Published in final edited form as:

Gastroenterology. 2014 March ; 146(3): 822–834.e7. doi:10.1053/j.gastro.2013.11.052.

Mapk Signaling Is Required for Dedifferentiation of Acinar Cells and Development of Pancreatic Intraepithelial Neoplasia in Mice

Meredith A. Collins¹, Wei Yan^{2,3}, Judith S. Sebolt-Leopold⁴, and Marina Pasca di Magliano^{1,5,6}

¹Program in Cellular and Molecular Biology, University of Michigan, Ann Arbor, Michigan

²Department of Pathology, University of Michigan, Ann Arbor, Michigan

³Michigan Center for Translational Pathology, University of Michigan, Ann Arbor, Michigan

⁴Department of Radiology, University of Michigan, Ann Arbor, Michigan

⁵Department of Surgery, University of Michigan, Ann Arbor, Michigan

⁶Department of Cell and Developmental Biology, University of Michigan, Ann Arbor, Michigan

Abstract

BACKGROUND & AIMS—Kras signaling via mitogen-activated protein kinase (MAPK) is highly up-regulated in pancreatic cancer cells. We investigated whether Mapk signaling is required for the initiation and maintenance of pancreatic carcinogenesis in mice.

METHODS—We studied the formation and maintenance of pancreatic intraepithelial neoplasia (Pan-INs) in *inp48Cre*; *TetO-KrasG12D*; *Rosa26rtTa-IRES-EGFP* (*iKras**) mice and *LSL-KrasG12D* mice bred with *p48Cre* mice (KC mice). Mice were given oral PD325901, which is a small-molecule inhibitor of Mek1 and Mek2 (factors in the Mapk signaling pathway), along with injections of cerulein to induce pancreatitis. Other mice were given PD325901 only after Pan-INs developed. Pancreatic tissues were collected and evaluated using histologic, immunohistochemical, immunofluorescence, and electron microscopy analyses. Acinar cells were isolated from the tissues and the effects of Mek1 and 2 inhibitors were assessed.

RESULTS—PD325901 prevented PanIN formation, but not pancreatitis, in *iKras** and KC mice. In *iKras** or KC mice given PD325901 at 5 weeks after PanINs developed, PanINs regressed and acinar tissue regenerated. The regression occurred through differentiation of the PanIN cells to acini, accompanied by re-expression of the acinar transcription factor *Mist1*.

© 2014 by the AGA Institute

Address requests for reprints to: Marina Pasca di Magliano, PhD, Departments of Surgery and Cell and Developmental Biology, 1500 E Medical Center Drive, Ann Arbor, Michigan 48109-5936. marinapa@umich.edu; fax: (734) 647-9654.

Current affiliation of W.Y.: Department of Pathology, Xijing Hospital, Fourth Military Medical University, Shaanxi Province, China.

Conflicts of interest

The authors disclose no conflicts.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <http://dx.doi.org/10.1053/j.gastro.2013.11.052>.

CONCLUSIONS—In iKras* and KC mice, Mapk signaling is required for the initiation and maintenance of pancreatic cancer precursor lesions. Mapk signaling promotes formation of PanINs by enabling dedifferentiation of acinar cells into duct-like cells that are susceptible to transformation.

Keywords

Pancreatic Cancer; Signal Transduction; Mouse Models; Acinar Differentiation

Pancreatic ductal adenocarcinoma is the most common form of pancreatic cancer, and the fourth leading cause of cancer death in the United States (<http://seer.cancer.gov> and <http://www.cancer.org/Research/CancerFactsFigures>). Mutations in Kras, most commonly Kras^{G12D} (Kras*), characterize more than 95% of human pancreatic ductal adenocarcinoma samples,^{1,2} and more than 90% of precancerous pancreatic intraepithelial neoplasia (PanIN) lesions.³ By using genetically engineered mouse models, our group and others previously have shown that oncogenic Kras* expression is necessary for pancreatic cancer initiation and maintenance.^{4–6} However, the biological mechanisms underlying Kras*-driven carcinogenesis are not fully understood.

Kras activates numerous downstream effector pathways. One of the best-studied effector pathways is the mitogen-activated protein kinase (MAPK) cascade. Under normal conditions, MAPK signaling is tightly regulated and is activated primarily by extracellular growth factor stimulation. After receptor phosphorylation, Kras binds guanosine triphosphate and becomes active. Kras then activates the serine/threonine kinase Raf, which phosphorylates, and subsequently activates, mitogen-activated protein kinase kinase (MEK1/2). In turn, MEK1/2 phosphorylates extracellular signal-regulated kinase (ERK1/2), which translocates to the nucleus where it promotes transcription and cell-cycle progression (for review see Dhillon et al⁷). Here, we investigated the role of MAPK signaling during the initial steps of pancreatic carcinogenesis.

Interestingly, in human beings, mutant Kras* expression initiates carcinogenesis inefficiently, as underscored by findings that the mutation occurs at a much higher rate than pancreatic cancer.^{8,9} Similarly, in genetically engineered mouse models of pancreatic cancer, pancreas-wide expression of oncogenic Kras that begins during embryogenesis results in sporadic PanIN formation postnatally over the course of several weeks.⁶ Chronic pancreatitis is a well-known risk factor for pancreatic cancer in human beings.¹⁰ In mice, mutations in Kras have been shown to work synergistically with both chronic and acute pancreatitis to drive tumorigenesis.^{11,12} Pancreatitis is accompanied by up-regulation of MAPK signaling in isolated acinar cells,^{13,14} and in vivo.¹⁵ The up-regulation of MAPK signaling is transient in wild-type animals but becomes sustained in the presence of oncogenic Kras*. However, whether MAPK signaling mediates pancreatitis-induced carcinogenesis has not been established.

In the current study, using 2 genetically engineered mouse models of pancreatic cancer, we show that MAPK signaling is required for the initiation and maintenance of PanIN lesions. Furthermore, we identify a new role for the MAPK pathway in regulating pancreatic acinar cell differentiation.

Materials and Methods

Mice

Mice were housed in specific pathogen-free facilities at the University of Michigan Comprehensive Cancer Center. This study was approved by the University of Michigan's University Committee on Use and Care of Animals. p48Cre (Ptf1aCre) mice¹⁶ were intercrossed with TetO-Kras^{G12D17} (expressing the murine mutant form Kras4B^{G12D}) and Rosa26^{rtTa-IRES-EGFP18} to generate p48Cre; TetO-Kras^{G12D}; Rosa26^{rtTa-IRES-EGFP} (iKras*) mice. LSL-Kras^{G12D} mice were bred with p48Cre mice to create KC mice. Combinations of single or double mutants were used as littermate controls. The mice used in this study were of mixed genetic background.

Immunohistochemistry/Immunofluorescence

Histology and immunohistochemistry/immunofluorescence were performed as previously described.⁴

Histopathologic Analysis

Histopathologic analysis was performed on de-identified slides that were examined by a pathologist (W.Y.) as previously described.^{4,19} The data are expressed as a percentage of total counted clusters. Error bars represent the SEM.

Three-Dimensional Culture

iKras* mice were treated with doxycycline 72 hours before harvest for 3-dimensional (3D) culture (see also the Supplementary Materials and Methods section and Zhang et al²⁰). Acinar clusters were treated with U0126 (10 μ mol/L) or PD325901 (100 nmol/L) over a period of 3–4 days.

Transmission Electron Microscopy

Tissue was harvested and minced into 1-mm³ pieces and then fixed in 2.5% glutaraldehyde in 0.1 mol/L Sorensen's buffer. Processing for transmission electron microscopy was performed by the University of Michigan Microscopy Image Analysis Core. Images were taken on a Philips CM 100 transmission electron microscope with AMT V600 software.

For detailed protocols, see the Supplementary Material.

Results

Up-Regulation of the MAPK Pathway After Acute Pancreatitis

We first investigated the activation of the MAPK pathway after the induction of acute pancreatitis in 2 genetically engineered mouse models of pancreatic cancer expressing oncogenic Kras*; iKras* and KC, and their littermate controls. Although both models have pancreas-specific expression of Kras*, in iKras* mice the expression can be induced at will,⁴ whereas in KC mice the expression begins during embryonic development.⁶ Control, iKras*, and KC mice (n = 3–4/genotype) were 4–6 weeks old, which is when most KC animals present with a normal pancreas.⁶ Doxycycline was administered to iKras* mice to

induce expression of oncogenic Kras*. Pancreatitis then was induced through 8 hourly cerulein injections over a period of 2 consecutive days.^{4,11} Tissues were harvested 2 days, 1 week, and 3 weeks after pancreatitis, corresponding to acute pancreatitis-induced damage, recovery (in wild-type) or onset of fibrosis (in Kras mutant animals), and PanIN formation (in Kras mutant mice), respectively (Supplementary Figure 1A). At 2 days, we observed tissue-wide acinar-ductal metaplasia (ADM) accompanied by inflammatory cell infiltration and edema in all 3 cohorts (Supplementary Figure 1B). Pancreatic damage was accompanied by up-regulation and nuclear translocation of pERK1/2, as previously described,^{13–15} indicating MAPK pathway activation in pancreatic epithelial cells (Supplementary Figure 1C). Tissue damage and MAPK pathway activation were transient in the control animals; 1 week after pancreatitis the tissue had recovered and expressed basal levels of pERK1/2 (Supplementary Figure 1B and C). Conversely, mice that express oncogenic Kras* displayed impaired tissue repair. One week after the induction of pancreatitis, the pancreata of both iKras* and KC cohorts had extensive ADM and fibrosis (Supplementary Figure 1B). Three weeks after pancreatitis, we observed tissue-wide PanIN surrounded by abundant stroma in both iKras* and KC mice (Supplementary Figure 1B). These dysplastic changes were accompanied by a continued increase of nuclear and cytoplasmic pERK1/2 levels (Supplementary Figure 1C). Thus, up-regulation of MAPK signaling upon induction of pancreatitis was transient in control animals,^{13–15} but became sustained in the presence of oncogenic Kras*.

Inhibition of MEK1/2 Prevents Inflammation-Driven ADM/PanIN Formation

In the next set of experiments, we examined whether sustained activation of the MAPK pathway was required for the initiation of tumorigenesis. To block activation of the MAPK pathway after induction of pancreatitis, we used the small-molecule MEK1/2 inhibitor PD325901. First, we investigated whether treatment with PD325901 prevented cerulein-induced pancreatitis. Control animals (n = 3/treatment group) were treated with either vehicle or PD325901 (10 mg/kg) every 12 hours, starting 24 hours before pancreatitis and then throughout the duration of the experiment (Supplementary Figure 2A). Two days after pancreatitis, the pancreata of PD325901-treated animals were indistinguishable from vehicle-treated animals. Both cohorts displayed ADM, inflammatory cell infiltration, and edema (Supplementary Figure 2B). Animals treated with the vehicle had increased levels of pERK1/2 compared with untreated animals, and animals treated with PD325901 had low pERK1/2 (Supplementary Figure 2B and C), showing that the MEK1/2 inhibitor effectively blocked MAPK signaling, but did not prevent the induction of pancreatitis.

Next, we inhibited MEK1/2 in both iKras* and KC mice before the induction of pancreatitis. iKras* animals were administered doxycycline starting at 4–6 weeks of age and then continuously, to activate and maintain Kras* expression. Control littermates also were administered doxycycline water. Concurrently, the animals were treated with either vehicle or PD325901 every 12 hours to inhibit MAPK signaling (n = 3/treatment group). No adverse effects were observed in any of the treatment groups. Age-matched KC animals (n = 3/treatment group) were treated with vehicle or PD325901 every 12 hours. Pancreatitis was induced 72 hours after the initiation of treatment and the pancreata were harvested 1 week later (Figure 1A) when we expected the tissue to display ADM and early PanIN lesions

(Supplementary Figure 1B). Inhibition of MEK1/2 activity, measured as pERK1/2 levels by Western blot, was confirmed in PD325901-treated control, iKras*, and KC mice (Figure 1B). Control animals treated with either vehicle or PD325901 showed normal tissue morphology 1 week after pancreatitis (Figure 1C). Vehicle-treated iKras* and KC animals presented with frequent ADM and some PanIN lesions (Figure 1D and Supplementary Figure 2D). KC mice had more PanINs than iKras* animals, possibly because oncogenic Kras* had been active since early in development. As expected, vehicle-treated animals had increased pERK1/2 levels (Figure 1E). Strikingly, both iKras* and KC animals treated with PD325901 showed a morphologically normal pancreas, with rare ADM where cells may have escaped MEK1/2 inhibition (Figure 1E, compare with control animals in Figure 1C), and no PanINs (Figure 1D and Supplementary Figure 2D).

Pancreatitis induces acinar cell dedifferentiation to a duct-like state that has been hypothesized to be permissive for transformation.^{21,22} Therefore, we performed co-immunofluorescence for amylase (an acinar cell marker), K19 (a ductal/PanIN cell marker), and smooth muscle actin (SMA) (a marker for activated fibroblasts). In both iKras* and KC animals treated with vehicle we observed cells expressing both amylase and K19, a feature of ADM. K19-positive PanIN lesions were observed prevalently in KC mice (Figure 1F). The areas of ADM were surrounded by SMA-positive fibroblasts, indicating an active stromal compartment. In contrast, immunofluorescence of iKras* and KC animals treated with the MEK1/2 inhibitor showed no co-localization of K19 and amylase (Figure 1F, compare with control in Figure 1C), indicating MAPK signaling is necessary for the dedifferentiation of acinar cells into duct-like cells.

The induction of acute pancreatitis results in the influx of numerous inflammatory components into the tissue.²³ To investigate the effect of inhibition of MAPK signaling in a cell-autonomous manner, we inhibited MEK1/2 using 2 different inhibitors, PD325901 and U0126, in an in vitro 3D culture system. Control, iKras*, and KC pancreata were harvested and digested with collagenase P to yield clusters of acinar cells (Figure 2A and Supplementary Figure 3A). These acinar clusters were suspended in a 3D Matrigel matrix and treated with either the MEK1/2 inhibitor (100 nmol/L PD325901 or 10 μ mol/L U0126) or dimethyl sulf-oxide. After 3–4 days in culture, control cells treated with dimethyl sulfoxide maintained their acinar morphology, as shown by the H&E staining and amylase expression, with some of the cell clusters developing into small duct-like structures expressing both K19 and amylase (Figure 2B and Supplementary Figure 3B). In contrast, almost all the cells expressing oncogenic Kras* formed large ducts that had lost most of their amylase expression and were K19 positive, or co-expressed both K19 and amylase (Figure 2B). MEK1/2 inhibition with both U0126 and PD325901 prevented ADM of iKras* and KC cells, resulting in amylase-expressing clusters with acinar-like morphology, similar to the control cells (Figure 2B and Supplementary Figure 3B and C). Thus, MAPK signaling is required for the dedifferentiation of acinar cells into duct-like cells and the subsequent initiation of tumorigenesis.

MAPK Signaling Is Required for PanIN Maintenance

We previously have shown that PanIN lesions are continuously dependent on oncogenic Kras* expression for their maintenance.⁴ Here, we sought to determine whether MAPK signaling is a key Kras* effector for PanIN maintenance. Therefore, we treated PanIN-bearing iKras* and KC mice with the MEK1/2 inhibitor PD325901. First, Kras* was activated in adult (4–6 week) iKras* mice, then pancreatitis was induced in iKras* and age-matched KC animals (n = 3–5/genotype/treatment group). Five weeks later, when tissue-wide PanINs were expected,⁴ we administered either PD325901 or vehicle. iKras* animals were treated for 1.5, 2, 3, 5, or 7 days, whereas the KC animals were treated for 2, 3, or 5 days (Figures 3A and 4A). In iKras* animals, inhibition of MAPK signaling had no effect on oncogenic Kras* expression (Supplementary Figure 4A), and Western blot analysis confirmed a decrease in pERK1/2 levels over the course of treatment (Figure 3B). As expected, both iKras* and KC animals treated with vehicle had widespread PanIN formation (Figures 3C and 4C, with quantification in Figures 3D and 4B). The lesions had abundant intracellular mucin, a characteristic of PanIN cells, identified by periodic acid–Schiff staining and expressed Claudin-18^{24,25} (Supplementary Figure 4C). Moreover, the lesions were surrounded by a collagen-rich desmoplastic stroma, measured by trichrome staining (Supplementary Figure 4D), and expressed high levels of pERK1/2 (Figures 3C and 4C). In iKras* mice, at 1.5 days after PD325901 administration, PanINs still were prevalent, although they showed dramatically reduced pERK1/2 levels (Figure 3C). Acinar cells were apparent as early as 2 and 3 days after PD325901 treatment and increased in number over the duration of MEK1/2 inhibition. Strikingly, by 5 days, very few PanIN lesions remained, and after 7 days the epithelial compartment consisted of only acinar cells and ADM (Figure 3C and quantification in D). These acinar cells derived from cells that had undergone Cre recombination and therefore expressed oncogenic Kras, as shown by expression of the lineage tracing marker EGFP (Figure 3C, insets). The question then arose of whether carcinogenesis would resume upon release of MEK1/2 inhibition. Thus, we followed a similar experimental design as previously described (5 weeks on doxycycline after acute pancreatitis, then 5 days of treatment with PD325901), then stopped administering the MEK1/2 inhibitor while keeping the mice on doxycycline for an additional 7 days (Figure 3F). Analysis of the tissues showed that ADM and PanIN formation had resumed, and pERK1/2 expression was increased (Figure 3F).

KC mice treated with the MEK1/2 inhibitor also displayed evidence of recovery in the epithelial compartment. However, this recovery was delayed because acinar cells did not arise until after 5 days of treatment, and PanIN lesions still persisted at this time point (Figure 4B and C). Interestingly, the delay in tissue repair of KC pancreata was not caused by insufficient inhibition of MAPK signaling because pERK1/2 levels were reduced at all time points (Figure 4C). The difference in recovery between the 2 models may be attributed to their Kras* expression: although both models expressed active Kras* for the duration of the experiment, Kras* was active in KC animals for multiple weeks before the induction of pancreatitis. However, alternative explanations are possible, such as the use of different promoters between the 2 models driving Kras* expression. This difference may result in driving Kras* expression at higher or lower levels on a per-cell basis. Even with the

difference in the dynamics of tissue recovery between the 2 models we show that MAPK signaling was required to maintain PanIN lesions.

PanIN Cells Redifferentiate to Acinar Cells on MEK1/2 Inhibition

Notwithstanding the different kinetics of tissue recovery in KC and iKras* mice, the 2 sets of samples shared some key characteristics. Upon MEK1/2 inhibition, both iKras* and KC samples showed a decrease in the number of proliferating PanIN cells (Figures 3D and 4B) as measured by both proliferating cell nuclear antigen and Ki67 immunostaining (Figures 3C and 4C, and Supplementary Figure 4E), despite the continued expression of Kras* (continuous doxycycline administration). Strikingly, upon MEK1/2 inhibition we observed an increase in proliferation in the acinar compartment, as shown by proliferating cell nuclear antigen and amylase co-staining (Figure 3C). In addition, examination of the stroma showed the loss of SMA expression in the fibroblasts (Figures 4D and 5), thus indicating the fibroblasts had lost their active status upon MEK1/2 inhibition.

To determine if the loss of PanIN lesions after PD325901 treatment was owing to cell death, we performed immunohistochemistry for cleaved caspase-3, a marker of apoptosis, as well as terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling staining. We observed rare positive staining at all time points in iKras mice (Figure 3C and Supplementary Figure 4F). Quantification of cleaved caspase-3 confirmed that the death of PanIN cells was unlikely to explain their elimination from the tissue over time (Figure 3E). In KC animals there was a modest increase in cell death after 2–3 days of MEK1/2 inhibition, but this appeared to be a transient effect because by 5 days after inhibition very few dying cells were observed (Figure 4C). We previously described redifferentiation of PanIN cells to the acinar lineage upon Kras* inactivation at the very initial stages of carcinogenesis.⁴ To investigate whether a similar phenomenon was occurring upon MEK1/2 inhibition, we performed co-immunofluorescence for amylase and K19. Both iKras* and KC animals treated with vehicle presented with PanIN lesions positive for K19 and the rare amylase-positive cell (Figure 4D, left column, and 5A, top row). After only 1.5 days of treatment with PD325901, iKras* tissues showed cells co-staining for both K19 and amylase, and few cells exclusively expressing amylase (Figure 5B, top row, and F). Twelve hours later, at the 2-day time point, amylase-positive acinar cells represented approximately 30% of the epithelial cells. Cells within the PanIN lesions that co-expressed K19 and amylase were identified easily (Figure 5C, 2d top row, and F). The percentage of acinar cells increased to almost 50% after 3 days of PD325901 administration (Figure 5D, 3d top row, and F). At this time point, almost all of the PanIN-like cells expressed amylase within the cytoplasm and K19 along the membrane (Figure 5D, 3d top right). By 5 and 7 days after treatment, the epithelial compartment was composed primarily of amylase-positive acinar cells, with occasional K19/amylase co-expressing cells (Figure 5E, top row, and F). A similar redifferentiation process also was observed in KC tissues, albeit with a slower dynamic of recovery than observed in iKras* mice (Figure 4D).

To further investigate the PanIN-acinar transition in iKras* tissues, we used transmission electron microscopy. Normal acinar cells have 2 very distinct features when observed under transmission electron microscopy; first, the nucleus is round and located basally, and,

second, the cytoplasm is filled with large zymogen granules.^{26,27} In contrast, PanIN cells had few small zymogen granules in their cytoplasm, highly atypical nuclei, and microvilli on their luminal edge (Figure 5A, bottom row). At 1.5 days of MEK1/2 inhibition, we observed rare cells presenting with an increased number of zymogen granules (Figure 5B, bottom row). After 2 days of treatment, the tissue was heterogeneous with acinar-like cells containing large zymogen granules and surrounding a smaller lumen (Figure 5C, bottom left) intermixed with residual cells with PanIN morphology. Interestingly, among the latter, a subset of cells had abundant vesicles in the cytoplasm (Figure 5C, bottom right). After 3 days, acinar cells characterized by large granules, small lumens, and rounder nuclei became prevalent (Figure 5D, bottom left). Within the residual PanIN-like cell population, most contained large, increased zymogen granules (Figure 5D, bottom right). By 5 and 7 days after MEK1/2 inhibition, the emerging acinar cells appeared morphologically normal with large, numerous zymogen granules, round nuclei, and surrounded a small lumen (Figure 5E). The increase in zymogen granules correlated with the increased expression of amylase and elastase over the course of PD325901 treatment (Figure 5F), providing further evidence that the PanIN cells were redifferentiating into acinar cells.

To further characterize the mechanism of epithelial reprogramming, we looked at the expression of pancreatic differentiation and progenitor markers. *Mist1* has been shown to be responsible for the induction and maintenance of the acinar cell differentiation, even in the context of oncogenic *Kras** expression.^{28–30} *Mist1* is not expressed in PanIN lesions (Figure 6A, top). Inhibition of MAPK signaling resulted in rapid up-regulation of *Mist1* messenger RNA in both iKras* and KC tissues (Figures 4E and E), and re-expression of *Mist1* in PanIN lesions (Figure 6A). The number of *Mist1*-positive cells increased over the course of treatment until most of the acinar-epithelial cells expressed *Mist1* at 7 days (Figure 6A). In parallel with the increase in *Mist1* expression, we observed an increase in *Gata6*, a transcription factor shown to regulate *Mist1*, at the early time points (Figure 6E and Supplementary Figure 4B).³¹ We also analyzed the expression of the pancreatic progenitor markers *Hes1*, *Pdx1*, and *Sox9*.^{32–34} All 3 of these markers are up-regulated in PanIN lesions upon *Kras** activation.⁴ MEK1/2 inhibition did not initially affect the expression of these factors. However, once PanIN regression had occurred and acinar cells had formed, their expression was reduced (Figure 6B–F). Expression of *Pdx1* and the progenitor markers *Foxa1* and *Foxa2*, which regulate *Pdx1* expression during pancreatic development,³⁵ also decreased upon MEK1/2 inhibition (Figure 6E and Supplementary Figure 4B). Interestingly, *Sox9* protein still was evident at 7 days (Figure 6D), but reverse-transcription quantitative polymerase chain reaction analysis showed that *Sox9* messenger RNA levels were reduced (Figure 6E and F). Taken together, our data show that the MAPK pathway was required for maintaining PanIN lesions by promoting acinar dedifferentiation.

Discussion

We previously showed that oncogenic *Kras** is required throughout pancreatic carcinogenesis, from precursor lesions to metastatic tumors.^{4,36} Among downstream effectors of *Kras*, the MAPK and phosphatidylinositol-3 kinase pathways have been implicated in pancreatic cancer progression; however, the relative importance of these 2 pathways during different stages of pancreatic carcinogenesis is still a matter of debate.^{37,38}

This question is highly clinically relevant because inhibitors to both pathways are being tested in clinical trials against a variety of solid tumors.^{39,40} Moreover, the biological role of each of these pathways in the normal pancreas and in pancreatic disease is poorly understood. Here, we focused on the MAPK pathway and endeavored to understand the effects of its activation during the initial stages of pancreatic carcinogenesis.

The MAPK pathway is up-regulated during pancreatitis and the initiating stages of tumorigenesis after pancreatitis.^{4,13,14} Previous studies have shown that MAPK activity is sufficient³⁷ and required⁴¹ for ADM and PanIN formation; however, the goal of our study was to explore the biological function of MAPK signaling during the early stages of pancreatic carcinogenesis.

Because Kras* is required for PanIN maintenance,⁴ we investigated the requirement for MAPK signaling in PanIN lesions. Inhibition of MEK1/2 in both iKras* and KC animals harboring PanINs resulted in regression of the lesions and the recovery of acinar cells. Interestingly, the newly formed acinar cells were highly proliferative, despite the continued inhibition of MAPK signaling, possibly because of c-Jun-N-terminal kinase/AP1 signaling.⁴² Thus, the MAPK signaling pathway is not required for acinar cell proliferation, at least in the context of PanIN regression. However, current evidence does not point at MAPK inhibition promoting acinar differentiation, rather at it being permissive for proliferation when other stimuli are present. The differential effect of MAPK inhibition on different cell types, both epithelial and mesenchymal, warrants further investigation.

Intriguingly, the mechanism underlying the recovery upon MAPK inhibition differed from that of Kras* inactivation at the same time point. Removal of Kras* expression in iKras* animals at the late PanIN stage (Kras* ON for 5 weeks) resulted in extensive cell death.⁴ In contrast, inhibition of MEK1/2 in iKras* animals yielded little to no apoptosis. It is likely that the difference observed between the inactivation of Kras* vs MEK1/2 inhibition in established PanINs occurred because blocking Kras* signaling shuts down numerous downstream pathways at once, many of which promote cell survival, subsequently resulting in cell death. Thus, MAPK inhibition recapitulates some aspects of Kras* inactivation, namely the regression of PanINs, but not others, such as the reduced survival. Thus, MAPK inhibition should be combined with the use of agents that block PanIN/tumor cell survival. Failing that, MAPK inhibition might result in quiescence of the redifferentiated cells, but release of the inhibition might lead to rapid relapse. Our results after transient MAPK inhibition do indeed indicate that carcinogenesis resumes once the inhibition is released. These findings should be considered when planning to use MAPK inhibitors as therapeutic agents in pancreatic cancer.

Interestingly, our results showed a previously unrecognized role of MAPK signaling during the dedifferentiation of pancreatic acinar cells to duct-like cells, a process that has been hypothesized to constitute the first step of pancreatic carcinogenesis.^{21,43} This process is mediated by MAPK-dependent changes in the expression of several pancreatic transcription factors that play opposing roles, with Sox9 promoting,²¹ and Nr5a2 and Mist1 preventing, dedifferentiation.^{30,44} MEK1/2 inhibition resulted in the rapid up-regulation of the acinar transcription factor Mist1 in PanIN cells, which preceded their redifferentiation to acinar

cells (Figure 7). Our data support previous findings indicating that forced expression of *Mist1* in neoplastic tissue results in the recovery of acinar cells.³⁰ In contrast, transcription factors such as *Hes1* and *Pdx1* that are expressed in PanINs were repressed during acinar redifferentiation.

Taken together, our data show that MAPK is required for both the formation and maintenance of PanIN lesions, and that it functions by promoting dedifferentiation of acinar cells. Therefore, during the initiation and progression of pancreatic cancer, the MAPK pathway hijacks the plasticity of the tissue to promote tumorigenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Dr Yaqing Zhang, Esha Mathew, Arthur L. Brannon III, and Kevin T. Kane for scientific discussion and reading of the manuscript; Marsha Thomas for laboratory support; and Dr Yaqing Zhang for help with tissue pathology and quantification. The p48-Cre mouse and *Pdx1* antibody were generous gifts from Dr Chris Wright (Vanderbilt University), the *Mist1* antibody was generously provided by Dr Stephen Konieczny (Purdue University), and the *Hes1* antibody was a generous gift from Dr Ben Stanger (University of Pennsylvania). The K19 antibody (Troma III) was obtained from the Iowa Developmental Hybridoma Bank.

Funding

This project was supported by the University of Michigan Biological Scholar Program, the University of Michigan Comprehensive Cancer Center, GI-SPORE P50CA13810 and NCI-1R01CA151588-01, a University of Michigan Program in Cellular and Molecular Biology training grant (National Institutes of Health T32 GM07315), and by a University of Michigan Center for Organogenesis training grant (5-T32-HD007515) (M.A.C.), and also was supported by R01 CA155198-02 (J.S.S.L.). The sponsors had no role in the study design.

Abbreviations used in this paper

ADM	acinar-ductal metaplasia
ERK1/2	extracellular signal-regulated kinase1/2
iKras*	inducible-Kras ^{G12D} (p48Cre, TetO-Kras ^{G12D} , Rosa26 ^{rtTa-IRES-EGFP})
KC	p48Cre, LSL-Kras ^{G12D}
Kras*	inp48Cre
TetO-KrasG12D	Rosa26 ^{rtTa-IRES-EGFP}
MAPK	mitogen-activated protein kinase
MEK1/2	mitogen-activated protein kinase kinase
PanIN	pancreatic intraepithelial neoplasia
SMA	smooth muscle actin
3D	3-dimensional

References

1. Jones S, Zhang X, Parsons DW, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science*. 2008; 321:1801–1806. [PubMed: 18772397]
2. Appels NM, Beijnen JH, Schellens JH. Development of farnesyl transferase inhibitors: a review. *Oncologist*. 2005; 10:565–578. [PubMed: 16177281]
3. Kanda M, Matthaei H, Wu J, et al. Presence of somatic mutations in most early-stage pancreatic intraepithelial neoplasia. *Gastroenterology*. 2012; 142:730–733. e9. [PubMed: 22226782]
4. Collins MA, Bednar F, Zhang Y, et al. Oncogenic Kras is required for both the initiation and maintenance of pancreatic cancer in mice. *J Clin Invest*. 2012; 122:639–653. [PubMed: 22232209]
5. Ying H, Kimmelman AC, Lyssiotis CA, et al. Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. *Cell*. 2012; 149:656–670. [PubMed: 22541435]
6. Hingorani SR, Petricoin EF, Maitra A, et al. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell*. 2003; 4:437–450. [PubMed: 14706336]
7. Dhillon AS, Hagan S, Rath O, et al. MAP kinase signalling pathways in cancer. *Oncogene*. 2007; 26:3279–3290. [PubMed: 17496922]
8. Yan L, McFaul C, Howes N, et al. Molecular analysis to detect pancreatic ductal adenocarcinoma in high-risk groups. *Gastroenterology*. 2005; 128:2124–2130. [PubMed: 15940643]
9. Parsons BL, Meng F. K-RAS mutation in the screening, prognosis and treatment of cancer. *Biomark Med*. 2009; 3:757–769. [PubMed: 20477713]
10. Lowenfels AB, Maisonneuve P, Cavallini G, et al. Pancreatitis and the risk of pancreatic cancer. International Pancreatitis Study Group. *N Engl J Med*. 1993; 328:1433–1437. [PubMed: 8479461]
11. Morris JP, Cano DA, Sekine S, et al. Beta-catenin blocks Kras-dependent reprogramming of acini into pancreatic cancer precursor lesions in mice. *J Clin Invest*. 2010; 120:508–520. [PubMed: 20071774]
12. Guerra C, Collado M, Navas C, et al. Pancreatitis-induced inflammation contributes to pancreatic cancer by inhibiting oncogene-induced senescence. *Cancer Cell*. 2011; 19:728–739. [PubMed: 21665147]
13. Duan RD, Williams JA. Cholecystokinin rapidly activates mitogen-activated protein kinase in rat pancreatic acini. *Am J Physiol*. 1994; 267:G401–G408. [PubMed: 7943237]
14. Duan RD, Zheng CF, Guan KL, et al. Activation of MAP kinase kinase (MEK) and Ras by cholecystokinin in rat pancreatic acini. *Am J Physiol*. 1995; 268:G1060–G1065. [PubMed: 7611406]
15. Mazzone E, Impellizzeri D, Di Paola R, et al. Effects of mitogen-activated protein kinase signaling pathway inhibition on the development of cerulein-induced acute pancreatitis in mice. *Pancreas*. 2012; 41:560–570. [PubMed: 22228051]
16. Kawaguchi Y, Cooper B, Gannon M, et al. The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. *Nat Genet*. 2002; 32:128–134. [PubMed: 12185368]
17. Fisher GH, Wellen SL, Klimstra D, et al. Induction and apoptotic regression of lung adenocarcinomas by regulation of a K-Ras transgene in the presence and absence of tumor suppressor genes. *Genes Dev*. 2001; 15:3249–3262. [PubMed: 11751631]
18. Belteki G, Haigh J, Kabacs N, et al. Conditional and inducible transgene expression in mice through the combinatorial use of Cre-mediated recombination and tetracycline induction. *Nucleic Acids Res*. 2005; 33:e51. [PubMed: 15784609]
19. Kojima K, Vickers SM, Adsay NV, et al. Inactivation of Smad4 accelerates Kras(G12D)-mediated pancreatic neoplasia. *Cancer Res*. 2007; 67:8121–8130. [PubMed: 17804724]
20. Zhang YQ, Morris JP, Yan W, et al. Canonical Wnt signaling is required for pancreatic carcinogenesis. *Cancer Res*. 2013; 73:4909–4922. [PubMed: 23761328]
21. Kopp JL, von Figura G, Mayes E, et al. Identification of Sox9-dependent acinar-to-ductal reprogramming as the principal mechanism for initiation of pancreatic ductal adenocarcinoma. *Cancer Cell*. 2012; 22:737–750. [PubMed: 23201164]

22. Stanger BZ, Hebrok M. Control of cell identity in pancreas development and regeneration. *Gastroenterology*. 2013; 144:1170–1179. [PubMed: 23622126]
23. Zheng L, Xue J, Jaffee EM, et al. Role of immune cells and immune-based therapies in pancreatitis and pancreatic ductal adenocarcinoma. *Gastroenterology*. 2013; 144:1230–1240. [PubMed: 23622132]
24. Ray KC, Bell KM, Yan J, et al. Epithelial tissues have varying degrees of susceptibility to Kras(G12D)-initiated tumorigenesis in a mouse model. *PLoS One*. 2011; 6:e16786. [PubMed: 21311774]
25. Karanjawala ZE, Illei PB, Ashfaq R, et al. New markers of pancreatic cancer identified through differential gene expression analyses: claudin 18 and annexin A8. *Am J Surg Pathol*. 2008; 32:188–196. [PubMed: 18223320]
26. Bendayan M, Roth J, Perrelet A, et al. Quantitative immunocytochemical localization of pancreatic secretory proteins in subcellular compartments of the rat acinar cell. *J Histochem Cytochem*. 1980; 28:149–160. [PubMed: 7354212]
27. Bendayan M, Londono I, Paransky E. Compartmentalization of pancreatic secretory zymogen granules as revealed by low-voltage transmission electron microscopy. *J Histochem Cytochem*. 2011; 59:899–907. [PubMed: 21832147]
28. Drenzo D, Hess DA, Damsz B, et al. Induced Mist1 expression promotes remodeling of mouse pancreatic acinar cells. *Gastroenterology*. 2012; 143:469–480. [PubMed: 22510200]
29. Shi G, Zhu L, Sun Y, et al. Loss of the acinar-restricted transcription factor Mist1 accelerates Kras-induced pancreatic intraepithelial neoplasia. *Gastroenterology*. 2009; 136:1368–1378. [PubMed: 19249398]
30. Shi G, DiRenzo D, Qu C, et al. Maintenance of acinar cell organization is critical to preventing Kras-induced acinar-ductal metaplasia. *Oncogene*. 2013; 32:1950–1958. [PubMed: 22665051]
31. Martinelli P, Canamero M, Del Pozo N, et al. Gata6 is required for complete acinar differentiation and maintenance of the exocrine pancreas in adult mice. *Gut*. 2013; 62:1481–1488. [PubMed: 23002247]
32. Murtaugh LC, Stanger BZ, Kwan KM, et al. Notch signaling controls multiple steps of pancreatic differentiation. *Proc Natl Acad Sci U S A*. 2003; 100:14920–14925. [PubMed: 14657333]
33. Seymour PA, Freude KK, Tran MN, et al. SOX9 is required for maintenance of the pancreatic progenitor cell pool. *Proc Natl Acad Sci U S A*. 2007; 104:1865–1870. [PubMed: 17267606]
34. Criscimanna A, Speicher JA, Houshmand G, et al. Duct cells contribute to regeneration of endocrine and acinar cells following pancreatic damage in adult mice. *Gastroenterology*. 2011; 141:1451–1462. 1462 e1–6. [PubMed: 21763240]
35. Gao N, LeLay J, Vatamaniuk MZ, et al. Dynamic regulation of Pdx1 enhancers by Foxa1 and Foxa2 is essential for pancreas development. *Genes Dev*. 2008; 22:3435–3448. [PubMed: 19141476]
36. Collins MA, Brisset JC, Zhang Y, et al. Metastatic pancreatic cancer is dependent on oncogenic Kras in mice. *PLoS One*. 2012; 7:e49707. [PubMed: 23226501]
37. Collisson EA, Trejo CL, Silva JM, et al. A central role for RAF→MEK→ERK signaling in the genesis of pancreatic ductal adenocarcinoma. *Cancer Discov*. 2012; 2:685–693. [PubMed: 22628411]
38. Eser S, Reiff N, Messer M, et al. Selective requirement of PI3K/PDK1 signaling for Kras oncogene-driven pancreatic cell plasticity and cancer. *Cancer Cell*. 2013; 23:406–420. [PubMed: 23453624]
39. Santarpia L, Lippman SM, El-Naggar AK. Targeting the MAPK-RAS-RAF signaling pathway in cancer therapy. *Expert Opin Ther Targets*. 2012; 16:103–119. [PubMed: 22239440]
40. Downward J. Targeting RAS and PI3K in lung cancer. *Nat Med*. 2008; 14:1315–1316. [PubMed: 19057554]
41. Ardito CM, Gruner BM, Takeuchi KK, et al. EGF receptor is required for KRAS-induced pancreatic tumorigenesis. *Cancer Cell*. 2012; 22:304–317. [PubMed: 22975374]
42. Guo L, Sans MD, Hou Y, et al. c-Jun/AP-1 is required for CCK-induced pancreatic acinar cell dedifferentiation and DNA synthesis in vitro. *Am J Physiol Gastrointest Liver Physiol*. 2012; 302:G1381–G1396. [PubMed: 22461029]

43. Morris JP, Wang SC, Hebrok M. KRAS, Hedgehog, Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma. *Nat Rev Cancer*. 2010; 10:683–695. [PubMed: 20814421]
44. von Figura G, Morris JP, Wright CV, et al. Nr5a2 maintains acinar cell differentiation and constrains oncogenic Kras-mediated pancreatic neoplastic initiation. *Gut*. 2013 Epub ahead of print.

Figure 1.

Inhibition of the MAPK pathway prevents initiation of tumorigenesis. (A) Experimental design (n = 3/genotype/treatment group). (B) Western blot for pERK1/2 and total ERK1/2 in control, iKras*, and KC tissues treated with vehicle (-) or PD325901(+). (C) H&E staining (scale bars: 50 μ m), pERK1/2 immunohistochemistry (inset, scale bar: 20 μ m), and co-immunofluorescence for K19 (PanIN/ducts), amylase (acinar cells), and SMA (activated fibroblasts), with nuclei denoted by 4',6-diamidino-2-phenylindole (DAPI) for control animals treated with either vehicle or PD325901. (D) H&E staining (scale bars: 50 μ m), (E) pERK1/2 immunohistochemistry (scale bars: 20 μ m), and (F) K19, amylase, SMA co-immunofluorescence (scale bars: 20 μ m) shows MEK1/2 is required for the initiation and formation of PanIN lesions in iKras* and KC pancreata.

Figure 2. MEK1/2 inhibition blocks ADM in 3D culture. Bright-field, H&E staining, and K19/ amylase co-immunofluorescence of 3D acinar clusters from control, iKras*, and KC pancreata (A) 1 day and (B) 3 days after dimethyl sulfoxide (DMSO) or PD325901 treatment. Scale bars: 20 μm.

Figure 3.

MAPK signaling is required for the maintenance of PanIN lesions in iKras* mice. (A) Experimental design (n = 3–5/treatment group). (B) Western blot for pERK1/2 and total ERK1/2 from control and iKras* mice treated with vehicle (–) or PD325901. (C) H&E staining (scale bars: 50 μ m) and immunostainings for pERK1/2, proliferating cell nuclear antigen (PCNA)/K19/amylose (nuclei denoted by DAPI, yellow arrows highlight PCNA/K19-positive cells and white arrows indicate PCNA/amylose-positive cells), and cleaved caspase 3 in iKras* tissues treated with either vehicle or PD325901 (scale bars: 20 μ m). (D) Histopathologic analysis, data represent mean $\pm$ SEM. (E) Quantification of the average

number of cleaved caspase 3–positive cells per 20× field of view, each point indicates one animal. (F) Experimental design, H&E (*scale bar*: 50 μ m) and pERK1/2 (*scale bar*: 20 μ m) staining for iKras (n = 3) mice after the release of MEK1/2 inhibition.

Figure 4. MEK1/2 inhibition in KC animals results in PanIN regression. (A) Experimental design (n = 3–5/treatment group). (B) Histopathologic analysis, data represent mean $\pm$ SEM. (C) H&E staining (scale bars: 50 μ m) and immunohistochemistry for pERK1/2, Ki67, and cleaved caspase 3 in KC pancreata treated with vehicle or PD325901 (scale bars: 20 μ m). (D) Co-immunofluorescence for K19, amylase, and SMA in KC tissues upon MEK1/2 inhibition (scale bars: 20 μ m). Quantification of K19-positive, amylase-positive, and K19/amylase double-positive epithelial cells.

Figure 5. Redifferentiation of PanIN cells into acinar cells upon MAPK inhibition in iKras* mice. Co-immunofluorescence for K19, amylase, and SMA (top rows, scale bar: 20 μ m) and transmission electron microscopy (TEM) (bottom rows, scale bar: 2 μ m) for iKras* animals treated with (A) vehicle or (B) PD325901 for 1.5 days, (C) 2 days, (D) 3 days, (E) 5 days and 7 days. Luminal spaces (L), and zymogen granules (ZG and arrows) are highlighted in the TEM images. (F) Quantification of K19-positive, amylase-positive, and K19/amylase double-positive epithelial cells and reverse-transcription quantitative polymerase chain reaction for acinar markers amylase and elastase relative to the epithelial marker E-cadherin.

Figure 6.

MAPK signaling regulates the expression of pancreatic transcription factors.

Immunohistochemistry for (A) *Mist1*, (B) *Hes1*, (C) *Pdx1*, and (D) *Sox9*, for iKras animals treated with either vehicle or PD325901 (scale bars: 20 μ m). Reverse-transcription quantitative polymerase chain reaction for *Mist1*, *Hes1*, *Pdx1*, and *Sox9* for (E) iKras* and (F) KC tissues.

Figure 7.

MAPK signaling prevents acinar differentiation in iKras* mice. Proposed model for the role of the MAPK pathway during pancreatic carcinogenesis. Oncogenic Kras* expression results in active MAPK signaling. Up-regulation of MAPK transduction in acinar cells results in acinar-ductal metaplasia and eventually dedifferentiation of acini into PanIN lesions. Inhibition of the MAPK in PanIN lesions results in a redifferentiation of the precancerous cells back into acinar cells.