[CANCER RESEARCH 64, 229–235, January 1, 2004]

Roles of Phosphatidylinositol 3′-Kinase and Mammalian Target of Rapamycin/p70 Ribosomal Protein S6 Kinase in K-Ras-Mediated Transformation of Intestinal Epithelial Cells

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ABSTRACT

Phosphatidylinositol 3′-kinase (PI3K) activity is required for Ras-mediated transformation of intestinal epithelial cells (IECs). The mammalian target of rapamycin (mTOR) and its downstream pathways control the translation of specific mRNAs that are required for cell proliferation and transformation. Here, we elucidated the roles of PI3K and mTOR in K-Ras-mediated transformation of IECs (IEC-6). Induction of K-Ras activated PI3K and mTOR in IECs. p70 ribosomal protein S6 kinase activity was induced by K-Ras in a PI3K- and mTOR-dependent manner. K-Ras did not significantly alter the phosphorylation of eukaryotic initiation factor 4E-binding protein 1. Treatment with either LY-294002 or rapamycin inhibited IEC proliferation and resulted in G_1 growth arrest. However, it was noted that inhibition of mTOR enhanced K-Ras-mediated morphological transformation and increased invasiveness of IECs in a mitogen-activated protein/extracellular signal-regulated kinase-dependent manner. Furthermore, inhibition of PI3K or mTOR impaired the growth of an array of colon cancer cells. Spindle transformation, reduced E-cadherin, and increased invasiveness were observed in LY-294002-treated Moser cells. Thus, our results suggest that K-Ras-mediated transformation of IECs involves activation of the PI3K/mTOR pathway. Inhibition of PI3K/mTOR activity leads to G_1 growth arrest of transformed IECs. On the other hand, inhibition of PI3K or mTOR may induce the epithelial to mesenchymal transdifferentiation of IECs under certain circumstances.

INTRODUCTION

About 50% of colorectal carcinomas contain K-*Ras* mutations (1), and the K-*Ras* oncogene plays a key role during the adenoma to carcinoma sequence of events involved in the neoplastic transformation of colonic epithelial cells (2). The mechanism governing Ras-mediated transformation is complex and involves a number of downstream signaling pathways. The Raf/mitogen-activated protein/extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway has been implicated as a critical effector of Ras function (3). Activation of Raf/MEK/ERK is known to be sufficient to transform NIH 3T3 fibroblasts (4, 5); however this activation is not sufficient for transformation of rat intestinal epithelial (RIE) cells (6). The phosphatidylinositol 3′-kinase (PI3K)/protein kinase B (Akt) pathway lies downstream of receptor tyrosine kinases and can be directly activated by Ras (7). The serine/threonine kinase, Akt, regulates gene transcription by direct phosphorylation of some of the forkhead transcription factors, such as FKHR, FKHRL, and AFX (8–10), or indirectly by modifying the cAMP-responsive element binding protein (11, 12), E2F (13), or nuclear factor κB (14). Activation of the PI3K/Akt pathway is important for Ras transformation of mammalian cells and essential in Ras-induced cytoskeletal reorganization (15). We have demonstrated that inhibition of PI3K activity significantly blocks Ha-Ras-mediated transformation of RIE cells (16). Ectopic expression of both active Raf and active Akt results in RIE cell transformation mimicking the transforming action of oncogenic Ras. These findings indicate the critical role of the PI3K pathway in Ras-mediated transformation of IECs. Further elucidating the molecular mechanisms mediating PI3K oncogenic activity will add insight to the understanding of oncogenic Ras signaling system.

Mammalian target of rapamycin (mTOR) is a member of phosphatidylinositol kinase-related kinase family and regulates protein translation, cell cycle progression, and cell proliferation (17). mTOR cooperates with effectors of PI3K to phosphorylate the p70 ribosomal protein S6 kinase (p70S6K), which is involved in protein translation of a family of mRNA transcripts that encode essential components of the protein synthetic apparatus (18, 19). Activated p70S6K phosphorylates the 40S ribosomal subunit protein S6 and promotes the translation of 5′ oligopyrimidine tract containing mRNAs (17). PI3K/mTOR activation also leads to phosphorylation of eukaryotic translation initiation factor 4E (eIF-4E)-binding proteins [4E-BPs (20, 21)]. Hypophosphorylated 4E-BPs bind and inhibit eIF-4E activity. Hyperphosphorylation of 4E-BPs liberates eIF-4E, which then binds to the cap structure present at the 5′ end of mRNA and initiates translation of specific subsets of mRNAs. Rapamycin, a bacterially derived drug with potent antitumor properties, inhibits mTOR activity and leads to dephosphorylation of p70S6K and 4E-BPs and therefore blocks protein synthesis (22, 23). Rapamycin decreases the translation of cyclin D1 mRNA and increases the turnover of cyclin D1 mRNA and protein (24, 25). Furthermore, rapamycin increases expression of $p27^{kip1}$ at both mRNA and protein levels and facilitates the formation of cyclin/cyclin-dependent kinase-$p27^{kip1}$ complex (24, 26).

In the present study, we further elucidated roles of PI3K and mTOR in K-Ras-mediated transformation of IECs. We show the critical roles of PI3K/mTOR in intestinal epithelial growth and transformation; therefore, this pathway can be targeted for cancer treatment. On the other hand, inhibition of mTOR enhanced K-Ras-mediated morphological transformation and invasion in a MEK-dependent manner, suggesting that transformed IECs may use substituted signaling pathways in response to inhibition of certain signaling pathways and result in proneoplastic phenotypes.

MATERIALS AND METHODS

Cell Culture and Chemicals. The IEC-i-K-Ras cell line with an inducible $K\text{-}Ras^{Val12}$ cDNA was generated by using LacSwitch eukaryotic expression system (Stratagene, La Jolla, CA) and grown in DMEM containing 10% fetal bovine serum (27). The $K\text{-}Ras^{Val12}$ cDNA is under the transcriptional control of the Lac operon. Isopropyl-1-thio-β-D-galactopyranoside (IPTG; Life Technologies, Inc., Gaithersburg, MD) at a concentration of 5 mM was used to induce the expression of mutated K-Ras. The RIE-i-myr-p110α cell line has been described previously (28). The expression of active p110α can be induced by 5 mM IPTG. Human colon cancer cell lines Lovo, SW-480, and Moser were purchased from American Type Culture Collection (Manassas, VA). The growth of cells in Matrigel (Collaborative Biomedical, Bedford, MA) was carried out as described previously (29). PD-98059, rapamycin, and LY-294002 were purchased from Calbiochem (San Diego, CA).

Received 6/24/03; revised 9/30/03; accepted 10/24/03.

Grant support: John Sealy Memorial Endowment Fund for Biomedical Research and the Gastrointestinal Research Interdisciplinary Program.

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Immunoblot Analysis and Antibodies. Immunoblot analysis was performed as described previously (30). Cells were lysed for 30 min in radioimmunoprecipitation assay buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 mM sodium orthovanadate), and then clarified cell lysates were denatured and fractionated by SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membrane. The filters were then probed with the indicated antibodies and developed by the enhanced chemiluminescence system (ECL; Amersham, Arlington Heights, IL). The anti-pan-Ras antibody was purchased from Calbiochem (La Jolla, CA). The anti-cyclin D1 antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-cyclin-dependent kinase 2 and $p27^{kip1}$ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-phosphorylated Akt antibody, anti-phosphorylated p70S6K antibody, anti-phosphorylated 4E-BP antibody, and the anti-active ERK1/2 antibody were obtained from Cell Signaling (Beverly, MA). Anti-Bcl-2 and anti-Bcl-xL antibodies were purchased from Transduction Laboratories (Lexington, KY). Anti-E-cadherin antibody was purchased from Transduction Laboratories.

p70S6K Kinase Assay. p70S6K kinase activity was measured by determining the transfer of the phosphate group of ATP to a specific peptide modeled after the major phosphorylation sites in S6 kinase (Upstate Biotechnology). The experiment was carried out according to the manufacturer's instructions.

Flow Cytometry. Cells were seeded into 100-mm plates and treated as indicated for 48 h. Cells were fixed in 70% EtOH, digested in 1 ml of 0.1% RNase (Sigma, St. Louis, MO), and stained with propidium iodide (Sigma). The DNA was analyzed by a flow cytometer, and the cell cycle profile was expressed as percentage of cells in each cell cycle stage.

Immunofluorescence. Cells were grown in 35-mm tissue culture plates and fixed in methanol/acetone at −20°C for 10 min. Fixed cells were incubated with 10% normal donkey serum for 1 h and then incubated with anti-E-cadherin antibody for 2 h at room temperature. After washing the cells three times with PBS, they were incubated with Cy3-conjugated donkey antimouse IgG (Jackson ImmunoResearch, West Grove, PA) for an additional hour. The stained cells were washed with PBS, mounted, and observed under fluorescent microscopy with appropriate filters.

Apoptosis. Subconfluent or confluent IEC-i-K-Ras cell cultures were established and subjected to the indicated treatment for 48 h. Floating and attached cells were collected and lysed in lysis buffer [1% NP40 in 20 mM EDTA and 50 mM Tris (pH 7.5)]. The supernatant containing fragmented DNA was clarified by centrifugation for 5 min at 1600 × *g*. The cell lysates were treated with a solution containing RNase A (5 mg/ml) and proteinase K (2.5 mg/ml). The DNA was then separated on 1.6% agarose gels.

RESULTS

Downstream Pathways Involved in K-Ras Transformation of IECs. Oncogenic Ras activates downstream signaling pathways that induce various cellular responses and result in transformation. We have shown that induction of Ha-Ras activates PI3K/Akt, which is critical for Ras-mediated growth and transformation of RIE cells (16). A conditionally K-Ras-transformed IEC-6 cell line has been established (IEC-i-K-Ras; Ref. 31) and was used for the present study. Expression of mutated $K\text{-}Ras^{Val12}$ induced the activation of MEK/ERK, PI3K, and p70S6K (Fig. 1*A*). Addition of IPTG increased the levels of phosphorylated MEK1/2 and ERK1/2. Phosphorylation of Akt [phosphorylated Akt (pAkt)], which reflects PI3K activity (32), was elevated; levels of phosphorylated p70S6K (pp70S6K-Thr^{389}), which is closely correlated with p70S6K activity (18), were increased as well. In contrast, levels of total MEK, ERK, Akt, and p70s6K were slightly altered by induction of K-Ras. p70S6K kinase activity was increased after expression of oncogenic K-Ras; this induction was attenuated by a mTOR inhibitor, rapamycin (100 nM; Fig. 1*B*). The K-Ras-induced pAkt was significantly blocked by treatment with a selective PI3K inhibitor, LY-294002 (20 μM), but was not inhibited by rapamycin (Fig. 1*C*). In contrast, the K-Ras-mediated phosphorylation of p70S6K was completely blocked by either LY-294002 or rapamycin. To confirm that phosphorylation of p70S6K was dependent on PI3K activity, we established a RIE-i-myr-p110α cell line in which the myristoylated PI3K catalytic subunit, p110α, was induced by the addition of IPTG. Expression of active p110α resulted in increased levels of pAkt; levels of pp70S6K were elevated as well (Fig. 1*D*).

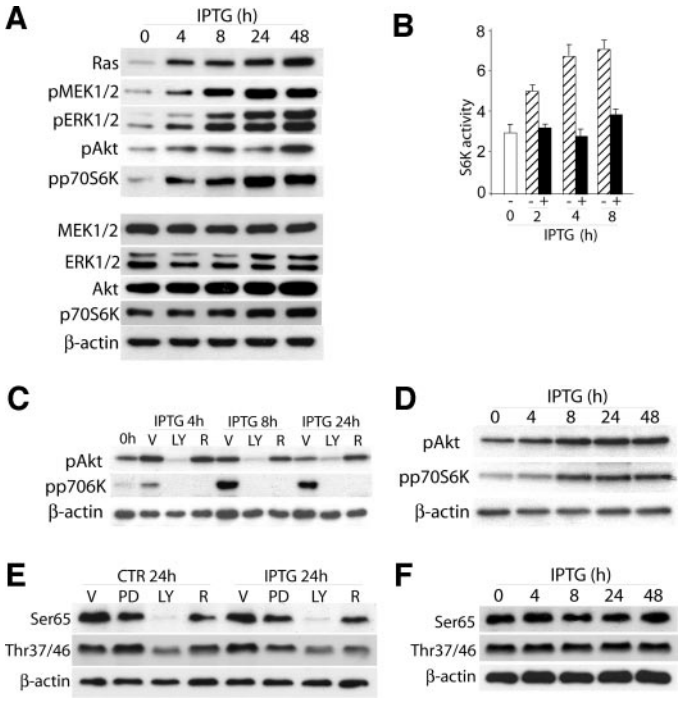
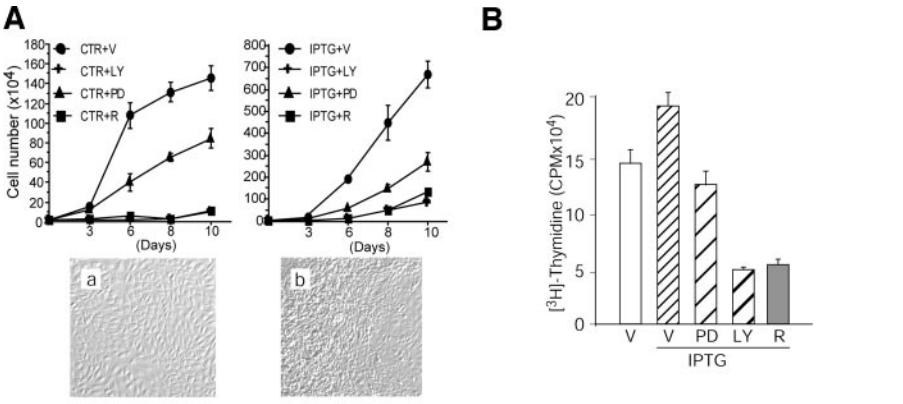


Fig. 1. K-Ras induction of downstream effectors. *A*, Western analysis for Ras, mitogen-activated protein kinase, phosphorylated Akt (pAkt), and p70 ribosomal protein S6 kinase (p70S6K). IEC-i-K-Ras cells were treated with isopropyl-1-thio-β-D-galactopyranoside and lysed in radioimmunoprecipitation assay buffer at the indicated time points. After electrophoresis, the proteins were transferred to nitrocellulose filters, and the filters were blotted with the indicated antibody. The relative expression levels were determined by autoradiography using the enhanced chemiluminescence (ECL) system. The results were similar in three independent experiments. *B*, determination of K-Ras-induced p70S6K kinase activity. IEC-i-K-Ras cells were treated with IPTG in the presence (+) or absence (−) of rapamycin (100 nM). Cell lysates were extracted, and p70S6K kinase activity was determined using the method described in "Materials and Methods." Results shown are representative of three separate experiments. *C*, roles of phosphatidylinositol 3′-kinase (PI3K) and mammalian target of rapamycin in K-Ras induction of Akt and p70S6K. IEC-i-K-Ras cells were treated with IPTG in the presence of DMSO (*V*), 20 μM LY-294002 (*LY*), or 100 nM rapamycin (*R*). *0h*, 0 h of IPTG treatment. Levels of pAkt and phosphorylated p70S6K were determined by immunoblot analysis. The results were similar in three independent experiments. *D*, induction of p70S6K phosphorylation by PI3K. PI3K activity was induced by IPTG in RIE-i-myr-p110α cells. Levels of pAkt and phosphorylated p70S6K were analyzed by Western blotting. Results shown are representative of three separate experiments. *E*, PI3K and mammalian target of rapamycin in eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) phosphorylation. IEC-i-K-Ras cells were treated with DMSO (*V*), 50 μM PD 98059 (*PD*), 20 μM LY-294002 (*LY*), or 100 nM rapamycin (*R*) in the presence (*IPTG*) or absence (*CTR*) of IPTG for 24 h. Levels of phosphorylated 4E-BP1 were determined by immunoblot analysis. *Ser65*, 4E-BP1 phosphorylated on Ser^{65}; *Thr37/46*, 4E-BP1 phosphorylated on $Thr^{37/46}$. *F*, phosphorylation of 4E-BP1 in K-Ras transformation. IEC-i-K-Ras cells were treated with IPTG for the indicated times. Levels of phosphorylated 4E-BP1 were determined by immunoblot analysis. Results were similar in three independent experiments.

PI3K/mTOR phosphorylates 4E-BPs, and hyperphosphorylation of 4E-BPs liberates eIF-4E, which then initiates translation of mRNAs with cap structure present at the 5′-untranslated region. Phosphorylation of $Thr^{37/46}$ is followed by Thr^{70} phosphorylation, and Ser^{65} is phosphorylated last (33). Because expression of mutated K-Ras induced PI3K and mTOR activities, we next determined whether K-Ras induced phosphorylation of 4E-BP1. The basal levels of phosphorylated 4E-BP1 were relatively high; LY-294002 completely attenuated the phosphorylation of 4E-BP1 on Ser^{65} and partially blocked the phosphorylation of $Thr^{37/46}$ (Fig. 1*E*). Rapamycin modestly reduced



C

Treatment	CTR	IPTG	IPTG+PD	IPTG+LY	IPTG+R
G0/G1 (%)	55	43	50	69	68
G2/M (%)	7	7	8	6	0
S (%)	38	50	42	25	32

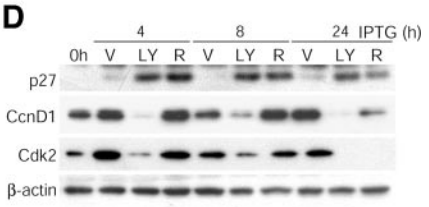


Fig. 2. Regulation of intestinal epithelial cell growth by K-Ras effectors. *A*, growth of IEC-i-K-Ras cells. *Top panels*, cells (2×10^4) were seeded in 12-well plates and subjected to the treatments as indicated (*CTR*, no IPTG; *V*, DMSO; *PD*, 50 μM PD-98059; *LY*, 20 μM LY-294002; *R*, 100 nM rapamycin; *IPTG*, 5 mM IPTG). Fresh media with treatments were replaced every 48 h. Cell numbers were counted at the indicated days, and values are means ± SD from triplicate wells. Data are representative of two separate experiments. *Bottom panels*, IEC-i-K-Ras cells (5×10^4) grown in DMEM with 10% fetal bovine serum were treated with vehicle (*a*) or IPTG (*b*) for 96 h and photographed (×100). *B*, [^{3}H]thymidine incorporation in IEC-i-K-Ras cells. Cells (5×10^4) were grown in DMEM containing 10% fetal bovine serum in the presence or absence of IPTG for 48 h. DMSO (*V*), 50 μM PD-98059 (*PD*), 20 μM LY-294002 (*LY*), or 100 nM rapamycin (*R*) was added with IPTG. [^{3}H]Thymidine incorporation was measured after a 3-h pulse. Results are expressed as cpm/well. Values are the means ± SD from quadruplicate wells. Results are representative of three separate experiments. *C*, cell cycle analysis. IEC-i-K-Ras cells were treated with DMSO (*V*), 50 μM PD-98059 (*PD*), 20 μM LY-294002 (*LY*), or 100 nM rapamycin (*R*) in the presence (*IPTG*) or absence (*CTR*) of IPTG for 48 h. DNA was analyzed by a flow cytometer, and cell cycle profiles were presented as percentage of cells in each stage of the cell cycle. *D*, phosphatidylinositol 3′-kinase and mammalian target of rapamycin regulation of cell cycle-related proteins. IEC-i-K-Ras cells were treated with DMSO (*V*), 20 μM LY-294002 (*LY*), or 100 nM rapamycin (*R*) in the presence of IPTG for the indicated times. *0h*, 0 h of IPTG treatment. Levels of cyclin D1, $p27^{kip1}$, and cyclin-dependent kinase 2 protein were analyzed.

levels of phosphorylated 4E-BP1. It was noted that expression of oncogenic K-Ras did not significantly induce the phosphorylation of 4E-BP1; levels of both 4E-BP1(Ser^{65}) and 4E-BP1($Thr^{37/46}$) were not altered by induction of K-Ras.

Critical Roles of PI3K and mTOR in IEC-i-K-Ras Cell Growth. PI3K activity is critical for the proliferation of nontransformed RIE cells (28) and Ha-Ras-transformed RIE cells (16). Because mTOR plays an important role in the G_1-S transition of the cell cycle and is regulated by PI3K activity, it was of interest to determine the involvement of mTOR in PI3K growth-regulatory action. The number of K-Ras-transformed IECs was ~5-fold of the number of nontransformed IEC-i-K-Ras cells after they were grown for 10 days (Fig. 2*A*, *top panels*). Both transformed and nontransformed IECs grew at a similar rate before reaching confluence. Thereafter, transformed IECs grew continuously, whereas the growth of nontransformed cells almost ceased. It was noted that expression of oncogenic K-Ras resulted in an overlapping growth of IECs, whereas nontransformed IECs grew as monolayers (Fig. 2*A*, *a* and *b*). Addition of PD-98059 (50 μM) inhibited the growth of both transformed and nontransformed IECs. Rapamycin (100 nM) and LY-294002 (20 μM) exerted similar growth-inhibitory effects on IEC-i-K-Ras cells and reduced cell number by ~90% in nontransformed IECs and by ~80% in K-Ras-transformed IECs. Induction of K-Ras stimulated the proliferation of IECs (Fig. 2*B*). [^{3}H]Thymidine incorporation assay demonstrated that DNA synthesis was increased ~35% after K-Ras was induced for 48 h. Treatment with PD-98059 attenuated the oncogenic Ras-stimulated cell proliferation. On the other hand, LY-294002 and rapamycin strongly inhibited DNA synthesis and resulted in a ~70% reduction of [^{3}H]thymidine incorporation in K-Ras-induced IECs. In agreement with these results, induction of K-Ras accelerated the G_1-S transition in IECs and increased cell number in the S phase by ~30%. Treatment with LY-294002 or rapamycin resulted in a growth arrest of IEC-i-K-Ras cells in the G_1 phase of the cell cycle (Fig. 2*C*). Western analysis revealed that levels of $p27^{kip1}$ protein were significantly induced by both LY-294002 and rapamycin (Fig. 2*D*). LY-294002 rapidly inhibited the expression of cyclin D1 and cyclin-dependent kinase 2; rapamycin exerted similar effects, however, at a relatively late time point (24 h).

K-Ras Promoted IEC Survival in a MEK- and PI3K-Dependent Manner. The PI3K/Akt pathway promotes growth factor-mediated cell survival and inhibits apoptosis (34, 35). To elucidate the signaling pathways that control programmed cell death in K-Ras-transformed IECs, DNA fragmentation assay was conducted in subconfluent (Fig. 3*A*, *left panel*) and confluent (Fig. 3*A*, *right panel*) IEC-i-K-Ras cells. PD-98059 induced DNA fragmentation in both nontransformed and transformed IECs in subconfluent cultures. In confluent cultures, nontransformed IEC-i-K-Ras cells spontaneously underwent apoptosis. PD-98059 treatment significantly increased DNA fragmentation. Induction of oncogenic K-Ras prevented IECs from cell death, noted by reduced DNA fragmentation in IPTG-treated IEC-i-K-Ras cells. Treatment with PD-98059 or LY-294002 restored apoptosis in these cells. In contrast, rapamycin treatment did not induce apoptosis in either nontransformed or transformed IECs. In agreement with these findings, floating cells were observed in confluent IEC-i-K-Ras cells; expression of K-Ras protected cells from detachment (Fig. 3*B*). Addition of either PD-98059 or LY-294002 significantly increased floating cells.

Induction of K-Ras increased the levels of Bcl-xL and Bcl-2; addition of LY-294002 attenuated the expression of Bcl-xL and blocked the expression of Bcl-2 after IEC-i-K-Ras cells were treated for 8 h (Fig. 3*C*). In contrast, rapamycin treatment increased the levels of Bcl-2 protein and did not significantly change Bcl-xL expression.

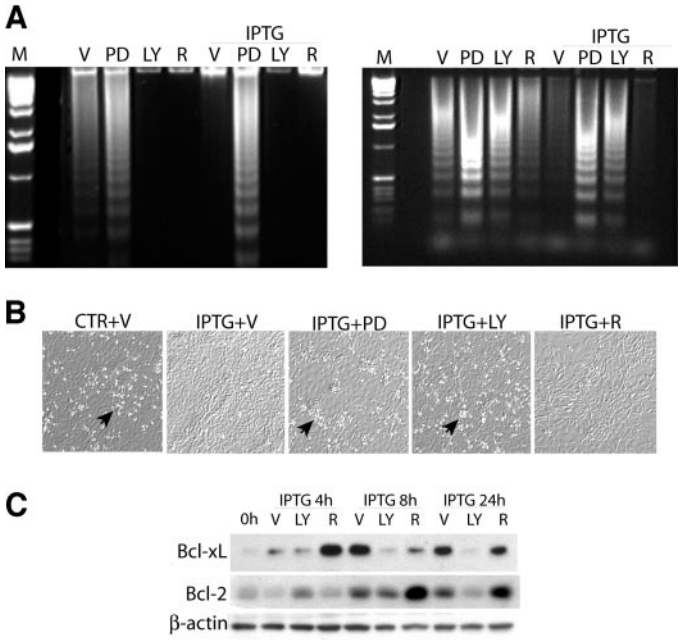


Fig. 3. Cell death signaling in IEC-i-K-Ras cells. *A*, DNA fragmentation assay. Subconfluent (*left panel*) or confluent (*right panel*) IEC-i-K-Ras cells were established in DMEM with 10% fetal bovine serum and subjected to the indicated treatments. *V*, DMSO; *PD*, 50 μM PD-98059; *LY*, 20 μM LY-294002; *R*, 100 nM rapamycin; *IPTG*, 5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Cells were incubated for 48 h after treatment, and then floating and attached cells were collected in lysis buffer, and the soluble DNA was isolated. DNA fragmentation was visualized on 1.6% agarose gel. The results were similar in three independent experiments. *B*, detection of floating cells. IEC-i-K-Ras cells were grown to confluence before the indicated treatments. Cells were photographed after 48 h of incubation (×100). *IPTG*, 5 mM IPTG; *V*, DMSO; *LY*, 20 μM LY-294002; *R*, 100 nM rapamycin; *CTR*, 48 h without IPTG. *Arrows* indicate examples of floating cells. *C*, phosphatidylinositol 3′-kinase and mammalian target of rapamycin regulation of Bcl-2 and Bcl-xL. IEC-i-K-Ras cells were treated with DMSO (*V*), 20 μM LY-294002 (*LY*), or 100 nM rapamycin (*R*) in the presence of IPTG for the indicated times. *0h*, 0 h of IPTG treatment. Levels of Bcl-2 and Bcl-xL proteins were analyzed.

Inhibition of mTOR Enhanced Morphological Transformation. Uninduced IEC-i-K-Ras cells displayed an epithelial morphology and grew as monolayer cultures. IEC-i-K-Ras cells acquired a spindle appearance when oncogenic K-Ras was induced by treatment with IPTG (Fig. 4*A*, compare *b* with *a*). Inhibition of MEK activity completely blocked K-Ras-mediated morphological transformation; inhibition of PI3K activity also reduced the spindle appearance of K-Ras-transformed IECs. Paradoxically, it was noted that while inhibiting the growth of IEC-i-K-Ras cells, rapamycin enhanced K-Ras-mediated morphological transformation (Fig. 4*A*, *e*). Addition of rapamycin significantly accelerated the transformation process and resulted in a more polarized fibroblast-like shape in IEC-i-K-Ras cells. Epifluorescence microscopy demonstrated that immunoreactivity of E-cadherin localized at cell junctions of nontransformed IEC-i-K-Ras cells. Induction of K-Ras resulted in significant loss of the junctional localization of E-cadherin. Rapamycin-treated IEC-i-K-Ras cells displayed spindle morphology; expression of E-cadherin was significantly reduced, and the junctional localization of E-cadherin was completely lost (Fig. 4*B*). These findings were confirmed by immunoblot analysis. Expression of mutated K-Ras reduced levels of E-cadherin; treatment with rapamycin further decreased the expression of E-cadherin in IEC-i-K-Ras cells (Fig. 4*C*).

To determine the roles of downstream effectors on the invasiveness of K-Ras-transformed IECs, IEC-i-K-Ras cells were grown in extracellular matrix (Matrigel). Nontransformed IECs were not able to grow in Matrigel; K-Ras-transformed IECs formed three-dimensional structures in Matrigel (Fig. 4*D*). PD-98059 blocked the growth of IEC-i-K-Ras cells in Matrigel (data not shown). Rapamycin treatment inhibited the growth of IPTG-treated IEC-i-K-Ras cells and reduced the number of colonies. It was noted that whereas K-Ras-transformed IECs formed round, noninvasive colonies; rapamycin induced these cells to invade Matrigel and grow as disordered chords. LY-294002 strongly blocked the growth of K-Ras-transformed IECs in Matrigel. Number and size of colonies were significantly reduced (Fig. 4*E*). However, after a prolonged incubation, LY-294002- and IPTG-treated IEC-i-K-Ras cells formed invasive chords in Matrigel.

Neoplastic transformation is dependent on complex oncogenic signals. Inhibition of a specific signaling pathway may, therefore, enhance other signaling pathways in tumor cells. Western analysis demonstrated that induction of K-Ras increased the levels of phosphorylated ERK (pERK); treatment with rapamycin also increased levels of pERK. In combination, IPTG and rapamycin strongly induced the phosphorylation of ERK (Fig. 5*A*). Treatment with PD-98059 completely blocked the phosphorylation of ERK in uninduced IEC-i-K-Ras cells and significantly blocked the IPTG- and rapamycin-induced phosphorylation of ERK. Similar results were observed when another MEK1/2 inhibitor, UO-126, was used. Addition of PD-98059 completely reversed IPTG- and rapamycin-induced spindle morphology (Fig. 5*B*). In contrast, LY-294002 did not block IPTG- and rapamycin- induced morphological transformation. PD-98059 treatment also reversed the rapamycin-induced invasion of IEC-i-K-Ras cells. Rapamycin stimulated K-Ras-transformed IEC-i-K-Ras cells to form disorganized chords and invade Matrigel; a relatively noninvasive morphology was restored 48 h after the addition of PD-98059 (Fig. 5*C*). Thus, MEK/ERK activity appeared to be critical for morphological transformation and invasion of IEC-i-K-Ras cells.

PI3K and mTOR in Human Colon Cancer Cells. To determine the potential clinical significance of our observations, it was critical to examine the functional roles of PI3K and mTOR in human colon cancer cells. As demonstrated in Fig. 6*A*, MEK activity was critical to the growth of Lovo, SW-480, and Moser colon cancer cells. Lovo cells were very sensitive to the growth-inhibitory effects of LY-294002 and rapamycin. Both LY-294002 and rapamycin inhibited the growth of SW-480 cells; a 50% reduction of cell number was observed by day 7. Whereas PD-98059 strongly blocked Moser cell growth, LY-294002 and rapamycin modestly inhibited the growth of Moser cells. It was noted that prolonged inhibition of PI3K activity with LY-294002 resulted in a spindle appearance in Moser cells (Fig. 6*B*). The spindle morphology of LY-294002-treated Moser cells was completely reversed by 48 h after addition of 10 μM PD-98059, suggesting the critical role of MEK in LY-294002-induced spindle morphology of Moser cells. Epifluorescence microscopy of Moser cells showed a patchy distribution of E-cadherin; immunoreactivity of E-cadherin was reduced in LY-294002-treated Moser cells (Fig. 6*C*). Western analysis revealed that expression of E-cadherin was low in Moser cells; treatment with LY-294002 further decreased levels of E-cadherin expression.

To determine the roles of MEK, PI3K, and mTOR in transformed phenotype of colon cancer cells, Lovo, SW-480, and Moser cells were grown in Matrigel. Lovo cells formed noninvasive colonies in Matrigel. Addition of PD-98059, LY-294002, or rapamycin significantly reduced the size and number of cell colonies; morphological alteration was not noted (data not shown). SW-480 cells grew poorly in Matrigel; LY-294002 and rapamycin slightly inhibited their growth (data not shown). Moser cells formed invasive colonies in Matrigel (Fig. 6*D*). PD-98059 inhibited cell growth and reduced the invasion of Moser cells, whereas LY-294002 treatment resulted in fibroblast-like transformation and significantly increased the invasiveness of Moser cells.

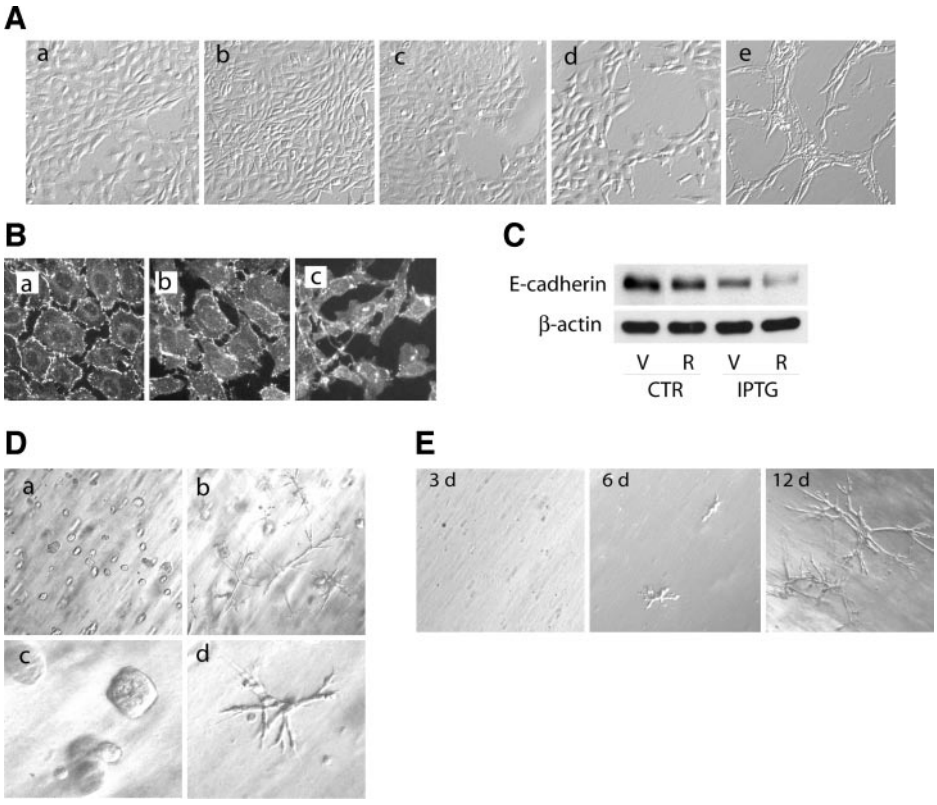


Fig. 4. Morphological transformation of IEC-i-K-Ras cells. *A*, rapamycin enhanced spindle morphology. IEC-i-K-Ras cells were treated with vehicle (*a*), 5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG; *b*), IPTG plus 50 μM PD-98059 (*c*), IPTG plus 20 μM LY-294002 (*d*), or IPTG plus 100 nM rapamycin (*e*) for 72 h and photographed (×200). *B*, immunofluorescence. IEC-i-K-Ras cells were treated with vehicle (*a*), 5 mM IPTG (*b*), or IPTG plus 100 nM rapamycin for 48 h (*c*). Fixed cells were stained with anti-E-cadherin antibody. After washing the cells with PBS, they were incubated with Cy3-conjugated donkey antimouse IgG for an additional hour. The dishes were washed with PBS, mounted, and observed under fluorescence microscopy with appropriate filters (×400). The results were similar in three independent experiments. *C*, rapamycin inhibition of E-cadherin. IEC-i-K-Ras cells were treated with vehicle (*V*) or 100 nM rapamycin (*R*) in the presence (*IPTG*) or absence (*CTR*) of 5 mM IPTG for 48 h; levels of E-cadherin were analyzed by immunoblot analysis. *D*, rapamycin and IEC-i-K-Ras cell invasion. Cells (10^4) were mixed in 0.1 ml of 1:1 diluted Matrigel. IPTG (5 mM) and DMSO (*a* and *c*) or 100 nM rapamycin (*b* and *d*) were added to the cover media. Cells were incubated for 6 days and photographed (*a* and *b*, ×40; *c* and *d*, ×200). *E*, phosphatidylinositol 3′-kinase and IEC-i-K-Ras cell invasion. Cells (10^4) were mixed in 0.1 ml of 1:1 diluted Matrigel; IPTG and 20 μM LY-294002 were added to the cover media. Cells were photographed at the indicated days (×100).

DISCUSSION

K-Ras-mediated transformation of IECs requires a number of downstream signaling pathways that, in combination, result in the transformed phenotype. Using a conditionally K-Ras-transformed IEC model, we delineated the functional contributions of individual Ras effectors to K-Ras-mediated transformation of IECs. The PI3K pathway predominantly controlled cell proliferation and was critical for cell cycle transition from the G_1 phase to the S phase. Furthermore, PI3K activity was crucial for IEC survival after reaching confluence. Blocking of PI3K activity significantly inhibited K-Ras-induced transformation of IECs. The roles of PI3K in established colon cancer cells varied. Growth of Lovo and other colon cancer cells (28) was greatly dependent on PI3K activity. In contrast, PI3K activity appeared to be dispensable for the growth of Moser cells. These results suggest that PI3K activation may be critical for intestinal epithelial transformation but may not be required for preservation of transformed phenotypes in a subpopulation of colon cancer cells.

We found that oncogenic K-Ras activated p70S6K in a PI3K- and mTOR-dependent manner, suggesting the involvement of activation of the translation of specific mRNA subpopulations. Interestingly, the 4E-BP/eIF-4E pathway appeared to be less involved in K-Ras-mediated transformation of IECs. Inhibition of either PI3K or mTOR suppressed IEC proliferation and led to growth arrest in the G_1 phase of the cell cycle. LY-294002 and rapamycin regulated the expression of cell cycle-related proteins (*e.g.*, cyclin D1, $p27^{kip1}$, and cyclin-dependent kinase 2) in a similar fashion. These results suggest that blocking the activation of mTOR may partially account for the mechanism by which inhibition of PI3K suppresses the proliferation and growth of K-Ras-transformed IECs.

Cumulative evidence suggests that rapamycin inhibits cell cycle progression from the G_1 to the S phase and may be a potent anticancer drug (23, 36). Rapamycin and its derivatives, CCL-779 and RAD-001, are being developed as anticancer drugs and are undergoing clinical trials (37). Our results show that rapamycin exerted a potent cytostatic effect on K-Ras-transformed IECs by blocking cell cycle progression from the G_1 to the S phase and, moreover, inhibited the growth of a number of human colon cancer cells. These results suggest that rapamycin may be a potent anticancer drug in colorectal cancers and may exert cytostatic effects on other tumor types that contain *Ras* mutations. Thus, further elucidating the molecular mechanisms by which rapamycin exerts its antitumor action has important clinical considerations.

An important observation from this study is that blocking of a specific pathway may disrupt the balance between signaling pathways and enhance other oncogenic signals. As a result, tumor cells acquire alterative phenotypes that may promote tumorigenicity. We found that inhibition of mTOR by rapamycin enhanced the spindle morphology and invasiveness of IEC-i-K-Ras cells. Rapamycin treatment increased the expression of Bcl-2, which may protect IECs from apoptosis and promote overlapping growth. The rapamycin-induced morphological alterations of IECs were associated with an increased MEK activity and were completely attenuated by the MEK inhibitor PD-

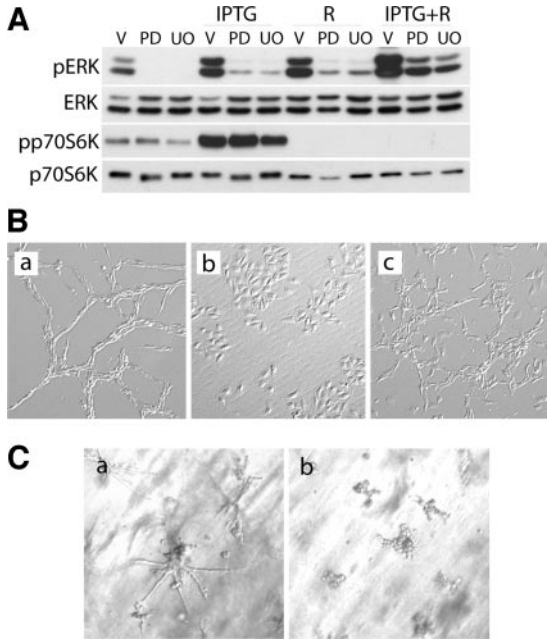


Fig. 5. Mitogen-activated protein/extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinase (ERK) and morphological transformation of IEC-i-K-Ras cells. *A*, rapamycin induction of phosphorylated ERK. IEC-i-K-Ras cells were subjected to the indicated treatment for 48 h. *V*, DMSO; *IPTG*, 5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG); *R*, 100 nM rapamycin; *PD*, 75 μM PD-98059; *UO*, 10 μM UO-126. Levels of phosphorylated ERK and phosphorylated p70S6K were analyzed using immunoblot analysis. This experiment was repeated three times. *B*, PD-98059 inhibition of spindle appearance. IEC-i-K-Ras cells were treated with IPTG in the presence of 100 nM rapamycin for 72 h (*a*). After IEC-i-K-Ras cells were treated with IPTG in the presence of 100 nM rapamycin for 72 h, 25 μM PD-98059 (*b*) or 10 μM LY-294002 (*c*) was added for 24 h (×100). *C*, PD-98059 reverses rapamycin-induced invasion. Cells (10^4) were mixed in 0.1 ml of 1:1 diluted Matrigel. IPTG (5 mM) and 100 nM rapamycin were added to cover media every 48 h. After 6 days of culture, vehicle (*a*) or 50 μM PD-98059 (*b*) was added. Cells were incubated for an additional 48 h and photographed (×100).

98059. Interestingly, while completely attenuating phosphorylation of ERK in nontransformed IEC-i-K-Ras cells, PD-98059 did not completely block rapamycin-induced pERK, suggesting that rapamycin induction of pERK may involve MEK1/2-dependent and independent mechanisms, and the MEK1/2-dependent pERK appeared to be critical for rapamycin-induced spindle morphology. Our results also suggest that a cross-talk between mTOR and the Raf/MEK/ERK pathway in K-Ras induced IECs. Activated Akt can inhibit the Raf/MEK/ERK pathway through phosphorylation of a highly conserved serine residue (Ser^{259}; Ref. 38) or by forming an inhibitory Akt-Raf complex (39). Additional experiments are required to elucidate the mechanism by which mTOR regulates the Raf/MEK/ERK pathway.

Induction of oncogenic K-Ras resulted in epithelial-mesenchymal transdifferentiation (EMT), which is characterized by the acquisition of spindle shape, delocalization of E-cadherin from cell junctions, and increased motility (40). The EMT of K-Ras-transformed IECs was dependent on induction of MEK/ERK activity. Our results show that inhibition of mTOR strongly increased MEK/ERK activity and resulted in a more fibroblastic appearance. Junctional localization of E-cadherin was not apparent, suggesting loss of cell-cell contact. Cell invasion was increased by the presence of rapamycin. These observations provide evidence that rapamycin treatment enhanced K-Ras-mediated EMT in IECs. Furthermore, treatment with LY-294002 resulted in spindle morphology, reduced expression of E-cadherin, and increased invasiveness in Moser cells, suggesting that inhibition of PI3K may enhance EMT in certain colon cancer cells. Previous studies have shown that transforming growth factor β (TGF-β) negatively regulates the growth of an array of epithelial cells; however, in certain circumstances, TGF-β mediates EMT (41–45). It was demonstrated that TGF-β-mediated EMT in pancreatic cancer cells requires ERK2 activation (43). Ha-Ras cooperates with TGF-β to cause EMT in mammary epithelial cells; a hyperactivation of Raf/MEK/ERK is required for this EMT (45). Furthermore, in the context of TGF-β-induced EMT, a subset of target genes that are activated strongly and specifically through the ERK/mitogen-activated protein kinase pathway promotes cell motility and modulate cell-matrix adhesion (44). In agreement with our results, these findings emphasize the critical role of the MEK/ERK pathway in EMT of epithelial cells.

A recent study (46) has shown that rapamycin, at a relatively low concentration (1.5 mg/kg/day), promotes regression in primary and metastatic CT-26 colon cancers in mice. Inhibition of tumor angiogenesis through a decrease in the production of vascular endothelial growth factor was identified as the underlying mechanism. A higher concentration of rapamycin (15 mg/kg/day) caused a more pronounced delay in tumor growth in the first 3 weeks, but after this time point, the tumors began to grow rapidly, and the mice died shortly thereafter. Apparently, more complete inhibition of mTOR resulted in increased rapamycin resistance and acquisition of a more aggressive phenotype in CT-26 tumors. Our study shows that inhibition of mTOR

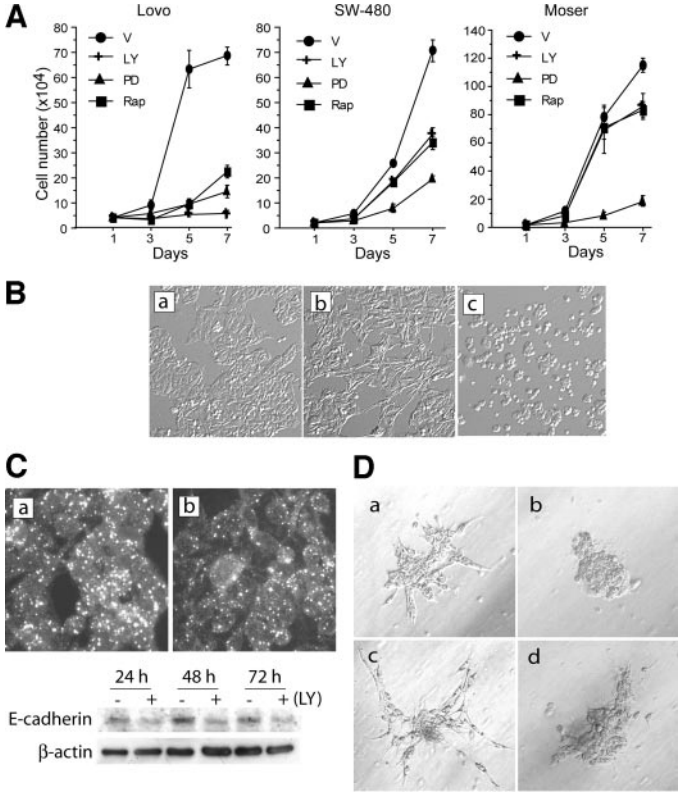


Fig. 6. Phosphatidylinositol 3′-kinase and mammalian target of rapamycin in human colon cancer cells. *A*, growth inhibition of LY-294002 and rapamycin in colon cancer cells. Cells (4×10^4 Lovo cells; 2×10^4 SW-480 and Moser cells) were grown in McCoy's 5A medium with 10% fetal bovine serum in 12-well plates and treated as indicated (*V*, DMSO; *LY*, 20 μM LY-294002; *PD*, 50 μM PD-98059; *R*, 100 nM rapamycin). Cell numbers were counted at the indicated days, and values are means ± SD from triplicate wells. All growth data are representative of two separate experiments. *B*, LY-294002 and the morphology of Moser cells. Moser cells were grown in McCoy's 5A medium with 10% fetal bovine serum and treated with vehicle (*a*) or 20 μM LY-294002 (*b*) for 10 days and photographed (×100). The LY-294002-treated cells were then subjected to a treatment with LY-294002 plus 10 μM PD-98059 for an additional 48 h (*c*; ×100). *C*, expression of E-cadherin in Moser cells. *Top panels*, Moser cells were treated with vehicle (*a*) or 20 μM LY-294002 for 10 days (*b*) and placed in 35-mm plates. Fixed cells were stained with anti-E-cadherin antibody (×400). *Bottom panels*, Moser cells were grown in the presence or absence of 20 μM LY-294002 for the indicated times; levels of E-cadherin were analyzed by immunoblot analysis. *D*, growth of Moser cells in Matrigel. Moser cells (10^3) were mixed in 0.1 ml of 1:1 diluted Matrigel. DMSO (*a*), 50 μM PD-98059 (*b*), 20 μM LY-294002 (*c*), or 100 nM rapamycin (*d*) was added to cover media. Cells were incubated for 6 days and photographed (×200).

may increase the activity of other oncogenic signaling pathways (*i.e.*, MEK/ERK and PI3K) and enhance the EMT in tumor cells that is linked with invasion and metastasis of epithelial carcinomas.

In summary, inhibition of either PI3K or mTOR results in cytostatic effects on K-Ras-transformed IECs; therefore, inhibitors for PI3K or mTOR may be potent anticancer drugs for colorectal carcinomas. However, blocking these signaling pathways may emphasize other oncogenic signals and result in pro-oncogenic phenotypes. Thus, determination of appropriate dosages and the design of combined treatments may significantly improve the results of therapies that target specific signaling pathways.

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