Senolytic elimination of Cox2-expressing senescent cells inhibits the growth of premalignant pancreatic lesions

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ABSTRACT

Objective Cellular senescence limits tumourigenesis by blocking the proliferation of premalignant cells. Additionally, however, senescent cells can exert paracrine effects influencing tumour growth. Senescent cells are present in premalignant pancreatic intraepithelial neoplasia (PanIN) lesions, yet their effects on the disease are poorly characterised. It is currently unknown whether senolytic drugs, aimed at eliminating senescent cells from lesions, could be beneficial in blocking tumour development.

Design To uncover the functions of senescent cells and their potential contribution to early pancreatic tumourigenesis, we isolated and characterised senescent cells from PanINs formed in a Kras-driven mouse model, and tested the consequences of their targeted elimination through senolytic treatment.

Results We found that senescent PanIN cells exert a tumour-promoting effect through expression of a proinflammatory signature that includes high Cox2 levels. Senolytic treatment with the Bcl2-family inhibitor ABT-737 eliminated Cox2-expressing senescent cells, and an intermittent short-duration treatment course dramatically reduced PanIN development and progression to pancreatic ductal adenocarcinoma.

Conclusions These findings reveal that senescent PanIN cells support tumour growth and progression, and provide a first indication that elimination of senescent cells may be effective as preventive therapy for the progression of precancerous lesions.

INTRODUCTION

Cellular senescence is a major roadblock to cancer development, preventing the proliferation of potentially transformed cells.1–4 Senescence of cells undergoing tumourigenic transformation is induced mainly by oncogene activity and DNA damage responses, and is mediated by several main pathways, including the p16INK4A and p53 tumour suppressors. Senescent cells are detected within tumours, often in premalignant stages, consistent with a tumour-inhibiting role.4 However, it is now clear that senescent cells can influence tumourigenesis through additional, non-cell-autonomous mechanisms, mostly mediated by the senescence-associated secretory phenotype (SASP), which involves the secretion of a host of cytokines, growth factors and remodelling enzymes.5,6 SASP composition and levels are highly context dependent, promoted by DNA damage, and mediated by several regulators, including nuclear factor-kB (NFkB).5,6

The non-cell-autonomous actions of senescent cells in cancer are complex. In some settings senescent cells...
were shown to inhibit tumourigenesis, by recruitment of immune cells or propagation of the senescence phenotype,\textsuperscript{7,8} while in other settings such cells promoted tumourigenesis, by secretion of tumour stimulatory cytokines or the generation of an immune protective environment.\textsuperscript{10–13} There is, however, overall little understanding of the prevalence of senescent cells in different tumour types, stages and cellular compartments and of their functions.

Senescent cells were shown to substantially contribute to ageing and associated diseases in mouse models, suggesting that their elimination could be therapeutically beneficial.\textsuperscript{14,15} Several drugs showing senolytic activity—preferentially eliminating senescent cells—have been identified, including inhibitors of the Bcl2 anti-apoptotic protein family, a FOXO4-derided peptide, a combination of dasatinib and quercetin, cardiac glycosides and others.\textsuperscript{3,14,15} These drugs, when administered in a variety of preclinical models, ameliorate a wide array of disease phenotypes in various tissues, and extend lifespan.\textsuperscript{3,16,57} However, the potential benefits and specific uses of senolytic drugs in cancer treatment or prevention are still largely unknown, and depend on the roles played by senescent cells in different disease settings.

Malignant pancreatic ductal adenocarcinoma (PDAC) represents one of the most deadly tumour types, with little recent progress in treatment options.\textsuperscript{18,19} Inflammation is thought to promote PDAC formation, as pancreatitis is a risk factor for the disease,\textsuperscript{20} and experimentally induced pancreatitis accelerates progression to malignancy in mouse models.\textsuperscript{31–34} Senescent cells are detected within pancreatic intraepithelial neoplasias (PanINs), the most common precursor to PDAC.\textsuperscript{23,25,26} Whether the presence of senescent cells within PanIN lesions influences PanIN growth and progression to PDAC is largely unknown. PanIN lesions are found in a substantial fraction of the general population, as assessed by autopsies,\textsuperscript{27,28} and populations at risk for PDAC, such as patients suffering from hereditary pancreatitis or from obesity, show high rates of PanIN formation.\textsuperscript{29,30} This suggests that premalignant pancreatic disease with potential progression risk may be prevalent, and therefore, understanding the effects of senescence within these lesions is of importance.

Here, we set out to uncover the functions of senescent cells within premalignant pancreatic lesions, and to test the potential effects of their elimination through senolytic treatment. Our findings reveal that these non-dividing cells express high Cox2 levels, providing essential support to PanIN development and progression, and that senolytic treatment eliminating these cells is effective in blocking PanIN growth and PDAC formation.

**RESULTS**

**Senescent and proliferating cells co-reside in PanINs**

To track senescent cell appearance in a rapid model for PanIN formation, we used mice carrying an inducible Cre under the control of the acinar-cell-specific Ptf1a promoter, a conditional Kras\textsuperscript{G12D} allele, and a conditional tdTomato reporter (Ptf1a-CreER\textsuperscript{+/-}; LSL-Kras\textsuperscript{G12D}; LSL-tdTomato mice). These triple-transgenic mice allow activation of the mutant Kras together with the tdTomato reporter in acinar cells by tamoxifen treatment. We treated triple-transgenic mice aged 6–8 weeks old with tamoxifen and collected their pancreas at different subsequent time points (figure 1A). Recombination activating tdTomato and Kras was detected in approximately 50% of acinar cells 10 days after tamoxifen treatment (figure 1B and online supplemental figure 1A).

Formation of acinar to ductal metaplasias (ADM) and PanIN-1 lesions was first observed 1 month after Kras activation, and these lesions were abundant 90 days after Kras activation, with few more advanced lesions (figure 1C,D). As expected, lesions were comprised of tdTomato-expressing cells (online supplemental figure 1B). Within PanINs, ~30% of cells stained positive for the Ki67 proliferation marker, and an average of 12% of cells stained for the apoptosis marker cleaved caspase 3 (CC3) (figure 1E–G). Staining for the senescence marker SA-βGal indicated the presence of senescent cells within the PanINs, which was supported by positive staining of a subset of cells for p16\textsuperscript{ink4a} and p53, the two main activators of this state (figure 1I). FACS quantification revealed that ~20% of the tdTomato\textsuperscript{+} cells were SA-βGal-positive (figure 1H,I). Consistent with their senescent state, these cells were also larger in size (figure 1J). These results indicate that PanINs contain dividing, apoptotic and senescent cells which are interspersed within lesions. Interestingly, SA-βGal\textsuperscript{+} cells were detected also in the tdTomato-negative fraction (figure 1H). Further analysis of the non-epithelial cell fraction revealed the presence of SA-βGal\textsuperscript{+} fibroblasts, which expressed elevated Cdkn2a levels, as well as immune cells, including macrophages (online supplemental figure 2). Thus, in addition to their presence within PanINs, senescent cells appear to reside in the stroma of these lesions.

**Senescent PanIN cells express Cox2**

To gain insights into the traits of the senescent PanIN cells, we dissociated pancreata of triple-transgenic mice 90 days after Kras activation, and isolated by FACS tdTomato\textsuperscript{+} cells that were either SA-βGal\textsuperscript{+} or SA-βGal\textsuperscript{−} (online supplemental figure 3A). Comparison of the transcripts of these two cell populations revealed an increase in the levels of Cdkn2a, encoding p16\textsuperscript{ink4a}, in SA-βGal\textsuperscript{+} cells (figure 2A and online supplemental figure 2C), and changes in gene sets associated with cytoskeletal structure, cell adhesion and other pathways associated with senescence (online supplemental figure 3B). Sets of cytokine-encoding genes were upregulated in the senescent cells, consistent with SASP activation (figure 2B and online supplemental figure 3C). Upregulated cytokine-encoding genes included Cxcl1, Cxcl5, Cxcl14, Tnf, Ccl2, Il18 and Il6, all known components of the SASP (figure 2A).

Among the upregulated SASP-associated genes was Ptgs2, encoding the enzyme Cox2 (cyclooxygenase 2), which is responsible for prostaglandin E2 synthesis, and is a central driver of inflammation (figure 2A). Cox2 was previously shown to be upregulated in PanINs and PDACs, and its proinflammatory activity promotes disease progression.\textsuperscript{31,32} However, the identity of Cox2-expressing cells within lesions was not determined. We stained PanIN sections for Cox2 and found that 20%–30% of cells expressed the protein (figure 2C–F). The majority of stained cells were epithelial cells in PanIN lesions, yet occasional staining of stromal fibroblasts was also observed (figure 2C). To further establish whether Cox2 expression is preferentially elevated in senescent PanIN cells, we sought sections for Cox2 and the proliferation markers Ki67 and bromodeoxyuridine (BrdU). This revealed a mutually exclusive pattern, indicating that Cox2\textsuperscript{+} cells are non-proliferating (figure 2E,F). Cox2-expressing cells within PanINs were larger on average than Cox-negative cells, consistent with a senescent state (figure 2G). Staining for SA-βGal and Cox2 on consecutive cryosections revealed overlapping patterns (figure 2H). These results indicate that Cox2 expression is specifically associated with the senescent fraction of PanIN cells.

To obtain further assessment of the senescent cell subpopulation we turned to single-cell transcriptomics. We recently conducted single cell RNA-Seq of pancreata of the Ptf1a-CreER\textsuperscript{+/-}; LSL-Kras\textsuperscript{G12D}; LSL-tdTomato mice at several time points after Kras activation, an analysis that identified a subcluster of senescent PanIN cells (figure 2I and online supplemental figure 4). Further analysis of this data revealed that expression of Ptgs2 (encoding Cox2) was highly specific to the senescent subcluster of cells, which also expressed authors.
Figure 1  Kras-driven PanINs contain intermixed dividing, apoptotic and senescent cells. (A) Diagram showing the timeline of Kras activation in Ptf1a-CreER<sup>Cre</sup> ; LSL-Kras<sup>G12D</sup> ; LSL-tdTomato mice by tamoxifen injection and subsequent analysis time points. (B) tdTomato fluorescence (red) in sections of pancreata collected 10 days after tamoxifen administration and control double-transgenic mice (left) 35 or 90 days after tamoxifen treatment, showing PanIN1 development in Kras-activated mice. (C) Percentages of tissue area occupied by acinar to ductal metaplasia (ADM) and PanIN lesions at indicated time points after tamoxifen treatment. Values indicate mean across mice (dots) per group ±SE. n=3 for 15 and 35 days, n=5 for 90 days. (D) Pancreas sections from Kras-activated PanIN1-bearing (right) and control (left) mice 3 months after tamoxifen treatment, stained for the proliferation marker Ki67, the apoptosis marker cleaved caspase 3 (CC3), the senescence markers SA-βGal and p16<sub>INK4a</sub> and for p53. (F) Percentage of Ki67<sup>+</sup> cells at indicated time points after tamoxifen treatment in pancreas of control mice (black), in regions outside of PanINs in Kras-activated mice (pink), and within PanIN lesions of Kras-activated mice (red). Values indicate mean across mice (dots) in each group ±SE. n=5 for ≤10 days and n=4 for 90 days. (G) Percentage of CC3<sup>+</sup> cells in the same samples scored and presented as in (F). (H) Representative FACS analysis of SA-βGal activity, detected with the C<sub>j</sub>FDG fluorescent substrate and tdTomato fluorescence, in pancreas cells isolated 3 months after tamoxifen treatment from control and Kras-activated mice. Gates indicate tdTomato<sup>-</sup> SA-βGal<sup>-</sup> (red) and tdTomato<sup>+</sup> SA-βGal<sup>+</sup> (green) cell fractions. (I) Percentage of SA-βGal<sup>+</sup> cells out of tdTomato<sup>+</sup> cells in control and Kras-activated mice, analysed by FACS as in (H), n=4 control and n=5 Kras-activated mice, collected in two independent experiments. (J) FACS histogram of cell size (forward scatter area, FSC-A) of tdTomato<sup>-</sup> SA-βGal<sup>-</sup> (red) and tdTomato<sup>+</sup> SA-βGal<sup>+</sup> (green) cell fractions isolated from pancreata of KRas-activated mice. ***P<0.001, t-test. Scale bars=50 µm. PanINs, pancreatic intraepithelial neoplasia.
Figure 2  Cox2 is preferentially expressed in non-dividing senescent PanIN cells. (A) SASP-associated genes upregulated in SA-βGal+ vs SA-βGal− PanIN cells isolated from Ptf1a-CreER+/−; LSL-Kras+/G12D; LSL-tomato mice 3 months after tamoxifen treatment, determined by mRNA-seq. The senescence activator Cdkn2a is shown on right. n=3 mice, P<0.1 for all genes. (B) Gene sets upregulated in SA-βGal+ vs SA-βGal− PanIN cells. Values indicate −log_{10} of adjusted P value as determined by ingenuity pathway analysis. (C) Cox2 stain (brown) of pancreas sections of control and Kras-activated mice, 3 months after tamoxifen treatment. (D) Percentage of Cox2+ cells at indicated time points after tamoxifen treatment in pancreas of control mice (black), in regions outside of PanINs in Kras-activated mice (pink), and within PanINs in Kras-activated mice (red). Values indicate mean across mice (dots) in each group ±SE. n=3 for both time points. (E) Co-staining of pancreas sections from control and Kras-activated mice for Cox2 (yellow) and the proliferation marker Ki67 (green), showing lack of overlap. (F) Percentage of Cox2+ cells, Ki67+ cells and cells expressing both proteins, in pancreas sections of Kras-activated mice (left); graph on right shows same analysis for BrdU+ cells. Values indicate mean percentages ±SE. Scored in >10 section areas from 5 or 3 mice (for Ki67 and BrdU, respectively), with a total of >1500 PanIN cells scored. (G) Mean area ±SE of Cox2− and Cox2+ PanIN cells. n=127 cells per group, from two mice. (H) Serial pancreas cryosections from Kras-activated mice stained for Cox2 (brown) or SA-βGal (blue). Arrows indicate examples of regions showing overlapping stain. (I) Uniform manifold approximation and projection (Umap) cell clustering of the scRNA-seq expression profiles of epithelial cells from Ptf1a-CreER+/−; LSL-Kras+/G12D; LSL-tomato mice, derived from Schlesinger et al. Shown are cells combined from 17D, 6 wk and 3–6 months after Kras activation, as well as cells from Ptf1a-CreER+/−; LSL-tomato control mice (see online supplemental figure 4). The senescent cell cluster is labelled as cluster 18. (J) Expression levels of Cdkn2a, Ptgs2, and Mki67 (encoding the proliferation marker Ki67) across cells. Red—high relative expression, grey—low expression. ***P<0.001, t-test. scale bars=50 μm. BrdU, bromodeoxyuridine; IGF, insulin-like growth factor; VEGF, vascular endothelial growth factor; IL-6, interleukin 6; HGF, hepatocyte growth factor; NFκB, nuclear factor-κB; PanINs, pancreatic intraepithelial neoplasia; SASP, senescence-associated secretory phenotype; TGF, transforming growth factor.
Cox2 is expressed in senescent PanIN cells, and that its activity facilitates the progression of low-grade PanIN lesions, which, upon treatment with the pancreatitis-inducing agent caerulein, with ABT-737 for two consecutive days, was observed in treated mice, versus untreated littermates (figure 4B). Using imaging flow cytometry we found that SA-βGal+ cells comprised ~15% of CK19+ cells in caerulein-treated mice (figure 4E). This suggested that the Bcl2-family-expressing cells in PanIN lesions could be targeted by ABT-737 for elimination. We did not detect increased mRNA levels of the Bcl2 family-encoding genes in the senescent cell subpopulation (data not shown), consistent with our previous demonstration that the upregulation of these proteins occurs largely at the post-transcriptional level.37

We next set out to test whether elimination of senescent PanIN cells can be achieved through pharmacological senolytic treatment, and whether this would affect PanIN development and progression to carcinoma. To do this we used Pdx1-Cre+; LSL-KrasG12D mice, which, upon treatment with the pancreatitis-inducing agent caerulein, develop inflammation-facilitated PanINs that contain senescent cells, and progress more rapidly and synchronously to the carcinoma stage.35 We and others have shown that senescent cell survival is dependent on the upregulation of Bcl2-family antiapoptotic proteins, and that the small-molecule ABT-737, which inhibits Bcl2, Bclxl and Bclw, induces apoptosis of senescent cells in culture and in vivo.13 16 36–38

Cox2 inhibition by sulindac treatment suppresses PanIN development. (A) H&E (top) and Alcian blue (bottom) stains of pancreas sections from Kras-activated mice treated with the Cox2 inhibitor sulindac for 3 weeks starting at 11 weeks of age, or untreated. (B) Percentage of PanIN-occupied area in untreated and mice treated with sulindac for 3 weeks, measured by image analysis of Alcian blue stained regions. n=3 mice (dots). (C) Percentage of PanIN cells staining positive for BrdU incorporation, indicating S phase, in mice treated for 3 days with sulindac and in control mice. n=3 mice. (D) Gene sets downregulated in tdTomato+ cells isolated from PanIN-bearing mice treated with sulindac for 1 week, relative to untreated mice. values indicate –log10 of adjusted P value. Graph values indicate mean±SE. *P<0.05, t-test. Scale bar=50µm. BrdU, bromodeoxyuridine; PanIN, pancreatic intraepithelial neoplasia.

Figure 3

We first tested whether Bcl2 family proteins are expressed in PanINs and if their expression was associated with senescence. Pdx1-Cre+; LSL-KrasG12D mice, but not control mice, developed large areas of low grade PanINs at the age of 3 months, 2 weeks after caerulein treatment (figure 4A,B). These PanINs were occupied by SA-βGal+ cells, and contained cells expressing Bcl2, Bclxl and Bclw proteins, contrasting with normal ductal and acinar compartments in control mice (figure 4B). Using imaging flow cytometry we found that SA-βGal+ cells comprised ~15% of CK19+ cells in caerulein-treated Pdx1-Cre+; LSL-KrasG12D mice (figure 4CD), consistent with the Ptf1a-CreER driven model (figure 1). The distribution of expression of the three Bcl2-family proteins was heterogeneous between PanIN regions. Bclxl expression was most consistently upregulated in PanIN lesions and was colocalised with Cox2 (figure 4E).

This analysis supports the identification of the senescent, non-proliferating PanIN cell subpopulation (data not shown), consistent with our previous demonstration that the upregulation of these proteins occurs largely at the post-transcriptional level.37 To test whether Bcl2 protein family inhibition eliminates senescent PanIN cells, we treated Pdx1-Cre+; LSL-KrasG12D mice, subsequent to caerulein administration, with ABT-737 for two consecutive days at the age of 3 months, and sacrificed the mice 1 day later (figure 3A). We observed increased numbers of cells expressing the apoptosis marker CC3 in PanINs of ABT-737-treated mice, indicating that Bcl2 family inhibition led to apoptosis of PanIN cells (figure 3B,C). These apoptotic cells were often shed into PanIN duct lumens. We did not detect CC3+ cells in stromal fibroblasts, macrophages, or other stromal components following treatment (online supplemental figure S). Importantly, ABT-737 treatment led to a significant decrease in the percentage of SA-βGal+ cells, and, concordantly,
Pancreas

of Cox2-expressing cells (which were colocalised), consistent with preferential elimination of senescent cells by Bcl2-family inhibition (figure 5D–G). Furthermore, the numbers of Bclxl-positive cells were also reduced, indicating that they were preferentially targeted (figure 6H,I). Interestingly, concurrent with the removal of the senescent cells, we observed at this immediate time point an increase in the division rates of remaining PanIN cells (online supplemental figure 6). This indicates that dividing PanIN cells are not preferentially targeted by the treatment. The elimination of the non-dividing senescent cells could, in part, reflect the proportional increase in the Ki67+ cells when the non-dividing, senescent cells, are removed. Together these results indicate that Bcl2 family inhibition by ABT-737 can eliminate approximately 50% of the senescent and Cox2+ cells within PanIN lesions, a rate that is consistent with our previous studies.13 16 37

Removal of senescent cells decreases PanIN formation and progression to PDAC

We next assessed whether targeting of the senescent PanIN cells influences disease development. To do this, we tested the effects of periodic ABT-737 treatment during the course of tumorigenesis. Starting at the age of 3 months, we injected caerulein-treated Pdx1-Cre+/−; LSL-Kras+/-;G12D mice with ABT-737 for two consecutive days each month, over 4 months, and then euthanised the animals (figure 6A, point 1). We reasoned that such intermittent treatment may be informative as to the effects of senescent cell clearance on the tumourigenic process. ABT-737-treated mice showed dramatically reduced lesion formation compared with vehicle-treated mice, with no evident systemic toxicity (figure 6B,C). Along with the marked reduction in PanIN formation, a significant drop in the expression of inflammatory cytokines, detected by cytokine arrays, was observed in the treated mice (figure 6D). We did not detect changes in the percentage of cells expressing Dclk1, which marks a putative cancer-initiating cell subpopulation39 (online supplemental figure 7), indicating that the decrease in presence of PanINs following ABT-737 treatment was not due to removal of this population. These findings indicate that targeting senescent cells through Bcl2 family inhibition suppresses PanIN development.

We next tested whether targeting of senescent cells by ABT-737 during early stages of tumourigenesis influences the ability of PanINs to progress to carcinoma. We treated Pdx1-Cre+/−; LSL-Kras+/-;G12D mice, subsequent to caerulein exposure, with monthly ABT-737 injections for 4 months, and then maintained the mice untreated.
Pancreas for an additional 3.5 months (figure 6A, point 2). At this time point 71% of vehicle-treated mice developed PDAC, displaying large solid tumours and increased pancreas size; in contrast, only 25% of the ABT-737-treated mice developed carcinomas (figure 6E–G and online supplemental figure 8). Histological analysis revealed a dramatic decrease in the pancreatic area occupied by PDAC in the treated mice (figure 6H). Together these findings indicate that senescent cells provide essential support to the development and progression of PanINs to carcinoma, at least in part through their expression of Cox2, and that these cells can be effectively eliminated through Bcl2-family inhibition leading to reduction in tumourigenesis.

**DISCUSSION**

Mortality rates from PDAC remain extremely high, due to late-stage diagnosis and limited treatment options. This dire state calls for further detailed dissection of the course of development of the disease, so that new therapeutic windows and methods for disease
Figure 6  Elimination of senescent cells blocks PanIN development and reduces PDAC incidence. (A) Timeline of caerulein and repeated ABT-737 treatments during PanIN and PDAC formation period. ABT-737 was administered for 2 days every month, for four consecutive months, and mice were either sacrificed after the last treatment (point 1), or maintained for a further 3.5 months (point 2). (B) Representative images of pancreatic sections from time point one stained for CK19, SA-βGal and CD45, labelling hematopoietic cells. (C) Percentage of tissue area containing ADM and PanIN lesions in mice treated with ABT-737 (n=6) or vehicle (n=3). Values indicate mean per group ±SE. (D) Levels of cytokines and chemokines in pancreata of Pdx1-Cre^-^; LSL-Kras^G12D^ mice treated with ABT-737, as measured by cytokine array. Values are shown relative to levels in vehicle-treated mice (pool of 3 mice per group). (E) Percentages of mice treated with ABT-737 or with vehicle that showed PDAC formation upon sacrifice at time point 2. Images show representative whole pancreas from mice in each group. Arrow indicates a large PDAC lesion. *P<0.05, χ^2^ test between ABT-737 and vehicle treated groups. (F) Pancreas to body-weight ratio in same mice as in (E). Values indicate mean across mice ±SE, n numbers as in (E). (G) Representative images of pancreatic sections from time point two stained as indicated. (H) Percentage of tissue area occupied by ADM, PanINs or PDAC in the same mice. *P<0.05, t test. ADM, acinar to ductal metaplasias; ns, not significant; PanIN, pancreatic intraepithelial neoplasia; PDAC, pancreatic ductal adenocarcinoma.
prevention can be identified. Here, we provide findings suggesting that senolytic treatment could represent a novel preventive treatment course for PDAC.

We uncovered a collaborative interaction between cell subsets within PanINs, in which senescent, non-dividing cells, stimulate lesion growth and progression, mediated, at least in part, by the proinflammatory Cox2 enzyme. Blocking this activity, or eliminating the senescent cells, dramatically reduced the growth of PanINs, indicating that this interaction is essential at this early phase of tumorigenesis. These findings shed light on the dual role of senescent cells in the development of pancreatic cancer: while PanIN cells entering senescence are likely unable to convert to dividing tumour cells, their retention in the microenvironment provides essential support to the non-senescent precancerous cells. As patient lesions progress to malignancy, however, tumour cells may become independent of this necessary support and inactivating mutations in the senescence-inducers p53 and p16 provide a proliferative advantage.

We show here that treatment with the Bcl2 family inhibitor ABT-737, one of the most effective senolytic courses identified to date,14 15 can target Cox2-expressing senescent cells within PanINs, and that periodic treatment dramatically reduces PanIN formation and progression. This is particularly striking in light of the intermittent nature of the treatment, as opposed to continuous therapy: 2 days of treatment monthly were sufficient to achieve a strong antitumourigenic effect.

The importance of inflammatory signalling pathways in pancreatic cancer has been described in multiple studies, and chronic pancreatitis is a major risk factor for the disease.20 21 The NFkB pathway was shown to provide important contributions to proinflammatory activation in the context of Ras-driven disease.24 40 Cox2, its direct target,31 appears to be a key component of protumourigenic inflammation in the pancreas, and its overexpression is sufficient to induce the formation of premalignant lesions in mice.23 24 32 We show here that the protumourigenic contribution of Cox2 is provided by a subset of premalignant cells, namely non-dividing senescent cells, rather than proliferating tumour cells, indicating a ‘division of labour’ at this early stage of tumorigenesis. Cox2 was described as a component of the SASP, and it represents a contribution of senescence to the disease at this early stage. Consistent with previous work,26 however, we found that senescence markers did not fully overlap at the individual cell level, with a somewhat different distribution of expression of Cdkn2a and Ptg2s observed in the scRNA-Seq analysis, suggesting that the Cox2+ cells are a subset among several types of non-dividing stromal cells within lesions.31

The Ptf1a-CreER model, in which oncogenic Kras is activated in acinar cells, provides rapid PanIN formation in the absence of external induction of inflammation. In this model, we found that pharmacologic inhibition of Cox2 through sulindac, a non-steroid anti-inflammatory drug, dramatically inhibited PanIN growth, suggesting that the Cox2+ cells provide the major protumourigenic inflammatory drive, essential for disease development. The Pdx1-Cre driven model requires external induction of inflammation, by acute 2-day treatment with caerulein, for timely and synchronous PanIN formation. These lesions contained senescent cells in similar numbers as those in the Ptf1a-CreER mice. While we did not test the effects of subsequent sulindac treatment in this model, it is remarkable that the senolytic treatment had a similar effect to that of sulindac and reduced inflammatory cytokine secretion, indicating that even subsequent to a tumour-promoting acute pancreatitis event, continuous production of inflammatory signals by senescent cells is essential for lesion growth. Previous work showed that when Kras-expressing mice are exposed to chronic caerulein-induced pancreatitis, conducted over 3 months, senescent cells do not appear with lesions, and lesions progress more rapidly.23 In the presence of chronic inflammation, senescent cells may, therefore, offer no added inflammatory benefit, yet limit lesion growth, and as such are out-selected. Interestingly, this work also found that the senescent cells reappear within lesions following cessation of pancreatitis,23 consistent with our findings.

The overall contribution of senescence and the SASP to lesion development could occur through direct stimulation of neighbouring cells, or indirectly through modification of the tumour microenvironment. While we did not detect increased proliferation rates of cells in the immediate vicinity of the Cox2+ cells (data not shown), direct stimulation cannot be excluded. The presence of senescent stromal cells may also represent a form of interaction of senescent cells with the microenvironment and a potential contribution to tumour formation, areas that require further dissection. Due to the systemic delivery of the treatments we cannot exclude their potential effects on additional cell types, including senescent stromal cells. However, our tissue section stains indicate that the primary cells undergoing apoptosis in response to ABT-737 treatment are the senescent PanIN cells.

Overall, further studies should provide a more detailed analysis of the identity and characteristics of the senescent cell subtypes that exert protumourigenic effects, and of their mechanisms of action, through Cox2, SASP or other means. Future work should address the potential effects of the senolytic treatments on additional cell types, such as senescent Cox2-negative cells, and cells in the tumour microenvironment.

Senolytic treatments are currently entering clinical trials in several age-related diseases.3 The potential for senolytic drug treatment in cancer is currently beginning to be explored. Usage of senolytics in various cancer settings requires a detailed understanding of the context in which senescence occurs among tumour cells and stromal components, and of the roles senescent cells carry out, knowledge that is currently lacking. We recently showed that senolytic treatment ameliorates senescence-induced epidermal hyperplasia and dysplasia reminiscent of actinic keratosis.13 The current study suggests that senolytic treatment, under a periodic protocol, could be considered as preventive therapy in patient populations at high risk for PDAC development, or in which early disease is suspected due to other conditions, such as pancreatitis. The high prevalence of PanIN lesions in the healthy ageing population, and their increased rates in hereditary pancreatitis patients,27 28 suggest that specific populations could benefit from therapy aimed at reducing the growth and progression potential of PanINs, a concept that could potentially be extended to other premalignant diseases.

METHODS

Mice

The following mouse lines were obtained from The Jackson Laboratory, maintained in a specific pathogen-free environment throughout the study, and crossed as indicated: Ptf1a-CreER (C57Bl6), Pdx1-Cre (C57Bl6), LSL-KrasG12D (mixed C57Bl6, 129sv), LSL-tdTomato (Strain Ai14, C57Bl6). For inducible Kras activation in acinar cells, Ptf1a-CreER; LSL-KrasG12D, LSL-tdTomato triple transgenic mice and controls were injected at 6 weeks of age subcutaneously with two doses of tamoxifen (Sigma) at 400 mg/Kg to activate Cre-mediated recombination. Assessment of the recombination of the LSL-KrasG12D allele was performed by semi-quantitative PCR on genomic DNA extracted from FACS-isolated tdTomato+ cells and calculation of recombined vs. non-recombined product fraction, measured by image analysis of gel using ImageJ software. For BrdU labelling mice were injected intraperitoneal in combination with 100 mg/kg BrdU 2 hours prior to sacrifice. For Cox2 inhibition following Kras activation, Ptf1a-CreER; LSL-KrasG12D; LSL-tdTomato mice received 0.6 mg/mL of sulindac (Sigma S4429) dissolved in drinking water, supplemented
by 5 mg/mL sucrose to compensate for bitterness, starting 15 weeks after Kras activation. For the induction of pancreatitis, Pdx1-Cre; LSL-Kras\(^{G12D}\) mice and controls received 7 hourly intraperitoneal injections of caerulein per day for 2 days with a 1 day recession in between. Caerulein (Sigma, C9026) was diluted in phosphate buffered saline (PBS) and injected at 50 μg/kg body weight. For senescent cell elimination, Pdx1-Cre; LSL-Kras\(^{G12D}\) mice, starting 2–3 weeks after caerulein treatment, received intraperitoneal injections of ABT-737 (25 mg/kg) on two consecutive days. Where indicated, injections were repeated in 1-month intervals. ABT-737 (Wuxi AppTec) was dissolved in DMSO and prepared in a working solution of 30% propylene glycol, 5% Tween 80, 3.3% dextrose in water pH 4–5. was dissolved in DMSO and prepared in a working solution of 30% propylene glycol, 5% Tween 80, 3.3% dextrose in water pH 4–5.

Both male and female mice were used in experiments. Genomic DNA sequence was performed for the Tprp53 locus encoding p53 and no mutations were identified by BLAST comparison to the reference genome.

Histology, immunohistochemistry and cytokine array

Dissected pancreata were either formalin fixed and paraffin embedded, or frozen in OCT solution for cryosectioning. The 5–7 μm paraffin sections were stained by standard procedures using ImmPress reagent kit (Vector #MP-7401/7402) and DAB Peroxidase Substrate Kit (Vector Laboratories), or conjugated fluorescent secondary antibodies (Jackson). The following antibodies were used: Ki67 (NeoMarkers RM910650), E-cadherin (BD Pharmingen 610182), tdTomato (anti dsRed, Clontech 632496), BrdU (BioRad MCA2060), Cox2 (Abcam ab179800), p16 (Santa Cruz sc-1207), Bclw (Cell signalling 31H4), Bclxl (Cell Signalling 31H4), Bclxl (Cell Signalling 31H4), and trimmed for adapters and poly-A tails were sequenced. R sequences were isolated tdTomato\(^{+}\)SA-βGal\(^{-}\) and tdTomato\(^{+}\)SA-βGal\(^{+}\) fractions from three mice, gated to exclude endothelial and immune cells (CD31\(^{+}\), CD45\(^{+}\)). Total RNA from FACS-sorted cells was isolated by TRIzol (Invitrogen) extraction followed by RNeasy Plus Micro Kit (Qiagen), using >10,000 cells of each sample. Profiling was conducted using an adapted CEL-Seq2 protocol: 3’ cDNA was synthesised and barcoded, followed by RNA synthesis, amplification by in vitro transcription and library generation for paired-end sequencing. Reads were demultiplexed, quality filtered and trimmed for adapters and poly-A tail using Cutadapt and aligned with the mouse genome (GRCm38) using TopHat2. Differentially expressed genes were determined by DESeq2, using a Padj < 0.1 cut-off. Enrichment for upregulated and downregulated gene sets was conducted by Ingenuity Pathway Analysis (Qiagen) using Padj < 0.1, and by GSEA, testing gene sets from the hallmark collection of the molecular signatures database and gene sets of upregulated genes in senescent Hras-expressing human IMR90 fibroblasts\(^{8}\) and senescent mouse stellate cells.\(^{8}\) The same analysis was conducted on tdTomato\(^{+}\) cells isolated from Kras-expressing mice treated with sulindac for 3 days or untreated. Single cell RNA Seq data acquisition and analysis was described in Schlesinger et al.,\(^{42}\) and was conducted on pancreata isolated from Ptf1a-Cre;ER; LSL-Kras\(^{G12D}\); LSL-tedTomato mice at indicated time points after Kras activation, as well as from control Ptf1a-Cre;ER; LSL-tedTomato mice. Data shown includes only the epithelial cell clusters. We used Seurat R package (V2.3.4) to perform graph-based unsupervised clustering, uniform manifold approximation for data analysis and visualisation in two-dimensional space.

Statistical analysis

Two-tailed unpaired Student’s t-test and analysis of variance were used for mouse group comparisons.

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REFERENCE


**Supplementary Figures**

Figure S1. Recombination rates of LSL-Kras<sup>ΔG12D</sup> allele. **A.** Percentage of LSL-Kras<sup>ΔG12D</sup> alleles showing recombination, in tdTomato<sup>+</sup> cells isolated by FACS from pancreata of 3 Ptf1a-CreER<sup>+</sup>; LSL-Kras<sup>ΔG12D</sup>; LSL-tdTomato<sup>+/−</sup> mice (dots) 5 days following tamoxifen treatment, as assessed by semi-quantitative PCR conducted on genomic DNA, comparing the ratio of recombined to non-recombined flox alleles. **B.** Representative images (low magnification – top, high magnification – middle and bottom) of PanIN tissues stained with an antibody against tdTomato, showing tdTomato expression in lesion cells and in surrounding acinar cells. Size markers = 50μm.
Figure S2. PanIN stroma contains senescent fibroblasts. A. Representative FACS analysis of cells from Ptfla-CreER
to LSL-Kras mice 3 months after Kras activation (Kras, right panels) and control Ptfla-CreER
to mice (left panel, Cont). The tdTomato transgene was replaced by staining for the epithelial marker Epcam. Co-staining of Epcam and SA-βGal, conducted using the fluorescent substrate C14:FDG, allowed the characterization and isolation of Epcam+ SA-βGalhigh cells, whose numbers were substantially increased in the Kras-expressing mice versus control mice (gate labeled blue). Staining with the cancer-associated fibroblast markers Pdpn and Cd140a (Pdgfra)
(right panel) revealed that the majority of Epcam+ SA-βGalhigh cells are fibroblasts. Percentages on right panel dot plot are of total Pdpn+, or of Cd140a+, out of Epcam+ SA-βGalhigh cells. B. Percentages of indicated subpopulations out of Epcam+ SA-βGalhigh cells. The Cd140a+ fibroblast subpopulation is included in the Pdpn+ population; the F4/80+ macrophage subpopulation is included in the Cd45+ immune population. Values represent mean across individual mice (dots) ± S.E.M. C. Expression levels of the Cdkn2a senescence marker measured by qRT-PCR in SA-βGal positive and negative subpopulations of Epcam+ epithelial compartment and Cd140+ fibroblasts, isolated from Kras activated mice, or in corresponding SA-βGal-negative cells isolated from control mice (Cont). Values indicate average of cells sorted from two mice (dots).

**A**

- EIF2 Signaling
- RhoGDI Signaling
- -Log
- P
- 0 1 2 3 4 5 6 7 8 9
- Integrin Signaling
- Signaling by Rho Family GTPases
- ILK Signaling
- Actin Cytoskeleton Signaling
- Remodeling of Epithelial Adherens Junctions
- RhoA Signaling
- ERK/MAPK Signaling
- Pancreatic Adenocarcinoma Signaling
- p38 MAPK Signaling
- JAK/Stat Signaling
- Apoptosis Signaling
- Death Receptor Signaling

**B**

- Hallmark inflammatory response
- NES=3.6
- FDR=0
- Higher expression
- Lower expression
- Sen IMR90-Ras fibroblasts
- NES=4.7
- FDR=0
- Sen mouse liver stellate cells
- NES=5.3
- FDR=0

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Figure S3. Isolation and profiling of the tdTomato+ SA-βGalhigh population. A. Sorting procedure for the isolation of senescent and non-senescent PanIN cells, shown on a representative FACS of cells from triple-transgenic Ptf1a-CreER<sup>+/−</sup>; LSL-Kras<sup>G12D</sup>; LSL-tdTomato<sup>+/−</sup> mice 3 months after Kras activation. Following FSC and SSC gating to eliminate cell doublets, cells were gated to exclude CD45<sup>+</sup> (hematopoietic) and CD31<sup>+</sup> (endothelial) fractions; tdTomato<sup>+</sup> SA-βGal<sup>−</sup> cells (red, top left), and tdTomato<sup>+</sup> SA-βGal<sup>high</sup> (green, top right) were gated for isolation as indicated. B. Additional gene sets upregulated (left) and downregulated (right) in SA-βGal<sup>+</sup> versus SA-βGal<sup>−</sup> PanIN cells isolated from Kras-activated mice 3 months after tamoxifen treatment. Values indicate –log<sub>10</sub> of Adj P value as determined by Ingenuity Pathway Analysis. C. Gene set enrichment analysis (GSEA) of expression profiles of SA-βGal<sup>+</sup> versus SA-βGal<sup>−</sup> PanIN cells indicating enrichment for inflammatory (top) and senescent cell (bottom) gene sets.
**Figure S4. scRNA-Seq of Kras-driven PanINs.** **A.** Uniform manifold approximation and projection (Umap) cell clustering of the scRNA-seq expression profile of epithelial cells from Ptf1a-CreER<sup>Cre</sup> /; LSL-Kras<sup>G12D</sup>; LSL-tdTomato mice, as shown in Fig. 2I,J. Colors indicate different time points after Kras activation in which cells were collected, as indicated. Cells from Ptf1a-CreER<sup>Cre</sup> /; LSL-tdTomato control mice (CTRL) are labeled red. Numbers of sequenced cells at each time point are indicated in parentheses. **B.** Expression of tdTomato across cells (red – high, grey - low), observed in normal acinar cells as well as PanIN cells.
Figure S5. Bcl2-family protein inhibition by ABT-737 does not induce apoptosis in stromal cells. Pdx1-Cre<sup>+/–</sup>;LSL-Kras<sup>+/G12D</sup> mice were treated by caerulein at 2.5 months of age, followed by treatment with ABT-737 two weeks subsequently, and analyzed two days later. A. Co-staining of pancreas sections from vehicle and ABT-737 treated mice for CC3 (red), and the fibroblast marker vimentin (green), showing no overlap in expression, with CC3 stain detected only within PanIN epithelial ductal structures. B. Co-staining of pancreas sections from vehicle and ABT-737 treated mice for CC3 (red), the macrophage marker F4/80 (green) showing no overlap in expression.
Figure S6. Bcl2-family protein inhibition by ABT-737 increases the percentage of Ki67* cells in PanINs. *Pdx1-Cre\textsuperscript{+/-};LSL-Kras\textsuperscript{G12D} mice were treated by caerulein at 2.5 months of age, followed by treatment with ABT-737 two weeks subsequently, and analyzed two days later. A. Representative images of pancreas sections from vehicle or ABT-737 treated mice stained for Ki67 (brown). B. Percentage of Ki67* cells in PanINs from same mice. *, P < 0.05, **, P < 0.005, t-test.
Figure S7. Bcl2-family protein inhibition by ABT-737 does not eliminate Dclk1+ cells. *Pdx1-Cre<sup>Cre<sup>–/–</sup>;LSL-Kras<sup>G12D</sup> mice were treated by caerulein at 2.5 months of age, followed by treatment with ABT-737 two weeks subsequently, and analyzed two days later. 

A. Representative staining for Dclk1 in pancreas sections from vehicle and ABT-737 treated mice.

B. Percentage of Dclk1+ cells in PanINs from same mice. *, P < 0.05, **, P < 0.005, t-test.
Figure S8. High grade PanINs and pancreatic carcinoma in Pdx1-Cre<sup>+/−</sup>;LSL-Kras<sup>G12D</sup> mice. Representative images of PanIN2 and PanIN3 lesions (panel A) and pancreatic ductal carcinoma (PDAC, panel B) observed in caerulein-treated Pdx1-Cre<sup>+/−</sup>;LSL-Kras<sup>G12D</sup> mice at 9 months of age. Lesions are from mice that did not receive ABT-737. Size marker = 200 µm.