K-Ras Promotes Growth Transformation and Invasion of Immortalized Human Pancreatic Cells by Raf and Phosphatidylinositol 3-Kinase Signaling

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Abstract

Mutational activation of the K-Ras oncogene is well established as a key genetic step in the development and growth of pancreatic adenocarcinomas. However, the mechanism by which aberrant Ras signaling promotes uncontrolled pancreatic tumor cell growth remains to be fully elucidated. The recent use of primary human cells to study Ras-mediated oncogenesis provides important model cell systems to dissect this mechanism. We have used a model of telomerase-immortalized human pancreatic duct-derived cells (E6/E7/st) to study mechanisms of Ras growth transformation. First, we found that human papillomavirus E6 and E7 oncogenes, which block the function of the p53 and Rb tumor suppressors, respectively, and SV40 small t antigen were required to allow mutant K-Ras(12D) growth transformation. Second, K-Ras(12D) caused growth transformation *in vitro*, including enhanced growth rate and loss of density dependency for growth, anchorage independence, and invasion through reconstituted basement membrane proteins, and tumorigenic transformation *in vivo*. Third, we determined that the Raf, phosphatidylinositol 3-kinase (PI3K), and Ral guanine nucleotide exchange factor effector pathways were activated, although extracellular signal-regulated kinase (ERK) activity was not up-regulated persistently. Finally, pharmacologic inhibition of Raf/mitogen-activated protein kinase/ERK and PI3K signaling impaired K-Ras–induced anchorage-independent growth and invasion. In summary, our studies established, characterized, and validated E6/E7/st cells for the study of Ras-induced oncogenesis. [Cancer Res 2007;67(5):2098–106]

Introduction

The majority of pancreatic cancers arise from cells of ductal origin, and one of the earliest genetic events in the progression of these normal ductal epithelia to premalignant pancreatic intraepithelial neoplasia is mutation of the K-Ras oncogene (1). Mutational activation of Ras proteins is seen with high frequency (90%) in pancreatic ductal adenocarcinomas (2). Hence, there has been considerable interest in the development of Ras inhibitors for pancreatic cancer treatment (3). However, despite our knowledge of Ras regulation and signaling (4), exploiting that information for defining effective approaches for blocking aberrant Ras function has not been accomplished. This task has been complicated by the realization that the mechanisms of Ras signaling important for oncogenesis exhibit significant cell context differences and therefore may be variable for different cancer types (5). Thus, there remains a strong need for delineating the mechanisms by which mutated Ras promotes and maintains pancreatic cancer growth.

K-Ras functions as a binary switch that cycles between an active GTP-bound and inactive GDP-bound state, with active Ras binding and activating a functionally diverse spectrum of downstream effector proteins (4). K-Ras is mutated in pancreatic cancers, leading to persistent effector activation. The best-characterized effector pathway in Ras function is the Raf/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) cascade. However, B-Raf mutations are not commonly seen in pancreatic cancers (6), and surprisingly, up-regulated ERK activation was not seen in most pancreatic cancer cell lines or patient tumors (7–9). Therefore, it is likely that other effector pathways may also serve critical roles in Ras-mediated promotion of pancreatic cancer growth.

Currently, four additional Ras effector families have been implicated in Ras-mediated oncogenesis (4). Of these, the phosphatidylinositol 3-kinases (PI3K), which activate the Akt serine/threonine kinases, and the Ral small GTPase guanine nucleotide exchange factors (RalGEF) have received the most attention (4). Two other effector pathways (Tiam1 and phospholipase Cε) have also been implicated in Ras-mediated oncogenesis (4), but their roles in pancreatic cancer growth have not been determined. Finally, additional effectors are likely to contribute to Ras-mediated oncogenesis. Therefore, to facilitate ongoing efforts to target Ras signaling for pancreatic cancer treatment, a more definitive determination of the role of specific effector pathways in pancreatic cancer growth is needed.

Recent advances have established new experimental systems to study pancreatic cancer and include the development of K-Ras–driven mouse models for pancreatic cancer (10–13). Although such models will surely provide powerful approaches for the dissection of Ras-mediated oncogenesis, whether mouse models accurately recapitulate human cancers remains to be fully validated (14). Therefore, cell culture models remain an important complement to mouse models. The advent of RNA interference (RNAi) has allowed the further development of human tumor cell culture models to study the mechanism of oncogenic Ras, and silencing of mutant K-Ras function in pancreatic carcinoma cell lines has provided

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further validation for the importance of continuous Ras function for tumor cell maintenance (15, 16). However, an obvious limitation of this approach is that established pancreatic carcinoma cell lines harbor many unknown genetic lesions, including some that may have been acquired during their long passage history in cell culture.

Another important breakthrough in the investigation of cancer progression uses human primary cells to study the role of Ras in malignant transformation (17). Weinberg et al. and others used ectopic expression of the catalytic subunit of telomerase (hTERT) and viral antigen inactivation of p53 and Rb tumor suppression function to create unique *in vitro* systems for the study of cancer (18–20). These genetically defined immortalized human cells are sensitive to growth transformation by ectopic expression of oncogenic forms of Ras. The well-defined nature of these cell systems allows for the dissection of mechanisms of oncogenesis caused by specific and discrete genetic events.

To date, several pancreatic cell systems have been described to study Ras oncogenesis (21–26). Although Lohr et al. (22) generated immortalized cells expressing SV40 large T antigen and mutant K-Ras that formed contact-independent colonies *in vitro* and orthotopic tumors in mice, these were of bovine and not human origin. Tsao et al. (26) investigated human pancreatic ductal epithelial (HPDE) cells immortalized with human papillomavirus (HPV) 16 E6 and E7 oncogenes to inactivate p53 and Rb, respectively, and further transformed these cells with mutant K-Ras. The K-Ras–transformed HPDE cells formed tumors in nude mice but, interestingly, showed no indication of *in vitro* transformation as indicated by proliferation or contact-independent growth. Hence, they concluded that additional genetic alterations were required for full malignant growth transformation of HPDE cells. Therefore, the need for well-defined genetic human cell culture models for the study of Ras-mediated pancreatic cancer development remains.

The goal of our studies was to develop a novel human cell system to study the mechanisms of K-Ras–mediated pancreatic cancer development. Here, we report on the biological and signaling consequences of K-Ras activation on an immortalized cell line derived from pancreatic duct epithelia (E6/E7/st). Previous reports indicate that pancreatic adenocarcinoma may in some cases arise from acinar-to-ductal metaplasia (27–29) via a precursor intermediary. Our immortalized duct-derived cell line seems to represent a population of this precursor (30). Similar to other human cell types, ectopic hTERT expression and suppression of p53 and Rb function were not sufficient to render these human pancreatic cells sensitive to transformation by oncogenic K-Ras. Instead, the additional introduction of SV40 small t (st) antigen was required for Ras-mediated growth transformation. Ectopic expression of mutant K-Ras at physiologic levels promoted anchorage-independent growth and invasion *in vitro* and tumorigenic growth *in vivo*. Sustained activation of MEK (but not ERK), Akt, and RalA was associated with Ras transformation, and inhibitors of both the Raf-MEK-ERK and PI3K effector pathways blocked the growth of Ras-transformed E6/E7/st cells. These results establish E6/E7/st cells as an important model cell system to delineate the signaling mechanisms for Ras-mediated oncogenesis and growth of pancreatic ductal epithelial cells.

Materials and Methods

Cell line generation and characterization. Pancreatic duct-derived cells were immortalized as described previously (23). Briefly, primary cell cultures isolated from pancreatic ducts were sequentially infected with retroviral vectors to express human telomerase (hTERT) and the E6 and E7 proteins of HPV16 (Fig. 1). From this precursor cell line, a matched pair was generated with or without expression of the constitutively activated K-Ras (G12D) mutant (designated E6/E7 and E6/E7/Ras, respectively). Finally, these two cell lines were infected with retrovirus to express viral SV40 st antigen (designated E6/E7/st and E6/E7/Ras/st, respectively). The resulting mass populations were maintained at 5% CO_2 in M3:5 growth medium [4 parts high-glucose DMEM (Life Technologies, Carlsbad, CA) to 1 part M3F (INCELL, San Antonio, TX) supplemented with 5% FCS].

To verify expression of ectopically introduced cDNA sequences, total RNA was extracted from cells using standard Trizol (Invitrogen, Carlsbad, CA) protocols and reverse transcription-PCR (RT-PCR) was done using the following sequences: K-Ras, 5′-CTTGCTGAATTCCTGCTGAAAATGACTGAATATA-3′ and 5′-GCTACTCGAGGTATGCCTTAAGAAAAAAGTACAA-3′; β-actin, 5′-CGGGACCTGACTGACTACCT-3′ and 5′-CAGCACTGTGTTGGCGTACA-3′; E6, 5′-GAACAGCAATACAACAAACCG-3′ and 5′-GCAACAAGACATACATCGACC-3′; E7, 5′-AGGAGGAGGATGAAATAGATGG-3′ and 5′-TGGTTTCTGAGAACAGATGGG-3′; and SV40 st, 5′-GAAGCAGTAGCAATCAACCC-3′ and 5′-GCTTCTTCCTTAAATCCTGGTG-3′. PCR products of K-Ras cDNA were digested with *Bcc*I to determine samples expressing wild-type and mutant K-Ras. Visualization of K-Ras protein expression and activation was done by pull-down assay using the glutathione *S*-transferase (GST)-bound Raf-Ras-binding domain as described previously (31), with expression of β-actin as a loading control (AC-15; Sigma-Aldrich, St. Louis, MO).

Growth transformation assays. For analysis of anchorage-independent growth, log-phase growing cells were trypsinized, and triplicates of 3×10^3 cells per well were suspended in enriched medium (supplemented with 10% FCS) supplemented with 1.5% agar and plated onto the agar-coated six-well plates. Standard medium (1 mL) was added to the top of the gelled matrix, and colonies were grown for 21 days. Stock solution of inhibitors was dissolved in DMSO (vehicle) and added to both the agar containing the cells and the feeding medium at the following final concentrations: MCP110/MCP122 (32), BAY 43-9006 (33), and LY294002 (Promega, Madison, WI) at 10 μmol/L or U0126 (Promega) at 30 μmol/L. After 21 days in culture, colonies were counted in five random three-dimensional fields per well and photographed.

For tumorigenicity analysis, cells were injected into the flanks of Hsd:Athymic Nude-*Foxn1*^{nu} nude mice (Harlan, Indianapolis, IN) at seeding densities of 0.2, 0.5, 1, or 2×10^6 cells per site. Tumor measurements were taken by calipers thrice weekly over 8 weeks or until tumor burden reached 1 cm^3. Tumor volumes were calculated by estimation of an ellipsoid using $(4/3)\pi(x/2)(y/2)(z/2)$, where x is length, y is width, and z is depth of the tumor.

Signaling protein expression and activation. Cells growing in log phase were incubated for 24 h in starvation medium (normal medium lacking serum but with 1 g/mL bovine serum albumin and 10 mmol/L HEPES) before adding inhibitors for an additional 24 h. Western blot analyses were done using primary antibodies against ERK1 and ERK2 (Cell Signaling), phosphorylated ERK1/2 (Cell Signaling, Danvers, MA), phosphorylated MEK1 and MEK2 (Cell Signaling), MAPK phosphatase-2 (MKP-2; Santa Cruz Biotechnology, Santa Cruz, CA), Akt (Cell Signaling), and phosphorylated Akt (Cell Signaling). Pull-down assays to detect formation of active GTP-bound RalA were done as described previously (34).

Migration assays. Cells grown to 90% confluence were starved as above for 24 h. At $t = 0$, cells were treated with inhibitors of the Raf-MEK-ERK or PI3K-Akt pathways as described above, and a wound was created by scratching the monolayer of cells with a pipette tip (35). At 12 h, cells were fixed and stained with Diff-Quik (Dade Behring, Newark, DE) and micrographs were taken with a 40× objective.

For transwell migration assays, pancreatic-derived cells were starved and treated with inhibitors as above for 24 h. Cells were suspended from adherent cultures with 1 mL TrypLE trypsin-free dissociation solution (Invitrogen) and counted. Twenty thousand cells were loaded into 500 μL of starvation medium and inhibitors in the top well of BioCoat chambers that contain growth factor reduced Matrigel extracellular basement membrane over a polyethylene terephthalate membrane with 8 μm pores (BD Biosciences, Bedford, MA). The bottom chamber contained the same

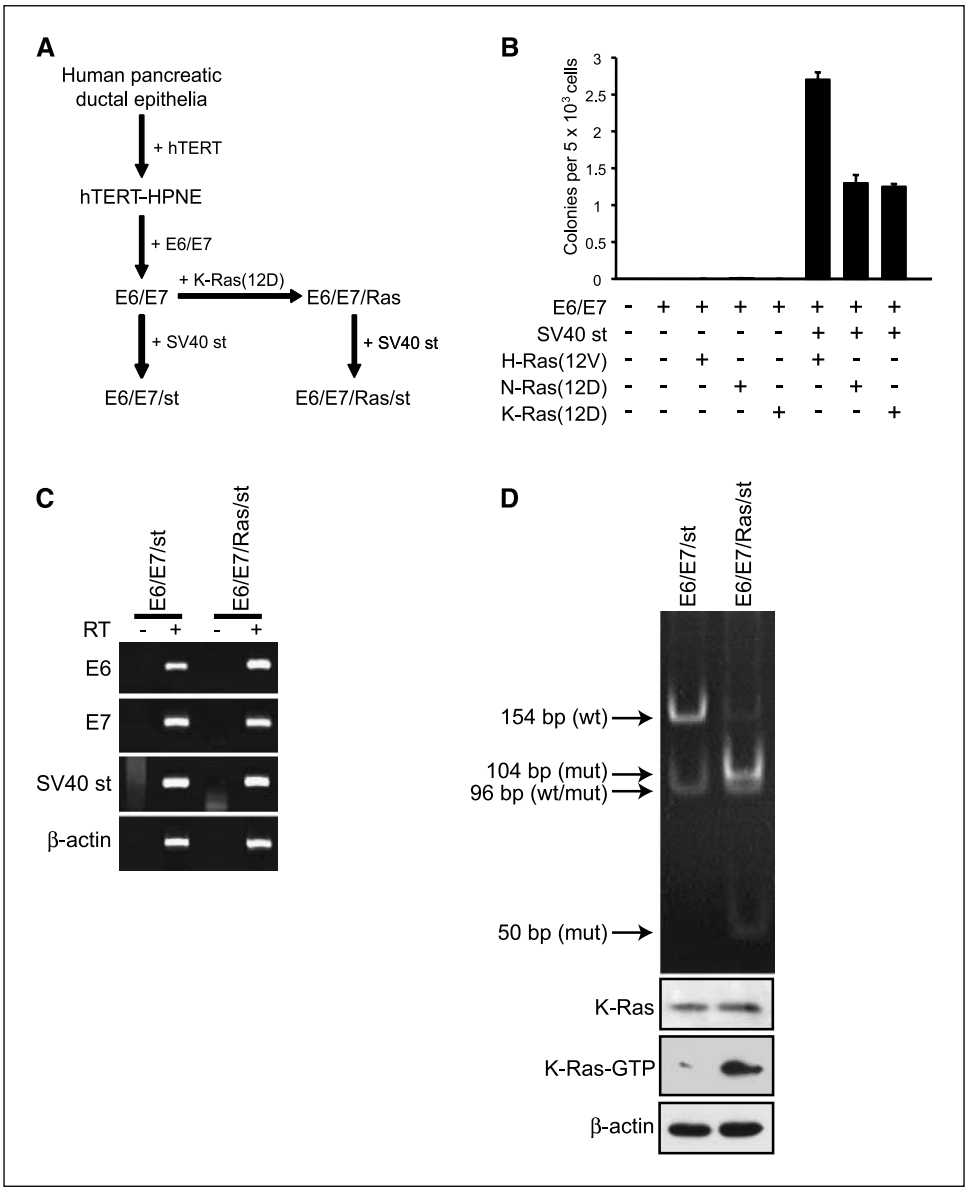

Figure 1. Expression of activated K-Ras in immortalized human pancreatic duct-derived cells. *A,* scheme for immortalization and Ras transformation of human pancreatic duct cells. As described previously (23), ectopic expression of hTERT was used to immortalize a primary pancreatic duct cell culture to generate hTERT-HPNE cells. These cells were then sequentially infected with retroviral constructs to express first the HPV proteins E6 and E7 (*E6/E7*) and then constitutively active K-Ras(12D). The resultant stably selected E6/E7 and E6/E7/Ras cells were then infected with retrovirus encoding SV40 st to establish E6/E7/st and E6/E7/Ras/st cells, respectively. *B,* requirement for SV40 st expression for K-Ras–induced growth in soft agar. E6/E7 cells were established that stably expressed activated mutants of three human Ras isoforms (E6/E7/Ras). E6/E7/Ras cells were then used to establish cells stably expressing SV40 st. The resulting mass populations of cells were then suspended in soft agar to monitor anchorage-independent growth. *C,* expression of stably introduced genes in E6/E7/st and E6/E7/Ras/st cells. RNA transcripts from cell populations were reverse transcribed (*+/– RT*), and PCR was done with specific primers to confirm expression of the indicated genes; β-actin was used as a control for equivalent mRNA. *D,* verification of the presence of the mutant *K-Ras* gene and expression in E6/E7/Ras/st cells. Genomic K-Ras primers were used to amplify a DNA fragment, which was then digested with *Bcc*I to distinguish wild-type K-Ras (*wt*) from mutant K-Ras(12D) (*mut*). Pull-down analyses and Western blot analyses with K-Ras–specific antiserum were done to determine the level of activated and total K-Ras protein expression. Data are representative of one to three independent experiments.

medium and inhibitor concentration. Cells were allowed to invade for 24 h before the Matrigel was removed, and invaded cells were fixed and stained with Diff-Quik. Cells adhering to the bottom surface of the membrane were counted under microscopy.

Results

Human pancreatic duct-derived cell immortalization. From previously established immortalized human pancreatic duct-derived cells (23), we then stably introduced constitutively active K-Ras(12D) into the E6/E7 cells (designated E6/E7/Ras) to create a matched pair of cells (Fig. 1*A*). However, our analyses of the E6/E7/Ras cohort found that these cells retained anchorage dependence for growth and did not form colonies when suspended in soft agar (Fig. 1*B*). Therefore, because studies with other human cell types identified the requirement for SV40 st antigen function to facilitate Ras transformation (19, 36, 37), we also established E6/E7 and E6/E7/Ras cells stably expressing SV40 st, designated E6/E7/st and E6/E7/Ras/st, respectively. We first did RT-PCR analyses to verify the expression of each of the introduced genes in the matched cells (Fig. 1*C*). To distinguish wild-type K-Ras (G12; wt) from mutant K-Ras (G12D; mut), we PCR amplified DNA from the pair of cells and then digested the K-Ras fragments with *Bcc*I. Although both cell lines express wild-type endogenous K-Ras (Fig. 1*D*), only the E6/E7/Ras/st cells expressed the mutant K-Ras(12D) transcript. Western blot analyses showed that wild-type and mutant K-Ras cells expressed similar levels of total K-Ras protein (Fig. 1*D*). However, pull-down analyses illustrated that constitutive K-Ras-GTP activation was seen only in E6/E7/Ras/st cells. This result contrasts with other human model cell systems described, where the ectopically introduced *Ras* gene resulted in significant protein expression at levels higher than is typically seen in human tumor cells. Thus, the matched pair of E6/E7/st and E6/E7/Ras/st cells provided us with a well-defined genetic model system to assess the biochemical and biological repercussions of mutant K-Ras when expressed at physiologic levels and without the potential confounding influences of other unknown genetic changes inherent in tumor cell lines.

Expression of oncogenic K-Ras leads to aberrant growth but not morphology. We next evaluated the growth properties of E6/E7/st and E6/E7/Ras/st cells to determine the consequences of Ras activation. Initially, we seeded the E6/E7/st and E6/E7/Ras/st cells onto plastic to observe differences in morphology and growth characteristics. At subconfluent densities, whereas cells expressing mutant K-Ras(12D) showed a similar morphology to their wild-type matched pair (Fig. 2*A*), oncogenic K-Ras(12D) expression caused a loss of density-dependent inhibition of growth at confluent densities (Fig. 2*A*, superconfluence). Whereas E6/E7/st cells formed a confluent cobblestone monolayer reminiscent of other epithelial cells, E6/E7/Ras/st cells continued to divide, forming multilayered cultures. Consistent with this observation, we observed that E6/E7/Ras/st cells showed both increased rate of growth and higher saturation densities when compared to E6/E7/st cells (Fig. 2*B*). Our results differ with a recent study that found that activated K-Ras did not alter the anchorage-dependent growth properties of immortalized pancreatic ductal epithelial cells (26).

In addition to differences in adherent cell proliferation, we also investigated the role of oncogenic Ras expression on anchorage-independent growth. Whereas E6/E7/st cells did not form colonies in soft agar, E6/E7/Ras/st cells showed spherical colony formation within 1 week of seeding and robust proliferating colonies that were visible to the naked eye within 2 weeks (Fig. 2*C*). Similarly, E6/E7 cells expressing activated H-Ras(12V) or N-Ras(12D), together with SV40 st, also formed colonies in soft agar, indicating that all Ras isoforms could cause growth transformation (Fig. 1*B*). Our observations are in contrast with another study that found that activated K-Ras did not promote the anchorage-independent growth of immortalized pancreatic ductal epithelial cells (26).

Finally, we determined if mutant K-Ras expression could alter the tumorigenicity of E6/E7/st cells when introduced s.c. into nude mice. Whereas E6/E7/st cells did not exhibit any tumor growth when monitored for up to 54 days, E6/E7/Ras/st cells resulted in palpable tumors within 1 week, and progressive tumor growth was seen in all of the injected mice (Fig. 2*D*). Taken together with our *in vitro* analyses, we conclude that constitutively active K-Ras causes multiple changes in growth characteristics similar to those attributed to mutant K-Ras in human pancreatic carcinoma cell lines.

Oncogenic K-Ras(12D) activates multiple downstream effector pathways. Although the Raf-MEK-ERK cascade is the best-characterized effector pathway involved in Ras transformation (4), previous studies found that ERK activity is not consistently elevated in pancreatic carcinoma cell lines and that other effector pathways may be important for Ras transformation (38). Therefore, we next investigated whether three key effector pathways important for Ras transformation are activated in E6/E7/Ras/st cells.

We first determined if the Raf-MEK-ERK pathway is persistently activated in E6/E7/Ras/st cells. Surprisingly, E6/E7/Ras/st and E6/E7/st cells showed comparable low levels of phosphorylated, activated ERK1/2. This contrasts with observations made in the majority of cell models of Ras transformation, where ectopic expression of mutant Ras causes persistent ERK activation (39, 40). However, these data are similar to those reported for pancreatic carcinoma cell lines, where elevated MEK, but not ERK, was seen (9). We also found a significant increase in the phosphorylated forms of MEK1 and MEK2. It has been reported previously that a lack of ERK activation seen in pancreatic adenocarcinoma cell lines was due to a concomitant increase in MEK-dependent MKP-2 activity (38). We too observed a modest increase in MKP-2 expression in E6/E7/Ras/st cells, which may contribute to the lack of ERK activation seen in these cells (Fig. 3).

We next determined the activation of two other Ras effector pathways. Compared with E6/E7/st cells, the mutant Ras-expressing counterparts showed elevated phosphorylated Akt levels (Fig. 3). We used a pull-down assay with a GST fusion protein

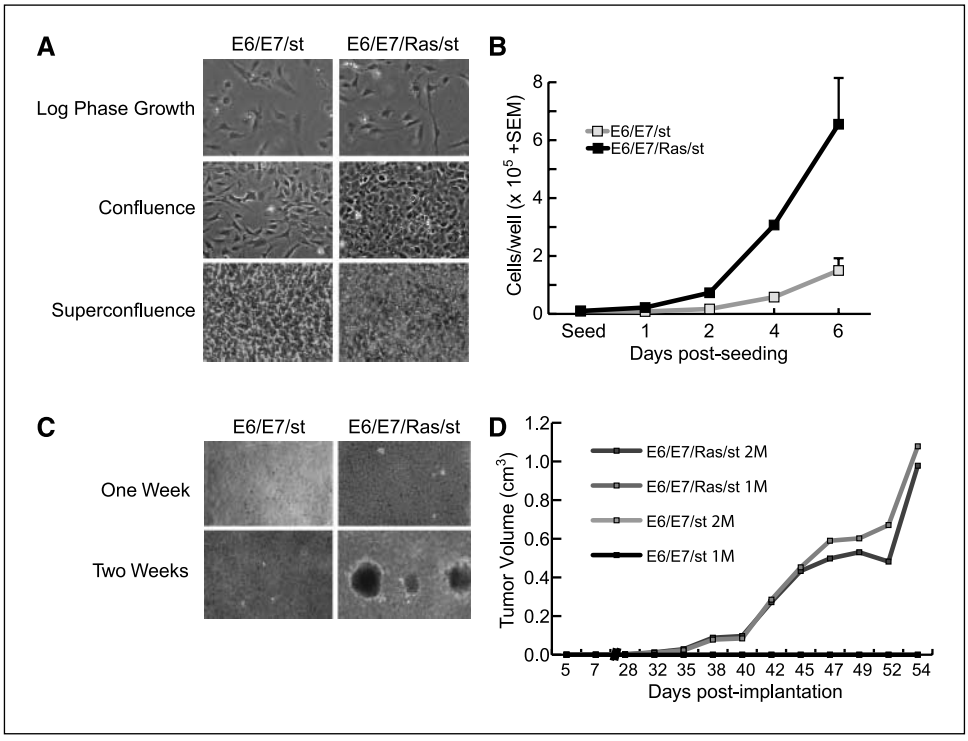

Figure 2. Expression of mutant K-Ras(12D) promotes growth transformation of E6/E7/st cells. *A,* mutant K-Ras causes loss of density-dependent inhibition of growth. Cell and culture morphology of E6/E7/st and E6/E7/Ras/st cells were monitored at the indicated cell densities. *B,* K-Ras accelerates E6/E7/st anchorage-dependent growth. Cells were seeded onto plastic to evaluate the rate of proliferation and saturation density. *Points,* mean of triplicate counts; *bars,* SE. *C,* K-Ras promotes anchorage-independent growth. Cells were suspended in soft agar to evaluate anchorage-independent growth potential over 21 d. *D,* K-Ras causes tumorigenic transformation of E6/E7/st cells. E6/E7/Ras/st cells [1×10^6 (*1M*) or 2×10^6 (*2M*)] were implanted s.c. into nude mice (n = 3–4 per group) and monitored for tumor formation for up to 54 d.

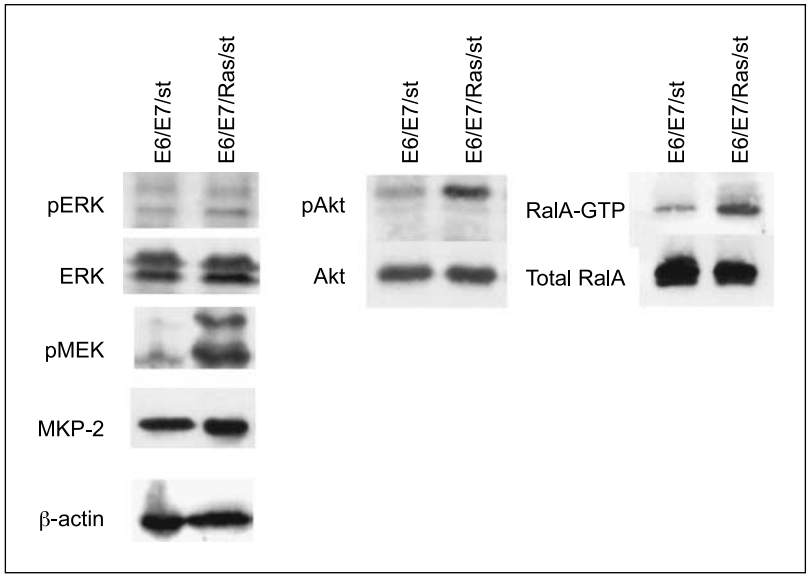

Figure 3. Ras activation of effector signaling pathways in E6/E7/st cells. Serum-starved whole-cell lysates were separated by SDS-PAGE, and Western blot analyses for the phosphorylated and activated forms of ERK1 and ERK2, MEK1 and MEK2, and Akt were done. Pull-down analysis was done to determine the level of activated RalA-GTP. Blot analyses were also done to determine total MEK1/2, ERK1/2, Akt, RalA, and MKP-2 protein expression, with blot analyses for β-actin to verify equivalent total protein for each cell line. Data are representative of one to three independent experiments.

carrying the Ral-GTP-binding domain of the Rlf RalGEF to quantify the levels of activated RalA-GTP and found that E6/E7/Ras/st cells showed greater activation of RalA when compared with E6/E7/st cells (Fig. 3). Thus, at least two additional Ras effector pathways known to promote Ras transformation are persistently activated in E6/E7/Ras/st cells.

Raf-MEK and PI3K activation promotes Ras growth transformation of E6/E7/st cells. We next used pharmacologic approaches to determine the contribution of specific effector pathways to Ras transformation of E6/E7/st cells. First, we used inhibitors that target different components of the Raf-MEK-ERK pathway. MCP110 is an inhibitor of Ras interaction with Raf and has been shown to block Ras transformation of NIH 3T3 cells (32). The compound MCP122 is a weak Ras-Raf interaction inhibitor and served as a negative control for MCP110. BAY 43-9006 (sorafenib) was developed originally as an inhibitor of Raf kinases, although it also has inhibitory activity for other protein kinases involved in tumor angiogenesis (33). U0126 is a highly specific inhibitor of MEK1- and MEK2-dependent activation of ERK. Second, we also used LY294002 to block PI3K activity.

E6/E7/Ras/st cells were seeded in soft agar with growth medium supplemented with vehicle (DMSO) or each inhibitor. Anchorage-independent colony formation was inhibited significantly by treatment with MCP110 but not by MCP122 (Fig. 4*A* and *B*). A similar level of reduction was seen with U0126 treatment, whereas complete inhibition of colony formation was seen in BAY 43-9006–treated cells. Thus, although the steady-state level of ERK activity was not elevated in E6/E7/Ras/st cells, these results indicate that the Raf-MEK pathway contributes to Ras-mediated anchorage-independent growth. Finally, LY294002 treatment also significantly reduced soft agar colony formation, indicating that the PI3K-Akt pathway also contributes to Ras-mediated anchorage-independent growth.

Oncogenic K-Ras stimulates migration, which is blunted by Raf pathway and PI3K inhibitors. Because Ras activation has been shown to promote altered cell motility and increased invasion in other cell systems, we wanted to use our model system to investigate whether oncogenic K-Ras causes an increase in pancreatic duct-derived cell motility. To assess migration, we did a scratch wound-healing assay for E6/E7/st and E6/E7/Ras/st cells, where a wound was created on 70% to 90% confluent monolayers of serum-starved cells to stimulate migration. We then treated the cells with the same inhibitors used in the contact-independent growth assay. We lysed parallel plates of cells to observe signaling protein activation for the Raf-MEK-ERK and PI3K-Akt pathways by Western blotting. For both E6/E7/st and E6/E7/Ras/st cells, migration from the border of the wound into the void occurred rapidly over 12 h. Whereas the negative control MCP122-treated cells showed similar rates of migration compared with DMSO-treated cells, cells treated with BAY 43-9006, MCP110, U0126, and LY294002 were slowed in their ability to migrate (Fig. 5*A* and *B*). All inhibitor treatments decreased motility of both cell types by 45% to 70%. That almost complete phosphorylated ERK inhibition by U0126 did not change motility significantly from BAY 43-9006–treated E6/E7/Ras/st cells (Fig. 5*C*) indicates that, although the increased activity of the Raf-MEK cascade promotes the migration of these cells, ERK phosphorylation is not required.

Oncogenic K-Ras(12D) induces an invasive phenotype. We next investigated whether constitutively active K-Ras stimulated the invasive properties of the E6/E7/st cells. For these analyses, we compared the ability of E6/E7/st and E6/E7/Ras/st cells to invade through Matrigel. In the absence of growth factor stimulation, E6/E7/st cells did not invade through Matrigel, whereas E6/E7/Ras/st cells migrated readily (Fig. 6*A* and *B*). Treatment with inhibitors of the Raf-MEK-ERK pathway as well as PI3K all decreased the invasive ability of E6/E7/Ras/st cells, with BAY 43-9006 causing a complete block. Thus, the activities of both the Raf and the PI3K effectors are involved in Ras promotion of E6/E7/st invasion *in vitro*.

Discussion

The frequent mutational activation of K-Ras in pancreatic cancers, when coupled with experimental mouse and cell culture studies that show a critical role for Ras in pancreatic tumor maintenance, provides strong validation for Ras as an important target for novel therapies for pancreatic cancer (1). Complicating the development of such therapies is the lack of a clear delineation

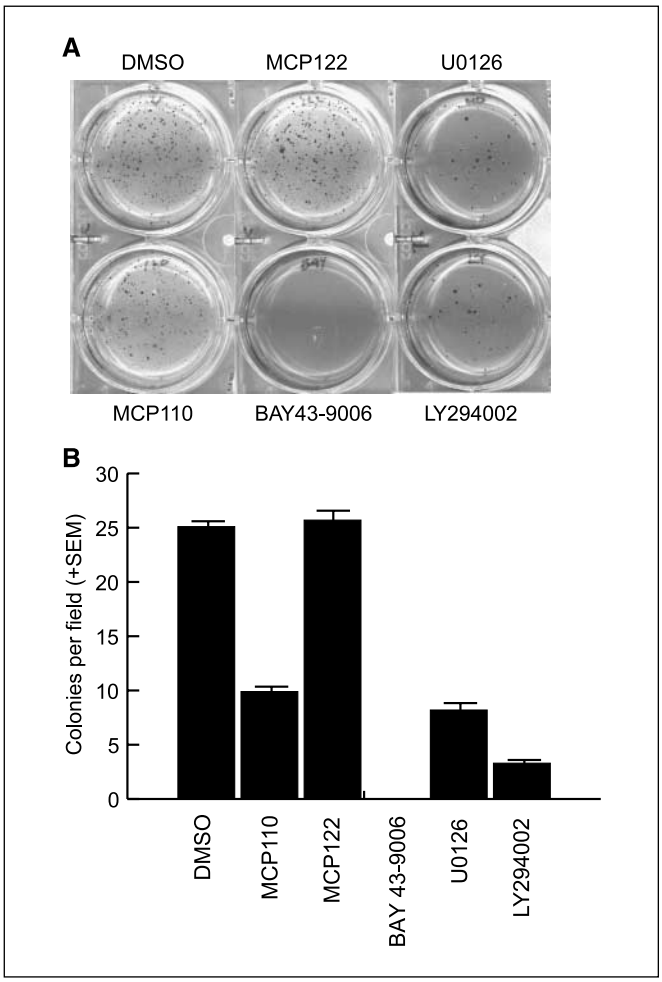

Figure 4. The Raf-MEK-ERK and PI3K-Akt pathways are required for Ras transformation of E6/E7/st cells. E6/E7/Ras/st cells were seeded into soft agar supplemented with complete growth medium containing the indicated inhibitors. *A,* cultures were monitored for up to 21 d and stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. *B,* quantification of colony formation. *Columns,* average of five random fields per dish from triplicate dishes of two independent experiments; *bars,* SE.

of the effector signaling mechanisms by which Ras drives the transformation of pancreatic cancer cells. In addition to the Raf-MEK-ERK cascade, at least four other effector pathways have been implicated in the oncogenic functions of Ras (4). Furthermore, it is evident that cell context differences are seen with mechanisms of Ras-mediated oncogenesis (5) and that observations made with the classic rodent fibroblast models may not accurately predict what Ras does in human epithelial tumor cells. In this study, we have established and characterized a model cell system to study Ras-mediated oncogenesis in pancreatic ductal cells. We showed that ectopic expression of hTERT can immortalize pancreatic duct-derived cells and although disruption of p53 and Rb tumor suppressor function did not result in transformation of these immortal cells, additional expression of the SV40 st antigen was required for rendering primary pancreatic cells sensitive to Ras-mediated transformation. We also showed that despite a lack of elevated ERK activation in Ras-transformed E6/E7/st cells, various inhibitors of the Raf-MEK-ERK MAPK cascade are nevertheless effective in blocking the aberrant growth of Ras-transformed E6/E7/st cells. Thus, we have validated E6/E7/st cells as an important model cell system to evaluate Ras-mediated oncogenesis.

Similar to other primary human cell types, we found that ectopic expression of SV40 st was required to facilitate Ras growth transformation *in vitro* (17). This need of st for Ras transformation in a variety of human cell types argues that st function defines a critical and general step in Ras-mediated oncogenesis. One mechanism by which st may contribute to transformation involves its interaction with and inactivation of the serine-threonine protein phosphatase 2A (PP2A; ref. 41). Hahn et al. (42) showed that suppression of PP2A B56γ expression could substitute for SV40 st to facilitate Ras transformation of human embryonic kidney epithelial cells. Conversely, overexpression of PP2A B56γ3 partially reversed the tumorigenicity of these cells. Although PP2A has many possible substrates, its role in tumorigenesis may involve suppression of the function of the Myc transcriptional factor oncogene. In support of this possibility, Counter et al. (43) recently showed that the requirement for st could be replaced by expression of a stabilization mutant of Myc. However, Hahn et al. (44) showed that st function can be replaced by PI3K activation, and in our system, the additive stimulation of PI3K by both constitutively active Ras and st may drive the activation of this kinase beyond the threshold necessary for transformation. Thus, our future analyses will involve determining whether loss of PP2A function or gain of Myc or PI3K function can also facilitate Ras transformation of E6/E7/st cells and whether loss of PP2A function is important for pancreatic cancer cell growth.

Our studies found that in contrast to other primary human cell model systems for Ras transformation (17), overexpression of mutant K-Ras protein was not required for growth transformation. Previously, we have found that increased levels of oncogenic K-Ras were not well tolerated in some nontransformed immortalized human cells (e.g., MCF-10A breast epithelia).[4] Additionally, others have indicated that high levels of ectopic activated Ras protein may result in premature senescence (45). Therefore, there may exist a selection against cells overexpressing K-Ras(12D), leaving a population of cells with moderate expression of constitutively activated K-Ras. Thus, for our current model system, an important advantage of E6/E7/st cells for studying mechanisms of Ras-mediated oncogenesis is that artifactual observations due to Ras overexpression will be avoided. This concern about Ras overexpression is emphasized by studies in mouse models where endogenous K-Ras activation resulted in different cellular responses when compared with ectopic expression of mutant K-Ras (46). Another important strength of E6/E7/st cells is that we observed many of the growth transformation assays to represent all-or-nothing phenotypes. This contrasts significantly with other recently described human cell model systems (17), where the immortalized cell population had already acquired properties of growth transformation (e.g., growth in soft agar), and the subsequent expression of activated Ras simply enhanced these properties. We found that the immortalized E6/E7/st cells failed to form both colonies in soft agar and tumors in nude mice and showed no invasion through Matrigel. In contrast, E6/E7/Ras/st cells showed very robust activities in all three assays. The potent growth transformation caused by Ras in E6/E7/Ras/st cells provides a very useful model system to identify both genetic and pharmacologic attenuators of Ras-mediated oncogenesis.

[4] A. McFall and C. Der, unpublished observation.

Tsao et al. (26) recently described K-Ras transformation of immortalized HPDE cells. Whereas activated K-Ras did cause tumorigenic growth transformation, surprisingly, Ras-transformed HPDE cells did not exhibit *in vitro* transformation properties as indicated by the failure to show enhanced anchorage-dependent or anchorage-independent growth. Although HPDE cells were immortalized by E6 and E7 expression, no ectopic expression of hTERT or SV40 st was done. Hence, the additional genetic alterations used to generate E6/E7/st cells may account for the ability of K-Ras to cause both *in vitro* and *in vivo* growth transformation of pancreatic duct-derived cells. Alternatively, the specific pancreatic cell precursor for the development of E6/E7/st and HPDE cells may be different. Because both studies established immortalized populations from pancreatic ductal tissue, it remains possible that HPDE and E6/E7/st cells represent models for distinct subsets of pancreatic cancers. We are currently evaluating HPDE cells to address this possibility.

Our analyses of effector signaling found sustained activation of Akt and RalA but not ERK. However, we did find elevated MEK activation in E6/E7/Ras/st cells. These results mirror the situation seen with K-Ras mutation-positive pancreatic carcinoma cell lines, where MEK, but not ERK, activation was consistently seen. The lack of ERK activation in pancreatic carcinoma cells was attributed to increased expression of the MKP-2 phosphatase (38), and we similarly saw an up-regulation of MKP-2 expression in E6/E7/Ras/st cells. Thus, activated K-Ras does seem to cause persistent activation of the Raf-MEK-ERK cascade, but a compensatory up-regulation of MKP-2 to dampen the activity of this pathway may be evident. Because hyperactivation of the Raf-MEK-ERK pathway can be deleterious to cell proliferation, a mechanism to attenuate ERK activity may be necessary to facilitate cell proliferation. The importance of the Raf-MEK-ERK cascade in Ras transformation of E6/E7/st cells is supported by the ability of mechanistically different inhibitors of this signaling cascade to block cell growth. For E6/E7/Ras/st cells, this transformation inhibition occurred despite the fact that ERK phosphorylation was not reduced for BAY 43-9006 and MCP110 treatments. These data suggest that this effector pathway is not simply a linear one and that signaling cross-talk is evident. Indeed, treatment of E6/E7/Ras/st cells with inhibitors that target the Raf-MEK-ERK pathway (U0126, BAY 43-9006, and, to a lesser extent, MCP110) resulted in decreases in Akt phosphorylation. BAY 43-9006 has been shown to have affinity for a variety of other kinases, including platelet-derived growth factor receptor, vascular endothelial growth factor receptors, Flt, and c-Kit (33), and although it is possible that the above data are due to off-target action of these drugs, the fact that all three inhibitors, which have different targets in this signaling cascade, caused reduction of activated Akt lends credence to the hypothesis that interaction between at least the Raf-MEK-ERK and PI3K-Akt-mammalian target of rapamycin pathways is relevant in the Ras-mediated transformation of human cells. The specificity of MCP110 was previously shown by the fact that this compound could block Raf-1 activity and phenotypic transformation resulting from oncogenic Ras activation but not ectopic Raf expression (32). Thus, recently developed inhibitors of Raf and MEK may be effective for pancreatic cancer treatment (47), but because we and

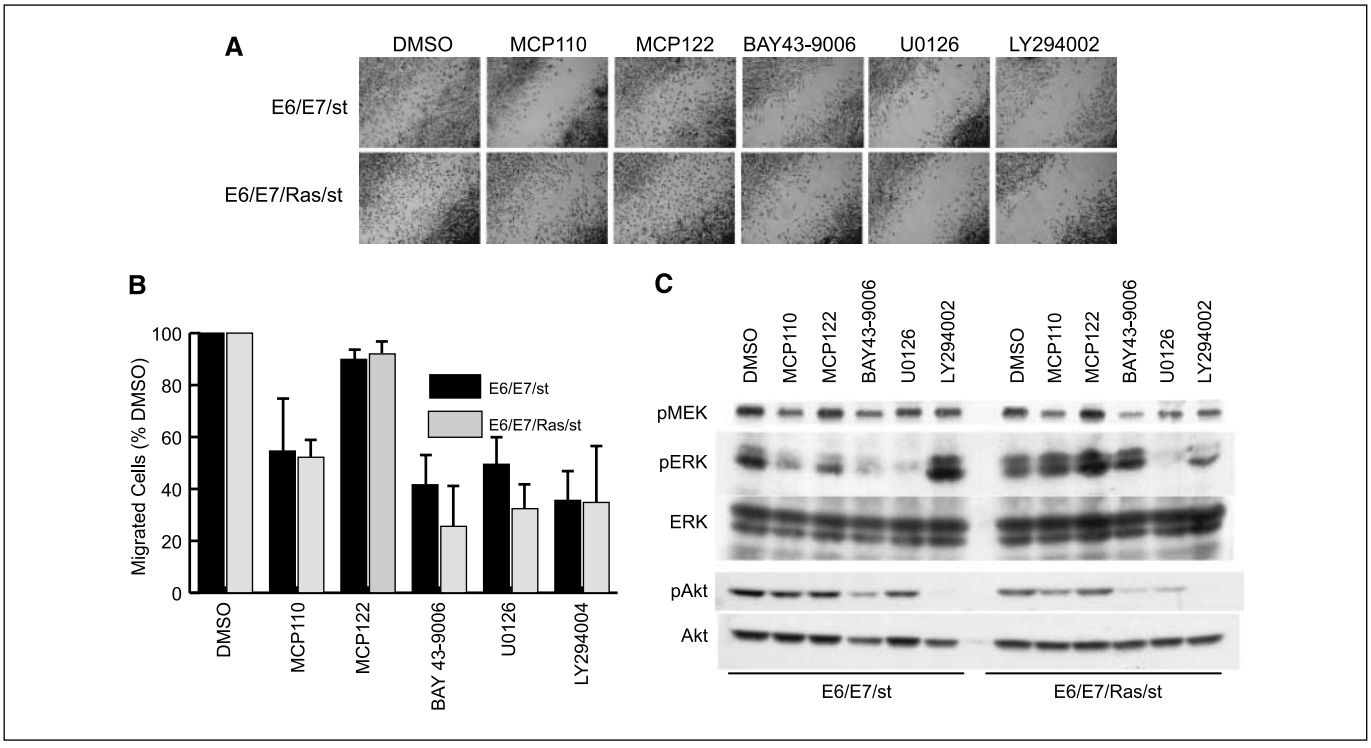

Figure 5. *In vitro* motility of human pancreatic duct-derived cells. *A* and *B,* E6/E7/Ras/st cells show enhanced migration. Immortalized pancreatic duct-derived cells treated with inhibitors of Ras-Raf interaction (MCP110/MCP122), MEK1/2 (U0126), Raf kinase (BAY 43-9006), or PI3K (LY294002) were grown to 90% confluence in serum starvation conditions. Plates were scratched, and after 12 h, cells were fixed, stained, and photomicrographed (*A*) and quantified (*B*) by counting the number of cells that had migrated into the void compared with $t = 0$ h (data not shown). *C,* E6/E7/st and E6/E7/Ras/st cell migration is dependent on ERK and Akt activity. Parallel cultures similarly treated with the indicated drugs at the same concentrations for 12 h were lysed for Western blot analysis of phosphorylated and total MEK and ERK proteins. Data are representative of two to five independent experiments.

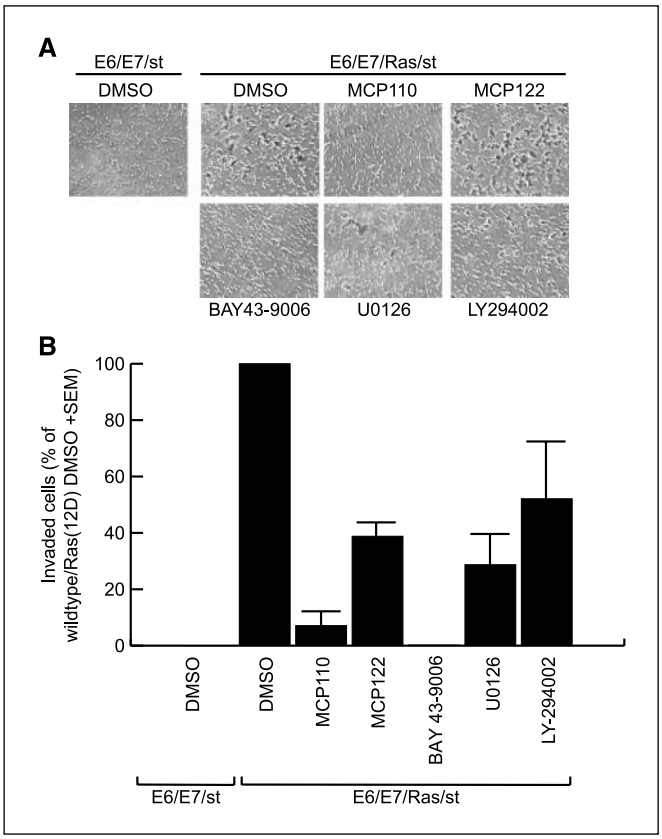

Figure 6. Oncogenic K-Ras(12D) induces E6/E7/st invasion *in vitro. A,* serum-starved cells were treated for 24 h with the indicated inhibitors and seeded into Matrigel invasion chambers. After an additional 24 h, invaded cells were fixed, stained, and counted. *B,* quantification of analyses shown in (*A*). *Columns,* representative images of triplicates from three independent experiments; *bars,* SE.

others have not seen consistent ERK activation in pancreatic cancer cell lines (8, 9) or patient tumors (7), ERK activation and inhibition may not be a reliable biomarker for monitoring patient selection or drug response.

Because the role of specific effectors in Ras-mediated growth transformation remains to be resolved, Ras transformation of E6/E7/st cells provides a powerful model system to address this question for pancreatic cell oncogenesis. We did find that two other key Ras effector pathways, the PI3K-Akt and the RalGEF-Ral axes, are activated persistently in E6/E7/Ras/st cells. Interestingly, our analyses of pancreatic carcinoma cell lines and patient tumors found infrequent activation of Akt (8), whereas we did find Akt activation in our cell system and PI3K inhibition did block Ras-mediated growth transformation. In contrast to ERK and Akt, we did find frequent activation of Ral GTPases (8). Our further analyses of Ral GTPases found that RNAi suppression of Ral GTPase expression impaired the soft agar growth and invasion *in vitro* and tumorigenicity and metastatic growth *in vivo* for most pancreatic carcinoma cell lines (7). Thus, our ongoing long-term studies will evaluate the sufficiency and necessity of the RalGEF-Ral pathway in Ras-mediated growth transformation of E6/E7/st cells. In particular, we will determine if the related RalA and RalB proteins contribute to tumorigenicity and metastasis, respectively, as we have seen in pancreatic carcinoma cells. If we find a similar important role for this effector pathway in E6/E7/st cells, it would provide further validation of these cells for the study of Ras-mediated pancreatic tumor cell growth.

In summary, our studies provide important justification for the use of E6/E7/st cells in the study of Ras-mediated growth transformation. Our validation of this model cell system adds to the growing roster of primary human cell types that have been developed using genetically defined steps to study oncogenesis. Concurrently, we are also using RNAi approaches to silence mutant K-Ras in pancreatic carcinomas to address similar questions. The use of these two complementary cell culture models will provide powerful model systems to delineate the key signaling mechanisms by which mutant K-Ras causes and maintains growth transformation of pancreatic epithelial cells.

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