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Pancreatitis-Induced Inflammation Contributes to Pancreatic Cancer by Inhibiting Oncogene-Induced Senescence

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SUMMARY

Pancreatic acinar cells of adult mice (≥P60) are resistant to transformation by some of the most robust oncogenic insults including expression of K-*Ras* oncogenes and loss of *p16Ink4a/p19Arf* or *Trp53* tumor suppressors. Yet, these acinar cells yield pancreatic intraepithelial neoplasias (mPanIN) and ductal adenocarcinomas (mPDAC) if exposed to limited bouts of non-acute pancreatitis, providing they harbor K-*Ras* oncogenes. Pancreatitis contributes to tumor progression by abrogating the senescence barrier characteristic of low-grade mPanINs. Attenuation of pancreatitis-induced inflammation also accelerates tissue repair and thwarts mPanIN expansion. Patients with chronic pancreatitis display senescent PanINs, providing they have received antiinflammatory drugs. These results support the concept that antiinflammatory treatment of people diagnosed with pancreatitis may reduce their risk of developing PDAC.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the tumors with worst prognosis, with <5% of patients surviving 5 years after diagnosis. During the last few years there have been important advances in our understanding of the molecular events responsible for the development of PDAC (Jones et al., 2008; Hruban and Adsay, 2009). Yet, progress in prevention, early diagnosis and therapeutic treatment of PDAC has not experienced major advances (Hidalgo, 2010).

The development of genetically modified mouse tumor models of PDAC offers the possibility to replicate in experimental systems the multiple events that lead to this complex disease (Hingorani et al., 2003; Guerra et al., 2007). In these models, inducible expression of a resident K-*Ras* oncogene during embryonic development triggers preneoplastic pancreatic intraepithelial lesions (mPanINs), which can progress into invasive mPDAC. Addition of other mutations observed in human PDAC, including inactivation of the *P16INK4A/P19ARF*, *TRP53*, or *SMAD4* tumor suppressors as well as activation of the HEDGEHOG signaling pathway, significantly accelerate tumor development leading to acquisition of a metastatic phenotype (Aguirre et al., 2003; Hingorani et al., 2005; Bardeesy et al., 2006; Ijichi et al., 2006; Pasca di Magliano et al., 2006).

Human PDAC is likely to originate from somatic mutations in K-*RAS* during adulthood rather than during embryonic development. We have previously shown that activation of a resident K-*Ras* oncogene in adult mice does not induce mPanINs and mPDAC (Guerra et al., 2007). Recently, it has been shown that rat adult acinar cells are also refractory to

Significance

Pancreatic ductal adenocarcinoma (PDAC) is one of the tumor types with worst prognosis. Efforts to understand its etiology and early development may help to save lives. Here we report that brief bouts of asymptomatic pancreatitis in adult mice lead to mPDAC as long as the acinar cells express K-Ras oncogenes. K-Ras mutations occurring after pancreatitis also induce mPDAC providing that the inflammatory response has not subsided. Inflammation contributes to mPDAC by eliminating the senescence program characteristic of benign lesions. Samples obtained from pancreatitis patients also display senescence markers but only if they have received antiinflammatory therapy. Thus, antiinflammatory treatment of patients diagnosed with even mild bouts of pancreatitis may prevent, or at least reduce the risk of developing PDAC.

transformation by Ras oncogenes (Tanaka et al., 2010). The resistance of adult acinar cells to K-*Ras* oncogenes may stem from the exhaustion of permissive acinar progenitors or from intrinsic changes in the biology of acinar cells during adulthood.

In this study, we have examined whether the resistance of adult acinar cells to malignant transformation by K-*Ras* oncogenes may result from the existence of a proliferative and/or senescence barrier mediated by p16Ink4a, a gene frequently inactivated in human PDAC, or by other tumor suppressors also implicated in PDAC development such as Trp53 (Collado et al., 2005). In addition, we have examined whether limited episodes of pancreatitis may cooperate with K-*Ras* oncogenes to induce mPDAC and have unveiled some of the mechanisms by which pancreatitis contributes to mPanIN development. Finally, we have extended these observations to human biopsies obtained from pancreatitis and PDAC patients.

RESULTS

Adult Acinar Cells Are Resistant to Multiple Oncogenic Insults

Expression of an endogenous K-*Ras* oncogene in acinar cells of K-*Ras*$^{+/LSLG12Vgeo}$ mice (designated from now on as K-*Ras*$^{+/G12V}$) during late embryonic development (E16.5) leads to the generation of acinar-to-ductal metaplasias followed by the appearance of low and high-grade mPanINs that occasionally progress to mPDAC (Guerra et al., 2007). The neoplastic nature of these lesions has been validated by other investigators (Hruban et al., 2006). The percentage of K-*Ras*^{G12V}-expressing acinar cells susceptible of inducing mPanIN lesions become significantly reduced during early postnatal development (see Figure S1A available online). In agreement with other laboratories (Gidekel Friedlander et al., 2009; Morris et al., 2010), induction of K-*Ras*^{G12V} expression at 3 (P21) and 6 (P42) weeks of age resulted in progressive reduction in the number of mice that developed mPanINs and to a significant delay in the onset of mPDAC. Acinar cells become resistant to mPanIN development when mice become 2 months old (P60) (Figure S1). Other groups have described frequent mPanIN induction on expression of K-*Ras* oncogenes in acinar cells of 6-week-old mice (De La O et al., 2008; Habbe et al., 2008). Although the bases for these variations are unknown, it is possible that genetic background may determine the precise timing at which postnatal acinar cells become resistant to transformation by K-*Ras* oncogenes.

Next, we examined whether this resistance could be overcome by inactivation of the oncogene-induced senescence (OIS) barrier likely to be mediated by tumor suppressors such as *p16Ink4a/p19Arf* or *Trp53* (Collado and Serrano, 2010). Compound K-*Ras*$^{+/G12V}$;*Elas*-tTA/*tetO*-Cre;p16*Ink4a*/p19*Arf*$^{lox/lox}$ (n = 13) and K-*Ras*$^{+/G12V}$;*Trp53*$^{lox/lox}$;*Elas*-tTA/*tetO*-Cre (n = 7) mice were exposed to doxycycline from conception until P60 to prevent expression of the *Elastase*-driven Cre recombinase. Removal of doxycycline from the drinking water allowed K-*Ras*^{G12V} expression in adult acinar cells along with concomitant ablation of the *p16Ink4a/p19Arf*^{lox} and *Trp53*^{lox} conditional alleles, respectively (Figures 1A and 1B). Mice were examined for the presence of pancreatic lesions 6 and 12 months after removal of doxycycline. Surprisingly, none of the pancreata presented detectable lesions including metaplasias or low-grade mPanINs in spite of serial analysis of the entire organ (Figures 1A and 1B). Excision of the conditional *p16Ink4a/p19Arf*^{lox} and *Trp53*^{lox} alleles in K-*Ras*^{G12V}-expressing acinar cells was confirmed by PCR analysis of DNA extracted from β-galactosidase expressing cells, the surrogate marker for K-*Ras*^{G12V} expression (Guerra et al., 2007), with the help of a laser-capture microscope (Figures S1B and S1C). These observations indicate that adult acinar cells are extremely resistant to malignant transformation and can tolerate some of the most robust genetic insults responsible for neoplastic development.

Loss of *p16Ink4a/p19Arf* and *Trp53* in Adult Acinar Cells Only Contribute to mPanIN and mPDAC Development in the Presence of K-*Ras* Oncogenes

In addition to K-*RAS* mutations, most human PanINs display inactivation of the *P16INK4a/P14ARF* locus. Thus, we examined whether pancreatitis may also cooperate with loss of these tumor suppressors to initiate mPanIN formation in the absence of K-*Ras* mutations. We induced pancreatitis by treating mice with daily doses of caerulein, a decapeptide analog of the pancreatic secretagogue cholecystokinin, as previously described (Guerra et al., 2007). K-*Ras*$^{+/+}$;p16*Ink4a*/p19*Arf*$^{lox/lox}$;*Elas*-tTA/*tetO*-Cre mice exposed to doxycycline until P60 were treated with caerulein and examined at 14 months of age. Systematic analysis of their pancreata (n = 8) by serial sectioning failed to reveal any mPanINs (Figures 1C and 1D). Because *TRP53* is also lost in many human PDACs, we examined whether pancreatitis cooperated with loss of this tumor suppressor to induce mPanIN formation in adult mice. K-*Ras*$^{+/+}$;*Trp53*$^{lox/lox}$;*Elas*-tTA/*tetO*-Cre mice (n = 4) exposed to doxycycline until P60 and treated with caerulein also failed to display mPanINs in spite of serially sectioning of all pancreata (Figures 1C and 1D). As expected, control K-*Ras*$^{+/G12V}$;*Elas*-tTA/*tetO*-Cre mice (8/8) submitted to the same treatments developed abundant low and high-grade mPanINs (data not shown). These observations indicate that activation of K-*Ras* oncogenes is an essential event to initiate mPanIN formation that cannot be replaced by loss of either *p16Ink4a/p19Arf* or *Trp53* tumor suppressors.

Loss of the p16*Ink4a*/p19*Arf* tumor suppressors in early pancreatic precursors during embryonic development efficiently cooperated with K-*Ras* oncogenes to induce invasive and metastatic mPDAC (Aguirre et al., 2003). Activation of K-*Ras* oncogenes along with concomitant loss of *p16Ink4a/p19Arf* in embryonic acinar cells also resulted in anaplastic carcinomas that metastasized to liver, stomach, diaphragm, lung, lymph nodes and spleen (Figures 2D and 2E). Thus, we examined whether loss of *p16Ink4a/p19Arf* could cooperate with K-*Ras*^{G12V} in adult mice. P90 K-*Ras*$^{+/G12V}$;*p16Ink4a/p19Arf*$^{lox/lox}$;*Elas*-tTA/*tetO*-Cre mice exposed to doxycycline until P60 were treated with caerulein to induce pancreatitis. As illustrated in Figure 2A, the lifespan of these mice was considerably shortened compared to mice that retained the *p16Ink4a/p19Arf* locus. Survival of heterozygous *p16Ink4a/p19Arf*$^{+/lox}$ mice was similar to that of wild-type controls (data not shown). After 3 months of caerulein treatment, the number of low-grade mPanINs did not differ significantly between *p16Ink4a/p19Arf* wild-type, heterozygous, or *null* mice (Figures 2B and 2C). However, the latter contained more high-grade lesions and developed mPDAC (Figures 2B and 2C;

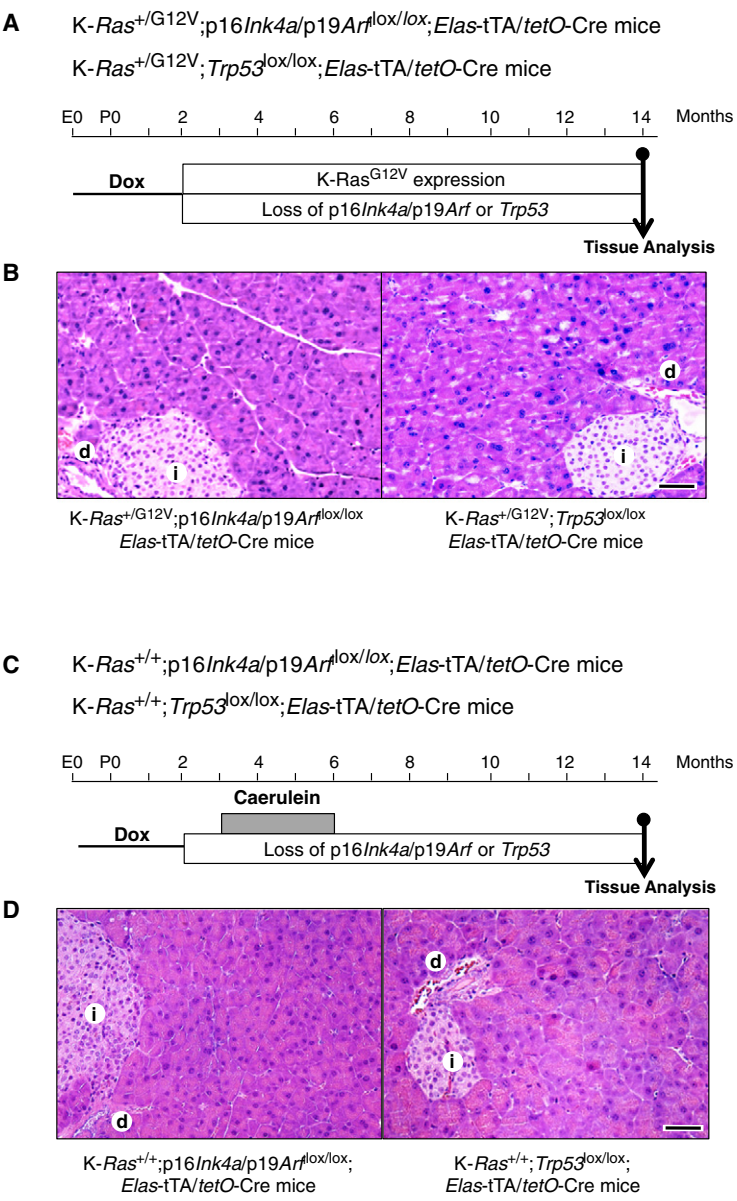

Figure 1. Loss of *p16Ink4a/p19Arf* or *Trp53* Tumor Suppressors Only Contribute to mPanIN and mPDAC Development in the Presence of K-*Ras* Oncogenes and Pancreatitis

(A) K-*Ras*$^{+/G12V}$;p16*Ink4a*/p19*Arf*$^{lox/lox}$;*Elas*-tTA/*tetO*-Cre and K-*Ras*$^{+/G12V}$;*Trp53*$^{lox/lox}$;*Elas*-tTA/*tetO*-Cre mice were exposed to doxycycline (Dox, thin line) until P60. At this time, doxycycline was removed from the drinking water to allow expression of the K-Ras^{G12V} oncogene and to ablate the p16*Ink4a*/p19*Arf* and the *Trp53* tumor suppressors in adult acinar cells.

(B) Hematoxylin and eosin (H&E) staining of paraffin sections obtained from 14-month-old (left) K-*Ras*$^{+/G12V}$;*p16Ink4a/p19Arf*$^{lox/lox}$;*Elas*-tTA/*tetO*-Cre mice and (right) K-*Ras*$^{+/G12V}$;*Trp53*$^{lox/lox}$;*Elas*-tTA/*tetO*-Cre mice shows normal parenchyma with no mPanIN lesions 1 year after turning on K-Ras^{G12V} expression and ablating the p16*Ink4a*/p19*Arf* and the *Trp53* tumor suppressors. i, islets; d, normal ducts. Scale bar represents 50 μm.

(C) K-*Ras*$^{+/+}$;p16*Ink4a*/p19*Arf*$^{lox/lox}$;*Elas*-tTA/*tetO*-Cre and K-*Ras*$^{+/+}$;*Trp53*$^{lox/lox}$;*Elas*-tTA/*tetO*-Cre mice were exposed to doxycycline (Dox, thin line) until P60. At this time, doxycycline was removed from the drinking water to allow ablation of the p16*Ink4a*/p19*Arf* and the *Trp53* tumor suppressors in adult acinar cells. Mice were subsequently treated with caerulein for three months (P90-P180) (gray box).

(D) H&E staining of paraffin sections obtained from 14-month-old (left) K-*Ras*$^{+/+}$;*p16Ink4a/p19Arf*$^{lox/lox}$;*Elas*-tTA/*tetO*-Cre mice and (right) K-*Ras*$^{+/+}$;*Trp53*$^{lox/lox}$;*Elas*-tTA/*tetO*-Cre mice shows normal parenchyma with no mPanIN lesions 8 months after finishing caerulein treatment. i, islets; d, normal duct. Scale bar represents 50 μm.

See also Figure S1.

Figures S2A–S2H). Analysis of these tumors at humane end point revealed that many of these mPDAC (Figures S2I–S2L) had also metastasized to liver and diaphragm (Figures S2M–S2P). Moreover, we also observed an occasional sarcomatoid tumor when mice were sacrificed at humane end point (data not shown). These observations indicate that loss of p16*Ink4a*/p19*Arf* contributes to pancreatic cancer in adult mice, providing that the mice express K-Ras oncogenes and have suffered from pancreatitis.

Episodic Pancreatitis Is Sufficient to Induce mPanINs and mPDAC in K-*Ras*^{G12V} Expressing Adult Acinar Cells

Chronic pancreatitis is one of the highest risk factors for the development of PDAC in humans (Lowenfels et al., 1993; Malka et al., 2002). In adult mice ($p \geq 60$), chronic pancreatitis is essential to induce mPanIN lesions and mPDAC in acinar cells expressing a resident K-*Ras*^{G12V} oncogene (Guerra et al., 2007). Yet, it is not known whether short episodes of pancreatitis may also represent a risk for PDAC development. To explore this possibility, we exposed adult (P90) K-*Ras*$^{+/G12V}$;*Elas*-tTA/*tetO*-Cre mice to caerulein for 3 months, 30 days after turning on K-*Ras*^{G12V} expression. This treatment had no detectable consequences on the overall health of the animals. In spite of its asymptomatic nature, this treatment led to significant atrophy in the parenchyma including mucinous metaplasia, edema and fibrosis regardless of whether the mice expressed a K-*Ras* oncogene (Figure S3A). In addition, all animals exhibited an inflammatory response throughout the entire parenchyma, primarily made up of macrophages and T lymphocytes (data not shown). At 8 months of age, 2 months after cessation of the caerulein treatment, all K-*Ras*$^{+/G12V}$;*Elas*-tTA/*tetO*-Cre mice (n = 15), but not control K-*Ras*$^{+/+}$;*Elas*-tTA/*tetO*-Cre animals (n = 7), displayed low and high-grade mPanIN lesions (Figure 3A). At this time, the pancreata of control mice showed limited improvement of atrophic areas and decreased numbers of inflammatory cells. Mutant mice, however, displayed increased atrophy and fibrosis, whose levels were directly related to the number and extent of the mPanIN lesions (data not shown). In these mutant mice, the inflammatory cells, mainly macrophages and T lymphocytes, were abundant and closely associated with mPanIN lesions (Figure 4B).

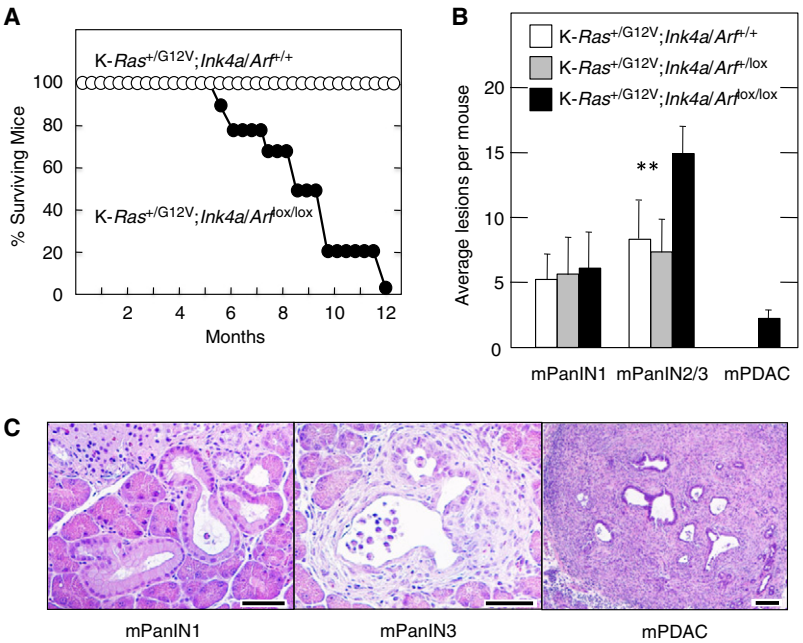

Figure 2. Loss of p16*Ink4a*/p19*Arf* Tumor Suppressors Accelerates mPanIN and mPDAC Development and Induces Anaplastic Sarcomatoid Tumors

(A) Survival of (solid circles) K-*Ras*$^{+/G12V}$;p16*Ink4a*/p19*Arf*$^{lox/lox}$;*Elas*-tTA/*tetO*-Cre and (open circles) K-*Ras*$^{+/G12V}$;p16*Ink4a*/p19*Arf*$^{+/+}$;*Elas*-tTA/*tetO*-Cre mice exposed to doxycycline until P60. At this time, doxycycline was removed from the drinking water to activate K-*Ras*^{G12V} expression and to ablate the p16*Ink4a*/p13*Arf* tumor suppressors. Caerulein treatment started at P90.

(B) Average number of mPanIN1, mPanIN2/3 and mPDAC lesions displayed at 6 months of age by (solid bars) K-*Ras*$^{+/G12V}$;p16*Ink4a*/p19*Arf*$^{lox/lox}$;*Elas*-tTA/*tetO*-Cre (n = 5), (gray bars) K-*Ras*$^{+/G12V}$;p16*Ink4a*/p19*Arf*$^{+/lox}$;*Elas*-tTA/*tetO*-Cre (n = 4) and (open bars) K-*Ras*$^{+/G12V}$;p16*Ink4a*/p19*Arf*$^{+/+}$;*Elas*-tTA/*tetO*-Cre (n = 5) mice treated as described in (A). Data shown represent mean ± SD. $^{**}p < 0.01$.

(C) H&E stained paraffin sections depicting representative (left) low-grade mPanIN1, (middle) high-grade mPanIN3, and (right) mPDAC lesions observed in the K-*Ras*$^{+/G12V}$;p16*Ink4a*/p19*Arf*$^{lox/lox}$;*Elas*-tTA/*tetO*-Cre mice described in (B). The scale bars represent (left and center) 20 μm and (right) 50 μm.

See also Figure S2.

When control K-*Ras*$^{+/+}$;*Elas*-tTA/*tetO*-Cre mice (n = 15) were examined 6 months later, their pancreata had recovered almost completely and we only observed few areas of inflammatory cells. K-*Ras*^{G12V}-expressing pancreata had also recovered except for areas of atrophy intimately associated with the mPanIN lesions. The levels of inflammatory cells had also subsided except for those closely associated with the mPanIN lesions (data not shown). At this time, all mice displayed low and high-grade mPanIN lesions (Figure 3A; Figure S3B). Moreover, 3 of the 15 K-*Ras*$^{+/G12V}$;*Elas*-tTA/*tetO*-Cre animals had developed mPDAC, of which two showed invasion of the proximal parenchyma and one displayed metastasis in liver and lung (Figure 3A; Figures S3C–S3I). These mPDACs displayed high levels of stroma similar to those observed in human patients (Figures S3C and S3D). In these lesions, inflammatory cells persisted but were located in the periphery of the stroma that surrounded the glandular structures of the tumor (Figure 4C; Figure S3F).

Next, we reduced the time of caerulein exposure to 1 month (Figure 3B). This shorter treatment also induced parenchyma atrophy and recruitment of inflammatory cells, although the

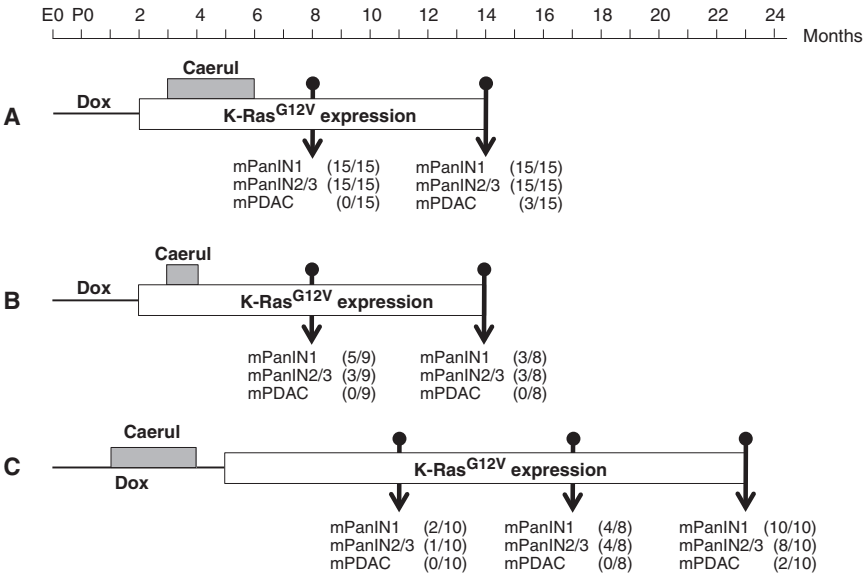

Figure 3. Episodic Pancreatitis Induces mPanINs and mPDAC in K-*Ras*$^{+/G12V}$;*Elas*-tTA/*tetO*-Cre Mice

K-*Ras*$^{+/G12V}$;*Elas*-tTA/*tetO*-Cre mice were exposed to doxycycline (thin line) and caerulein (gray box) for the indicated periods of time. The open box indicates the time of K-Ras^{G12V} expression. The number of animals positive for low-grade mPanIN1, high-grade mPanIN2/3, and mPDAC is indicated for each protocol and time point.

(A) Mice exposed to doxycycline (Dox) from conception (E0) to P60 were treated with caerulein (Caerul) for 3 months, from P90 to P180. Mice were sacrificed at 8 and 14 months of age, that is 6 and 12 months after turning on K-Ras^{G12V} expression.

(B) Mice exposed to doxycycline from conception (E0) to P60 were treated with caerulein for 1 month, from P90 to P120. Mice were sacrificed at 8 and 14 months of age, that is 6 and 12 months after turning on K-Ras^{G12V} expression.

(C) Mice exposed to doxycycline from conception (E0) to P150 were treated with caerulein for 3 months, from P30 to P120. Mice were sacrificed at 11, 17, and 23 months of age, that is, 6, 12, and 18 months after turning on K-Ras^{G12V} expression.

See also Figure S3.

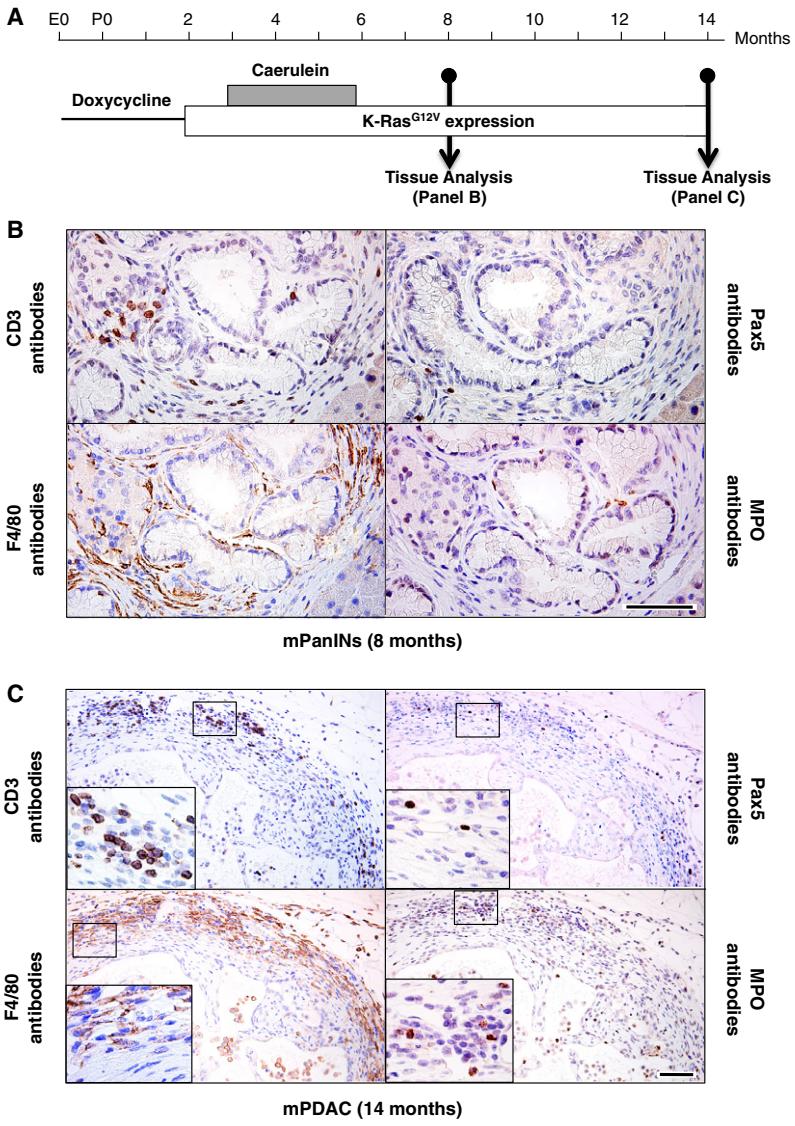

Figure 4. Inflammatory Infiltrates in mPanIN Lesions and mPDAC in K-*Ras*^{G12V} Expressing Adult Mice Treated with Caerulein for Three Months

(A) K-*Ras*$^{+/G12V}$;*Elas*-tTA/*tetO*-Cre mice were exposed to doxycycline (thin line) and caerulein (gray box) for the indicated periods of time. Expression of the K-*Ras*^{G12V} oncogene (open box) is indicated. Mice were sacrificed at 8 and 14 month of age.

(B and C) Immunostaining of inflammatory cells surrounding (B) mPanIN lesions in 8-month-old-mice (2 months after cessation of caerulein) and (C) mPDAC in 14-month-old-mice (8 months after cessation of caerulein treatment) using antibodies against T lymphocytes (CD3 antibodies), B lymphocytes (Pax5 antibodies), macrophages (F4/80 antibodies), and neutrophils (MPO antibodies). Insets show detailed areas containing the corresponding immune cells. Scale bars represent 50 μm.

extent of atrophic areas and inflammatory foci were significantly reduced (data not shown). When mice were analyzed at 8 months of age, 4 months after cessation of caerulein exposure, >50% of the mice (5/9) had developed low-grade mPanINs and three of them displayed high-grade lesions (Figure 3B; Figure S3J). At this time, the parenchyma was well preserved with few mucinous metaplasia and small focal areas of inflammatory cells, mostly associated with mPanIN lesions (data not shown). Analysis of K-*Ras*$^{+/G12V}$;*Elas*-tTA/*tetO*-Cre mice at 14 months of age revealed a similar percentage of animals with high-grade mPanINs (3/8) (Figure 3B; Figure S3J). However, none of them had developed mPDAC at this time. When we allowed these mice (n = 11) to age (1.5 and 3 years of age), we observed mPanIN lesions in most of them (10.2 ± 5.1 low-grade and 4.8 ± 2.6 high-grade mPanINs/mouse, respectively) with only one mouse failing to show any lesions. Yet, only two mice developed mPDAC (Figures S3K–S3N). These observations indicate that development of high-grade mPanIN and mPDAC in adult mice depends on the extent of tissue damage and the inflammatory response.

We also interrogated whether PDAC development required the presence of K-*Ras* oncogenes at the time of pancreatic damage. To this end, K-*Ras*$^{+/G12V}$;*Elas*-tTA/*tetO*-Cre mice were maintained in doxycycline until P150 while they were treated with caerulein from P30 to P120. Thus, mice had 1 month to recover from caerulein treatment before expressing their resident K-*Ras*^{G12V} oncogene (Figure 3C). Analysis of 10 pancreata at 6 months after turning on K-*Ras*^{G12V} expression (11 months of age) revealed the presence of mPanINs in few mice (Figure 3C). Six months later (17 months of age), the percentage of mice depicting mPanINs had increased to 50% (4/8) (Figure 3C). All animals (n = 10) displayed mPanINs on 18 months of continuous K-Ras^{G12V} expression (23 months of age) (Figure 3C; Figure S3O), and two of them had mPDACs that invaded the adjacent parenchyma (Figure 3C; Figure S3P). No lesions were observed when K-*Ras*^{G12V} expression was activated at P150 in mice not exposed to caerulein (Figure S1A). These observations indicate that K-*Ras* oncogenes can initiate mPanIN and mPDAC development in adult mice providing that there is preexisting pancreatic damage and an inflammatory response.

Pancreatitis induces expression of genes characteristic of progenitor cells such as Pdx1 (Jensen et al., 2005; Fendrich et al., 2008) and Sox9 (Seymour et al., 2007; Yoshida et al., 2008), hence raising the possibility that the susceptibility to K-Ras oncogenic signaling might be due to the induction of a less mature differentiated state. As illustrated in Figure S3Q, caerulein treatment induces high levels of Pdx1 and Sox9 in acini as well as in metaplasias. However, Pdx1 expression faded away from the acinar cells within 30 days after caerulein withdrawal and was not expressed (except in very few cells) at the time (P150) when K-Ras^{G12V} expression was turned on (Figure S3Q). As previously reported, Pdx1 becomes reexpressed in low-grade mPanINs (Hingorani et al., 2003; Guerra et al., 2007; Morris et al.,

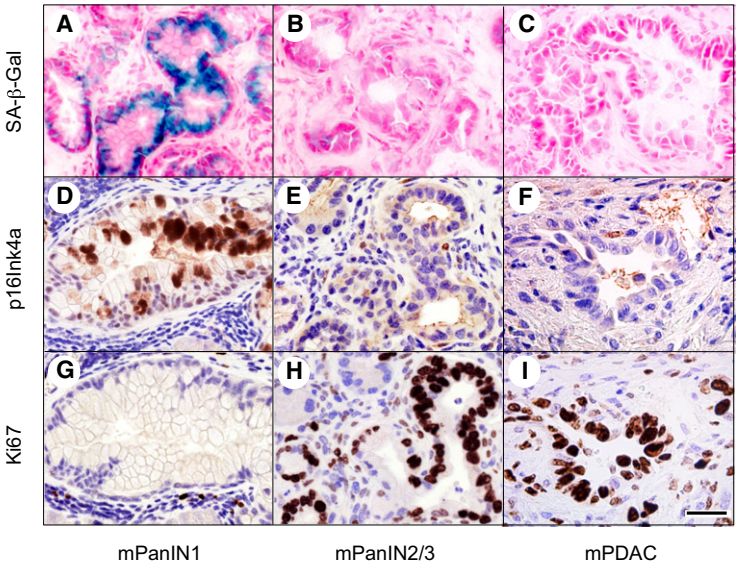

Figure 5. Senescence Markers Are a Feature of Low-Grade mPanINs, but Disappear in High-Grade mPanIN2/3 and mPDAC

(A–C) Senescence associated β-galactosidase (SA-β-Gal) staining (blue) in (A) low-grade mPanIN1 but not in (B) high-grade mPanIN2/3 or (C) mPDAC.

(D–F) p16Ink4a immunostaining (brown) in (D) low-grade mPanIN1 but not in (E) high-grade mPanIN2/3 or (F) mPDAC.

(G–I) Ki67 immunostaining (brown) inversely correlates with the expression of the above senescence markers. Ki67 is detected in a low percentage of cells of low-grade mPanIN1 (G) and in a high percentage of cells of high-grade mPanIN2/3 (H) and mPDAC (I). Note that (D–G), (E–H), and (F–I) correspond to serial sections. Scale bar represents 50 μm.

See also Figure S4.

2010). Instead, Sox9 expression was maintained throughout the transformation process including metaplasias (Figure S3Q) and mPanINs (Morris et al., 2010). As expected, the expression pattern of acinar cell markers such as chymotrypsin and elastase was retained at the time of K-Ras^{G12V} expression as well as after a month of continuous oncogene expression (Figure S3Q).

The inflammatory response observed immediately after cessation of caerulein exposure (P120) (Figure S3R) significantly subsided 30 days later in both in wild-type and mutant mice (Figure S3S) at a time, P150, in which induction of K-Ras^{G12V} expression has not yet taken place (Figure 3C; Figure S3Q). At this time, we could only observe the presence of T lymphocytes (Figure S3S). Occasionally, isolated B lymphocytes, macrophages and neutrophils were also observed (Figure S3S). At P180, that is 30 days after turning on K-Ras^{G12V} expression, the inflammatory response remained at similar levels and no significant differences were observed between wild-type and mutant mice (Figure S3T). No mPanIN lesions were observed at either P150 or P180 (Figures S3S and S3T). Whether the presence of these inflammatory cells plays a key role in the subsequent development of mPanIN lesions remains to be determined.

The Senescence Program Present in Low-Grade mPanIN Is Abrogated during Progression to High-Grade mPanIN Lesions

Metaplasias and low-grade mPanINs observed in mice expressing K-RasG12V since embryonic development display senescence markers similar to those present in preneoplastic stages of other tumor types (Collado and Serrano, 2010). However, OIS is not an immediate consequence of K-Ras^{G12V} expression because morphologically normal acinar cells expressing this oncogene do not display senescence markers, regardless of whether K-*Ras*^{G12V} expression takes place in embryonic or adult acinar cells (Figure S4A; data not shown). Thus, induction of OIS must require additional changes likely to be involved in the acinar-ductal transdifferentiation process required to generate low-grade mPanIN lesions. Low-grade mPanINs developing in K-*Ras*$^{+/G12V}$;*p16Ink4a/p19Arf*$^{lox/lox}$;*Elas*-tTA/*tetO*-Cre mice not exposed to doxycycline and hence lacking the *p16Ink4a/p19Arf* tumor suppressors since embryonic development did not display senescence markers (Figure S4B). Thus, indicating that these tumor suppressors play a key role in the induction of senescence in low-grade mPanIN lesions.

The senescence phenotype of these low-grade mPanINs disappeared during tumor progression. As illustrated in Figure 5, high-grade mPanIN2/3 lesions as well as mPDAC were negative for senescence markers including endogenous senescence-associated β-galactosidase (SA-β-Gal) and expression of the p16Ink4a tumor suppressor. Expression of these senescence markers inversely correlated with the appearance of proliferative markers such as Ki67. Whereas Ki67 was expressed in <10% of the cells in SA-β-Gal positive mPanIN1 lesions, it was detected in >50% of the cells of high-grade mPanINs and mPDAC (Figure 5).

Pancreatitis-Induced Inflammation Contributes to mPanIN Development by Inhibiting OIS

Surprisingly, senescence markers were not present in low-grade mPanINs induced by K-*Ras*^{G12V} expression in adult mice, a process that requires exposure to caerulein. Thus, we decided to ascertain whether senescence was a property of lesions initiated in the embryonic pancreas or was inhibited by caerulein-induced pancreatitis. K-*Ras*$^{+/G12V}$;*Elas*-tTA/*tetO*-Cre mice expressing K-Ras^{G12V}, since late embryonic development were either allowed to develop mPanINs by themselves or were treated with caerulein for three months. Whereas the low-grade mPanINs present in control mice (not treated with caerulein) contained senescent cells (Figures 6A–6C), none of those mPanINs present in animals exposed to caerulein displayed senescence markers, including SA-β-Gal and p16Ink4a (Figures 6D–6F).

As described above, the pathological features characteristic of pancreatitis disappeared on withdrawal of caerulein. Thus, we examined whether progressive disappearance of the inflammatory response had any effect on senescence. Adult K-*Ras*$^{+/G12V}$;*Elas*-tTA/*tetO*-Cre mice starting K-*Ras*^{G12V} expression at P60 were treated with caerulein for 3 months and either immediately sacrificed or allowed to recover for 3 additional months. Mice sacrificed at the end of the caerulein treatment

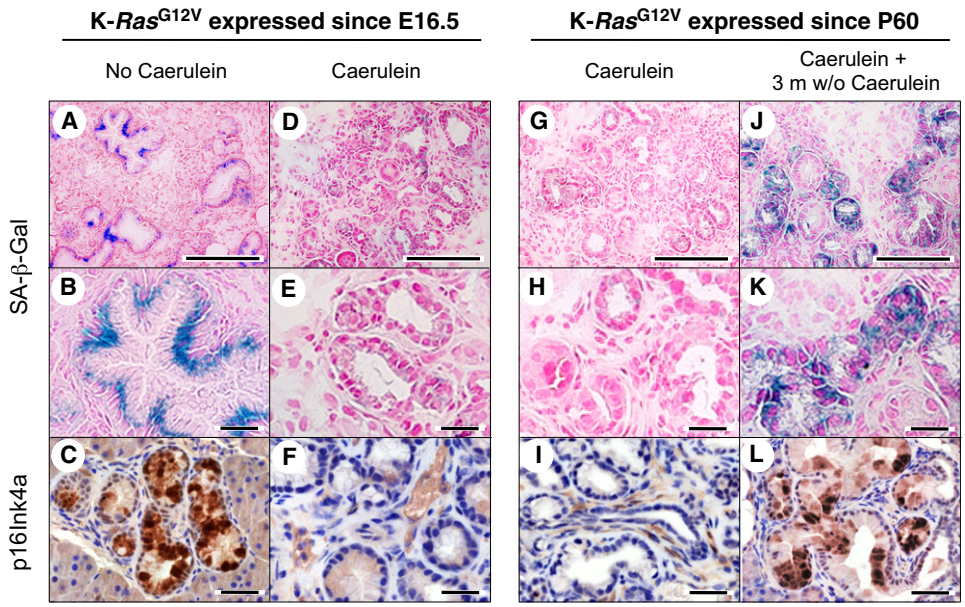

Figure 6. Senescent Low-Grade mPanINs Reappear on Partial Recovery from Pancreatitis Injury

(Left) K-*Ras*$^{+/G12V}$;*Elas*-tTA/*tetO*-Cre mice not exposed to doxycycline, thus expressing the endogenous K-*Ras*^{G12V} oncogene since embryonic development (E16.5).

(A–C) Pancreatic sections showing low-grade mPanIN1 lesions. Sections were stained for (A and B) SA-β-Gal (blue), or (C) p16Ink4a (brown) expression. (B) Amplified version of (A) to better illustrate SA-β-Gal expression in mPanIN1 lesions.

(D–F) Pancreatic sections of mice treated with caerulein for 3 months (P60 to P150) depicting low-grade mPanIN1 lesions. Sections were stained for (D and E) SA-β-Gal (blue) or (F) p16Ink4a (brown) expression. (E) Amplified version of (D) to better illustrate the absence of SA-β-Gal expression in mPanIN1 lesions.

(Right) K-*Ras*$^{+/G12V}$;*Elas*-tTA/*tetO*-Cre mice exposed to doxycycline from conception to P60, thus expressing the endogenous K-*Ras*^{G12V} oncogene since P60.

(G–I) Pancreatic sections of mice treated with caerulein for 3 months (P90–P180) showing low-grade mPanIN1 lesions. Sections were stained for (G and H) SA-β-Gal (blue) and (I) p16Ink4a (brown) expression. (H) is an amplified version of (G) to better illustrate the absence of SA-β-Gal expression in mPanIN1 lesions.

(J–L) Pancreatic sections of mice treated with caerulein for 3 months (P90–P180) and allowed to recover for 3 additional months showing low-grade mPanIN1 lesions. Sections were stained for (J and K) SA-β-Gal (blue) and (L) p16Ink4a (brown) expression. (K) Amplified version of (J) to better illustrate the reappearance of SA-β-Gal expression in mPanIN1 lesions. Scale bars represent 50 μm (A, D, G, and J) and 20 μm (B, C, E, F, H, I, K, and L).

See also Figure S5.

displayed low-grade mPanINs devoid of senescence markers (Figures 6G–6I). In contrast, those that were allowed to recover for 3 months had low-grade mPanINs positive for SA-β-Gal staining and p16Ink4a (Figures 6J–6L) as well as for PAI-1 and Sprouty-4 expression (Figure S5A). Indeed, senescence reappeared as shortly as 1 month after the cessation of caerulein exposure, a time when the inflammatory response had not completely subsided (Figure S5B). These observations indicate that OIS can be inhibited by limited episodes of pancreatitis but can reappear after the pancreatitis-induced damage has partially subsided.

Antiinflammatory Treatment Reverts Tissue Damage and Delays Progression of Pancreatitis-Induced mPanIN Lesions

To evaluate the overall contribution of inflammation to tumor progression, we studied the effect of Sulindac on mPanIN/mPDAC development. Sulindac is a nonsteroidal antiinflammatory drug thought to act on COX-1 and COX-2 enzymes (Smith et al., 1994). K-*Ras*$^{+/G12V}$;*Elas*-tTA/*tetO*-Cre animals raised in the presence of doxycycline until P60 were treated with caerulein at P90 for 3 months. At the end of the treatment, mice were allowed to recover for 3 additional months either without further treatment or treated with Sulindac. Mice not exposed to Sulindac displayed the typical lesions induced by pancreatitis such as parenchyma atrophy, edema and infiltration of inflammatory cells (Figure 7A; Figure S6A). Moreover, their pancreata exhibited multiple diffuse mPanIN lesions, ranging from low-grade mPanIN1A to invasive mPDAC, although most of them were high-grade mPanIN2/3 lesions (Figures 7A and 7B). In contrast, mice treated with Sulindac had well-preserved pancreata with few areas of parenchyma atrophy and limited infiltration of inflammatory cells (Figure 7A; Figure S6B). Moreover, we observed a dramatic reduction (75%) in the number of high-grade lesions (Figures 7A and 7B; Figure S6B). Perhaps more importantly, those lesions present in the Sulindac treated animals were considerably smaller (Figures 7A and 7C; Figure S6B). Whereas the average size of the high-grade mPanINs in control mice was 5.21 mm^2, those present in Sulindac-treated animals was only 0.14 mm^2, a dramatic 95% reduction (Figure 7C). These results strongly implicate inflammation as a key contributor to the effect of pancreatitis not only in promoting mPanIN formation, but also in inducing progression to mPDAC.

Pancreatitis-Induced Inflammation Blocks Senescence in Human PanINs

Finally, we examined whether some of these observations could be extended to human patients. To this end, we

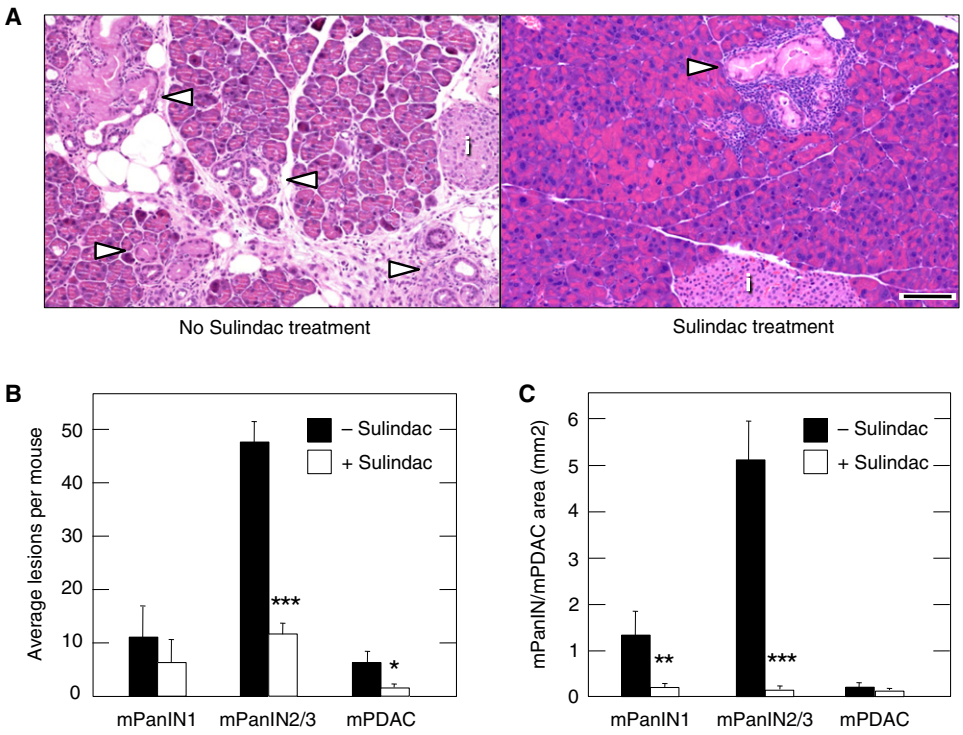

Figure 7. Inhibition of the Inflammatory Response by Sulindac Reverts Tissue Damage and Delays Progression of mPanIN Lesions

K-*Ras*$^{+/G12V}$;*Elas*-tTA/*tetO*-Cre mice were exposed to doxycycline until P60. At this time, doxycycline was removed from the drinking water to achieve expression of K-*Ras*^{G12V}. Mice were subsequently treated with caerulein for 3 months (P90–P180). Half the mice were allowed to recover for 3 months without further treatment whereas the other half was treated with Sulindac.

(A) H&E-stained paraffin sections of representative pancreata of mice (left) not treated or (right) treated with Sulindac. Note that the pancreata of the untreated mice displayed high levels of edema, fibrosis, parenchyma atrophy, and abundant large mPanIN lesions. In contrast, mice that underwent Sulindac treatment showed a well-preserved parenchyma and contained few small mPanINs. Arrowheads point to mPanIN lesions, (i) indicates an islet. Scale bar represents 50 μm.

(B) Average lesions per mouse. Solid bars indicate mice not treated with Sulindac (n = 3). Open bars correspond to mice treated with Sulindac (n = 3). Data shown represent mean ± SD. ***$p < 0.00017$, *$p < 0.036$.

(C) Area of mPanIN1, mPanIN2/3 and mPDAC lesions observed in serial pancreata sections. Solid bars indicate mice not treated with Sulindac (n = 3). Open bars correspond to mice treated with Sulindac (n = 3). Data shown represent mean ± SD. **$p < 0.0037$, ***$p < 0.00012$.

See also Figure S6.

examined the expression of P16INK4a as a marker of senescence in samples obtained from pancreata of six patients with PDAC that also contained low-grade PanINs. Whereas none of the cells within the PDAC expressed detectable levels of this tumor suppressor, all six low-grade PanINs presented a very strong nuclear signal when probed with anti P16INK4a antibodies (Figure 8A). Parallel analysis of Ki67 staining, a marker for proliferation, revealed an inverse correlation with P16INK4a immunostaining (Figure 8C), an observation highly reminiscent of the results obtained in mice. These findings indicate that human low-grade PanINs also contain senescent cells.

Next, we examined the presence of senescent cells in PanINs present in surgically removed samples from nine patients suffering from chronic pancreatitis. The clinical histories of these patients are summarized in Table S1. To our surprise, low-grade PanINs were strongly positive for P16INK4a expression only in four of the nine samples (Figure 8B). Examination of the clinical history of these patients revealed that the four patients whose samples displayed senescence markers had received antiinflammatory treatments. Whereas two of them had been treated with prednisolone, the other two had received NSAIDs. In contrast, none of the five patients that lacked of P16INK4a expression had received antiinflammatory therapy (Figure 8B; Figure S7). These pancreatitis-derived biopsies also displayed significant differences when they were analyzed for Ki67 expression. As illustrated in Figure 8C, PanINs present in biopsies derived from pancreatitis patients that have received antiinflammatory treatment were essentially negative for Ki67. In contrast, those PanINs present in biopsies derived from untreated patients contained high levels of Ki67. These results strongly suggest that inhibition of the inflammatory response induced by pancreatitis helped to maintain senescence, possibly contributing to the clinical benefit provided by antiinflammatory drugs.

DISCUSSION

Most cancers arise from somatic mutations during adulthood. Thus, proper understanding of how tumors are initiated requires modeling cancer in adult animals. In pancreas, the susceptibility to transformation by a resident K-*Ras* oncogene displayed by

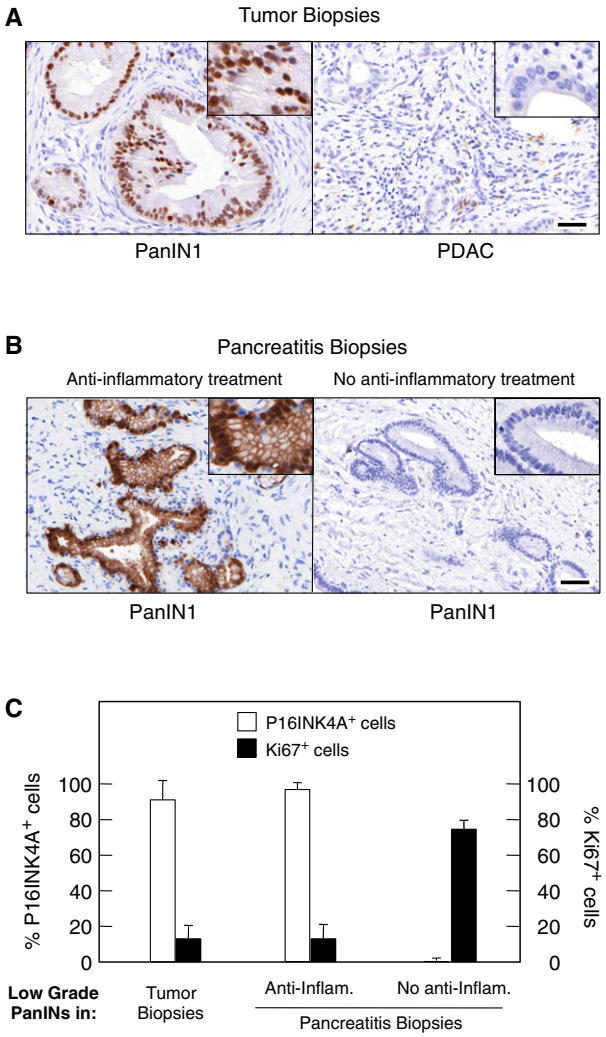

Figure 8. Senescence Markers in Human Low-Grade PanINs

(A) Low-grade PanINs present in biopsies from PDAC patients display senescence markers. (Left) Sections showing low-grade PanIN1s are positive for P16INK4a immunostaining. (Right) Sections depicting PDAC lesions are negative for P16INK4a immunostaining. Insets show amplified images to illustrate nuclear staining in PanIN1 lesions. Scale bar represents 50 μm.

(B and C) Antiinflammatory treatment restores senescence in low-grade PanIN of chronic pancreatitis patients. (B) P16INK4a immunostaining of biopsies obtained from patients suffering from chronic pancreatitis. (Left) Representative section displaying low-grade PanIN1s positive for P16INK4a staining present in a biopsy obtained from a patient treated with antiinflammatory drugs. (Right) Representative section displaying low-grade PanIN1s negative for P16INK4a staining present in a biopsy obtained from a patient not-treated with antiinflammatory drugs. Insets show amplified images to illustrate nuclear staining in PanIN1 lesions. Scale bar represents 50 μm. (C) Quantification of the percentage of cells present in low-grade PanIN1 lesions positive for (open bars) P16INK4a immunostaining or (solid bars) Ki67 staining. Samples were obtained from (left) PDAC patients, (center) chronic pancreatitis patients treated with antiinflammatory drugs, and (right) chronic pancreatitis patients not treated with antiinflammatory drugs. Data shown represent mean ± SD. See also Figure S7 and Table S1.

embryonic pancreatic precursors is lost in adult mice ($p \geq 60$) (Guerra et al., 2007). Indeed, the resistance of the adult pancreas to malignant transformation extends beyond K-Ras oncogenic signaling. As illustrated here, adult acinar cells are also refractory to transformation by concomitant expression of a resident K-*Ras* oncogene and loss of the *p16Ink4a/p19Arf* or *Trp53* tumor suppressors, some of the most robust mutational events known in cancer.

In spite of their resistance to mutational insults, adult acinar cells retain their capacity to proliferate in response to certain pathological insults and to acquire tumorigenic properties (Slater et al., 1998; Jura et al., 2005). Exposure of adult mice to caerulein-induced pancreatitis induces proliferation of their acinar cells in order to repair tissue damage but does not result in formation of mPanINs or other preneoplastic lesions (Strobel et al., 2007). However, caerulein treatment restores permissiveness of adult acinar cells to malignant transformation by K-*Ras* oncogenes (Guerra et al., 2007; Gidekel Friedlander et al., 2009; Morris et al., 2010). Additional loss of the *p16Ink4a/p19Arf* or *Trp53* (unpublished results) tumor suppressors exacerbates the transformation process. Interestingly, pancreatitis does not cooperate with loss of either of these tumor suppressors. Thus, indicating that K-*Ras* oncogenes are essential for initiation of mPDAC.

mPDACs generated by a combination of oncogenic insults including K-*Ras* oncogenes, loss of *p16Ink4a/p19Arf* or *Trp53*, and caerulein-induced pancreatitis often display invasive and metastatic properties, albeit those induced in adult animals require longer latencies (Aguirre et al., 2003; Hingorani et al., 2005; Guerra et al., 2007). Moreover, a combination of K-*Ras* oncogenes and loss of *p16Ink4a/p19Arf* in early pancreatic precursors (Aguirre et al., 2003) or in embryonic acinar cells (this study) frequently result in anaplastic carcinomas, an aggressive tumor type seldom observed when these mutations are induced in adult animals. These results suggest that tumors that originate in the embryo develop more aggressively than those initiated during adulthood. Moreover, the more frequent occurrence of anaplastic carcinomas suggests that these oncogenic insults may engage pathways in embryonic acinar cells that are downregulated in their adult counterparts.

In humans, chronic pancreatitis represents one of the highest risk factors for developing PDAC (Lowenfels et al., 1993). However, most PDAC patients do not have a history of chronic pancreatitis. Thus, it is important to determine whether less aggressive forms of pancreatitis, including sporadic or even asymptomatic pancreatitis, may also increase the risk of developing PDAC. As illustrated here, limited bouts of mild pancreatitis lasting as little as 1 month were sufficient to elicit mPanIN formation, providing that the injured acinar cells expressed a K-*Ras* oncogene. Longer pancreatitis events resulted in increased incidence of mPanIN formation along with the appearance of high-grade mPanIN lesions and mPDAC with reduced latencies. The number and size of the lesions observed in these two protocols were considerably lower than those observed in mice suffering from chronic pancreatitis (Guerra et al., 2007). Finally, mPDAC can also develop if pancreatitis occurs before activation of K-*Ras* oncogenes, providing that tissue damage and the inflammatory response have not subsided. These observations raise the possibility that PDAC development in humans may also stem from limited bouts of pancreatitis providing that tissue repair and the inflammatory response coexist with sporadic K-*Ras* mutations.

Pancreatitis, most likely through its inflammatory component, contributes to mPanIN progression by abrogating OIS, a natural defense mechanism against tumor development (Collado and Serrano, 2010). Postmortem analysis of pancreatic tissue obtained from autopsies of healthy individuals has revealed the presence of PanIN lesions carrying K-*Ras* oncogenes that, presumably had not progressed to PDAC due to an active senescence program (Terhune et al., 1998; Lüttges et al., 1999; Löhr et al., 2005). Low-grade mPanIN lesions induced in the embryonic pancreas do not display senescence markers if the mice suffered from pancreatitis. In adult animals, which require pancreatitis for mPanIN development, senescence markers can only be detected once the inflammatory response has subsided. These observations suggest that one of the mechanisms by which pancreatitis-induced inflammation contributes to the progression of mPDAC is by eliminating the senescence barrier that prevents progression of low-grade mPanINs into more aggressive lesions.

In addition, the inflammatory response delays repair of the nontransformed pancreata and stimulate expansion of mPanIN lesions. Whether the latter effect is solely mediated by inhibiting senescence, remains to be determined. Animals treated with the dual COX-1/2 inhibitor Sulindac for 3 months after exposure to caerulein displayed almost normal pancreata with only few areas of atrophy and limited numbers of inflammatory cells. Whereas Sulindac had limited effect on the number of low-grade lesions, it caused a significant reduction (up to 75%) in the number of high-grade mPanINs and mPDAC. Yet, the most dramatic effect of this antiinflammatory treatment was observed on the extent of the area occupied by mPanIN lesions, which displayed a dramatic 95% reduction. These observations suggest that, in addition to eliminating the senescence barrier, the inflammatory response induced by episodic pancreatitis may contribute to mPDAC development by additional mechanisms.

How relevant are these observations to human patients? As illustrated in this study, low-grade PanINs present in samples surgically removed from PDAC patients displayed senescence markers. No such markers were observed in pancreatic tumor cells. Yet, the most informative observations correlating PanIN development with senescence came from the analysis of samples surgically removed from chronic pancreatitis patients. Although the number of samples analyzed is still small, we found an intriguing correlation between the occurrence of senescence in low-grade PanINs and prior exposure of the patients to antiinflammatory agents. The results were independent of whether the antiinflammatory treatment was based on steroids or on NSAIDs. These observations suggest that pancreatitis patients may benefit from antiinflammatory treatments by retaining the senescent phenotype in their low-grade PanIN lesions, possibly preventing their progression into high-grade lesions.

K-*RAS* mutations have been identified in the pancreas of healthy individuals (Terhune et al., 1998; Lüttges et al., 1999; Löhr et al., 2005). In addition, patients with chronic pancreatitis have over 10-fold increased risk of developing PDAC (Lowenfels et al., 1993; Malka et al., 2002). As illustrated in this study, mPDAC develop in adult mice expressing a resident K-*Ras* oncogene if they undergo episodic events of pancreatitis, even under asymptomatic conditions. These observations raise the possibility that in humans, at least some PDACs may originate from mild bouts of pancreatitis in individuals that unknowingly carry K-*RAS* mutations. Because the identification of K-*RAS* oncogene containing cells within the pancreas of healthy individuals is not possible, it will be important to monitor people that have been diagnosed with pancreatitis for the development of pancreatic lesions, that if unattended may lead to PDAC. Likewise, it will be important to develop biomarkers that can identify individuals that have suffered from asymptomatic pancreatitis, at least in high-risk populations.

The correlation between inflammation and loss of OIS also has important implications for the prevention of PDAC development. Treatment with antiinflammatory drugs maintained senescence markers in low-grade PanINs present in chronic pancreatitis patients. Interestingly, recent epidemiological studies have suggested a beneficial correlation between NSAIDs and pancreatic cancer risk (Bonifazi et al., 2010; Bradley et al., 2010; Rothwell et al., 2011). Our results suggest that antiinflammatory treatments might decrease the risk of PDAC development by maintaining the senescence phenotype of early PanIN lesions. Now, it will be necessary to determine whether inhibition of the inflammatory response will have a beneficial response in patients already carrying fully developed PDAC. The mouse models utilized here should contribute to address this question.

EXPERIMENTAL PROCEDURES

Mice

K-*Ras*$^{+/LSLG12Vgeo}$;*Elas*-tTA/*tetO*-Cre (Guerra et al., 2007), p16*Ink4a*/p19*Arf*$^{lox/lox}$ (Krimpenfort et al., 2001), and *Trp53*$^{lox/lox}$ (Jonkers et al., 2001) strains have been described. All experiments were approved by the CNIO Ethical Committee and performed in accordance with the guidelines for Ethical Conduct in the Care and Use of Animals as stated in The International Guiding Principles for Biomedical Research involving Animals, developed by the Council for International Organizations of Medical Sciences (CIOMS).

Human Samples

Human samples were obtained from the histopathology files of University College Hospital from patients who had undergone pancreatoduodectomy, distal pancreatectomy or total pancreatectomy with a primary diagnosis of chronic pancreatitis or ductal adenocarcinoma from 2004 to 2008 after approval by the institutional Research Ethics Committee (Central London REC 3, Reference 06/Q0512/106) and informed consent was obtained from all patients.

Histopathology and Immunohistochemistry

Specimens were fixed in 10% buffered formalin and embedded in paraffin. For histopathological analysis, pancreata were serially sectioned (3 μm) and every 10 sections stained with hematoxylin and eosin. Remaining sections were kept for immunohistochemical studies. Antibodies used for mouse samples include CD3 (1/250, goat polyclonal, Santa Cruz 1127), Cytokeratin19 (rat monoclonal, Troma III, Developmental Studies Hybridoma Bank), Chymotrypsin (1/50, mouse monoclonal 4E1, ABD Serotec, 2100-0657), Elastase (1/50, rabbit polyclonal, ABCAM, ab21590), F4/80 (1/25, rat monoclonal BM8, BMA Biomedicals), Ki67 (prediluted rabbit monoclonal SP6, Master Diagnóstica 0003110QD), MPO (1/1250, rabbit polyclonal, DAKO A0398), PAI-1 (1/50, rabbit polyclonal, Santa Cruz sc-8979), Pax5 (1/500, goat polyclonal, Santa Cruz 1974), phospho-Histone H3-Ser10 (1/200, rabbit polyclonal, MILLIPORE 06-570), prosurfactant protein C (SPC) (1/50, rabbit polyclonal, MILLIPORE AB3786), p16Ink4a (1/75, rabbit polyclonal, Santa Cruz 1207), Sox9 (1/800, rabbit polyclonal, MILLIPORE AB5535), and Sprouty-4 (1/50, goat polyclonal, Santa Cruz 18607) antibodies. Antibodies against Pdx1 were kindly provided by A. Skoudy. Positive cells were visualized using 3,3-diaminobenzidine tetrahydrochloride plus (DAB+) as a chromogen. For human samples, P16INK4a

immunolabeling was performed according to the manufacturer's protocol (CINtec Histology Kit, mtmlabs, Heidelberg, Germany). Ki67 staining was performed with mouse monoclonal Anti-Human Ki-67 (Dako).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures and one table and can be found with this article online at doi:10.1016/j.ccr.2011.05.011.

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