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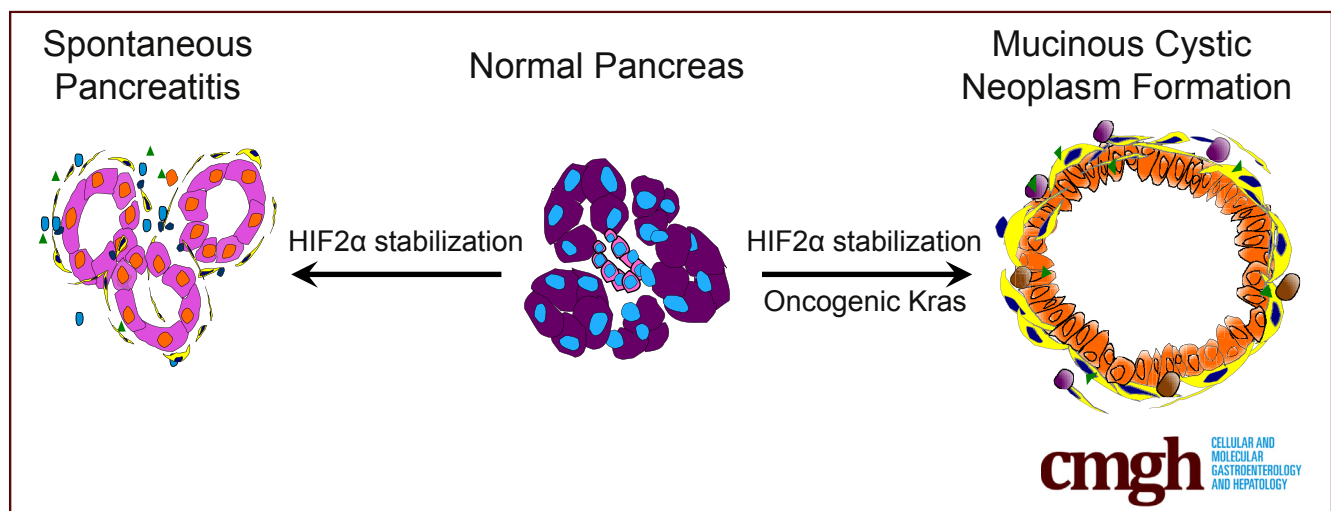
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ORIGINAL RESEARCH

Pancreatic HIF2 α Stabilization Leads to Chronic Pancreatitis and Predisposes to Mucinous Cystic Neoplasm

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SUMMARY

Tissue hypoxia controls cell differentiation in the embryonic pancreas. Expression of the hypoxia-inducible factor 2 α is induced in chronic pancreatitis. Stabilization of hypoxia-inducible factor 2 α in mouse pancreata leads to the development of chronic pancreatitis and in the presence of oncogenic Kras drives the formation of mucinous cystic neoplasm.

BACKGROUND & AIMS: Tissue hypoxia controls cell differentiation in the embryonic pancreas, and promotes tumor growth in pancreatic cancer. The cellular response to hypoxia is controlled by the hypoxia-inducible factor (HIF) proteins, including HIF2 α . Previous studies of HIF action in the pancreas have relied on loss-of-function mouse models, and the effects of HIF2 α expression in the pancreas have remained undefined.

METHODS: We developed several transgenic mouse models based on the expression of an oxygen-stable form of HIF2 α , or indirect stabilization of HIF proteins through deletion of von Hippel-Lindau, thus preventing HIF degradation. Furthermore, we crossed both sets of animals into mice expressing oncogenic Kras^{G12D} in the pancreas.

RESULTS: We show that HIF2 α is not expressed in the normal human pancreas, however, it is up-regulated in human chronic pancreatitis. Deletion of von Hippel-Lindau or stabilization of HIF2 α in mouse pancreata led to the development of chronic pancreatitis. Importantly, pancreatic HIF1 α stabilization did not disrupt the pancreatic parenchyma, indicating that the chronic pancreatitis phenotype is specific to HIF2 α . In the presence of oncogenic Kras, HIF2 α stabilization drove the formation of cysts resembling mucinous cystic neoplasm (MCN) in humans. Mechanistically, we show that the pancreatitis phenotype is linked to expression of multiple inflammatory cytokines and activation of the unfolded protein response. Conversely, MCN formation is linked to activation of Wnt signaling, a feature of human MCN.

CONCLUSIONS: We show that pancreatic HIF2 α stabilization disrupts pancreatic homeostasis, leading to chronic pancreatitis, and, in the context of oncogenic Kras, MCN formation. These findings provide new mouse models of both chronic pancreatitis and MCN, as well as illustrate the importance of hypoxia signaling in the pancreas. (*Cell Mol Gastroenterol Hepatol* 2018;5:169–185; <https://doi.org/10.1016/j.jcmgh.2017.10.008>)

Keywords: Pancreas; Hypoxia; HIF2 α ; Kras^{G12D}; Chronic Pancreatitis; Mucinous Cystic Neoplasm.

See editorial on page 165.

During pancreas development, oxygen levels positively control β -cell differentiation.¹ Pancreatic cancer, the third leading cause of cancer-related death,² is extensively hypoxic.³ Hypoxia has been shown to promote pancreatic tumor growth, invasion, and metastasis.^{4,5} At the cellular level, the response and adaptation to hypoxia is controlled by hypoxia-inducible factors (HIFs). In vertebrates, the HIF family contains 3 isoforms: HIF1 α , HIF2 α , and HIF3 α . The HIF proteins are transcription factors, activating genes containing a hypoxia response element in response to low levels of cellular oxygen.^{6,7} In normal oxygen conditions, HIF proteins are hydroxylated post-translationally, allowing association with the von Hippel-Lindau (VHL) tumor suppressor and tagging for proteasomal degradation.⁸ In hypoxia, VHL is unable to tag the HIF proteins for degradation and the HIFs accumulate intracellularly, translocate to the nucleus, and activate target genes.⁸

Hypoxia induces HIF1 α and HIF2 α expression in the pancreas.⁹ Furthermore, a number of studies have associated perturbation of the HIF factors with pancreatic abnormalities/disease. HIF1 α is expressed during the development of pancreatic cancer, and its deletion promotes pancreatic tumorigenesis in a Kras-driven model of pancreatic cancer.¹⁰ HIF2 α expression is required for the embryonic development of the pancreas, and a lack of HIF2 α expression in developing mice leads to smaller pancreata and decreased branching.¹¹ In the presence of oncogenic Kras, HIF2 α inactivation inhibits the progression of precancerous lesions.¹²

Here, we show that pancreas-specific inactivation of VHL, or stabilization of HIF2 α (but not HIF1 α), induces chronic pancreatitis in mice. Although many mouse models of pancreatitis recover over time (for review, see Lerch and Gorelick¹³), mice overexpressing HIF2 α have bona fide chronic pancreatitis that persists until most of the pancreatic parenchyma is substituted by fibrotic or fatty tissue. The link between HIF2 α expression and pancreatitis is strengthened further by the observation that this protein accumulates in a subset of human chronic pancreatitis samples.

Different precursor lesions are described for human pancreatic cancer. Although pancreatic intraepithelial neoplasia (PanIN) is the most common, intraductal papillary mucinous neoplasms and mucinous cystic neoplasms (MCNs) can progress to malignancy (for review see Hezel et al¹⁴ and Ying et al¹⁵). The molecular drivers underpinning progression to each of these specific precursor lesions are only partially understood. Here, we show that stabilization of HIF2 α in the presence of oncogenic Kras specifically drives formation of MCN, through activation of Wnt signaling. Notably, Wnt signaling is a common feature of human MCN.

Methods

Mice

Mice were housed in the specific pathogen-free facility at the University of Michigan Comprehensive Cancer Center. This study was approved by the University of Michigan

Committee on the Use and Care of Animals, and the University of Pittsburgh Institutional Animal Care and Use Committee. Pdx1-Cre, Ptf1a-Cre, LSL-Kras^{G12D}, R26-^{LSLHif2a/+}, and VHL floxed mice have been described previously.¹⁶⁻¹⁸

Glucose Tolerance Testing

Glucose tolerance testing was performed as previously described.¹⁹ Before testing, animals were fasted for 4 hours during the light cycle. Initial blood glucose levels were measured using tail blood samples. Then, animals were administered glucose at a dose of 2 g glucose per kilogram of body weight by intraperitoneal injection. Tail blood samples then were measured for blood glucose levels at 15, 30, 60, 90, and 120 minutes after glucose injection. Blood glucose level was measured using the Accu-Chek Aviva diabetes monitoring kit and Accu-Chek (Roche Diabetes Care, Indianapolis, IN) Aviva Plus testing strips.

Glucose-Stimulated Insulin Secretion

Overnight fasted mice were anesthetized by an intraperitoneal injection of Avertin (Sigma-Aldrich, St. Louis, MO). Anesthetized mice then were injected intraperitoneally with glucose at 3 g/kg body weight and blood was collected retro-orbitally at 0, 2, 7, 15, and 30 minutes. Serum was separated by centrifuging the blood at 8000 rpm for 8 minutes at 4°C. Serum insulin was measured using the Ultra-sensitive mouse insulin ELISA kit (CrystalChem, Downers Grove, IL), following the manufacturer's recommendation.

Immunohistochemistry and Immunofluorescence

Histology and immunohistochemistry studies, as well as periodic acid-Schiff and Gomori trichrome staining, were performed as previously described.²⁰ To prepare for staining, tissue was collected and fixed overnight in 10% neutral buffered formalin. Tissue then was embedded in paraffin and sectioned. The University of Michigan Cancer Center Histopathology Core performed embedding and sectioning. Sections were imaged using an Olympus (Olympus, Center Valley, PA) BX-51 microscope, Olympus DP71 digital camera, and CellSens (Olympus) Standard software. Primary antibodies used are included in [Supplementary Table 1](#).

Quantitative Reverse-Transcription Polymerase Chain Reaction

Tissue for RNA extraction was collected in lysis buffer (Ambion, Foster City, CA) and RNA was isolated using the PureLink RNA Mini Kit (Ambion). Reverse transcription was

[§]Authors share co-senior authorship.

Abbreviations used in this paper: ER, endoplasmic reticulum; HIF2 α , hypoxia-inducible factor 2 α ; KC, Pdx1-Cre;LSLkras^{G12D}; MCN, mucinous cystic neoplasm; PanIN, pancreatic intraepithelial neoplasia; qPCR, quantitative polymerase chain reaction; UPR, unfolded protein response; VHL, von Hippel-Lindau.

 Most current article

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performed using the High-Capacity Complementary DNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Primers were optimized for amplification conditions of 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Melt curve analysis was performed for all samples. *Cyclophilin A* was used as the control housekeeping gene for normalization. The primer sequences for genes analyzed are included in [Supplementary Table 2](#). Quantitative polymerase chain reaction (qPCR) array was performed using the Mouse Th17 Response PCR Array (Qiagen, Frederick, MD) according to the manufacturer's instructions.

Western Blot

Tissue for protein extraction was collected in radioimmunoprecipitation assay buffer with protease inhibitor. Equal amounts of protein were added per lane, run by electrophoresis in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes. Each membrane was blocked with 5% milk. Primary antibody incubation was performed overnight at 4°C. Secondary antibody incubation was performed for 2 hours at room temperature. Protein bands were detected using Western Lightning Plus Enhanced Chemiluminescence (NEL103001EA; PerkinElmer, Waltham, MA) and film.

Statistics

Statistical significance for reverse-transcription qPCR results was determined by an unpaired 2-tailed *t* test, with the threshold for statistical significance determined to be $P < .05$.

Study Approval

The University of Michigan Institutional Review Board approved all human studies. Informed consent was received from all human patients before inclusion in the studies detailed in this work. All animal studies were approved by the University of Michigan Committee on the Use and Care of Animals.

Results

HIF2 α Accumulates in Human Chronic Pancreatitis and its Forced Stabilization Causes Chronic Pancreatitis in Mice

HIF2 α protein expression is not detectable in the normal human pancreas. However, we observed abundant HIF2 α expression in human samples of chronic pancreatitis ($n = 5$ chronic pancreatitis samples) ([Figure 1A](#)). Overexpression of HIF2 α was observed in 3 of 5 human chronic pancreatitis samples, with lower levels of expression seen in the other 2 samples, but still higher than observed in the normal pancreas. This may indicate variability in HIF2 α expression in chronic pancreatitis, or suggest that high HIF2 α expression levels represent a subset of human chronic pancreatitis. Because a functional role for HIF2 α had not been described in this disease, we generated Pdx1-Cre;Rosa26^{LSL-HIF2 α /+}

mice. In these animals, an oxygen-stable form of HIF2 α ¹⁶ is expressed in the pancreas upon Cre recombination ([Figure 1B](#)). We observed that pancreata with stabilized HIF2 α were smaller than in their littermate controls (shown at 9 weeks of age) ([Figure 1C](#)). However, there was no difference in total body weight between the controls and HIF2 α stabilized mice at all ages analyzed. From 7 weeks to 1 year of age HIF2 α stabilized animals showed similar total body weight to age-matched littermate control animals ($n = 6$ pairs of age-matched littermates, 1 control, and 1 HIF2 α stabilized) ([Figure 1D](#)).

At 2 weeks of age ($n = 2$), animals expressing HIF2 α had atrophic pancreatic parenchyma resembling end-stage chronic pancreatitis ([Figure 1E](#)). By 4 weeks of age, shortly after weaning, HIF2 α stabilized animals had developed further signs of chronic pancreatitis ($n = 2$) ([Figure 1F](#)). These changes progressed over time, with few residual acini and significant inflammatory infiltrates at 9 weeks of age ($n = 5$) ([Figure 1G](#)). In older mice ($n = 4$ analyzed at 1 year of age), pancreata were mostly replaced by adipose tissue with small remnant clusters of acinar cells, dilated ducts, and intermixed inflammatory infiltration ([Figure 1G](#)), indicating chronic disease and lack of recovery from pancreatitis. This feature distinguishes this model from most other mouse models of pancreatitis, in which repair is observed over time.^{13,21} To analyze the molecular changes underlying the onset of pancreatitis, we extracted RNA from 2-week-old mouse pancreata and performed qPCR analysis ($n = 3$ mice per genotype). By 2 weeks of age, HIF2 α stabilized animals were not expressing amylase (*Amy2b*), indicating loss of acinar differentiation. At the same age, cytokeratin 19 (*CK19*) expression was unchanged compared with littermate controls. Finally, smooth muscle actin (*SMA*) expression was increased in HIF2 α stabilized mice, indicating an increase in activated fibroblasts ([Figure 1I](#)).

To validate HIF2 α stabilization in experimental animals, we performed a Western Blot. As expected, HIF2 α accumulated in Pdx1-Cre;Rosa26^{LSL-HIF2 α /+} pancreata but not in littermate controls ($n = 2$ pancreata per genotype) ([Figure 2A](#)). Although immunostaining for HIF2 α is technically challenging, our results were in line with an increase in HIF2 α protein in the experimental mice ([Figure 2B](#)). Gene expression analysis by qPCR showed up-regulation of HIF targets, including *Pdk1*,²² indicating functional up-regulation of the HIF signaling pathway in HIF2 α stabilized animals ([Figure 2C](#)).

To expand our analysis, we generated Ptf1a^{+Cre}; Rosa26^{LSL-HIF1 α /+} mice in which a different Cre recombinase was used to activate the same stabilized HIF2 α in the pancreas ([Figure 3A](#)). Similar to our previous results, Ptf1a^{+Cre};Rosa26^{LSL-HIF2 α /+} animals showed inflammatory infiltration and pancreatic atrophy typical of chronic pancreatitis ([Figure 3B](#)). Possibly owing to differences in Cre expression, the phenotype developed more slowly, with small pockets of inflammation at 1 month, progressing to full pancreatic involvement by 7 months of age ($n = 1$ at 1 month, $n = 3$ at 3 months, and $n = 2$ at 7 months) ([Figure 3B](#)).

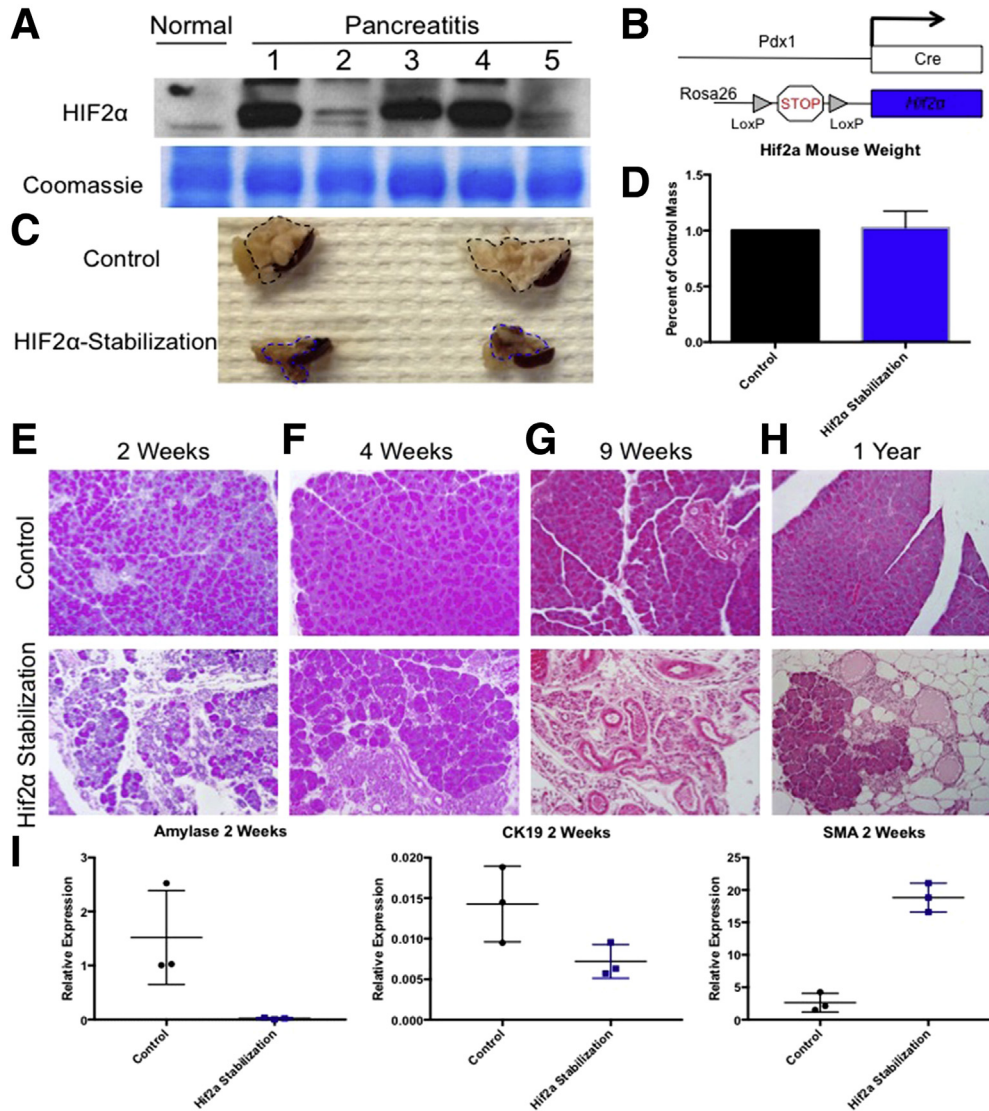


Figure 1. HIF2 α expression is associated with chronic pancreatitis in human beings and mice. (A) Western blot analysis of HIF2 α expression in lysates from human normal and chronic pancreatitis pancreata (n = 5 chronic pancreatitis samples). (B) Transgenic mouse scheme. (C) Gross morphology of control and HIF2 α stabilized pancreata. (D) Average weight of mice with HIF2 α stabilization compared with age-matched littermate controls (n = 6 pairs of age-matched littermates). H&E evaluation of (E) 2-week (n = 2), (F) 4-week (n = 2), (G) 9-week (n = 5), and (H) 1-year-old (n = 4) pancreata. (I) qPCR analysis of gene expression levels in 2-week-old pancreata. SMA, smooth muscle actin.

Disruption of the Negative Hif Regulator VHL Mimics HIF2 α Stabilization in Mice

To determine whether the phenotype observed upon HIF2 α stabilization was indeed owing to activation of the hypoxia pathway, we generated mice lacking VHL expression in the pancreas. Under normoxic conditions, VHL acts to inhibit HIF function by tagging HIF proteins for degradation in the proteasome.⁸ Thus, deletion of VHL prevents the degradation of HIF proteins, leading to stabilization of the endogenous HIF proteins. We crossed mice bearing $Ptf1a^{+/Cre}$ with mice carrying a floxed allele of VHL¹⁷ (Figure 3C) to generate $Ptf1a^{+/Cre};VHL^{fl/fl}$ (termed VHL^{PanKO}) animals. Histologic analysis of VHL^{PanKO} mice showed that HIF stabilization by this method phenocopied the chronic pancreatitis observed in HIF2 α stabilization, observable from as early as 3 months of age (n = 5 at 3 months, n = 2 at 5 months, and n = 2 at 7 months) (Figure 3D). Taken together, these data further show that

activation of the HIF pathway in the pancreas through stabilization of HIF2 α leads to the development of a spontaneous fibroinflammatory response resembling human chronic pancreatitis.

HIF1 α Stabilization Has no Effect on Pancreas Morphology and Function

To determine whether HIF1 α , a factor related to HIF2 α , was similarly implicated in human pancreatitis, we probed HIF1 α protein expression in the same human chronic pancreatitis samples in which we observed HIF2 α up-regulation. In human chronic pancreatitis HIF1 α was up-regulated dramatically in only 1 of 5 samples by Western blot (Figure 4A). We then generated $Pdx1-Cre;Rosa26^{LSL-HIF1\alpha/+}$ and $Ptf1a^{+/Cre};Rosa26^{LSL-HIF1\alpha/+}$ mice, in which HIF1 α stabilization is stabilized in a tissue-specific manner. Consistent with the human findings

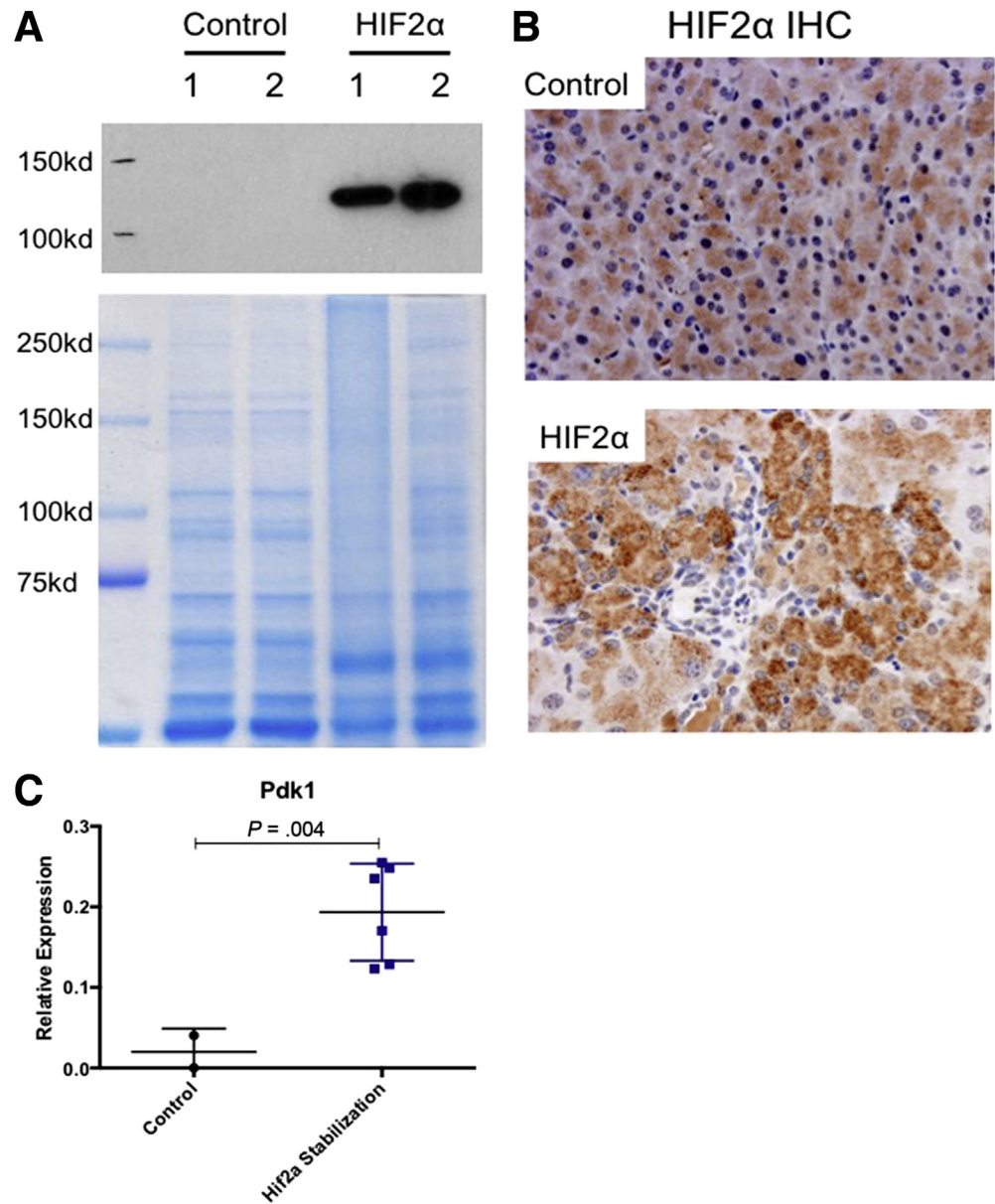


Figure 2. Validation of HIF2 α stabilization in mouse pancreata. (A) Western blot for HIF2 α in lysates from control and HIF2 α stabilized pancreata, with Coomassie stain for loading control (n = 2 samples per condition). (B) Immunohistochemistry for HIF2 α in control and HIF2 α stabilized pancreata. (C) qPCR analysis of HIF target gene expression levels in 9-week-old pancreata.

indicating no strong correlation between HIF1 α and pancreatitis, animals with pancreatic HIF1 α stabilization, independently from the Cre driver used, had normal pancreata (Pdx1-Cre;Rosa26^{LSL-HIF1 α /+}; n = 10 mice, histology analyzed at ages 6 wk to 1 y; Ptf1a^{+/-}/Cre;Rosa26^{LSL-HIF1 α /+}; n = 2) (Figure 4B and C). This indicates that the chronic pancreatitis phenotype is specific to HIF2 α expression, and not HIF pathway activation in general.

Pancreatitis Occurs Postnatally in HIF2 α Stabilized Mice

We next sought to determine whether the pancreatitis phenotype caused by HIF2 α expression was caused by impaired embryonic development, thus analyzed pancreata from newly born animals. At 1 day of age, we observed no

discernible differences in histology between HIF2 α stabilized animals and their control littermates (n = 2) (Figure 5A) via H&E staining. HIF2 α stabilization was confirmed by immunohistochemistry, in which both control and HIF2 α stabilized animals showed positive HIF2 α expression in the developing islets, as expected,¹¹ and only HIF2 α stabilized pancreata showed positive HIF2 α staining throughout the pancreas (Figure 5B). Control and HIF2 α stabilized animals showed high levels of proliferation, as evidenced by Ki67 staining, with no differences between the 2 groups (Figure 5C). Similarly, apoptotic cells, as measured by immunostaining for cleaved caspase 3, were equally infrequent in both groups (Figure 5D). Thus, mice expressing stabilized HIF2 α are born with a normal pancreas.

In comparison, at 2 weeks of age, when the phenotype is clearly evident, changes in proliferation and apoptosis

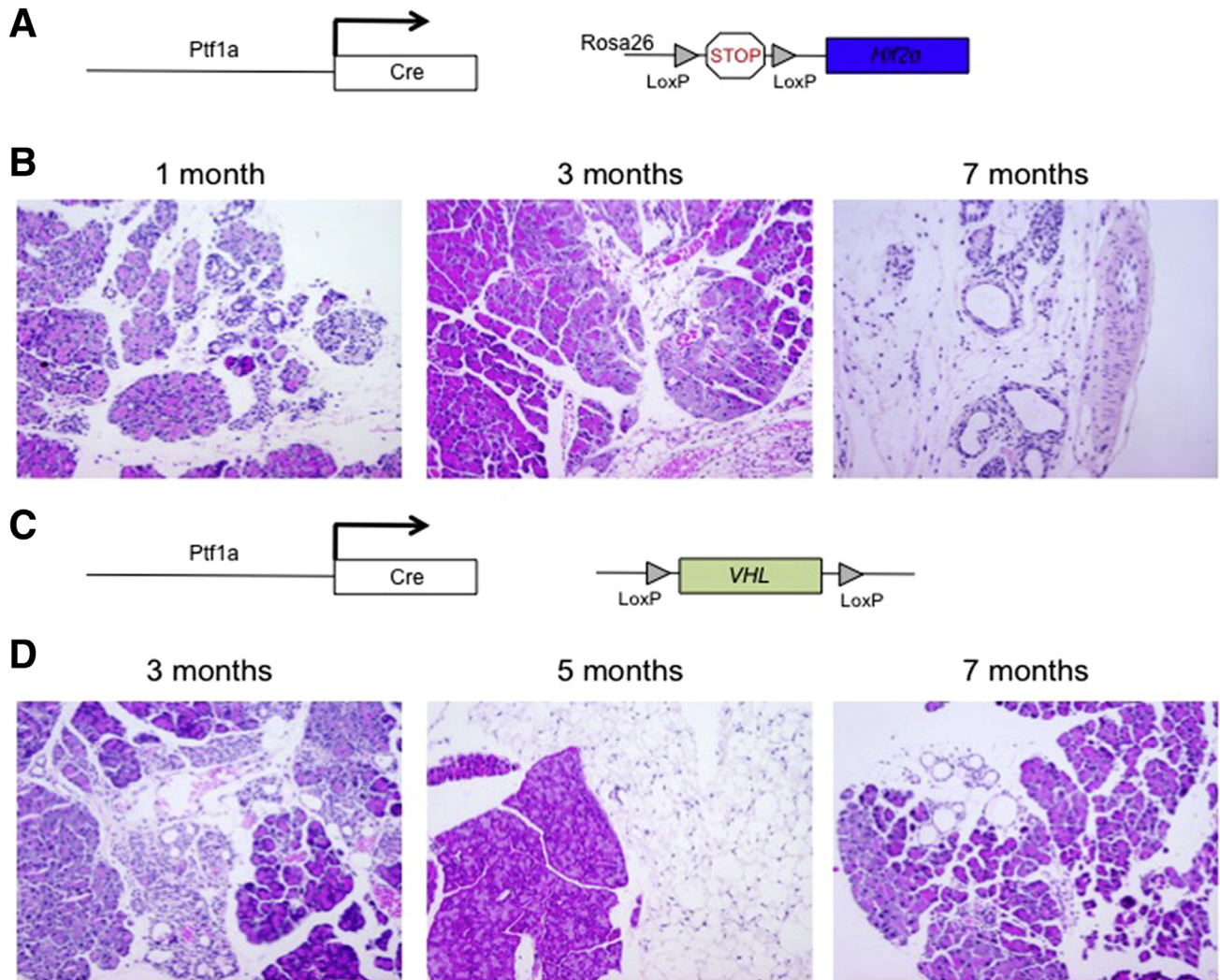


Figure 3. HIF2 α stabilization causes chronic pancreatitis in multiple mouse models. (A) Transgenic mouse scheme for Ptf1a-Cre; HIF2 α -LSL/+ (HIF2 α stabilized) animals. (B) H&E analysis of HIF2 α stabilized animals at 1, 3, and 7 months of age (n = 1 at 1 month, n = 3 at 3 months, and n = 2 at 7 months). (C) Transgenic mouse scheme for Ptf1a-Cre; VHL^{fl/fl} animals (VHL^{PanK0}). (D) H&E analysis of VHL^{PanK0} animals at 3, 5, and 7 months of age (n = 5 at 3 months, n = 2 at 5 months, and n = 2 at 7 months).

became apparent. At this age, the pancreas is undergoing active proliferation. Although the acinar cell population was reduced in the HIF2 α animals, if acinar cells were present they were highly proliferative; furthermore, when considering proliferation across all cell types, there was an increase in the HIF2 α pancreata (Figure 6A). At the same time, apoptosis was increased in HIF2 α pancreata, both in areas of acinar-to-ductal metaplasia and in the remaining acini (Figure 6B). We next analyzed animals shortly after weaning, at 4 weeks of age (n = 2) (histology in Figure 1F). In these animals, proliferation was higher in HIF2 α stabilized animals compared with control littermates (Figure 6C). Similarly, apoptotic cell death was increased in HIF2 α animals compared with controls (Figure 6D). Thus, in both 2- and 4-week-old mice, the process of pancreatitis is actively ongoing.

At 9 weeks of age, the pancreatitis had progressed so that most of the pancreas parenchyma was severely

disrupted. However, clusters of acini persisted within the tissue: immunostaining for Mist1, an acinar lineage marker, showed reduced expression even in those areas (Figure 7A). The tubular structures common across the tissue expressed Sox9, a ductal marker also expressed in acinar-to-ductal metaplasia and a promoter of pancreatic carcinogenesis²³ (Figure 7B). Even in areas of ongoing inflammation and acinar cell loss, chromogranin A staining confirmed that islets persisted in the tissue (Figure 7C) (n = 3 animals per genotype for each immunostain). Other features of chronic pancreatitis, such as extensive fibrosis (Gomori trichrome, n = 5 pancreata per genotype) (Figure 7D) and abundant infiltration of immune cells (immunostaining for CD45, n = 3 pancreata per genotype) (Figure 7E) were similarly common across HIF2 α pancreata. Molecular markers of fibrosis detected in human pancreatitis were similarly increased. In addition, HIF2 α stabilized pancreata expressed higher levels of

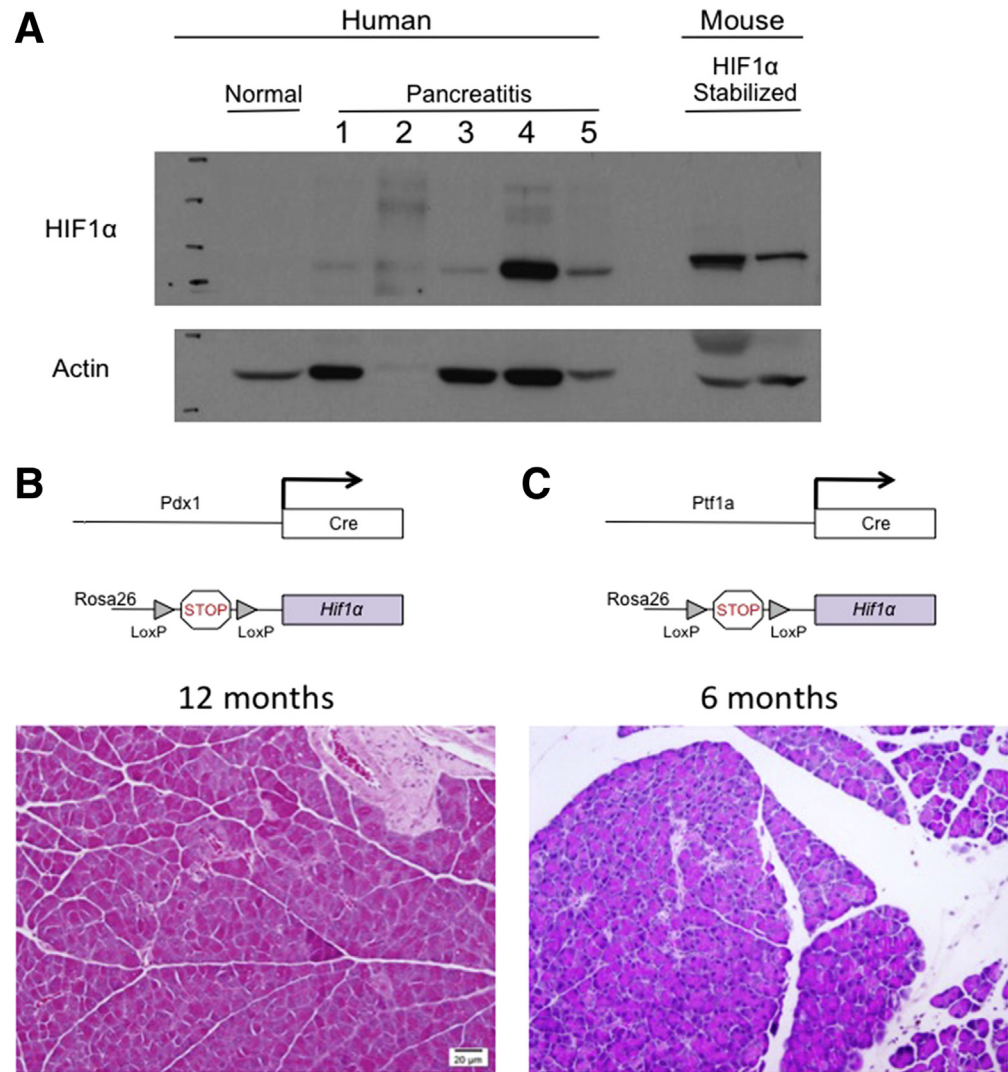


Figure 4. HIF1 α stabilization does not disrupt the pancreatic parenchyma.

(A) Western blot for HIF1 α levels in lysates from human normal and chronic pancreatitis samples, as well as mouse HIF1 α stabilized pancreata as positive control. (B) Transgenic mouse scheme and H&E for Pdx1-Cre; HIF1 α -LSL/+ (HIF1 α stabilized) pancreata (n = 10 mice, histology analyzed from ages 6 wk to 1 y). (C) Transgenic mouse scheme and H&E for Ptf1a-Cre; HIF1 α -LSL/+ pancreata (n = 2 at 6 months of age).

genes that are associated with the fibrosis present in chronic pancreatitis, including *TGF β* and *MMP9*^{24,25} (Figure 8A).

Pancreatitis in HIF2 α Stabilized Mice Is Linked to Activation of the Unfolded Protein Response and Overexpression of Inflammatory Cytokines

We then sought to gather a mechanistic understanding connecting HIF2 α and chronic pancreatitis. In cells exposed to a hypoxic environment, HIF pathway activation occurs in parallel to activation of other stress response pathways, such as the unfolded protein response (UPR) and its resulting endoplasmic reticulum (ER) stress. In hypoxia, HIF signaling and the UPR interact directly, cooperating to activate some of the same downstream targets, to control outcomes such as metabolism and cell survival during tumor growth.^{26–29} In the exocrine pancreas, disruption of the UPR via acinar-specific deletion of one of its main signaling components, *Xbp1*, resulted in ER stress and led to extensive apoptosis and expansion of tubules expressing Sox9, similar to the phenotype observed in HIF2 α stabilized

animals.²⁸ In addition, ER stress is an early pathologic event in chronic pancreatitis and persists throughout the course of the disease, in both human beings and mice.³⁰ We thus performed qPCR analysis of HIF2 α or control pancreata. Consistent with activation of the unfolded protein response and subsequent ER stress, we found increased expression of 2 key components of the ER stress pathway, *Bip* and *Chop*,²⁸ in HIF2 α stabilized pancreata (n = 3) (Figure 8B). Therefore, HIF2 α stabilized animals show higher levels of ductal markers and ER stress compared with control littermates, both consistent with chronic pancreatitis.

Chronic pancreatitis is associated with a specific profile of cytokine expression. To conduct an unbiased analysis of cytokine profiles we used a qPCR array to compare expression between control and HIF2 α stabilized animals at 6 weeks of age. The 6-week age was chosen because it corresponds to an actively developing phenotype (Figure 8C, and Supplementary Table 3, online only). Cytokines that were different among the groups in the array we then validated by qPCR analysis using different sets of 6-week-old animals for each genotype. Interestingly, HIF2 α

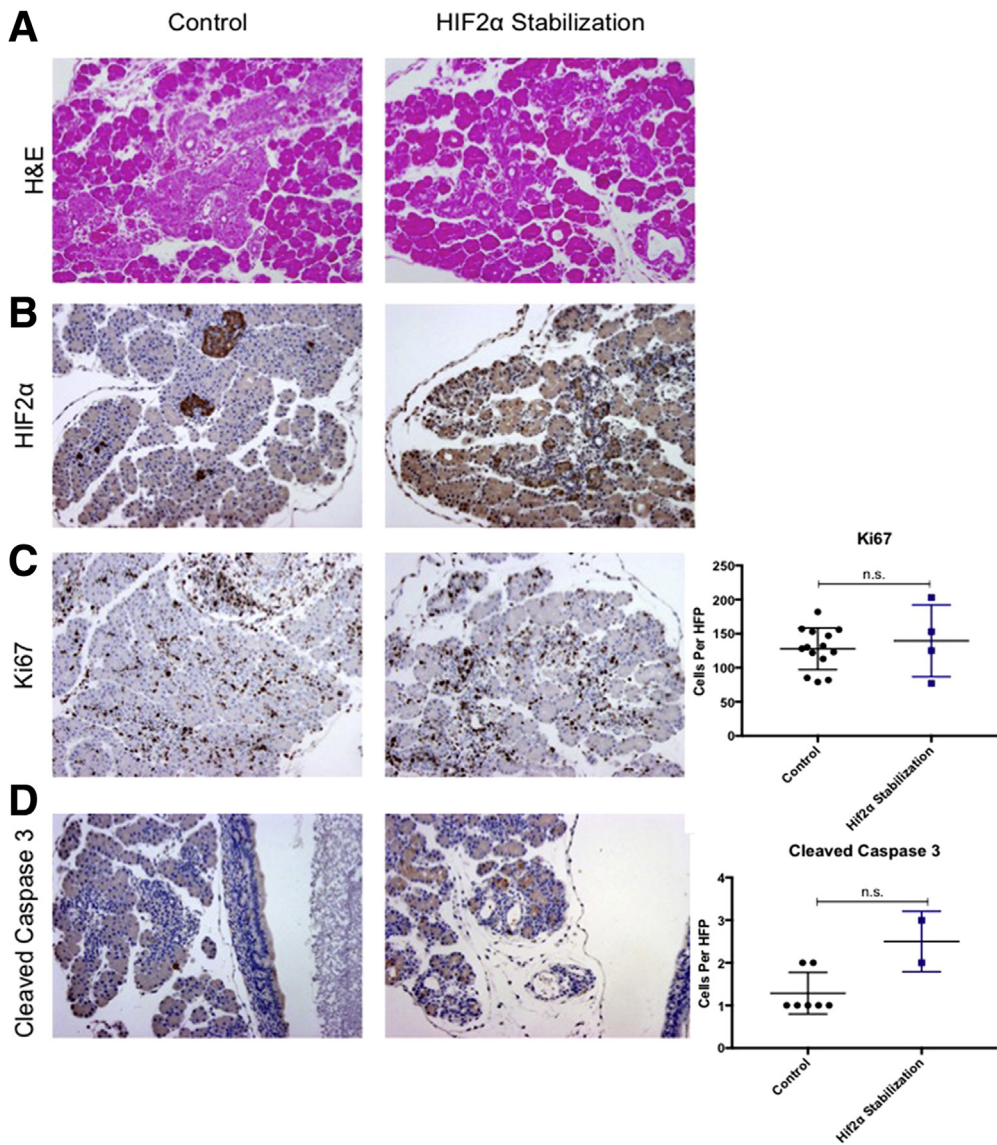


Figure 5. Pancreas histology in 1-day-old HIF2 α stabilized pancreata. (A) H&E analysis in 1-day-old animals (n = 2 HIF2 α stabilized, n = 3 control). (B) Immunohistochemistry for HIF2 α in 1-day-old animals. (C) Immunohistochemistry and quantification of positive cells per high-power field (HFP) for Ki67 and (D) cleaved caspase 3 in 1-day-old animals.

stabilized animals expressed higher levels of cytokines associated with human chronic pancreatitis such as *Icam1*, *Ccr2*, and *Il6r*³¹⁻³³ (Figure 8D). Although many mouse models of chronic pancreatitis recover over time,¹³ HIF2 α stabilized mice recapitulate many aspects of human chronic pancreatitis, including molecular and histologic aspects, making them an exciting new experimental model.

HIF2 α Stabilized Mice Develop Type 3c Diabetes

Chronic pancreatitis patients develop type 3c diabetes over time.^{34,35} To determine whether HIF2 α stabilization recapitulated this aspect of chronic pancreatitis, we analyzed the endocrine islets in 9-week-old mice. The islets in HIF2 α stabilized pancreata were morphologically normal, containing insulin-positive β -cells (n = 3 animals per genotype) (Figure 9A). To measure islet function, we subjected 9-week-old animals to glucose tolerance testing. HIF2 α stabilized animals had impaired glucose tolerance, with a

sharper initial increase and sustained increase of blood glucose levels compared with controls (n = 5 animals per genotype) (Figure 9B). To test for insulin secretion, we measured blood insulin levels in mice after a glucose challenge. Unlike wild-type, HIF2 α stabilized animals had no increase in blood insulin levels after glucose challenge (Figure 9C), indicating β -cell dysfunction (n = 3 animals per genotype). Multiple mechanisms for diabetes development in type 3c diabetes have been postulated, but one likely mechanism is decreased insulin secretion from β -cells, which would mirror the state seen in the HIF2 α stabilized animals.³⁶

Activation of HIF Signaling in the Context of Oncogenic Kras Causes Formation of Mucinous Cystic Neoplasm

Chronic pancreatitis is a risk factor for the development of pancreatic cancer.³⁷ In addition, animals lacking HIF2 α

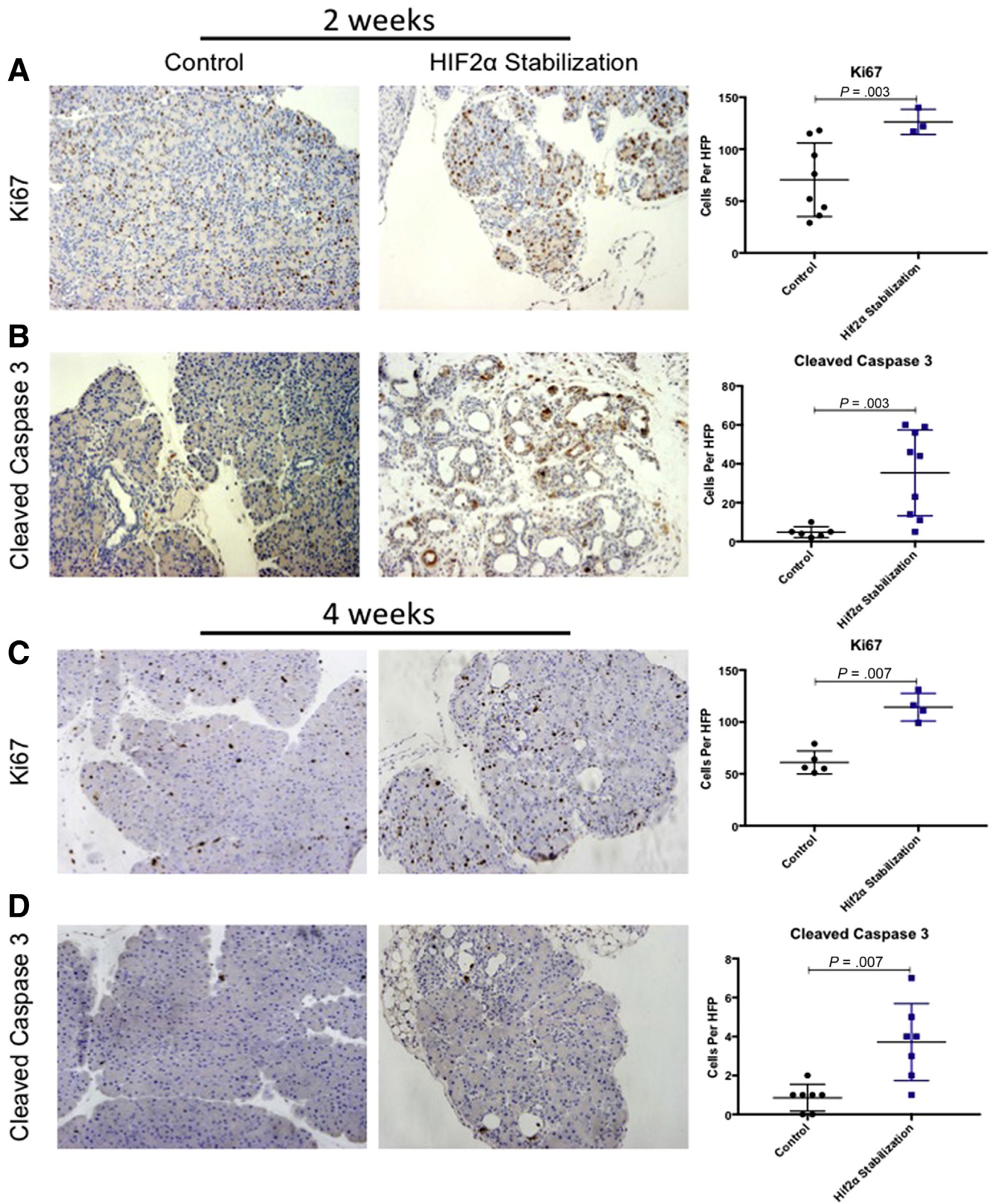


Figure 6. HIF2 α stabilization-induced pancreatitis in young mice. Immunohistochemistry and quantification of positive cells per high-power field for (A) Ki67 and (B) cleaved caspase 3 in 2-week-old animals (n = 2 animals per genotype). Immunohistochemistry and quantification of positive cells per high-power (HFP) field for (C) Ki67 and (D) cleaved caspase 3 in 4-week-old animals (n = 2 animals per genotype).

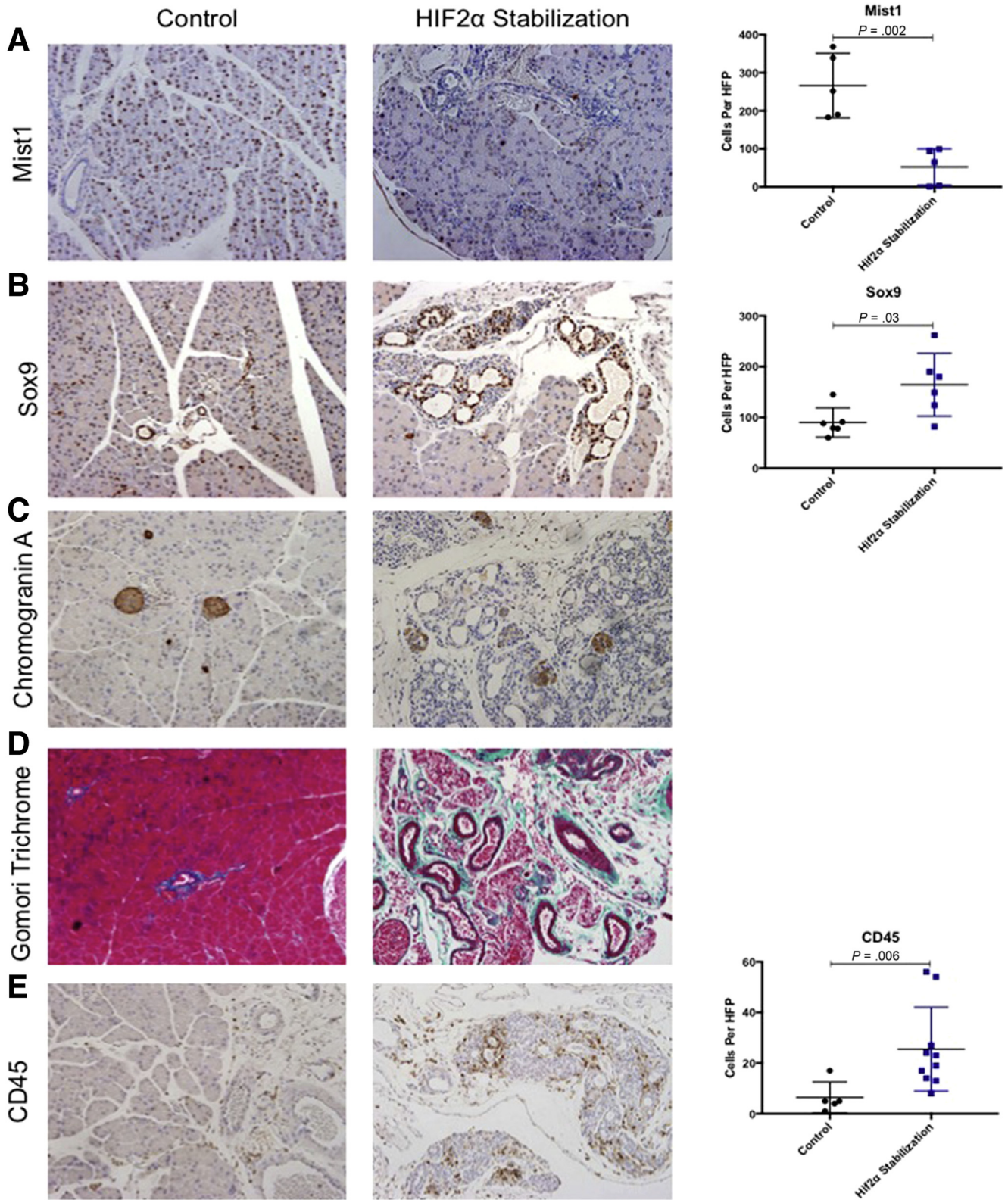


Figure 7. Immunostaining for lineage markers in 9-week-old HIF2 α stabilized pancreata. Immunohistochemistry and quantification of positive cells per high-power field (HFP) for (A) Mist1 and (B) Sox9. (C) Immunohistochemistry for the endocrine marker chromogranin. (D) Gomori trichrome staining and (E) CD45 immunohistochemistry and quantification. All staining was in 9-week-old animals (n = 5).

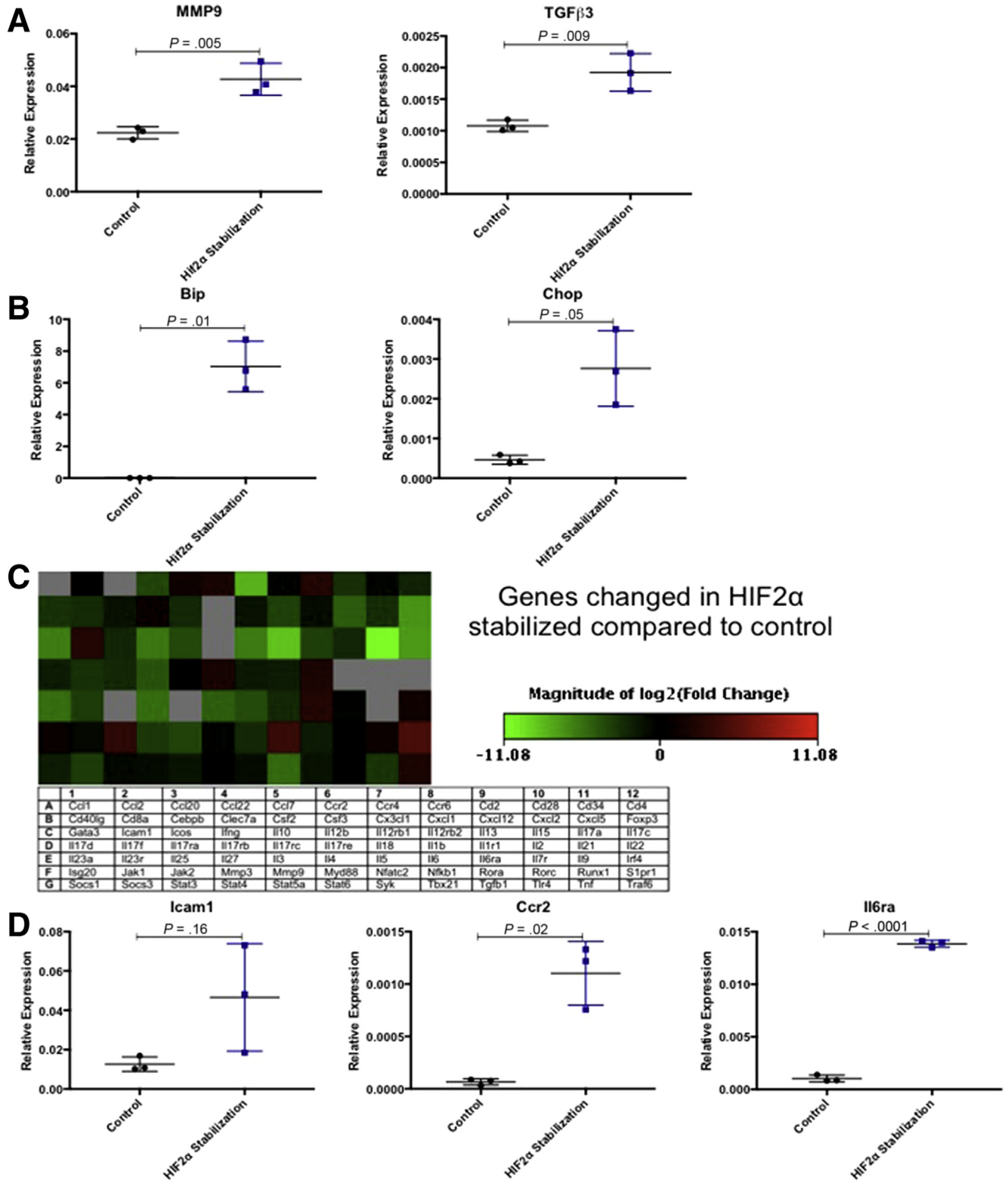


Figure 8. HIF2 α stabilization causes gene expression changes consistent with chronic pancreatitis. qPCR for (A) markers of fibrosis and (B) ER stress in control and HIF2 α stabilized animals. (C) qPCR array results, including table of genes analyzed. All data represented as levels in HIF2 α stabilized vs control pancreas. Green = higher expression in control; red = higher expression in Hif2 α . All changes represented as magnitude of log₂(fold-change). (D) qPCR validation of changed targets from qPCR array in control and HIF2 α stabilized animals.

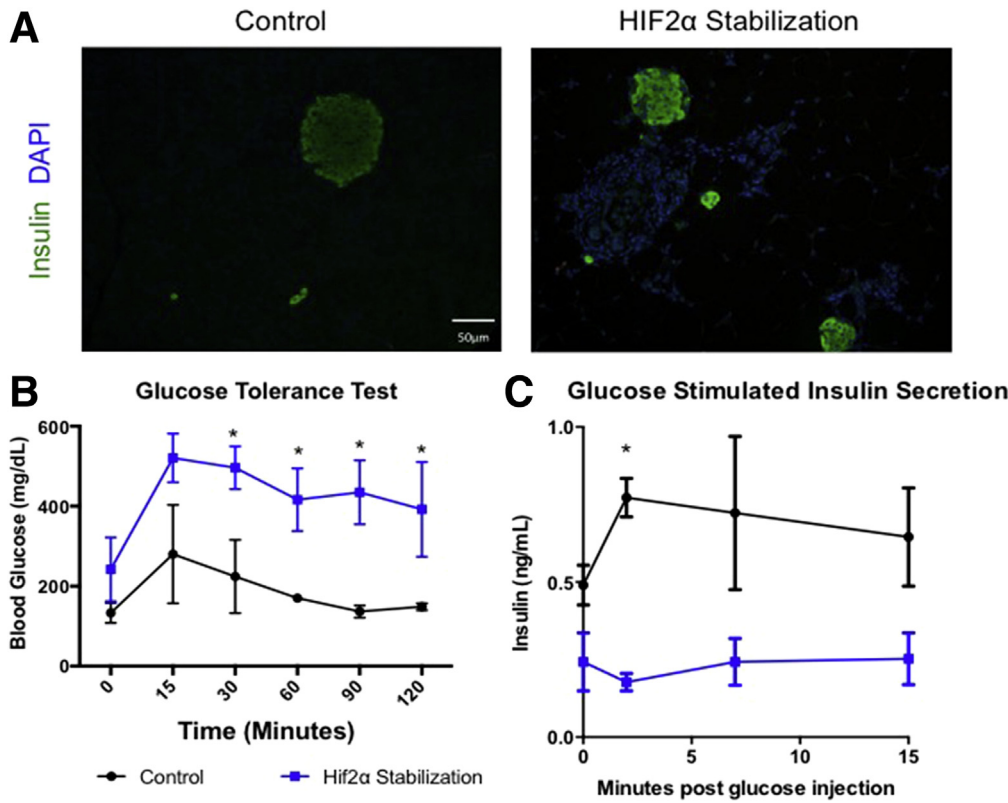


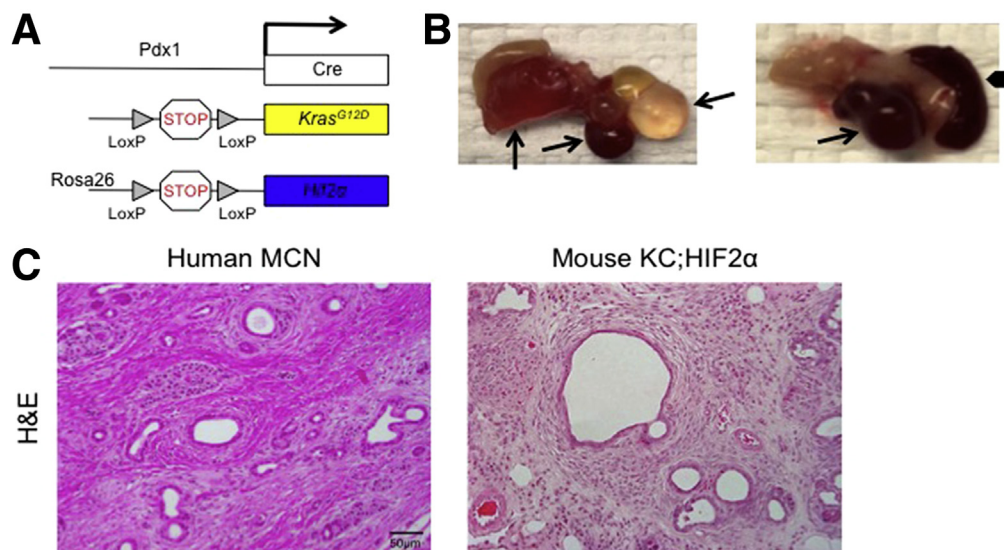
Figure 9. HIF2α stabilization causes endocrine pancreas dysfunction. (A) Immunofluorescence for insulin and 4',6-diamidino-2-phenylindole (DAPI) in 9-week-old animals (n = 3 animals per genotype). (B) Glucose tolerance test (n = 5 animals per genotype) and (C) glucose-stimulated insulin secretion (n = 3 animals per genotype) in 9-week-old animals.

expression in a mouse model of pancreatic cancer develop lesions that fail to progress to cancer, suggesting a role for HIF2α in pancreatic cancer progression.¹²

Because HIF2α stabilization caused pancreatitis, we hypothesized that it might similarly promote carcinogenesis in the presence of oncogenic Kras. Mice that express oncogenic Kras in the pancreas, such as Pdx1-Cre;Kras^{+/LSL-G12D}

or Ptf1a-Cre;Kras^{+/LSL-G12D} (KC) develop PanIN, a precursor lesion to pancreatic cancer.¹⁸ We crossed both Pdx1-Cre;Kras^{+/LSL-G12D} and Ptf1a-Cre;Kras^{+/LSL-G12D} mice with the Rosa26^{LSL-Hif2a/+} mice to generate mice that express both oncogenic Kras and stabilized HIF2α in the pancreas, here named KC;HIF2α (Figures 10A and 11C). At 9–12 weeks, KC mice had, as expected, sporadic PanINs

Figure 10. HIF2α stabilization during pancreatic cancer initiation leads to MCNs. (A) Transgenic mouse scheme. (B) Gross morphology of 2 separate KC;HIF2α animals at 9 weeks of age. Arrows indicate cysts, arrowhead indicates spleen. (C) H&E evaluation of human MCN and KC; HIF2α pancreata (n = 7 KC;HIF2α pancreata and 3 human MCN samples).



interspersed within a largely normal pancreas. In contrast, in age-matched KC;HIF2 α mice we observed large cystic lesions. These lesions developed with full penetrance (Figure 10B, arrows) ($n = 7$ mice), a pathologic evaluation recognized them as corresponding to human MCN (Figure 10C). We then aged mice of each genotype to obtain a time course of MCN development (Figure 11A). As expected, KC animals developed PanINs, with their prevalence increasing over time from sporadic at 1 month of age to prevalent in the pancreas by 9 months ($n = 14$) (Figure 11C). Conversely, age-matched KC;HIF2 α animals developed cystic lesions resembling human MCN. These cysts were small at 1 month of age and increased in size over time ($n = 10$) (Figure 11D).

We then used a complementary approach to stabilize HIF2 α , by deleting *VHL* in KC mice (Figure 11B). Similar to KC;HIF2 α , KC;VHL^{fl/fl} mice developed MCN-like lesions that progressed over time, from small lesions at 1 month of age

to large cysts in older animals ($n = 9$) (Figure 11E). Thus, activation of the HIF pathway, independently from the mode of activation, cooperates with oncogenic *Kras* to drive formation of cystic lesions.

We then compared the cystic lesions in our mouse model with human MCN. Similar to human histology, in KC;HIF2 α mice the cystic lesions were lined by flat cuboidal epithelial cells with no papillary architecture and surrounded by a fibrotic reaction. The lesions presented with apical expression of mucin (periodic acid-Schiff staining) and expression of CK19 in the epithelial cells, similar to human MCN³⁸ (Figure 12A and B). Furthermore, we observed positive staining for ER surrounding the lesions, a characteristic of ovarian-type stroma (Figure 12C), a diagnostic feature of human MCN. Other histologic features characteristic of human MCN in KC;HIF2 α mice include expression of mesenchymal markers such as vimentin in the stroma but not the epithelium (Figure 12D). Analysis

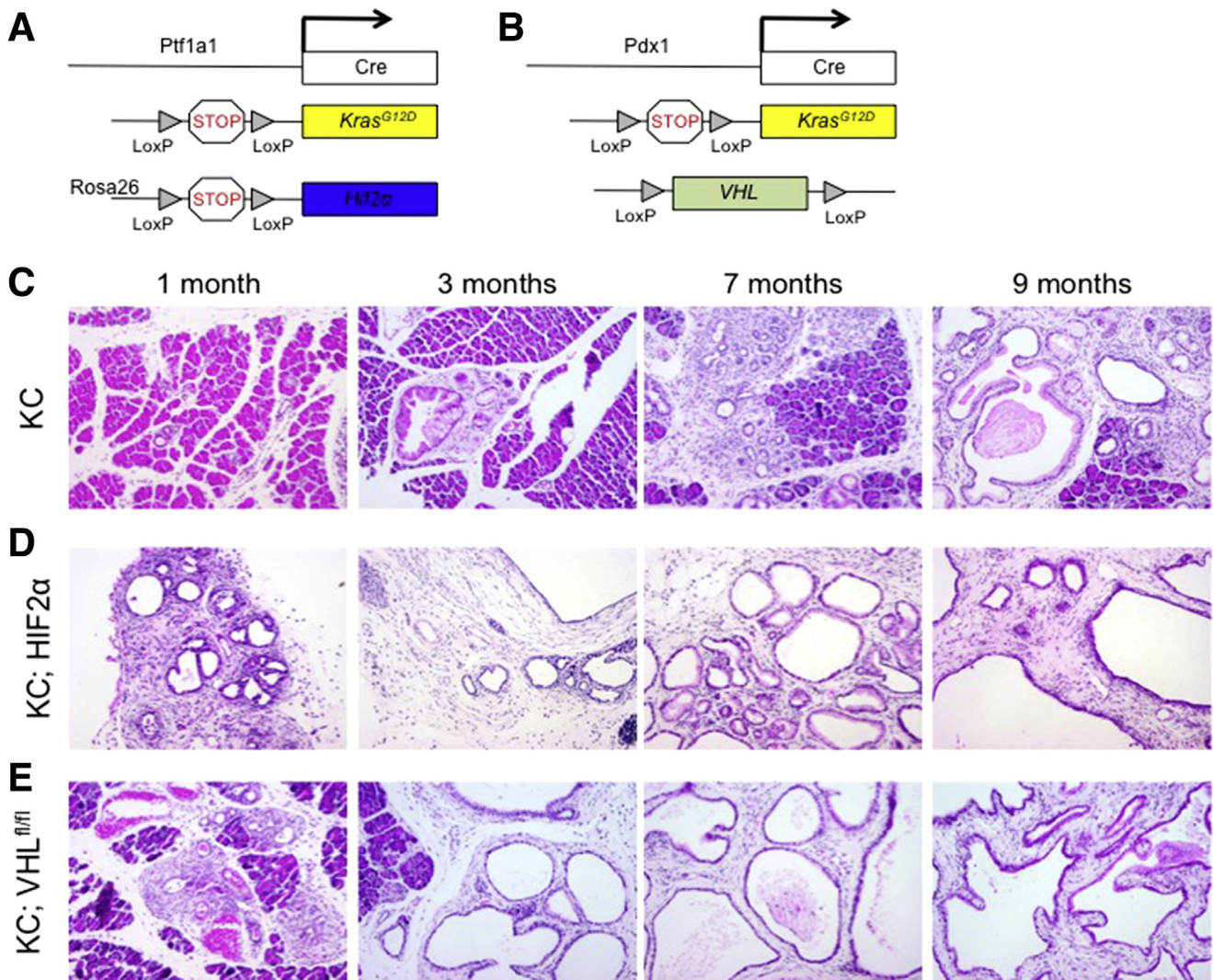


Figure 11. HIF2 α stabilization results in mucinous cystic neoplasm formation in multiple mouse models. Transgenic mouse scheme for (A) Ptf1a-Cre; LSL-Kras^{G12D}; HIF2 α -LSL/+ (KC;HIF2 α) and (B) Ptf1a-Cre; LSLKras^{G12D}; VHL^{fl/fl} (KC;VHL^{fl/fl}) animals. H&E histology analysis at 1, 3, 7, and 9 months in (C) KC ($n = 14$), (D) KC;HIF2 α ($n = 10$), and (E) KC;VHL^{fl/fl} animals ($n = 9$).

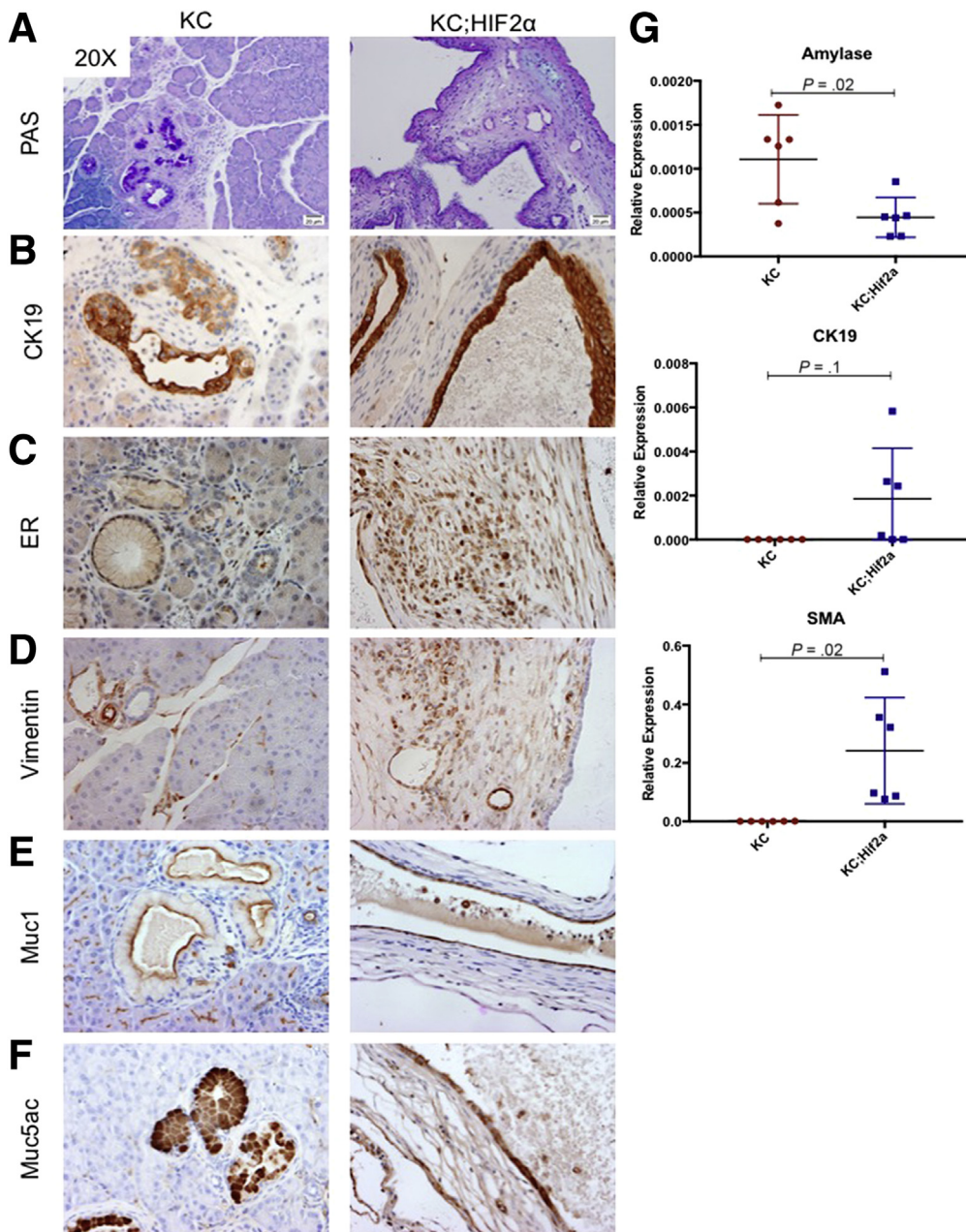


Figure 12. KC;HIF2 α animals have MCN-like features. (A) Periodic acid-Schiff (PAS) staining, and immunohistochemistry for (B) CK19, (C) ER, (D) vimentin, (E) Muc1, and (F) Muc5ac in 9- to 10-week-old KC and KC;HIF2 α animals (n = 2–4 pancreata per genotype for each immunostaining condition). (G) qPCR analysis of gene expression levels in KC and KC;HIF2 α pancreata at 9–10 weeks of age (n = 6).

of mucin expression in the animals showed expression of Muc1 in both KC and KC;HIF2 α animals, as expected in both PanIN and MCN type lesions³⁹ (Figure 12E). In addition, both the PanINs in KC animals and the cystic lesions in KC;HIF2 α mice were positive for Muc5ac expression (Figure 12F). This pattern of Muc1 and Muc5ac expression has been described previously in human MCNs.⁴⁰ In addition, we used qPCR to analyze gene expression analysis of pancreatic cell types in KC and KC;HIF2 α animals. We observed lower levels of *amylase*, and higher levels of *CK19* and *SMA* in KC;HIF2 α mice compared with controls (n = 6) (Figure 12G). Thus, stabilization of HIF2 α in the presence of oncogenic *Kras* directed formation of MCN-like lesions rather than PanINs.

We then investigated whether molecular underpinnings of MCN formation were found in our model. Human MCN is associated with de-regulated Wnt signaling⁴¹ and mouse experiments have shown Wnt signaling to be a driver of this disease.⁴¹ HIF2 α modulates Wnt expression during the development of PanINs in the KC mouse model. Accordingly, our analysis showed increased levels of Wnt target genes (*Lef1*, *MYC*, and *Axin*) in KC;HIF2 α animals compared with KC at 9 weeks of age (n = 3) (Figure 13A). We then performed immunohistochemistry for the Wnt target *Lef1* in both human MCN (n = 2 human MCN samples) and in MCN of KC;HIF2 α animals compared with KC (n = 2 per genotype) (Figure 13B). In both human and mouse MCN we observed stromal *Lef1* expression (Figure 13B and C). Thus, our model mimics

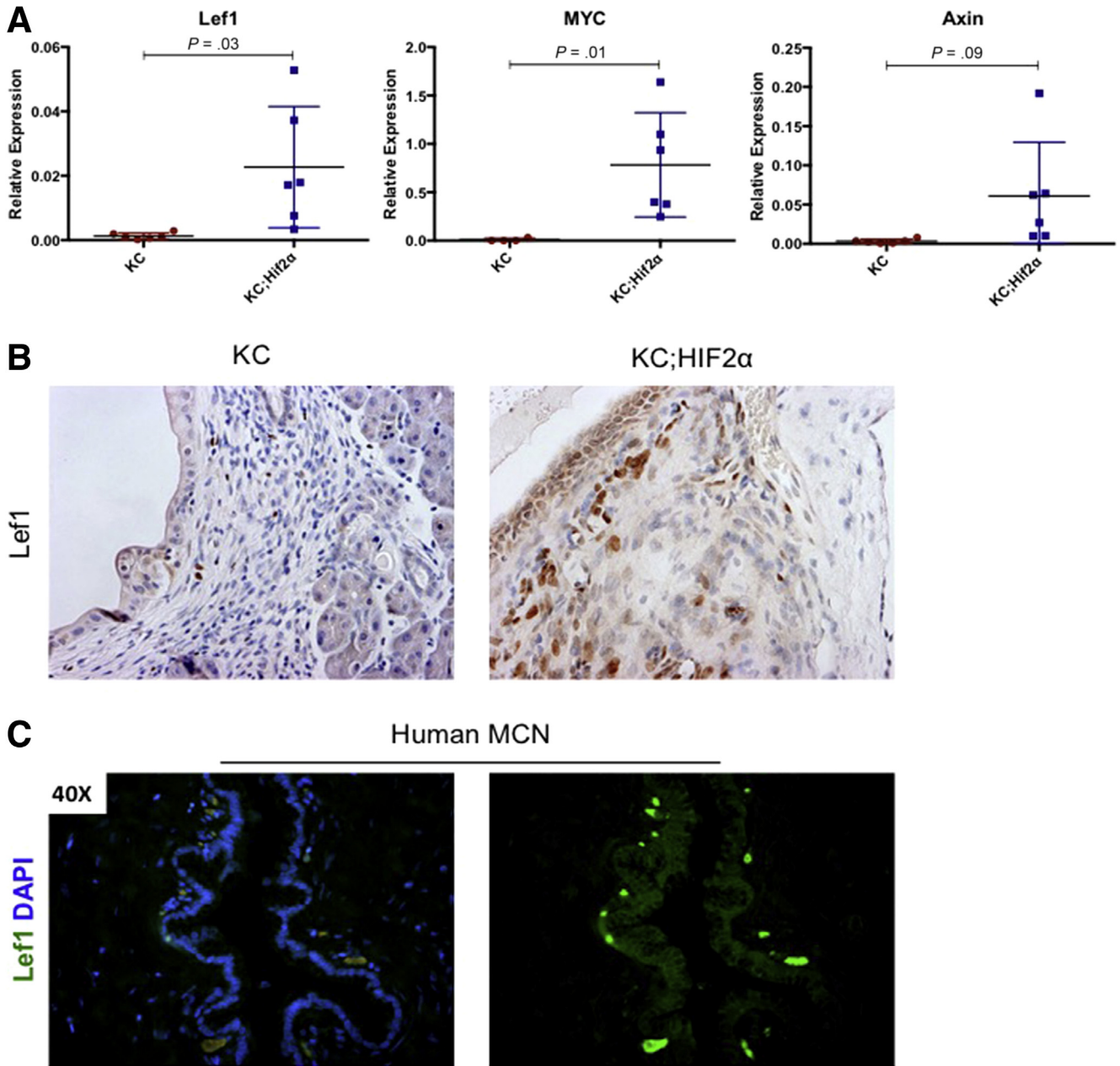


Figure 13. Wnt pathway is activated in KC;HIF2 α animals. (A) qPCR analysis for gene expression levels of Wnt pathway targets in KC and KC;HIF2 α animals at 9–10 weeks of age (n = 3). (B) Immunohistochemistry for Lef1 in KC and KC;HIF2 α animals (n = 2 pancreata per condition). (C) Immunofluorescence for Lef1 in human MCN tissue (n = 2 human MCN samples). DAPI, 4',6-diamidino-2-phenylindole.

both the histology and molecular features of human MCN and will be useful to study this disease in the future. Of note, human MCN is prevalent in females,⁴⁰ although we observed no difference in incidence among female and male mice, a finding likely reflecting the different hormonal regulation.

Discussion

Here, we show that HIF2 α protein accumulates in human chronic pancreatitis. The expression of an oxygen-stable

form of HIF2 α in the mouse pancreas results in chronic pancreatitis and atrophy, with loss of acini and increased chronic inflammation in the lobule. In addition, stabilization of HIF2 α along with oncogenic Kras expression recapitulates human MCN. Notably, these effects were restricted to HIF2 α expression and not to expression of the closely related family member HIF1 α . Thus, we describe 2 new models of human disease, and provide new insight into the role of hypoxia signaling, specifically through HIF2 α , in the pancreas.

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Author contributions

Heather K. Schofield, Yatrik M. Shah, M. Bishr Omary, Farzad Esni, and Marina Pasca di Magliano conceived the study concept and design; Heather K. Schofield, Manuj Tandon, Min-Jung Park, Sadeesh K. Ramakrishnan, Esther C. Kim, and Christopher J. Halbrook acquired and analyzed data; Heather K. Schofield wrote the initial draft of the manuscript; and Jiaqi Shi, Yatrik M. Shah, M. Bishr Omary, Farzad Esni, and Marina Pasca di Magliano edited and finalized the manuscript.

Conflicts of interest

The authors disclose no conflicts.

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Supplementary Table 1. Antibodies Used

Antibody	Supplier	Catalog number	IHC dilution	IF dilution	Western blot dilution
Hif2 α	Novus (Littleton, CO)	Nb100-122	1:100		1:1000
Estrogen receptor	Millipore (Burlington, MA)	06935	1:300		
Mist1	Stephen Konieczny, Purdue University (West Lafayette, IN)		1:500		
Sox9	Millipore	AB5535	1:500		
Chromogranin A	Immunostar (Hudson, WI)	20085	1:300		
CD45	BD Biosciences (San Diego, CA)	553076	1:200		
Ki67	Vector Laboratories (Burlingame, CA)	VP-RM04	1:100		
Cleaved caspase 3	Cell Signaling (Danvers, MA)	9961	1:300		
Insulin	Abcam (Cambridge, England)	AB7842		1:300	
Vimentin	Cell Signaling	5741S	1:100		
CK19	Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA)	TROMA-III	1:100		
HIF1 α	Santa Cruz (Dallas, TX)		1:100		
Muc1			1:100		
Muc5ac	Thermo Scientific (Waltham, MA)	MS-145-P1	1:100		
Lef1	Cell Signaling		1:100	1:300	

IF, immunofluorescence; IHC, immunohistochemistry.

Supplementary Table 2. Primers Used

Gene	Forward primer sequence 5' to 3'	Reverse primer sequence 5' to 3'
<i>Amylase</i>	AGGAACATGGTTGCCTTCAG	CTGACAAAGCCCAGTCATCA
<i>CK19</i>	CGCGGTGGAAGTTTTAGTGGG	AGGCGAGCATTGTCAATCTGTA
<i>Smooth muscle actin</i>	GCTGGTGATGATGCTCCCA	GCCCATCCAACCATTACTCC
<i>Pdk1</i>	TACTCAGTGGAACACCGCC	GTTTATCCCCGATTCAGGT
<i>Bip</i>	GTGTCCTCTCTGGTGATCAGG	TGTCITTTTGTAGGGGTCGTT
<i>Chop</i>	CCTGAGGAGAGAGTGTCCAG	CAGATCCTCATACCAGGCTTC
<i>MMP9</i>	CTGGACAGCCAGACACTAAAG	CTCGCGGCAAGTCTTCAGAG
<i>TGFβ3</i>	CAGGCCAGGGTAGTCAGAG	ATTTCCAGCCTAGATCCTGCC
<i>Icam1</i>	TCCGCTGTGCTTTGAGAACT	GGCTCAGTATCTCCTCCCA
<i>Ccr2</i>	ATCCACGGCATACTATCAACATC	CAAGGCTCACCATCATCGTAG
<i>Il6ra</i>	CCTGAGACTCAAGCAGAAATGG	AGAAGGAAGTCCGGCTTCAGT
<i>Lef1</i>	AGTGCAGCTATCAACCAGATCCT	TTTCCGTGCTAGTTCATAGTATTTGG
<i>MYC</i>	TGAGCCCCTAGTGCTGCAT	AGCCCGACTCCGACCTCTT
<i>Axin</i>	GCCAATGGCCAAGTGTCTCT	GCGTCATCTCCTGGGCA