

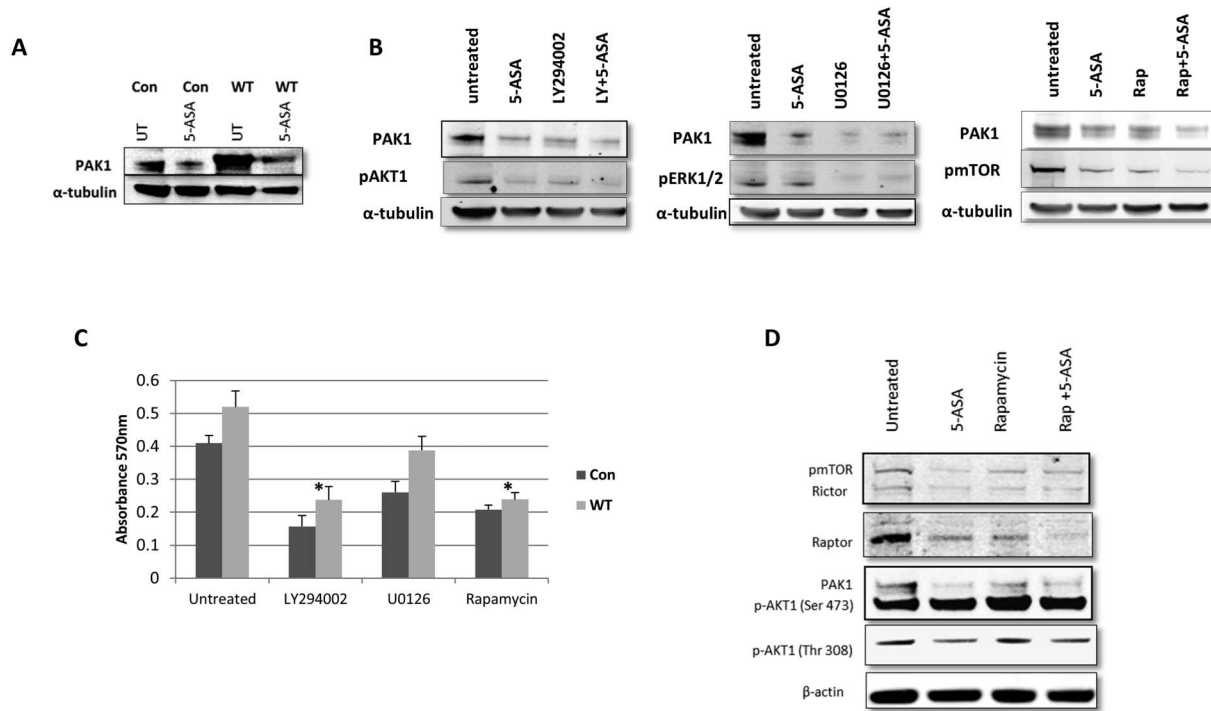


FIGURE 3. PAK1 contributes to PI3K/mTOR and MAPK pathways in HCEC-1CT. A, In the cells overexpressing WT-PAK1 or Con, 5-ASA treatment (20 mM; 24 hours) effectively inhibited PAK1 and PAK1-WT overexpression. Alpha-tubulin was used as a loading control. B, Treatment of cells with PI3K inhibitor LY294002 and 5-ASA reduced PAK1 expression. 5-ASA also reduced p-AKT levels. MEK inhibitor U0126 inhibited PAK1 expression. 5-ASA treatment did not alter p-ERK levels in HCEC-1CT. Rapamycin (mTOR inhibitor) downregulated PAK1 expression and 5-ASA exhibited additional effect in rapamycin pretreated HCEC-1CT. C, In control and PAK1-WT overexpressing HCEC-1CT, all inhibitors (LY294002, U0126, and rapamycin) reduced cell proliferation, the functional consequence of PAK1 overexpression. PI3K inhibition by LY294002 and inhibition of mTOR signaling by rapamycin were more efficient in reducing cell proliferation in HCEC-1CT overexpressing WT-PAK1 compared with MEK inhibitor U0126. * $P < 0.01$. D, Effect of 5-ASA on mTOR pathway in HCEC-1CT. 5-ASA inhibited p-mTOR and raptor indicating inhibition of mTORC1 complex through inhibition of p-AKT. Increase in p-AKT (Ser473) is a known consequence of activation of feedback loop on mTOR inhibition. Beta-actin was used as a loading control.

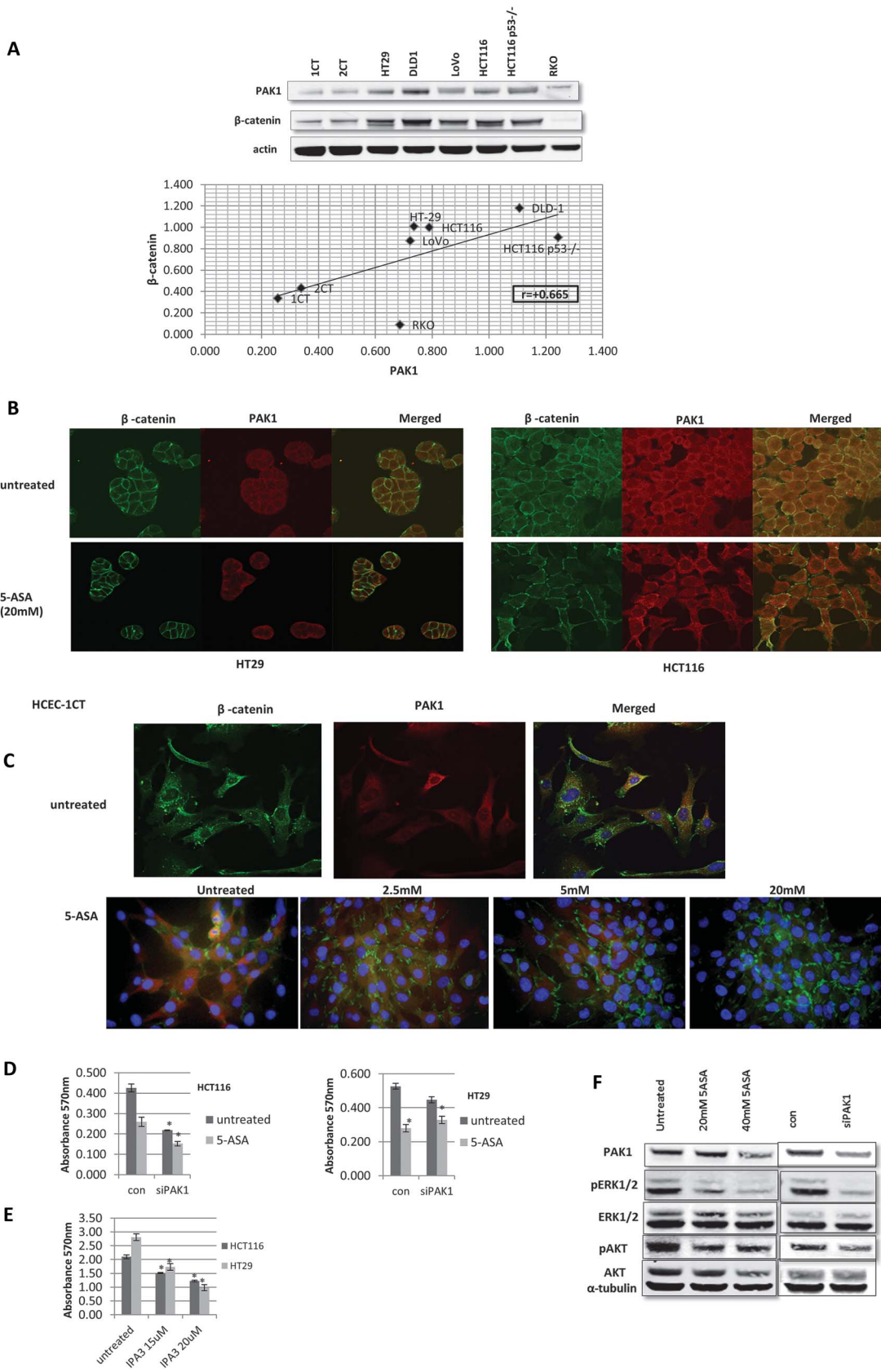
more effective in counteracting the increased cell proliferation on PAK1-WT overexpression. These data suggested that blocking mTOR and PI3K signaling can antagonize functional consequence of PAK1 overexpression.

Inhibition of p-AKT1 and mTOR by 5-ASA has not been investigated in normal colon epithelial cells previously. We further examined the effect of 5-ASA on mTORC1 and mTORC2 and performed Western blot analysis of p-mTOR, raptor (representing mTORC1 complex), rictor (representing mTORC2 complex), and AKT phosphorylation (Ser473, Thr 308). 5-ASA and rapamycin inhibited p-mTOR and raptor (Fig. 3D). In rapamycin pretreated cells, 5-ASA further inhibited raptor expression. Since, mTOR in complex with rictor (mTORC2) has been identified as PDK2 phosphorylating AKT at Ser473,²² we examined the effect of 5-ASA on Ser473-AKT phosphorylation. Ser473-p-AKT was increased on 5-ASA and rapamycin treatment indicating activation of feedback loop reported earlier.²³ 5-ASA, however, inhibited p-AKT1 (Thr 308) as was observed previously (Fig. 3B). These observations suggest that 5-ASA inhibits mTOR through

inhibition of AKT (Thr 308) phosphorylation and potentiates the effect of rapamycin on mTORC1 inhibition.

PAK1 Interacts with β -Catenin in Colon Epithelial Cells and Its Overexpression Correlates with β -catenin Expression in CRC Cell Lines

To investigate somatic mutations that might drive PAK1 overexpression, we examined PAK1 expression in a panel of CRC cell lines (see Fig. A, Supplemental Digital Content 3, <http://links.lww.com/IBD/A681>). Normal diploid HCEC-1CT and 2CT cells²⁴ were also included for the comparison. CRC cell lines mutated in K-Ras (DLD-1, LoVo, HCT116) and p53 (HT29, HCT116 p53^{-/-}) expressed higher levels of PAK1 compared with HCEC-1CT and 2-CT and RKO (Fig. 4A). In CRC, Wnt/ β -catenin pathway contributes to cell proliferation and survival; therefore, beta-catenin levels were also examined in this panel. It was interesting to note that PAK1 overexpression was associated with increased β -catenin expression in these cell lines ($r = +0.665$; Pearson's correlation).



Inhibition of Wnt/ β -catenin is a known mechanism of 5-ASA activity.^{7,25} PAK1 contributes to this pathway by phosphorylating β -catenin and facilitating its nuclear localization.^{26,27} Immunofluorescence was performed on CRC cells to examine the PAK1- β -catenin localization. HT29 and HCT116 cells displayed both nuclear and cytoplasmic expression and colocalization of PAK1 and β -catenin (Fig. 4B). 5-ASA treatment reduced β -catenin and PAK1 in the nucleus and disrupted their interaction and colocalization. Moreover, as reported previously, 5-ASA induced membranous localization of β -catenin.⁷ The interaction between PAK1 and β -catenin was also tested in HCEC-1CT (Fig. 4C). PAK1 was mostly cytoplasmic, whereas β -catenin was both membranous and cytoplasmic. PAK1 colocalized with β -catenin in the cytoplasm (Fig. 4C). A dose-dependent effect of 5-ASA was observed on PAK1- β catenin expression and localization. 5-ASA treatment increased membranous β -catenin and decreased cytoplasmic PAK1 and β -catenin. PAK1 was also detected in β -catenin immunoprecipitate and treatment with 5-ASA abolished this interaction likely due to reduction in total β -catenin and PAK1 (see Fig. B, Supplemental Digital Content 3, <http://links.lww.com/IBD/A681>).

Next, we examined the effect of PAK1 inhibition on cellular proliferation in PAK1 overexpressing CRC cells. Cell proliferation was decreased on inhibition of PAK1 on 5-ASA treatment and silencing PAK1 using siRNA (Fig. 4D) or inhibition of kinase activity using IPA3 (Fig. 4E). HCT116 and HT29 exhibited dose-dependent inhibition of cellular proliferation on IPA3 treatment (Fig. 4E). Normal HCEC-1CT cells were rather resistant to IPA3 and a higher dose of IPA3 (20 μ M) resulted in cytotoxicity (see Fig. C, Supplemental Digital Content 3, <http://links.lww.com/IBD/A681>).

5-ASA has been previously shown to interfere with the PI3K/AKT and MAPK/ERK pathways.^{5,7} To further examine whether 5-ASA and targeted inhibition of PAK1 (by silencing PAK1 with siRNA) have similar molecular effects, ERK1/2 and AKT1 phosphorylation was examined in CRC cells (HT-29). Cells treated with 5-ASA exhibited decreased phosphorylation of ERK1/2 and p-AKT (Fig. 4F). Silencing PAK1 (siPAK1) elicited similar effect as 5-ASA, suggesting that molecular effects of 5-ASA on ERK1/2 and AKT1 phosphorylation are mediated by inhibition of PAK1. These observations led us to conclude that in CRC cells, PAK1 contributes to MEK and AKT signaling and 5-ASA likely downregulates both pathways through inhibition of PAK1.

FIGURE 4. PAK1 interacts and colocalizes with β -catenin in colon epithelial cells. A, CRC cell lines were examined for PAK1 expression. PAK1 expression was increased in CRC cells compared with normal diploid human colon epithelial cells HCEC 1-CT and 2-CT. β -actin was used as a loading control. Pearson's correlation analysis revealed a positive relationship between β -catenin and PAK1 expression in HCEC and CRC cell lines ($r = +0.665$). B, In CRC cells HT29 and HCT116, PAK1 and β -catenin colocalized in both cytoplasm and nucleus. 5-ASA treatment reduced nuclear β -catenin and PAK1 and disrupted their interaction. C, In HCEC-1CT, both PAK1 and β -catenin showed predominantly cytoplasmic localization and interaction. 5-ASA treatment exhibited a dose-dependent effect on reduction of PAK1 and β -catenin. Membranous β -catenin at cell junctions was retained by 5-ASA. D, MTT assay showing that 5-ASA and silencing PAK1 (siPAK1) decreased cell proliferation in CRC cells HT29 and HCT116. E, PAK1 kinase inhibitor IPA3 reduced cell proliferation in CRC cell lines in a dose-dependent manner ($*P < 0.05$). F, 5-ASA and PAK1 silencing elicited similar molecular effects in HT29 and reduced p-ERK1/ERK2 and p-AKT (Thr308) signaling that was activated in untreated cells. Alpha-tubulin was used as a loading control.

DISCUSSION

In this study, PAK1 was found to be overexpressed in the intestinal epithelium of IBD and CAC. Functional consequences of PAK1 overexpression were examined in primary colonic epithelial cells (overexpressing WT-PAK1), and the data demonstrated that PAK1 overexpression stimulated cell proliferation and decreased apoptosis. When examined for the activation of growth pathways in parallel to PAK1 expression, IBD and CAC samples showed an increase in epithelial AKT1 and mTOR phosphorylation suggesting that activation of AKT/mTOR pathway and PAK1 overexpression are early events in the disease progression to cancer. Our data demonstrating an association and interaction of PAK1 with β -catenin in primary colonic epithelium suggests its role in Wnt/ β -catenin signaling and intestinal homeostasis. 5-ASA treatment effectively inhibited PAK1 on overexpression and also AKT and mTOR activation. Taken together, the data demonstrate that PAK1 is overexpressed in IBD and CAC, contributing to AKT/mTOR cell survival pathway, and implicates it as a target of 5-ASA (Fig. 5).

In the absence of oncogenic signaling, what drives PAK1 overexpression is not yet clear as PAK1 acts as a scaffolding protein and a nodal kinase in multiple signaling cascades.¹⁹ PAK1 overexpression in IBD and CAC might be a direct influence of proinflammatory cytokines as preliminary findings suggest that PAK1 contributes to TNF- α /NF- κ B signaling.²⁸ It is known that TNF- α signaling activates AKT for NF- κ B activation,²⁹ and PAK1 might be another player in the activation of NF- κ B pathway. It was previously shown that expression of active PAK1 in NIH3T3 cells stimulated NF- κ B through nuclear translocation of p65.¹⁸ It is likely that inflammation-driven PAK1 overexpression promotes cell proliferation and survival contributing to PI3K/AKT and mTOR signaling, which is activated in IBD, as was observed in this study. However, role of stress-activated signaling cascade (SAPK/JNK/p38) and regulation of PAK1 requires further investigation in the context of chronic gut inflammation.

Activation of mTOR pathway in IBD has not been clearly demonstrated, although mTOR activity has been recently implicated in impairment of epithelial autophagy in IBD.³⁰ We observed an increased mTOR activity in IBD but not in CAC indicating that unlike PAK1 and p-AKT, mTOR activation was not sustained throughout the disease progression. Interestingly, p-mTOR was found to be mostly nuclear both in IBD and CAC.

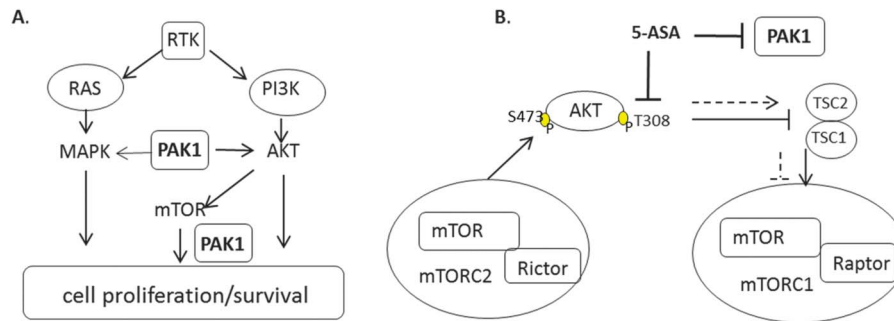


FIGURE 5. Model representing PAK1 as a viable target for chemoprevention in CAC. A, PAK1 contributes to MAPK and AKT pathways downstream of growth factor induced receptor tyrosine kinases (RTK) signaling. PAK1 also contributes to mTOR signaling, promoting growth signals, and decreasing apoptosis. Inhibition of PAK1 can be a beneficial strategy to mitigate survival pathways. B, 5-ASA inhibits PAK1 and AKT/mTOR axis. Inhibition of p-AKT by 5-ASA can suppress mTORC1 activation. Additionally, inhibition of PAK1 by 5-ASA could also attenuate PI3K/mTOR signaling.

Nuclear-cytoplasmic shuttling of mTOR has been implicated in the activation of its targets eIF4E and S6K1,³¹ involved in the initiation of translation and protein synthesis and thereby promoting cell growth and survival.

Both, PI3K and MAPK signaling regulate mTORC function.^{32,33} In HCEC, overexpression of PAK1 per se did not activate either of these pathways; however, downregulation of PAK1 by inhibitors of these pathways indicates that PAK1 expression is regulated downstream of these molecules. This implicates that PAK1 contributes to these prosurvival pathways in colonic epithelium which are activated in response to mucosal insult. In CRC, as a consequence of activation of oncogenic signaling, PAK1 activity might be critical in tumor progression. Multiple signaling pathways including Wnt/ β -catenin, ERK, and AKT exhibit diminished activation in the PAK1-knockout mice and on inhibition of PAK1 in CRC cells,^{14,15,34} supporting its role in full activation of these pathways.

This study provides a novel insight of signaling pathways regulating PAK1 expression in colon epithelial cells that can be used for targeting PAK1 overexpression in gastrointestinal diseases including IBD and CRC. Inhibition of PI3K/AKT and mTOR pathway can potentially curtail PAK1 activity and inhibition of PAK1 can impair AKT/mTOR activation. Remarkably enough, 5-ASA inhibited both of these pathways. In the context of UC and CAC, 5-ASA has been previously shown to reduce p-AKT.⁵ In CRC cells, 5-ASA caused phospholipase-D dependent loss of mTOR signaling.³⁵ We have previously identified eIF4b and IF4e as targets of 5-ASA, which are members of the cellular translation machinery regulated by PI3K/mTOR.³⁶ Nevertheless, activation (and potential inhibition) of the mTOR pathway in IBD warrants further investigations.

As a component of adherens junctions and canonical Wnt signaling, β -catenin serves a dual role in intestinal physiology. It has been recently demonstrated that in CRC cells, PAK1 phosphorylates β -catenin and promotes its nuclear functions, whereas PAK1 inhibition increased intercellular adhesion through membranous translocation of β -catenin and E-cadherin.^{7,27} Since,

PI3K/AKT signaling is known to activate β -catenin transcriptional activity, it is seemingly possible that PAK1 cooperates with PI3K/AKT signaling to sustain Wnt/ β -catenin activity. Our data in HCEC-1CT demonstrate an interaction of PAK1 and β -catenin and substantiates physiological relevance of such association in normal colon epithelial cells. Disruption of this interaction by 5-ASA might contribute to downregulation of nuclear functions of β -catenin, thereby inhibiting canonical Wnt signaling and consequently promoting membranous translocation of β -catenin at adherens junctions. This activity of 5-ASA is in accordance to its clinical benefit in UC. It can be speculated that as a multifunctional kinase, PAK1 might have additional roles in the regulation of gut homeostasis including epithelial–stromal interactions and epithelial–mesenchymal transition during epithelial restitution.

Overexpression of PAK1 in IBD and CAC is a novel finding that implicates PAK1 in chronic gut inflammation. Since PAK1 overexpression is an early event in disease progression from colitis to cancer, it can be used as a predictive marker of disease activity and remission. Activation of survival pathways and an increase in cellular proliferation as a consequence of PAK1 overexpression makes PAK1 a viable target for chemoprevention in inflammation-driven colon cancer. This study also underscores the molecular mechanism of anti-inflammatory drug 5-ASA that can confer chemopreventive benefits in CAC. Specific targeting of PAK1 may open an alternative approach in the treatment of IBD.

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