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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Spontaneous Pancreatitis Caused by Tissue-Specific Gene Ablation of *Hhex* in Mice

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SUMMARY

Hhex is expressed in developing and mature pancreatic ductal cells. Embryonic *Hhex* ablation leads to chronic pancreatitis, yet Hhex is not required for mature exocrine compartment maintenance. Hhex represses G-protein coupled receptor *Npr3*, and thus likely ductal cell secretion.

BACKGROUND & AIMS: Perturbations in pancreatic ductal bicarbonate secretion cause chronic pancreatitis. The physiologic mechanism of ductal secretion is known, but its transcriptional control is not. We determine the role of the transcription factor hematopoietically expressed homeobox protein (*Hhex*) in ductal secretion and pancreatitis.

METHODS: We derived mice with pancreas-specific, Cre-mediated *Hhex* gene ablation to determine the requirement of Hhex in the pancreatic duct in early life and in adult stages. Histologic and immunostaining analyses were used to detect the presence of pathology. Pancreatic primary ductal cells were isolated to discover differentially expressed transcripts upon acute *Hhex* ablation on a cell autonomous level.

RESULTS: Hhex protein was detected throughout the embryonic and adult ductal trees. Ablation of *Hhex* in pancreatic progenitors resulted in postnatal ductal ectasia associated with acinar-to-ductal metaplasia, a progressive phenotype that ultimately resulted in chronic pancreatitis. *Hhex* ablation in adult mice, however, did not cause any detectable pathology. Ductal ectasia in young mice did not result from perturbation of expression of Hnf6, Hnf1β, or the primary cilia genes. RNA-seq analysis of *Hhex*-ablated pancreatic primary ductal cells showed mRNA levels of the G-protein coupled receptor natriuretic peptide receptor 3 (*Npr3*), implicated in paracrine signaling, up-regulated by 4.70-fold.

CONCLUSIONS: Although Hhex is dispensable for ductal cell function in the adult, ablation of *Hhex* in pancreatic progenitors results in pancreatitis. Our data highlight the critical role of *Hhex* in maintaining ductal homeostasis in early life and support ductal hypersecretion as a novel etiology of pediatric chronic pancreatitis. *(Cell Mol Gastroenterol Hepatol 2015;1:550–569; http://dx.doi.org/10.1016/j.jcmgh.2015.06.007)*

Keywords: Npr3; Pancreatic Ducts; Primary Cilia.

The exocrine pancreas, composed of acinar and ductal cells, plays a crucial role in digestion by delivering alkaline, isotonic pancreatic juice containing digestive enzymes to the duodenum. Pancreatic zymogens, released from acini in response to postprandial enterohormonal and neural signals, traverse an intricate network of ducts of increasing size.[1–3] Rather than merely serving as conduits, the pancreatic ducts actively aid in digestion by secreting bicarbonate against an immense concentration gradient.[4] Similar to acinar cells, ductal cells are stimulated to secrete in response to enterohormonal and neural inputs via the cyclic adenosine 5′-monophosphate/protein kinase A and calcium/phospholipase C-β signaling pathways.[5–9] Additionally, various paracrine factors released from acinar cells have been identified that augment ductal cell stimulation, ensuring a coordinated pancreatic response.[10,11]

Bicarbonate secretion serves to solubilize intraluminal zymogens and neutralize acidic chyme in the duodenum.[12] Impairment of ductal cell functioning, such as what is

Abbreviations used in this paper: **ADM, acinar-to-ductal metaplasia; ANP, atrial natriuretic peptide; CFTR, cystic fibrosis transmembrane conductance regulator; DAPI, 4,6-diamidino-2-phenylindole; E, embryonic development day; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; H&E, hematoxylin and eosin stain; HCP, hereditary chronic pancreatitis; Hhex, hematopoietically expressed homeobox protein; ICP, idiopathic chronic pancreatitis; Npr3, natriuretic peptide receptor 3; P, postnatal day; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDCs, pancreatic primary ductal cells; PRSS1, cationic trypsinogen (protease, serine, 1); PSCs, pancreatic stellate cells; SMA, smooth muscle actin; SPINK1, serine peptidase inhibitor, Kazal type 1; YFP, yellow fluorescent protein.**

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frequently observed in patients harboring mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), contributes to the pathogenesis of pancreatic insufficiency and chronic pancreatitis, an important risk factor for pancreatic ductal adenocarcinoma.[13–15] Although the mechanism by which bicarbonate is transported across the pancreatic ductal epithelium has been elucidated, the transcriptional control governing this process remains poorly understood.

Recently, we reported that the transcription factor Hhex (hematopoietically-expressed homeobox protein), initially described as Prh (proline-rich homeodomain) in chicken, is expressed in the pancreatic ductal epithelium;[16,17] however, its function in this cell type and its potential contribution to pancreatic disease pathogenesis have not been determined. Previous studies indicate that Hhex is critical for proper development of structures derived from all three germ layers, including liver, thyroid, forebrain, heart, hematopoietic progenitors, and endothelium.[18–20] In the endoderm, *Hhex* is first expressed throughout the primitive endoderm but is later restricted to the visceral endoderm before gastrulation.[21,22] At embryonic development day 7 (E7.0), *Hhex* transcripts are localized to the anterior endoderm and the ventral-lateral foregut, the site of ventral pancreatic and liver organogenesis. *Hhex* mRNA is expressed at E10.0 in precursors of the thymus, liver, thyroid, dorsal pancreatic bud, and gallbladder.[23] By E13.5, endodermal *Hhex* expression is limited to the thyroid, liver, epithelial cells of the pancreas and extrahepatic biliary ducts, and most cell types of the lung, and it is notably high in the epithelia of the extrahepatic bile ducts and pancreas at E16.5.[23] In the adult mouse, Hhex gene activity has been previously described in the lung, thyroid, and liver;[23] moreover, Hhex has been shown to regulate directly functional genes in various mature cell types, such as somatostatin in δ-cells.[17]

The expression pattern of Hhex in the ventral-lateral foregut prior to pancreas specification suggests that it may serve an essential function in pancreatic development. Indeed, $Hhex^{-/-}$ mice fail to specify the ventral pancreatic bud, and they exhibit variable forebrain truncation, thyroid hypoplasia, and cannot expand the hepatic primordium.[20,24,25] Importantly, the failure of ventral pancreatic morphogenesis was determined to be the result of lack of proliferation of the definitive endoderm, thus compromising cell migration and subjecting these cells to morphogenetic inhibition by signaling from the cardiac mesoderm.[24] This cell-extrinsic mechanism was confirmed by the proper induction of the pancreatic progenitor gene *Pdx1* and the proendocrine genes *Isl1*, *Ngn3*, and *NeuroD* when $Hhex^{-/-}$ endodermal explants were grown away from the cardiac mesoderm.[24] Embryonic lethality of $Hhex^{-/-}$ mice, however, precluded any further analysis of the role of Hhex in pancreatic development or function.

Here, we characterized the expression dynamics of Hhex within the ductal compartment of the pancreas and determined its requirement for ductal development and function by employing conditional gene ablation in mice. Ablation of *Hhex* in pancreatic progenitors resulted in postnatal ductal ectasia that progressed to chronic pancreatitis later in life, consistent with a published model of ductal hypertension.[26] Moreover, we identified the G-protein coupled receptor natriuretic peptide receptor 3 (*Npr3*), the activation of which is reported to potentiate secretin signaling to increase pancreatic flow rate, as regulated by Hhex and likely contributing to the pathogenesis of chronic pancreatitis in this genetic model.[27]

Table 1. Primers Used for Genotyping Analysis

Primer	Sequence (5′→3′)	Product Size (bp)
Hhex-F	ATTGACGGAAATGTTGCCATA	WT: 473
Hhex-R	CCAAGTGACACGATCCAGAAC	loxP: 605
CreERT2-F	TTTCAATACCGGAGATCATGC	550
CreERT2-R	ATTCCTGTCCAGGAGCAAGTT	
Cre-F	GCGGCATGGTGCAAGTTGAAT	232
Cre-R	CGTTCACCGGCATCAACGTTT	
YFP1	AAGACCGCGAAGAGTTTGTC	WT: 600
YFP2	GGAGCGGGAGAAATGGATATG	YFP+: 320
YFP3	AAAGTCGCTCTGAGTTGTTAT	

Materials and Methods

Mice

The derivation of the $Hhex^{loxP}$ allele has been described previously elsewhere.[28] $Pdx1\text{-}Cre^{Early}$ mice were kindly provided by Dr. Guoqiang Gu and Dr. Doug Melton, and $Sox9\text{-}CreER^{T2}$ mice were kindly provided by Dr. Maike Sander.[29,30] The mice were maintained on a 129SvEv/C57BL/6 mixed background. Genotyping was performed by polymerase chain reaction (PCR) analysis using genomic DNA isolated from toe snips of newborn mice. The genotyping primers are provided in Table 1, and the thermocycler conditions were as follows: $Hhex^{loxP}$ and $CreER^{T2}$: 94°C for 4 minutes [94°C for 35 seconds, 60°C for 35 seconds, 72°C for 50 seconds] 33 times, 72°C for 7 minutes, 4°C indefinitely; *Cre*: 94°C for 5 minutes [94°C for 30 seconds, 56°C for 45 seconds, 72°C for 60 seconds] 30 times, 72°C for 10 minutes, 4°C indefinitely; and *YFP*: 94°C for 3 minutes, [94°C for 30 seconds, 50°C for 60 seconds, 72°C for 60 seconds] 35 times, 72°C for 2 minutes, 4°C indefinitely. Experimental mice were derived from crossing $Hhex^{loxP/loxP}$ animals with either $Hhex^{loxP/loxP}$;$Pdx1\text{-}Cre^{Early}$ or $Hhex^{loxP/loxP}$;$Sox9\text{-}CreER^{T2}$ mice; $Hhex^{loxP/loxP}$ littermates were used as controls for all experiments. For timed matings, the morning at which a vaginal plug was present was considered day E0.5.

For experiments with tamoxifen induction, adult mice (>9 weeks of age) were administered 5 mg of tamoxifen (T5648, Lot SLBF8049V; Sigma-Aldrich, St. Louis, MO) per 40 g of body mass for 3 consecutive days by oral gavage. Tamoxifen was suspended in a 10% ethanol/90% sunflower seed oil (S5007; Sigma-Aldrich) (v/v) mixture at 20 mg/mL and rotated at 42°C for 2 hours until completely dissolved. All procedures involving mice were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.