Hif1α deletion limits tissue regeneration via aberrant B cell accumulation in experimental pancreatitis

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SUMMARY

Pancreatitis is an inflammatory disease of the exocrine pancreas and ranks among the most common gastrointestinal disorders. Inflamed tissues frequently experience conditions of insufficient oxygen availability, or hypoxia. Here we demonstrate that hypoxia and consequent stabilization of the hypoxia-inducible factor 1α (HIF1α) transcription factor occur in murine and human pancreatitis. Mice lacking pancreas-specific HIF1α expression displayed markedly impaired pancreatic regeneration following cerulein-induced pancreatitis, which was associated with excessive intrapancreatic B cell accumulation. Notably, B cell depletion in mice with established pancreatitis significantly enhanced tissue regeneration. Our study reveals a crosstalk between pancreatic HIF1α expression and B cell trafficking that regulates tissue regeneration, and identifies plausible molecular targets for treating pancreatitis patients.

Keywords

HIF1α; hypoxia; pancreatitis; inflammation; B cells; αCD20; injury; regeneration

INTRODUCTION

Tissue injury often results in inflammation and subsequent regenerative responses to restore tissue integrity and homeostasis. However, persistent inflammation and tissue remodeling

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AUTHOR CONTRIBUTIONS
K.E.L. and M.C.S. conceived and directed the study, designed the experiments, and wrote the manuscript. K.E.L. performed the majority of the experiments. M.S. and R.M. contributed to the experimental work. K.E.L., R.H.V. and M.C.S. analyzed data. All authors discussed the results and commented on the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.
can also lead to chronic inflammatory diseases, leading to increased likelihood of tumor initiation. Pancreatitis, or inflammation of the pancreas, is one of the most prevalent gastrointestinal conditions resulting in hospitalization (Forsmark et al., 2016). Recurrent acute pancreatitis can develop into chronic pancreatitis, marked by progressive tissue destruction and persistent inflammation and fibrosis (Braganza et al., 2011). Chronic pancreatitis is highly linked with an increased risk of pancreatic cancer in humans (Lowenfels et al., 1993), and mice with experimentally induced pancreatitis exhibit accelerated tumorigenesis (Guerra et al., 2007).

Inflammatory diseases are often characterized by tissue hypoxia, or oxygen (O$_2$) deprivation, caused by vascular damage, edema, and intense metabolic activity of both regenerating tissue and infiltrating immune cells (Eltzschig and Carmeliet, 2011). Transcriptional adaptation to hypoxic stress is largely coordinated by hypoxia-inducible factors (HIFs) (Nakazawa et al., 2016). Specifically, hypoxic stress inhibits normal proteasomal degradation of HIFα subunits (HIF1α and HIF2α), resulting in HIFα protein accumulation. Once stabilized, HIF1α activates the transcription of numerous genes mediating metabolic adaptations, angiogenesis, cell survival, metastasis, and inflammation (Lee and Simon, 2015; Schito and Semenza, 2016). HIF1α protein is detected in multiple inflammatory disorders, such as inflammatory bowel disease and acute lung injury, and has profound effects on disease pathogenesis and outcomes (Palazon et al., 2014). HIF1α is also highly expressed in human chronic pancreatitis (Crnogorac-Jurcevic et al., 2005). Moreover, pancreas-specific Hif1α deletion promotes Kras$^{G12D}$-driven pancreatic tumorigenesis with dramatic stromal expansion (Lee et al., 2016). However, the impact of HIF1α on pancreatitis has remained largely unknown.

Here, using cerulein-induced experimental pancreatitis and pancreas-specific HIF1α-deficient mice, we show that HIF1α ablation in pancreatic epithelium impairs tissue regeneration following inflammatory injury, accompanied by excessive intrapancreatic accumulation of B lymphocytes. Our study reveals that B cells are a negative regulator of tissue regeneration and a critical mediator of hypoxic influences on pancreatitis.

**RESULTS**

**Pancreatitis induces tissue hypoxia and HIF1α accumulation**

To explore a possible role for HIF1α in the pathogenic progression of pancreatitis, we first ascertained HIF1α expression patterns, using an established cerulein-based mouse model of experimental pancreatitis. Cerulein is an analog of the gastrointestinal hormone cholecystokinin, which stimulates acinar cells to produce and secrete digestive enzymes. Consistent with published observations (Fukuda et al., 2011; Mallen-St Clair et al., 2012), mice receiving repetitive administration of cerulein over 3 weeks exhibited severe exocrine pancreatic injury, including extensive acinar cell loss, edema, and leukocyte infiltration within 1–3 days after final cerulein administration (Figures 1A and 1B). This cerulein model results in a more chronic pancreatitis-like phenotype diagnosed in humans, and exhibits fibrosis (Lerch and Gorelick, 2013). However, unlike human chronic pancreatitis, due to enhanced capacity for pancreatic regeneration in experimental rodents, injury resolves over time after cerulein withdrawal (Figure 1B) (Halbrook et al., 2017; Murtaugh and Keefe,
Hypoxic cells, identified by positive immunostaining with Hypoxyprobe and HIF1α antibodies, were barely detectable in the normal pancreas, as expected (Figure 1B). In contrast, Hypoxyprobe+ cells and HIF1α accumulation were widely observed in damaged pancreata from cerulein-treated mice (Figure 1B). Concurrent with gradual tissue recovery, pancreatic Hypoxyprobe and HIF1α staining steadily decreased (Figure S1), and disappeared by 21 days after the final cerulein injection (Figure 1B). Significantly, human chronic pancreatitis patient samples were also positive for HIF1α immunostaining (7 of 10 samples) (Figure 1C), implicating hypoxia and HIF1α as potential contributors to this disease and its resolution.

**Hif1α deletion impedes pancreatic regeneration following cerulein-induced pancreatitis**

To investigate the in vivo functions of HIF1α in pancreatitis, we generated cerulein-mediated pancreatitis in p48-Cre;Hif1α0/0 and Hif1α0/0 control mice. We previously reported that p48-Cre;Hif1α0/0 (henceforth Hif1αKO) animals exhibit efficient pancreas-specific Hif1α deletion and develop normally with no obvious signs of pancreatic dysfunction (Lee et al., 2016). Based on hematoxylin-eosin staining, HIF1α ablation did not result in major histological differences in comparison with controls 3 days after cerulein treatment was discontinued, which corresponds to the “injury phase” (Figure 2A). The initial acinar tissue destruction and stromal response to pancreatic injury were comparable between Hif1αKO and controls, as evidenced by Amylase immunofluorescent staining for acinar cell mass, Picrosirius Red staining for fibrosis, and immunohistochemical and flow cytometric analyses of CD45+ cells for leukocyte infiltration (Figures 2B–2G). As expected, control animals demonstrated nearly complete regeneration of pancreatic tissue by day 21 (Figures 2A–2G). In direct contrast, Hif1αKO mice failed to complete the regenerative response to cerulein-mediated inflammatory injury, as demonstrated by persistent acinar cell loss, severe interlobular/intralobular spaces, immune infiltrates, and fibrosis (Figures 2A–2G). Moreover, a pronounced decrease in proliferating epithelial cells was detected in regenerating Hif1αKO pancreata (Figures S2A and S2B). Interestingly, both Hif1αKO and Hif1α0/0 mice displayed abundant metaplastic lesions (Figure 2A) and comparable numbers of Sox9+ progenitor cells (Figures S2C and S2D), suggesting that HIF1α expression is not required for acinar-to-ductal metaplasia. An additional 21 day recovery period, i.e., 42 days post-cerulein treatment, eventually prompted substantial regeneration of Hif1αKO pancreata, albeit with incomplete resolution of inflammation (Figures S2E-S2J). Together, these data indicate that HIF1α is dispensable for initial pancreatic injury caused by cerulein, but required for efficient pancreatic regeneration and resolution of inflammation and fibrosis.

**Hif1α deletion leads to excessive accumulation of B lymphocytes in the pancreas during tissue regeneration**

To gain mechanistic insights into how HIF1α loss compromises pancreatic regeneration, we characterized immunomodulation by HIF1α during inflammatory injury and recovery. Flow cytometric analysis of pancreatic tissue revealed that relative percentages of macrophages, myeloid-derived suppressor cells (MDSC), dendritic cells, T cells, and B cells among all CD45+ immune cells are comparable between Hif1αKO and controls during the injury phase (Figure S3A), suggesting that pancreatic HIF1α is largely dispensable for initial immune responses to cerulein-mediated injury. However, at day 7, which corresponds to the
“regeneration phase”, the relative proportions of macrophages and B cells were significantly different between \( Hif1^\alpha KO \) and control pancreata (Figure 3A). \( Hif1^\alpha \) deletion did not change the frequencies of CD4\(^+\) and CD8\(^+\) T cells (Figure S3B), or expression of lineage-specific transcription factors for CD4\(^+\) T-cell subsets (e.g., \( Tbx21 \) for Th1, \( Gata3 \) for Th2, \( Rorc \) for Th17, and \( Foxp3 \) for Treg) (Figure S3C). Whereas the absolute numbers of macrophages were comparable (Figure S3D), both the percentage and absolute number of B cells were markedly increased in \( Hif1^\alpha KO \) pancreata compared with their \( Hif1^\alpha \) fl/fl counterparts (Figures 3A and 3B), implicating selective modulation of B cell recruitment upon HIF1\( ^\alpha \) ablation during the regeneration phase. Of note, \( Hif1^\alpha KO \) mice also exhibited increased numbers of CD45\(^+\) pancreatic leukocytes compared to controls at day 7 (Figure S3E), although this difference did not achieve statistical significance (\( P = 0.06 \)). Elevated numbers of intrapancreatic B cells in \( Hif1^\alpha KO \) samples at day 7 were preceded by increased levels of Cxcl13, a key B-cell chemoattractant, at day 3 (Figure 3C). Pancreatic Cxcl13 expression decreased over the course of tissue regeneration (Figure S3F), consistent with diminution of hypoxia (Figure 1B) and B cell numbers (Figure S3G). Importantly, B cells were found within the injured pancreatic parenchyma from both murine (Figure 3D) and human pancreatitis (9 of 10 samples) (Figures 3E and 3F), supporting a potential role for B cells in the progression of human pancreatitis.

**B cell depletion enhances pancreatic regeneration**

The contribution of B cells to tissue injury and regeneration has been addressed in multiple experimental models (Ammirante et al., 2013; de Visser et al., 2005; Jang et al., 2010; Novobrantseva et al., 2005; Zouggari et al., 2013); however, B cell function(s) in the setting of pancreatic injury and repair have remained obscure. As impaired pancreatic regeneration upon \( Hif1^\alpha \) deletion coincides with a selective accumulation of B cells, we evaluated the effects of B cell depletion on pancreatic regeneration in cerulein-treated mice. Administration of \( \alpha \)CD20 monoclonal antibodies (\( \alpha \)CD20 mAb) at days 1 and 7 following the final cerulein injection efficiently depleted B cells (>90%) in the pancreas, spleen, and peripheral blood of both \( Hif1^\alpha KO \) and \( Hif1^\alpha \) fl/fl mice (Figures 4A–4D). When comparing isotype control IgG2a-treated and \( \alpha \)CD20 mAb-treated cohorts at a later stage of regeneration (day 14 after the cessation of cerulein administration), it became apparent that B cell depletion significantly accelerated regeneration of both \( Hif1^\alpha KO \) and \( Hif1^\alpha \) fl/fl pancreatic tissues, as shown by increased acinar cell area and decreased fibrosis and immune infiltration (Figures 4E–4H). Notably, intrapancreatic B cell numbers and the extent of tissue regeneration in \( Hif1^\alpha \) fl/fl and \( Hif1^\alpha KO \) animals treated with isotype control antibodies in this phase of recovery (day 14) were similar (Figures 4B and 4E–4H). Thus, while hypoxic regulation of B cell trafficking peaks at day 7 and fades, anti-regenerative phenotypes due to excessive B cell accumulation in \( Hif1^\alpha KO \) pancreata take 21 days to be fully manifested. Importantly, a reduction in total pancreatic immune infiltrates was 2-fold greater than B cell loss upon \( \alpha \)CD20 mAb treatment (14x10\(^5\) CD45\(^+\) leukocyte reduction vs 7x10\(^5\) CD19\(^+\) B cell depletion, irrespective of HIF1\( ^\alpha \) status) (Figures 4B and 4H), indicating that decreased immune cell infiltration is not merely a reflection of B cell depletion. \( \alpha \)CD20 mAb treatment led to an increase in the percentage (but not absolute number) of intrapancreatic macrophages (Figures S4A and S4B) due to a prominent decrease in the absolute number of total pancreatic immune cells (Figure 4H). B cell depletion had no effect on MDSC.
dendritic cell, CD4+ T cell, and CD8+ T cell percentages (Figures S4A and S4C), or the relative proportions of M1-like (CD86+MHCII+F4/80+CD11b+) and M2-like (CD206+F4/80+CD11b+) macrophages (Figures S4D and S4E) in this context. Interestingly, splenic B cell numbers were increased in Hif1αKO mice at day 14 (Figure 4C) and returned to baseline at day 21 (Figure S4F), implying a systemic immune reaction to pancreatic HIF1α ablation. Collectively, our findings suggest that B cell depletion enhances pancreatic regeneration and that HIF1α deficiency impedes regeneration by promoting intrapancreatic B-cell accumulation.

DISCUSSION

Hypoxia is a common trait of tissue injury, and serves as a pivotal regulator of tissue responses to injury and repair. Previous studies demonstrated that hypoxia and subsequent HIF1α induction accelerate tissue regeneration in the heart and axon (Cho et al., 2015; Nakada et al., 2017). We show here that hypoxia and HIF1α accumulation occur during experimental pancreatitis, and that HIF1α is critical for optimal pancreatic regeneration following injury. Our observations support the notion that HIF1α, a key regulator of hypoxic adaptations, promotes the regenerative processes of damaged, inflamed, and hypoxic pancreata to regain tissue integrity and homeostasis. Of note, two recent studies have reported contribution of HIF1α to intrapancreatic coagulation and injury response during experimental acute pancreatitis (Park et al., 2018), and onset of chronic pancreatitis by HIF2α overexpression in mice (Schofield et al., 2018). These results, along with our findings suggest that hypoxia affects multiple biological aspects of pancreatitis, and the two HIFα isoforms play distinct roles in this disease.

Interestingly, we found that delayed tissue regeneration by pancreatic HIF1α deficiency is coupled to aberrant B cell accumulation in the pancreas. B cells have been shown to interfere with reparative processes in certain tissue types, including heart, liver, and kidney (Jang et al., 2010; Novobrantseva et al., 2005; Zouggari et al., 2013); however, the importance of B cells in pancreatic regeneration has remained obscure. B cell depletion following pancreatitis revealed that B cells inhibit pancreatic regeneration, and that HIF1α regulates regenerative processes by limiting intrapancreatic B cell accumulation. Future studies will define the molecular mechanisms responsible for anti-regenerative properties of B cells in the injured pancreas.

Our recent work showed that HIF1α ablation dramatically accelerates early pancreatic tumorigenesis, accompanied by significantly increased numbers of B lymphocytes within neoplastic lesions (Lee et al., 2016). While this was somewhat surprising, HIF1α can function as a tumor suppressor in other cancers as well (Kaelin, 2017). The finding that HIF1α ablation augments B cell infiltration in the context of pancreatitis thus reinforces a link between HIF1α and B cell trafficking. We observed that the expression of CXCL13 (a B-cell chemoattractant) is elevated upon cerulein-induced pancreatitis, and further increased by HIF1α deficiency. This is also consistent with our previous demonstration of increased CXCL13 downstream of Hif1α deletion during pancreatic oncogenesis (Lee et al., 2016), implicating CXCL13 as a critical chemotactic factor for B cell recruitment to diseased
pancreata. The precise mechanism for CXCL13 regulation by HIF1α remains to be determined.

Failure to resolve acute inflammation following tissue injury triggers chronic inflammation that contributes to tumor development and progression (Shalapour and Karin, 2015). Given the pro-tumorigenic impact of B cells on pancreatic cancer (Gunderson et al., 2016; Lee et al., 2016; Pylayeva-Gupta et al., 2016), as well as our observation that B cell depletion increases exocrine tissue regeneration with substantially reduced overall immune infiltration and fibrosis, B cells are likely a crucial immune component for establishing chronic inflammatory states, which in turn promote tumorigenesis.

In conclusion, our study unveils HIF1α as an important pro-regenerative factor in the pancreas. We also show that B cells suppress pancreatic regeneration, and their trafficking and/or function are modulated by hypoxia and HIF1α in the setting of pancreatitis. Importantly, targeting B cells via αCD20 monoclonal antibodies or Bruton tyrosine kinase (BTK) inhibitors represents potential therapeutic avenues to treat pancreatitis patients. As FDA-approved B cell-targeting agents already exist (Kipps et al., 2017), their potential for the treatment of pancreatitis could be rapidly evaluated. Notably, the BTK inhibitor ibrutinib improved survival rates in mouse models of pancreatic cancer (Gunderson et al., 2016; Masso-Valles et al., 2015), and B cell-targeted approaches could thus have beneficial actions on both pancreatitis and pancreatic cancer.

**EXPERIMENTAL PROCEDURES**

**Mice and Treatments**

All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. The Hif1α<sup>fl/fl</sup> and p48-Cre strains have been previously described (Kawaguchi et al., 2002; Ryan et al., 2000), and wild-type mice were purchased from Jackson Laboratory. All experimental mice were on the C57BL/6 genetic background, and a mix of male and female. Pancreatitis was induced in 6–8 week old mice by 7 hourly intraperitoneal injections of 50 ng/g body weight cerulein (Sigma-Aldrich) per day, 3 days a week, for 3 weeks. Where indicated, mice received intraperitoneal injections with isotype control mouse IgG2a (C1.18.4, #BE0085, BioXcell) or mouse IgG2a anti-mouse CD20 mAb (5D2, a kind gift from Genentech) at 10 mg/kg at day 1 and day 7 after the final cerulein injection. Pancreata were harvested at the indicated time points.

**Histology, Immunofluorescence, Immunohistochemistry, and Microscopy**

Pancreata were fixed in 4% paraformaldehyde/PBS (4°C, overnight) and processed for paraffin-embedding. For histological analysis, deparaffinized sections (5 μm) were stained with Harris hematoxylin and eosin or Picric acid-Sirius red (all from Sigma-Aldrich) followed by alcohol dehydration series and mounting.

For immunohistochemistry, slides were quenched in 0.6% hydrogen peroxide/methanol for 15 min, and boiled for 20 min in 10 mM sodium citrate (pH 6.0) for antigen retrieval. Sections were blocked with 5% serum/1% BSA/0.5% Tween-20 for 1 hour. Slides were incubated with primary antibodies diluted in blocking buffer overnight at 4°C. Following the...
primary antibody, slides were incubated with biotinylated secondary antibodies followed by ABC solution and developed with 3,3’-diaminobenzidine (all from Vector Laboratories). Slides were counterstained with hematoxylin, dehydrated, and mounted with Permount (Fisher), and then examined on a Leica DM5000B microscope. The following antibodies were used: rat anti-B220 (RA3–6B2, #557390, 1:400, BD Biosciences), rat anti-CD45 (30-F11, #550539, 1:200, BD Biosciences), and rabbit anti-HIF1α (#ab2185, 1:5000, Abcam).

For immunofluorescence, slides were boiled for 20 min in 10 mM sodium citrate (pH 6.0) for antigen retrieval and blocked with 5% serum/0.3% Triton X-100 for 1 hour. Sections were incubated with primary antibodies diluted in 1% BSA/0.3% Triton X-100 overnight at 4°C, and were then incubated with Alexa Fluor-labeled secondary antibodies (Invitrogen) diluted in 1% BSA/0.3% Triton X-100 for 1 hour. Slides were counterstained with Hoechst 33342 (Invitrogen), and mounted in Prolong Gold antifade reagent (Invitrogen). Fluorescence images were acquired using an Olympus IX81 microscope. The following antibodies were used: rabbit anti-Amylase (#A1978, 1:200, Sigma-Aldrich), mouse anti-E-cadherin (#610181, 1:200, BD Biosciences), rabbit anti-Ki67 (#ab15580, 1:1000, Abcam), and rabbit anti-Sox9 (#AB5535, 1:4000, Millipore).

For quantification of acinar cell mass and fibrosis, Amylase and Picrosirius Red positive areas were measured respectively via morphometric analysis using ImageJ.

**Hypoxprobe**

Mice received injection intraperitoneally with 60 mg/kg of Hypoxprobe (pimonidazole hydrochloride, Hypoxprobe, Inc) and sacrificed 1.5–2 hours later. To detect the formation of pimonidazole adducts, pancreatic tissue sections (paraffin-embedded) were immunostained with Hypoxyprobe-1-MAb1 (Hypoxyprobe-1 Plus kit, Hypoxyprobe, Inc) following the manufacturer’s instructions.

**Human Pancreas Specimen**

Human pancreatic tissue sections (3 normal and 10 chronic pancreatitis samples) were obtained from the Cooperative Human Tissue Network (CHTN) and handled at the University of Pennsylvania with the approval of its institutional review board committees. All tissues were collected with the donor being informed and given consent, and anonymized prior to being provided to the investigators. Immunohistochemical analysis was performed as described above. The following antibodies were used: mouse anti-human CD20 (FB1, #555677, 1:1000, BD Biosciences) and mouse anti-human HIF1α (HA111, #NB100–296, 1:250, Novus).

**Flow Cytometry**

Single cell suspensions from mouse pancreas, spleen, and blood were prepared as previously described (Lee et al., 2016). Cells were stained in PBS/0.5% FBS/2 mM EDTA with the following fluorochrome-conjugated antibodies: V450-conjugated anti-CD3 (500A2, #560801, 1:100), APC-Cy7-conjugated anti-CD11b (M1/70, #561039, 1:50), V450-conjugated anti-CD11c (HL3, #560521, 1:100), APC-conjugated anti-CD19 (1D3, #550992, 1:200), PE-Cy7-conjugated anti-CD45 (30-F11, #552848, 1:100), V500-conjugated anti-
CD45 (30-F11, #561487, 1:100), V450-conjugated anti-Gr1 (RB6–8C5, #560454, 1:200) (from BD Biosciences); APC-conjugated anti-CD86 (GL1, #17–0862, 1:100), PE-conjugated anti-F4/80 (BM8, #12–4801, 1:100) (from eBioscience); FITC-conjugated anti-CD206 (MR5D3, #MCA2235FT, 1:50) (from AbD Serotec); PE-Cy7-conjugated anti-MHCII (M5/114.15.2, #107629, 1:100) (from BioLegend). The viability marker 7-aminoactinomycin D (7-AAD) was purchased from BD Biosciences. Flow cytometry was performed on a FACSCanto flow cytometer (BD Biosciences), and data was analyzed using FlowJo software.

Quantitative RT–PCR

Total RNA was isolated from pancreatic tissues using the RNeasy mini kit (Qiagen). cDNA was synthesized using a High Capacity RNA-to-cDNA Master Mix (Applied Biosystems). PCR reactions were performed using TaqMan Universal PCR regents mixed with indicated cDNAs and TaqMan primers in a ViiA7 Real-Time PCR system (Applied Biosystems). Expression levels were normalized by 18S rRNA.

Statistical Analyses

Data were analyzed using GraphPad Prism 7 software. Statistical tests with normally distributed variables included 2-tailed Student’s t test and 2-way ANOVA. D’Agostino and Pearson test and/or Shapiro–Wilk test was used to test the normality of sample distribution. When variables were not normally distributed, we performed non-parametric Mann-Whitney test. Bonferroni correction was applied for multiple comparisons. P value < 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Hypoxia and stabilization of HIF1α occur during pancreatitis.
(A) Schema of pancreatitis induction with cerulein and tissue harvest. Cerulein was administered to WT mice as 7 hourly intraperitoneal injections (50 ng/g of body weight/injection) three times a week for 3 weeks.
(B) Immunohistochemical staining for Hypoxyprobe or HIF1α in pancreata from WT mice treated with cerulein or PBS.
(C) HIF1α immunohistochemical staining of human normal pancreatic or chronic pancreatitis tissues.
Arrowheads point to representative positive staining. Scale bars, 50 μm.
See also Figure S1.
Figure 2. HIF1α is required for efficient regeneration of the pancreas.

Cerulein was administered to Hif1α^fl/fl and Hif1α^KO mice as described in Figure 1A. 

(A) Hematoxylin and eosin (H&E) staining of pancreata. Yellow arrowheads indicate metaplastic lesions; insets show higher magnified view of the same field. Scale bars, 300 μm.

(B and C) Immunofluorescence staining for Amylase in pancreata (B) and corresponding quantification (C) (n ≥ 5 mice per group, n = 3 FOV per animal). Scale bars, 100 μm.
(D and E) Picrosirius Red staining of pancreata (D) and corresponding quantification (E) (n ≥ 4 mice per group, n = 3 FOV per animal). Scale bars, 100 μm.

(F) Immunohistochemical staining for CD45 in pancreata. Scale bars, 50 μm.

(G) Absolute numbers of CD45+ immune cells in the pancreas as analyzed by flow cytometry (n ≥ 5 mice per group). Symbols represent individual mice with a horizontal bar at the mean.

The data in (C) and (E) are shown as the mean ± s.e.m. FOV, fields of view. NS, not significant. *P < 0.05, **P < 0.01; Mann Whitney test with Bonferroni post-test (C and G), two-way ANOVA with Bonferroni post-test (E).

See also Figure S2.
Figure 3. Elimination of HIF1α promotes B cell accumulation in the injured pancreas during regeneration.

Cerulein was administered to Hif1α^+/fl and Hif1α^KO mice as described in Figure 1A. (A and B) Flow cytometry analysis of immune infiltrates from pancreata harvested at day 7 after final cerulein injection. Percentage of CD11b^+ F4/80^+ macrophages (MΦ), Gr1^+ CD11b^+ myeloid-derived suppressor cells (MDSC), CD11c^+ F4/80^- dendritic cells (DC), CD3^+ T cells (T), and CD19^+ B cells (B) among live CD45^+ immune cells (A) and absolute numbers of CD19^+ B cells (B) (n ≥ 6 mice per group). Symbols represent individual mice with a horizontal bar at the mean.

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(C) Quantitative RT-PCR analysis of Cxcl13 in pancreata harvested at day 3 after final cerulein injection (n \geq 7 mice per group).

(D) Immunohistochemical staining for B220 in pancreata harvested at day 7 after final cerulein injection.

(E and F) CD20 immunohistochemical staining of human normal pancreatic or chronic pancreatitis tissues (E) and corresponding quantification (F) (n = 3 normal, n = 10 chronic pancreatitis samples, n = 3 FOV per sample). N, normal pancreas. CP, Chronic pancreatitis. Scale bars, 50 μm. The data in (C) and (F) are shown as the mean ± s.e.m. NS, not significant. **P < 0.01, ***P < 0.001; Student’s t test (A and B), Mann Whitney test with Bonferroni post-test (C), Mann Whitney test (F). See also Figure S3.
Figure 4. B cell depletion accelerates pancreatic regeneration.

(A) Schema of pancreatitis induction, B cell depletion, and tissue harvest. Hif1α<sup>fl/fl</sup> and Hif1α<sup>KO</sup> mice treated with 7 hourly injections of cerulein (50 ng/g of body weight/injection) three times a week for 3 weeks, received either isotype control antibody or αCD20 mAb (10 mg/kg) at day 1 and 7 after the final cerulein injection, and were analyzed at day 14 after the cessation of cerulein administration.
(B-D) Absolute numbers of CD19+ B cells in the pancreas (B) and spleen (C), and the percentage of CD19+ B cells in peripheral blood among live CD45+ immune cells (D) as analyzed by flow cytometry (n ≥6 mice per group).

(E) H&E, Amylase immunofluorescence staining, or Picrosirius Red staining of pancreata. Insets show higher magnified view of the same field. Scale bars for H&E, 300 μm. Scale bars for Amylase and Picrosirius Red, 100 μm.

(F and G) Quantification of Amylase immunofluorescence staining (F) and Picrosirius Red staining (G) (n ≥6 mice per group, n = 3 FOV per animal).

(H) Absolute numbers of CD45+ immune cells in pancreata as analyzed by flow cytometry (n ≥6 mice per group).

Symbols in (B-D and H) represent individual mice with a horizontal bar at the mean. The data in (F and G) are shown as the mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; two-way ANOVA with Bonferroni post-test (B-D, F, and H), Mann Whitney test with Bonferroni post-test (G).

See also Figure S4.