The Role of HNF6 on Pancreatic Cancer

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BRIEF. The effects of the acinar-specific inactivation of the *Hnf6* transcription factor in pancreatic cancer as well as the correlation between PanIN progression to pancreatic cancer and HNF6 expression were studied here.

ABSTRACT. In cancer, the pancreas progresses through various disease stages, PanINs, before the development of malignant tumor cells in the epithelium of the pancreatic ducts. Hnf6 is a gene that is normally expressed in two cell types of the adult pancreas (acinar and duct) but preliminary data suggested that it is turned off in pancreatic cancer. In this study, Hnf6 was inactivated in only the acinar cells in mice to discern the cell type in which its inactivation is most correlated with cancer. The results showed that the mice had developed the initial symptoms of pancreatitis, a disease of pancreatic inflammation. Furthermore, HNF6 expression in different PanIN stages of human pancreatic cancer was also analyzed to determine when the gene is turned off in the progression to cancer. Results showed that the percentage of HNF6 expressing cells decreases with increasing PanIN stages. In cancerous tissues, HNF6 was not detected. Here, the negative correlation between HNF6 expression and cancer suggests that HNF6 is an important factor for maintaining healthy pancreatic tissue, and that the loss causes the progression into pancreatitis and possibly cancer. Insight on the role of Hnf6 in pancreatic cancer development could lead to its use as a biomarker for early detection.

INTRODUCTION.

Every year, more than 30,000 people in the United States alone are diagnosed with pancreatic cancer—the fourth leading cause of cancer mortality [1]. Because there is a paucity of symptoms specific to the disease until late in its progression, diagnosis is severely delayed, and the five-year survival rate is a mere 5% [2]. In its advanced stages, pancreatic cancer induces various physical symptoms, including exhaustion, loss of appetite, abdominal pain, and jaundice. These external symptoms are translated from abnormalities at the molecular level in the pancreas, a glandular organ where the cancer originates, develops, and ultimately metastasizes.

Located beneath the stomach and connected to the rostral duodenum, the pancreas is a crucial component of both the endocrine and digestive systems. The endocrine pancreas, also known as the islets of Langerhans, accounts for ~2% of adult pancreatic mass and is subdivided into five main hormone-producing cell types, including insulin-producing β cells and glucagon-secreting α cells [3]. The remaining 98% exocrine pancreas is comprised of acinar cells that secrete digestive enzymes into the pancreatic ducts, where they are subsequently transported into the duodenum. Disruption in the homeostasis of either pancreatic component increases the likelihood of disease development.

In human pancreatic disease, precancerous lesions, known as PanINs (pancreatic intraepithelial neoplasms), progress through characteristic stages into invasive pancreatic ductal adenocarcinoma (PDAC) [4], the most common form of pancreatic tumors [5]. Each PanIN stage (Figure S1) is graded based on the degree of ductal epithelial neoplasms, location of nuclei, and architectural atypia [4]. Within the realm of pancreatic cancer, there has been some controversy as to whether the disease stems from the acinar or the duct cells. Although tumorigenesis originates from the aberrant proliferation of the ductal epithelium, many argue that these duct cells arise as the result of conversion of metaplastic acinar cells [6] that become relatively insensitive to apoptosis compared to their healthy counterparts [5]. The terminal tumor cells induce further physiological abnormalities such as the formation of cysts, severe fibrosis, necrosis, increased ductal proliferation, and long term inflammation of peripheral acini [4]. These symptoms contribute to the rapid deterioration of the cancerous pancreas.

Various genes in pancreatic development have been studied due to their potential roles in disease. Hepatic Nuclear Factor 6, *Hnf6*, (also known as ONECUT-1 or OC-1) is a transcription factor crucial for endocrine differentiation and function, as well as duct morphogenesis. As transcription factors initiate expression of target genes, *Hnf6* activates the expression of neurogenin 3 (*Ngn3*), a pro-endocrine gene responsible for hormone-cell differentiation [7]. Initially expressed in all pancreatic cell progenitors during development, *Hnf6* is silenced in the endocrine lineage post-differentiation at late gestation, when expression becomes confined to the ducts and acini, where it is still expressed in adults [8]. When a global gene inactivation of *Hnf6* is generated in mice, 75% of mice die between the ages of postnatal day 1 and postnatal day 10 due to impaired liver function, while the remaining 25% develop severe diabetes as a result of an insulin imbalance [7]. Loss of *Hnf6* also contributes to ductal architectural atypia and the formation of cystic structures within the pancreatic ducts [9]. Subsequent studies showed that when *Hnf6* is inactivated in the pancreatic epithelium during embryogenesis, the mice displayed ductal dysplasia and developed chronic pancreatitis [3] (Figure S2), a disease characterized by pancreatic exocrine inflammation and an increased risk factor for pancreatic cancer. Additionally, preliminary experiments utilizing human pancreatic tissue samples that revealed the loss of HNF6 in 100% of pancreatic adenocarcinoma samples (Figure S3) have been conducted. Together, these two exciting findings are highly suggestive of the negative correlation between HNF6 expression and pancreatic cancer, and thus, lend focus to the following study.

As pancreatic adenocarcinoma occurs through progressive precursor stages, it was hypothesized that the inactivation of HNF6 occurs early within a progression timeline, potentially beginning at acinar-to-ductal metaplasia and possibly pancreatitis. In the current study, we analyzed the expression of HNF6 in normal adult human pancreas, different stage PanIN lesions, and cancer in human tissue samples obtained via collaborators. Furthermore, in respect to the debate concerning the loci associated with pancreatic cancer, this study also explored the inactivation of *Hnf6* in the acinar cells to discern its potential cell-specific role in carcinogenesis. Previously obtained pancreata from mice with an acinar cell-specific inactivation of *Hnf6* ($HNF6^{\Delta acini}$) were examined using a series of histological tests to determine the effects on pancreatic morphogenesis and function. It was further hypothesized that loss of *Hnf6* solely in the acini would result in pancreatitis and the initial stages of pancreatic adenocarcinoma.

Because of its direct application to humans, the study of the role of HNF6 in pancreatic cancer development could provide crucial scientific insight on the malignant nature of the disease. Such knowledge could potentially aid in the development of clinical diagnostic tests that could provide for early detection and a higher five-year survival rate.

MATERIALS AND METHODS.

Genotype of Mice.

The elastase-Cre transgene contains an acinar-cell specific promoter sequence, located upstream of the bacteriophage DNA recombinase called Cre [10]. Within the endogenous Hnf6 gene, specific DNA sequences were introduced flanking the critical DNA binding domain. These sequences, called loxP sites, are bound by the Cre recombinase, deleting the intervening DNA sequence. Thus, in cells that express elastase (the acinar cells), the Hnf6 DNA binding domain is deleted by Cre, resulting in a null mutation. Transgenic elastase-Cre mice were previously interbred with mice containing the loxP-marked Hnf6 gene ($HNF6^{flox/flox}$) to inactivate the gene in acinar cells (Figure S4). The result-

ing generation of mice, HNF6$^{flox/flox}$; elastase-Cre, are interchangeably referred to as HNF6$^{\Delta acini}$ mice, whose pancreata were obtained and used in this study.

Tissue Processing.

Pancreatic tissue from 4-week-old mice was fixed using 4% paraformaldehyde at 4°C for 1 hour and washed twice in phosphate buffered saline (PBS). The tissue was incubated in 70% ethanol at 4°C and subsequently dehydrated and paraffin embedded. The paraffin blocks were cut into 5 micron sections using a microtome, and mounted with tissue bonding agent Sta-on (Surgipath) onto glass slides. Finished slides were set on a slide warmer and left for 4 hours to solidify the bonding process.

Deparaffinizing and Rehydrating.

Prior to any staining or antibody labeling, all tissues were deparaffinized in xylene and rehydrated through a decreasing ethanol series. Slides were incubated in xylene three times for 3 minutes, 100% ethanol two times for 2 minutes, 95%, 90%, and 70% ethanol successively for 2 minutes each, 50% ethanol for 1 minute, and deionized water for 5 minutes on a rotating platform. In the dehydrating that preceded paraffin embedding, this process was reversed.

Hemotoxylin & Eosin (H&E) Staining.

H&E staining was initially performed to analyze the general morphology of the pancreas samples. Eosin is a contrast stain that allows for a general visualization of tissue morphology, staining acinar cells dark pink, and islets and blood vessels a lighter shade of pink, while Hematoxylin is a purple nuclear stain. Following rehydration, tissue was incubated in hematoxylin for 3 minutes and subsequently washed in running deionized water. Excess staining was removed by repeated dipping of the sections into an acid-alcohol mixture that consisted of 1% hydrochloric acid in 70% ethanol. Tissues were washed in deionized water and were slowly dipped twice in eosin (Surgipath) into ethanol, from 100% to 70%, at 30 second intervals in each successive concentration. Once at 70%, the excess eosin was removed and the sections were dehydrated into xylene and mounted with Permount (Fisher).

Sirius Red Staining.

Sirius Red allows for an overall visualization of the amount of collagen deposition within tissues, staining collagen red and acinar cells and islets green. Sections were deparaffinized, rehydrated, washed in PBS and deionized water, while on a rotating platform. Solution A (0.1% Direct Red and 0.1% Fast Green FCF in saturated picric acid) was then mixed and incubated on sections for 2 hours at room temperature. Solution B (1% glacial acetic acid in distilled water) was then mixed, and slides were exposed to Solution B for 5 minutes. The tissues were then dehydrated and mounted with Permount.

HNF6 with Eosin Counterstain.

Tissue sections were deparaffinized and rehydrated to deionized water. Sections were exposed to antigen retrieval using sodium citrate buffer in a pressure cooker for 15 minutes, and afterwards, they were left to cool to room temperature for 2 hours, followed by washing in PBS. Endogenous peroxidase activity was then quenched by exposing slides to 3% hydrogen peroxide for 20 minutes. Slides were washed further and blocked for 1 hour at room temperature in 5% normal donkey serum (NDS) and 1% BSA in PBS. Primary antibody rabbit anti-HNF6 (Santa Cruz Biotechnology, Inc.) was used in a 1:300 dilution in 1% BSA in PBS and left overnight at 4°C. After this interval, sections were washed in PBS and incubated for 1 hour at room temperature in secondary antibody biotinylated goat anti-rabbit (Abcam Inc.) in a 1:500 dilution in 1% BSA in PBS. The sections were washed in PBS, and the Vectastain ABC reagent (20 µl/ml reagent A, 20 µl/ml reagent B, in PBS)(Vector Laboratories) was then mixed and incubated on the tissue at room temperature for 30 minutes. The slides were washed again and the DAB Peroxidase Substrate kit was used to visualize the antibody labeling for a maximum 5 minute period, until the sections were placed into deionized water to quench the reaction. Following the addition of secondary antibody, HNF6 protein localization appeared as a brown precipitate. Tissues were counterstained with eosin for contrast and mounted with Permount.

Microscope Imaging.

Stained tissue samples were viewed under bright-field illumination and immunofluorescently labeled tissue was viewed under epifluorescence using appropriate optical filters on an Olympus BX41 microscope and digital camera with the Magnafire program (Optronics, Inc.). Imaging of H&E, HNF6 against eosin contrast, Sirius Red, and F4/80 against eosin contrast staining were all performed under light microscopy at both 10X and 20X magnifications. Imaging of the HNF6 and DBA immunofluorescently labeled sections was performed under fluorescent microscopy using three optical filters: HNF6 (green), DBA (red), and DAPI (blue) at 10X and 20X magnifications.

RESULTS.

Loss of HNF6 expression is correlated with PanIN and PDAC in human tissues

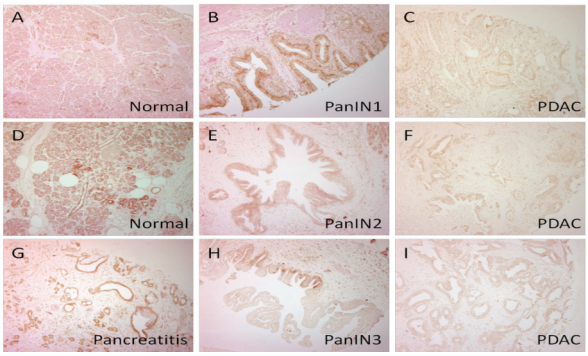

Figure 1. HNF6 Expression in human PanINs and pancreatic adenocarcinoma. HNF6 protein (brown) expression is shown against eosin contrast (pink). (**A**,**D**) Normal tissue, (**G**) pancreatitis and acinar-to-ductal metaplasia, and (**B**) PanIN1 indicate positive expression, while (**E**) PanIN2, (**H**) PanIN3, and (**C**,**F**,**I**) PDAC show negative expression.

To examine the correlation between HNF6 expression and the progression to pancreatic cancer, immunohistochemistry labeling of HNF6 on tissue samples from diseased human pancreata was performed to assay for the presence of the HNF6 protein. The microscopic images were examined to determine a correlation of HNF6 expression with pancreatic morphology. Analyses of images suggested that there was a general negative trend in increasing PanIN progression and HNF6 expression, while all of the tumor sections were completely negative. Within the samples, 17% of acinar-to-ductal metaplasia, 14% of pancreatitis, 52% of PanIN1, 70% of PanIN2, 92% of PanIN3, and 100% of cancer sections displayed negative HNF6 expression.

H&E staining revealed unhealthy acinar tissue in HNF6$^{\Delta acini}$ mice.

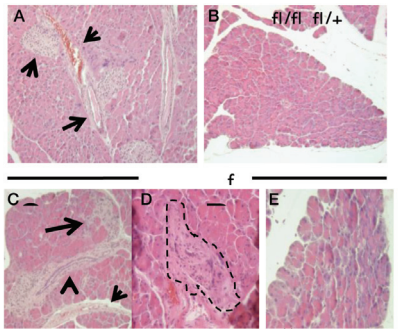

Figure 2. Unhealthy acinar tissue in HNF6$^{\Delta acini}$ pancreata. (**A**,**B**)Acinar tissue, duct (**D**), and islet (**I**) in control mice appear healthy (bv denotes blood vessel). Mutant mice, HNF6$^{fl/fl}$;elastase-Cre, exhibit (**C**: **ab**) the possibility of acinar tissue trandifferentiating into ductal tissue and fibrotic tissue infiltration of acini; (Outline in **D**) abnormal architecture of the duct-like structure that appears to have been derived from acinar cells; and (**E**) increased spacing between acinar cells.

H&E stainingwas initially performed to visualize the general morphology of the tissue from the pancreata of the HNF6$^{\Delta acini}$ mice to detect for signs of atypia. The results revealed the presence of unhealthy acinar tissue, of which

several areas indicated the appearance of inflammation, based on darker nuclear appearance (Figure S5), and abnormal spacing between acinar cell clusters. The possibility of acinar cells transdifferentiating into duct cells was also evident by morphology in many tissue areas. Furthermore, the staining also showed fatty infiltration in several areas (Figure S5). The tissue is thought to have replaced the acinar tissue originally located in these areas because it is observed to only surround duct-like structures, a characteristic that is absent in typical adipose.

Increase in periductal collagen deposition.

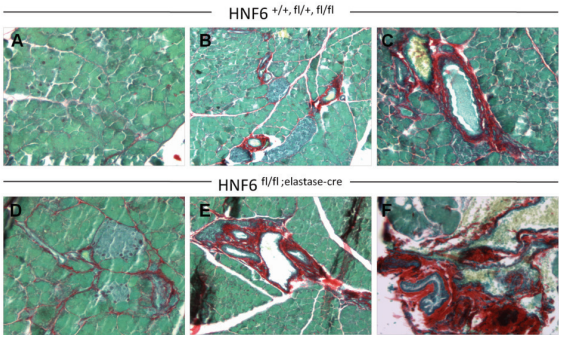

Figure 3. Increase in periductal collagen deposition. Sirius Red staining shows (**A**) healthy acinar tissue (green) and normal levels of collagen (red) surrounding (**B**) small and (**C**) large ducts in control mice. HNF6$^{\Delta acini}$ mice displayed abnormal duct architecture and a significant increase in periductal collagen (**E**-**F**). (**D**) Furthermore, there was an increase in collagen between the acini. Overall, collagen increase is indicative of fibrosis.

Sirius Red staining was performed in order to determine whether there was an increase in collagen deposition around the ducts—a characteristic observed in pancreatitis and pancreatic cancer. The results indicated that HNF6$^{\Delta acini}$ mice did display an increase in levels of collagen both around the ducts and between acinar cells, relative to control mice. Furthermore, this excess in collagen was almost always accompanied by abnormal ductal architecture. Within control pancreata, there exists a moderate level of collagen in order to connect and support the pancreatic ducts and tissue. An increase relative to this normal amount is indicative of fibrosis, a characteristic of chronic pancreatitis.

DISCUSSION.

Loss of HNF6 may occur in a progression from acinar-to-ductal metaplasia to cancer.

The original hypothesis in this study was that the inactivation of HNF6 occurs in a progression that begins at acinar-to-ductal metaplasia, advances to pancreatitis and to the PanIN stages, and eventually develops into invasive carcinoma. Observation of the percentage of negative expression in the data at each stage in the predicted pathway supports this hypothesis and the largely negative expression of HNF6 in the pancreatic cancer tissue samples, shown in Figure 1, strongly reiterates the negative correlation between HNF6 expression and cancer. The scale of HNF6 expression in human pancreatic disease development developed here may potentially indicate the course of the development of pancreatic adenocarcinoma, which is a subject that currently remains unclear.

Loss of Hnf6 in acini in mice displays initial stages of pancreatitis.

Figure 2 shows that the pancreata of four-week-old HNF6$^{\Delta acini}$ mice displayed chronic acinar-cell damage and possibly acinar-to-ductal metaplasia, indicators of the onset of disease. In many areas, there existed an infiltration of fatty tissue (Figure S5). As adipose tissue replaces healthy acinar cells, it expands within the cell, subsequently displacing organelles and ultimately inducing necrosis. This suggested that adipose replacement is succeeded by macrophage infiltration, as the resulting cellular debris from acinar cell death requires phagocytic removal. This reasoning lead to the further marking of the tissue with macrophage marker F4/80 to detect for the presence of macrophages, which in combination with cell death, is a hallmark of not only pancreatitis but also cancer.

Furthermore, Sirius Red staining revealed increased levels of collagen around the ducts and between the acinar cells. Within a normal pancreas, there exist fair amounts of collagen between cells, as collagen is a main component of connective tissue that links the various cell types within the pancreas. However, this excess collagen displayed in the knockout mice (Figure 3) is indicative of fibrosis, a telltale characteristic of pancreatitis.

After observing these results from HNF6$^{\Delta acini}$ pancreas, a phenotype for these acinar cell-specific knockout mice was characterized. It can be concluded that loss of *Hnf6* from the acini does contribute to the abnormal morphogenesis of the pancreas and the initial stages of pancreatitis. Upon comparison of this phenotype to that of the conditional pancreatic epithelial knockout of *Hnf6*, it was observed that although highly similar, the HNF6$^{\Delta acini}$ mice displayed less severe symptoms. As these mice were relatively young upon examination, the next step is to follow HNF6$^{\Delta acini}$ mice at later stages to see whether this phenotype worsens or develops into full-blown pancreatitis and possibly pancreatic cancer.

Most of the existing research on *Hnf6* in pancreatic disease development has examined the effects of either its manipulation or its target genes. The present study, however, observes not only manipulation of the gene, but also the possible source(s) of its manipulation that induces such severe results as pancreatic cancer. As *Hnf6* is expressed only in duct and acinar cells in adults [8], and the effects of its *acinar*-specific inactivation were examined here, another goal for future studies is to determine the pancreatic symptoms of a *ductal*-specific *Hnf6* gene inactivation. Such results would then be compared with those from the pancreatic epithelial *Hnf6* knockout in Zhang et al. [3] and the acinar-cell inactivation in the present study to possibly localize the inactivation of *Hnf6* to a specific cell type in the development of pancreatic cancer. The resulting implications may, in turn, help pinpoint the yet unknown cell-type of origin of pancreatic ductal adenocarcinoma. Thus, the loss of the expression of the *Hnf6* gene could potentially be used as a biomarker for pancreatic cancer. With these possibilities, the diagnosis of pancreatic cancer could eventually occur significantly early enough to halt the metastases of tumor cells—a crucial step in extenuating the threat of a disease in which the current incidence rate nearly parallels the mortality rate.

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SUPPORTING INFORMATION.

Figure S1. Progression to pancreatic adenocarcinoma
Figure S2. Pancreatitis and ductal atypia in HNF6$^{\Delta panc}$ pancreata
Figure S3. Loss of HNF6 in human pancreatitis and PDAC tissues examined
Figure S4. Elastase-Cre mediated inactivation of *Hnf6;* Figure S5. Fatty infiltration.

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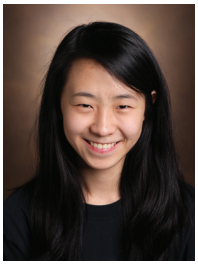

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