Pancreatic HIF2α 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 2α is induced in chronic pancreatitis. Stabilization of hypoxia-inducible factor 2α 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 HIF2α. Previous studies of HIF action in the pancreas have relied on loss-of-function mouse models, and the effects of HIF2α expression in the pancreas have remained undefined.

METHODS: We developed several transgenic mouse models based on the expression of an oxygen-stable form of HIF2α, 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 KrasG12D in the pancreas.

RESULTS: We show that HIF2α 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 HIF2α in mouse pancreata led to the development of chronic pancreatitis. Importantly, pancreatic HIF1α stabilization did not disrupt the pancreatic parenchyma, indicating that the chronic pancreatitis phenotype is specific to HIF2α. In the presence of oncogenic Kras, HIF2α 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 HIF2α 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.2017.10.006)

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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: HIF1α, HIF2α, and HIF3α. 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 proteosomal 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 HIF1α and HIF2α expression in the pancreas.9 Furthermore, a number of studies have associated perturbation of the HIF factors with pancreatic abnormalities/disease. HIF1α is expressed during the development of pancreatic cancer, and its deletion promotes pancreatic tumorigenesis in a Kras-driven model of pancreatic cancer.10 HIF2α expression is required for the embryonic development of the pancreas, and a lack of HIF2α expression in developing mice leads to smaller pancreata and decreased branching.11 In the presence of oncogenic Kras, HIF2α inactivation inhibits the progression of precancerous lesions.12

Here, we show that pancreas-specific inactivation of VHL, or stabilization of HIF2α (but not HIF1α), induces chronic pancreatitis in mice. Although many mouse models of pancreatitis recover over time (for review, see Lerch and Gorelick13), mice overexpressing HIF2α have bona fide chronic pancreatitis that persists until most of the pancreatic parenchyma is substituted by fibrotic or fatty tissue. The link between HIF2α 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 HIF2α 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-KrasG12D, R26LSLHif2a/+, 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 intra-peritoneal 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 performed as previously described.20 For qPCR, reverse transcription and qPCR were performed using the Quantitect SYBR Green RT-PCR kit (Qiagen) and the CFX96 System (Bio-Rad, Hercules, CA) with the following conditions: 1 cycle at 50°C for 20 minutes, 1 cycle at 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The relative abundance of each target was calculated using the 2−ΔΔCT method.21 Primers are listed in Supplementary Table 2.
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. Cyclin 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 radiography 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

**HIF2α Accumulates in Human Chronic Pancreatitis and its Forced Stabilization Causes Chronic Pancreatitis in Mice**

HIF2α protein expression is not detectable in the normal human pancreas. However, we observed abundant HIF2α expression in human samples of chronic pancreatitis (n = 5 chronic pancreatitis samples) (Figure 1A). Overexpression of HIF2α 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 HIF2α expression in chronic pancreatitis, or suggest that high HIF2α expression levels represent a subset of human chronic pancreatitis. Because a functional role for HIF2α had not been described in this disease, we generated Pdx1-Cre;Rosa26LSL-HIF2α+/− mice. In these animals, an oxygen-stable form of HIF2α is expressed in the pancreas upon Cre recombination (Figure 1B). We observed that pancreata with stabilized HIF2α 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 HIF2α stabilized mice at all ages analyzed. From 7 weeks to 1 year of age HIF2α stabilized animals showed similar total body weight to age-matched littermate control animals (n = 6 pairs of age-matched littermates, 1 control, and 1 HIF2α stabilized) (Figure 1D).

At 2 weeks of age (n = 2), animals expressing HIF2α had atrophic pancreatic parenchyma resembling end-stage chronic pancreatitis (Figure 1E). By 4 weeks of age, shortly after weaning, HIF2α 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. To analyze the molecular changes underlying the onset of pancreatitis, were extracted RNA from 2-week-old mouse pancreata and performed qPCR analysis (n = 3 mice per genotype). By 2 weeks of age, HIF2α 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 HIF2α stabilized mice, indicating an increase in activated fibroblasts (Figure 1).

To validate HIF2α stabilization in experimental animals, we performed a Western Blot. As expected, HIF2α accumulated in Pdx1-Cre;Rosa26LSL-HIF2α+/− pancreata but not in littermate controls (n = 2 pancreata per genotype) (Figure 2A). Although immunostaining for HIF2α is technically challenging, our results were in line with an increase in HIF2α protein in the experimental mice (Figure 2B). Gene expression analysis by qPCR showed up-regulation of HIF targets, including Pdk1 (Figure 2A), indicating functional up-regulation of the HIF signaling pathway in HIF2α stabilized animals (Figure 2C).

To expand our analysis, we generated Ptf1a+/Cre; Rosa26LSL-HIF1α+/− mice in which a different Cre recombinase was used to activate the same stabilized HIF2α in the pancreas (Figure 3A). Similar to our previous results, Ptf1a+/Cre; Rosa26LSL-HIF2α+/− 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).
Disruption of the Negative Hif Regulator VHL Mimics HIF2α Stabilization in Mice

To determine whether the phenotype observed upon HIF2α 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. 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 VHL (Figure 3C) to generate Ptf1a+/Cre;VHLfl/fl (termed VHL PanKO) animals. Histologic analysis of VHL PanKO mice showed that HIF stabilization by this method phenocopied the chronic pancreatitis observed in HIF2α 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 HIF2α leads to the development of a spontaneous fibroinflammatory response resembling human chronic pancreatitis.

HIF1α Stabilization Has no Effect on Pancreas Morphology and Function

To determine whether HIF1α, a factor related to HIF2α, was similarly implicated in human pancreatitis, we probed HIF1α protein expression in the same human chronic pancreatitis samples in which we observed HIF2α up-regulation. In human chronic pancreatitis HIF1α was up-regulated dramatically in only 1 of 5 samples by Western blot (Figure 4A). We then generated Pdx1-Cre;Rosa26LSL-HIF1α+/+ and Ptf1a+/Cre;Rosa26LSL-HIF1α+/+ mice, in which HIF1α stabilization is stabilized in a tissue-specific manner. Consistent with the human findings...
indicating no strong correlation between HIF1α and pancreatitis, animals with pancreatic HIF1α stabilization, independently from the Cre driver used, had normal pancreata (Pdx1-Cre;Rosa26LSL-HIF1α/++; n = 10 mice, histology analyzed at ages 6 wk to 1 y; Ptf1α+/Cre;Rosa26LSL-HIF1α/++; n = 2) (Figure 4B and C). This indicates that the chronic pancreatitis phenotype is specific to HIF2α expression, and not HIF pathway activation in general.

Pancreatitis Occurs Postnatally in HIF2α Stabilized Mice

We next sought to determine whether the pancreatitis phenotype caused by HIF2α 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 HIF2α stabilized animals and their control littermates (n = 2) (Figure 5A) via H&E staining. HIF2α stabilization was confirmed by immunohistochemistry, in which both control and HIF2α stabilized animals showed positive HIF2α expression in the developing islets, as expected, and only HIF2α stabilized pancreata showed positive HIF2α staining throughout the pancreas (Figure 5B). Control and HIF2α 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 HIF2α are born with a normal pancreas.

In comparison, at 2 weeks of age, when the phenotype is clearly evident, changes in proliferation and apoptosis
became apparent. At this age, the pancreas is undergoing active proliferation. Although the acinar cell population was reduced in the HIF2α animals, if acinar cells were present they were highly proliferative; furthermore, when considering proliferation across all cell types, there was an increase in the HIF2α pancreata (Figure 6A). At the same time, apoptosis was increased in HIF2α 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 HIF2α stabilized animals compared with control littermates (Figure 6C). Similarly, apoptotic cell death was increased in HIF2α 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 HIF2α pancreata. Molecular markers of fibrosis detected in human pancreatitis were similarly increased. In addition, HIF2α stabilized pancreata expressed higher levels of

![Figure 3](image-url)
genes that are associated with the fibrosis present in chronic pancreatitis, including TGFβ and MMP9. 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. We thus performed qPCR analysis of HIF2α 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, in HIF2α stabilized pancreata (n = 3) (Figure 8B). Therefore, HIF2α 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 HIF2α 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, HIF2α
stabilized animals expressed higher levels of cytokines associated with human chronic pancreatitis such as Icam1, Ccr2, and Il6.

Although many mouse models of chronic pancreatitis recover over time, HIF2α stabilized mice recapitulate many aspects of human chronic pancreatitis, including molecular and histologic aspects, making them an exciting new experimental model.

**HIF2α Stabilized Mice Develop Type 3c Diabetes**

Chronic pancreatitis patients develop type 3c diabetes over time. To determine whether HIF2α stabilization recapitulated this aspect of chronic pancreatitis, we analyzed the endocrine islets in 9-week-old mice. The islets in HIF2α 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. HIF2α 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, HIF2α 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 HIF2α stabilized animals.

**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. In addition, animals lacking HIF2α
Figure 6. HIF2α 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 HIF2α 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. HIF2α stabilization causes gene expression changes consistent with chronic pancreatitis. qPCR for (A) markers of fibrosis and (B) ER stress in control and HIF2α stabilized animals. (C) qPCR array results, including table of genes analyzed. All data represented as levels in HIF2α stabilized vs control pancreas. Green = higher expression in control; red = higher expression in HIF2α. All changes represented as magnitude of log2(fold-change). (D) qPCR validation of changed targets from qPCR array in control and HIF2α stabilized 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<sup>+</sup>/LSL-G12D or Ptf1a-Cre;Kras<sup>+</sup>/LSL-G12D (KC) develop PanIN, a precursor lesion to pancreatic cancer.18 We crossed both Pdx1-Cre;Kras<sup>+</sup>/LSL-G12D and Ptf1a-Cre;Kras<sup>+</sup>/LSL-G12D mice with the Rosa26LSL-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...
interspersed within a largely normal pancreas. In contrast, in age-matched KC;HIF2α 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;HIF2α 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 HIF2α, by deleting VHL in KC mice (Figure 11B). Similar to KC;HIF2α, KC;VHLfl/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;HIF2α 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 MCN10 (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;HIF2α mice include expression of mesenchymal markers such as vimentin in the stroma but not the epithelium (Figure 12D). Analysis

Figure 11. HIF2α stabilization results in mucinous cystic neoplasm formation in multiple mouse models. Transgenic mouse scheme for (A) Ptf1a-Cre; LSL-KrasG12D; HIF2α-LSL/+ (KC;HIF2α) and (B) Ptf1a-Cre; LSL-Kras-G12D; VHLfl/fl (KC;VHLfl/fl) animals. H&E histology analysis at 1, 3, 7, and 9 months in (C) KC (n = 14), (D) KC;HIF2α (n = 10), and (E) KC;VHLfl/fl animals (n = 9).
of mucin expression in the animals showed expression of Muc1 in both KC and KC;HIF2α animals, as expected in both PanIN and MCN type lesions (Figure 12E). In addition, both the PanINs in KC animals and the cystic lesions in KC;HIF2α 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;HIF2α animals. We observed lower levels of amylase, and higher levels of CK19 and SMA in KC;HIF2α mice compared with controls (n = 6) (Figure 12G). Thus, stabilization of HIF2α 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 signaling\(^ {41}\) and mouse experiments have shown Wnt signaling to be a driver of this disease.\(^ {42}\) HIF2α 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;HIF2α 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;HIF2α 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...
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, 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 HIF2α protein accumulates in human chronic pancreatitis. The expression of an oxygen-stable form of HIF2α 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 HIF2α along with oncogenic Kras expression recapitulates human MCN. Notably, these effects were restricted to HIF2α expression and not to expression of the closely related family member HIF1α. Thus, we describe 2 new models of human disease, and provide new insight into the role of hypoxia signaling, specifically through HIF2α, in the pancreas.

**Figure 13. Wnt pathway is activated in KC;HIF2α animals.** (A) qPCR analysis for gene expression levels of Wnt pathway targets in KC and KC;HIF2α animals at 9–10 weeks of age (n = 3). (B) Immunohistochemistry for Lef1 in KC and KC;HIF2α 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.
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### Supplementary Table 1. Antibodies Used

| Antibody     | Supplier                      | Catalog number | IHC dilution | IF dilution | Western blot dilution |
|--------------|-------------------------------|----------------|--------------|-------------|-----------------------|
| Hif2α        | 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        |             |                       |
| HIF1α        | 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.
| Gene                  | Forward primer sequence 5’ to 3’ | Reverse primer sequence 5’ to 3’ |
|-----------------------|-----------------------------------|----------------------------------|
| Amylase               | AGGAACATGGTTGCTTCAG               | CTGACAAAGCCCAAGTCTCA             |
| CK19                  | CGCGGTGGAGTTTAGTGCTGGG            | AGGCGAGCAATTGCAAATCTCTGA         |
| Smooth muscle actin   | GCTGTGTGATGCTGGCTCCCA             | GCCCATCACCATATTACTCC             |
| Pdk1                  | TTACTCAGTGGACACGCGCC              | GTTTATCCCCAGTTCGGT              |
| Bip                   | GTGTCTCCTCTCTGATCATCAG            | TGTCTTTTGTATAGGGGTCTGTT          |
| Chop                  | CCTGAGGAGAGAGCTTTCAG              | CAGATCTCACTACAGGCTCC             |
| MMP9                  | CTGGACAGCCAGACCTAAAG              | CTCGGGCAAGTGTTCCAGAG             |
| TGFβ3                 | CAGGCCAGGAGCTGTCAGA               | ATTTCCAGCCTAGATCCTGCC            |
| Icam1                 | TCCGTGTGCTTTTGAGAAGTA            | GGCTCAGATCTCCTCCTCCTC            |
| Ccr2                  | ATCCACGGGACACTACTCAACC           | CAAGGCTCACTACATCCTGTA            |
| Il6ra                 | CCGAGACTCAACAGAAGAATGG           | AGAGGAGAAGTCGCTTCCAGT            |
| Lef1                  | AGTGCACGTATCAACAGATCCT            | TTTCGACCTGATGTCATAGTTG          |
| MYC                   | TGAGCCCCAGTGCTGCA               | AGCCCGACTCCGACGCTT              |
| Axin                  | GCCAATGCGCAACTGCTCTT             | GCATCTCCTTTGAGCA               |