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IL17 Functions through the Novel REG3β–JAK2–STAT3 Inflammatory Pathway to Promote the Transition from Chronic Pancreatitis to Pancreatic Cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) offers an optimal model for discovering "druggable" molecular pathways that participate in inflammation-associated cancer development. Chronic pancreatitis, a common prolonged inflammatory disease, behaves as a well-known premalignant condition that contributes to PDAC development. Although the mechanisms underlying the pancreatitis-to-cancer transition remain to be fully elucidated, emerging evidence supports the hypothesis that the actions of proinflammatory mediators on cells harboring Kras mutations promote neoplastic transformation. Recent elegant studies demonstrated that the IL17 pathway mediates this phenomenon and can be targeted with antibodies, but the downstream mechanisms by which IL17 functions during this transition are currently unclear. In this study, we demonstrate that IL17 induces the expression of REG3β, a well-known mediator of pancreatitis, during acinar-to-ductal metaplasia and in early pancreatic intraepithelial neoplasia (PanIN) lesions. Furthermore, we found that REG3β promotes cell growth and decreases sensitivity to cell death through activation of the gp130-JAK2-STAT3-dependent pathway. Genetic inactivation of REG3β in the context of oncogenic Kras-driven PDAC resulted in reduced PanIN formation, an effect that could be rescued by administration of exogenous REG3β. Taken together, our findings provide mechanistic insight into the pathways underlying inflammation-associated pancreatic cancer, revealing a dual and contextual pathophysiologic role for REG3β during pancreatitis and PDAC initiation. *Cancer Res; 75(22); 4852–62. ©2015 AACR.*

Introduction

Pancreatic ductal adenocarcinoma (PDAC) has been recognized by the scientific community, advocacy groups, and government agencies as an important national health priority because of its physically and morally painful impact and dismal outcome. Interestingly, in the recent past, most research efforts have primarily focused on how genetic and epigenetic alterations lead to the aberrant activation of key oncogenes and inactivation of tumor suppressor pathways to consequently confer the transforming pancreatic cells with growth and survival advantages. The most common genetic abnormality in pancreatic cancer is oncogenic *KRAS* mutation, which is the initial key event for the initiation phase of pancreatic carcinogenesis. However, mutation of *KRAS* alone is not sufficient for frank cancer progression, but rather additional aberrations, such as inactivation of tumor suppressor genes and signals from the tumor microenvironment, are required for tumor promotion and progression. In this regard, pancreatic cancer that originates in the setting of inflammation (chronic pancreatitis) offers an optimal model to study these events. Chronic pancreatitis is a known premalignant disease with a 53-fold lifetime cumulative risk of developing pancreatic cancer (1). Notably, oncogenic *KRAS* mutations are also found in this disease and are believed to contribute to its transformation into cancer. In fact, emerging data indicate that proinflammatory mediators can act on pancreatic cells carrying *KRAS* mutation to complete their process of neoplastic transformation through modulation of differentiation, growth, survival, and senescence.

In this regard, our laboratory has focused on characterizing druggable proinflammatory pathways that function as mediators of the pancreatitis–cancer transition. The current study, therefore, focuses on characterizing the function of REG3β, one of the best known pancreatitis mediators, in the initiation of pancreatic

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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cancer by KRAS. This molecule, also known as pancreatitis-associated protein (PAP), p23, or hepatocarcinoma-intestine-pancreas protein, was originally discovered in the pancreatic juice of rats with acute pancreatitis, but was absent in the pancreatic juice from healthy rats (2). The PAP/REG3β gene and its mRNA were subsequently cloned from several species (3–10), indicating that *REG3β* is an evolutionarily conserved gene. REG3β expression is found in a limited number of healthy tissues, such as in Paneth cells of the small intestine (11), in luminal epithelial cells of the uterus in estrus (12), in alpha cells of the Langerhans islets (13), and in somatotropic cells of the pituitary gland (14). In contrast, REG3β is overexpressed in various diseased tissues, such as the pancreas with acute pancreatitis (3, 15), transformed hepatocytes (9), the brain with Alzheimer disease (16), regenerating motoneurons (17), the brain in response to a traumatic injury (18), inflamed (19, 20) and transformed epithelial colonic cells (21), cholangiocarcinoma cells (13, 22), regenerating islet beta cells (23), the myocardium of rats with decompensated pressure-overload hypertrophy (24), pheochromocytoma cells (25), bladder cancer cells (26), and psoriatic keratinocytes (27). Structurally, REG3β is a 16-kDa secretory protein related to C-type lectins, although a classical lectin-related function has not been reported yet, apart from a study suggesting that REG3β binds to bacterial proteoglycans (28). Moreover, several pro- and anti-inflammatory cytokines are able to induce REG3β expression, which can also be self-induced through the canonical JAK2/STAT3-dependent pathway (29). Thus, based on this knowledge, we predict that REG3β may mediate procarcinogenic effects downstream of proinflammatory pathways with known transforming abilities. To test the validity of this hypothesis, we investigated whether REG3β modulates the neoplastic effects of the IL17A pathway. This idea was recently stimulated by elegant studies, which demonstrated an essential role of IL17A in the development of PDAC after induction via activated oncogenic Kras (30). In fact, *in vivo* gain-of-function and loss-of-function studies from these investigations both demonstrated that IL17A promotes the initiation and progression of PDAC. *In vivo* neutralization of IL17A led to a rapid change in the gene expression program of PDAC cells, including the loss of IL6 expression and STAT3 activation, two key regulators of PDAC development (30). These results suggest that signals through the IL17RA can alter pancreatic cell transformation. However, the full repertoire of molecular mediators that work downstream of IL17A to modulate the inflammation-to-cancer transition remain to be defined.

In the current study, we report for the first time that REG3β is activated by the IL17A in pancreatic epithelial cells with $Kras^{G12D}$ mutations. In addition, we observe that REG3β expression is induced during acinar-to-ductal metaplasia (ADM) and in early pancreatic intraepithelial neoplasia (PanIN) lesions, which are the histopathologic hallmarks of pancreatic cancer initiation. Moreover, in this process, REG3β primarily acts as a paracrine factor to stimulate the development of PanIN downstream of IL17RA. This effect of REG3β reflects its ability to promote cell growth and decreases sensitivity to apoptosis by coupling to the gp130-JAK2-pSTAT3 signaling pathway. Furthermore, REG3β boosts interactions between epithelial cells and immune cells, triggering the expression of some mediators, such as IL10 and TGFβ, and activating the CXCL12/CXCR4 axis. Finally, we describe results from two antibody-based preclinical trials, which demonstrate the efficacy of neutralizing either IL17 or REG3β in antagonizing KRAS-induced pancreatic cancer initiation. Thus, in addition to providing valuable mechanistic information, this study offers new knowledge of significant biomedical relevance, which should be taken into consideration for the future design of strategies aimed at preventing inflammation-associated pancreatic cancer development.

Materials and Methods

Mice

Descriptions of the relevant strains *Pdx1*-cre;*LSL*-$Kras^{G12D}$, *Pdx1*-cre;*LSL*-$Kras^{G12D}$;Ink4a/$Arf^{fl/fl}$, and REG3β$^{-/-}$ can be found elsewhere (31–33). Because animals originate from different genetic backgrounds, we systematically relied on littermate control and experimental mice. Pancreatitis was induced through the intraperitoneal administration of cerulein (Sigma) at 250 μg/kg body weight twice daily for one week. The antibody anti-IL17RA was purchased from R&D Systems and anti-REG3β was obtained from Dynabio S.A.; both were intraperitoneally injected twice weekly. Recombinant REG3β was obtained from Dynabio S.A. and intraperitoneally injected daily. Mice were kept in the Experimental Animal House of the Centre de Cancérologie de Marseille (CRCM), pole Luminy, following institutional guidelines.

Cell culture

AR-42J, MiaPaCa2, Panc1, and THP-1 cells obtained from the ATCC were maintained in DMEM (Invitrogen) supplemented with 10% FBS at 37°C with 5% CO_2. Recombinant IL17A was purchased from R&D Systems; anti-STAT3 and anti-phospho-STAT3 antibodies and neutralizing anti-gp130 were obtained from Cell Signaling. The JAK2 inhibitor AG-490 was purchased from Sigma. THP-1 cells were differentiated from macrophages through an initial incubation with 100 nmol/L phorbol 12-myristate 13-acetate (PMA; Sigma) for 48 hours at 37°C in 12-well plastic Petri dishes (Nunclon; Nunc Inc.). PMA was removed 24 hours before the experiments. For coculture experiments, THP-1 cells were seeded at the lower compartment of the Transwell system, whereas MiaPaCa2 cells were cocultured for 24 hours in the physically separated upper chamber (Thincert 12-well plate cell culture inserts, 0.4 μm pore size, Greiner Bio-One BVBA).

Histology

Pancreatic sections were fixed in 4% paraformaldehyde and embedded in paraffin. Hematoxylin and eosin (H&E) was performed using standard procedures. Sections were probed with primary antibodies against REG3β and IL17RA. Samples were examined with a Nikon Eclipse 90i microscope.

Quantification of REG3β-positive lesions per tissue

The number of peritumoral acini (low- and high-grade PanIN) is expressed as REG3β-positive lesions per field and was calculated as follows: the number of REG3β-positive lesions, which were determined by IHC, was counted and a percentage was calculated according to the total number of lesions.

Quantification of lesions per mouse

The number of lesions per field was counted, and lesion types were classified on H&E-stained slides. Quantification represents the average of 15 to 20 20× fields of view for 6 mice of each genotype as previously reported (34).

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qRT-PCR

RNAs were prepared from AR-42J, THP-1, and MiaPaCa2 cells using TRIzol Reagent (Life Technologies) and reverse transcribed using Go Script (Promega) according to the manufacturer's instructions. Quantitative RT-PCR was performed in a Stratagene cycler using Takara reagents. Primer sequences are available upon request.

Immunoblotting

Protein extraction was performed on ice with a total protein extraction buffer: 50 mmol/L HEPES pH 7.5, 150 mmol/L NaCl, 20% SDS, 1 mmol/L EDTA, 1 mmol/L EGTA, 10% glycerol, 1% Triton, 25 mmol/L NaF, 10 μmol/L $ZnCl_2$, and 50 mmol/L DTT. Before lysis, a protease inhibitor cocktail was added at 1:200 (Sigma-Aldrich, NUPR1340) composed of 500 μmol/L PMSF, 1 mmol/L sodium orthovanadate, and 1 mmol/L β-glycerophosphate. Protein concentrations were measured using a BCA Protein Assay Kit (Pierce Biotechnology). Protein samples (60 μg) were denatured at 95°C and subsequently separated by SDS-PAGE. After transfer to nitrocellulose, membrane blocking was performed with 1% BSA; samples were probed with the primary antibody followed by a horseradish peroxidase-coupled secondary antibody. Images were obtained with a Fusion FX image acquisition system (Vilber Lourmat).

Cell viability

The PrestoBlue reagent was added and cell viability was estimated after a 3-hour incubation time, following the PrestoBlue cell viability reagent protocol provided by the supplier (Life Technologies).

Cell proliferation

Cell proliferation was assessed with a commercial kit (Cell proliferation ELISA, BrdUrd; Roche Diagnostics) according to the manufacturer's instructions. Following a 24-hour incubation with the recombinant REG3β, cells were BrdUrd-labeled during 3 hours at 37°C. Cells were fixed and incubated with a peroxidase-conjugated anti-BrdUrd antibody for 90 minutes at room temperature. Then, the peroxidase substrate 3,3′,5,5′-tetramethylbenzidine was added, and BrdUrd incorporation was quantitated by measuring the differences in absorbance at a wavelength of 370 minus 492 nm.

Caspase-3/7 activity

To induce cell death by starvation, human PDAC cells were cultured in nonsupplemented Earle's Balanced Salt Solution (EBSS) medium (free of glucose, amino acids, lipids, and growth factors). To detect caspase-3/7 activation, MiaPaCa2 and Panc1 cells were plated in 96-well plates, starved, and treated with recombinant REG3β in the presence and absence of either the anti-gp130 antibody or the JAK2 inhibitor and analyzed using ApoONE Homogeneous Caspase-3/7 Assay (Promega) according to the manufacturer's instructions. After 1 hour of incubation, a fluorescence plate reader, using respective wavelengths of 480 and 535 nm was used to measure excitation and emission in the samples with the caspase substrate.

Statistical analysis

One-way variance analysis was used to calculate the significances of REG3β-positive lesions per tissue (percentage values), as previously described (34). To compare REG3β mRNA expressions, BrdUrd incorporation, and caspase-3/7 activity after treatment with different concentrations of the REG3β, we used the one-way variance analysis; the P value was calculated using ΔC_t, as described by Yuan and colleagues (35). Two-way variance analysis was used to compare lesion numbers per field in $Kras^{G12D}$ mice carrying the indicated REG3β ($REG3\beta^{+/+}$ or $REG3\beta^{-/-}$) at 14 weeks of age. Values are expressed as mean ± SEM. All tests of significance were two-tailed and the level of significance was set at 0.05. The examined cell lines represent at least three independent experiments. All statistical tests were performed using IBM SPSS statistics 21.

Results

The inducible REG3β expression characterizes the transition period between inflammation and cancer

Our laboratory has been critically involved in characterizing the role of REG3β in acute pancreatitis (32). Chronic pancreatitis results from repeated episodes of acute pancreatitis, and this prolonged inflammatory condition is accompanied by the continued synthesis of REG3β. Thus, as chronic pancreatitis precedes PDAC, we hypothesized that REG3β may fulfill a previously unsuspected role in mechanistically linking both disease processes. We were also guided by the recent discovery that the proinflammatory cytokine IL17A promotes PDAC development and sought to investigate if REG3β is a mediator of this phenomenon. Consequently, we first performed IHC to examine the expression of REG3β and IL17RA proteins in the pancreas of Pdx1-Cre; $Kras^{G12D}$ mice. Figure 1A shows that REG3β and IL17RA proteins are barely detectable in normal pancreatic exocrine cells and within pancreatic islets, REG3β, but not IL17RA, is only evident in the glucagon-producing cells. In the pancreas of Pdx1-Cre; $Kras^{G12D}$ animals, IL17RA is expressed in almost all acinar cells, reaches its highest levels in both ADM and early PanIN lesions, and is lost in more advanced PanIN lesions. Notably, we also found that the expression of REG3β in the exocrine pancreas follows the same pattern as IL17RA.

Treatment of the AR-42J cultured acinar cell line with IL17A increases the levels of both REG3β mRNA and protein in a dose-dependent manner (Fig. 1B and C), revealing that they are part of the same pathway. Thus, both IL17RA and REG3β appear to be induced by the oncogenic activation of KRAS to act early during the process of neoplastic transformation. Next, we sought to gain insight into the causal relationship between IL17RA activation, in KRAS mutant carrying cells, and the induction of REG3β. For this purpose, we neutralized IL17RA receptor activity in Pdx1-Cre; $Kras^{G12D}$ and Pdx1-Cre;$Kras^{wt}$ mice through the systemic administration of a specific anti-IL17RA antibody and measured the expression of the REG3β protein in the pancreas by Western blot. The result of this experiment, shown in Fig. 1D, demonstrates that the levels of REG3β protein is higher in the $Kras^{G12D}$ pancreas compared with the $Kras^{wt}$ pancreas. Moreover, Fig. 1D shows that the neutralization of IL17RA receptor in the pancreas of $Kras^{G12D}$ mice almost completely inhibited the upregulation of REG3β protein levels. Combined, these results suggest that, in the context of oncogenic KRAS mutation, REG3β functions downstream of the IL17A.

Then, we examined whether the expression of REG3β is dependent on IL17RA activation in response to pancreatitis. Therefore, we repeatedly injected mice with supramaximal doses of cerulein, which induces a strong pancreatic inflammation (36). IL17RA was again neutralized through the systemic injection of anti-IL17RA

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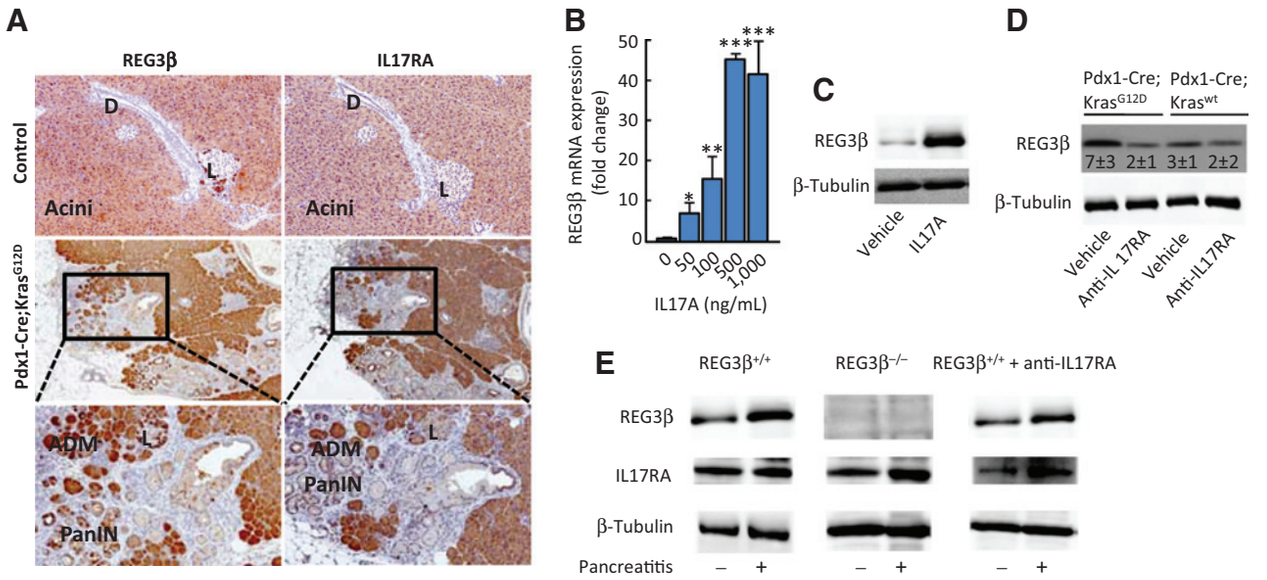

Figure 1.
Expression of REG3β and IL17RA in the pancreas. A, IHC detection of REG3β and IL17RA proteins in control and for 14-week-old Pdx1-Cre;Kras^{G12D} mice. D, duct; L, Langerhans Islet. B, AR-42J cells were treated with increasing concentrations of recombinant IL17A and the expression of REG3β was measured by qRT-PCR. C, AR-42J cells were treated with 500 ng/mL of recombinant IL17A and the expression of REG3β was measured by Western blotting. D, Pdx1-Cre; Kras^{G12D} and Pdx1-Cre;Kras^{wt} mice were treated with a systemic injection of anti-IL17RA antibody and the pancreatic expression of the REG3β protein was measured by Western blotting. E, pancreatitis was induced in REG3β$^{-/-}$, REG3β$^{+/+}$, and REG3β$^{+/+}$ treated with anti-IL17RA antibody through systematic injection and the pancreatic expression of the REG3β protein was measured by Western blotting. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

antibodies. Interestingly, the expression of REG3β in the Kras^{G12D} positive pancreas seemed almost completely dependent on IL17RA activation (Fig. 1D). However, the expression of REG3β is not dependent on IL17RA in the pancreas with pancreatitis, as IL17RA immunoneutralization does not change the expression of REG3β. However, IL17RA expression is induced by pancreatitis in a REG3β-independent manner (Fig. 1E). Thus, we hypothesize that increased levels of REG3β, likely caused by various inflammatory mediators, accompany the development and progression of pancreatitis until initiating oncogenic KRAS mutation triggers this event to be exclusively dependent on IL17A signaling.

REG3β promotes KRAS-mediated PDAC initiation in both mice and humans

To investigate the role of REG3β in the formation of KRAS-induced PanIN lesions, we crossed REG3β$^{-/-}$ mice with Pdx1-Cre;Kras^{G12D} animals. Control Pdx1-Cre;Kras^{G12D};REG3β$^{+/+}$ mice exhibited ADM, as well as low- and high-grade PanIN, at 14 weeks (Fig. 2; average lesions per type and power field were ADM = 8.3 and PanIN 1a = 6.4, PanIN 1b = 5.9, PanIN 2 = 2.3, and PanIN 3 = 0.6, $n = 6$). In contrast, Pdx1-Cre;Kras^{G12D}; REG3β$^{-/-}$ animals displayed a reduced number of both ADM and PanIN lesions (Fig. 2; average lesions per type and field were ADM = 2.2 and PanIN 1a = 0.6, PanIN 1b = 0.3, PanIN 2 = 0.3, and PanIN 3 = 0.1, $n = 6$). These differences were statistically significant for various genotypes and lesion types ($P < 0.001$). Thus, we next investigated whether exogenous administration of recombinant REG3β protein is able to rescue PanIN development in Pdx1-Cre;Kras^{G12D};REG3β$^{-/-}$ animals. We performed daily intraperitoneal injections of 1 μg recombinant REG3β protein beginning at 5 weeks of age. The results of this experiment, shown in Fig. 3, demonstrate that the recombinant REG3β increases the number of PanIN lesions in Pdx1-Cre;Kras^{G12D};REG3β$^{-/-}$ mice closer to the number found in Pdx1-Cre;Kras^{G12D};REG3β$^{+/+}$ animals. The average lesions per type and field were ADM =

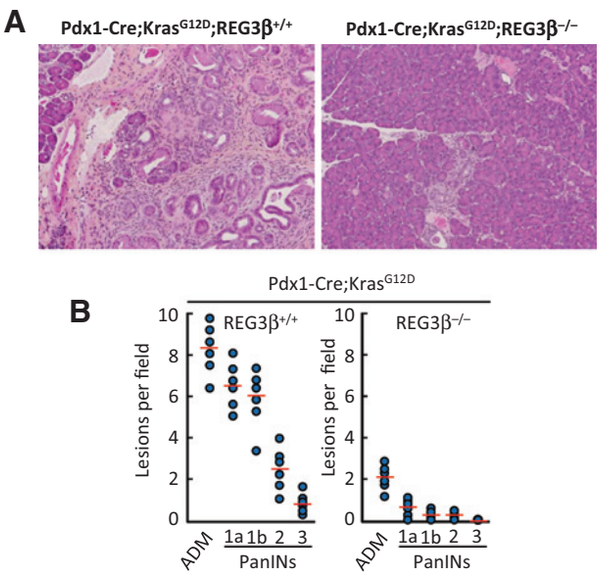

Figure 2.
REG3β expression is required for the Kras^{G12D} oncogene to induce PanIN. A, images representative of the pancreas in Pdx1-cre;LSL-Kras^{G12D};REG3β$^{-/-}$ and Pdx1-cre;LSL-Kras^{G12D};REG3β$^{+/+}$ mice. H&E staining. B, number of ADM and PanIN lesions per ×20 tissue field in Pdx1-cre;LSL-Kras^{G12D};REG3β$^{-/-}$ and Pdx1-cre;LSL-Kras^{G12D};REG3β$^{+/+}$ mice at 14 weeks of age. Means are denoted by horizontal bars. Differences between genotypes and lesion types were significant ($P < 0.001$).

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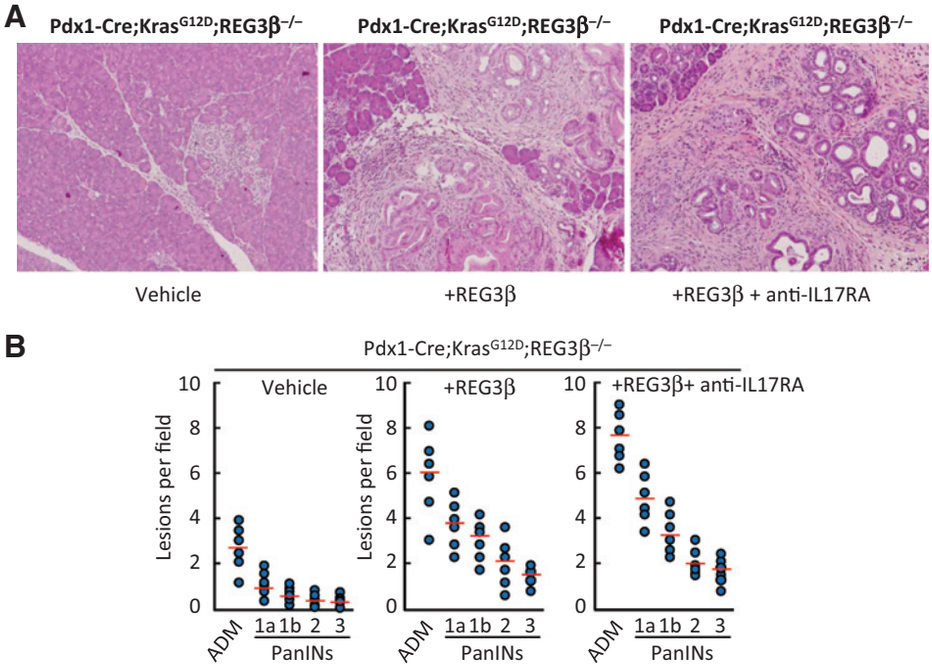

Figure 3.
Systemic REG3β injection rescues PanIN development in Pdx1-cre;LSL-Kras^{G12D};REG3β$^{-/-}$ mice. A, images representative of the pancreas in Pdx1-cre;LSL-Kras^{G12D};REG3β$^{-/-}$ mice treated with vehicle, with recombinant REG3β, and with recombinant REG3β in combination with neutralizing anti-IL17RA antibody. B, number of ADM and PanIN lesions per ×20 tissue field in Pdx1-cre;LSL-Kras^{G12D};REG3β$^{-/-}$ mice treated with vehicle, with recombinant REG3β, and with recombinant REG3β in combination with the anti-IL17RA antibody at 14 weeks of age. Means are denoted by horizontal bars. Differences between genotypes and lesion types were significant ($P < 0.01$) for Pdx1-Cre;Kras^{G12D};REG3β$^{-/-}$ mice treated with vehicle and Pdx1-cre;LSL-Kras^{G12D};REG3β$^{-/-}$ mice treated with recombinant REG3β and with a combination of recombinant REG3β and the anti-IL17RA antibody; no difference was found for Pdx1-Cre;Kras^{G12D};REG3β$^{-/-}$ mice treated with recombinant REGβ protein and with a combination of recombinant REGβ and the neutralizing anti-IL17RA antibody ($P > 0.05$).

2.8 and PanIN 1a = 0.9, PanIN 1b = 0.5, PanIN 2 = 0.4, and PanIN 3 = 0.2 ($n = 6$) for Pdx1-Cre;Kras^{G12D};REG3β$^{-/-}$ mice treated with the vehicle; whereas average values were ADM = 6.1 and PanIN 1a = 3.8, PanIN 1b = 3.5, PanIN 2 = 2.0, and PanIN 3 = 1.6 ($n = 6$) for Pdx1-Cre;Kras^{G12D};REG3β$^{-/-}$ mice treated continually with recombinant REG3β. These differences were statistically significant for various genotypes and lesion types ($P < 0.01$). Consequently, we conclude that the expression of REG3β is necessary for PanIN development induced by the Kras^{G12D} oncogene during the initiation period of pancreatic carcinogenesis.

To determine whether the effect of REG3β on stimulating KRAS-induced PanIN formation is dependent upon IL17RA signaling, we treated a cohort of Pdx1-Cre;Kras^{G12D};REG3β$^{-/-}$ mice with recombinant REG3β protein, although simultaneously inhibiting IL17RA with a specific neutralizing antibody. The neutralizing anti-IL17RA antibody had no significant influence on either the number of lesions or the grade of PanIN promoted by recombinant REG3β (Fig. 3; average lesions per type and field were ADM = 7.6 and PanIN 1a = 4.9, PanIN 1b = 3.3, PanIN 2 = 1.9, and PanIN 3 = 1.7, $n = 6$). These differences were statistically significant for Pdx1-Cre; Kras^{G12D};REG3β$^{-/-}$ mice treated with vehicle ($P < 0.01$), but no significant differences were observed for Pdx1-Cre;Kras^{G12D};REG3β$^{-/-}$ mice that received the combined treatment of recombinant REG3β protein and neutralizing anti-IL17RA antibodies ($P > 0.05$). Therefore, recombinant REG3β is able to rescue the development of PanIN in Pdx1-Cre; Kras^{G12D}; REG3β$^{-/-}$ mice, suggesting that REG3β plays a paracrine effect on stimulating the formation of PanIN lesion by KRAS containing pancreatic cells.

Because the systemic administration of recombinant REG3β protein rescues PanIN formation in REG3β deficient mice, we next sought to determine whether, conversely, the systemic neutralization of either REG3β or IL17RA potentially inhibits PanIN development in Pdx1-Cre; Kras^{G12D};REG3β$^{+/+}$mice. As shown in Fig. 4, the treatment of these mice with either anti-REG3β or anti-IL17RA antibodies (beginning at 5 weeks old and for 9 consecutive weeks) had a significant effect on the development of PanIN lesions. Average lesions per type and field decreased as follows: ADM = 7.6 to 2.1 and PanIN 1a = 5.8 to 1.7, PanIN 1b = 5.3 to 1.2, PanIN 2 = 2.3 to 0.8, and PanIN 3 = 1.6 to 0.3 for animals treated with anti-IL17RA ($n = 6$); and ADM = 7.6 to 2.6 and PanIN 1a = 5.8 to 1.5, PanIN 1b = 5.3 to 0.8, PanIN 2 = 2.3 to 0.6, and PanIN 3 = 1.6 to 0.2 for animals treated with anti-REG3β antibodies ($n = 6$). These differences were statistically significant for Pdx1-Cre;Kras^{G12D};REG3β$^{+/+}$ mice treated with the vehicle and those treated with anti-IL17RA and anti-REG3β antibodies ($P < 0.01$). We found no significant differences for mice treated with anti-IL17RA and anti-REG3β antibodies ($P > 0.05$). These findings indicate that neutralizing either IL17RA or REG3β with specific antibodies is a useful strategy to inhibit PanIN development in Pdx1-Cre;Kras^{G12D};REG3β$^{+/+}$ mice. Because therapeutic modalities to block IL17RA are actively being tested in clinical trials for many human diseases, our findings support the idea that

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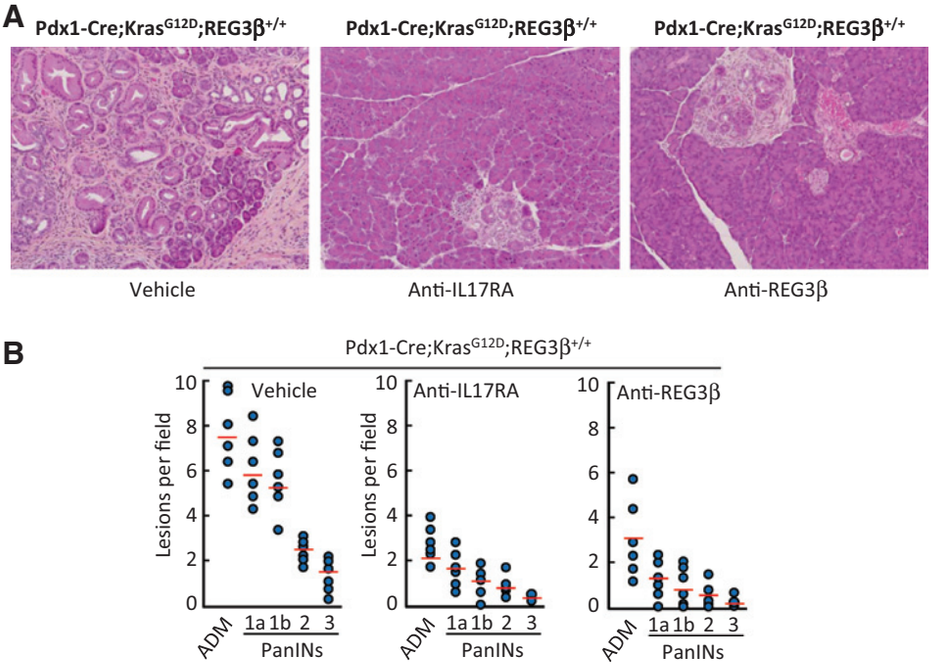

Figure 4.
Systemic injection of anti-REG3β and anti-IL17RA antibodies inhibits PanIN development in Pdx1-cre;LSL-Kras^{G12D};REG3β$^{+/+}$ mice. A, images representative of the pancreas in Pdx1-cre;LSL-Kras^{G12D};REG3β$^{+/+}$ mice treated with vehicle, with anti-REG3β, and with the neutralizing anti-IL17RA antibodies. B, number of ADM and PanIN lesions per ×20 tissue field in Pdx1-cre;LSL-Kras^{G12D};REG3β$^{+/+}$ mice treated with vehicle, with anti-REG3β, and with the neutralizing anti-IL17RA antibodies at 14 weeks of age. Means are denoted by horizontal bars. Differences between genotypes and lesion types were significant ($P < 0.01$) for Pdx1-Cre;Kras^{G12D};REG3β$^{+/+}$ mice treated with vehicle and Pdx1-cre;LSL-Kras^{G12D};REG3β$^{+/+}$ mice treated with anti-REG3β and with the neutralizing anti-IL17RA antibodies; no difference was found for Pdx1-Cre;Kras^{G12D};REG3β$^{+/+}$ mice treated with anti-REG3β and with the neutralizing anti-IL17RA antibodies ($P > 0.05$).

that this strategy may have beneficial effects in preventing or treating preneoplastic pancreatic cancers, which arise in the setting of chronic pancreatitis.

We next evaluated the role of REG3β at the postinitiation step, namely, on frank PDAC. Toward this end, we first used the Pdx1-Cre;Kras^{G12D};Ink4a/Arf$^{fl/fl}$ mouse model, which develops PDAC at a few weeks of age and evolves according to a natural history that is similar to that seen in humans (37). Using pancreatic samples from 8-week-old mice in which PDAC has already developed, we observed a strong reactivity for REG3β with the following percentages of positive lesion staining: 82 ± 13% for ADM, 46 ± 6% for PanIN 1a, 41 ± 3% for PanIN 1b, 11 ± 2% for PanIN 2, 3 ± 2% for PanIN 3, and 0% in well-established PDACs (Fig. 5). Thus, these results led us to characterize the expression of

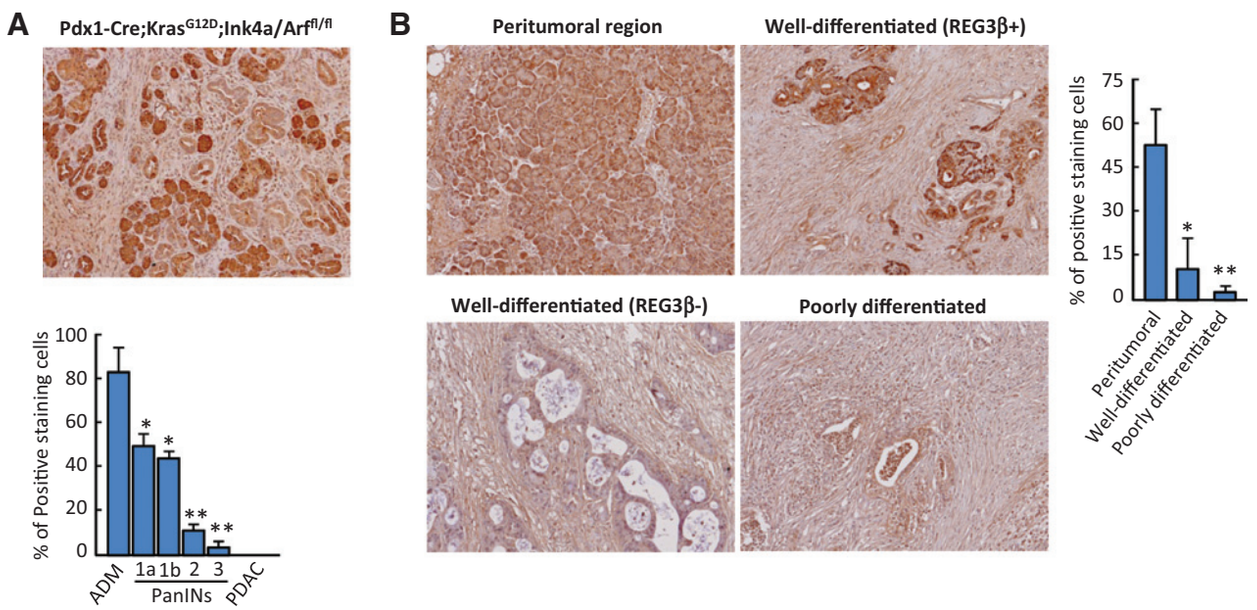

Figure 5.
REG3β is expressed in early PDAC lesions in mice and humans. A, IHC image representative of REG3β detection in the pancreas of Pdx1-Cre;Kras^{G12D};Ink4a/Arf$^{fl/fl}$ mice; quantification of positive REG3β lesions expressed as the percentage of positive staining cells. B, IHC detection of REG3β in human PDAC and quantification of positive REG3β lesions expressed as the percentage of positive staining cells. *, $P < 0.05$; **, $P < 0.01$.

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REG3β in human PDAC samples carrying mutations in KRAS. We found that the expression of REG3β in human tumors appears as a strong reaction in the nontransformed peri-tumor region as well as in some, but not all, well-differentiated PDACs. In contrast, REG3β staining was negative in poorly differentiated PDACs (Fig. 5). Thus, altogether, these results further confirm that REG3β is expressed in the early phases of PDAC development.

REG3β promotes pancreatic cell growth by inducing cell proliferation and inhibiting apoptosis

To gain insight into both the cellular and molecular mechanisms underlying the function of REG3β during pancreatic cancer initiation, we treated two PDAC-derived cell lines (MiaPaCa2 and Panc1) with increasing concentrations of recombinant REG3β at 24-hour intervals for a period of 72 hours and measured BrdUrd incorporation. When compared with control, cells that were treated with REG3β at a concentration of 50 ng/mL had an increase in BrdUrd incorporation of 2.3-fold and 1.7-fold ($P < 0.05$) for MiaPaCa2 and Panc1 cells, respectively (Fig. 1B). Moreover, at a concentration of 100 ng/mL for REG3β, the values for BrdUrd incorporation increased to 2.3-fold and 3.9-fold ($P < 0.01$). These results demonstrate that an important effect of REG3β, as it relates to cancer cells, is its ability to stimulate their proliferation. Thus, we complemented these studies by measuring the effects of REG3β on pancreatic cell death. For this purpose, we activated the apoptotic program of pancreatic cancer cells by serum starvation for 24, 48, and 72 hours. At the same time, cells were also incubated with or without recombinant REG3β protein at a concentration of 100 ng/mL. Apoptosis was determined by measuring cell viability and caspase-3/7 activities at the end of the experiment. Figures 6B and 7C show that the treatment of both MiaPaCa2 and Panc1 cells with REG3β for 48 and 72 hours increased their resistance to apoptosis as evidenced by increased cell viability and decreased caspase-3/7 activity. Thus, we conclude that REG3β promotes cell growth and protects against cell death in PDAC cells. These data are relevant to better understanding the role of this protein during the process of initiation, because it is well-established that both of these processes are crucial for normal cells to acquire the ability to undergo ADM and form PanIN lesions in response to oncogenic KRAS.

A novel IL17-regulated paracrine pathway involving REG3β-JAK2-STAT3 signaling promotes KRAS-mediated PDAC initiation

Our results demonstrate that REG3β is a mediator of both inflammation and cancer. This finding is reminiscent of the effects recently described by several laboratories for STAT3, which, similar to REG3β, also works downstream of proinflammatory pathways. Thus, we hypothesized that REG3β may signal *via* this intracellular signaling pathway. To evaluate the influence of this protein on STAT3 phosphorylation, which is a marker for its activation, we treated pancreatic cells with 100 ng/mL of recombinant REG3β for 1 hour, which demonstrated that REG3β significantly increased the phosphorylation-dependent activation of STAT3 (Fig. 6D). Next, we extended our mechanistic investigations to determine whether this effect occurs via gp130, a protein that functions as a receptor for REG3β. Interestingly, STAT3 activation in response to REG3β was almost completely inhibited by pretreating the cells with an anti-gp130 neutralizing antibody. Similar results were obtained using the JAK2 inhibitor AG-490, indicating that this kinase links the REG3β-gp130 interaction to STAT3 activation. Thus, we formally assessed whether the activation of this pathway was linked to the effects of REG3β on BrdUrd incorporation and cell death. Cells were treated with 100 ng/mL of recombinant REG3β for 24 hours either in the presence or absence of the anti-gp130 antibody and proliferation was measured by BrdUrd incorporation. Similar experiments were also performed using the JAK2 inhibitor AG-490. The results of these experiments shown in Fig. 6E demonstrate that both the anti-gp130 antibody and AG-490 readily inhibit the growth-promoting effect of REG3β on MiaPaCa2 and Panc1 cells. Finally, we also evaluated whether gp130 and JAK2 were involved in REG3β-mediated apoptosis resistance. Accordingly, PDAC-derived MiaPaCa2 and Panc1 cells were treated with recombinant REG3β in the presence and absence of either the anti-gp130 antibody or AG-490. Figure 6F shows that the anti-apoptotic effect of recombinant REG3β was almost completely inhibited by the anti-gp130 antibody, as well as the AG-490 compound. Based on these results, we conclude that REG3β is, at least partly, responsible for the activation of the intracellular pathway led by the gp130 receptor, JAK2, and phosho-STAT3, which therefore promotes pancreatic cell growth and increases resistance to cell death.

REG3β regulates the phenotype of both PDAC-derived cells and macrophages in a cell-to-cell-dependent interaction

The experiments described above have demonstrated that REG3β is a paracrine factor released by the Kras-transformed as well as inflamed pancreatic epithelial cells, similar to many other cancer promoting proteins that are synthesized and released by the epithelial compartment of the initiating PanIN lesions. Recent studies, however, indicate that inflammatory mediators also play a role in stimulating protumoral or inhibiting antitumoral responses in the tumor microenvironment, which led us to establish a coculture model to evaluate whether REG3β has the ability to function in this manner. This coculture model involved the use of human pancreatic cancer cells (MiaPaCa2) and macrophage-differentiated THP-1 monocytes, both grown in a single chamber that contains a separation to avoid cell-to-cell contact. Because we along with others have shown that the CXCL12-CXCR4 chemokine signaling pathway is key in promoting pancreatic cancer, we first analyzed whether REG3β affects the activation of this signaling pathway. As shown in Fig. 7, REG3β induced an increase in the expression of CXCR4 mRNA in macrophages (1.0 ± 0.1 vs. 1.5 ± 0.1 folds, $P < 0.01$). This increase in CXCR4 mRNA was unaltered whether macrophages were cultured alone or in the presence of pancreatic cancer cells. Parallel experiments showed that REG3β did not modify the expression of CXCL12 in pancreatic cancer cells when cultured alone. In contrast, REG3β did, however, induce a marked increase in the expression of CXCL12 when pancreatic cancer cells were cocultured with macrophages (0.6 ± 0.1 vs. 9.7 ± 1.2 folds, $P < 0.01$). Similarly, pancreatic cancer cells exhibited a comparable effect in the expression of TGFβ (2.1 ± 0.2 vs. 3.8 ± 0.4 folds, $P < 0.05$) and IL10 (0.6 ± 0.1 vs. 4.3 ± 0.3 folds, $P < 0.01$), other cytokines, which like CXCL12 modulate pancreatic cancer development. Control experiments demonstrated that, in the absence of REG3β, cocultured cells did not change the expression of these mediators. Notably, REG3β also induced THP-1 macrophages from the coculture to increase the synthesis of IL10 (2.4 ± 0.4 vs. 7.2 ± 0.8 folds, $P < 0.01$) and MRC-1 (mannose receptor; 1.1 ± 0.2 vs.

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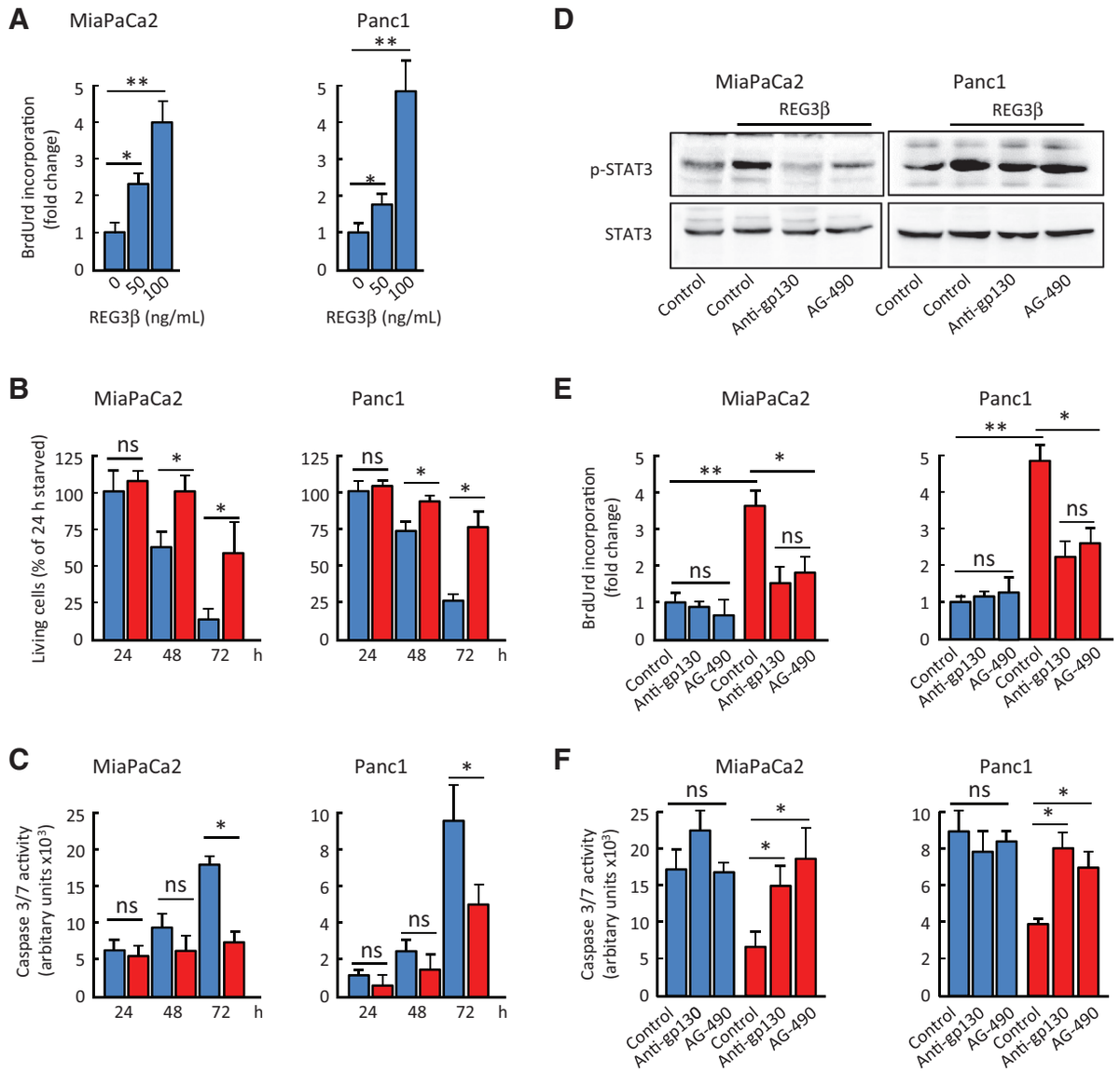

Figure 6.
REG3β promotes cellular growth and increases cell resistance to cell death through a gp130-, JAK2-, and STAT3-dependent mechanism. A, MiaPaCa2 and Panc1 cells were treated with 50 and 100 ng/mL of recombinant REG3β protein and BrdUrd incorporation was measured after 24 hours. Data are expressed as fold changes relative to untreated cells. B and C, MiaPaCa2 and Panc1 cells were cultivated in EBSS media for 24, 48, and 72 hours in either the absence (blue) or presence (red) of recombinant REG3β protein. Cell viability (B) and caspase 3/7 activity (C) were measured. D, MiaPaCa2 and Panc1 cells were cultivated in the presence of recombinant REG3β protein together with either the anti-gp130 or the AG-490 compound and the expression of phosphoSTAT3 or STAT3 was measured by Western blotting. E and F, MiaPaCa2 and Panc1 cells were cultivated in EBSS media for 72 hours in the absence (blue) and presence (red) of recombinant REG3β protein together with either the anti-gp130 or the AG-490 compound and BrdUrd incorporation (E) and caspase 3/7 activity (F) were measured. *, $P < 0.05$; **, $P < 0.01$. ns, nonsignificant.

2.4 ± 0.2 folds, $P < 0.01$), two typical markers of the M2 phenotype. Taken together, these results demonstrate that REG3β can induce robust communication between pancreatic cancer cells and cells, which, like macrophages, are commonly found in the desmoplastic microenvironment where they regulate many cytokine-mediated immunotumoral responses. These effects of REG3β appear to act independently yet complementary to its role in promoting PanIN development by acting on the gp130 receptor present in epithelial cells.

Discussion

Elegant studies, primarily performed during the last decade, demonstrate that inflammatory cells and other stromal elements exert a potent protumoral effect on oncogenic Kras-activated epithelial cells (38). Noteworthy, many of the stimuli found to promote tumor growth are also activated during pancreatitis. In fact, chronic pancreatitis is known to accelerate the process of pancreatic tumorigenesis in both humans and mice (1, 39). Recently, using a sophisticated combination of transgenic mice

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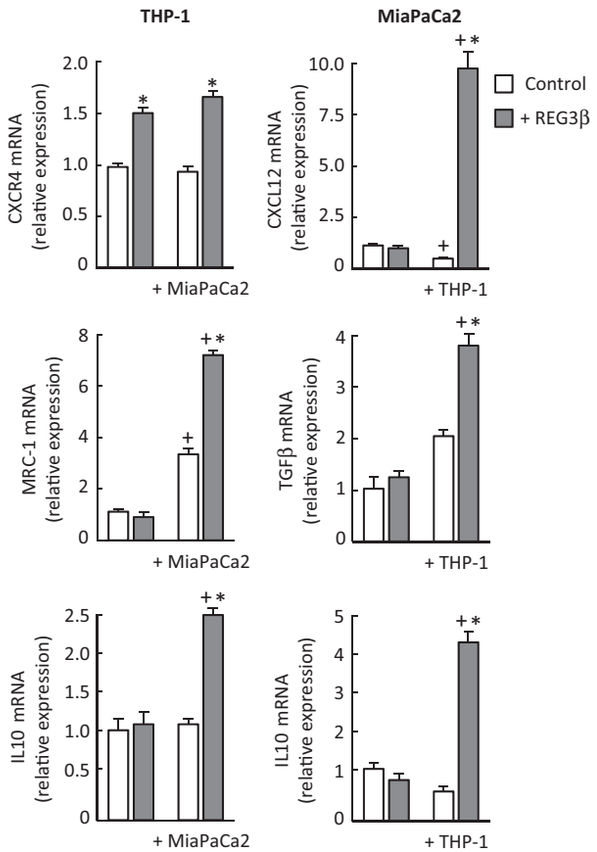

Figure 7.
REG3β induces changes in gene expression of macrophages and PDAC cells when they are cocultured. Macrophage-differentiated THP-1 cells (left) and MiaPaCa2 cells (right) were cultured alone or in combination using transwell inserts. Cells were treated with 500 ng/mL of recombinant REG3β protein and RNA was obtained after 24 hours. THP-1 cells increased the expression of CXCR4 in response to REG3β; a similar increase was observed in the presence of MiaPaCa2 cells. By contrast, REG3β induced the expression of MRC-1 and IL10 only when THP-1 cells were cocultured with MiaPaCa2 cells. Coculture also promoted the expression of MRC-1 even in the absence of REG3β. On the other hand, REG3β had no effect on MiaPaCa2 cells alone, but strongly induced the expression of CXCL12, TGFβ, and IL10 when cocultured with THP-1 cells.*, $P < 0.05$ versus its corresponding control; +, $P < 0.05$ versus no coculture.

with specific neutralizing antibodies, McAllister and colleagues demonstrated that T_H17 and $IL17^+/\gamma\delta T$ cells, infiltrating the pancreatic stroma, produce IL17A so as to promote PanIN development through the activation of the STAT3 signaling pathway (30). This study also determined that the IL17RA receptor is expressed in pancreatic cells upon oncogenic Kras-activation. Combined, therefore, these observations reveal that inflammation induces immune cells to migrate into the pancreatic stroma where they produce and release the master cytokine IL17A. Simultaneously, the expression of the IL17RA is induced in epithelial pancreatic cells after oncogenic Kras activation, thereby setting the conditions for this pathway to be activated in a paracrine fashion. However, the mechanisms that work downstream of this pathway to promote KRAS-induced pancreatic carcinogenesis remain to be fully understood. In this study, we used several complementary approaches to demonstrate that the expression of REG3β is induced downstream of the IL17RA receptor and that REG3β is necessary for PanIN development in mice. In addition, we were able to show that REG3β activates a gp130-, JAK2-, and STAT3-dependent signaling pathway to promote cell growth and increase cell resistance in PDAC-derived cells. Unexpectedly, we also found that REG3β promotes the expression of mediators in both PDAC-derived cells and macrophages in a cell-to-cell-dependent manner. The Pdx1-Cre; $Kras^{G12D}$;$REG3\beta^{+/+}$ mice, in which the REG3β protein was neutralized with a specific antibody against REG3β (Fig. 4), were unable to develop PanIN, demonstrating that REG3β is an essential factor for PanIN development. Therefore, REG3β should be considered a potential target for the treatment of patients with inflammation-associated PDAC. In summary, we have established that REG3β is a key effector of the IL17A/IL17RA receptor pathway acting during both the inflammation and transformation steps of inflammation-induced pancreatic cancer, which functions according to the model shown in Supplementary Fig. S1.

Our study also demonstrates that in addition to working on receptors of KRAS-transformed pancreatic epithelial cells, REG3β modulates the function of inflammatory and PDAC-derived cells when both cells were cocultivated (Fig. 7). This immunomodulatory activity of REG3β on THP-1 macrophages occurs completely independent of the cell-to-cell contact, because, in our experimental set-up, these cells were cocultured in chambers carrying a barrier that prevents their direct interactions. We find that REG3β activates the CXCL12/CXCR4 signaling cascade, which sustains tumor cell growth, induces angiogenesis, facilitates tumor escape through evasion of immune surveillance, and promotes metastasis with neural invasion (40). Moreover, we show that REG3β induces the expression of IL10, TGFβ and MRC-1, which together behave as immunomodulators and increase the synthesis of the pancreatic desmoplastic reaction (41). Thus, these results underscore the fact that, in addition to its effects on pancreatic cancer cells, REG3β can regulate the timely release of chemokines that support the dialogue between tumor cells and cells from its microenvironment (42). Thus, it is likely that in the complex chemokine-rich milieu of chronic pancreatitis, REG3β facilitates the transforming function of KRAS by acting on both pancreatic cells and its microenviroment. We are optimistic that future studies using state-of-the-art conditional knockout models for deleting REG3β in different cell types will extend these observations and further define the relative importance of each of these mechanisms to the development of inflammation-associated pancreatic cancer.

Notably, studies seeking to better understand the mechanisms that underlie the transition from pancreatitis into cancer have recently gained popularity since a model for studying this process was established by Guerra and colleagues (39). We have recently used this model to demonstrate that an EGF-dependent proinflammatory NFATc1-STAT3 pathway promotes the transition of chronic pancreatitis into cancer (43). Similarly, Liou and colleagues found that RANTES and TNFα produced by activated stromal macrophages have a significant effect on the transformation of ADM following activation of NF-κB signaling (44). Moreover, McAllister and colleagues discovered that IL17-secreting cells play a similar role (30). Thus, from considering these studies and others in aggregate, it appears that more than one proinflammatory mediator can promote PanIN development by KRAS. It is even likely that epithelial cells from the pancreatic parenchyma or mesenchymal cells from the stroma synthesize mediators

that can act at different times during the initiation process (early vs. late PanINs), though this idea has remained experimentally unexplored. In addition, similar to what we observe with REG3β, which expression disappears after the cancer initiation step, different factors secreted in either an autocrine manner by KRAS mutant pancreatic cells or a paracrine manner by cells from an inflammatory-protumoral microenvironment may be required at subsequent steps during carcinogenesis. In fact, decades of genome-wide expression profiling studies have clearly demonstrated that with each additional mutation or epigenetic alteration acquired during the transformation process, pancreatic cancer cells switch on and off the synthesis of different cytokines and growth factors. However, studies that seek to define which of these factors are operational during the pancreatitis–cancer transition, like the one reported here, have recently begun. These types of studies are of paramount importance because patients with chronic pancreatitis are the largest group of individuals at risk for dying of pancreatic cancer. More importantly, these patients also display the highest score among all high-risk populations for acquiring this disease (1).

Thus, the current study was designed to define whether one of the best-characterized mediators of pancreatitis, REG3β, also promotes the development of pancreatic cancer. Indeed, our results not only support this hypothesis, but also discovered mechanisms by which REG3β promotes cancer development at the molecular level through its involvement in a IL17A-REG3β-gp130-JAK2-STAT3 pathway and at the cellular level by increasing cell proliferation and survival. Moreover, at the whole organism level, our antibody-based preclinical trials demonstrate that inhibition of the IL17A-REG3β pathway antagonizes KRAS-induced pancreatic cancer initiation. Thus, this new knowledge changes the previously established paradigm, which considered REG3β as an exclusive pancreatitis-associated protein, into a new paradigm that describes this protein as a druggable link between pancreatitis and pancreatic cancer. Furthermore, we also have studied several molecules within this pathway, namely, IL17, REG3β, JAK2, and STAT3, for which inhibitors are being tested in clinical trials, thereby contributing to build the foundation of future combinatorial therapies. In conclusion, the experiments reported here support the idea that premalignant diseases of the pancreas actively establish mechanisms that can self-perpetuate inflammation, and, more importantly, also lead to neoplastic transformation. Fortunately, we demonstrate that antagonizing these mechanisms have a beneficial effect in preventing this malignant switch.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: E. Folch-Puy, E. Chuluyan, R. Urrutia, D. Closa, J.L. Iovanna

Development of methodology: L. Bonjoch, M.B. Lopez-Millan

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Loncle, M.B. Lopez-Millan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Loncle, M.B. Lopez-Millan, S. Lac, P. Cordelier, P. Dubus, R. Urrutia, D. Closa, J.L. Iovanna

Writing, review, and/or revision of the manuscript: C. Loncle, L. Bonjoch, E. Folch-Puy, M.B. Lopez-Millan, M.I. Molejon, E. Chuluyan, P. Cordelier, P. Dubus, G. Lomberk, R. Urrutia, D. Closa, J.L. Iovanna

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.B. Lopez-Millan, S. Lac, G. Lomberk, R. Urrutia

Study supervision: G. Lomberk, R. Urrutia, J.L. Iovanna

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IL17 Functions through the Novel REG3β–JAK2–STAT3 Inflammatory Pathway to Promote the Transition from Chronic Pancreatitis to Pancreatic Cancer

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