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Oncogenic K-ras Drives Cell Cycle Progression and Phenotypic Conversion of Primary Pancreatic Duct Epithelial Cells

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ABSTRACT

We have established a primary pancreatic duct epithelial cell culture (PDEC) system to investigate the relationship between oncogenic activation of K-ras and pancreatic ductal tumorigenesis. We have found that the acute introduction of physiological levels of oncogenic K-ras (K-ras^{V12}) into quiescent PDECs stimulates S-phase entry and induces a pronounced increase in cell size. Both effects are dependent on the functional integrity of the phosphatidylinositol 3′-kinase (PI3K)/mammalian target of rapamycin (mTOR) signaling pathway. In addition, K-ras^{V12} promotes the loss of epithelial E-cadherin and the gain of mesenchymal N-cadherin in PDEC. Our observations indicate that the oncogenic activation of K-ras is sufficient to elicit mitogenic and morphogenic responses in pancreatic ductal cells and hence is likely to play an instructive role in the initiation of pancreatic ductal adenocarcinoma.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDA) is the fourth most common cause of cancer-related mortality (1). PDA is thought to arise from proliferative premalignant lesions of the ductal epithelium, termed PanINs for pancreatic intraepithelial neoplasms, through a series of genetic alterations (2). These include activating mutations in the K-*ras* gene and the loss of the tumor suppressor genes *p16^{Ink4a}*, *p53*, and *DPC4* (3). Because activating K-*ras* mutations are the first genetic changes to be detected coincidently with the earliest PanIN lesions, it is generally thought that the deregulated signaling activity of mutated K-ras is an essential determinant in the evolution of PDA. This notion is supported by the findings that targeted expression of oncogenic K-ras in the pancreas triggers the development of hyperproliferative ductal lesions resembling those observed in early PanINs (4–7), and, conversely, blocking K-ras function attenuates the growth and tumorigenicity of human pancreatic cancer cell lines (8, 9). However, the presence of K-ras mutations in normal ductal epithelium, in ductal lesions within normal pancreatic tissue, and in ductal lesions adjacent to K-ras-negative carcinomas challenges the idea of this genetic alteration having a causative role in the pathogenesis of PDA (10). To examine the role of mutational activation of K-ras in the genesis of PDA, we have investigated the effects of a mutant form of K-ras that has been identified in approximately 40% of PDA, K-ras^{V12}, on primary pancreatic ductal cells (11). We show that physiological levels of K-ras^{V12} promote cell cycle progression in quiescent rat pancreatic ductal cells. Furthermore, in the presence of K-ras^{V12}, pancreatic ductal cells undergo marked changes in cell size, shape, and display altered differentiation characteristics. Both the proliferative and morphological effects of K-ras^{V12} depend on mammalian target of rapamycin (mTOR) signaling. These findings indicate that oncogenic activation of K-ras incites cellular responses that may bear direct relevance to the onset of PDA.

MATERIALS AND METHODS

Pancreatic Duct Epithelial Cell Isolation and Cell Culture. Main pancreatic ducts were dissected from male Fisher CDF rats (Charles River) and subjected to 2 mg/ml collagenase Type XI (Sigma) for 12 min at 37ºC. Using a dissecting scope (Stemi DV4 Stereomicroscope; Zeiss), main ducts were manually separated from surrounding tissue. Subsequently, pancreatic duct fragments were subjected to another collagenase digest, 2 units/ml dispase I (Sigma) treatment for 8 min, and serial trypsinization [six successive 5-min treatments with 0.1% trypsin I-300 (ICN)]. Isolated PDECs were resuspended in pancreatic medium [1:1 Hams F12/DMEM mix supplemented with 100 ng/ml EGF (Austral Biologicals), 40 μg/ml dexamethasone (Sigma), 2.5 mg/ml bovine pituitary extract (Life Technologies), 50 μM triiodo-L-thyronine (Sigma), 100 μg/ml cholera toxin (Sigma), insulin/transferring/selenium (Sigma), 1 μg/ml soybean trypsin inhibitor (ICN), and 10% FBS (Life Technologies)], plated onto gridded coverslips coated with 2–5 μg/cm^2 laminin I (Sigma), and incubated in 37ºC, 5% CO_2. Pancreatic medium minimal medium for serum starvation consisted of 100 μg/ml cholera toxin, 1 μg/ml soybean trypsin inhibitor, and 0.25% FBS and was added to cells for 48 h.

Protein Purification and Microinjection. K-RasV12 and K-RasV12A35 were expressed in bacteria and were purified using standard Ni-affinity chromatography. PDEC microinjection experiments were initiated 4 days postisolation. The injection solution contained 0.5–4 mg/ml Ras proteins and 4 mg/ml dog IgG in injection buffer consisting of 200 mM NaCl, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), and 5 mM $MgCl_2$ in PBS. To calculate the number of K-Ras molecules injected per cell, we estimated that each injection pulse introduced 1×10^{-15} liters of injection solution into each cell (12). Endogenous Ras levels are based on estimates provided previously (13). The following pharmacological inhibitors at indicated working concentrations were used in our studies: 20 μM phosphatidylinositol 3′-kinase (PI3K) inhibitor LY294002 (Calbiochem); 10 μM p38 inhibitor SB203580 (Calbiochem); and 10 ng/μl mTOR inhibitor rapamycin (Calbiochem).

Immunofluoresence. Immunofluorescence was carried out using standard protocols. The following primary antibodies were used: bromodeoxyuridine (BrdU; Roche); cytokeratin 19 (Novocastra); E-cadherin (Becton Dickinson); and N-cadherin (Becton Dickinson). Secondary antibodies used were: fluorescein-conjugated antidog IgG (Rockland); rhodamine-conjugated goat anti-mouse (ICN); and 4′,6-diamidino-2-phenylindole (DAPI; Sigma).

RESULTS AND DISCUSSION

To analyze the effects of oncogenic K-ras (K-Ras^{V12}) in a cellular context that is relevant to the initiation of PDA, we have established a cell culture system for primary pancreatic duct epithelial cells (PDEC). The epithelial identity of the cells was confirmed by their cobblestone-like morphology and positive staining for the epithelial cell markers cytokeratin 19 and E-cadherin (Fig. 1, *A* and *B*). Under the culture conditions we have used, PDEC proliferated with a doubling time of approximately 24 h up to 7 days after plating. By day 8–10, the growth rate diminished, and the cells eventually underwent senescence as determined by the detection of senescence-associated-β-galactosidase approximately 2 weeks after isolation (data not shown). In the present study, we have used PDEC isolated from rats to maximize cell yields. However, the same protocol can be used to derive PDEC from mice (Fig. 1*C*).

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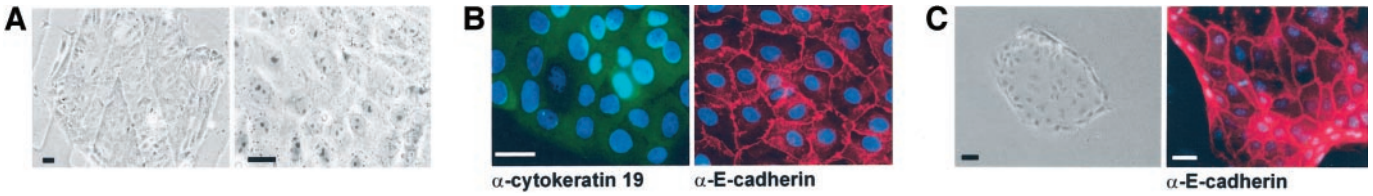

Fig. 1. Characterization of primary pancreatic ductal epithelial cells (PDEC). *A*, phase contrast micrographs of PDEC at low (*left panel*) and high (*right panel*) magnification. *B*, immunofluorescent staining of PDEC for epithelial-specific markers cytokeratin 19 (*left panel*) and E-cadherin (*right panel*). Cell nuclei are counterstained with DAPI. *C*, a phase contrast micrograph (*left panel*) and E-cadherin immunofluoresent staining (*right panel*) of PDEC isolated from mice. *Scale bars* = 20 μm in all panels.

Although PanIN lesions that harbor oncogenic K-ras display enhanced proliferation (14, 15), the cause-and-effect relationship between the mutational activation of K-ras and ductal cell proliferation is not known. To determine the effect of oncogenic K-ras on PDEC mitogenesis, quiescent serum-deprived PDEC were microinjected with different concentrations of purified K-ras^{V12} protein, and cell cycle progression was assessed by BrdU incorporation. As illustrated in Fig. 2, *A* and *B*, K-ras^{V12} induced a significant stimulation of S-phase entry when introduced into quiescent PDEC. We have calculated (see "Materials and Methods") that the concentration of microinjected K-ras^{V12} required to obtain maximal stimulation of DNA synthesis corresponds to approximately 1×10^4 molecules of Ras introduced into each cell, which is comparable with endogenous amounts of Ras (13). In contrast, when the level of microinjected protein was increased to approximately 4×10^4 molecules/cell, a marked reduction of DNA synthesis was observed, implying that amounts of K-ras^{V12} that surpass endogenous levels are unable to elicit a mitogenic effect. These results indicate that aberrant activation of physiologically relevant levels of K-ras might be sufficient to confer a growth advantage on pancreatic ductal cells *in vivo*.

It has been demonstrated that high-intensity Ras signaling can lead to antiproliferative responses such as cell cycle arrest or premature senescence (16, 17). To determine the effect of excessive Ras signaling on PDEC proliferation, steady-state growing PDEC were microinjected with increasing amounts of K-ras^{V12}, and DNA synthesis was monitored by BrdU incorporation. As illustrated in Fig. 2, *C* and *D*, the introduction of approximately 1×10^5 K-ras^{V12} molecules/cell, roughly 10-fold over physiological levels, resulted in the inhibition of S-phase entry. In contrast, microinjection of the same amount of a mutant form of K-ras^{V12} that is defective in effector interactions, K-ras^{V12A35}, had no effect on PDEC proliferation (Fig. 2, *C* and *D*). This indicates that the antiproliferative response to supraphysiological

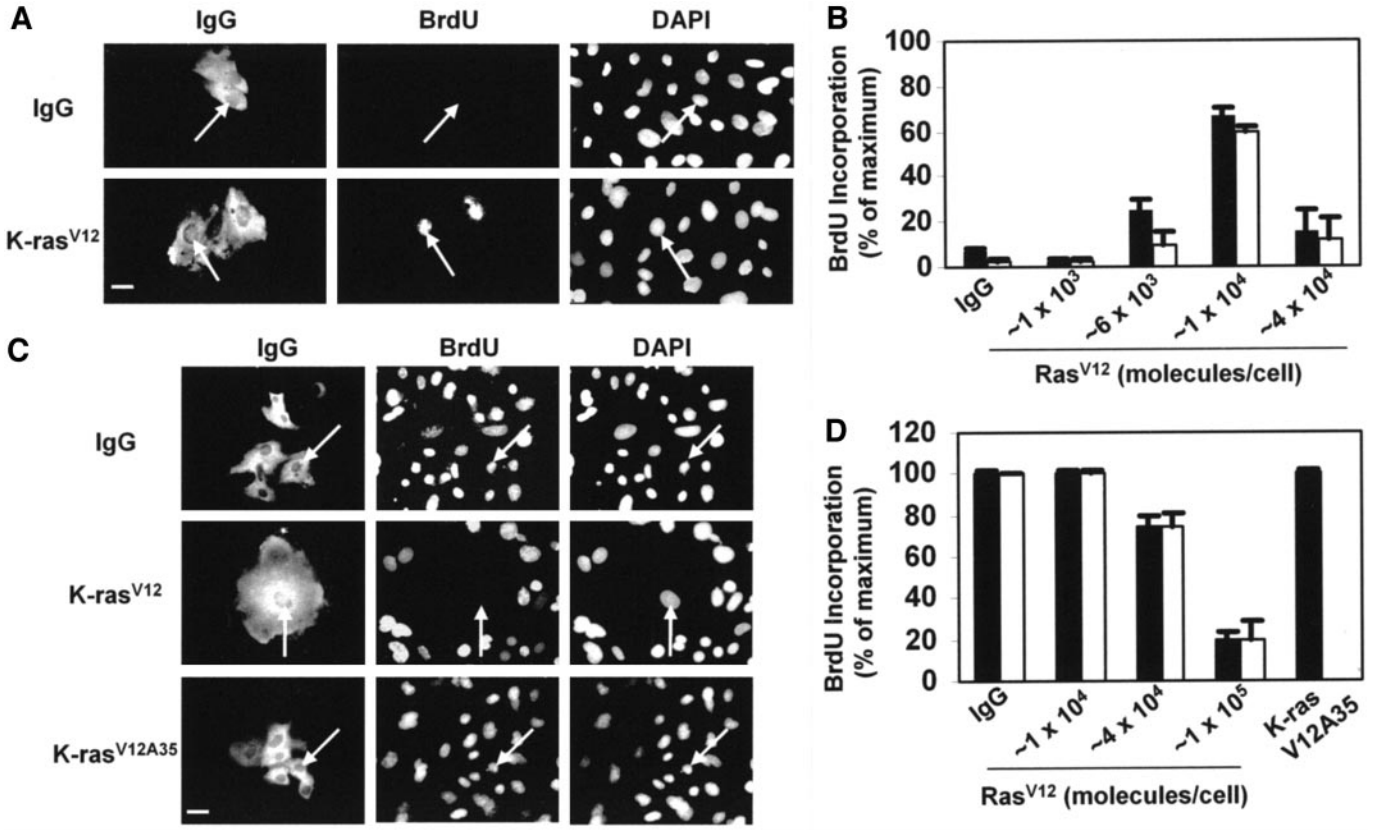

Fig. 2. Effects of oncogenic Ras on PDEC cell cycle progression. *A*, quiescent PDEC were co-injected with IgG marker and the indicated amounts of K-ras^{V12} protein per cell. Cells were fixed and stained for IgG, BrdU incorporation (*BrdU*), and DAPI 40 h postinjection. *B*, dose dependence of Ras-induced cell cycle progression. Maximal BrdU incorporation corresponds to the number of IgG-injected cells that incorporate BrdU in response to 10% serum stimulation. Numbers reflect mean ± SD of six independent experiments with at least 40 cells scored per experiment. ■, K-ras^{V12}; □, H-ras^{V12}. *C*, PDEC in 20% serum were co-injected with IgG marker and the indicated amount of K-ras^{V12} protein. Cells were fixed 40 h after injection and stained for IgG, BrdU, and DAPI. *D*, dose dependence of Ras^{V12}-induced cell cycle arrest. BrdU incorporation was calculated as the percentage of BrdU-positive cells out of total injected cells. The mean ± SD from three independent experiments with at least 90 cells injected per experiment is shown. ■, K-ras^{V12}; □, H-ras^{V12}. *Scale bars* = 20 μm.

levels of K-ras^{V12} is a direct consequence of deregulated Ras-dependent signals. An increase in K-ras levels, principally through gene amplification mechanisms, has been also observed in advanced stages of PDA in humans, as well as in mouse models (7, 18). In primary fibroblasts, the growth arrest response to high levels of activated Ras has been shown to be mediated by the up-regulation of p16^{Ink4a} and p53 (16). Significantly, *Ink4a* loss occurs at nearly 100% frequency in moderately advanced stage of the disease, and *p53* loss is detected in over 50% of late-stage pancreatic adenocarcinomas (3). The growth constraining effect of high levels of oncogenic K-ras signaling on ductal cells may therefore underlie the requirement for *Ink4a* or *p53* deficiency in the progression of human PDA. It should be noted that the responses of ductal cells to the introduction of the oncogenic form of H-ras, H-ras^{V12}, were essentially identical to the responses induced by K-ras^{V12} in terms of both the mitogenic effect at physiological levels and antiproliferative effect at supraphysiological levels (Fig. 2, *B* and *D*). These observations suggest that the high prevalence of K-ras mutations in ductal adenocarcimoma does not reflect an intrinsic difference between the signaling function of H-ras and K-ras in pancreatic ductal cells.

The introduction of K-ras^{V12} into PDEC led to a significant increase in cell size (Fig. 2, *A* and *C*). Morphometric analysis of the cell perimeter revealed a 3-fold increase in surface area of PDEC injected with physiological levels of K-ras^{V12} and 7–10-fold increase in surface area in PDEC injected with supraphysiological levels of K-rasV12 (Fig. 3*B*). We sought to determine the signaling pathways involved in the K-ras^{V12}-induced morphogenic effects focusing on the pronounced changes seen following microinjection of supraphysiological levels of K-ras^{V12}. The phosphotidylinositol-3-kinase (PI3K) pathway is well known for its role in mediating changes in cell morphology. Microinjection of K-ras^{V12} in the presence of the PI3K-specific inhibitor LY294002 failed to promote an increase in cell size, indicating an essential role for PI3K in oncogenic K-ras-induced changes in ductal cell morphology (Fig. 3, *A* and *B*). The serine/threonine kinase mTOR is a well-established downstream component of PI3K-dependent signals that control cell size (19). K-ras^{V12}-injected cells treated with rapamycin, which specifically blocks mTOR, displayed a partial increase in cell size relative to untreated K-ras^{V12}-injected cells (Fig. 3, *A* and *B*), suggesting the existence of additional pathways that contribute to the effect of K-ras^{V12} on morphogenesis.

The effects of the PI3K pathway on cell size are mediated by the activation of translational effectors that promote protein synthesis, the best characterized of which are S6K1 and 4EBP1/eIF-4E (19). S6K1 and 4EBP1 are direct targets of mTOR, whereas eIF-4E activity is regulated, at least in part, through phosphorylation by MNK1 kinase, a substrate of ERK and p38 MAPK (19). The Rac GTPase is a downstream effector of PI3K and has been identified as a regulator of the p38 pathway (20). This raises the possibility that the LY294002-sensitive increase in cell size in response to K-ras^{V12} might be dependent on p38 signaling. To test this idea, PDEC were microinjected with K-ras^{V12} in the presence of SB203580, a specific inhibitor of p38. Similar to the effect of rapamycin, treatment with SB203580 alone only partially inhibited the K-ras^{V12}-induced increase in cell size. In contrast, cotreatment with SB203580 and rapamycin abolished the K-ras^{V12}-induced increase in PDEC size (Fig. 3, *A* and *B*). These observations indicate that the constitutive activation of the Ras effector PI3K in PDEC leads to an increase in cell size through the synergistic contribution of mTOR- and p38-dependent signals. Because alterations in epithelial architecture including an increase in cell size are frequently observed at early stages of neoplastic conversion, our observations indicate that K-ras activation of the PI3K/mTOR

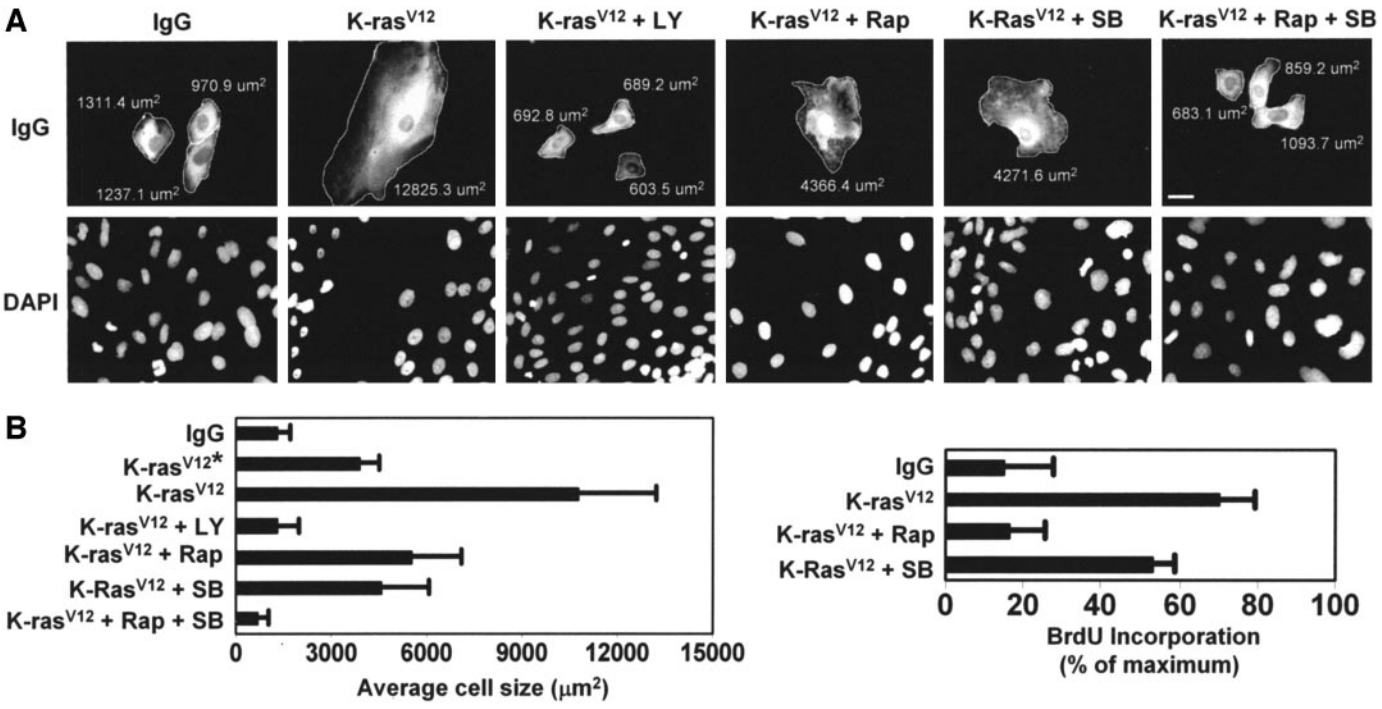

Fig. 3. Effects of K-ras^{V12} on PDEC morphogenesis. *A*, PDEC maintained in growth medium containing 10% serum were co-injected with IgG marker and 1×10^5 molecules of K-ras^{V12} per cell. Cells were treated with the following concentrations of pharmacological inhibitors as indicated: 20 μM PI3K inhibitor LY294002 (*LY*), 10 μM p38 inhibitor SB203580 (*SB*), and 10 μM mTOR inhibitor Rapamycin (*Rap*). After 24 h, PDEC were fixed and immunostained to detect IgG and counterstained with DAPI. The boundries of injected PDEC are marked by a white solid line, and cell size calculated as surface area is shown. *Scale bar* = 20 μm. *B*, quantification of cell size measurements described in *A*. Values are the averages of PDEC surface areas from three independent experiments with at least 30 cells measured per experimental condition. SD is indicated by *error bars*. *, physiological levels of K-ras^{V12}. *C*, quiescent PDEC were co-injected with IgG and K-ras^{V12} in the presence or absence of pharmacological inhibitors in pancreatic minimal medium. BrdU assay, immunostaining, and quantitation were performed as described in Fig. 2. The mean SD of five independent experiments with at least 40 cells scored per experiment is shown. Pharmacological inhibitors had no effects on IgG-control-injected PDEC (data not shown).

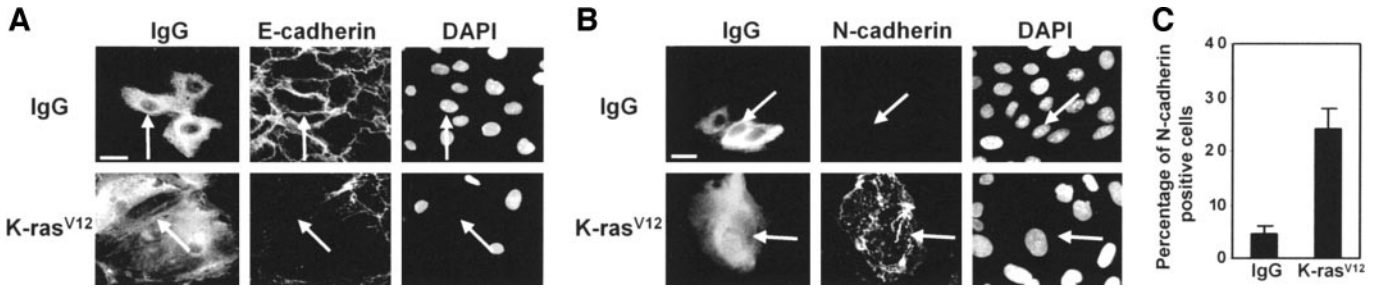

Fig. 4. Oncogenic K-Ras drives phenotypic conversion of PDEC. PDEC were co-injected with IgG marker and 1×10^5 molecules of K-ras^{V12} per cell and immunostained for E-cadherin (*A*) and N-cadherin (*B*) 24 h after injection. Injected cells were identified by the IgG marker. *Scale bars* = 20 μm. *C*, quantification of N-cadherin staining is presented as the percentage of injected cells that are N-cadherin positive. Values are the averages from five independent experiments with at least 40 cells injected. SD is indicated by the *error bars*.

pathways may contribute to the histological aberrations in the ductal epithelium associated with early stages of PDA.

Both mTOR and p38 MAPK signaling pathways have been implicated in the regulation of cell cycle progression (19, 21). However, neither mTOR nor p38 have been directly linked to Ras-induced mitogenesis. Microinjection of K-ras^{V12} in the presence of rapamycin inhibited S-phase entry in quiescent PDEC, whereas SB203580 had no effect on cell cycle progression (Fig. 3*C*). These results suggest a role for mTOR signaling in coordinating the effects of oncogenic K-ras on PDEC cell-cycle progression and cell growth. The relevance of mTOR-mediated signals to PDA is further supported by reports demonstrating that mTOR and its downstream components are constitutively active in pancreatic cancer cell lines and tissues and that rapamycin inhibits the proliferation of pancreatic cancer cells (22, 23).

Features of high-grade PanINs include prominent changes in tissue architecture as a result of alterations in cell-cell and cell-matrix adhesion. E-cadherin is an epithelial specific member of the cadherin family that plays a critical role in mediating Ca^{2+}-dependent cell-cell adhesion (24). E-cadherin loss occurs at high frequency in many types of human carcinomas and is causally linked to tumor progression, invasion, and metastasis (24). Moreover, reduced expression of E-cadherin has been observed in advanced pancreatic tumor tissue and has been correlated with increased tumor de-differentiation and poor prognosis (25, 26). To examine whether constitutive K-ras activity alters the cell-cell adhesion properties of PDEC, cells were microinjected with K-ras^{V12} and visualized for E-cadherin using immunofluorescence staining. As illustrated in Fig. 4*A*, a significant decrease in E-cadherin staining was observed in the junctions of neighboring PDEC injected with K-ras^{V12} denoting loss of cell-cell contact. This observation indicates that oncogenic activation of K-ras may contribute to the loss of normal ductal architecture by promoting the disruption of cell-cell adhesion. Our results are similar to those observed upon expression of oncogenic ras in MDCK epithelial cells, which induces dispersal of E-cadherin from intercellular junctions (27).

In many human cancers, the loss of E-cadherin is accompanied by the inappropriate expression of nonepithelial cadherins such as the mesenchymal cadherin N-cadherin (28). This phenomenon, referred to as cadherin switching, is thought to be responsible for the increase in cellular motility and invasion during malignant tumor progression (28). Immunofluorescent analysis of PDEC microinjected with K-ras^{V12} revealed the presence of N-cadherin in the cell borders of approximately 25% of injected cells (Fig. 4, *B* and *C*). The induction of N-cadherin expression by oncogenic K-ras has also been observed in pancreatic ductal cells isolated from transgenic mice in which the expression of oncogenic K-ras is specifically targeted to the duct epithelial cells (4). Together, these results suggest that oncogenic K-ras may enhance the metastatic properties of pancreatic cancer cells by promoting cadherin switching. Because cadherin switching is most frequently observed at advanced stages of tumor progression, our findings suggest a role for oncogenic activation of Ras in mediating ductal changes that contribute to late stages of pancreatic cancer tumorigenesis. Indeed, activating K-ras mutations occur with increasing frequency in progressively later stages of PanINs (15), and inhibition of oncogenic K-ras decreases the invasiveness of pancreatic cancer cell lines (29).

The progression model of pancreatic adenocarcinoma indicates that the evolution of the disease proceeds through the cooperative effects of multiple genetic lesions (2). This synergy has recently been modeled in several mouse models of pancreatic cancers (7, 30–32). Although, these mouse models have been extremely successful in recapitulating the majority of histological changes observed during the initiation and progression of PDA, they remain inadequate in terms of identifying the cellular origin of this malignancy. The complexities in addressing this question are posed by the findings that pancreatic neoplasia can arise from acinar, islet, ductal cells, or pancreatic stem cell progenitors, as well as by the inherent plasticity of these various pancreatic cell lineages (33). The experimental system described in this study has enabled us to determine the direct effects of oncogenic activation of K-ras on pancreatic ductal cells, the presumed progenitor of PDA. Our observations that physiological levels of K-ras^{V12} enhance the proliferative capacity of PDEC and drive their phenotypic conversion indicate that the activation of K-ras in mature postmitotic ductal cells is sufficient and potentially necessary to elicit cellular responses that can contribute to the initiation of PDA. The ability to manipulate the growth properties of pancreatic ductal cells in culture should provide a powerful approach to dissect the effector mechanisms by which specific genetic events contribute to the genesis of pancreatic adenocarcinoma.

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