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RELATIONSHIP BETWEEN CHRONIC INFLAMMATION AND CANCER: INTERLEUKIN-1β OVEREXPRESSION INDUCES PANCREATIC DUCTAL ADENOCARCINOMA IN ONCOGENIC KRAS MICE

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SUMMARY

Chronic pancreatitis is an established risk factor for pancreatic ductal adenocarcinoma (PDAC) development. Polymorphisms in the pro-inflammatory cytokine gene interleukin 1β (IL-1β), as well as high IL-1β or low IL-1 receptor antagonist (IL-1RA) serum levels, are associated to worse prognosis in PDAC patients. To characterize the role of IL-1β in pancreatic tumorigenesis, we generated a transgenic mouse model bearing $KRAS^{G12D}$ mutation combined to chronic inflammation induced by pancreatic overexpression of human IL-1β (KC-IL-1β). We found that IL-1β overexpression induced PDAC onset in 6 out of 13 $KRAS^{G12D}$ bearing animals (46%), with a median overall survival of 10.5 months, compared to only 1 out of 13 mice carrying $KRAS^{G12D}$ mutation alone (KC)(7.7% p= 0.02).

In primary pancreatic $KRAS^{G12D}$ organoid cultures, IL-1β exposure increased the number of spheroids and induced gene expression changes consistent with epithelial to mesenchymal transition (EMT), as shown by increased expression of vimentin, Zeb1, Snail. All these changes were counteracted using a recombinant human IL-1receptor antagonist (IL1-RA). Consistently, immuno-histochemical analysis confirmed that in KC-IL-1β tumor epithelial cells and metastasis were strongly positive for vimentin.

The relevance of these findings was confirmed in human PDAC, showing higher IL-1 receptor I (IL1-RI) and vimentin expression in tumor tissue compared with adjacent normal pancreas.

Regarding the mechanism involved in EMT activation, IL-1β exposure was found to induce an up-regulation of ribosome biogenesis rate, with consequent down-regulation of p53 protein expression which has been shown to be responsible for EMT changes.

The finding that IL-1β/IL1-RI inflammatory pathway stimulates acinar cell proliferation and promotes EMT provides the rationale for a therapeutic strategy based on IL-1β receptor blockade to counteract inflammation-induced pancreatic tumorigenesis

1. INFLAMMATION AND CANCER

In 1863 Rudolf Virchow hypothesized the presence of a relationship between inflammation and cancer by observing that cancers frequently originate at sites of chronic inflammation, and suggesting that the inflammatory milieu may enhance cell proliferation (1, reviewed in 2). The link between inflammatory process and cancer is now well established (3,4). Chronic inflammation greatly increases the risk to develop cancer in many tissues: indeed the development of about 15% of the global cancers is attributable to infectious agents, with inflammation as a major component of these chronic infections (5,6).

The mechanisms underlying the link between inflammation and cancer can be summarized in two main pathways. In the intrinsic pathway, primary genetic alterations of oncogenes and tumor suppressors drive cancer onset and determine the expression of inflammation-related programs resulting in pro-inflammarory circuits supporting cancer development. In the extrinsic pathway chronic inflammation drives cancer onset, determining genomic instability, facilitating genetic alterations or directly editing the host genome. For example, Helycobacter Pylori infection determines gastric cancer, HPV and HCV infections cause cervical and hepatic cancer respectively (reviewed in 7). Inflammatory cytokines, which can be produced either by cancer cells or tumor-associated leukocytes, are the main factors involved in the molecular pathways linking inflammation to cancer. In particular, three cytokines, IL-1, IL-6, and TNF-α have been demonstrated to have a major role in this setting through paracrine or autocrine loops.

1.1 INTERLEUKIN 1 (IL-1)

IL-1 includes a family of closely related genes: IL-1α, IL-1β, and IL-1 receptor antagonist (IL1-RA), an endogenous inhibitor of IL-1. IL-1α and IL-1β act on the same receptor, but whereas IL-1β is active in its secreted form, IL-1α is mainly active in cellular associated form, being rarely secreted (8). Upon binding of IL-1, IL-1 receptor activates multiple downstream signaling pathways including NF-KB, which regulates multiple cellular processes involved in inflammation and immunity (7,8). Multiple studies indicate an important role of IL-1 in carcinogenesis. IL-1 can augment the metastatic capacity of cancer cells by modulating inflammatory pathways (9,10). IL-1β has been demonstrated to increase tumor invasiveness, adhesiveness, and angiogenesis in murine models of carcinogen-induced cancers (11). IL-1 has been implicated in hepato-carcinogenesis and pancreatic tumorigenesis (12,13). IL-1β polymorphisms are associated with increased risk of gastric cancer onset (14). Furthermore gastric specific IL-1β expression in IL-1β transgenic mice, leads to chronic gastric inflammation and cancer (15). Taken together these data strongly support a role of IL-1 signaling in the pathogenesis of gastrointestinal tract cancers.

1.2 INTERLEUKIN 6 (IL-6)

Besides NF-KB, JAK/STAT signaling is one of the main pathways activated in inflammation-associated tumorigenesis. Importantly these two pathways are tightly interconnected, since STAT3 signaling is required for the maintenance of NF-KB activation (16). The main activator of JAK/STAT signaling in inflammation is IL-6, which in turn is a NF-KB target gene (17). The NF-KB-IL-6-STAT3 axis, leads to

increased transcription of several known oncogenes, such as bcl-2, MCL-1, MYC, Cyclin D, which protect from apoptosis and drive proliferation of malignant cells (18). JAK/STAT signaling can be involved also in tumor–associated immune suppression, by regulating the expression of cytokines affecting the polarization of T-helper subsets, and programmed death ligands (inhibiting the function of cytotoxic T cells) (19,20).

Recently, a new pathway connecting IL-6 and cancer has been described. IL-6 has been shown to up-regulate ribosome biogenesis thus decreasing the binding of ribosomal proteins to MDM2 with the consequent increased MDM2-mediated p53 degradation. The down-regulation of p53 was responsible for the activation of the epithelial-mesenchymal-transition program (21). The pathogenetic role of IL-6 signaling is well established in several types of cancer, including hepatocellular carcinoma, multiple myeloma and lymphoma (22-24).

1.3 TUMOR NECROSIS FACTOR α (TNF-α)

Increased expression of the proinflammatory cytokine TNF-α is detected in multiple types of tumors, including ovarian, breast, bladder, prostate, colorectal, and lymphoid cancers (reviewed in 7). TNF-α regulates angiogenesis and metastasis in solid tumors, and facilitates epithelial to mesenchymal transition (25). High levels of circulating TNF-α have been associated with poor prognosis in hematologic malignancies of both lymphoid or myeloid origin (26). Importantly, transgenic mice lacking the TNF-α gene are resistant to skin carcinogenesis, further supporting a role of TNF-α in the pathogenesis human cancer (27).

2. PANCREATIC CANCER

Pancreatic ductal adenocarcinoma (PDAC) will become the second leading cause of cancer deaths in the United States by 2030 (28).

Surgical resection is the most effective treatment, but just few patients are eligible for a radical procedure at the time of diagnosis, and even among the resected ones, survival is very poor, with less than 20% of patients alive after 5 years. Systemic chemotherapy provides only a modest benefit for advanced disease, and cure is still elusive. More reliable early detection methods are needed, as well as increased understanding of the mechanisms of metastasis and therapeutic resistance.

Despite the high biological complexity of PDAC, four genes are recurrently mutated in the majority of patients. Mutation in KRAS occurs in more than 90% of cases, and tumor suppressors CDKN2A, p53, DPC4/SMAD4 are altered in the 95%, 70% and 55% of patients, respectively (29).

Point mutations in codon 12 of KRAS gene ($KRAS^{G12D}$ or $KRAS^{G12V}$) are the most commonly identified in human pancreatic adenocarcinoma (30). This and other mutation that activate KRAS have been found in precancerous lesions, like acinar to ductal metaplasia (ADM) and pancreatic intraepithelial neoplasia (PanIN), ranging from low-grade PanIN (PanIN-1A/1B) to high grade PanIN (PanIN-2/3) (31).

Cellular responses to Ras activation vary and depend on the cell type, Ras isoform, expression level and wild-type allele status. For example, targeted overexpression of activated K-ras in the pancreas often leads to the development of pancreatic acinar hyperplasia or dysplasia, but not invasive cancer. Conversely, expression of activated $KRAS^{G12D}$ at a physiological level induced by Pdx1-cre is sufficient to initiate the development of murine PanIN. The oncogenic function of mutant K-ras protein is therefore significantly influenced by the expression level of the mutant KRAS allele or the ratio of the wild-type to mutant K-ras protein (32-34).

Given the frequency of KRAS mutations in PDAC and their presence in early precursor lesions, ADM and PanINs, the activation of oncogenic KRAS has been recognized to be the first event in pancreatic carcinogenesis, playing a pivotal role in the ADM-to PanIN-to PDAC progression.

Nevertheless, since KRAS mutation alone is sporadically sufficient to drive the progression to full-blown pancreatic cancer in transgenic mice (35,36) additional oncogenic events must take place in order to accelerate pancreatic carcinogenesis.

3. INFLAMMATION AND PANCREATIC CANCER

The potential association between chronic pancreatitis (CP) and pancreatic cancer is matter of debate since many years. The risk of PDAC development in patients affected by chronic pancreatitis is difficult to assess, due to the difficult diagnosis of the disease itself. Indeed diagnostic criteria for CP vary from center to center, the disease shows often low symptoms and pancreatic biopsy or direct visualization of the organ is difficult (37).

Most of the studies, case control series or studies relying on data case register, reported relative risk of PDAC development patients diagnosed with chronic pancreatitis variable from 2.3 to 18.5 (38,39).

More recently, a prospective single center study confirmed the association between chronic pancreatitis and pancreatic cancer, with a standardized incidence ratio of 26.7 (40).

However, the mechanisms underlying the risk of pancreatic cancer in CP patients are unclear. Ductal epithelial hyperplasia, metaplasia and dysplasia and KRAS mutations have been described in CP patients, suggesting an oncogenic multistep sequence. Chronic pancreatic inflammation may account for the increased risk of PDAC in the course of CP whether induced by environmental, genetic, or other causes, as is the case for other premalignant disease. The duration of CP may influence the magnitude of the increase in the risk of pancreatic cancer, as suggested by the estimated cumulative risk of 40% found in patients with hereditary pancreatitis. However, only 1.1% of CP patients developed PDAC, and CP may account for only 0.1% to 5% of cases of pancreatic cancer compared with an attributable risk of approximately 30% for smoking (40).

Taken together, these evidences show that patients affected by any form of CP have an increased risk of developing PDAC: patients with an early onset pancreatitis, like hereditary or tropical pancreatitis have rates of pancreatic cancer that are at least 50-fold greater that in the healthy population. However, a

policy of pancreatic cancer screening in CP patients does not seems to be justified. Moreover, diagnosing PDAC in CP patients remains challenging and more reliable screening tests will need to be developed before screening for pancreatic cancer in patients with chronic pancreatitis becomes feasible.

Moreover, the connection between chronic inflammation and epithelial-to-mesenchymal transition (EMT) process has recently been established in pancreatic cancer, starting from early stages of tumorigenesis (41).

Polymorphisms in pro-inflammatory cytokine genes such as IL-1β, TNF-α and IL-6 are known to increase the risk of human pancreatic cancer, and high serum levels of IL-1β and IL-6 are associated to worse prognosis in advanced PDAC patients (42-45).

IL-1β is a pleiotropic pro-inflammatory cytokine, produced by monocytes, macrophages, and epithelial cells, and it is part of the interleukin-1 family, [which includes IL-1α and IL-1 receptor antagonist (IL1-RA). IL-1β is involved in many cellular activities, including cell proliferation, differentiation, and apoptosis and it plays a crucial role during pathogenesis of pancreatitis in humans and mice (46,47). Moreover, high IL-1β or low IL1-RA serum levels are associated with worse survival in human PDAC (48). Taken together these evidences confirm that inflammation could play a major role in pancreatic tumorigenesis. Nevertheless, the specific role of IL-1β is still poorly defined.

4. GENETICALLY ENGINEERED MOUSE MODELS (GEMMs) OF PANCREATIC CANCER

Over the past 20 years the adoption of molecular biological techniques for the genetic manipulation of the mouse has resulted in a vast surge of interest in using the mouse as a model system for the investigation of almost all facets of mammalian biology. Remarkably, it has now become possible to genetically alter the mouse genome with nucleotide precision, obtaining genetically engineered mouse models (GEMMs). Incorporation of exogenous DNA into the mouse genome to produce a transgenic animal can be achieved by pronuclear injection of DNA in the fertilized zygote. Transgenic animals produced by this method are generally gain-of-function mutants since the transgene is designed either to express a novel gene product or to deregulate a normal gene. When gene targeting (precise alteration of endogenous genes) is accomplished by homologous recombination in embryonic stem cells it is possible to generate null or knockout mutations.

More recently, strategies exploiting site-specific DNA recombination have been incorporated into transgenic and gene-targeting procedures to allow in vivo manipulation of DNA in embryonic stem cells or living animals. A large number of site-specific DNA recombinases have been described from bacteria and yeast, and the recombination reactions that they catalyze span a wide range of complexity. The relative simplicity and efficiency of Cre recombinase from bacteriophage P1 has made it particularly useful for the generation of transgenic mice. Cre recognizes a 34-bp site on the P1 genome called loxP and efficiently catalyzes reciprocal conservative DNA recombination between pairs of loxP sites: Cre-mediated recombination between two directly repeated loxP sites results in excision of the DNA between them as a covalently closed circle. To obtain a recombinant activation of gene expression it is advantageous to design a dormant transgene that can be activated after establishment of the transgenic

line, since often the introduction of a transgene into the mouse results in either morbidity or such reduced viability that it is difficult to maintain the transgenic mouse line by breeding. One way of doing this is by inserting a lox STOP cassette between the potentially toxic transgene and its promoter. After establishment of a transgenic line, the STOP signal can be removed by Cre-mediated excision, by intercrossing with a second mouse expressing Cre, to activate the transgene as desired.

The tissue specificity of expression for the recombinant activated dormant transgene is a function both of the promoter specificity of the target transgene and of the promoter specificity of the Cre transgene. Moreover, the spatial and temporal occurrence of recombination can be completely specified by placing Cre under the control of a promoter having the desired spatial and temporal pattern of expression.

A variety of genetically engineered mouse models of pancreatic cancer have been developed over the last decade using the Cre/lox System.

Hingorani and colleagues (35) developed a mouse model of PanIN lesions, Pdx-1cre;LSL-$KRAS^{G12D}$ (KC), targeting the expression of $KRAS^{G12D}$ to pancreatic progenitor cells, through the construction of a conditionally expressed allele (35). The targeting vector contained Lox-STOP-Lox (LSL) construct inserted into the mouse genomic KRAS locus upstream of a modified exon 1 engineered to contain a G to A transition in codon 12. This mutation results in a glycine to aspartic acid substitution in the expressed protein, compromising both the intrinsic and extrinsic GTPase activities and resulting in constitutive downstream signaling activation of Ras effector pathways. The expression of the mutated allele is achieved by interbreeding LSL- $KRAS^{G12D}$ mice with animals that express Cre recombinase from the pancreatic-specific promoters PDX-1, a pancreatic progenitor marker expressed since E8.5, and its homozygous deletion is an embryonic lethal event (49). In the same way, Hingorani and colleagues (50) developed a metastatic cancer mouse model, targeting the expression of both $KRAS^{G12D}$ and $Trp53^{R172H}$ Pdx1-cre;LSL- $KRAS^{G12D}$; LSL- $Trp53^{R172H}$ (KPC), an ortholog of one of the most common TP53 mutation in human PDAC, to progenitor cells of the mouse pancreas (50).

In conclusion, one of the principle advantages of GEMMs is the stepwise evolution of a nascent tumor from normal precursors that results in a more authentic tissue architecture compared to xenografts that are reconstituted from cell lines, or are lacking in immune cells. Thus, the innate complexities of GEMMs make them ideal tools to study mechanisms of tumorigenesis and test the efficacy of cancer therapies, playing an increasingly prominent role in identifying the relevant patient population most likely to benefit from any given treatment (51,52).

5. AIMS

The aim of the present study was to investigate the role of chronic inflammation induced by IL-1β in PDAC development. For this purpose, we generated a novel transgenic mouse model characterized by $KRAS^{G12D}$ mutation combined to a pancreatic overexpression of human IL-1β (KC- IL-1β). We followed longitudinally a cohort of KC-IL-1β mice up to 20 months of age for evidence of disease progression. Moreover, we studied the effect of IL-1β on primary epithelial pancreatic cells from $KRAS^{G12D}$ mice by evaluating the very first step of carcinogenesis, as identified in ADM process. We found that IL-1β-induced chronic pancreatitis accelerated pancreatic carcinogenesis in $KRAS^{G12D}$ mice and stimulated ADM and promoted EMT in organoid cultures from pancreas of $KRAS^{G12D}$ mice through IL-1β/IL1-RI pathway. The latter changes were counteracted by treatment with a human recombinant IL-1 receptor antagonist, able to competitively block the IL1-RI receptor. We also investigated the mechanism underlying EMT induction following IL-1β stimulation.

6. RESULTS

6.1 Generation of KC-IL-1β mice: functional and histo-pathological characterization

To generate the new KC-IL-1β murine model we took advantage of the constitutive model of chronic pancreatitis, Elastase sshIL-1β mice line 124 (47) inter-breeded to LSL-KRAS^{G12D} mice (53). Double positive animals Elastase sshIL-1β; LSL-KRAS^{G12D} were further crossed to homozygous PDX-1-Cre mice, to ensure the excision of the silencing cassette at the LoxP sites and the subsequent expression of mutant allele KRAS^{G12D} in pancreatic progenitor cells, starting from embryonic day 8 in fetal mouse (49). Thus, we obtained mutant PDX-1-Cre;LSL-KRAS^{G12D};Elastase sshIL-1β animals (KC-IL-1β), characterized by mutant KRAS^{G12D} expressed under the PDX-1 promoter (35) and human IL-1β expression driven by rat elastase promoter (Figure 1A).

To confirm the functional activity of KC-IL-1β, we performed enzyme-linked immunosorbent assay (ELISA) in murine pancreatic protein extracts, validating the increased secretion of hIL-1β in KC- IL-1β mice at 7 weeks of age (Figure 1B).

Figure 1.

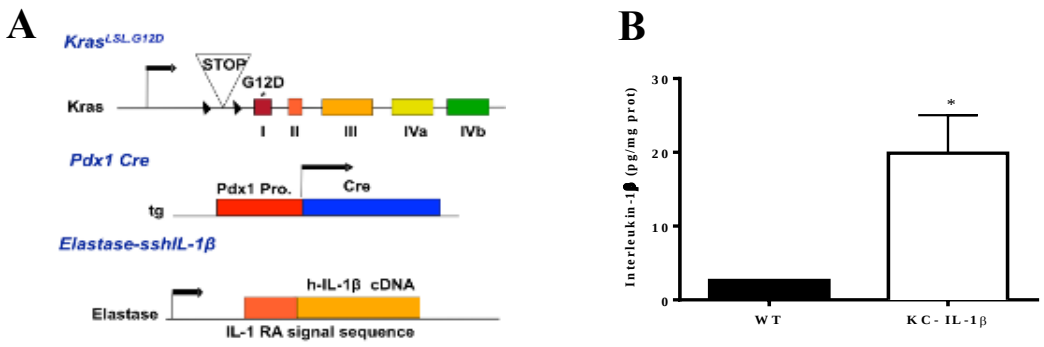
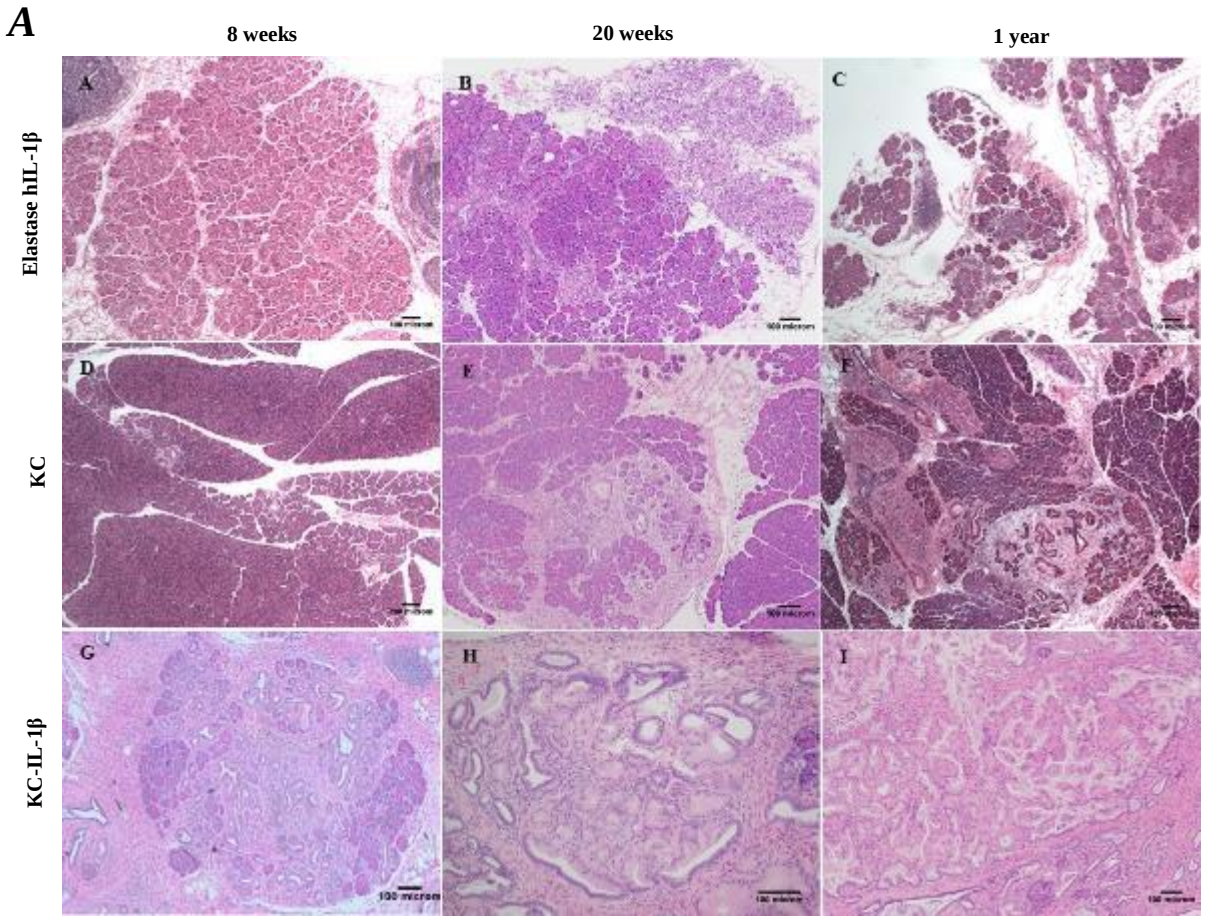


Figure 1. A) Genetic design of KC-IL-1β transgenic mice. B) ELISA from KC-IL-1β pancreatic tissues confirming increased IL-1β protein expression compared with wild type mice.

Histologically, KC-IL-1β mice displayed features of mild chronic inflammation, periductal fibrosis, acinar atrophy, ADM and PanIN1A-1B lesions since 8 weeks of age (Figure 2A: panel A,D,G). These changes progressed in severe chronic pancreatitis, with increased stromal reaction and loss of regular pancreatic parenchyma, replaced completely by dysplastic lesions in mice of 20 weeks of age and older (Figure 2A: panel B). At the same age, 75% of KC-IL-1β mice showed PanIN3 lesions and 25% invasive PDAC (Figure 2A: panel J,K). None of the age-matched control mice, Elastase hIL-1β and KC mice, developed pancreatic invasive lesions (Figure 2A: panel A-F).

One-year old KC-IL-1β mice developed malignant lesions: tumors were histologically characterized and covered a broad histopathological aspect, ranging from mucinous cystic type to carcino-sarcoma-like features (Figure 2A, panel I). Instead, one-year old Elastase hIL-1β and KC mice showed respectively severe pancreatic atrophy with adipose substitution, tubular complex and ADM the first, and diffuse PanIN 1-2 lesions the latter, both without a clear malignant evolution (Figure 2A: panel C,F). These data confirmed that IL1β overexpression cooperates with KRAS mutations accelerating the progression of precursor lesions and the PDAC development in KC- IL-1β.

Figure 2



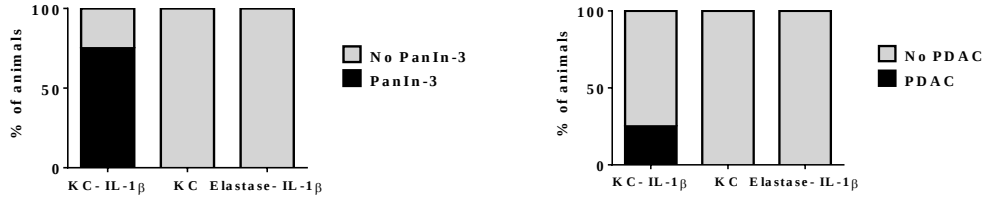


Figure 2. A) Histological sections from Elastase-IL-1β (panel A-C), KC (panel D-F) and KC-IL-1β (panel G-I). B) Number of PanIN-3 lesions and PDAC in KC-IL-1β mice and controls mice at 20 weeks of age.

6.2 IL-1β pancreatic overexpression accelerates the development of pancreatic invasive tumors in KRAS^{G12D} mice

We followed longitudinally a cohort of 13 KC-IL-1β mice up to 20 months of age for evidence of disease progression. 46% of animals (6/13) developed invasive pancreatic tumors, with a global median overall survival of 10.5 months. As internal controls, we bred both Elastase hIL-1β and KC mice. As previously published, the chronic pancreatitis model never developed invasive PDAC, rather a progressive acinar atrophy and pancreatic adipose substitution (Figure 2A: panel A-C) and none of the Elastase hIL-1β mice died during the period of observation. Differently, one mouse of the KC cohort developed PDAC and succumbed to cancer (1/13 – 7.7%) at 14 months of age. When directly compared, IL-1β overexpression mouse model significantly increased the rate of PDAC development in KRAS^{G12D} animals (Chi Square 0.02) (Figure 3A, 3B)

PDX-1-Cre;LSL-KRAS^{G12D};LSL-Trp53^{R172H} mice (KPC) were followed as well as positive control of carcinogenesis, showing PDAC incidence (15/15) and global overall survival (5.4 months) consistent with published data (Figure 3A).

Figure 3

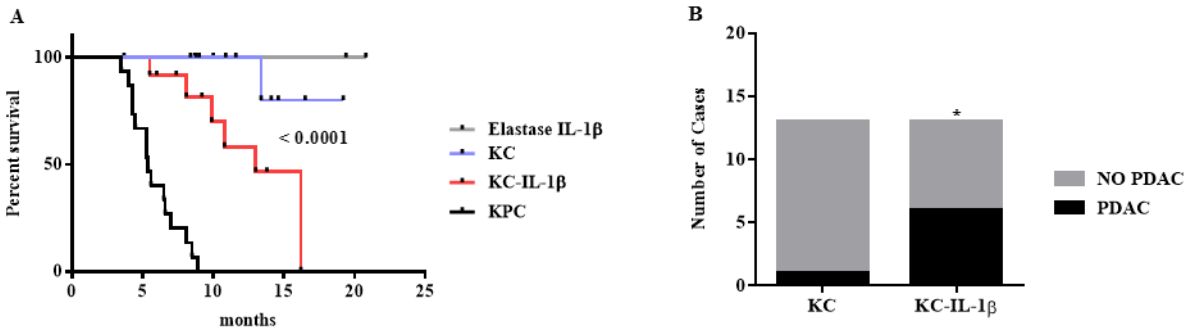


Figure 3. A) Overall survival (Kaplan-Meier) curve of Elastase IL-1β, KC, KC-IL-1β, KPC mice. B) Contingency graph indicating a significant increase in KC-IL-1β tumor incidence in comparison to KC.

All the tumors were histologically confirmed and KC-IL-1β neoplastic features are summarized in Table1.

ID	Age (mo)	Sex	PDAC	Histology	Liver	Lung	Ascites	PW/BW
D-1147	8.1	male	yes	invasive PanIn3	metastasis	no	no	1.01/28
E-2169	5.5	male	yes	adenocarcinoma	no	no	yes	2.05/35
D-7249	9.9	male	yes	anaplastic carcinoma	no	no	no	0.85/35
D-7247	13	male	yes	mucinous cystic neoplasm	metastasis	no	no	2.0/35
E-9054	10.8	male	yes	invasive PanIn3	metastasis	no	yes	2.01/35
D-7244	16.2	male	yes	adenocarcinoma	no	no	no	1.01/29

Liver metastasis were found in 50% of animals (3/6) whereas abdominal ascites in 33% of animals (2/6). Histologically, primary tumors were poorly differentiated PDAC, with malignant gland and infiltrating single cells, characterized by marked nuclear polymorfism and increased nuclear/cytoplasmatic ratio, and surrounded by a prominent periductal stromal reaction of spindle-like cells (Figure 4). One case showed features of mucinous-cystic-like neoplasm with low grade of cytological atypia, but characterized by cribriform architecture complexes and extensive malignant mucinous metaplasia with invasive capability.

Figure 4

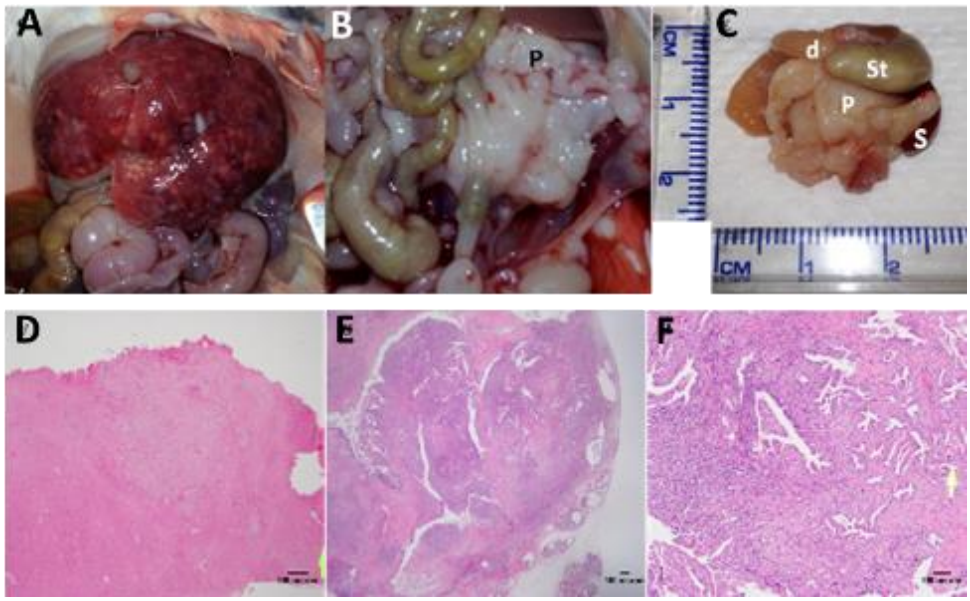
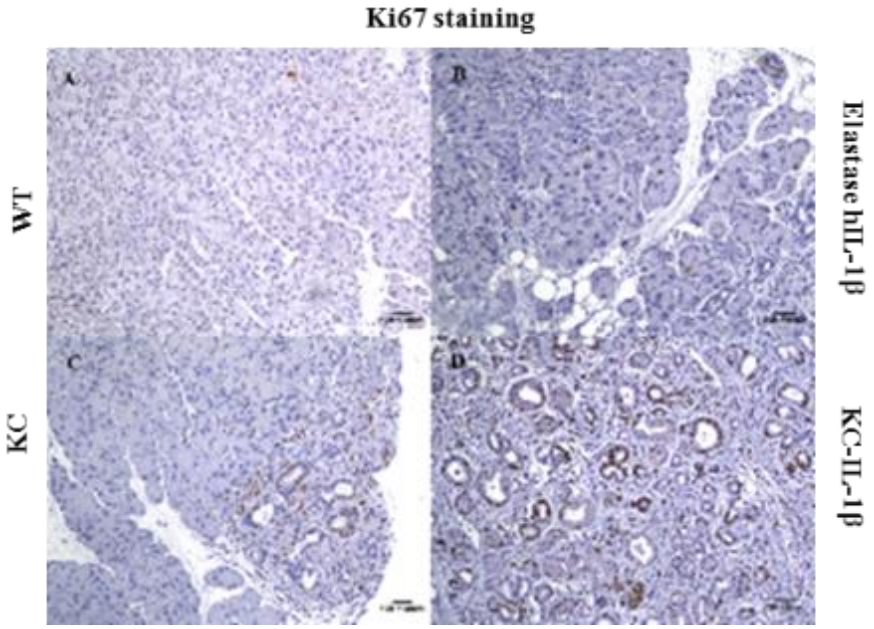


Figure 4. A) Macroscopic morphological pattern of liver metastasis and primary tumors KC-IL-1β (A-C). Histological sections from metastatic (D) and primary tumors (E,F).

6.3 IL-1β pancreatic overexpression increases proliferation in KRAS^{G12D} mice and stimulates ADM in organoid cultures through IL-1β/IL1-RI pathway

To better understand the early steps of carcinogenesis in KC-IL-1β mice, we analyzed the proliferative activity in paraffin-embeeded pancreatic sections in 20 weeks old Elastase hIL-1β, KC and KC-IL-1β mice. Consistently with the overall survival trend, the proliferation index evaluated by Ki67 immunostaining was significantly higher in KC- IL-1β samples in comparison to age-matched control mice. In particular the majority of Ki67 expressing cells were found in pancreatic epithelial structures like ADM and PanINs lesions, suggesting a major effect of IL-1β-promoted pancreatitis on the pancreatic epithelial compartment (Figure 5: panel A,B).

Figure 5



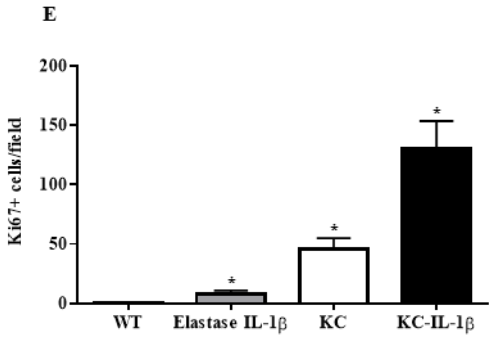


Figure 5. Ki-67 staining of samples from wild type pancreas (panel A), Elastase hIL-1β (panel B), KC (panel C) and KC-IL-1β (panel D). Quantitative evaluation of Ki-67 label index (panel E).

Thus, to test if IL-1β overexpression plays a role directly on epithelial cells or if its effect is mostly mediated by immune cells involved in inflammation, we studied IL-1β effect on primary epithelial pancreatic cells in the very first step of carcinogenesis, as identified in ADM process (54,55).

To study ADM we took advantage of 3D pancreatic organoid cultures, able to reproduce a regenerative and dedifferentiation program in vitro (56,57).

We isolated $KRAS^{G12D}$ epithelial cells from KC pancreata and we treated them as shown in Figure 6A. IL-1β exposure promoted the ADM process, as shown by the increased number of $KRAS^{G12D}$ organoids in comparison to the untreated $KRAS^{G12D}$ spheroids (Figure 6B-C). Then, to rule out unspecific effect of IL-1β administration in increasing organoid number, we treated cells with a human recombinant IL-1 receptor antagonist, able to competitively block the IL1-RI receptor. Thirty-minutes of RA pre-treatment, followed by 24 hours IL-1β administration, counteracted the cytokine effect on organoid number (Figure 6C), suggesting that its effect is primarily directed on epithelial cells and it is specifically mediated by IL-1RI.

No differences in organoids number were found between untreated $KRAS^{G12D}$ and $KRAS^{G12D}$ spheroids exposed to IL1-RA alone, proving that IL1-RA exposure itself has no toxic effects on primary pancreatic cells (Figure 6B-C).

Taken together, these data show that IL-1β acts directly on epithelial $KRAS^{G12D}$ cells increasing proliferation in vivo and inducing ADM process in pancreatic organoid cultures. This effect is mediated by IL1-RI on pancreatic cells, and it can be blocked using the human IL1-RA (anakinra), currently approved by FDA for rheumatoid arthritis treatment.

Figure 6

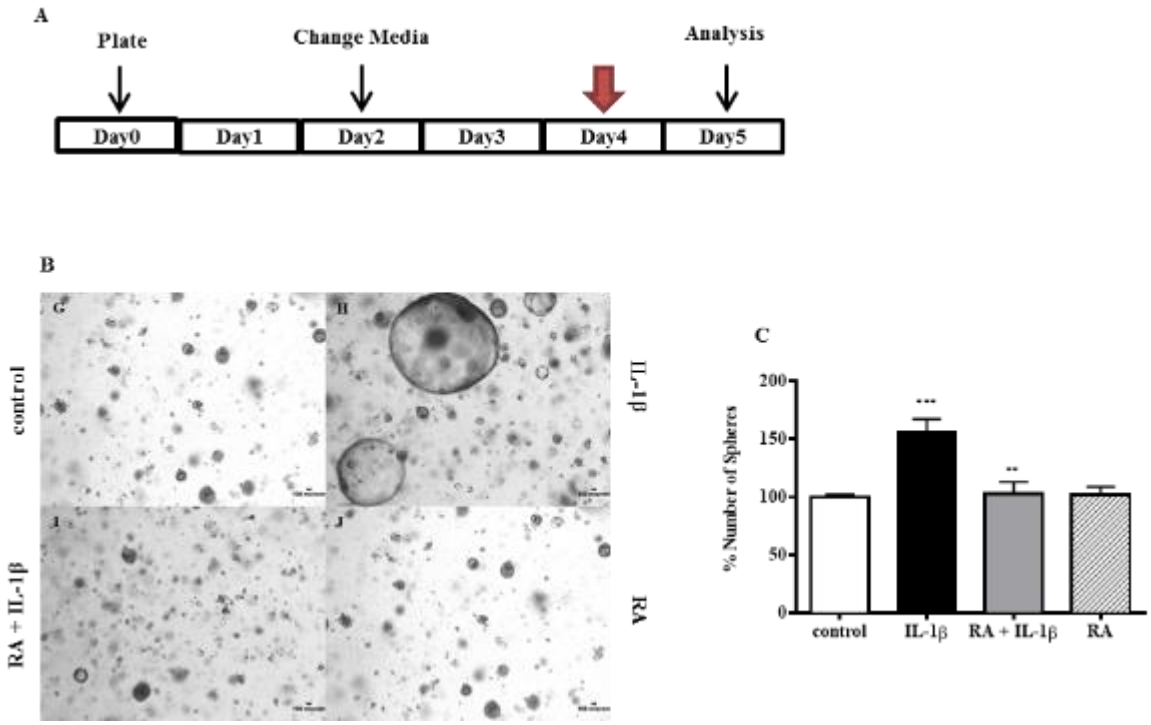


Figure 6. A) Schematic representation of IL-1β treatment B) Images of spheroid cultures C) Quantitative evaluation of the number of spheroids in untreated and IL- 1β and RA treated spheres.

6.4 IL-1β pancreatic overexpression promotes EMT activation in KC-IL-1β mice

Since inflammation is shown to be a promoter of EMT in many solid tumors (58) we looked at the role of chronic pancreatitis on EMT process during carcinogenesis in KC-IL-1β mice. Starting from 8 weeks and up to 20 weeks of age, we appreciated a progressive decrease of the number of cells stained by anti E-cadherin antibody in pancreatic tissue (Figure 7: panel A,B), up to a very low staining in cancer lesion (Figure 7: panel C). The opposite trend is observed after vimentin staining, showing a constantly increase of vimentin positive staining in epithelial cells (Figure 7: panel D-F). Notably, at 20 weeks of age and in full-blown pancreatic cancer not only the stromal microenvironment reacted to vimentin staining, but also single ductal cells were vimentin positive in PanIN lesions and in the tumor epithelial component. These data let us to speculate about a mesenchymal transformation of epithelial pancreatic cells in early pre-neoplastic stage, due to an early and chronic IL-1β exposure in pancreatic tissue.

Taken together, these data suggest that IL-1β overexpression stimulates the loss of epithelial characteristics in pancreatic cells, transforming acinar and ductal cells in fibroblast-like cells, potentially not distinguishable from the surrounding stroma during PDAC development.

Figure 7

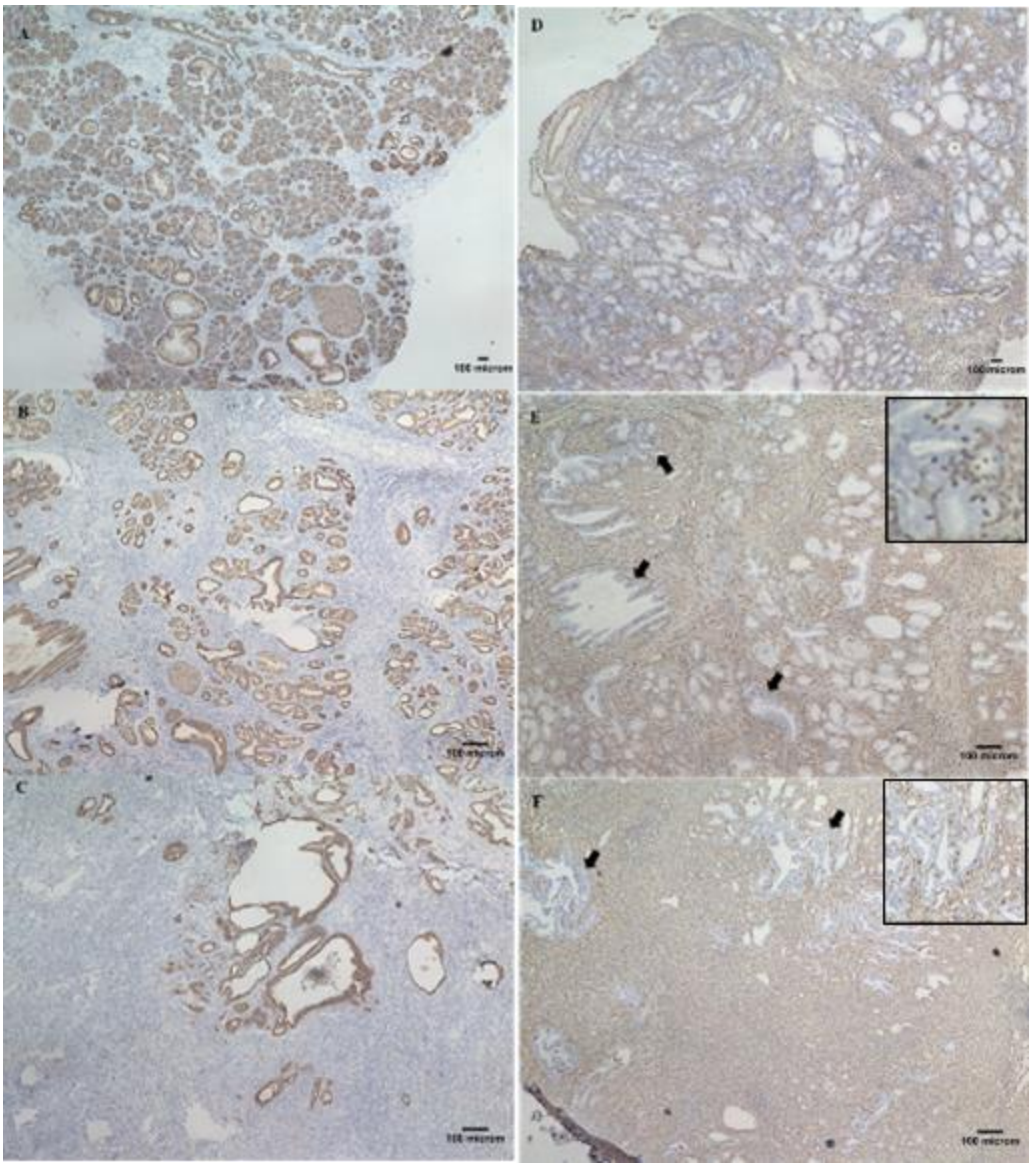


Figure 7. A-C) E-Cadherin staining on pancreatic sections from KC-IL-1β mice 8 weeks old (A), 20 weeks old (B) and KC-IL-1β mice affected by PDAC (C). D-F) Vimentin staining on pancreatic sections from KC-IL-1β mice 8 weeks old (C), 20 weeks old (D) and KC-IL-1β mice affected by PDAC (F).

6.5 IL-1β exposure promotes EMT in KRAS^{G12D} pancreatic organoid cultures through IL-1β/IL1-RI pathway

To better understand how IL-1β induces EMT in early steps of carcinogenesis, we studied the effect of IL1β exposure on primary KRAS^{G12D} organoids (see treatment protocol in figure 8A).

Vimentin expression was highly increased in spheroids culture after IL-1β exposure when compared to untreated organoids (Figure 8B, 8C: panel A-D). Pre-treatment with IL1-RA counteracted the IL-1β effect, suggesting that the EMT process induced by IL-1β, as well as its pro-proliferative effect, are mediated by IL-1R in primary KRAS^{G12D} organoids (Figure 8B, 8C: panel E-F).

Thus, we analyzed two important transcription factors able to mediate EMT in pancreas, Zeb1 and Snail: we showed that both of these markers greatly increased after IL-1β exposure. This effect, as well as vimentin expression, was antagonized by IL-1RA pre-treatment on KRAS^{G12D} organoids (Figure 8D, 8E), confirming the relevance of IL-1β/IL1-RI pathway in EMT induction in pancreatic organoids.

Interestingly, beside IL-1β effect on proliferation and EMT, we observed the increase of inducible Nitric Oxide Synthase (iNOS) on KRAS^{G12D} organoids after IL-1β treatment. iNOS gene activation induces NO production, a reactive oxygen species involved in immune response and angiogenesis in many cell types. Again, the effect of IL-1β on iNOS expression level was blocked by IL-1RA administration, proving that IL-1β plays a direct role on in oxidative stress induction, and this action is mediated by IL1-RI activation (Figure 8F).

Figure 8

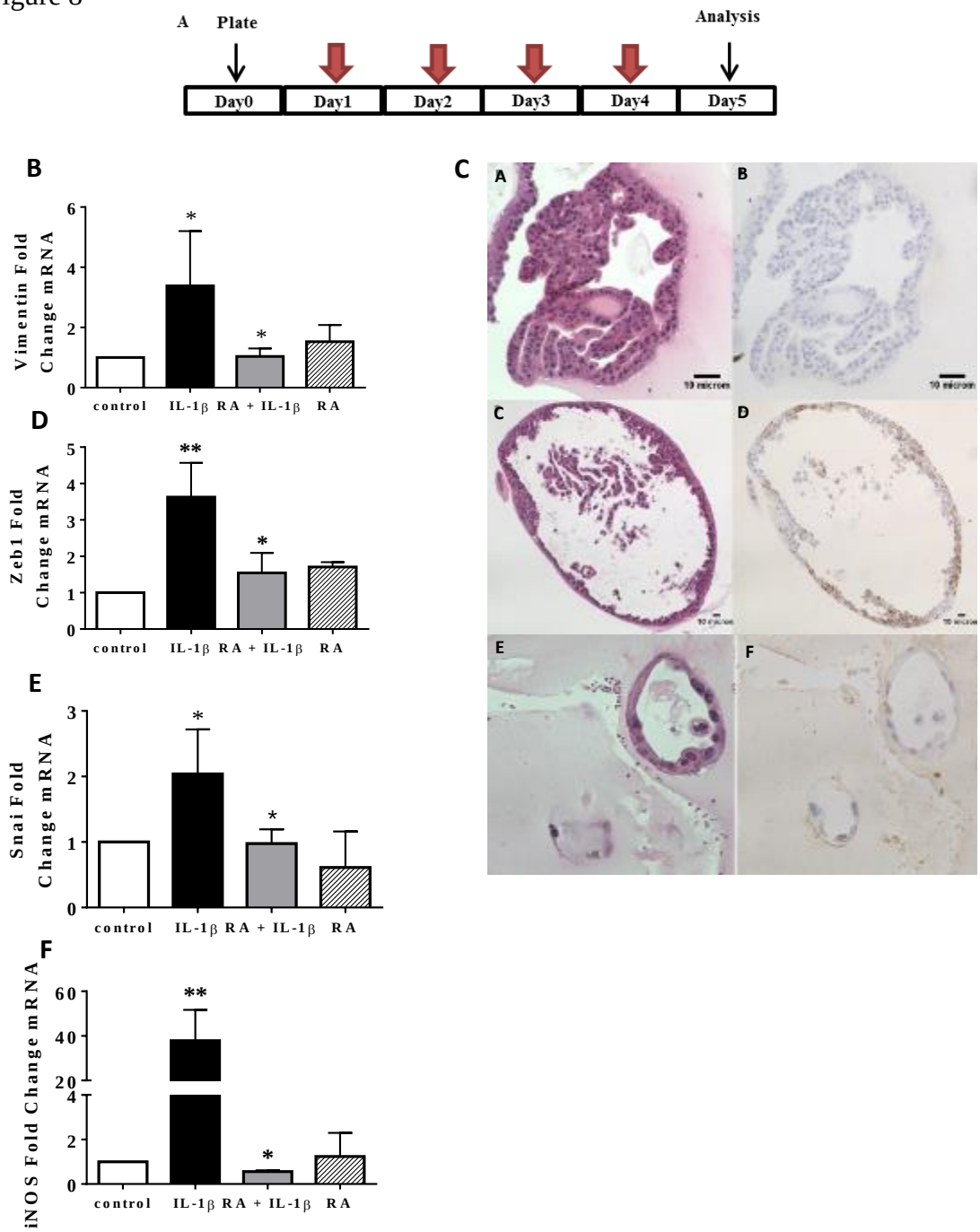


Figure 8. A) Schematic representation of IL-1β treatment. B) Vimentin mRNA expression level after IL-1β treatment, combination treatment of IL-1β and IL1-RA (IL-1 β + RA) and IL1-RA alone (RA). C) H&E sections (panel A-C) and Vimentin staining (panel D-F) of spheroids cultures: untreated spheres (panel A,D), IL-1β treated spheres (panel B,E), and combination treatment of IL-1β and IL1-RA (panel C,F). D) Zeb1 mRNA expression level after IL-1β, combination treatment IL-1 β + RA and RA treatments. E) Snail mRNA expression level after IL-1β, IL-1 β + RA and RA treatments. F) Zeb1 mRNA expression level after IL-1β, IL-1 β + RA and RA treatments.

Moreover, the crucial role of IL1-RI has been confirmed further in human PDAC samples, showing a higher gene expression level of the IL-1 receptor in tumor tissue, in comparison to the level found in the adjacent normal pancreatic areas (Figure 9A). Moreover we detected a strong IL1-RI and vimentin reactivity in human PDAC samples (Figure 9B: panel A-C). Notably, vimentin expression was found in both epithelial and stromal compartment in PDAC (Figure 9B: panel B).

This suggests a strict connection between IL1-RI signaling and EMT process. In particular the high expression of IL1-RI and the strong vimentin reaction, let us speculate that IL-1β/IL1-RI inflammatory pathway can promote EMT and dissemination in human pancreatic tissue, providing the rationale for a therapeutic strategy based on IL-1β receptor blockade in individuals at high risk for pancreatic cancer or affected by chronic pancreatitis.

Figure 9.

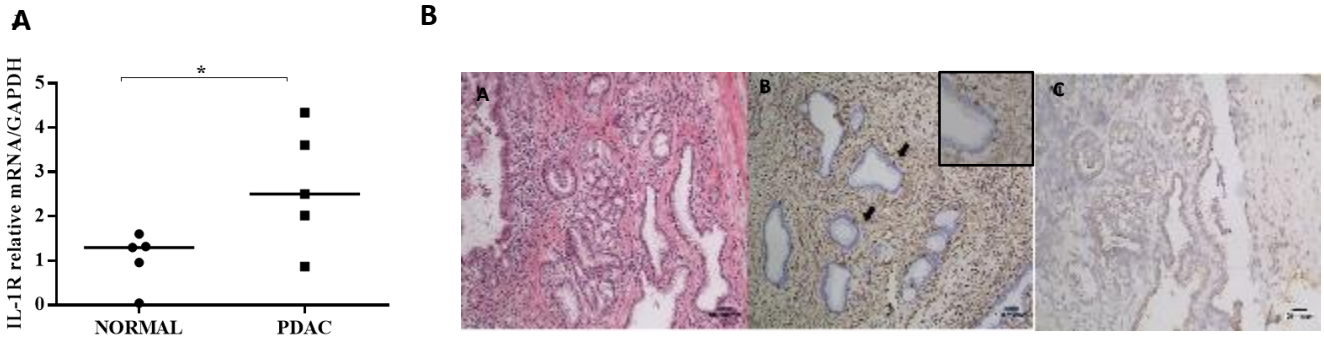


Figure 9. A) IL-1R mRNA expression level in normal and tumor human pancreatic tissues. B) H&E (panel A), vimentin (panel B) and IL-1R (panel C) staining on human PDAC samples.

6.6 IL-1β exposure up-regulates the ribosome biogenesis rate and down-regulates the expression of p53

The above reported results indicated that IL-1β was responsible for the induction of EMT both in the pancreas of KC-IL-1β mice and in pancreatic organoid cultures.

There is evidence that IL-6, one of the major interleukin involved in the relationship between inflammation and cancer, induces the acquisition of cellular phenotypic changes characteristic of EMT, in human cancerous and non-cancerous cell lines and in human colon epithelial cells in ulcerative colitis. These EMT changes are due to the fact that IL-6 enhances rRNA transcription which, by reducing the availability of ribosome proteins for MDM2 binding, increases the MDM2-mediated proteasomal degradation of p53, through the well- established ribosomal proteins/MDM2/p53 pathway (59,60). Therefore, we wondered whether the mechanism described for IL-6 induction of EMT might be valid also for IL-1β. For this purpose we analyzed the effect of IL-1β on ribosome biogenesis on $KRAS^{G12D}$ organoids. The changes in rRNA transcriptional activity in these cell exposed to IL-1β were ascertained by using real-time PCR analysis of the 45S pre-rRNA expression. This analysis revealed that IL-1β greatly enhanced rRNA transcription (Fig. 10A). Then, we evaluated the effect of IL-1β exposure on p53 protein expression in primary $KRAS^{G12D}$ cells by Western blot analysis. After IL-1β treatment the amount of p53 appeared to be markedly reduced in comparison not only with control cells but also with cells exposed to IL-6 (Fig. 10B).

Figure 10

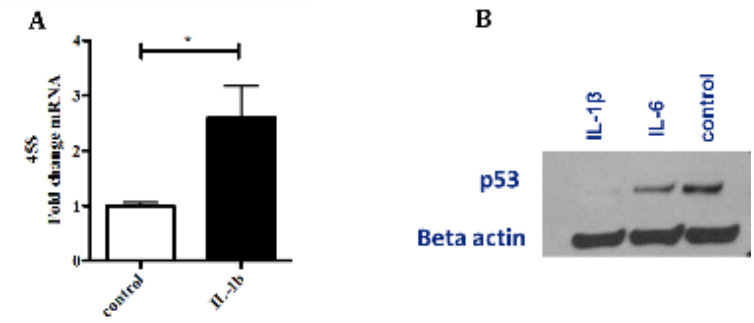
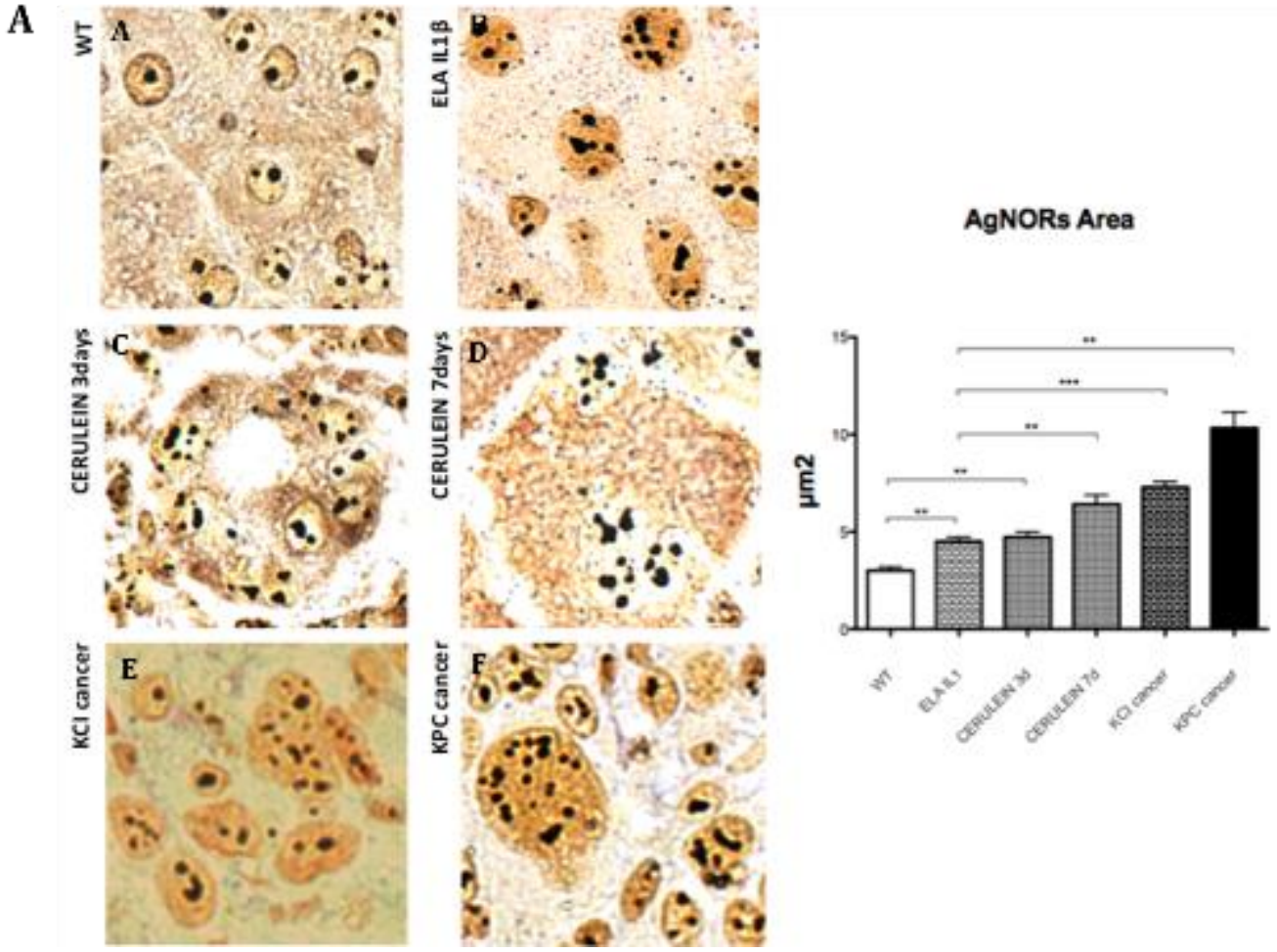


Figure 10. A) 45S pre-rRNA mRNA levels in untreated and IL-1β exposed (24 hours) KRAS^{G12D} spheres. B)Western Blot evaluation of p53 level in untreated, IL-1β and IL-6 exposed (24 hours) primary KRAS^{G12D} cells.

Therefore, these results indicated that IL-1β activated the same pathway that in IL-6 treated cells was responsible for activation of the EMT process. Since the enhancement of ribosome biogenesis was at the basis of EMT activation, we evaluated whether this metabolic change also occurred in the pancreas of Elastase hIL-1β mice and other mouse models of chronic pancreatitis (wild type mice treated with cerulein) and in KC-IL-1β and KPC mice. For this purpose we measured the size of nucleoli of acinar cells in histological sections stained with the silver procedure specific for nucleolar proteins on the nucleolar organizer, the AgNOR proteins (61). Since the distribution of the AgNOR proteins within the cell is directly proportional to the nucleolar size and to its ribosome biogenesis rate, their quantitative morphometric analysis allows precise information to be obtained on the rate of rRNA transcription of tissues in situ (61,62). As shown in Fig. 11A pancreatic acinar cells from Elastase hIL-1β mice, as well as KC-IL-1β, exhibited an increased amount of the silver-stained nucleolar structures which indicated an up-regulated ribosomal biogenesis rate in comparison with the corresponding cells from wild-type mice. Taken together, these observations may lead to conclude that factors produced in the inflammatory milieu, such as IL-6 and IL-1β, increase the ribosome biogenesis, the first step which activates the EMT process. For this reason, we also investigated whether an up-regulation of rRNA synthesis actually occurs

in human pancreatic acinar cells during chronic pancreatitis (Figure 11B). Quantitative morphometric analysis of the size of nucleoli in five cases of chronic pancreatitis carried out on histological section selectively silver-stained for AgNOR proteins, clearly showed that the amount of the nucleolar silver-stained structures of acinar cells from chronically inflamed pancreas was statistically higher than that of acinar cells from normal pancreas (5.23 µm ± 1.78 SD *vs* 3.47 µm ± 1.14 SD; $p<0.01$).


A
WT
A
ELA IL1β
B
CERULEIN 3days
C
CERULEIN 7days
D
KCl cancer
E
KPC cancer
F
AgNORs Area
µm2
15
10
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WT
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KCl cancer
KPC cancer

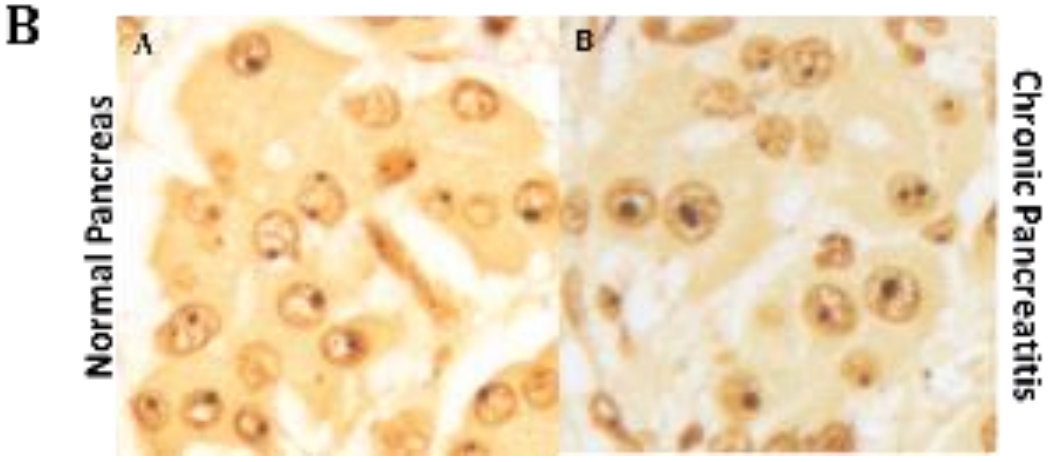
B
Normal Pancreas
A
B
Chronic Pancreatitis

Figure 11

Figure 11. A) AgNORs staining and quantitative evaluation in pancreatic samples from wild type (WT) (panel A), Elastase hIL-1β (panel B), wild type mice 3 days (panel C) and 7 days (panel D) after cerulean treatment, KC-IL-1β (panel E) and KPC mice (panel F) B) AgNORs staining of human normal pancreatic samples and chronic pancreatitis (panel A-B)

7. DISCUSSION

Many studies have proved that inflammation is a risk factor of pancreatic cancer onset. However the exact mechanism linking chronic pancreatitis and PDAC development has not been completely defined yet.

Here, we reported a new mouse model of pancreatic cancer arisen from chronic pancreatitis, characterized by a tumor incidence of more than 40%, created by combining pancreatic IL-1β overexpression and $KRAS^{G12D}$ mutation in PDX-1-Cre;LSL-$KRAS^{G12D}$;Elastase sshIL-1β mice. These animals showed an increase in pre-neoplastic epithelial cells proliferation, a faster development of PanINs and faster progression to invasive PDAC in comparison to KC model.

There is evidence that IL-1β level is elevated in many human cancers (8). IL-1β is mainly released by tumor-associated macrophages in the tumor microenvironment, where it can act promoting angiogenesis, tumor growth and metastasis (63). Moreover, the enforced expression of IL-1β is strictly correlated with tumor progression and advanced metastatic disease in cancer patients (64,65).

In this study we highlighted two critical events due to high IL-1β expression. First, we demonstrated the role of IL-1β on epithelial pancreatic cell malignant transformation, and second, we showed that IL-1β acts as EMT trigger, essential for cancer cell dissemination and metastatic spread. Both these crucial aspects, needed for survival and dissemination of PDAC, were blocked by antagonizing the IL1 receptor. Moreover, using a pure epithelial organoid pancreatic culture, we demonstrated that IL-1β stimulates directly the ADM process, thus proving the specific effect of IL-1β on PDAC initiation and identifying this cytokine as a crucial element, among all the tumor microenvironment elements, that was able to mediate inflammation in cancer initiation. In the same kind of culture we showed the direct role of IL-1β on pancreatic EMT, proved by the increased expression of Vimentin, Zeb1 and Snail, and restored

by IL1-RA treatment, to antagonize IL-1β effect. The EMT process induced by IL-1β was very likely the consequence of the down-regulation of p53 expression. In fact, IL-1β exposure greatly stimulated the rRNA transcription, which has been shown to increase the MDM2-mediated degradation of p53 (21,66), and p53, as it has been repeatedly demonstrated, negatively controls the activation of the EMT program (21, 67-69)

Another important effect of IL-1β stimulation we observed in the present study was the very high increase in iNOS gene expression in $KRAS^{G12D}$ pancreatic organoids. iNOS, the synthase isoform most commonly associated with malignant disease, is known to be responsible for the induction of the production of high level of NO, which in turn stimulates malignant transformation, angiogenesis, and metastasis spread (65). Thus, our observation suggests that IL-1β may play a key role also in the oxidative stress induction, with an inflammation-mediated production of NO. Moreover, since its known from literature that IL-1β pro-tumorigenic activity is mediated by NF-κB (70), and iNOS gene promoter is regulated by NF-κB dependent activation, we can argue about an IL-1β mediated stimulation of this transcript, highlighting its direct effect on oxidative stress induction.

In this study, every effect induced by IL-1β stimulation has been counteracted by using the human recombinant IL1-RA, highlighting a therapeutic strategy in blocking IL-1β pathway during tumorigenesis, as recently showed in pancreatic cancer cells and orthotopic nude mice (71).

Moreover, the IL1-RA anakinra is an FDA approved compound for severe rheumatoid arthritis treatment (72) and its use in combination with chemotherapy is currently underway in phase I trials for advanced breast and pancreatic cancer patients (clinicaltrials.gov/ ct2/show/ record/ NCT02021422; clinicaltrials.gov/ ct2/ show/record NCT02021422).

In conclusion, the crucial role of IL-1β we have shown here during PDAC tumorigenesis and progression, highlight the importance to develop new therapeutic drugs blocking the IL1 receptor signaling, especially for those patients affected by chronic inflammation whit high IL-1β level, in order to block

the cytokine action since the very first steps of carcinogenesis, where its inhibition may be of crucial effect in blocking disease progression towards metastatic PDAC.

8. MATERIALS AND METHODS

Pancreatic Tissue Preparation:

Pancreata were isolated and washed in cold DEPC water. Portions were cut and snap frozen in liquid nitrogen for protein and RNA extraction. Proteins were extracted using a tissue homogenizer and RIPA buffer + protein inhibitors (complete EDTA free, Roche Applied Science, Indianapolis, IN, USA) and protein quantification was performed using the Bradford method. For RNA isolation, frozen samples were ground using a pestle in liquid nitrogen and homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was extracted according to manufacturer's instructions and cleaned using RNeasy columns (Qiagen, Valencia, CA, USA). The remaining tissue was fixed overnight in 10% neutral buffered formalin before transfer to 70% ethanol. Specimens were then processed and embedded in paraffin for histologic analysis.

Interleukin IL-1β Quantification:

To quantify human interleukin-1β in pancreatic tissue, ELISA assays were performed with the Quantikine® Human IL-1β immunoassay (R&D systems, Minneapolis, MN, USA, #DLB50) according to the manufacturer's instructions.

Histology, immuno-histochemistry, immunofluorescence and microscopy:

5µm paraffin embedded were prepared for immunohistochemistry For immunohistochemical staining. Slides were deparaffinized in xylene and endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide in methanol for visualization using the peroxidase reaction. Antigen retrieval was performed by boiling the slides in citrate buffer S2 (10mM pH 6.0) in a water bath

for 20 min. Slides were rinsed in PBS Tween 0.05% and blocked for 30 min. with 2% BSA. Primary antibodies and biotinylated secondary antibodies (Jackson Immunoresearch) were diluted in 2% BSA and incubated overnight at 4°C. Subsequently, slides were incubated with alkaline phosphatase or peroxidase conjugated streptavidin (Dako) and either VectorRed substrate (Vector Laboratories) or 3,3'-diaminobenzidine (Sigma-Aldrich) as chromogens, respectively. Slides were counterstained with hematoxylin and mounted for viewing. Bright field and fluorescence images were acquired using an Eclipse TU2000-U microscope (Nikon) connected to a cooled color CCD camera (RTKE Diagnostic Instruments) using SPOT software (Spotimaging)

Quantification of immunohistochemical staining:

Ki67 positive cells were quantified in five high power fields from at least 3 mice per group.

RNA extraction and quantitative RT-PCR analysis:

RNA was extracted from each group using RNAqueous®-Micro Total RNA Isolation Kit (Thermo Sci,catalog no. AM1931). High-fidelity cDNA was generated from each RNA sample with the Superscript III cDNA amplification system (Invitrogen). Quantitative RT-PCR reaction samples were prepared as a mixture with Quantitect SYBR Green PCR kit (Qiagen). Reactions were performed using an Applied Biosystems Prism 9700 PCR machine.

Sphere Cultures:

3D cultures were performed as described previously (Wescott et al., 2009), after isolation of primary pancreatic epithelial cells from KC mice. Organoids were cultured for 5-7 days before analysis. Sphere number and size were analyzed using ImageJ software. Human IL-1β 50 ng/mL and human recombinant IL1-RA 50 ng/mL containing medium was changed every other day.

Western Blotting:

Protein extraction was performed on ice using RIPA buffer with protease inhibitor (Complete) (Roche) cocktail and phosphatase inhibitor (phosSTOP) (Roche). Protein samples were subsequently separated in 10%Bis-Tris Gel NuPAGE® electrophoresis using MES SDS Running Buffer (Invitrogen, CA, USA). After transfer to nitrocellulose, membranes were blocked with 5% BSA, and samples were probed with anti p53 primary antibody (Cell Signaling) followed by horseradish peroxidase-coupled secondary antibody. Images were acquired using X-ray film development, and bands were antified with ImageJ.

NOR Silver Staining

NOR silver staining was performed according to the guidelines of the "International Committee on AgNOR Quantitation" (73). In short, slides were moved from water to heat-resistant plastic Coplin jars, fully immersed in 10 mM sodium citrate buffer (pH 6.0) and autoclaved at 120C for 20 min. After cooling to room temperature in the sodium citrate buffer, slides were stained with silver for 13 min at 37C in the dark using a solution of one volume 2% gelatin in 1% aqueous formic acid and two volumes of 50% silver nitrate. Sections were finally dehydrated and mounted in Canada balsam without any counterstaining.

Image Cytometry

Quantitative evaluation of silver-stained nucleolar structures was carried out by image cytometry. Morphometric analysis of silver-stained NORs was carried out using the Image-Pro Plus program (Media Cybernetics, Silver Spring, MD). The main stages of image processing were as follows: a field was selected by the operator at the microscope under a ×40 objective lens. The selected image was then captured and stored in the digital memory and displayed on the color monitor. Here the operator

interactively defined the gray threshold that permitted automatic quantification of only the black dots corresponding to the silver- stained nucleoli. The morphometric analysis was then performed on a cell-to-cell basis by converging the window over the corresponding nucleus. For each case, the AgNOR area of at least 100 nuclei was measured and the mean AgNOR area (±SD) calculated.

Tissue samples:

Human pancreatitis and pancreatic cancer samples were provided by Dr. Helen Remotti (Dept. of Pathology – CUMC). Murine pancreatic cancer samples were provided by Dr. Helen Remotti, Dr. Gloria Su and Dr. Ken Olive. All samples were anonymized.

Statistical Analysis

Statistical testing and was done using GraphPad Prism software with the appropriate test for each experiment. For experiments with two experimental groups two-tailed, unpaired t-test were applied. Proportions were compared with the test for proportions (Tarca et al., 2009). For experiments with three or more groups, we applied 1way ANOVA testing with Bonferroni's Multiple Comparison Test. Statistical significance was depicted as follows:

*= $p < 0.05$, **= $p < 0.005$ and ***= $p < 0.000$

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